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

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Summary. Grapevine trunk diseases (GTDs) threaten the economic sustainability of viticulture, causing reductions of yield and quality of grapes. Biological control is 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 grapevine sap, cane and pith tissues, and evaluate their *in vitro* antagonistic activity against selected fungal GTD pathogens. Bacterial and fungal isolates were preliminarily screened in dual culture assays to determine their antifungal activity against *Neofusicoccum parvum* and *Eutypa lata*. Among the fungal isolates, *Trichoderma* spp. inhibited mycelium growth of *E. lata* by up to 64% and of *N. parvum* by up to 73%, with overgrowth and growth cessation being the likely antagonistic mechanisms. Among the bacterial isolates, *Bacillus* spp. inhibited mycelium growth of *E. lata* by up to 20% and of *N. parvum* by up to 40%. Selected antagonistic isolates of *Trichoderma, Bacillus* and *Aureobasidium* spp. were subjected to further dual culture antifungal analyses against *Diplodia seriata* and *Diaporthe ampelina,* with *Trichoderma* isolates consistently causing the greatest inhibition. Volatile organic compound antifungal analyses showed that these *Trichoderma* isolates inhibited mycelium growth of *N. parvum* (20% inhibition), *E. lata* (61% inhibition) and *Dia. ampelina* (71% inhibition). Multilocus sequence analyses revealed that the *Trichoderma* isolates were most closely related to *Trichoderma asperellum* and *Trichoderma hamatum*. This study had identified grapevine sap as a novel source of potential biological control agents for control of GTDs. Further testing will be necessary to fully characterize modes of antagonism of these microorganisms, and assess their efficacy for pruning wound protection *in planta*.

Keywords. Biological control**,** endophytes, microbial antagonism, antifungal.

INTRODUCTION

Fungal diseases are major biotic threats to future economic sustainability of table and wine grape production. Grapevine trunk diseases (GTDs) are prevalent in most viticulture regions causing significant yield and quality

reductions, and increasing crop management costs for cultural and chemical disease management (Siebert *et al.,* 2001; Gubler *et al.,* 2005; Úrbez-Torres *et al.,* 2006; Bertsch *et al.,* 2013; Kaplan *et al.,* 2016). GTDs lead to premature decline and dieback of grapevines and are caused by complexes of several taxonomically unrelated 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 botryosphaeriaceous taxa including *Botryosphaeria, Diplodia, Dothriorella, Lapsiodiplodia, Neofusicoccum, Neoscytalidium, Phaeobotryosphaeria,* and *Spencermartinsia* (Úrbez-Torres. 2011; Pitt *et al.,* 2013; Rolshausen *et al.,* 2013; Pitt *et al.,* 2015; Yang *et al.,* 2017). Another severe GTD is Eutypa dieback, caused by 24 species of Diatrypaceae, with the most virulent and common being *Eutypa lata* (Trouillas *et al.,* 2010; Pitt *et al.,* 2013; Luque *et al.,* 2014; Rolshausen *et al.,* 2014). Esca and Phomopsis dieback also comprise the GTD complex, and are of worldwide economic importance (Munkvold *et al.,* 1994). GTDs can occur simultaneously, 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 development of wedge-shaped cankers in infected grapevine trunks and cordons. From the infection sites, which are often pruning wounds, the fungi will grow downwards occupying vascular elements and adjacent cells. When affected vineyards are no longer economically sustainable, growers face no alternative other than replanting (Gramaje *et al.,* 2018). GTDs can also be found in dormant wood cuttings and young grafted plants, and thus spread to grapevines during plant propagation processes (Waite and Morton, 2007; Aroca *et al.,* 2010; Gramaje and Armengol, 2011; Billones-Baaijens *et al*., 2013).

Management of GTDs is difficult and influenced by the specific disease and/or pathogens involved, but a variety of preventative methods have been studied and implemented. These include cultural practices such as double pruning and application of fungicides (Bertsch *et al.,* 2013). However, these methods have highly variable efficacy, may not be environmentally sustainable, and can be costly (Zanzotto and Morroni, 2016). A promising approach is the use of biological control agents (BCAs) to control pathogens causing GTDs. This utilizes naturally occurring micro-organisms to suppress pests and pathogens (Heimpel and Mills, 2017; Martinez-Diz *et al.,* 2020). Grapevines can be colonized by many micro-organisms that can reside intercellularly or intracellularly as endophytes (West *et al.,* 2010; Gilbert *et al.,* 2014), or they can colonize surfaces of grapevine organs, especially leaves, as epiphytes (Hardoim *et al.,* 2015; Bruisson *et al.,* 2019). Endophytes have been shown to be valuable potential BCAs, as they have been associated with most plant species, and most are non-pathogenic bacteria or fungi that asymptomatically colonize their hosts (Strobel and Daisy. 2003).

Since about 2000, more than 40 BCAs have been isolated, identified and tested against the pathogens responsible for the GTD complex, and while the majority of cultured endophytes do not exhibit inhibitory activity, some *Trichoderma* spp. and *Bacillus* spp. have been highly efficient in protecting pruning wounds against various GTD pathogens *in vitro*, and in greenhouse and field trials (Schmidt *et al.,* 2001; Di Marco *et al.,* 2002; 2004; John *et al.,* 2008; Halleen *et al.,* 2010; Kotze *et al.,* 2011; Rezgui *et al.,* 2016; Mondello *et al.,* 2018; Martinez-Diz *et al.,* 2020) Several successful efforts have also been made to commercialize these organisms 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/or induce systemic resistance (John *et al.,* 2005; Harman, 2006; Mukherjee *et al.,* 2013). *Bacillus* spp. can antagonize GTDs through antibiotic production, competition for nutrients, and/or activation of host defense responses (Choudhary and Johri 2009; Cawoy *et al.,* 2011).

There have been no published reports of evaluation of grapevine sap inhabiting microbes for their antifungal activity against GTD pathogens. The majority of antagonistic endophyte studies in relation to GTDs have sourced microbes from grapevine bark or roots. The present study aimed to exploit this knowledge gap by isolating microbes from grapevine sap, both immediately after making fresh pruning cuts and 7 d later, and evaluating their *in vitro* antagonistic activity against several pathogens responsible for GTDs. Isolations of potential antagonists were also made from grapevine pith and cane tissues.

MATERIALS AND METHODS

Isolation of potential biocontrol organisms from grapevine

All microbial sampling was performed at the Plant Pathology Fieldhouse Facility, University of California, Davis in Yolo County (38°31'24.1"N, 121°45'43.3"W) from (*Vitis vinifera*, Cultivar Chenin Blanc – 10-yrs-old) in March 2019 prior to any standard pruning. A total of ten randomly selected apparently 'healthy' 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 1-year-old lignified spurs were sprayed with 70% ethanol for surface sterilization to avoid contamination, and once dry, a horizontal pruning cut was made in each spur 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 later spread by a sterile glass rod onto each of petri plates containing either potato dextrose agar amended with 100 mg L^{-1} tetracycline (PDA-T) or nutrient agar (NA). Growing fungal or bacterial cultures were subcultured for *in vitro* screening and molecular identifications. Epiphytic microbes were sampled by scraping dry sap from the pruning surfaces 7 d after the initial cut from the same grapevine canes, and the samples were plated as described above. After incubation at 25°C for approx. 7 d, sub-cultures of all growing microbes were made to fresh PDA-T or NA.

Grapevine endophytes were also isolated in September 2019 from the same vineyard, from untreated canes used in a pruning wound protection trial. The canes were each 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. These tissue pieces were then plated on PDA-T and NA plates. The plates were incubated at 25°C for approx. 7 d before subcultures were made of growing isolates.

Extractions of genomic DNA

Genomic DNA was extracted from fungi by scraping mycelium from each 1-week-old isolate subculture, and adding this to a 2 mL capacity tube containing 300 mL of Nuclei Lysis Solution and 1 mm diam. glass beads (bioSpec Products). Mycelium was homogenized for 40 s at 6 m s^{-1} 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 Corporation). Genomic DNA was extracted from each 1-week-old bacterial sub culture by collecting a loop of bacteria with a sterile pipette tip and inoculating a 0.2 mL capacity PCR tube containing 15 mL of Molecular Grade Water culturing for 15 min at 95°C in a thermal cycler.

PCR amplification and sequencing of fungal ITS, TEF-1a and b1-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, puH and rpoB genes

The 16S rRNA gene was amplified using the primers 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).

PCR assays

PCR assays were each carried out in a final volume of 25 mL, in a reaction mixture containing 0 mM Tris-HCl (pH 8.8), 50 mM KCl, 3 mM MgCl2, 0.2 mM of each dNTP, 1.0 mM of each primer and 1 unit of Go Taq polymerase (Promega Corporation). 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 Corporation). Purified PCR samples were sent to Quintarabio, Hayward 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 carried out with Mega X using the maximum composite likelihood model for estimating genetic differences. A phylogenetic tree was obtained using the neighbour-joining method with 1000 bootstrap replicates.

Dual culture assays

All fungal and bacterial isolates were tested in *in vitro* dual culture assays against the GTD pathogens *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 5 mm diam. plug from each isolate culture was then placed 1 cm from the edge of a 100 × 15 mm plate, and a 5 mm diam. plug of 1-week-old *N. parvum* or *E. lata* agar culture was placed 1 cm from the opposite edge of the plate. Plates with only the patho-

gens were used as experimental controls. *Neofusicoccum parvum* assays were incubated at 25°C for 4 d before the percentage of pathogen inhibition was recorded. The *E. lata* assays were incubated at 25°C for 14 d before being recorded. The percentage of inhibition of pathogen mycelium growth was calculated using the formula of Idris *et al.* (2007): % inhibition = [(C-T)/C] × 100), where C is the colony radius (mm) of the pathogen when plated by itself and T is the radius of the pathogen when plated with an isolate. There were a total of ten replicates per isolate in these assays. Representative isolates from each genus exhibiting potential biological control activity against *N. parvum* or *E. lata* were subsequently tested against the GTD pathogens *Diplodia seriata* and *Diaporthe ampelina* using the same assay protocol.

Assays for production of volatile compounds

Production of antifungal volatile organic compounds (VOCs) was assessed using the two-sealed-base-plates method described by Gotor-Vila *et al.* (2017), with modifications. Petri dishes (100 × 15 mm) were each half filled with PDA-T or PDA, and a 5 mm diam. mycelium plug of each 1-week-old isolate were placed in the centre of each base plate. A 5 mm diam. mycelium plug of a pathogen was placed in the centre of another base plate and the two base plates were immediately sealed together using parafilm. Plates with only the pathogen served as experimental controls. *Neofusicoccum parvum* and *D. seriata* assays were incubated at 25°C for 4 d before percentage of pathogen inhibition was recorded, and *E. lata* and *Dia. ampelina* assays were incubated at 25°C for 14 d. The percentage inhibition of pathogen mycelium growth was calculated using the formula of Idris *et al.* (2007) (above). Ten replicates were used for each isolate tested.

Statistical analyses

Data obtained from the dual culture assays were analyzed using one-way ANOVA, and means were separated using the *post hoc* Dunnett's test at $P = 0.05$.

RESULTS

Isolation and ITS/16s sequencing of potential biocontrol organisms

Eleven fungal isolates and two bacterial isolates were cultured on growth media from sampled grapevine tissues (Table 1). The majority of isolates were obtained from either cane tissue or sap collected immediately after pruning cuts were made. Only two isolates were obtained from sap 7 d after pruning, and one isolate was obtained from grapevine pith. PCR amplification of the ITS gene, sequencing and BLAST analyses showed that nine of the fungal isolates were *Aureobasidium* and two were *Trichoderma* (Table 1). PCR amplification of the 16S rRNA, sequencing and BLAST analyses showed that the two bacterial isolates were *Bacillus* genus (Table 1).

Table 1. Sources of isolated microorganisms and their 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 tissue	*Bacillus* (16S)
UCD 8368	Grapevine cane tissue	*Trichoderma* (ITS)
UCD 8717	Grapevine sap, collected after 7 days	*Trichoderma* (ITS)

Preliminary screening, dual culture assays with Neofusicoccum parvum *and* Eutypa lata

The *in vitro* antagonistic potential of all subcultured bacterial and fungal isolates (Table 1) was initially evaluated against the GTDs pathogens *N. parvum* and *E. lata* using dual culture assays. While the majority of isolates did not inhibit mycelium growth of *N. parvum*, two *Bacillus* spp. isolates (UCD 8745 and UCD 8347) and two *Trichoderma* isolates (UCD 8368 and UCD 8717) inhibited growth of this pathogen, by 35% to 64% ($P \leq 0.05$; Figure 1A) compared to the *N. parvum* control. When the isolates were tested for antagonistic potential

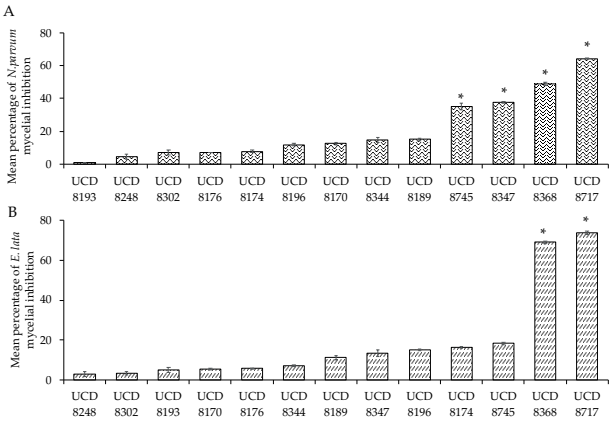

Figure 1. Preliminary *in vitro* dual culture evaluation of isolated microorganisms' ability to inhibit radial mycelium growth of the grapevine trunk disease pathogens (A) *Neofusicoccum parvum* and (B) *Eutypa lata.* Values are means (± standard errors) of ten replicates. * indicates differences compared with experimental controls (Dunnett's test; $P \leq 0.05$).

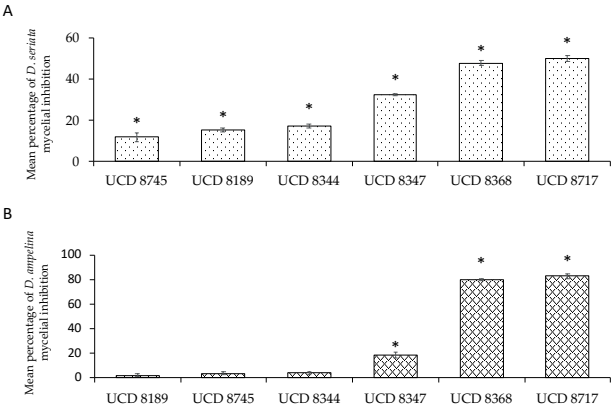

Figure 2. *In vitro* dual culture evaluation of selected microorganisms' ability to inhibit radial mycelial growth of the grapevine trunk disease pathogens (A) *Diplodia seriata* and (B) *Diaporthe ampelina.* Asterisk (*) indicates significant inhibition in comparison with a control (Dunnett's test $P \leq 0.05$).

against *E. lata*, only the *Trichoderma* isolates UCD 8368 and UCD 8717 radial mycelium growth, with both isolated reducing growth by more than 65% ($P \leq 0.05$; Figure. 1B).

Dual culture assays with Diplodia seriata *and* Diaporthe ampelina

The *Trichoderma* isolates UCD 8368 and UCD 8717 and *Bacillus* isolates UCD 8745 and UCD 8347 were further assessed in dual culture assays, as were the *Aureobasidium* isolates, UCD 8189 and UCD 8344, to evaluate these genera for suppression of *D. seriata* and *Dia. ampelina*. All isolates inhibited growth of *D. seriata* by 15% to 50% ($P \leq 0.05$; Figure 2A). Both *Trichoderma* isolates gave the greatest growth inhibition at approx. 50% compared to the controls. There was variation between the *Bacillus* isolates, with UCD 8347 causing approx. 32% inhibition and UCD 8745 causing approx. 11% inhibition. The *Aureobasidium* isolates UCD 8189 and UCD 8344 were similar in their antagonistic activity, causing, respectively, approx. 15% and 17% inhibition. When the isolates were tested against *Dia. ampelina*, the *Trichoderma* isolates UCD 8368 and UCD 8717 caused the greatest inhibition, in excess of 80%. The *Bacillus* isolate UCD 8347 also reduced mycelium radial growth of *Dia. ampelina*, though to a much lesser extent ($P \leq 0.05$; Figures 2B and 3)

Assays for volatile organic compounds

When the isolates were screened against *N. parvum* for antagonistic activity through production of antifungal volatile organic compounds (VOCs), only *Bacillus* isolate UCD 8347 (approx. 10% inhibition) and *Trichoderma* isolate UCD 8368 (approx. 20% inhibition) reduced growth of the pathogen ($P \leq 0.05$; Figure 4A). When the isolates were tested against *E. lata*, all but the *Aureobasidium* isolates reduced radial growth. The *Trichoderma* isolates UCD 8368 and UCD 8717 exhibited the greatest VOC effects, both causing at least 50% growth inhibition, while the *Bacillus* isolates UCD 8745 and UCD 8347 caused, respectively, approx. 37% and 39% inhibition ($P \leq 0.05$; Figure 4B). None of the isolates exhibited any VOC mediated inhibition of *D. seriata* (Figure 7C). However, against *Dia. ampelina*, all isolates gave VOC mediated inhibition, with UCD 8717 causing approx. 70% inhibition. *Trichoderma* isolate, UCD 8368 caused approx. 40% inhibition, while the *Bacillus* isolates UCD 8745 and UCD 8347 and the *Aureobasidium* isolates UCD 8189 and UCD 8344 all caused approx. 20% inhibition ($P \leq 0.05$; Figures 4D and 5).

Multilocus phylogenetic analyses of antagonistic isolates

Multilocus phylogenetic analysis of the *ITS* and *b1-tubulin* genes showed that isolates UCD 8344 and UCD 8189 were most closely related to *Aureobasidium pullulans* (Figure 6). Analysis of the *purH* and *rpoB* genes showed that isolates UCD 8347 and UCD 8745 were most closely related to *Bacillus velezensis* (Figure 7). Analysis of the *ITS* and *TEF-1a* genes showed that

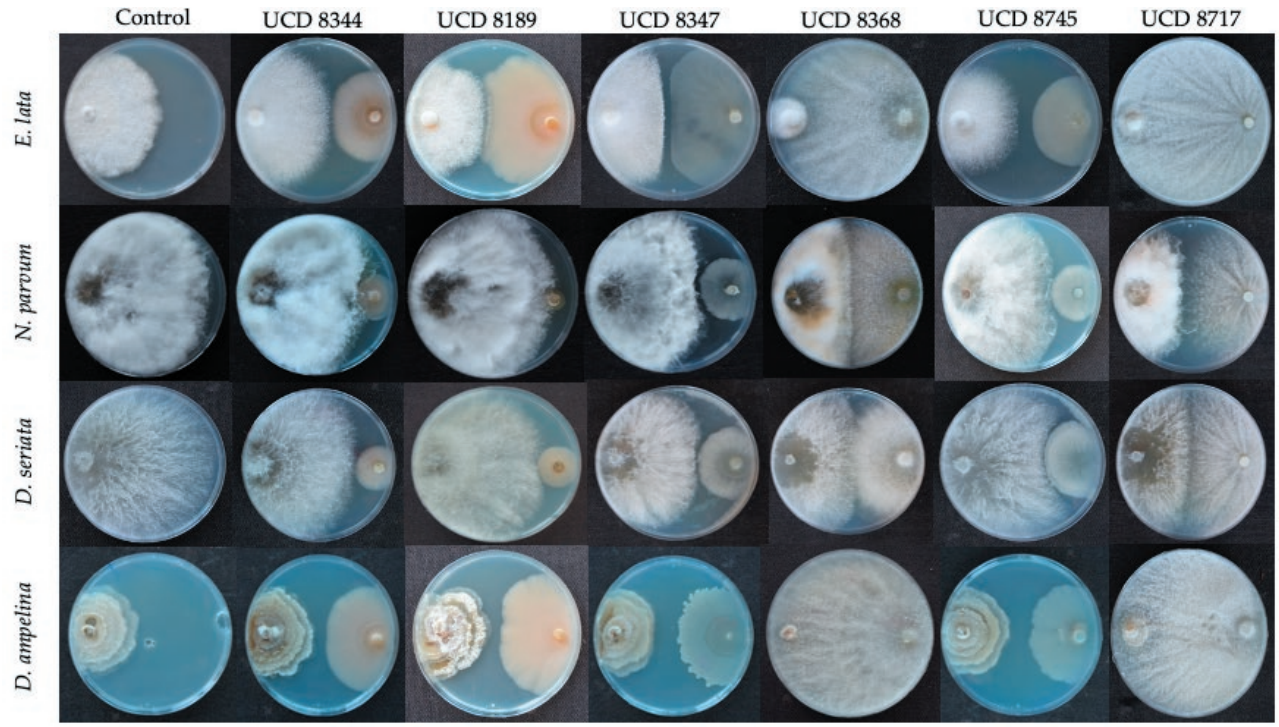

Figure 3. Representative visual summary of *in vitro* dual culture evaluation of selected isolates ability to inhibit radial mycelial growth of selected grapevine trunk disease pathogens.

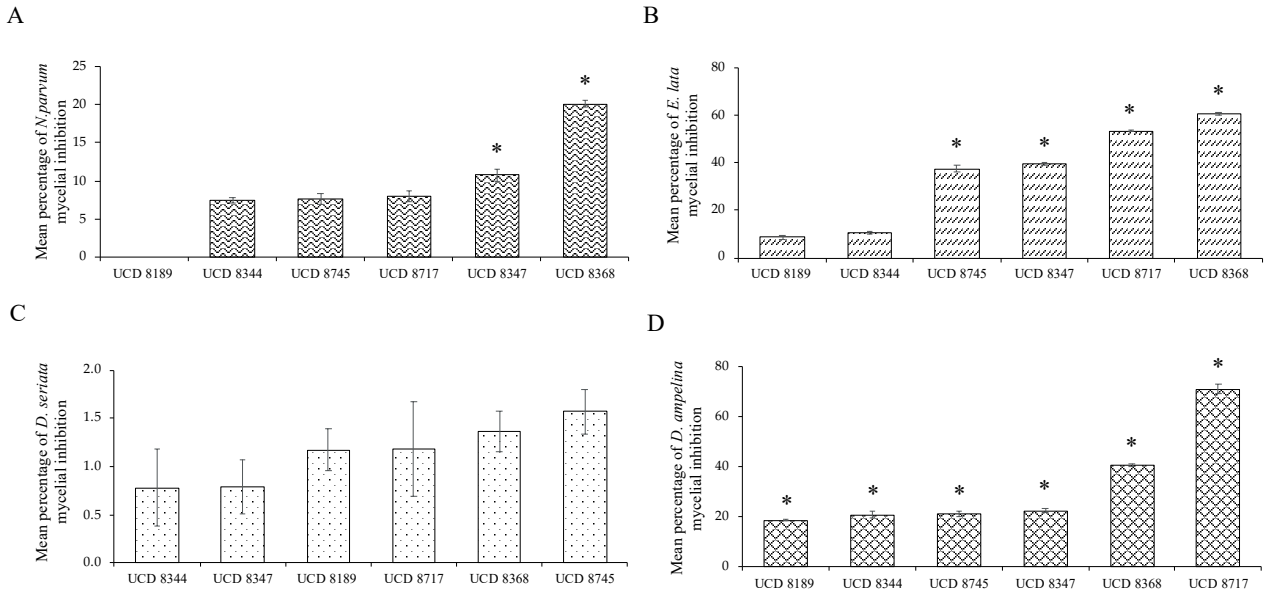

Figure 4. Mean proportions (%) of inhibition of radial mycelium growth of the grapevine trunk disease pathogens (A) *Neofusicoccum parvum,* (B) *Eutypa lata*, (C) *Diplodia seriata* and (D) *Diaporthe ampelina* in assays for volatile organic compound using the sealed-base-plates method with modifications. Values are means (± standard errors) of ten replicates. * indicates inhibition in comparison with controls (Dunnett's test; $P \leq 0.05$).

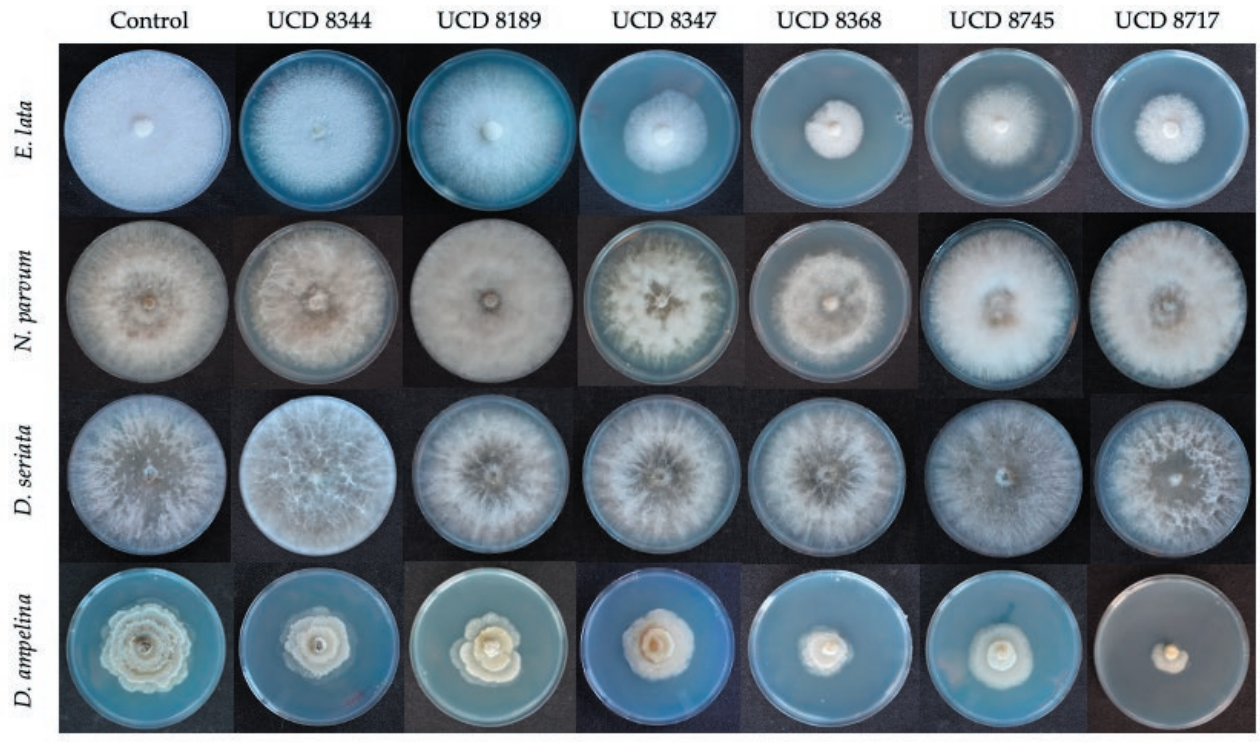

Figure 5. Representative summary of *in vitro* volatile evaluation of abilities of selected microorganisms (columns of culture) to inhibit radial mycelium growth of four grapevine trunk disease pathogens (rows of cultures).

isolates UCD 8368 was most closely related to *Trichoderma asperellum*, and UCD 8717 to *Trichoderma hamatum* (Figure 8).

DISCUSSION

Grapevine pruning wound protection has historically been mediated using synthetic chemicals which have dominated the crop protection industry since the 1980s. However, sustainability of crop production requires a shift towards low pesticide strategies, so there is increasing interest in novel solutions to prevent and control GTDs (Mondello *et al*., 2018). Biological control agents including *Trichoderma* spp. and *Bacillus* spp. have been shown *in vitro* to have potential for pruning wound protection against infections from GTDs (Schmidt *et al.,* 2001; Di Marco *et al.,* 2002; 2004; John *et al*., 2008; Halleen *et al.,* 2010; Kotze *et al.,* 2011; Rezgui *et al.,* 2016). However, microbial inhabitants of nutrient rich grapevine sap have not been previously evaluated for BCA ability against GTDs, so along with microbes isolated from grapevine pith and cane tissues, the present study evaluated microbes for *in vitro* activity against the GTD pathogens *N. parvum*, *E. lata*, *D. seriata* and *Dia. ampelina.*

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 mycelium growth inhibition against all the tested pathogens in dual culture assays, exhibiting at least 75% inhibition against the slow growing pathogens, *E. lata* and *Dia. ampelina* (Figures 1B and 2B). Isolate UCD 8368, which is closely related to *T. harzianum* (Figure 8) was also shown to similarly reduce *in vitro* growth of *E. lata* (Úrbez-Torres e*t al.,* 2020). Whilst *Trichoderma* spp. possess various antifungal mechanisms, this mycelium inhibition was likely attributed to overgrowth (Kotze e*t al.,* 2011), as the assessed isolates grew more rapidly and surrounded the pathogens in dual cultures (Figure 3). These results are similar to those from other studies, where *Trichoderma* spp. have been subjected to dual culture assays against *N. parvum, D. seriata* and *E. lata* (Mutawila e*t al.,* 2015; Silva-Valderrama e*t al.,* 2020; Úrbez-Torres et *al., 2*020). For example, *Trichoderma i*solates from Southern Italy inhibited N. *parvum r*adial growth by up to 74% (Úrbez-Torres et *al.,* 2020). It is hypothesized that this observed overgrowth by *Trichoderma* spp. *t*ranslates to competition for space and nutrients in

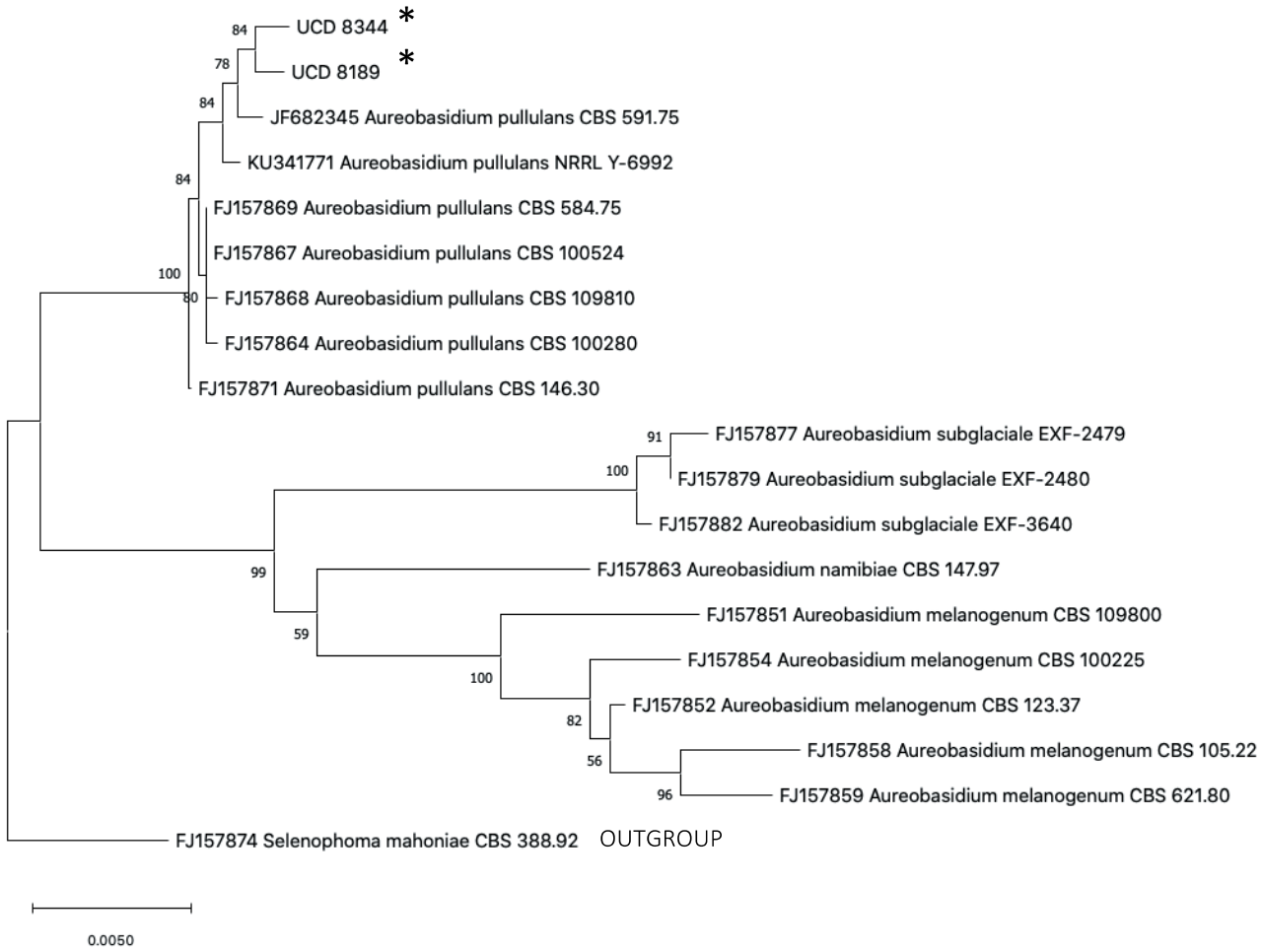

Figure 6. Maximum parsimony phylogenetic tree for isolates UCD 8344 and UCD 8189 based on a multigene data set of internal transcribed spacer rDNA (ITS) and b1-tubulin. Bootstrap support for the maximum-likelihood analysis is given at each node (1000 replicates). * indicates isolates evaluated in the present study. *Selenopoma mahoniae* FJ150872 was used as an outgroup.

grapevine pruning wounds as a mechanism to protect against GTDs (Úrbez-Torres et a*l., 202*0).

In the volatile assay, isolates UCD 8368 and UCD 8717 were still able to inhibit *E. lata* and *Dia. ampelina* (Figure 4B and D), which was probably due to the ability of *Trichoderma* spp. to produce volatile and non-volatile substances which have been shown to inhibit a range of fungi (Chambers and Scott, 1995; John *et al.,* 2004; Kexiang *et al*., 2002; Kucuk and Kivanc, 2004). John *et al.* (2004) showed that volatile compounds synthesized by *T. harzianum* AG1, AG2, and AG3 were inhibited growth of *E. lata*, and growth was completely inhibited by non-volatile compounds. In the present study, isolates UCD-8368 and UCD 8717 elicited a coconut odour which has previously been characterized as 6-n-pentyl-2H- pyran-2-one (Claydon *et al*., 1987), and reported to inhibit fungi including *Rhizoctonia solani*. The inhibition of *N. parvum* and *D. seriata* mycelium growth by isolates UCD 8368 and UCD 8717 in dual culture assays can likely be attributed to growth cessation, when microorganism and pathogens grow until they came in contact with one another and growth of both organisms ceases (Kotze *et al.,* 2011) (Figures 1A, 2A and 3). This mechanism as the primary method of inhibition was also indicated by the volatile assays because there was no inhibition of *N. parvum* and *D. seriata* by isolates UCD 8368 and UCD 8717 (Figures 4A, 4C and 5). The mycoparasitic reactions such as hyphal coiling, adhesion and penetration (Dos Reis Almeida *et al.,* 2007) have been shown to coincide with physical contact interactions; overgrowth and ceased growth. With isolate UCD 8717 being isolated from grapevine sap, this is the first report of a grapevine sap inhabiting microbe showing promising BCA *in vitro* activity against GTDs. Deyett and Rolshausen (2019)

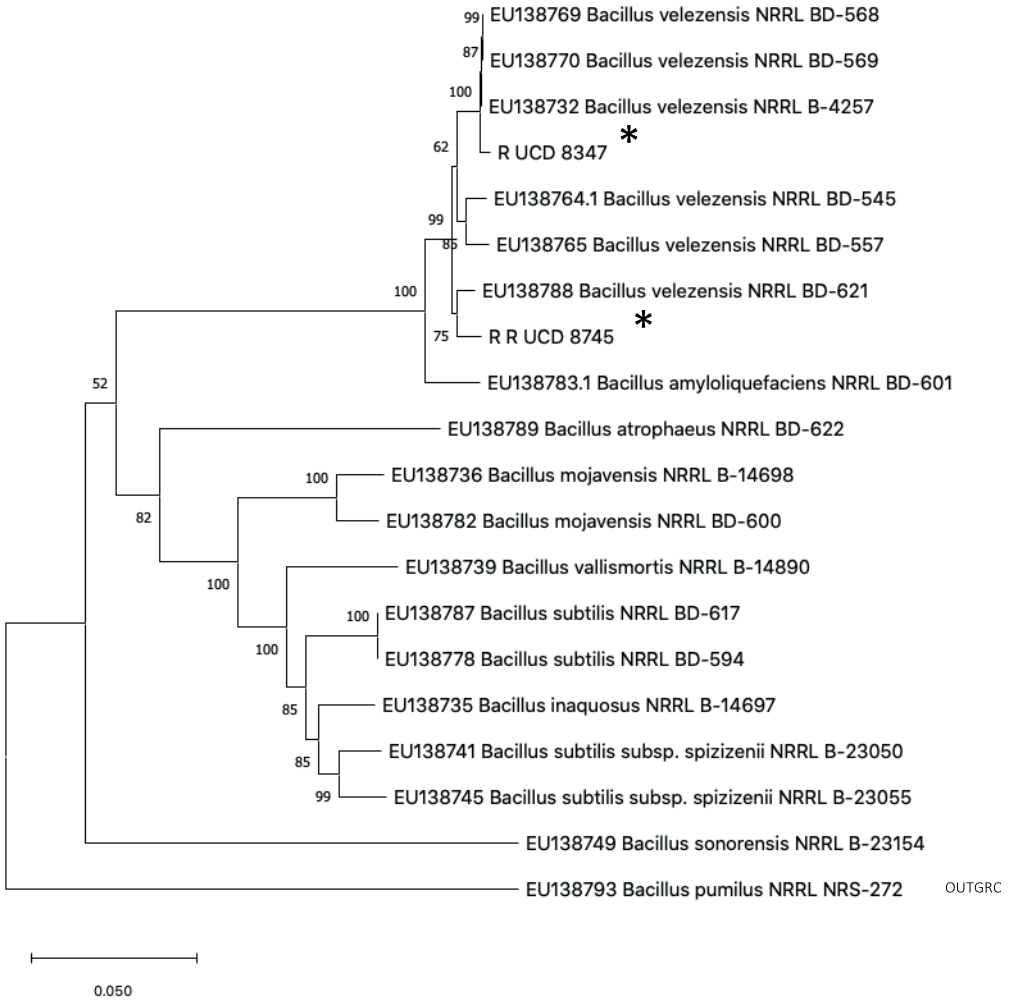

Figure 7. Maximum parsimony phylogenetic tree for isolates of UCD 8347 and UCD 8745 based on a multigene data set of purine biosynthesis (*purH*) and RNA polymerase subunit B (*rpoB*). Bootstrap support for the maximum-likelihood analysis is given at each node (1000 replicates). * indicates isolates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

utilized a culture-independent amplicon metagenomic approach to characterize the major bacterial and fungal taxa that comprise grapevine xylem sap microbial communities. They showed 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 these nutrients are remobilized to the vegetative parts of grapevines following winter dormancy, providing conducive environments to harbour beneficial microbes (Deyett and Rolshausen, 2019).

The bacterial isolates (*Bacillus* spp.) UCD 8347 and UCD 8745 exhibited varying antifungal ability and mechanisms in this study, depending on the GTD fungal pathogen. In the dual culture assays between UCD 8347 and *E. lata*, zones of inhibition were observed (Figure 3). Inhibition zones are most likely indicative of antibiotic production (Kotze, 2004) as a mechanism of mycoparasitism. Ferreira *et al*., (1991) identified at least two *Bacillus* isolates that produced antibiotic substances responsible for the inhibition of mycelium growth and ascospore germination. Kotze (2011) dual incubated (*in vitro*) *E. lata* with the same isolate and showed that the pathogen displayed little mycelium growth and clear inhibition zones between the cultures. Malformation of the hyphae, specifically swelling, was observed. Kotze (2011) showed that a *Bacillus subtilis* isolate gave clear zones of inhibition against *Phomopsis viticola.* In an assay for volatiles, isolate UCD 8347 inhibited *E. lata*, indicating that the antibiotic substance may have been volatile. Isolate UCD 8347 also gave small zones of inhibition against *N. parvum* in the dual culture assays (Figure 3), and slightly inhibited (by 10%) growth of *N. parvum*.in the assay for

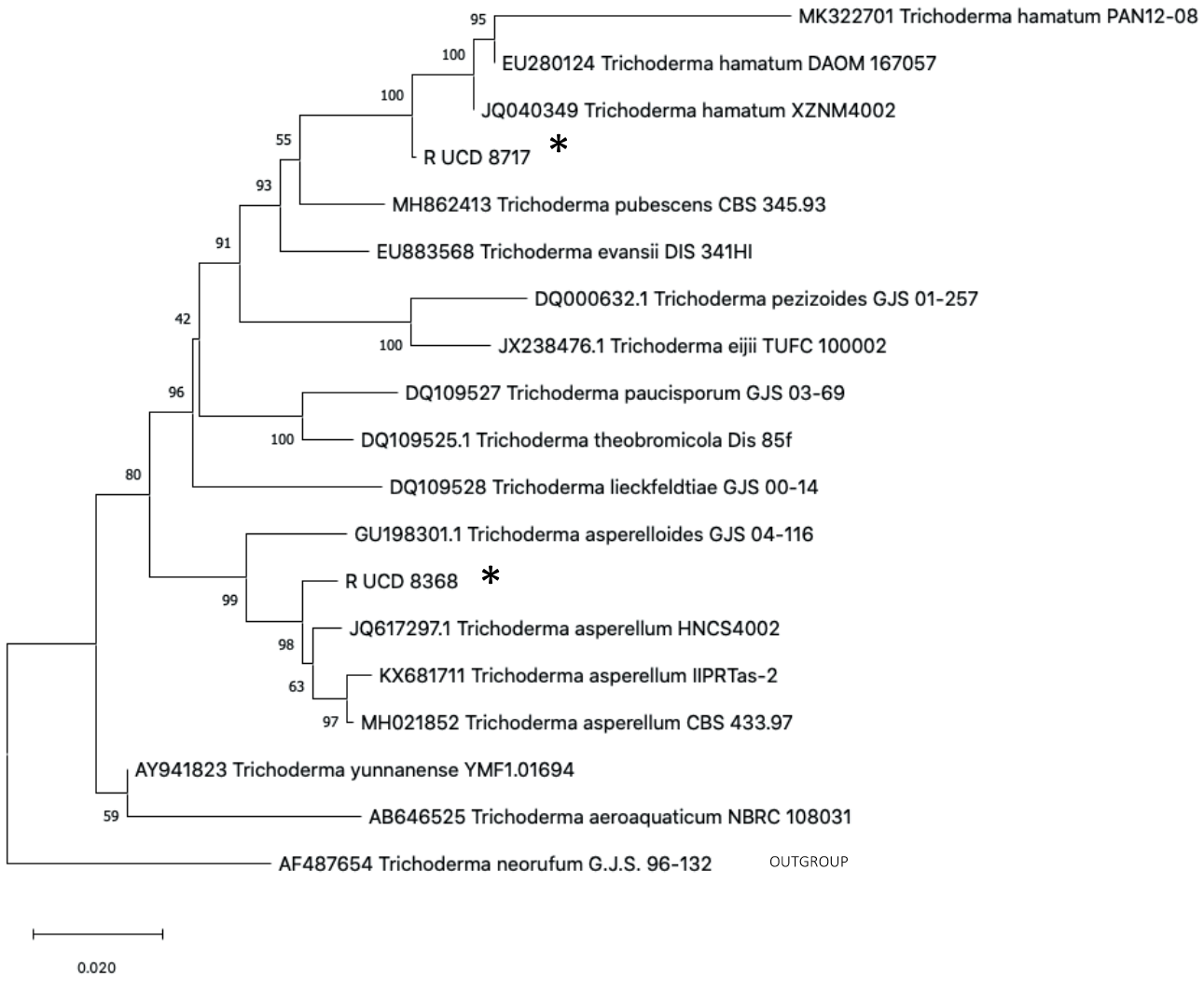

Figure 8. Maximum parsimony phylogenetic tree for isolates UCD 8368 and UCD 8717 based on a multigene alignment of the *Trichoderma* Hamatum/Asperellum clade using internal transcribed spacer rDNA (ITS), and translation elongation factor 1-alpha (TEF1). Bootstrap support for the maximum-likelihood analysis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

volatiles, indicating the antibiotic substance may be a volatile product (Figure 4A). Isolate UCD 8347 also gave inhibition of *D. seriata* and *Dia. ampelina* in the dual culture assay (Figure 2A and B), and of *Dia. ampelina* in the volatile assays (Figure 4D) but the mechanism of inhibition was unclear. Isolate UCD 8745 gave similar results to isolate UCD 8347, although with less inhibition in some assays, and the mechanism of inhibition is not as clear. Subsequent studies should investigate the VOC profiles of these isolates.

Studies of grapevine microbiomes have shown that *A. pullulans* is commonly distributed in grapevines, 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), so *A. pullulans* has promise as a potential BCA. In the present study, the *Aureobasidium* isolates UCD 8344 and UCD 8189 showed no antagonistic ability against *N. parvum*, *E. lata* or *Dia. ampelina* in dual culture assays, but these isolates inhibited mycelium growth of *D. seriata* in dual cultures (Figure 2A). This was probably due to ceased growth as these two isolates did not inhibit *D. seriata* in the VOC assays (Figure 4C). Similar results were obtained by Pinto *et al.* (2018), where *A. pullulans* strain Fito_F278 reduced mycelium growth of *D. seriata* F98.1 in a dual culture assay, and was postulated cause ceased growth of the pathogen.

Although different types of microorganisms were tested in the present study, only *Trichoderma* spp. have

been shown to be the most suitable agents for biological control of GTDs. This probably stems from the synergistic actions of different *Trichoderma* spp. biocontrol mechanisms, their ecological characteristics (saprotrophic, endophytic), and their positive effects induced on their host plants. Grapevines accommodate large pools of resident microorganisms embedded in complex micro-ecosystems (Pinto and Gomes, 2016), so further attempts should be made to identify novel strains of *Trichoderma* and other microorganisms to promote advances in GTD management.

With the need to make agricultural practices as sustainable as possible, novel solutions for GTD management are required, so that high quality grapes are produced that comply with the high standards of food safety. While *in vitro* BCA efficacy does not always translate to efficacy *in planta*, these microbes are the most promising, sustainable option for grapevine growers, because of restrictions and concerns with using chemical fungicides for disease control. The present study has identified potential BCAs with potential for simultaneous control of economically important pathogens responsible for GTDs, and has indicated that further studies to characterize BCA modes of antagonism and evaluate their efficacy in field trials. These potential BCAs may provide long lasting protection of grapevines against GTDs because they share the same host as the important GTD pathogens.

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