Isolation, Identification and Evaluation of Potential Biological Control Agents for Sustainable Grapevine Pruning Wound Protection

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Isolation, Identification and Evaluation of Grapevine Derived Microbes for Sustainable Grapevine Pruning Wound Protection

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Isolation, Identification and Evaluation of Grapevine Derived Microbes for Sustainable Grapevine Pruning Wound Protection

Abstract

Grapevine trunk diseases (GTDs), caused by many different fungal pathogens, threaten the economic sustainability of viticulture worldwide causing a significant reduction of both yields and quality of grapes. GTDs are referred to as a complex of diseases with Botryosphaeria dieback, Eutypa dieback and Esca being major contributors. Pruning wounds are the main point of entry for these fungal pathogens and majority of spore release occurs in the winter months when precipitation activates spore release. Traditionally, disease control is focused on cultural practices and preventative pruning wound protection by the application of chemical pastes and sprays. However, with an imperative need for sustainable agricultural practices there has been much interest in the use of biological control agents (BCAs) as pruning wound protectants. This study aimed to (i) identify naturally occurring potential BCAs from a variety of grapevine tissues, including sap, cane and pith and evaluate their antagonistic activity against selected fungal pathogens responsible for GTDs *in vitro*, (ii) take forward promising candidates to greenhouse and field trials to evaluate as pruning wound protectants alongside commercial chemical and biological protectants and (iii) determine the recovery rate of biologicals from treated canes at the end of greenhouse and field studies. Isolated bacterial and fungal isolates from grapevine structures were screened *in vitro* to determine their antifungal activity via a dual culture assay and volatile assay against *Eutypa lata*, *Diplodia seriata*, *Diaporthe ampelina* and *Neofusicoccum parvum*. Among the fungal isolates, *Trichoderma* spp. inhibited *E. lata* mycelial growth up to 64% and *N. parvum* mycelial growth up to 73% with overgrowth and stopped growth being the likely antagonistic mechanisms. Among the bacterial isolates, *Bacillus* spp. inhibited *E. lata* mycelial growth up to

20% and *N. parvum* mycelial growth up to 40%. Under greenhouse and field conditions, *Trichoderma asperellum* and *Trichoderma gamsii* consistently provided high pruning wound protection in greenhouse and field trials, with a mean percent disease control (MPDC) of 88% and 100% for *E. lata* and *N. parvum* respectively, when compared to the water treated-inoculated positive control. The chemical protectants, Thiophanate-methyl + Myclobutanil and Fluopyram + Trifloxystrobin were also able to effectively protect wounds with a MPDC of up to 86%. Lastly, when biological treatments were evaluated for recovery from treated canes at the end of the growing season, *Trichoderma*-based treatments had a rate of recovery between 0 and 100%.

Chapter 1

In vitro evaluation of grapevine endophytes, epiphytes and sap micro-organisms for potential use to control grapevine trunk disease pathogens

Abstract

Grapevine trunk diseases (GTDs) threaten the economic sustainability of viticulture worldwide causing a significant reduction of both yields and quality of grapes. Biological control presents a promising sustainable alternative to cultural and chemical methods to mitigate the effects of pathogens causing GTDs, including Botryosphaeria dieback, Eutypa dieback and Esca. This study aimed to identify naturally occurring potential biological control agents from a variety of grapevine tissues, including sap, cane and pith and evaluate their antagonistic activity against selected fungal pathogens responsible for GTDs *in vitro*. Bacterial and fungal isolates were preliminary screened *in vitro* to determine their antifungal activity via a dual culture assay against *Neofusicoccum parvum* and *Eutypa lata*. Among the fungal isolates, *Trichoderma* spp. inhibited *E. lata* mycelial growth by up to 64% and *N. parvum* mycelial growth by up to 73%. Among the bacterial isolates, *Bacillus* spp. inhibited *E. lata* mycelial growth up to 20% and *N. parvum* mycelial growth up to 40%. Select antagonistic isolates of *Trichoderma, Bacillus and Aureobasidium* spp. were subject to further dual culture antifungal analysis against *Diplodia seriata* and *Diaporthe ampelina,* with *Trichoderma* isolates consistently causing the greatest inhibition. Volatile organic compound antifungal analysis revealed that *Trichoderma* isolates significantly inhibited mycelial growth *of N. parvum, E.* lata and *D. ampelina* causing up to 20.11%, 60.55% and 70.9% inhibition

respectively ($P \leq 0.05$). Multilocus sequence analysis revealed that *Trichoderma* isolates are most closely related to *Trichoderma asperellum* and *Trichoderma hamatum*. This study identifies grapevine sap as a novel source of potential biological control agents for control of GTDs to support existing efforts to control GTDs. Further testing will be necessary to fully characterize these microbes' mode of antagonism and assess their efficacy for pruning wound protection *in planta*.

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Figure 8. Maximum parsimony phylogenetic tree of UCD 8368 and UCD 8717 based on a multigene alignment of the *Trichoderma* Hamatum/Asperellum clade using internal transcribed spacer rDNA (ITS), and translation elongation factor 1-alpha (TEF1). Bootstrap support for the maximum-likelihood analysis is given at each node (1000 replicates). Asterix (*) indicates subcultures evaluated in this study. AF487654 *Trichoderma neorufum* was used as an outgroup.

Introduction

Fungal diseases are a major biotic threat to the economic sustainability of table grapes and wine grapes worldwide. Grapevine trunk diseases (GTDs) are prevalent in most viticulture regions worldwide causing a significant reduction of both yields and quality of grapes, as well as increasing crop management costs for cultural and chemical preventative measures (Urbez-Torres *et al.*, 2006; Gubler *et al.,* 2005; Siebert *et al.,* 2001; Bertsch *et al.,* 2013; Kaplan *et al.,* 2016). GTDs lead to premature decline and dieback of grapevines and are caused by a complex of several taxonomically unrelated groups of Ascomycetes. Botryosphaeria dieback, also known as Black Dead Arm or 'Bot Canker' is one of the most severe GTDs and is currently associated with 26 botryosphaeriaceaous taxa in the genera *Botryosphaeria, Diplodia, Dothriorella, Lapsiodiplodia, Neofusicoccum, Neoscytalidium, Phaeobotryosphaeria, and Spencermartinsia* (Pitt *et al.,* 2013; Urbez-Torres. 2011; Pitt *et al.,* 2015; Yang *et al.,* 2017; Rolshausen *et al.,* 2013). Another devastating GTDs is Eutypa dieback, caused by 24 species in the Diatrypaceae family with the most virulent and common being *Eutypa lata* (Luque *et al.,* 2014; Pitt *et al.,* 2013; Rolshausen *et al.,* 2014; Trouillas *et al.,* 2010; Trouillas *et al*. 2011). Esca and Phomopsis dieback also comprise the GTDs complex and are of worldwide economic importance (Munkvold *et al.,* 1994; Gubler *et al.,* 1995). GTDs can occur simultaneously in all grapevine producing areas though severity may differ among regions (Mugnai *et al.,* 1999; Pascoe and Cottral, 2000; Halleen *et al.,* 2003; Gubler *et al.,* 2005). Characteristic symptoms of Botryosphaeria and Eutypa dieback are the formations of wedge-shaped cankers in infected trunks and cordons. From the infection site, which is often a pruning wound, the fungal pathogen will grow downwards occupying vascular elements as well as adjacent cells. When the affected vineyards are no longer economically sustainable to maintain, growers sadly face no alternative but to replant (Gramaje *et al.*, 2018). GTDs can also be found in

dormant wood cuttings and young grafted plants and thus spread to grapevines during the plant propagation process (Aroca *et al.,* 2010; Gramaje and Armengol, 2011; Waite and Morton, 2007; Billones-Baaijens *et al.,* 2013).

Management of GTDs is difficult and influenced by the specific disease and/or pathogens involved but over the years a variety of preventative methods have been studied and implemented, including cultural practices such as double pruning and application of chemical fungicides (Weber et al. 2007; Bertsch *et al.,* 2013). However, these methods are highly variable in efficacy, not environmentally sustainable and can be very costly (Zanzotto *et al.,* 2016). A promising new approach is the use of biological control agents (BCAs) to control pathogens causing GTDs. Biological control refers to the utilization of naturally occuring micro-organisms to suppress pests and pathogens (Martinez-Diz *et al.,* 2020; Heimpel and Mills, 2017). Grapevine, like perennial woody plants, can be colonized by an innumerable number of micro-organisms that can reside intercellularly or intracellularly within grapevine tissue and are called endophytes (Gilbert *et al.,* 2014; West *et al.,* 2010) or they can colonize the surface of grapevine organs, such as leaves and are called epiphytes (Bruisson *et al.,* 2019; Hardoim *et al.,* 2015). Endophytes have been shown to be a valuable source of potential BCAs as they are believed to be associated with all 300,000 plant species, most of them non-pathogenic bacteria or fungi that colonize plants asymptomatically (Strobel and Daisy. 2003). Since the turn of the century, more than 40 BCAs have been isolated, identified and tested against the pathogens responsible for the GTDs complex and whilst the majority of cultured endophytes do not exhibit inhibitory activity, some *Trichoderma spp*. and *Bacillus spp.* have proved highly efficient in protecting pruning wounds against various GTDs pathogens *in vitro,* greenhouse and field trials (Mondello *et al.,* 2018; Di Marco *et al.,* 2002; 2004; John *et al.,* 2008; Halleen *et al.,* 2010; Schmidt *et al.,* 2001; Kotze *et al.,* 2011; Rezgui *et al.,* 2016;

Martinez-Diz *et al.,* 2020) and several successful efforts have been made to commercialize these species as BCAs (Otoguro and Suzuki, 2018). *Trichoderma spp.* can stimulate plant growth and suppress pathogens by direct competition for nutrients and space, exhibit mycoparasitism and antibiosis and induce systemic resistance (Harman. 2006; Mukherjee *et al.,* 2013; John *et al.,* 2005). *Bacillus spp.* can antagonize GTDs via antibiotic production, competition for nutrients and activation of the plant defense response (Cawoy *et al.,* 2011; Choudhary and Johri 2009).

To our knowledge, there have been no published reports evaluating grapevine sap inhabiting microbes for their antifungal activity against pathogens causing GTDs. The majority of antagonistic endophyte studies related to GTDs have sourced microbes from grapevine bark and roots. Thus, our study aimed to exploit this gap in the knowledge by isolating microbes from grapevine sap both immediately after making fresh pruning cuts as well as seven days later and evaluate for their antagonistic activity against a variety of pathogens responsible for GTDs *in vitro*. We also made isolations from other grapevine sources including pith and cane tissue.

Materials and Methods

Isolation of potential biocontrol organisms from grapevine

All microbial sampling was performed at the University of California, Davis, Plant Pathology Fieldhouse Facility in Yolo County (38°31'24.1"N 121°45'43.3"W) from an 8-years old 'Sauvignon blanc' vineyard in March 2019 prior to any standard pruning. A total of 10 randomly selected 'healthy' looking vines were used in this study with samples taken from four randomly pruned spurs per vine. For collection of sap exudate, the cut points of one-year old lignified spurs were sprayed with 70% ethanol for surface sterilization to avoid contamination, and once dry, a

horizontal pruning cut was made with sterile pruning shears. A 100 µl sample of sap exudate was immediately collected from the bleeding wound with a pipette and stored on ice. A 20 µl aliquot of sap exudate from each spur was spread by a sterile glass rod onto potato dextrose agar amended with tetracycline at 100 mg/L (PDA-T) and nutrient agar (NA) plates. Growing fungal and bacterial cultures were sub-cultured for *in vitro* screening and molecular identification. Sampling for epiphytic microbes was performed by scraping dry sap from the pruning surface seven days after the initial cut from the same canes and plated as described above. After incubation at 25°C for roughly 7 days, sub-cultures of all growing microbes were made to fresh PDA-T and NA.

Grapevine endophytes were also isolated in September 2019 from the same vineyard from untreated control canes used in a pruning wound protection trial. The canes were split longitudinally, and isolations were made from the exposed wood and pith tissues. A total of ten canes were used and three pieces of tissue and three pieces of pith were collected from each cane and plated on PDA-T and NA plates. Plates were incubated at 25 °C for roughly 7 days before subcultures of growing isolates were performed.

Genomic DNA extraction

Genomic DNA was extracted by scraping fungal mycelium from 1 week old subcultures of isolates and added to a 2ml tube containing 300 µl of Nuclei Lysis Solution and 1mm diameter glass beads (bioSpec Products). Mycelium was homogenized for 40 seconds at 6 m/sec in a FastPrep-24™ 5G bead beating grinder and lysis system (MP Biomedicals). Genomic DNA was extracted using a DNA extraction kit (Wizard Genomic DNA Purification Kit; Promega Corp, Madison, WI). Genomic DNA was extracted from 1-week old bacterial sub cultures by collecting a loop of

bacteria with a sterile pipette tip and inoculating a 0.2 ml PCR tube containing 15 µl of Molecular Grade Water and ran in a thermal cycler for 15 minutes at 95 °C.

PCR amplification and sequencing of Fungal ITS, TEF-1a and β1-tubulin genes.

The internal transcribed spacer (ITS) region of the ribosomal RNA *(rRNA)* gene was amplified using the primers, ITS1 and ITS4 (White *et al.,* 1990). The translation elongation factor 1 alpha gene (TEF-1a) was amplified using the primers, EF1-728F and EF1-968R (Carbone and Kohn, 1999). The beta tubulin gene *(Bt)* was amplified using the primers, Bt2a and Bt2b (Glass and Donaldson. 1995).

PCR amplification and sequencing of Bacterial 16S rRNA, purH and rpoB genes.

The 16S rRNA gene was amplified using the primes 16S U1 and 16S U2 (Lu *et al.,* 2000). The purine biosynthesis gene was amplified using the primers, purH-70f and purH-1013r (Rooney *et al.,* 2009). The RNA polymerase subunit B (*rpoB*) gene was amplified using the primers, rpoB-229f and rpoB-3354Rr (Rooney *et al.,* 2009).

All PCR assays were performed in a final volume of 25 µl in a reaction mixture containing 0 mM Tris-HCl (pH 8.8), 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM of each dNTP, 1.0 µM of each primer and 1 unit of Go Taq polymerase, Promega Corp., Madison, WI. Primers and excess nucleotides were removed from the amplified DNA using a PCR clean-up kit (EXO SAP). New England BioLabs and DNA was quantified using a QuantiFluor dsDNA System, Promega Corp., Madison, WI. Purified PCR samples were sent to Quintarabio, Hayward, CA for Sanger Sequencing. Sequence chromatograms were analyzed, and the sequences were assembled using Sequencher version 5.4.6. Alignment was performed with Clustal W. Phylogenetic analysis was performed with Mega X

using the Maximum composite likelihood model for estimating genetic differences. A phylogenetic tree was obtained using the neighbor-joining method with 1000 bootstrap replicates.

Dual culture assay

All fungal and bacterial isolates were tested in an initial *in vitro* dual culture assay against the GTDs, *N. parvum* and *E. lata*. Fresh subcultures were made from each isolate and incubated at 25°C for 1 week on PDA-T plates for fungal isolates and PDA plates for bacterial isolates for the assay. A 5mm diameter plug from each isolate was then placed 1cm from the edge of a 100 x 15mm plate and a 5 mm diameter plug of 1 week old *N. parvum* or *E. lata* was placed 1cm from the opposite edge of the plate. Plates with only the pathogen served as controls. *N. parvum assays* were incubated at 25°C for 4 days before the percentage of pathogen inhibition was recorded whereas *E. lata* assays were incubated at 25°C for 14 days before being recorded. The percentage of inhibition of pathogen mycelial growth was calculated using the formula reported by Idris et al. (2007): % inhibition = [(C-T)/C] x 100) where C is the radius in mm of the pathogen when plated by itself and T is the radius of the pathogen when plated with an isolate. There was a total of 10 replicates per isolate. Representative isolates from each genus isolated exhibiting potential biological control ability against *N. parvum* and *E. lata* were subsequently tested against the GTD pathogens, *Diplodia seriata* and *Diaporthe ampelina* using the same assay.

Volatile assay

The production of antifungal volatile organic compounds (VOCs) was assessed using the two-sealed-base-plates method described in Gotor-Vila *et al.,* (2017) with modifications. 100 x 15mm petri dishes were half filled with PDA-T or PDA and a 5mm diameter mycelial plug of 1 week old isolates were placed in the center of a base plate. A 5mm diameter mycelial plug of a pathogen

was placed in the center of another base plate and the two base plates were immediately sealed together using parafilm. Plates with only the pathogen served as controls. *N. parvum* and *D. seriata* assays were incubated at 25°C for 4 days before percentage of pathogen inhibition was recorded whereas *E. lata* and *D. ampelina* assays were incubated at 25°C for 14 days The percentage of inhibition of pathogen mycelial growth was calculated using the formula reported by Idris *et al.,* (2007) as mentioned above. There was a total of 10 replicates per isolate tested.

Statistical analyses

Data obtained from the dual culture assay was analyzed by one-way ANOVA and means were separated by the post-hoc Dunnett's test at a 0.05 significance level.

Results

Isolation and ITS/16s sequencing of all potential biocontrol organisms from grapevine

In total, eleven fungal isolates and two bacterial isolates were cultured on growth media from all grapevine 'structures' sampled (Table 1). The majority of isolates were obtained from either grapevine cane tissue or sap collected immediately after pruning cuts were made. Only two isolates were obtained from sap seven days after pruning and one isolate was obtained from grapevine pith. PCR amplification of the ITS gene, sequencing and BLAST revealed that nine of the fungal isolates were members of the *Aureobasidium* genus and two were members of the *Trichoderma* genus (Table 1). PCR amplification of the 16S rRNA, sequencing and BLAST revealed that the two bacterial isolates were members of the *Bacillus* genus (Table 1).

Preliminary screening – Dual culture assay (N. parvum and E. lata)

The antagonistic potential of all subcultured bacterial and fungal isolates (Table 1) was initially evaluated against the GTDs pathogens *N. parvum* and *E. lata in vitro* using a dual culture assay. Whilst the majority of isolates showed no significant inhibition of *N. parvum* mycelial growth, the two bacterial isolates (*Bacillus* spp.*)*, UCD 8745 and UCD 8347 and the two *Trichoderma* isolates, UCD 8368 and UCD 8717 caused a significant inhibition of *N. parvum* mycelial growth, ranging from 35% to 64.4% (Fig. 1A, $P \leq 0.05$) compared to the *N. parvum* control. When the isolates were tested for antagonistic potential against *E. lata*, only the *Trichoderma* isolates, UCD 8368 and UCD 8717 were able to cause significant inhibition of *E. lata* radial mycelial growth, both resulting in excess of 65% mycelial inhibition compared to the control (Fig. 1B, $P \leq 0.05$).

Dual culture assay (D. seriata and D. ampelina)

The Trichoderma *isolates*, UCD 8368 and UCD 8717 and *Bacillus* isolates, UCD 8745 and UCD 8347 were taken forward for further dual culture analysis as were the *Aureobasidium* isolates, UCD 8189 and UCD 8344 so that each genus of microorganisms isolated were evaluated. The antagonistic potential of these isolates were next evaluated against the GTDs pathogens *D. seriata* and *D. ampelina* using the same dual culture assay as mentioned above. All isolates caused a significant inhibition of *D. seriata* radial mycelial growth, ranging from 15.23% to 50.2% (Fig. 2A, $P \leq 0.05$) compared to the control. Both *Trichoderma* isolates caused the greatest radial inhibition at roughly 50% compared to the control. There was variation between the *Bacillus* isolates as UCD 8347 caused roughly 32% radial inhibition whilst UCD 8745 only caused roughly 11% radial inhibition. The *Aureobasidium* isolates, UCD 8189 and UCD 8344 were similar in their antagonistic activity, causing roughly 15% and 17% radial inhibition respectively. When the isolates were tested against the GTDs pathogen, *D. ampelina*, the *Trichoderma* isolates, UCD 8368 and UCD 8717 caused the greatest inhibition, in excess of 80%. The *Bacillus* isolate UCD 8347

also significantly reduced mycelial radial growth of *D. ampelina*, though to a much lesser extent (Fig. 2B, P≤0.05 and Fig. 3).

Volatile organic compound (VOC) assay

When the isolates were screened for antagonistic activity via production of antifungal volatile organic compounds (VOCs) against *N. parvum*, only the *Bacillus* isolate, UCD 8347 and *Trichoderma* isolate, UCD 8368 caused significant inhibition of *N. parvum,* causing roughly 10% and 20% radial inhibition respectively (Fig. 4A, P≤0.05). When the isolates were tested against *E. lata*, all but the *Aureobasidium* isolates were capable of causing significant radial inhibition. The *Trichoderma* isolates, UCD 8368 and UCD 8717 isolates exhibited the greatest VOC effect, both causing at least 50% radial inhibition, whilst the *Bacillus* isolates, UCD 8745 and UCD 8347 isolates caused roughly 37% and 39% radial inhibition respectively (Fig. 4B, P≤0.05). No isolates exhibited any VOC mediated significant inhibition of *D. seriata* (Fig. 7C). However, against *D. ampelina*, all isolates exhibited VOC mediated significant inhibition with UCD 8717 causing roughly 70% inhibition. The other *Trichoderma* isolate, UCD 8368 caused roughly 40% inhibition, whilst the *Bacillus* isolates, UCD 8745 and UCD 8347 and *Aureobasidium* isolates, UCD 8189 and UCD 8344 all caused roughly 20% inhibition (Fig. 4D, P≤0.05 and Fig. 5).

Multilocus phylogenetic analysis of antagonistic isolates

Multilocus phylogenetic analysis of the *ITS* and *β1-tubulin* gene via maximum parsimony revealed that the isolates, UCD 8344 and UCD 8189 were most closely related to *Aureobasidium pullulans* (Figure 6). Multilocus phylogenetic analysis of the *purH and rpoB* gene via maximum parsimony revealed that the isolates, UCD 8347 and UCD 8745 were most closely related to *Bacillus velezensis* (Figure 7). Multilocus phylogenetic analysis of the *ITS* and *TEF-1a* gene via maximum

parsimony revealed that the isolates, UCD 8368 and UCD 8717 were most closely related to *Trichoderma asperellum* and *Trichoderma hamatum* respectively (Fig. 8).

Discussion

Grapevine pruning wound protection has historically been mediated by synthetic chemicals which have dominated the crop protection industry dating back to the 1980s. However, the longevity of crop production requires a greater shift towards sustainable practices so there is great interest in novel solutions to prevent and control grapevine trunk diseases (GTDs) (Mondello and Songy. 2018). Biological control agents (BCAs) including *Trichoderma spp*. and *Bacillus spp.* have been demonstrated to have excellent potential for pruning wound protection against infection from GTDs *in vitro* (Di Marco *et al.*, 2002, 2004; John et al., 2008; Halleen *et al.*, 2010; Schmidt *et al.*, 2001; Kotze *et al.*, 2011; Rezgui *et al.*, 2016). Microbial inhabitants of nutrient rich grapevine sap have not been evaluated for BCA ability against GTDs, so along with isolations from grapevine pith and cane tissue, we evaluated isolated microbes against the selected GTDs fungal pathogens, *Neofusicoccum parvum*, *Eutypa lata*, *Diplodia seriata* and *Diaporthe ampelina in vitro.*

In vitro dual culture assays are the primary means to detect antagonistic activity of microorganisms (Di Marco *et al.*, 2002; Haidar *et al.*, 2016). Both *Trichoderma* isolates UCD 8368 and UCD 8717 in this study exhibited significant mycelial inhibition against all pathogens in dual culture assays, exhibiting at least 75% mycelial inhibition against the slow growing pathogens, *E. lata* and *D. ampelina* (Fig. 1B and 2B). UCD 8368, which is most closely related to *T. harzianum* (Fig. 8) was also shown to be effective in a similar *in vitro* study at in inhibiting *E. lata* radial growth (Úrbez-Torres *et al.*, 2020). Whilst *Trichoderma spp.* possess various antifungal mechanisms, this

mycelial inhibition can be likely attributed to overgrowth (Kotze *et al.,* 2011) as they grew considerably faster and surrounded the pathogens in dual culture (Figure 3). These findings have been backed up by similar studies where various *Trichoderma spp*. have been subject to dual culture assays against *N. parvum, D. seriata* and *E. lata* (Mutawila *et al.,* 2015; Silva-Valderrama *et al.,* 2020; Úrbez-Torres *et al.,* 2020). For example, *Trichoderma* isolates from Southern Italy were able to inhibit *N. parvum* radial growth by up to 74.3% (Úrbez-Torres *et al.,* 2020). It is hypothesized that this observed overgrowth by *Trichoderma spp.* translates to competition for space and nutrients in grapevine pruning wounds and therefore a mechanism to protect against GTDs (Úrbez-Torres *et al.,* 2020).

However, in the volatile assay, UCD 8368 and UCD 8717 were still able to cause significant inhibition of *E. lata* and *D. ampelina* (Figures 4B and D) which is most likely due to the ability of *Trichoderma spp.* to produce volatile and non-volatile substances which have been shown to inhibit a range of fungi (John *et al.,* 2004; Kucuk and Kivanc, 2004; Kexiang *et al.,*. 2002; Dennis and Webster, 1971a; Ghisalberti and Sivasithamparam, 1991; Chambers and Scott, 1995). John *et al.,* (2004) showed that volatile compounds synthesized by *T. harzianum* AG1, AG2, and AG3 were able to inhibit growth of *E. lata* compared to a control and *E. lata* growth was completely inhibited by non-volatile compounds. In this study UCD-8368 and UCD 8717 elicited a coconut odor (detectable via smelling) which has previously been characterized as 6-n-pentyl-2H- pyran-2-one (Claydon *et al*., 1987), and reported to inhibit fungi such as *Rhizoctonia solani*. The significant mycelial inhibition of *N. parvum* and *D. seriata* by UCD 8368 and UCD 8717 in the dual culture assay can likely be attributed to stopped growth, a term which describes when microorganism and pathogen grow until they came in contact with one another, whereafter growth of both organisms seizes (Kotze *et al.,* 2011) (Fig. 1A, 2A and 3). This mechanism as the primary

method of inhibition can be supported in the volatile assay because there was no inhibition of *N. parvum* and *D. seriata* by UCD 8368 and UCD 8717 (Fig. 4A, C and Figure 5). The mycoparasitic reactions such as coiling, adhesion and penetration of pathogenic hyphae (Almeida *et al.*, 2007), have been shown to coincide with the physical contact interactions; overgrowth and stopped growth. With UCD 8717 being isolated from grapevine sap, this is to our knowledge the first report of a grapevine sap inhabiting microbe showing promising BCA ability against GTDs *in vitro*. In a recent study, Deyett and Rolshausen (2019) utilized a culture-independent amplicon metagenomic approach to characterize the major bacterial and fungal taxa that comprise grapevine xylem sap microbial communities, revealing that the core microbiome consisted of seven bacterial and five fungal taxa. Grapevine sap is a rich source of glucose, fructose and amino acids, especially in spring when nutrients are remobilized to the vegetative parts of the grapevine following winter dormancy and is thus a conducive environment to harbor beneficial microbes (Deyett and Rolshausen, 2019).

The bacterial isolates (*Bacillus* spp.) UCD 8347 and UCD 8745 exhibited varying antifungal ability and mechanisms of antifungal ability in this study depending on the GTDs fungal pathogen. In the dual culture assay between UCD 8347 and *E. lata*, a zone of inhibition was observed (Fig. 3). Inhibition zones are most likely indicative of antibiotic production (Kotze, 2004), a mechanism of mycoparasitism. Ferreira *et al.*, (1991) identified at least two *Bacillus* produced antibiotic substances that were responsible for the inhibition of mycelial growth and ascospore germination. In a recent study, Kotze, (2008) dual incubated (*in vitro*) *E. lata* with the same isolate and showed that *E. lata* displayed little mycelial growth and a clear inhibition zone between the cultures. Malformation of the hyphae, specifically swelling, was observed at a microscopic level. Another study by Kotze, (2011) showed that a *Bacillus subtilis* isolate exhibited a clear zone of inhibition

against *Phomopsis viticola.* In the volatile assay, isolate UCD 8347 caused significant inhibition against *E. lata* suggesting that the antibiotic substance may be a volatile product. Isolate UCD 8347 also exhibited a small zone of inhibition against *N. parvum* in the dual culture assay (Fig. 3) and it could also significantly inhibit *N. parvum* growth, albeit by only 10% in the volatile assay indicating the antibiotic substance may be a volatile product (Fig. 4A). Isolate UCD 8347 also exhibited significant inhibition of *D. seriata* and *D. ampelina* in the dual culture assay (Fig. 2A and B) and *D. ampelina* in the volatile assay (Fig. 4D) but the mechanism of inhibition is unclear. Isolate UCD 8745 had similar results to UCD 8347 albeit with less inhibition in some assays and the mechanism of inhibition is not as clear. It may be prudent in subsequent studies to investigate the VOC profile of these isolates.

Studies of the grapevine microbiome show that *Aureobasidium pullulans* is commonly distributed in grapevine, both in below and above ground structures (Sabate *et al.,* 2002; Martini *et al.,* 2009; Grube *et al.,* 2011; Barata *et al.*, 2012; Pinto *et al.,* 2014) and therefore, *A. pullulans* is an attractive micro-organism for investigating BCA potential. In this study, the *Aureobasidium* isolates UCD 8344 and UCD 8189, whilst possessing no antagonistic ability against *N. parvum*, *E. lata* and *D. ampelina* in the dual culture assay, were able to cause significant mycelial inhibition of *D. seriata* in dual culture (Fig. 2A). This is likely due to stopped growth as they had no inhibitory effect against *D. seriata* in the volatile assay (Fig. 4C). Similar results were obtained in a study by Pinto *et al.,* (2018), where *A. pullulans* strain Fito_F278 was able to significantly reduce the mycelial growth of *D. seriata* F98.1 in a dual culture assay and was also postulated to be as a result of stopped growth.

Although several different types of microorganisms were tested in this study, currently only *Trichoderma spp.* have been shown to be the most suitable agent for biological control of GTDs. The reason for this supremacy probably stems from the synergistic action of *Trichoderma spp.* various biocontrol mechanisms, in their ecological characteristics (saprotrophic, endophytic) and in the positive effects induced in their host plants. Considering that grapevines accommodate a large pool of resident microorganisms embedded in a complex micro-ecosystem (Pinto and Gomes, 2016), further attempts should be made to identify novel strains of *Trichoderma* and other microorganisms promoting advances in management of GTDs.

With the imperative need to make future agricultural practices as sustainable as possible we need novel solutions to control GTDs thus yielding high quality grapes that comply with the high standards of food safety. Whilst BCA efficacy *in vitro* does not always translate to efficacy *in planta*, they are at present the most promising, sustainable option for grapevine growers based on the restrictions and concerns of using chemical fungicides. This study has identified potential BCAs with great potential for simultaneous control of economically important pathogens responsible for GTDs and warrants further studies to characterize their modes of antagonism and evaluate their efficacy in field trials. There is hope these potential BCAs can provide long lasting protection of grapevine against GTDs because they share the same host.

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Tables and figures

Table 1. Source of isolated microorganisms and ITS/16S identification

Isolate	Source	Genus
UCD 8193	Grapevine cane tissue	*Aureobasidium* (ITS)
UCD 8248	Grapevine cane tissue	*Aureobasidium* (ITS)
UCD 8302	Grapevine sap, collected immediately	*Aureobasidium* (ITS)
UCD 8176	Grapevine cane tissue	*Aureobasidium* (ITS)
UCD 8174	Grapevine sap, collected immediately	*Aureobasidium* (ITS)
UCD 8196	Grapevine sap, collected immediately	*Aureobasidium* (ITS)
UCD 8170	Grapevine sap, collected immediately	*Aureobasidium* (ITS)
UCD 8344	Grapevine cane tissue	*Aureobasidium* (ITS)
UCD 8189	Grapevine sap, collected immediately	*Aureobasidium* (ITS)
UCD 8745	Grapevine sap, collected after 7 days	*Bacillus* (16S)
UCD 8347	Grapevine cane pith	*Bacillus* (16S)
UCD 8368	Grapevine cane tissue	*Trichoderma* (ITS)
UCD 8717	Grapevine sap, collected after 7 days	*Trichoderma* (ITS)

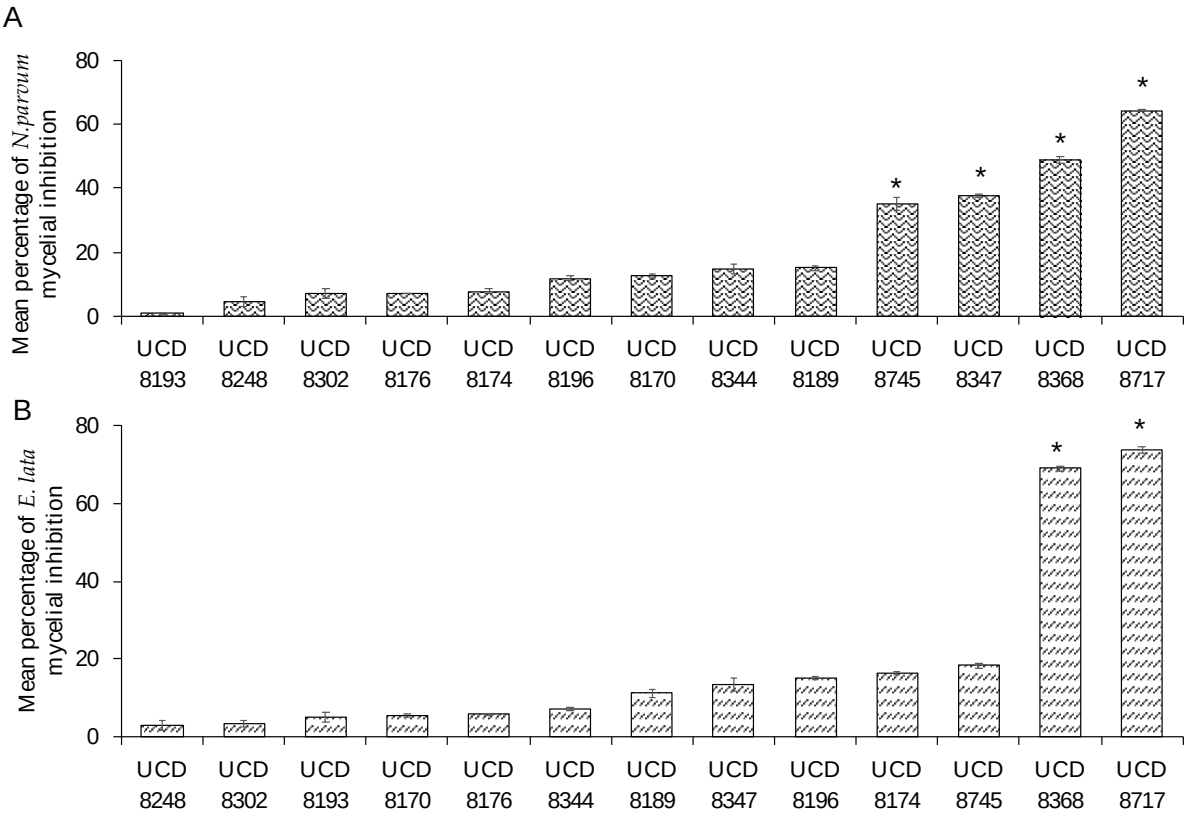

Figure 1. Preliminary *In vitro dual culture* evaluation of isolated micro-organisms ability to inhibit radial mycelial growth of the grapevine trunk disease pathogens (A) *Neofusicoccum parvum* and (B) *Eutypa lata.* The percentage of inhibition of pathogen mycelial growth was calculated using the formula: % inhibition = [(C-T)/C] x 100) (49)where C is the radius in mm of the pathogen when plated by itself and T is the radius of the pathogen when plated with a grapevine isolate. Values represent the average of ten replicates ± standard error. Asterisk (*) indicates significant inhibition in comparison with a control (Dunnett's test $P \leq < 0.05$).

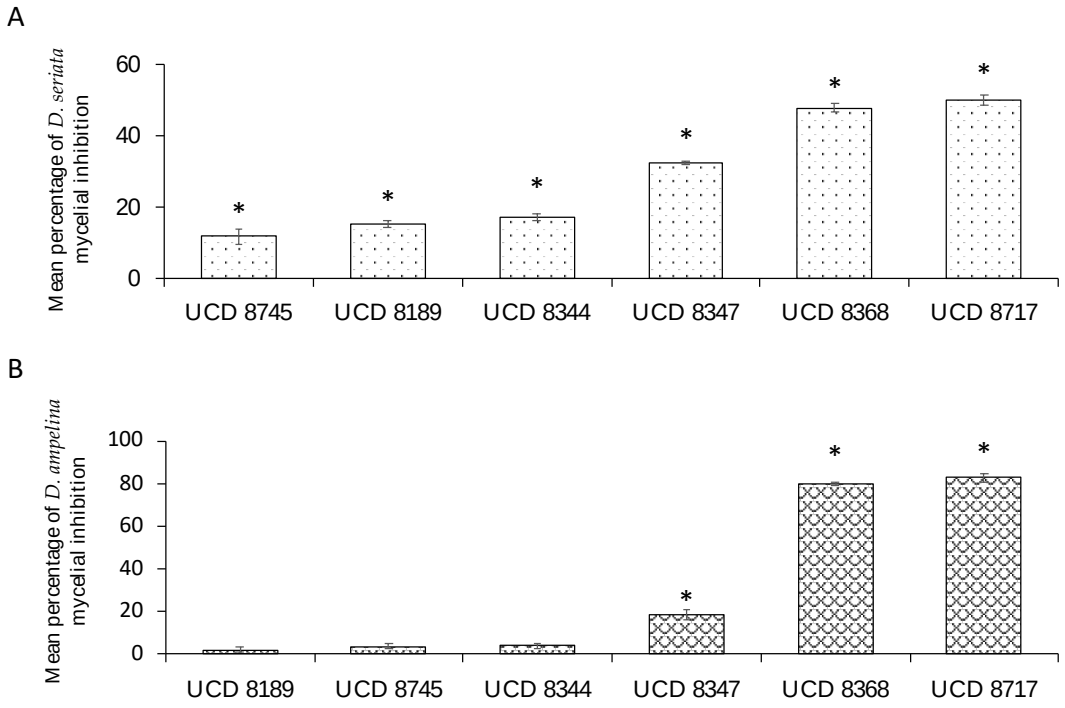

Figure 2. *In vitro dual culture* evaluation of selected micro-organisms ability to inhibit radial mycelial growth of the grapevine trunk disease pathogens (A)*) Diplodia seriata and (B) Diaporthe ampelina.* The percentage of inhibition of pathogen mycelial growth was calculated using the formula: % inhibition = [(C-T)/C] x 100) (49) where C is the radius in mm of the pathogen when plated by itself and T is the radius of the pathogen when plated with a grapevine isolate. Values represent the average of ten replicates ± standard error. Asterisk (*) indicates significant inhibition in comparison with a control (Dunnett's test $P \leq < 0.05$).

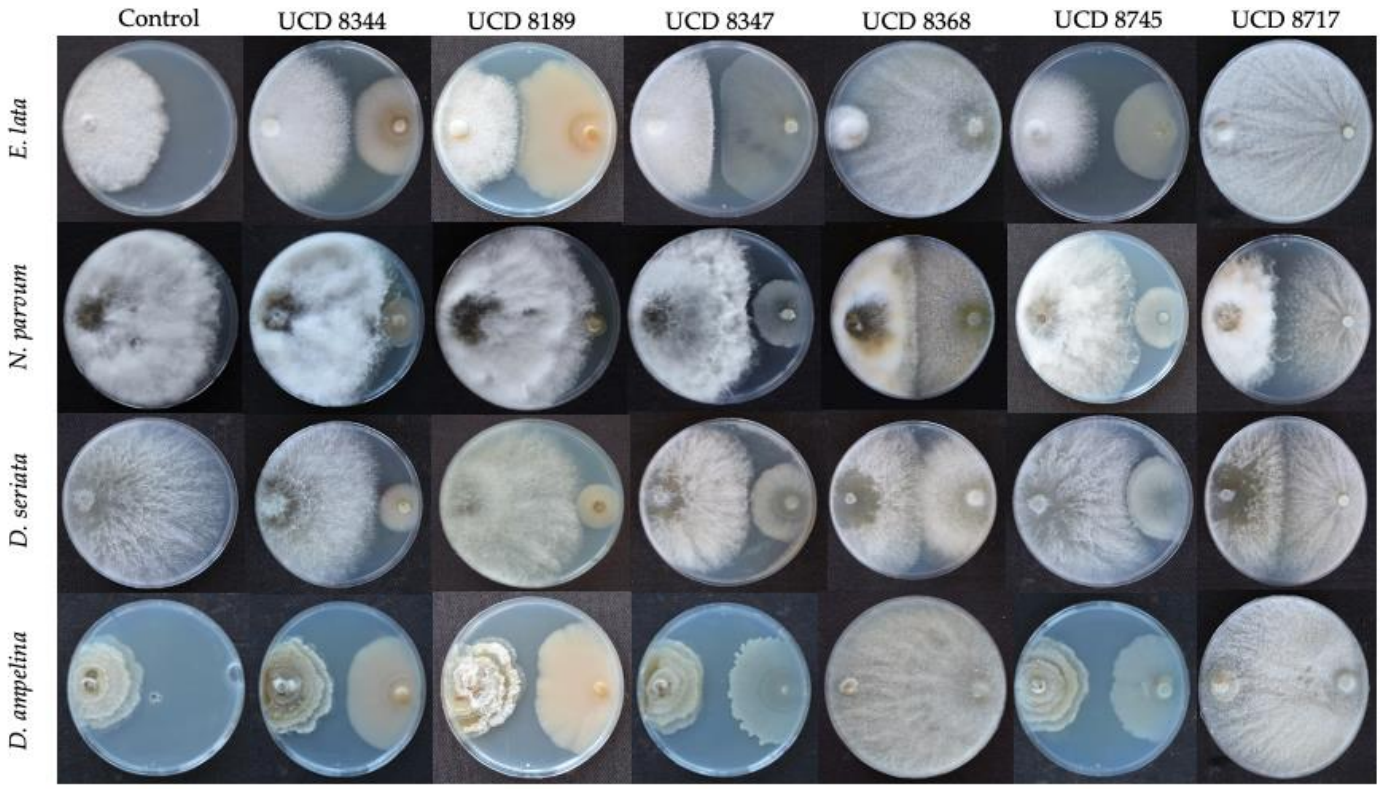

Figure 3. Representative visual summary of In vitro dual culture evaluation of selected isolates ability to inhibit radial mycelial growth of selected grapevine trunk disease pathogens.

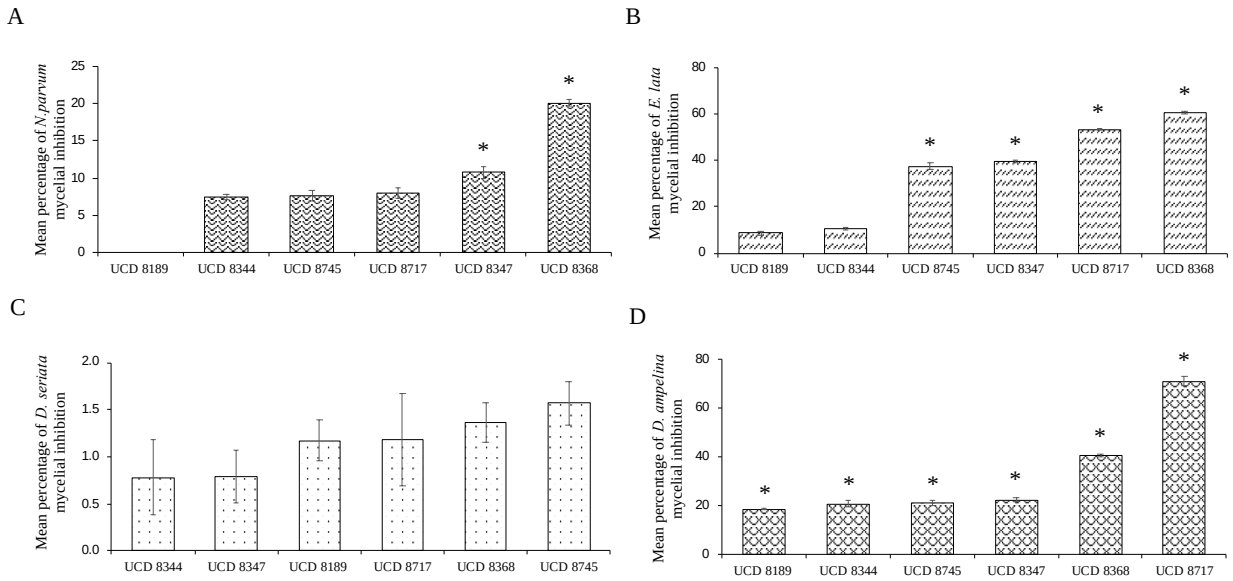

Figure 4. *In vitro volatile* evaluation of selected micro-organisms ability to inhibit radial mycelial growth of the grapevine trunk disease pathogens (**A**) *Neofusicoccum parvum,* (**B**) *Eutypa lata,* (**C**) *Diplodia seriata and* (**D**) *Diaporthe ampleina* using the sealed-base-plates method (50) with modifications . The percentage of inhibition of pathogen mycelial growth was calculated using the formula: % inhibition = [(C-T)/C] x 100) (49) where C is the radius in mm of the pathogen when plated by itself and T is the radius of the pathogen when plated with a grapevine isolate. Values represent the average of ten replicates ± standard error. Asterisk (*) indicates significant inhibition in comparison with a control (Dunnett's test $P \leq < 0.05$).

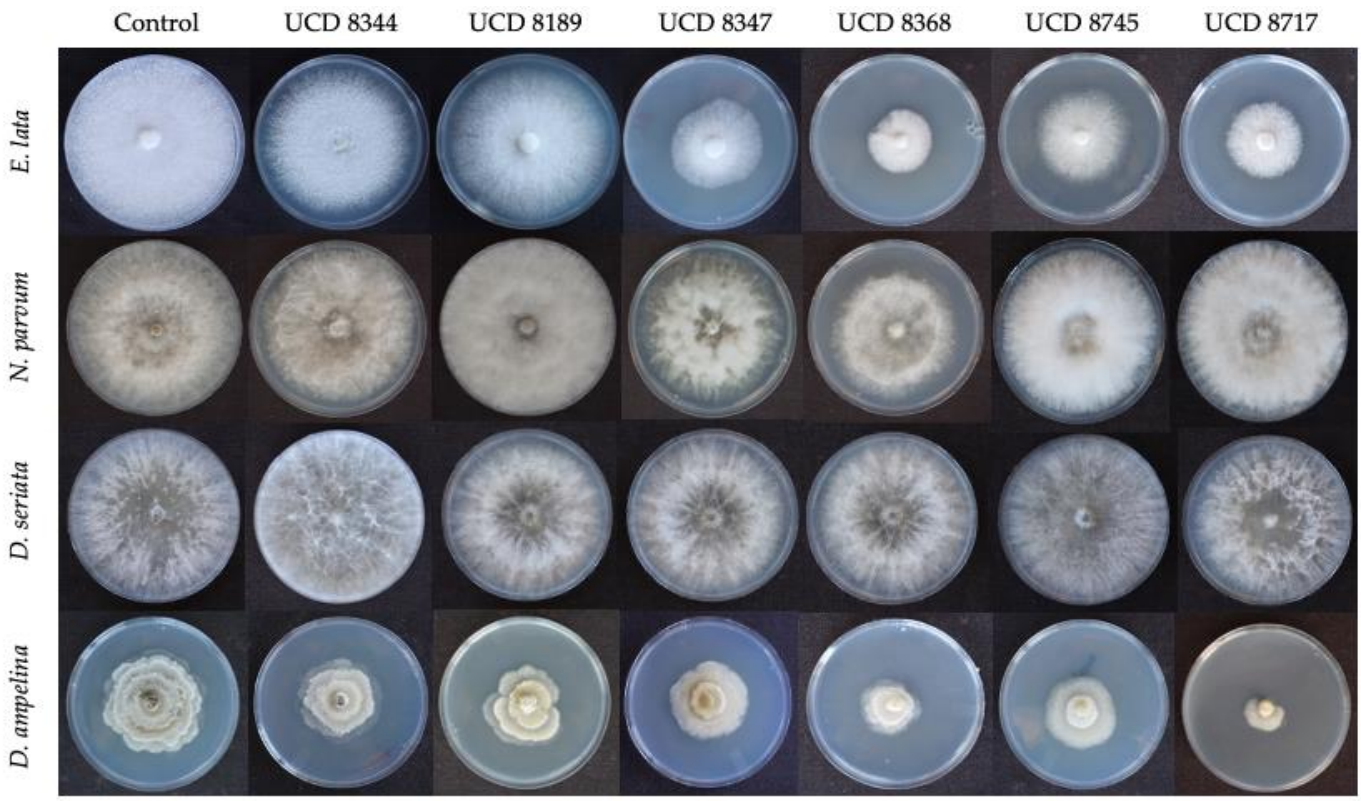

Figure 5. Representative visual Summary of *In vitro volatile* evaluation of selected micro-organisms ability to inhibit radial mycelial growth of the grapevine trunk disease pathogens

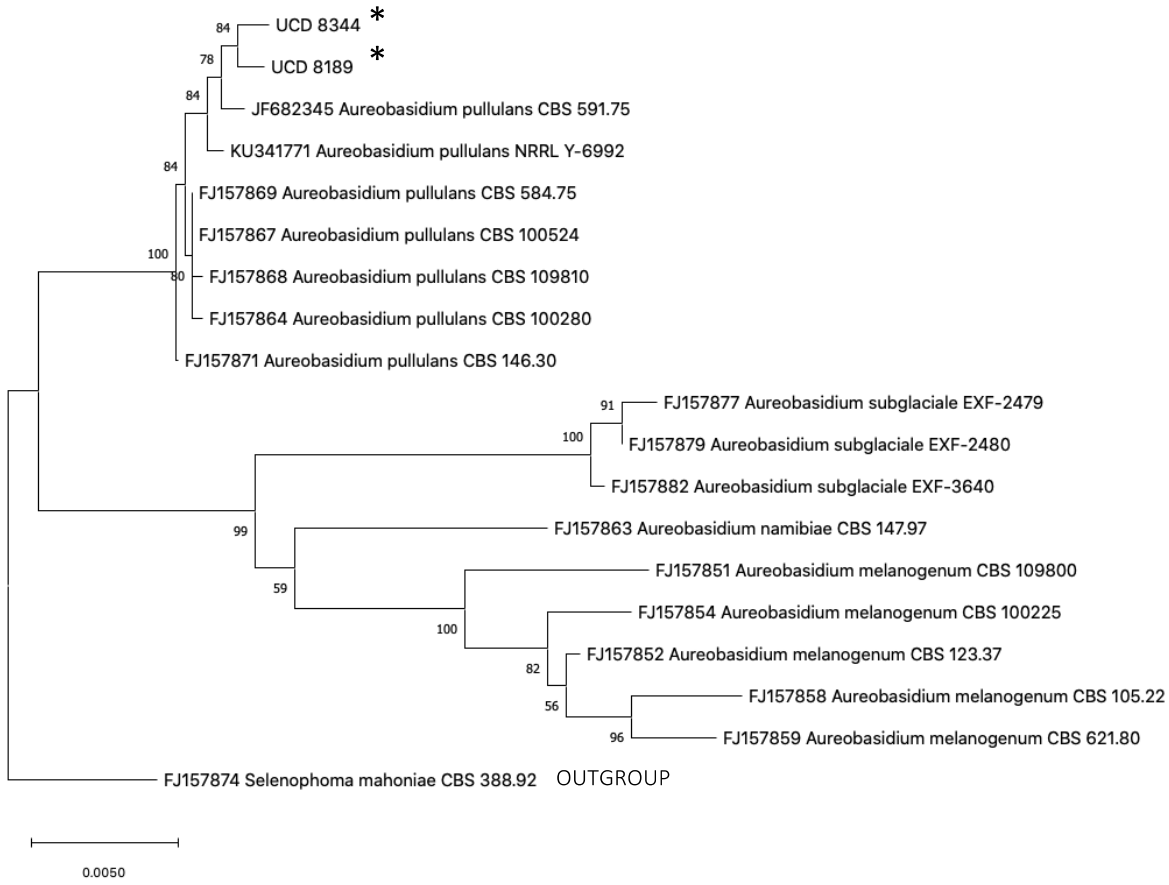

Figure 6. Maximum parsimony phylogenetic tree of UCD 8344 and UCD 8189 based on a multigene data set of internal transcribed spacer rDNA (ITS) and β1-tubulin. Bootstrap support for the maximum-likelihood analysis is given at each node (1000 replicates). Asterix (*) indicates isolates evaluated in this study. FJ150872 *Selenopoma mahoniae* was used as an outgroup.

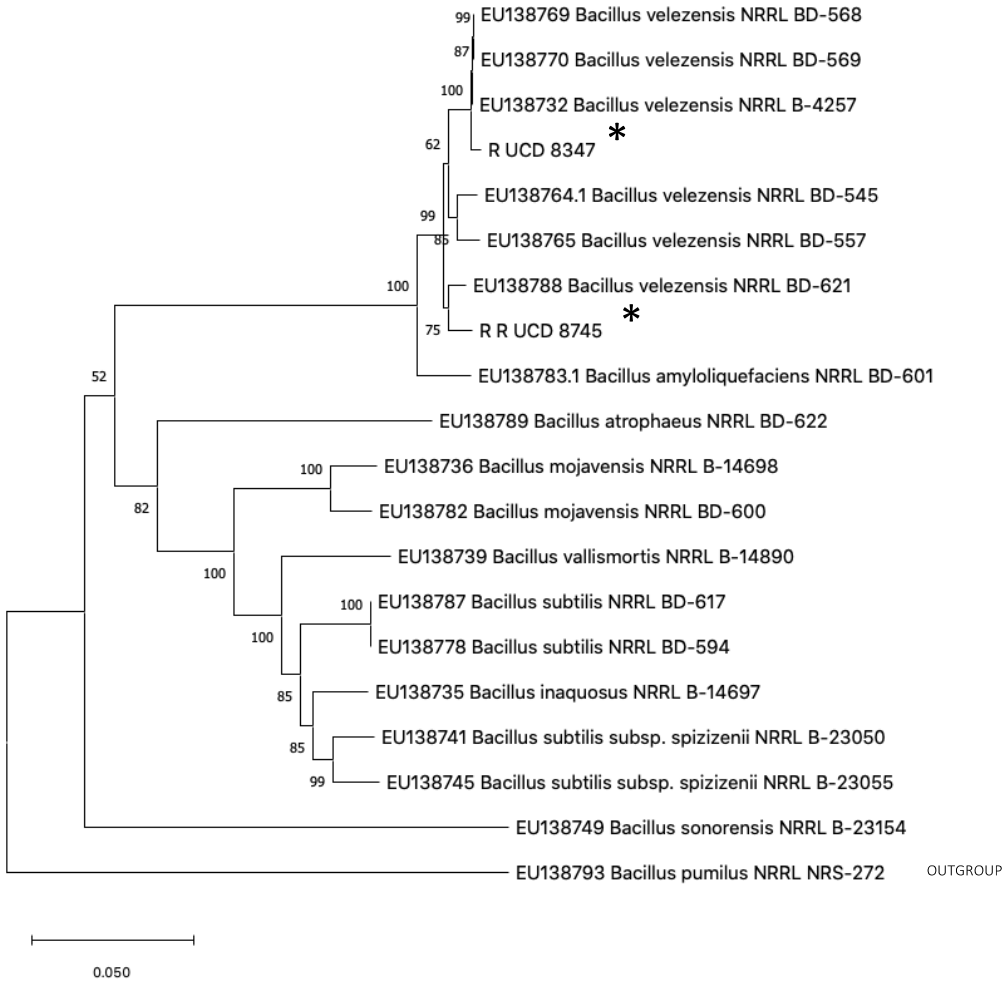

Figure 7. Maximum parsimony phylogenetic tree of UCD 8347 and UCD 8745 based on a multigene data set of purine biosynthesis (*purH*) and RNA polymerase subunit B (*rpoB*). Bootstrap support for the maximum-likelihood analysis is given at each node (1000 replicates). Asterix (*) indicates isolates evaluated in this study. EU138793 *Bacillus pumilus* was used as an outgroup.

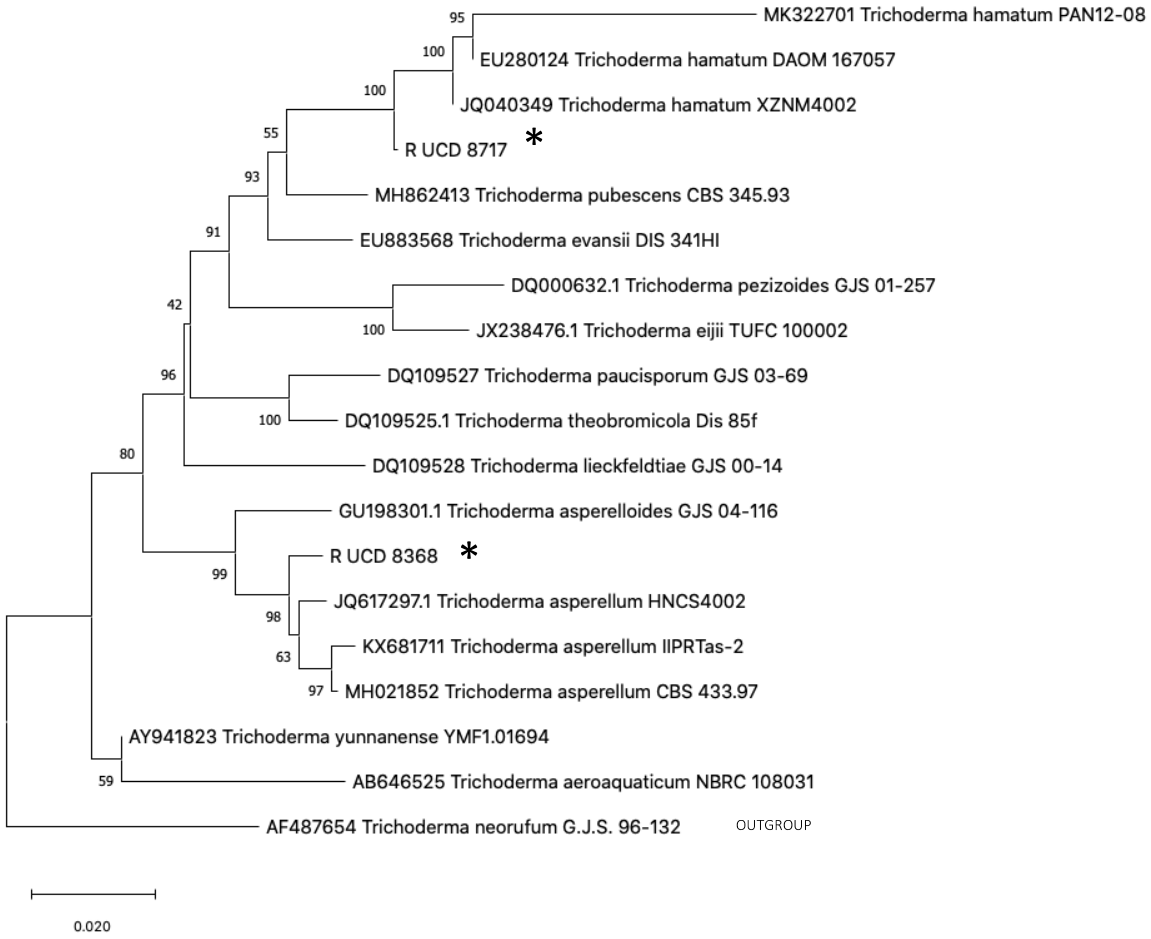

Figure 8. Maximum parsimony phylogenetic tree of UCD 8368 and UCD 8717 based on a multigene alignment of the *Trichoderma* Hamatum/Asperellum clade using internal transcribed spacer rDNA (ITS), and translation elongation factor 1-alpha (TEF1). Bootstrap support for the maximum-likelihood analysis is given at each node (1000 replicates). Asterix (*) indicates subcultures evaluated in this study. AF487654 *Trichoderma neorufum* was used as an outgroup.

Chapter 2

Evaluation of Pruning Wound Protectants to Control Grapevine Trunk Diseases Pathogens *Eutypa Lata* and *Neofusicoccum Parvum*

Abstract

Grapevine trunk diseases, caused by many different fungal pathogens, are one of the most economically important diseases affecting the grapevine industry worldwide. Pruning wounds are the main point of entry for these fungal pathogens and thus, disease control is focused on preventative pruning wound protection by chemical products and/or biological control agents (BCAs). In this study we evaluated a broad variety of already registered or at the experimental stage of chemical and BCAs in greenhouse and in field trials for the protection of table- and wine-grape vines against infection of *Eutypa lata* and *Neofusicoccum parvum*, major pathogens responsible for Eutypa and Botryosphaeria dieback, respectively. Our study showed that *Trichoderma asperellum* and *Trichoderma gamsii* consistently provided high pruning wound protection in greenhouse and field trials, with a mean percent disease control (MPDC) of 88% and 100% for *E. lata* and *N. parvum* respectively, when compared to the water treated-inoculated positive control. The chemical protectants, Thiophanate-methyl + Myclobutanil and Fluopyram + Trifloxystrobin were also able to effectively protect wounds with a MPDC of up to 86%. Lastly, when biological treatments were evaluated for recovery from treated canes at the end of the growing season, *Trichoderma*-based treatments had a rate of recovery between 0 and 100%.

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Introduction

Grapevines are one of the most extensively grown and economically important woody fruit crops in the world with approximately 7.4 million hectares cultivated and 77.8 million tons of fruit harvested in 2018 (OIV 2019). A variety of pests and pathogens cause significant annual losses in vineyards worldwide and grapevine trunk diseases (GTDs) are currently considered one of the main biotic threats limiting the profitable lifetime expectancy of vineyards (Siebert 2001; Bertsch et al. 2013; Kaplan et al. 2016; Gramaje et al. 2018). GTDs represent a complex of diseases with up to 133 xylem-colonizing fungal species from 34 genera contributing to this complex. These diseases are described as slow-progression diseases with symptoms often taking several years to exhibit following infection. The majority of GTDs exhibit generalized symptoms including delayed bud-break, leaf chlorosis, reduced vigor, stunted growth and canker formation (Agustí-Brisach and Armengol 2013; Bertsch et al. 2013; Gramaje et al. 2018; Gramaje and Armengol 2011; Mondello et al. 2018; Úrbez-Torres 2011). Esca, Eutypa dieback and Botryosphaeria dieback are GTDs affecting mature vineyards whilst Petri disease and Black-foot disease primarily affect young vineyards (Gramaje and Armengol 2011; Úrbez-Torres et al. 2008; Agustí-Brisach and Armengol 2013; Bertsch et al. 2013). The economic impact of Botryosphaeria dieback and Eutypa dieback in California was estimated to be $USD260 million annually whilst the incidence of Esca was reported to have reached 80% in regions of Southern Italy (Siebert et al. 2001; Romanazzi et al. 2009). Several taxonomically unrelated Ascomycete fungi represent each of the GTDs, with 26 botryosphaeriaceaous taxa in the genera *Botryosphaeria, Diplodia, Dothriorella, Lapsiodiplodia, Neofusicoccum, Neoscytalidium, Phaeobotryosphaeria, and Spencermartinsia* acting as causal agents of Botryosphaeria dieback for example (Pitt et al. 2013; Úrbez-Torres 2011; Pitt et al. 2015; Yang et al. 2017; Rolshausen et al. 2013). A total of 24 species in the Diatrypaceae

family with the most virulent and common being *Eutypa lata* have been reported to contribute to Eutypa dieback (Pitt et al. 2013; Luque et al. 2014; Trouillas et al. 2010). Esca, which has its own sub-complex itself is primarily caused by *Phaeomoniella chlamydospore, Phaeoacremonium minimum,* other *Phaeoacremonium spp.* and some basidiomycete fungi (Gramaje et al. 2015).

Infection of grapevines by the pathogens of GTDs largely occurs via pruning wounds, though any open wound, natural or manmade including those as a consequence of practices such as de-suckering, trimming and re-training can expose vulnerable vascular tissue to pathogen colonization and spread (Gramaje et al. 2018; Makatini et al. 2014). There are multiple sources of GTDs fungal inoculum including infected pruning debris left on vineyard floors, the surface of infected vines as well as native vegetation that surrounds vineyards (Trouills and Gubler. 2010). Inoculum release and dispersal is facilitated during precipitation events (Úrbez-Torres. 2011; Van Niekerk et al. 2010; Elena et al. 2016). For example, conidia release of *Botryosphaeriaceae spp.* and *P. chlamydospora* has been primarily correlated with rain events (Van Niekerk et al. 2010; Larignon et al. 2000; Eskalen et al. 2001; Úrbez-Torres et al. 2010; Valencia et al. 2015) and *E. lata* perithecia are thought to develop only in areas receiving more than 350 mm of rainfall annually (Carter. 1991). Once airborne, fungal inoculum and on fresh exposed pruning wounds and when optimal air temperatures and moisture are present, they begin to germinate in the xylem vessels and colonize the vine spur, cordon and trunk causing an irreversible loss of function to the xylem and phloem elements ultimately resulting in dieback and wilt symptoms (Mostert et al. 2006; Epstein et al. 2008; Moyo et al. 2014).

Management of GTDs is difficult and influenced by the specific disease and/or pathogens involved and therefore, disease control is primarily focused on preventative methods (Gramaje et al. 2018). Adoption of preventative practices in young, healthy vineyards is strongly recommended for vineyard longevity and this includes recommendations such as late pruning in California, as well as double pruning (Weber et al. 2007) and importantly pruning in dry conditions when the risk of spread of airborne inoculum is low and pruning wounds heal quicker (Hillis et al. 2017; Úrbez-Torres et al. 2010). Another approach is to apply pruning wound protectants which have been shown to confer long-term benefits to vineyards including reducing the incidence of GTDs and reducing the number of vines that need to be replaced and higher marketable fruit (Gispert et al. 2020; Rolshausen et al. 2010). The most effective pruning wound protectants historically have contained chemical active ingredients dating back to the early 1980s, when application of benomyl and thiabendazole to pruning wounds showed protection against *E. lata* (Moller et al. 1980; Rolshausen et al. 2010). However, the intensive utilization of chemical products has led to their persistence in soils worldwide, compromising the fragile microbiota as well as resulting in runoff, polluting water supplies. Their use has raised widespread concern about their possible risks towards consumers, winegrowers, and bystanders, including the operators of the sprayers (Leroux et al. 2007). With the imperative need to make future agricultural practices as sustainable as possible grapevine growers desperately need novel solutions to control GTDs thus yielding high quality grapes that comply with the high standards of food safety. In light of this, the use of biological control agents (BCAs) to protect pruning wounds against fungal pathogens, presents a viable, durable and sustainable alternative and should be considered a research priority (Gramaje et al. 2015). Since 2000, more than 40 BCAs have been identified, characterized and tested against some of the fungal pathogens associated with the Esca complex, Botryosphaeria dieback and

Eutypa dieback. Studies regarding the use of fungal BCAs for pruning wound protection have mainly been focused on *Trichoderma* spp. due to their ability to sense, invade and destroy other fungi via a variety of mechanisms including mycoparasitism, production of secondary metabolites and competition for resources and nutrients (Mukherjee et al. 2013). Isolates of *T. atroviride* have been shown to directly inhibit GTDs pathogens including *Ph. viticola, Pa. chlamydospora, D. seriata* and *L. theobromae* when applied to pruning wounds *in vitro* and to reduce pathogen incidence in pruning wounds *in vivo* (Kotze et al. 2011; Berbegal et al. 2020). Among the bacterial BCAs, *Bacillus subtilis* has been the most promising against GTDs. It's antagonistic *in vitro* activity against GTDs was confirmed as a wound protectant with varying biocontrol efficacy according to both the GTDs and the selected pathogen species including *N. australe, L. theobromae and D. seriata* (Kotze et al. 2011; Schmidt et al. 2001).

Whilst attempts to reduce antagonize GTDs pathogens using BCAs has been considerably successful *in vitro* (Mondello et al. 2018), there are comparatively few studies that investigate their efficacy in field trials under natural conditions and these studies have shown variable results for preventing infection from *Botryosphaeriaceae* spp. and Esca pathogens (Halleen et al. 2010). The main aims of this study were to (i) evaluate the effectiveness of a variety of chemical and biological registered and experimental pruning wound protectants for control of the GTDs *N.* parvum and *E. lata* in greenhouse and field trials, and (ii) to assess the ability of BCAs to colonize pruning wounds for the duration of the growing season.

Materials and Methods

Greenhouse detached cane assay (DCA)

Cane preparation

Following the method by Ayres et al. (2011), in February 2020, nine-year-old dormant grapevine canes cv. Cabernet Sauvignon, were collected from a vineyard in Sacramento County, CA. Canes were cut into ~11 cm long single-node sections with the tip of the cane being about 2 cm above the node and stored at 3-4^{o}C until used. The day of the experiment, a cut was made 1cm above the node to mimic a pruning wound. Cut canes were placed into holes in 1-inch thick polystyrene boards, with the bottom of the cane extending approximately 1cm below the board. The boards with canes were floated on tap water in plastic tubs on benches in a greenhouse at UC Davis and maintained at roughly 25^{0}C. The water was changed weekly throughout the duration of the experiment.

Inoculum preparation

Cankered grapevine wood with pycnidia for *N. parvum* was collected from naturally infected grapevine trunks in a vineyard in Lodi, California in 2019 and confirmed as *N. parvum* via morphological analysis. Pycnidia structures from wood segments were soaked in SDW in a petri dish overnight to allow the release of Pycnidiospores. The final concentration was adjusted to 1.5 x 10^5 conidia/mL using a Fuchs Rosenthal modified haemocytometer. (Neubauer, Weber Scientific International, Middlesex, England). A 0.05% Tween 20 solution (BDH Laboratory Supplies, Poole, Dorset, UK) was added as a surfactant.

E. lata mycelium was grown on APDA plates for 10 days. Mycelium was scraped off and homogenised in sterile distilled water using an OMNI Tissue Master 125 homogenizer. The fragmented mycelial solution was adjusted to a concentration of 1.5 x 10^5/ml using a Fuchs Rosenthal modified haemocytometer.

Experiment design and treatment preparation

This study was performed twice, with a total of 10 canes per treatment organized in a completely randomized block design. There were a total of sixteen treatments including a water treated, inoculated positive control and registered and experimental commercial chemical and biocontrol treatments. Also included were several biocontrol agents (Table 1) that were identified in a previous study (Blundell et al. 2021a). Of these isolates, the fungal isolates UCD 8717 (*Trichoderma hamatum*), UCD 8189 (*Aureobasidium pullulans*), and bacterial isolate UCD 8745 (*Bacillus sp.*) were grown for seven days from a mycelial plug on acidified potato dextrose agar medium (APDA) and subsequently prepared at a concentration of 1.5 x 10^5 spores/ml using a Fuchs Rosenthal modified haemocytometer. The bacterial isolate UCD 8745 was grown for 3 days at 25^{o}C using a rotary shaker by inoculating 800ml of Czapek Dox Broth (CDB) with a streak of UCD 8745 using a sterilized pipette. The bacterial cells were centrifuged at 3500 *g* for 20 min, and mineral oil (Pharmaoil 20, Mat-Chem, Durban, South Africa) added to achieve a cell suspension of 10^8 cells mL^{-1}. The registered and experimental commercial liquid formula treatments (Table 1) were prepared according to label recommendations. All treatments were sprayed with a 1-liter hand-held spray bottle onto the tip of the canes until run off (about 3 full squeezes). Treated canes received either one, two or three inoculations with a 20 µl solution (roughly 2000 conidia or mycelium fragments) of either *E. lata* or *N. parvum*. Canes that were inoculated once received inoculum at 24 hours after treatment application. Canes that were inoculated twice received

inoculum at 24 hours and 1 week after treatment application. Canes that were inoculated thrice received inoculum at 24 hours, 1 week and 2 weeks after treatment application. At each time point, a water treated, inoculated positive control was included. After 4 weeks from the treatment application, all canes were collected and stored at 3-4^{0C} until they were processed.

Evaluation of pruning canes

Four weeks after the initial inoculation event, each cane was split with a knife longitudinally exposing the pith and xylem, and six small tissue slices (three from the pith and three from the margin of the dead wood, or any area exhibiting discoloration) were excised aseptically and cultured on APDA (for the canes that are treated with fungal pathogens) and on PDA (for canes that were treated with biocontrol products that contains beneficial bacteria). After room temperature incubation for 5-14 days, recovery of fungal pathogens was recorded by their morphological characteristics. The recovery of the biological based treatments was also recorded by the morphological characteristics of the biological species.

Data analysis

The efficacy of the treatments controlling the GTDs was calculated as the Mean Percent of Infection (MPI). The following formula was used for the MPI calculation: Number of GTDs infected samples (canes from which the pathogen could be re-isolated)/total number of canes inoculated x 100. The mean percent disease control (MPDC) was calculated on the basis of MPI of the control treatments as (100x(1-(MPI treatment/MPI control))). Means comparisons were made using Dunnett's test α=0.05. All data analysis was performed using JMP software (SAS Institute, Cary, NC).

Field Trials

Trial design

Two independent field trials were set up in Sacramento County, CA (cv Cabernet Sauvignon-wine grape, 9 years old) and in Kern County, CA (cv Allison-table grape, 4 years old) using the treatments listed in table 1. At each field trial, there was a total of four vines per treatment with five spurs used per vine, organized in a completely randomized block design across four rows. Grapevines were trained to bilateral cordons on a horizontally divided trellis with typically eight spurs per cordon (Delano) and 20 spurs per cordon (Elk Grove). All vines were spur pruned (1 foot-long) in February, and within 24 hours of pruning, the liquid treatments were sprayed with a 1-liter hand-held spray bottle on the pruning wound until runoff (about 3 full squeezes). All treatments used in the DCA were included in the field trials as well as a water treated, uninoculated control (Table 1). Treatments and *E. lata* and *N. parvum* inoculum were prepared as described above in the greenhouse DCA. Canes treated with a chemical protectant were inoculated with a 20 µl solution (roughly 2000 spores) of either *E. lata* or *N. parvum*, 24 hours after treatment application. Canes treated with a biological protectant were inoculated with a 20 µl solution (roughly 2000 spores) of either *N. parvum* or *E. lata,* 7 days after treatment application.

Evaluation of treated canes

Eight months after inoculation, treated spurs were cut to about 15 cm in length and brought to the lab for their evaluation. The recovery of pathogen and biological treatment from treated canes was performed as described above in the greenhouse detached cane assay.

Data analysis

Prior to statistical analysis, data was assessed for normality and homogeneity of variances Means comparisons were made using Dunnett's test at $P<0.05$. All data analysis was performed using JMP software (SAS Institute, Cary, NC).

Results

Greenhouse Detached Cane Assay (DCA)

At the end of the 4-week DCA, *E. lata* was recovered from 40% of water treated, inoculated positive control canes that were inoculated at 24 hours, and at 24 hours and 1 week after treatment application (Table 2) whilst the recovery rate dropped to 30% when water treated, inoculated positive control canes were inoculated three times (Table 2). When canes were inoculated with *E. lata* just once, 12 out of 14 treatments provided at least 75% mean percentage disease control (MPDC) (Table 2) whereas treatment efficacy decreased with subsequent inoculation events as 8 out of 14 treatments and 2 out of 14 treatments provided at least 75% MPDC for two and three inoculation events respectively (Table 2). The chemical protectant Fluopyram and Trifloxystrobin and isolate UCD 8717 were the most consistently effective treatments being able to provide at least 75% MPDC of *E. lata* regardless of the number of inoculations (Table 2).

Neofusicoccum parvum was recovered from 60% of water treated, inoculated positive control canes that were inoculated once with *N. parvum* Pycnidiospores (Table 2). *N. parvum* recovery from water treated, inoculated control canes increased to roughly 80% when canes received two and three inoculation events (Table 2). Treatments exhibited considerable variation in efficacy, with *Trichoderma asperellum* and *Trichoderma gamsii*, a blend of crab and lobster shell powder and *Bacillus velezensis* conferring 100% MPDC of *N. parvum* when canes were inoculated once, but when canes were inoculated three times, decreased to 50% and 0% MPDC respectively. The

protectants, *Trichoderma asperellum and Trichoderma gamsii*, Thiophanate-methyl + Myclobutanil and isolate UCD 8717 were the most consistent protectants with 88% MPDC of *N. parvum* when canes were inoculated three times.

Recovery of biological agents from treated canes was highest when the treatment contained a *Trichoderma spp*. regardless of the pathogen or number of inoculation events (Table 3). *Trichoderma atroviride* and *Trichoderma asperellum and Trichoderma gamsii* exhibited 100% genus recovery from treated canes inoculated with *E. lata* across all inoculation events whilst isolates UCD 8717, and *Trichoderma atroviride* exhibited 100% genus recovery from treated canes inoculated with *N. parvum* across all inoculation events (Table 3). The biological treatments *Bacillus subtilis* strain QST 713, *Bacillus velezensis* and UCD 8745 had a considerably lower genus recovery rate regardless of the inoculated pathogen or number of inoculation events (Table 3).

Field trials

In both Sacramento and Kern County, all treatments were able to inhibit *E. lata* and *N. parvum* to some level compared to the water treated-inoculated positive control. Overall in Sacramento County, treatments were generally able to confer a greater wound protection against *N. parvum* than *E. lata* with 13 treatments significantly reducing *N. parvum* recovery versus only 9 treatments capable of significantly reducing *E. lata* recovery ($P < 0.05$) (Table 4). In Sacramento County, the superior protectants were the biological treatments, *Trichoderma asperellum and Trichoderma gamsii* and *Aureobasidium pullulan*s strain DSM14940/14941 both significantly reducing MPI of *E. lata* to roughly 5% ($P < 0.05$) (Table 4) compared to an MPI of 40% for the water treated-inoculated positive control. The superior chemical treatment was Thiophanate-methyl + Myclobutanil, conferring 75% MPDC (Table 4). In Sacramento County, *Trichoderma asperellum*

and Trichoderma gamsii was again the superior protectant against *N. parvum* along with *Trichoderma atroviride, Trichoderma asperellum and Trichoderma gamsii* + a blend of crab and lobster shell powder, *Bacillus velezensis* and Thiophanate-methyl + Myclobutanil with 100%, 93%, 86% and 86% MPDC respectively compared to the water treated-inoculated positive control.

In Kern County, whilst no treatments significantly reduced *E. lata* infection, a total of 8 treatments significantly reduced *N. parvum* colonization compared to the water treated-inoculated control canes ($P < 0.05$), the most effective being *Bacillus subtilis* strain QST 713 (5% MPI) (Table 4). Canes treated with *Trichoderma atroviride, Trichoderma asperellum and Trichoderma gamsii,* and Thiophanate-methyl + Myclobutanil were also very effective protectants resulting in an *N. parvum* MPI of roughly 10% (Table 4). Across both sites, biological treatments containing either *Trichoderma spp*. or *Aureobasidium spp*. had the highest rate of genus recovery, up to 100% regardless of the pathogen, whilst there was consistently low recovery of Bacillus based treatments across both sites (Table 5).

Discussion

Preventative protection of grapevine pruning wounds is currently the only way to limit the infection of grapevine trunk diseases (GTDs) pathogens since there are no mitigation options once infection has occurred. Pruning wound protection has historically been mediated by the application of synthetic chemical but with widespread concerns regarding their possible risks towards consumers, winegrowers, and operators and the imperative need to make future agricultural practices as sustainable as possible, alternative solutions are required to mitigate the effect of grapevine trunk diseases (GTDs) thus yielding high quality grapes that comply with the high standards of food safety. Biological control agents (BCAs) have been demonstrated to have

excellent potential for pruning wound protection against infection from GTDs, with *Trichoderma spp.* and *Bacillus spp.* exhibiting significant antifungal abilities *in vitro* (Blundell et al. 2021a; Mondello et al. 2018; Mutawila et al. 2015; Úrbez-Torres et al. 2020). This study aimed to compare the efficacy of a variety of chemical and biological treatments (registered and experimental) as pruning wound protectants in greenhouse and field trials and evaluate the recovery of BCA microbes.

Our detached cane assays (DCA) showed that a variety of biological and chemical treatments were highly effective in protecting grapevine canes against *E. lata* and *N. parvum* (Fig. 1; 2). Whilst the majority of treatments were less effective as multiple inoculation events were performed (to mimic natural infection in the field) some treatments including isolate *T. hamatum* UCD 8717, and Fluopyram and Trifloxystrobin were able to remain highly effective, both conferring 100% mean percent disease control (MPDC) against *E. lata* after three inoculation events (Table 2). Furthermore, our detached cane assay reports that all *Trichoderma*-based products had a minimum genus recovery rate of 70%, regardless of pathogen or number of inoculation events, many reaching 100% recovery (Table 3). Comparable results were obtained in a similar study performed by John et al. (2004), where autoclaved cane segments were treated with *T. harzianum* AG1 and inoculated with *E. lata* ascospores. *T. harzianum* AG1 had a recovery rate of 90% and significantly reduced the recovery of *E. lata*. When comparing this study to John et al. (2004), we chose not to autoclave our canes as it would eliminate the indigenous microbiome, which may influence the interaction of the treatments and the GTDs and sterilization removes natural host resistance reactions such as lignin accumulation and production of phenolics, thus we attempted to replicate natural conditions as much as possible. Another difference between the studies is the use of *E. lata* ascospores by John et al. (2004), whereas we used mycelium fragments. It would be prudent in

future studies for us to use ascospores to replicate natural infection. Other similar studies support our results including a DCA performed by Ayres et al. (2011) which showed that application of chemical fungicides was effective in reducing infection of pruning wounds by *E. lata*. A recent detached cane assay performed by Úrbez-Torres et al. (2020) revealed that some *Trichoderma* isolates protected pruning wounds for up to 21 days with 100% MPDC against *N.* parvum. A similar single-node plantlet study was performed by Mundy and Robertson (2010) which used pruning paste treatments to determine their efficacy in protecting grapevine canes from *E. lata* and *Neofusicoccum australis* infection. A commercial preparation of *Trichoderma spp*. greatly reduced lesion length of both *E. lata* and *N. australis* compared to the no pruning paste control. Whilst *Trichoderma*-based products generally provided consistently high levels of protection against both pathogens, *Bacillus* and *Aureobasidium* based products exhibited variable protection in our study. For example, *Bacillus velezensis* resulted in an *N. Parvum* mean percent of infection (MPI) of 0% when inoculated once at 24 hrs but only an MPI of 30% and 80% when inoculated two and three times respectively (Table 2). This variable efficacy correlates with low recovery rates of *Bacillus* (Table 3) suggesting that the ability of micro-organisms to colonize pruning wounds long term may be tied to their antagonistic activity GTDs fungal pathogens.

Species belonging to the fungal genus *Trichoderma* have been the most extensively investigated BCAs for pruning wound protection against GTDs (Blundell et al. 2021b; Del Pilar et al. 2020; John et al. 2005; Halleen et al. 2010; Kotze et al. 2011; Mutawila et al. 2015, 2016; Úrbez-Torres et al. 2020). Under field conditions, our results showed that the *Trichoderma asperellum and Trichoderma gamsii* combination was the superior protectant overall, providing a consistently high level of pruning wound protection compared to the water treated, inoculated positive control, with 100% MPDC against *N. parvum* and 88% MPDC against *E. lata* in Sacramento County and 100%

MPDC against *E. lata* in Kern County (Table 4). Whilst the majority of prior pruning wound protection field trials have not occurred in conditions representative of these field trials, results of a field trial in 2019 in Yolo County, California can be comparable (Blundell et al. 2021b), where *Trichoderma asperellum and Trichoderma gamsii* resulted in 92% MPDC of *E. lata,* but only 29% MPDC of *N. parvum*. A similar study by Kotze, (2011) showed that various *Trichoderma* protectants generally reduced the incidence of the inoculated GTDs compared to the untreated controls when given 7 days to colonize the pruning wounds prior to pathogen inoculation.

Trichoderma-based products generally exhibit high efficacy in wound protection against all GTDs, are able to colonize the wood of pruned canes and could be recovered up to eight months under field conditions (Di Marco et al. 2004; Halleen et al. 2010; John et al. 2008; Blundell et al. 2020). Our results support these findings, with at least 70% BCA recovery from canes treated with *Trichoderma atroviride* in Sacramento County (Table 5). Of the BCAs tested in these field trials, generally the *Trichoderma* and *Aureobasidium* based products had a high rate of recovery when compared to *Bacillus* based products, with the highest rate of recovery for canes treated with a *Bacillus* based product being 25% (Table 5). Interestingly, despite *Trichoderma asperellum and Trichoderma gamsii* being the superior protectant, it had a lower recovery rate than other less effective protectants and recovery rate varied between trials (Table 5) suggesting BCA incidence does not always correlate to GTD antagonism. Mutawila et al. (2011, 2016) also demonstrated how *Trichoderma* incidence on different cultivars was highly variable and not related to its biocontrol efficiency when challenged with GTDs. The age of vineyard, cultivar, environmental conditions, time of application and phenological grapevine stages are all likely factors responsible for variation in BCA efficiency and recovery (Elmer and Reglinski 2006; Mondello et al. 2018).

For example, Halleen et al. (2010) reported a range (20-76%) of E. lata *Trichoderma*-based products recovery depending on the year and cultivar.

However, we should be cautious in our conclusions regarding *E. lata* control in Kern county, because despite using *E. lata* inoculum at a higher level than would occur naturally, *E. lata* recovery from water treated, inoculated controls was only 25%. A study by Trouillas and Gubler, (2010) revealed that no *E. lata* perithecia were detected in Kern county nor other counties in the southern portion of the San Joaqin valley. This knowledge combined with the fact that there is a low average yearly rainfall in the southern portion of the San Joaqin Valley suggests the importance of precipitation in *E. lata* incidence and distribution. It will be useful in future studies to perform field trials in counties that are within the natural host range of *E. lata* (Trouillas and Gubler. 2010). Ascospores of *E. lata* are the primary form of inoculum so in future studies it may also be prudent to use *E. lata* ascospores derived from stroma as this method has been shown to be successful in other studies (Ayres et al. 2016; Rolshausen et al. 2010; Sosnowski et al. 2013; Trouillas et al. 2010; Weber et al. 2007) though some studies that used a high ascospore concentration also saw a low *E. lata* recovery rate from controls (Sosnowski et al. 2013). As we are relying on morphological identification for recording pathogen recovery, we may identify higher recovery by performing DNA-based detection methods in subsequent studies (Pouzoulet et al. 2013, 2017; Brown et al. 2021).

Thiophanate-methyl + myclobutanil and Fluopyram + Trifloxystrobin were also effective at providing simultaneous pruning wound protection of *E. lata* and *N. parvum* across both sites (Figures 3 and 4). Other studies have reported similar results (Rolshausen et al. 2010), including a long-term study over six years by Gispert et al. (2020) comparing application of Thiophanate-

methyl + myclobutanil versus no treatment which revealed that its application resulted in a decrease in GTDs incidence and a decrease in number of vine replants. Brown et al. (2021) reported a decrease in recovery of *N. parvum* and *Diaporthe ampelina* in canes treated with Thiophanate-methyl + myclobutanil compared to a water treated, inoculated control.

Trunk disease susceptibility varies from variety to variety, region to region and even vineyard to vineyard. Pruning wound protection is an important preventative strategy for the management of GTD and application of certain treatments can have numerous long-term benefits including a lower incidence of GTDs, less vines that need replanting, and a significant increase in total and marketable yield (Gispert et al. 2020). Considering the ability of *Trichoderma* to colonize pruning wounds and sustain its presence, long term protection of pruning wounds with *Trichoderma*-based products can be an effective, sustainable tool in the management of pruning wound pathogens in conjunction with effective chemical protectants including Fluopyram + Trifloxystrobin and Thiophanate-methyl + myclobutanil.

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Tables and figures

Table 1. A list of all treatments used in greenhouse detached cane assays and field trials in Sacramento County and Kern County to evaluate their ability to protect grapevine pruning wounds from infection of the grapevine trunk diseases, *E. lata and N. parvum* . Asterisks (*) indicate micro-organisms isolated from various grapevine structures and tested for antifungal ability prior to this experiment (Blundell et al. 2021). All other treatments are either registered commercial or have potential for registration as commercial pruning wound protectants. [a]All treatments were used in both the greenhouse and field trials except for the water control-non inoculated which was only included in the field trials. [b] Fungicide group (FRAC) names: triazoles (DMI), pyridinyl-ethyl-benzamides (SDHI), oximino-acetates (QoI), thiophanates (MBC), fungal *Trichoderma* spp. and bacterial *Bacillus* spp. (BM 02), and not classified (NC).

	Treatment or Trade Name	Active Ingredient(s)	Fungicide group (code number)[b]	Manufacturer	Application rate per acre (100gal)
[a]	Water treated - Non inoculated negative control	N/A	N/A	N/A	N/A
	Water treated - Inoculated positive control	N/A	N/A	N/A	N/A
	Terramera (Exp B)	Caprylic acid	NC	Terramera Inc.	2.4 (%v/v)
	Luna Sensation	Fluopyram + Trifloxystrobin	SDHI (7) + QoI (11)	Bayer CropScience	5.0 fl oz
	Topsin M + Rally	Thiophanate-methyl + Myclobutanil	MBC (1) + DMI (3)	United Phosphorous, Inc + DOW AgroSciences LLC	1.25 lbs + 2.25 oz
	BioTam + Crab Life-Powder	Trichoderma asperellum and Trichoderma gamsiis + a blend of crab and lobster shell powder	BM02	Isagro USA + Conchazul de Mexico	2 lb + 0.5 lb
	Crab Life Powder	A blend of crab and lobster shell powder	NC	Conchazul de Mexico	0.5 lb
	Biotam	*Trichoderma asperellum* and *Trichoderma gamsii*	BM 02	Isagro USA	2 lb
	GCM	*Bacillus velezensis*	NC	N/A	Apply fermented product
	Vintec	*Trichoderma atroviride*	BM 02	Bi-PA	2.8 oz
	Serenade	*Bacillus subtilis* strain QST 713	BM 02	Bayer CropScience	2 qt
	Botector	*Aureobasidium pullulans* strain DSM14940/14941	NC	Westbridge Agricultural Products	100 gal
*	UCD 8717	*Trichoderma hamatum*	NC	N/A	1x10 ^{8}/ml
*	UCD 8189	*Aureobasidium pullulans*	NC	N/A	1x10 ^{8}/ml
*	UCD 8745	*Bacillus sp.*	NC	N/A	Apply fermented product

Table 2. Results of greenhouse detached cane assays evaluating treatments control of the grapevine trunk diseases, *E. lata and N. parvum.* Canes were either inoculated at 24 hours, at 24 hours and 1 week, or at 24 hours, 1 week and 2 weeks after treatment application. MPI is mean percent infection (number of canes from which the GTDs could be re-isolated/total number of canes inoculated) x 100. MPDC is mean percent disease control calculated on the basis of MPI of the control treatments as (100x(1-(MPI treatment/MPI control))). Values followed by a different letter were significantly different according to Dunnett's test (p=0.05).

	Inoculum-24 hrs				Inoculum-24 hrs + 1 week				Inoculum-24 hrs + 1 week + 2 week			
	E. lata		*N. parvum*		*E. lata*		*N. parvum*		*E. lata*		*N. parvum*	
Treatment	MPI	MPDC	MPI	MPDC	MPI	MPDC	MPI	MPDC	MPI	MPDC	MPI	MPDC
Water treated-Inoculated positive control	40 a		60 a		40 a		80 a		30 a		80 b	
Caprylic acid	30 a	25	30 a	50	30 a	25	40 abc	50	30 a	0	60 ab	25
Fluopyram + Trifloxystrobin	0 b	100	30 a	50	10 a	75	10 bc	88	0 b	100	40 ab	50
Thiophanate-methyl + Myclobutanil	0 b	100	50 a	17	0 b	100	40 abc	50	10 a	67	10 a	88
Trichoderma asperellum and *Trichoderma gamsii* + a blend of crab and lobster shell powder	0 b	75	0 c	100	0 b	100	30	63	20 a	33	40 ab	50
A blend of crab and lobster shell powder	0 b	100	50 a	17	0 b	100	70 ab	13	10 a	67	40 ab	50
Trichoderma asperellum and *Trichoderma gamsii*	10 a	100	30 a	50	0 b	100	0 c	100	20 a	33	10 a	88
Bacillus velezensis	10 a	75	0 c	100	20 a	50	30 abc	63	20 a	33	80 b	0
Aureobasidium pullulans strain DSM14940/14941	0 b	100	60 a	0	20 a	50	60 abc	25	40 a	0	30 ab	63
Bacillus subtilis strain QST 713	0 b	100	10 b	83	0 b	100	20 abc	75	10 a	67	30 ab	63
Trichoderma atroviride	10 a	75	20 a	67	20 a	50	30 abc	63	10 a	67	30 ab	63
Trichoderma hamatum	0 b	100	30 a	50	10 a	75	10 bc	88	0 b	100	10 a	88
Aureobasidium pullulans	10 a	75	20 a	67	20 a	50	20 abc	75	20 a	33	40 ab	50
Bacillus sp.	0 b	100	10 b	83	10 a	75	40 abc	50	10 a	67	50 ab	38

Table 3. Mean percentage of biological treatment recovery from inoculated canes after 4 weeks from initial treatment application. Canes were either inoculated at 24 hours, at 24 hours and 1 week, or at 24 hours, 1 week and 2 weeks after treatment application. Values represent the average of twenty replicates.

	Recovery %					
	E. lata			*N. parvum*		
Treatment	24 hours	1 week	2 weeks	24 hrs	24 hrs + 1 week	24 hrs + 1 week + 2 weeks
Bacillus subtilis strain QST 713	0	10	0	10	20	50
Bacillus velezensis	30	0	0	70	40	20
Bacillus sp.	30	70	60	70	60	40
Aureobasidum pullulans strain DSM14940/14941	60	40	20	60	10	40
Trichoderma hamatum	70	100	70	100	100	100
Aureobasidum pullulans	80	40	50	80	80	80
Trichoderma atroviride	100	100	100	100	100	100
Trichoderma asperellum and *Trichoderma gamsii* + a blend of crab and lobster shell powder	100	90	80	90	70	90
Trichoderma asperellum and *Trichoderma gamsii*	100	100	100	90	90	80

Table 4. Results of field trials evaluating treatments control of the grapevine trunk diseases, *E. lata and N. parvum* in commercial vineyards in Sacramento and Kern County, 2020. MPI is mean percent infection (number of canes from which the GTDs could be re-isolated/total number of canes inoculated) x 100. MPDC is mean percent disease control calculated on the basis of MPI of the control treatments as (100x(1-(MPI treatment/MPI control))). Values followed by a different letter were significantly different according to Dunnett's test (p=0.05).

	Sacramento County				Kern County			
	E. lata		N. parvum		*E. lata*		N. parvum	
Treatment	MPI	MPDC	MPI	MPDC	MPI	MPDC	MPI	MPDC
Water treated-Inoculated positive control	40 a		70 a		25 a		45 a	
Trichoderma hamatum	35 ab	13	20 bcd	71	5 a	80	30 abcd	33
A blend of crab and lobster shell powder	30 abc	25	25 cd	64	15 a	40	35 abc	22
Caprylic acid	25 abcd	38	50 ab	29	15 a	40	25 abcd	44
Trichoderma asperellum and *Trichoderma gamsii* + a blend of crab and lobster shell powder	25 abcd	38	5 cd	93	10 a	60	30 abcd	33
Bacillus velezensis	20 abcd	50	10 cd	86	5 a	80	15 bcd	67
Fluopyram + Trifloxystrobin	15 bcd	63	25 cd	64	10 a	60	15 bcd	67
Trichoderma atroviride	15 bcd	63	5 cd	93	5 a	60	10 bcd	44
Bacillus subtilis strain QST 713	15 bcd	63	15 bcd	79	10 a	60	5 d	89
Aureobasidium pullulans	15 bcd	63	25 cd	64	10 a	60	40 abc	0
Water treated - Non inoculated negative control	10 cd	75	15 bcd	79	15 a	40	15 bcd	67
Thiophanate-methyl + Myclobutanil	10 cd	75	10 cd	86	5 a	80	10 bcd	78
Bacillus sp.	10 cd	75	25 bc	64	10 a	60	25 abcd	44
Aureobasidium pullulans strain DSM14940/14941	5 d	88	20 bcd	79	20 a	40	15 bcd	67
Trichoderma asperellum and *Trichoderma gamsii*	5 d	88	0 d	100	0 a	100	10 bcd	67

Table 5. Mean percentage of biological treatment recovery from inoculated canes following collection of canes in October 2020. Values represent the average of twenty replicates.

	Recovery %			
	Sacramento County		Kern County	
Treatment	***E. lata***	***N. parvum***	***E. lata***	***N. parvum***
Bacillus velezensis	0	25	25	5
Bacillus subtilis strain QST 713	0	5	0	0
Bacillus sp.	0	5	10	0
Trichoderma hamatum	0	20	20	15
Trichoderma asperellum and *Trichoderma gamsii* + a blend of crab and lonbster shell powder	35	10	30	30
Trichoderma asperellum and *Trichoderma gamsii*	60	45	20	30
Aureobasidum pullulans strain DSM14940/14941	65	100	25	30
Trichoderma atroviride	70	100	45	80
Aureobasidum pullulans	100	100	25	60

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