Phytopathologia Mediterranea
The international journal of the
Mediterranean Phytopathological Union

Firenze University Press
www.fupress.com/pm

**Citation:** E. Stempien, R.J.G. Pierron, I. Adendorff, W. J. Van Jaarsveld, F. Halleen, L. Mostert (2020) Host defence activation and root colonization of grapevine rootstocks by the biological control fungus *Trichoderma atroviride*. *Phytopathologia Mediterranea* 59(3): 615-626. doi: 10.14601/Phyto-11137

**Accepted:** October 20, 2020

**Published:** December 30, 2020

**Copyright:** © 2020 E. Stempien, R.J.G. Pierron, I. Adendorff, W. J. Van Jaarsveld, F. Halleen, L. Mostert. This is an open access, peer-reviewed article published by Firenze University Press (http://www.fupress.com/pm) and distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** All relevant data are within the paper and its Supporting Information files.

**Competing Interests:** The Author(s) declare(s) no conflict of interest.

**Editor:** Fontaine F., Université de Reims Champagne-Ardenne, Reims, France.

Short Notes

# Host defence activation and root colonization of grapevine rootstocks by the biological control fungus *Trichoderma atroviride*

Elodie STEMPIEN[1], Romain Jean Gaston PIERRON[1,2], Ilka ADENDORFF[1], Wynand Jacobus VAN JAARSVELD[1], Francois HALLEEN[1,3], Lizel MOSTERT[1,*]

[1] *Department of Plant Pathology, University of Stellenbosch, Private Bag X1, Matieland, 7602, South Africa*
[2] *Laboratoire Vigne, Biotechnologies et Environnement (LVBE, EA3991), Université de Haute-Alsace, Colmar, France*
[3] *Plant Protection Division, ARC Infruitec-Nietvoorbij, Private Bag X5026, Stellenbosch, 7599, South Africa*
*Corresponding author. E-mail: lmost@sun.ac.za

**Summary.** Several *Trichoderma* species can act as biocontrol agents and hold the potential to control soilborne diseases through different modes of action. Little is known about the colonization pattern of *Trichoderma atroviride* in grapevine roots and activation of induced systemic resistance *in planta*. A laboratory model was developed to assess root colonization and its impact on grapevine defence activation. Rootstock cuttings from 1-year-old dormant canes were inoculated with conidium suspensions of *T. atroviride* T-77 or *T. atroviride* USPP T1, and host and inoculum colonisation were assessed after 21 d. The two strains of *T. atroviride* were re-isolated from the treated plants (from 70% of the roots and 20% of crowns). Colonization rates did not depend on the *Trichoderma* strain or rootstock cultivar. However, up-regulation of targeted defence genes was dependent on the inoculated *Trichoderma* strain and rootstock cultivar. Furthermore, in leaves of rootstock cultivars 'US 8-7' and 'Paulsen 1103', genes were up-regulated which encode for PR proteins involved in plant defence or production of stilbenic phytoalexins. *Trichoderma atroviride* T-77 was transformed with tdTomato fluorescent protein to allow visualization by confocal laser scanning microscopy. These results give new insights into the mechanisms of grapevine-*Trichoderma* interactions, and allow detection of establishment of potential biocontrol agents within host tissues.

**Keywords.** *Vitis* spp., *Trichoderma* spp., defence response.

## INTRODUCTION

The worldwide drive for environmentally sustainable practices has influenced methods used to control plant diseases. Disease management needs to decrease reliance on synthetic chemicals and increase use of biological control

agents (BCAs) (Edreva, 2004; Woo *et al.*, 2014). *Trichoderma* species are widely used and well-known BCAs. Sixty percent of fungal available biocontrol products contain *Trichoderma* species (Verma *et al.*, 2007), as individual species or species mixtures (Harman *et al.*, 2004).

*Trichoderma* species are saprophytic fungi commonly found in soil, while some can also be endophytes in plants. Endophytes can colonize healthy plant tissues without causing host symptoms or losses, and have minimal environmental impacts. The mechanisms of action of *Trichoderma* spp. as BCAs include mycoparasitism (Harman *et al.*, 2004), secretion of mycolytic enzymes (Reino *et al.*, 2007), competition for limiting resources (Harman *et al.*, 1993; Haran *et al.*, 1995; Howell, 2006) and/or production of antibiotic metabolites (Harman and Kubicek, 2002; Vinale *et al.*, 2006; Mutawila *et al.*, 2016b). *Trichoderma* species also have positive effects on their hosts, including growth enhancement and resistance activation (Harman, 2006; Vinale *et al.*, 2008a; Gallou *et al.*, 2009; Parrilli *et al.*, 2019).

Activation of host defence genes by *Trichoderma* is an important component of biological control. As *Trichoderma* colonizes plants, endogenous compounds are released that lead to the recognition by the hosts and triggering of host defence reactions (Lorito *et al.*, 1994; Viterbo and Chet, 2006; Woo *et al.*, 2006; Brotman *et al.*, 2008; Vinale *et al.*, 2008b; Morán-Diez *et al.*, 2009; Hermosa *et al.*, 2013; Salas-Marina *et al.*, 2015; Contreras-Cornejo *et al.*, 2016; Guzmán-Guzmán *et al.*, 2017; Mendoza-Mendoza *et al.*, 2018; Nogueira-Lopez *et al.*, 2018). The different *Trichoderma* antagonisms, mechanisms and soil habitats make these fungi ideal candidates in controlling soilborne diseases.

Blackfoot disease (BFD) of grapevines is soilborne, and affects nursery grapevine plants and vines in newly established vineyards, causing economic losses in most grapevine industries (Halleen *et al.*, 2004; 2006a; Gramaje and Armengol, 2011; Probst *et al.*, 2012; Úrbez-Torres *et al.*, 2014). Management of BFD relies on integrated programmes that include the use of hot water treatments (HWT), cultural practices and BCAs (Gramaje *et al.*, 2010; Úrbez-Torres *et al.*, 2014; Halleen and Fourie, 2016). Application of *Trichoderma* for control of BFD has given variable results (Fourie *et al.*, 2001; Fourie and Halleen, 2006; Halleen *et al.*, 2007; dos Santos *et al.*, 2016; Halleen and Fourie, 2016; Berlanas *et al.*, 2018; Gramaje *et al.*, 2018). Improved understanding of host colonization and resistance activation by *Trichoderma* would aid utilisation of this biocontrol fungus for BFD control.

Grapevine rootstocks usually have American *Vitis* spp. origins, and include *V. riparia*, *V. berlandieri*, *V. champinii*, *V. aestivalis*, *V. riparia* or (sometimes) *Muscadinia rotundifolia*. This makes study of rootstock disease resistance difficult, because plant genomes could differ dependent on the rootstock cultivar. To date, molecular protocols developed for the study of *Vitis* resistance are rarely optimized for rootstocks.

*Trichoderma* species can activate either systemic acquired resistance (SAR) and/or induced systemic resistance (ISR) in host plants (Segarra *et al.*, 2007; Shoresh *et al.*, 2010; Rubio *et al.*, 2014; Martínez-Medina *et al.*, 2017; Manganiello *et al.*, 2018). ISR activated by *Trichoderma* species induces a state of priming by increasing the plant immune activation, leading to rapid and effective defence responses against pathogens in distal plant parts (Segarra *et al.*, 2009; Lorito *et al.*, 2010; Pieterse *et al.*, 2014; Conrath *et al.*, 2015; Martínez-Medina *et al.*, 2017). Mutawila *et al.* (2016a) showed that *Trichoderma* species triggered the activation of defence genes in an artificial system of grapevine cells. Di Marco and Osti (2007) showed that systemic activation of host resistance by *Trichoderma* root treatments of nursery vines reduced necrotic areas of *Botrytis cinerea*-inoculated grapevine leaves. Nevertheless, plant reactions when colonized by *Trichoderma*, at molecular and biochemical levels, are still not well understood (Contreras-Cornejo *et al.*, 2016; Guzmán-Guzmán *et al.*, 2017).

Endophytic growth and activation of grapevine rootstock defence genes by *Trichoderma* is not known, and these would aid understanding of the efficacy of this biocontrol fungus. Knowledge of root colonization and defence activation is an essential first step in evaluating potential use of *Trichoderma* spp. as BCAs for BFD or other diseases in nurseries and new vineyards. The aim of the present study was to increase understanding of the internal establishment of *T. atroviride* and activation of grapevine defence genes in different rootstock cultivars. The objectives were: i) to investigate the colonization by *T. atroviride* of different rootstock cultivars; and ii) to assess activation of host defence genes during colonization. This knowledge will help to ensure potential BCAs are well-established within host plants to prevent pathogen infections.

## MATERIALS AND METHODS

### *Evaluation of* Trichoderma atroviride *colonization of rooted rootstock plants by re-isolation*

#### Fungal isolates

*Trichoderma atroviride* isolate T-77 and *T. atroviride* isolate USPP T1 (Department of Plant Pathology, Stellenbosch University) were grown on Potato Dextrose Agar (PDA, 39 g $L^{-1}$) and subcultured every 21 d. *Tricho-*

*derma atroviride* T-77 is a commercial inoculum that was originally isolated from grapevine roots and is the component of Eco77® (Plant Health Products). Isolate USPP T1 originated from grapevine shoots.

## Plant material

One-year-old dormant canes of the grapevine rootstock cultivars 'Richter 110' (*V. berlandieri* × *V. rupestris*), 'US 8-7' (Jacquez: *V. aesativalis* × *V. cinerea* × *V. vinifera* and 'Richter 99') and 'Paulsen 1103' (*V. berlandieri* × *V. rupestris*) were collected from a nursery mother block near Wellington, South Africa. The cuttings were disinfected by soaking a didecyldimethylammonium chloride compound (Sporekill®) for 1 h followed by hot water treatment for 45 min at 50°C. The basal end of each two-bud cutting was dipped in 4-indole-3-butyric acid powder and rooted in a perlite-filled mist bed maintained at 26°C under daylight conditions. The cuttings were watered for 10 s every 15 min for the first 5 weeks, then reduced to 2 min twice each day for 1 week. The rooted cuttings were then transplanted into perlite-containing cups after 6 weeks. Plants were then maintained for 1 week, receiving water *ad libitum* to avoid potting stress, before application of experimental treatments.

## *Colonization by* Trichoderma atroviride

### Rootstocks inoculation

Plants of 'Richter 110', 'US 8-7' and 'Paulsen 1103' were inoculated by drenching with conidium suspensions of either *T. atroviride* T-77 or *T. atroviride* USPP T1 (final concentration of $1 \times 10^6$ conidia $mL^{-1}$) into the perlite. Inoculum was freshly prepared on the day of inoculation. *Trichoderma atroviride* cultures were grown on PDA plates for 2 weeks before covering each plate with 5 mL of tap water and scraping the culture to collect conidia. The resulting conidium suspension was filtered through a double layer of sterile cheesecloth and adjusted to the required concentration after counting with haemocytometer. Seventy-five plants were used per treatment, and a total of 225 plants were inoculated. For each trial, each plant was inoculated with 100 mL of conidium suspension or 100 mL of sterile water for the control plants.

### Re-isolation of *Trichoderma atroviride*

Roots were harvested for re-isolations 21 d after inoculation. The roots were then rinsed with water and surface sterilized (30 s in 70% ethanol, 1 min in 3.5% sodium hypochlorite and 30 s in 70% ethanol), to ensure that re-isolated fungi originated from the inner root tissues of the plant and not from rhizospheres. Four pieces of roots were plated onto each Petri plate containing PDA amended with streptomycin (40 mg.$L^{-}$1; PDA+) and two plates were used per plant. Four small sections from the internal tissue of the crown were plated onto one PDA+ plate per plant. The plates were then incubated at room temperature in day light conditions. Fungal growth from roots of each rootstock was determined as proportion (%) of the isolated segments colonized. Infection by *Trichoderma* isolates was scored based on the number of root pieces or crowns colonized on the plates.

## *Evaluation of* Trichoderma atroviride *colonization of rooted rootstock plants using an Agrobacterium-transformed* T. atroviride *isolate*

### *Agrobacterium* transformation of *Trichoderma atroviride* with tdTomato fluorescent protein

*Agrobacterium tumefaciens* AGL-1 was used as host for plasmid pBHt2-tdTom (Caasi et *al*., 2010). This strain contains a gene coding for a tdTomato (orange-red) fluorescent protein, under control of the *Pyrenophora tritici-repentis* toxA promoter, the hygromycin phosphotransferase hph for selection of fungal transformants, aminoglycoside phosphotransferase, and the kanamycin resistance marker for selection of bacterial transformants. Transformation was based on an optimized protocol from Gorfer *et al.* (2007). Briefly, the *Agrobacterium* vector was induced in a minimal medium (AtIND) containing acetosyringone (3', 5'-dimethoxy-4'-hydroxy-acetophenone 200 μM; AS) and kanamycin (50 mg $mL^{-1}$). *Trichoderma atroviride* T-77 conidia were inoculated into the AtIND medium, after induction of the *Agrobacterium*. After 2 d , 200 μL of medium containing *Agrobacterium* and *T. atroviride* T-77 were plated on a thin layer of MoserIND medium. Once the fungal thalli developed enough (2–3 d), they were overlayed with a layer of Moser medium supplemented with 200 mg $L^{-1}$ of hygromycin B. Transformants that crossed the selection layer were plated onto selective PDA and checked for fluorescence. Single conidium isolations were then repeated three times onto PDA plates supplemented with hygromycin B (200 μg $L^{-1}$) to avoid chimeras. The transformation of *T. atroviride* T-77 was confirmed by confocal laser microscopy.

### Confocal laser microscopy

Confocal laser microscopy was performed at the Confocal & Light Microscope Imaging Facility, UCT (University of Cape Town), using a LSM880 Airyscan (Carl Zeiss) and a Fast Airyscan module confocal. The EC "Plan-Neofluar"20×/0.5 M27 objective was used for low magnification at a zoom factor of 1. The objective used for high magnification was LCI "Plan-Apochromat"63×/1.4 Oil DIC M27 with a zoom factor of 1. A 561 nm solid-state red laser was used with emission detection from 566 nm to 609 nm, with 2% laser power and a pinhole size of 74 μm. The maximum intensity projections were obtained by capturing and processing Z-stacks. The imaging software ZEN 2.3 SP1 (Carl Zeiss) was used to process the images.

### Experimental design and re-isolation from rootstock plants

Plants of 'Richter 110', 'US 8-7' and 'Paulsen 1103' each with six fully developed leaves, were inoculated with conidium suspensions of *T. atroviride* strain T-77::*tdTomato* (finale concentration of $1 \times 10^6$ conidia $mL^{-1}$) for confocal laser microscopy and re-isolation. Each plant was inoculated with 100 mL of the conidium suspension, or 100 mL of water for the control plants. In total, 96 plants were inoculated, 36 plants for the control and 60 for the treatment with the conidium suspension. After 3, 7, 10 and 21 d post inoculation (dpi), re-isolations were carried out from the roots and crowns of the plants (as described above). *Trichoderma atroviride* T-77::*tdTomato* was confirmed by making slides of the cultures and visualisation with an epifluorescence Zeiss Axioscope microscope (Carl Zeiss). Colonization by *T. atroviride* T-77::*tdTomato* was scored based on the frequency of occurrence in plates, not number of plant pieces.

### *Induced systemic responses in leaves by* Trichoderma *atroviride root colonization*

### Experimental design

The plants of 'Richter 110', 'Paulsen 1103' and 'US 8-7' used to determine host defence activation in leaves were the same as those used for the colonization experiment (above). The top fully developed leaf from each of five plants were harvested at 21 dpi and then combined as one repeat of one treatment, resulting in a total of three biological repetitions. The leaves were then stored at -80°C for later analysis.

### RNA extraction, cDNA synthesis and qPCR from leaves

Frozen leaves (-80°C) were ground in liquid nitrogen with a mortar and pestle. Total RNA was extracted from 150 mg of ground leaf material using the RNeasy Plant Mini Kit (QIAGEN, Germany) following the manufacturer's instructions, and was quantified at 260 nm wavelength using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). Residual genomic DNA was removed by DNase1 digestion on an extraction column with the RNase-free DNase set (QIAGEN) at 25°C for 15 min during the RNA extraction. cDNAs were synthesized from 0.5 μg of DNase-treated RNA using the iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad). Real-time PCRs were carried out on a CFX96 Real Time System C1000 Touch™ Thermal Cycler (Bio-Rad). qPCR reactions were each carried out in a reaction buffer containing 2× iQ SYBR® Green Supermix, 0.2 mM of forward and reverse primers, and 10 ng of reverse transcribed RNA, in a final volume of 20 μL. Thermal cycling conditions were as follows: 30 s at 95°C followed by 40 cycles of 15 s at 94°C, 30 s at 60°C, and 30 s at 72°C. The defence genes and primer pairs associated with defence in *Vitis vinifera* (*Vv*) used for quantitative real-time PCR are listed in Table 1. The study included genes encoding for a wide range of defence reactions including *VvCAM* that encodes for cell signalling and calcium fluxes and *VvSOD* for reactive oxygen species (ROS) metabolism. *VvPR2*, *VvCHIT4C* and *VvPR6* are related to the production of different pathogenesis-related proteins (PR proteins), and *VvLTP* to the production of lipid transfer proteins and the PR14 proteins. *VvPAL* is associated with the phenylpropanoid pathway, *VvACO1* with the ethylene pathway, *VvLOX9* with the jasmonic pathway and *VvSTS* with the stilbene pathway. The data obtained were analyzed using CFX Manager Software (Bio-Rad). The results obtained for each gene of interest were normalized to the expression of two reference genes, *VvEF1-y*, an elongation factor 1 gene, and *VvActin* encoding actin proteins. Induction ratios compared to the controls were calculated according to Hellemans *et al.* (2007), as:

$$\text{Induction ratio} = [(1+E)\Delta CT(REF1)\times(1+E)\Delta CT(REF2)]^{0,5}$$

Gene expression was considered significant at an induction ratio of 4. The calculation takes the PCR efficiency (E) as well as the ΔCT, representing the difference between the Ct of the negative control and the Ct of the sample. For each sample, tests were carried out with two technical repetitions (the Ct results from two data values) and three biological repetitions (five leaves from five plants

**Table 1.** Sequences of the primer pairs derivative of *Vitis vinifera* and used for RT-qPCR.

| Gene (Target) | Primer sequences[a] | Reference | Gene ID/ Gene bank accession |
|---|---|---|---|
| *VvEF1-y* (Elongation factor 1) | F : 5'-CAAGAGAAACAATCCCTAGCTG-3'<br>R : 5'-TCAATCTGTCTAGGAAAGGAAG-3' | Rossdeutch *et al.*, 2016 | VIT_12s0035g01130 |
| *VvActin* (Actin) | F : 5'-CTTGCATCCCTCAGCACCTT-3'<br>R : 5'-TCCTGTGGACAATGGATGGA-3' | Rossdeutch *et al.*, 2016 | VIT_04s0044g00580 |
| *VvCAM* (Calmodulin) | F : 5'- TATTCCAGTAGTTTGGGTTGGTAGTG-3'<br>R : 5'-AAGAAGCACCAAACAAGAAAGGAG-3' | Perazzolli *et al.*, 2010 | GR911644.1 |
| *VvSOD* (Superoxide dismutase) | F : 5'-TGCCAGTGGTAAGGCTAAGTTCA-3'<br>R : 5'-GTGGACCTAATGCAGTGATTGA-3' | Stempien *et al.*, 2018 | AF056622 |
| *VvPR2* (PR protein 2) | F : 5'-GGGGAGATGTGAGGGGTTAT-3'<br>R : 5'-TGCAGTGAACAAAGCGTAGG-3' | Bellee *et al.*, 2017 | AF239617 |
| *VvCHIT4C* (Acidic class IV chitinase) | F : 5'-GTGTGTCCGGGAAGGATTACT-3'<br>R : 5'-TCAAGCCATCAAACCCAATGC-3' | Mutawila *et al.*, 2016a | XM002275480 |
| *VvPR6* (PR protein 6) | F : 5'-AACCATTAAGAGGGAGAATCCTCA-3'<br>R : 5'-CACGGACCCTAGTGCAGTAAA-3' | Mutawila *et al.*, 2016a | XM002284411 |
| *VvLTP* (PR protein 14) | F : 5'-CTGGCATCAATTTCGGTCTT-3'<br>R : 5'-AAGGCTGAGTGGTCCAAGTG-3' | Bruisson, 2015 | NM_001281191.1 |
| *VvACO1* (Aconitase 1) | F : 5'-GCCGGTTTGAAGTTCCAGGCCA-3'<br>R : 5'-ACTCAAACTGTGGCAATGGGACCC-3' | Bellee *et al.*, 2018 | XM_002273394.1 |
| *VvLOX9* (Lipoxygenase) | F : 5'-CCCTTCTTGGCATCTCCCTTA-3'<br>R : 5'-TGTTGTGTCCAGGGTCCATTC-3' | Perazzolli *et al.*, 2010 | AY159556 |
| *VvSTS* (Stilbene synthase) | F : 5'-AAGGGTCCGGCCACCATCCT-3'<br>R : 5'-ACGCAGTCATGTGCTCGCTCT-3' | Mutawila *et al.*, 2016a | XM002268806 |
| *VvPAL* (Phenylalanine ammonia-lyase-like) | F : 5'-GGTGAGCTTCACCCCTCCAGGT-3'<br>R : 5'-GGAGCTGCAGGGGTCATCAATGT-3' | Mutawila *et al.*, 2016a | XM002281763 |

[a] Primer efficiency of 1.8 used for all the sets of primers.

were used for each repetition). PCR amplification tests were each conducted in 20 µL final volume (10 µL of Taq DNA Polymerase Master Mix 2x [AMPLIQON], 1 µL of forward and reverse *VvActin* primers, 2 µL of cDNA and 6 µL of sterile water), to verify the quality of the cDNA.

*Statistical analyses*

For *Trichoderma* re-isolation and RT-qPCR gene expression analyses, data evaluation was performed with R 3.6.1. Software (R Development Core Team, 2016) through a multifactorial ANOVA, and multiple comparison of the means with the Tukey test ($P \leq 0.05$).

## RESULTS

*Evaluation of* Trichoderma *colonization of rooted rootstock plants by re-isolation*

The inner roots of all three tested rootstock cultivars were efficiently colonized at 21 dpi by both *Trichoderma* strains. The re-isolation percentages showed that the strains T-77 and USPP T1 colonized the plant parts to a similar extent, at an average of 70% in roots and 20% in the crowns (Figure 1). The roots were significantly more colonized than the crowns. The average colonization percentages for both strains for the roots were 78% for 'Richter 110' and 'Paulsen', and 74% for 'US 8-7'. The colonization percentages for the crowns were 25% for 'Richter 110', 30% for 'Paulsen' and 19% for 'US 8-7'. However, there were no statistically significant differences in root and crown colonization by the *T. atroviride* strains between the three rootstocks.

*Evaluation of* Trichoderma *colonization of rooted rootstock plants using an Agrobacterium-transformed* T. atroviride

*Trichoderma atroviride* T-77 was successfully transformed. The transformant *T. atroviride* T-77::*tdTomato* expressed the expected intensity of fluorescence. Mycelium and conidia were observed from pure cultures (Figure 2), allowing to test the ability of this strain to colonize rootstock roots. Observations *in planta* were hampered by the hardening of roots and required specialized

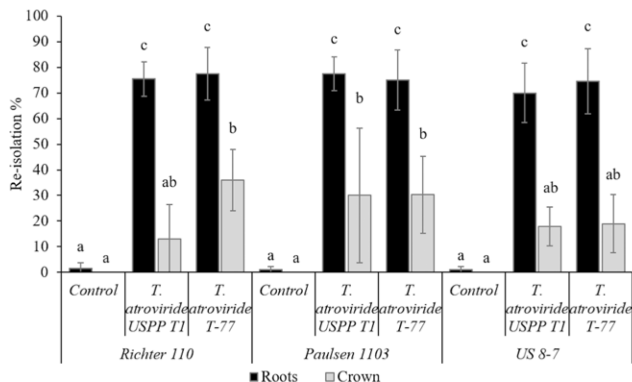


**Figure 1.** Mean proportions (%) of re-isolations of *Trichoderma atroviride* T-77 and *T. atroviride* USPP T1 from the grapevine rootstock cultivars 'Richter 110', 'Paulsen 1103' and 'US 8-7'. Re-isolations were made from plant roots and crowns 21 d post-inoculation. Each of the data points shows the average of 25 replicates (five biological replicates and five technical replicates). Error bars indicate standard deviations of the means. Different letters accompanying the means indicate significant differences ($P \leq 0.05$; Tukey Contrasts).

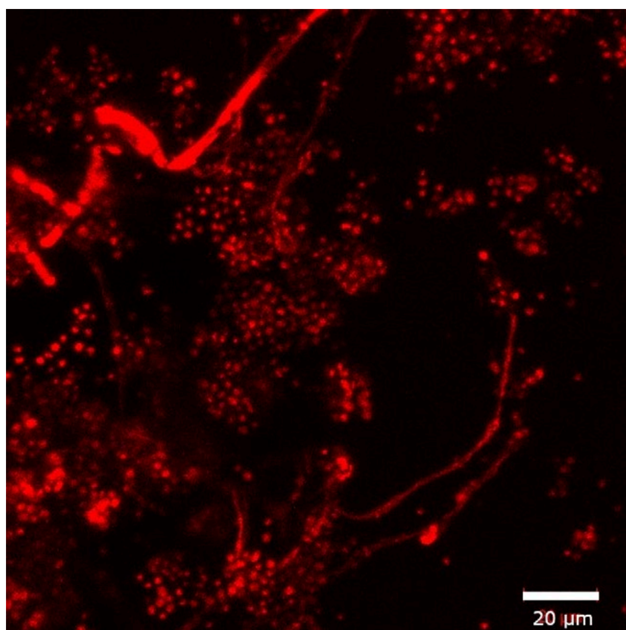


**Figure 2.** Confocal microscope image of *Trichoderma atroviride* T-77::*tdTomato* mycelia and conidia.

sectioning of plant material, so assessments of colonization were made based on re-isolation of the fluorescent strain.

Re-isolations of *T. atroviride* T-77::*tdTomato* were carried out (described above) from roots and crowns at the different time points: 3, 7, 11 and 21 dpi. Whereas *T. atroviride* T-77::*tdTomato* was absent from the control plants, it was present in all three cultivars after 3 dpi in the roots and crowns (Table 2). Colonization of roots was from 80% to 100%, and of crowns from 20% to 60%. After 21 dpi, colonization in the roots was 80% to 100% and 60% in the crown. However, in the roots and the crowns, the colonization was not significantly different ($P \leq 0.05$, Tukey Contrasts) between the three cultivars or between the four periods post-inoculation.

### *Systemic response in leaves induced by* Trichoderma atroviride *root colonization*

Firstly, primers designed for *Vitis vinifera* were evaluated to assess gene expression of rootstocks. Expression of defence related genes was assessed at 21 dpi in parallel with re-isolations for the two *T. atroviride* strains (Figure 3). In 'Paulsen 1103', only the defence genes encoding for PR proteins (*VvPR6*) and stilbene synthase (*VvSTS*) were significantly induced by both *Trichoderma* strains, although the induction was greater with *T. atroviride* T-77 than with *T. atroviride* USPP T1 (Figure 3 B). In 'US 8-7' leaves, *VvChit4c*, *VvLTP*, *VvACO1* and *VvSTS* were significantly up-regulated, but only by *T. atroviride* USPP T1 (Figure 3 C). For 'Richter 110', no modulation of defence gene expression was detected after 21 dpi (Figure 3A).

## DISCUSSION

Several *Trichoderma* species are well known BCAs, and these could have potential for use against grapevine trunk diseases via root applications. To use a BCA to prevent pathogen infection, the mechanisms of grapevine-BCA interactions and establishment of the potential BCA inside host plants must be well understood. However, how *Trichoderma* species colonize grapevine, and how they react on molecular or biochemical levels, are not well-understood. In the present study, a protocol to inoculate *Trichoderma* on grapevine was develop under laboratory conditions, to investigate colonization of roots and bases of grapevine rootstock plants by *Trichoderma*, and the abilities of these fungi to activate host defence in leaf tissues.

Strains of *Trichoderma* have been found to inhabit root systems of many plant species (Shoresh *et al.,* 2010; Hermosa *et al.,* 2012). However, to our knowledge there has been no laboratory study confirming early colonization of grapevine roots by these fungi. *Trichoderma* spp. readily colonize roots in field applications, as reported by Fourie *et al.* (2001), where less BFD pathogens were isolated from grapevine roots after treatment

**Table 2.** Numbers of plants from which *Trichoderma atroviride* T-77::*tdTomato* were re-isolated from roots and crowns after 3, 7, 10 or 21 d post-inoculation (dpi).

| Cultivar | Incidence in roots (per plant)[a] | | | | Incidence in crowns (per plant)[a] | | | |
|---|---|---|---|---|---|---|---|---|
| | 3 dpi | 7 dpi | 10 dpi | 21 dpi | 3 dpi | 7 dpi | 10 dpi | 21 dpi |
| 'Richter 110' | 4 | 5 | 5 | 5 | 1 | 5 | 3 | 3 |
| 'Paulsen 1103' | 5 | 5 | 5 | 5 | 2 | 4 | 3 | 3 |
| 'US 8-7' | 5 | 4 | 5 | 5 | 3 | 5 | 4 | 3 |

[a] Five plants for each treatment and time point.

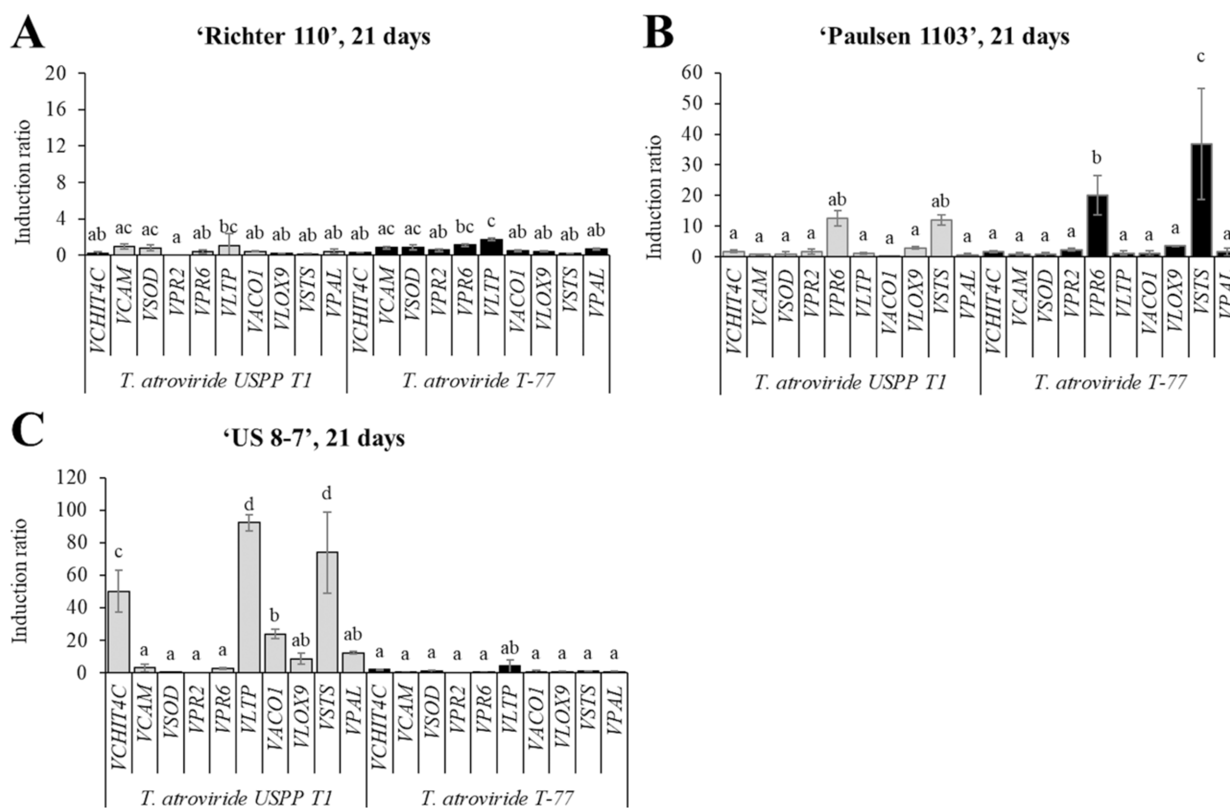


**Figure 3.** Mean gene induction ratios for different gene expressions (as indicated from RT-qPCR) in the leaves of grapevine rootstock cultivars 'Richter 110' (A), 'Paulsen 1103' (B) and 'US 8-7' (C) after 21 d after inoculations with *Trichoderma atroviride* T-77 (grey bars) or *T. atroviride* USPP T1 (black bars). Individual data points are means of two technical and three biological replicates. Error bars indicate standard deviations of the means. Different letters accompanying the means indicate significant differences ($P \leq 0.05$; Tukey Contrasts).

with *Trichoderma* species. In the present study, the ability of *Trichoderma* to colonized grapevine root tissues was evaluated with two *T. atroviride T. atroviride* strains inoculated onto three grapevine rootstock cultivars. After 21 dpi, approx. 70% of roots and 20% of crowns of inoculated plants were colonized by both strains. The lower colonization in crowns than in roots could be explained by the growth of *T. atroviride* from the roots to the crowns, but the infections could also have occurred directly into the crowns since these host tissues were not completely callused. The amount of colonization in the roots and crowns of the rootstock plants was neither dependent on the cultivar nor the *T. atroviride* strain. The *T. atroviride* transformed with tdTomato allowed assessment of colonization using re-isolations and confocal laser microscopy. Protocols for visual observation *in planta* need to be optimized. The results showed that *T. atroviride* successfully colonized the roots and crowns of grapevine plants after 3 dpi, and colonization was similar in the three studied cultivars.

This study has demonstrated rapid and high frequencies of colonization of grapevine roots by *T. atroviride*. However, several studies have showed low levels of field colonization of grapevine rootstocks by *Trichoderma* species. Ferrigo *et al.* (2017) reported low amounts of colonization of 'Richter 110' roots after a *T. atroviride* soil inoculation. However, these treatments were effective in controlling disease caused by *Agrobacterium vitis*. Berlanas *et al.* (2018) applied *Trichoderma* root dips to 'Richter 110' plants for 24 h, and colonization rate was less than 1% 2 months after inoculation.

After determining that the potential BCA was established in its host, the interactions with the host were assessed. The mechanism by which plants perceive *T. atroviride* is not well understood. Beneficial microbes only induce limited levels of host immune systems after recognition during local colonization of roots. Defence responses are activated in plant hosts due to Microbe-Associated Molecular Patterns (MAMPs) and Damage-Associated Molecular Patterns (DAMPs) perceived as danger indicators (Boller and Felix, 2009). *Trichoderma* spp. secrete structural proteins and secondary compounds that act as MAMPs, and their secreted enzymes that act against plant host cell walls operate as DAMPs (Hermosa *et al.,* 2013). In recent studies of the *Trichoderma* genome, it was demonstrated that the genome could encode for potential effector proteins that assist plant colonization (Mendoza-Mendoza *et al.,* 2018; Nogueira-Lopez *et al.,* 2018). Furthermore, *Trichoderma* spp. have been shown to form attachment structures that are similar to appressoria that penetrate through production of cellulolytic and proteolytic enzymes (Brotman *et al.,* 2008; Contreras-Cornejo *et al.,* 2016). These attachment structures were probably the results of the germinating conidia identified in the present study using confocal laser microscopy. Once recognized by hosts, *Trichoderma* spp. prime the plant defence systems (Guzmán-Guzmán *et al.,* 2018), and induce expression of defence genes, which confer local resistance (site inhabiting resistance) in roots and ISR against pathogens in distal plant parts (Pieterse *et al.,* 2014).

Development of a system to assess activation of grapevine defence genes after *Trichoderma* conidium drenching required considerable optimization. The extraction of mRNA from roots was abandoned after several failed attempts. Obtaining mRNA from roots is known to be difficult, most probably due to inhibitors present within the extraction. Extraction of mRNA from leaves is more reliable and this allowed demonstration of ISR by induction of defence genes in the leaves after only the roots were inoculated with *T. atroviride* T-77 or *T. atroviride* USPP T1 conidium suspensions. Induction of defence genes was studied after 21 dpi. Differences between rootstock cultivars were demonstrated. In leaves of 'Richter 110', no modulation of expression of the targeted genes was observed in response to *T. atroviride* inoculation with either of the fungus strains. This was confirmed by a separate assay (unpublished data), in which no impact of *T. atroviride* inoculation was detected for expression of defence genes at 10 dpi. Absence of induction in 'Richter 110' by *T. atroviride* could be explained by low sensitivity of the cultivar. In leaves of 'US 8-7', up-regulation was detected for the defence genes *VvChit4c* and *VvLTP* encoding two PR proteins, *VvACO1* encoding a protein involved in ethylene pathway synthesis, and *VvSTS* encoding a stilbene synthase. However, the expression of these genes was only induced in leaves of grapevine plants inoculated with strain USPP T1, suggesting that the modulation of defence gene expression is dependent on the *Trichoderma* strain. In leaf tissues of 'Paulsen 1103', two genes (*VvPR6* and *VvSTS*) were up-regulated by both strains of *Trichoderma*. However, the gene expression was slightly greater when induced by strain T-77 than strain USPP T1. Furthermore, these results can be correlated with information on rootstock susceptibility to pathogens. Sieberhagen (2017) inoculated rootstock cuttings with conidium suspensions of different pathogens, including those causing BFD, to determine the resistance or susceptibility of grapevine rootstocks used in South Africa. 'US 8-7' and 'Paulsen' presented the least disease severity against all the pathogens tested including BFD pathogens, whereas 'Richter 110' developed the greatest disease severity. The high susceptibility of 'Richter 110' to BFD pathogens could explain inefficient priming by *Trichoderma* due to a lack of host defence activation, in comparison to 'Paulsen 1103' and 'US 8-7'.

The two experiments carried out in the present study have demonstrated that the intensity of induction of defence genes was dependent on the *Trichoderma* strain and the grapevine rootstock cultivar. In both experiments, the *Trichoderma* inoculations lead to up-regulation of gene encoding PR proteins or proteins involved in stilbene synthesis. These compounds may represent an effective defense response to protect grapevine plants from BFD. High expression of the stilbene synthase gene (*VvSTS*) results in increased resistance responses to pathogens (Adrian and Jeandet, 2012). It is not certain that priming of host defence will be sufficient for protection against, for example, infection by black foot pathogens. Field experiments have shown that the use of *Trichoderma* did not consistently prevent black foot pathogen infections of nursery vines (Berlanas *et al.*, 2018; van Jaarsveld *et al*., 2020).

In conclusion, the present study has increased understanding of the mechanisms of grapevine-*Trichoderma* interactions. Knowledge of these interactions is important to assist screening for potential biocontrol agents that can be used against BFD and other grapevine trunk diseases. Although *T. atroviride* similarly colonized the three rootstock cultivars tested, activation of host defence was cultivar dependent and therefore needs to be evaluated for more rootstock cultivars. *Trichoderma* treatments need to be evaluated in combination with a pathogen with measurable symptoms, in controlled environment experiments and in the field. This will aid optimization of the use of *Trichoderma* spp. for grapevine root application.

## ACKNOWLEDGMENTS

This research was funded by Winetech and the Department of Science and Technology (DST), South Africa. The authors thank Dr Markus Gorfer (Fungal Genomics Unit, Austrian Research Centre and BOKU Vienna) for providing the bacterium and plasmid used in this study. Dr Philip Young assisted in the choice of reference genes examined.

## LITERATURE CITED

Adrian M., Jeandet P., 2012. Effects of resveratrol on the ultrastructure of *Botrytis cinerea* conidia and biological significance in plant/pathogen interactions. *Fitoterapia* 83, 1345–1350. https://doi.org/10.1016/j.fitote.2012.04.004

Alaniz S., Abad-Campos P., García-Jiménez J., Armengol J., 2011. Evaluation of fungicides to control *Cylindrocarpon liriodendri* and *Cylindrocarpon macrodidymum in vitro*, and their effect during the rooting phase in the grapevine propagation process. *Crop Protection* 30, 489–494. https://doi.org/10.1016/j.cropro.2010.12.020

Bellée A., Comont G., Nivault A., Abou-Mansour E., Coppin C., … Corio-Costet M.F., 2017. Life traits of four Botryosphae species and molecular responses of different grapevine cultivars or hybrids. *Plant Pathology* 66, 763–776. https://doi.org/10.1111/ppa.12623

Bellée A., Cluzet S., Dufour M.-C., Mérillon J.-M., Corio-Costet M.-F., 2018. Comparison of the impact of two molecules on plant defense and on efficacy against *Botrytis cinerea* in the vineyard: A plant defense inducer (Benzothiadiazole) and a fungicide (Pyrimethanil). *Journal of Agricultural and Food Chemistry* 66, 3338–3350. https://doi.org/10.1021/acs.jafc.7b05725

Berlanas C., Andrés-Sodupe M., López-Manzanares B., Maldonado-González M.M., Gramaje D., 2018. Effect of white mustard cover crop residue, soil chemical fumigation and *Trichoderma* spp. root treatment on black-foot disease control in grapevine. *Pest Management Science* 74, 2864–2873. https://doi.org/10.1002/ps.5078

Boller T., Felix G., 2009. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annual Review of Plant Biology* 60, 379–406. https://doi.org/10.1146/annurev.arplant.57.032905.105346

Brotman Y., Briff E., Viterbo A., Chet I., 2008. Role of swollenin, an expansin-like protein from *Trichoderma*, in plant root colonization. *Plant Physiology* 147, 779–789. https://doi.org/10.1104/pp.108.116293

Bruisson S., 2015. *Potentialisation de réactions de défense suite à la mycorhization de la Vigne et effets sur la production de stilbènes*. PhD Thesis. Université de Haute Alsace, Laboratoire Vigne, Biotechnologies et Environnement - Colmar, France.

Caasi O.C., Walker N.R., Marek S.M., Enis,J.N., Mitchell T.K., 2010. Infection and colonization of turf-type Bermudagrass by *Ophiosphaerella herpotricha* expressing green or red fluorescent proteins. *Phytopathology*, 100(5), 415-423. https://doi.org/10.1094/PHYTO-100-5-0415

Conrath U., Beckers G.J.M., Langenbach C.J.G., Jaskiewicz M.R., 2015. Priming for enhanced defense. *Annual Review of Phytopathology* 53, 97–119. https://doi.org/10.1146/annurev-phyto-080614-120132

Contreras-Cornejo H.A., Macías-Rodríguez L., del-Val E., Larsen J., 2016. Ecological functions of *Trichoderma* spp. and their secondary metabolites in the rhizosphere: interactions with plants. *FEMS Microbiology Ecology* 92. https://doi.org/10.1093/femsec/fiw036

Di Marco S., Osti F., 2007. Applications of *Trichoderma* to prevent *Phaeomoniella chlamydospora* infections in organic nurseries. *Phytopathologia Mediterranea* 46, 73–83.

dos Santos R.F., Heckler L.I., Lazarotto M., da Ressurreição Garrido L., Rego C., Blume E., 2016. *Trichoderma* spp. and *Bacillus subtilis* for control of *Dactylonectria macrodidyma* in grapevine. *Phytopathologia Mediterranea* 55, 293–300.

Edreva A., 2004. A novel strategy for plant protection: Induced resistance. *Journal of Cell and Molecular Biology* 3, 61–69.

Ferrigo D., Causin R., Raiola A., 2017. Effect of potential biocontrol agents selected among grapevine

endophytes and commercial products on crown gall disease. *BioControl*, 62(6), 821-833. https://doi.org/10.1007/s10526-017-9847-3

Fourie P.H., Halleen F., 2006. Chemical and biological protection of grapevine propagation material from trunk disease pathogens. *European Journal of Plant Pathology* 116, 255–265. https://doi.org/10.1007/s10658-006-9057-9

Fourie P.H., Halleen F., van der Vyver J., Schreuder W., 2001. Effect of *Trichoderma* treatments on the occurrence of decline pathogens in the roots and rootstocks of nursery grapevines. *Phytopathologia Mediterranea* 40, S473–S478.

Gallou A., Cranenbrouck S., Declerck S., 2009. *Trichoderma harzianum* elicits defence response genes in roots of potato plantlets challenged by *Rhizoctonia solani*. *European Journal of Plant Pathology* 124, 219–230. https://doi.org/10.1007/s10658-008-9407-x

Gorfer M., Klaubauf S., Bandian D., Strauss J., 2007. *Cadophora finlandia* and *Phialocephala fortinii*: Agrobacterium-mediated transformation and functional GFP expression. *Mycological Research* 111, 850–855. https://doi.org/10.1016/j.mycres.2007.05.002

Gramaje D., Armengol J., 2011. Fungal trunk pathogens in the grapevine propagation process: Potential inoculum sources, detection, identification, and management strategies. *Plant Disease* 95, 1040–1055. https://doi.org/10.1094/PDIS-01-11-0025

Gramaje D., Muñoz R.M., Lerma M.L., García-Jiménez J., Armengol J., 2010. Fungal grapevine trunk pathogens associated with Syrah decline in Spain. *Phytopathologia Mediterranea* 48, 396–402. https://doi.org/10.14601/Phytopathol_Mediterr-2934

Gramaje D., Úrbez-Torres J.R., Sosnowski M.R., 2018. Managing grapevine trunk diseases with respect to etiology and epidemiology: Current strategies and future prospects. *Plant Disease* 102, 12–39. https://doi.org/10.1094/PDIS-04-17-0512-FE

Guzmán-Guzmán P., Alemán-Duarte M.I., Delaye L., Herrera-Estrella A., Olmedo-Monfil V., 2017. Identification of effector-like proteins in *Trichoderma* spp. and role of a hydrophobin in the plant-fungus interaction and mycoparasitism. *BMC Genetics* 18, 16. https://doi.org/10.1186/s12863-017-0481-y

Guzmán-Guzmán P., Porras-Troncoso M.D., Olmedo-Monfil V., Herrera-Estrella A., 2018. *Trichoderma* species: versatile plant symbionts. *Phytopathology* 109, 6–16. https://doi.org/10.1094/PHYTO-07-18-0218-RVW

Halleen F., Fourie P.H., 2016. An integrated strategy for the proactive management of grapevine trunk disease pathogen infections in grapevine nurseries. *South African Journal of Enology and Viticulture* 37, 104–114. https://doi.org/10.21548/37-2-825

Halleen F., Schroers H.J., Groenewald J.Z., Crous, P.W., 2004. Novel species of *Cylindrocarpon* (*Neonectria*) and *Campylocarpon* gen. nov. associated with black foot disease of grapevines (Vitis spp.). *Studies in Mycology*, 50(2), 431-455.

Halleen F., Fourie P.H., Crous P.W., 2006a. A review of black foot disease of grapevine. *Phytopathologia Mediterranea* 45, S55–S67.

Halleen F., Fourie P.H., Crous P.W., 2007. Control of black foot disease in grapevine nurseries. *Plant Pathology* 56, 637–645. https://doi.org/10.1111/j.1365-3059.2007.01613.x

Haran S., Schickler H., Oppenheim A., Chet I., 1995. New components of the chitinolytic system of *Trichoderma harzianum*. *Mycological Research* 99, 441–446. https://doi.org/10.1016/S0953-7562(09)80642-4

Harman G.E., 2006. Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathology* 96, 190–194. https://doi.org/10.1094/PHYTO-96-0190

Harman G.E., Hayes,C.K., Lorito M., Broadway R.M., Di Pietro A., … Tronsmo A., 1993. Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase. *Phytopathology* 83, 313–318.

Harman G.E., Kubicek C.P., 2002. *Trichoderma* and *Gliocladium*: Basic biology, taxonomy and genetics. CRC Press, Boca Raton, FL, USA.

Harman G.E., Howell C.R., Viterbo A., Chet I., Lorito M., 2004. *Trichoderma* species – opportunistic, avirulent plant symbionts. *Nature Reviews Microbiology* 2, 43–56. https://doi.org/10.1038/nrmicro797

Hellemans J., Mortier G., Paepe A.D., Speleman F., Vandesompele J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biology* 8, R19. https://doi.org/10.1186/gb-2007-8-2-r19

Hermosa R., Viterbo A., Chet I., Monte E., 2012. Plant-beneficial effects of *Trichoderma* and of its genes. Microbiology, 158, 17–25. https://doi.org/10.1099/mic.0.052274-0

Hermosa R., Rubio M.B., Cardoza R.E., Nicolás C., Monte E., Gutiérrez S., 2013. The contribution of *Trichoderma* to balancing the costs of plant growth and defense. *International Microbiology* 16, 69–80. https://doi.org/10.2436/20.1501.01.181

Howell C.R., 2006. Understanding the mechanisms employed by *Trichoderma virens* to effect biological control of cotton diseases. *Phytopathology* 96, 178–180. https://doi.org/10.1094/PHYTO-96-0178

Lorito M., Peterbauer C., Hayes C.K., Harman G.E., 1994. Synergistic interaction between fungal cell wall degrading enzymes and different antifungal compounds enhances inhibition of spore germination. *Microbiology* 140, 623–629. https://doi.org/10.1099/00221287-140-3-623

Lorito M., Woo S.L., Harman G.E., Monte E., 2010. Translational research on *Trichoderma*: From 'omics to the field. *Annual Review of Phytopathology* 48, 395–417. https://doi.org/10.1146/annurev-phyto-073009-114314

Manganiello G., Sacco A., Ercolano M.R., Vinale F., Lanzuise S., … Woo S.L., 2018. Modulation of tomato response to *Rhizoctonia solani* by *Trichoderma harzianum* and its secondary metabolite harzianic acid. *Frontiers in Microbiology* 9. https://doi.org/10.3389/fmicb.2018.01966

Martínez-Medina A., Appels F.V.W., Wees S.C.M. van, 2017. Impact of salicylic acid- and jasmonic acid-regulated defences on root colonization by *Trichoderma harzianum* T-78. *Plant Signaling & Behavior* 12, e1345404. https://doi.org/10.1080/15592324.2017.1345404

Mendoza-Mendoza A., Zaid R., Lawry R., Hermosa R., Monte, E., … Mukherjee, P.K., 2018. Molecular dialogues between *Trichoderma* and roots: Role of the fungal secretome. *Fungal Biology Reviews* 32, 62–85. https://doi.org/10.1016/j.fbr.2017.12.001

Morán-Diez E., Hermosa R., Ambrosino P., Cardoza R.E., Gutiérrez S., … Monte E., 2009. The ThPG1 endopolygalacturonase is required for the *Trichoderma harzianum*–plant beneficial interaction. *Molecular Plant-Microbe Interactions* 22, 1021–1031. https://doi.org/10.1094/MPMI-22-8-1021

Mutawila C., Stander C., Halleen F., Vivier M.A., Mostert L., 2016a. Response of *Vitis vinifera* cell cultures to *Eutypa lata* and *Trichoderma atroviride* culture filtrates: Expression of defence-related genes and phenotypes. *Protoplasma* 254, 863–879. https://doi.org/10.1007/s00709-016-0997-4

Mutawila C., Vinale F., Halleen F., Lorito M., Mostert L., 2016b. Isolation, production and *in vitro* effects of the major secondary metabolite produced by *Trichoderma* species used for the control of grapevine trunk diseases. *Plant Pathology* 65, 104–113. https://doi.org/10.1111/ppa.12385

Nogueira-Lopez G., Greenwood D.R., Middleditch M., Winefield C., … Mendoza-Mendoza A., 2018. The apoplastic secretome of *Trichoderma virens* during interaction with maize roots shows an inhibition of plant defence and scavenging oxidative stress secreted proteins. *Frontiers in Plant Science* 9. https://doi.org/10.3389/fpls.2018.00409

Parrilli M., Sommaggio D., Tassini C., Di Marco S., Osti F., … A., Burgio, G., 2019. The role of *Trichoderma* spp. and silica gel in plant defence mechanisms and insect response in vineyard. *Bulletin of Entomological Research* 109, 771–780. https://doi: 10.1017/S0007485319000075 2019

Perazzolli M., Bampi F., Faccin S., Moser M., De Luca F., … Moser C., 2010. *Armillaria mellea* induces a set of defense genes in grapevine roots and one of them codifies a protein with antifungal activity. *Molecular Plant Microbe Interactions* 23, 485-496. https://doi.org/10.1094/MPMI-23-4-0485

Pieterse C.M.J., Zamioudis C., Berendsen R.L., Weller D.M., Van Wees S.C.M., Bakker P.A.H.M., 2014. Induced Systemic Resistance by beneficial microbes. *Annual Review of Phytopathology* 52, 347–375. https://doi.org/10.1146/annurev-phyto-082712-102340

Probst C., Jones E.E., Ridgway H.J., Jaspers M.V., 2012. Cylindrocarpon black foot in nurseries—two factors that can increase infection. *Australasian Plant Pathology* 41, 157–163. https://doi.org/10.1007/s13313-011-0103-5

R Development Core Team, 2016. R: A language and environment for statistical computing. Vienna, Austria.

Reino J.L., Guerrero R.F., Hernández-Galán R., Collado I.G., 2007. Secondary metabolites from species of the biocontrol agent *Trichoderma*. *Phytochemistry Reviews* 7, 89–123. https://doi.org/10.1007/s11101-006-9032-2

Rossdeutsch L., Edwards E., Cookson S.J., Barrieu F., Gambetta G.A., … Ollat N., 2016. ABA-mediated responses to water deficit separate grapevine genotypes by their genetic background. *BMC Plant Biology* 16, 91. https://doi.org/10.1186/s12870-016-0778-4

Rubio M.B., Quijada N.M., Pérez E., Domínguez S., Monte E., Hermosa R., 2014. Identifying beneficial qualities of *Trichoderma parareesei* for plants. *Applied and Environmental Microbiology* 80, 1864–1873. https://doi.org/10.1128/AEM.03375-13

Salas-Marina M.A., Isordia-Jasso M.I., Islas-Osuna M.A., Delgado-Sánchez P., Jiménez-Bremont J.F., … Casas-Flores S., 2015. The Epl1 and Sm1 proteins from *Trichoderma atroviride* and *Trichoderma virens* differentially modulate systemic disease resistance against different life style pathogens in *Solanum lycopersicum*. *Frontiers in Plant Science* 6. https://doi.org/10.3389/fpls.2015.00077

Segarra G., Casanova E., Bellido D., Odena M.A., Oliveira E., Trillas I., 2007. Proteome, salicylic acid, and jasmonic acid changes in cucumber plants inoculated with *Trichoderma asperellum* strain T34. *Pro-*

*teomics* 7, 3943–3952. https://doi.org/10.1002/pmic.200700173

Segarra G., Ent S.V. der, Trillas I., Pieterse C.M.J., 2009. MYB72, a node of convergence in induced systemic resistance triggered by a fungal and a bacterial beneficial microbe. *Plant Biology* 11, 90–96. https://doi.org/10.1111/j.1438-8677.2008.00162.x

Shoresh M., Harman G.E., Mastouri F., 2010. Induced Systemic Resistance and plant responses to fungal biocontrol agents. *Annual Review of Phytopathology* 48, 21–43. https://doi.org/10.1146/annurev-phyto-073009-114450

Sieberhagen M., 2017. Determining the resistance or susceptibility of grapevine rootstocks used in South Africa towards fungal trunk disease pathogens. MSc thesis. University of Stellenbosch, Faculty of AgriSciences - Stellenbosch, South Africa.

Stempien E., Goddard M.-L., Leva Y., Bénard-Gellon M., Laloue H., … Chong J., 2018. Secreted proteins produced by fungi associated with Botryosphaeria dieback trigger distinct defense responses in *Vitis vinifera* and *Vitis rupestris* cells. *Protoplasma* 255, 613–628. https://doi.org/10.1007/s00709-017-1175-z

Úrbez-Torres J.R., Haag P., Bowen P., O'Gorman D.T., 2014. Grapevine trunk diseases in British Columbia: Incidence and characterization of the fungal pathogens associated with Esca and Petri Diseases of grapevine. *Plant Disease* 98, 469–482. https://doi.org/10.1094/PDIS-05-13-0523-RE

van Jaarsveld W.J., Halleen F., Bester M.C., Pierron R.J.G., Stempien E., Mostert L., 2020. Investigation of *Trichoderma* species colonization of nursery grapevines for improved management of black foot disease. *Pest Management Science* https://doi.org/10.1002/ps.6030.

Verma M., Brar S.K., Tyagi R.D., Surampalli R.Y., Valéro J.R., 2007. Antagonistic fungi, *Trichoderma* spp. : Panoply of biological control. *Biochemical Engineering Journal* 37, 1–20. https://doi.org/10.1016/j.bej.2007.05.012

Vinale F., Marra R., Scala F., Ghisalberti E.L., Lorito M., Sivasithamparam K., 2006. Major secondary metabolites produced by two commercial *Trichoderma* strains active against different phytopathogens. *Letters in Applied Microbiology* 43, 143–148. https://doi.org/10.1111/j.1472-765X.2006.01939.x

Vinale F., Sivasithamparam K., Ghisalberti E.L., Marra R., Barbetti M.J., … Lorito M., 2008a. A novel role for *Trichoderma* secondary metabolites in the interactions with plants. *Physiological and Molecular Plant Pathology* 72, 80–86. https://doi.org/10.1016/j.pmpp.2008.05.005

Vinale F., Sivasithamparam K., Ghisalberti E.L., Marra R., Woo S.L., Lorito M., 2008b. *Trichoderma*–plant–pathogen interactions. *Soil Biology and Biochemistry* 40, 1–10. https://doi.org/10.1016/j.soilbio.2007.07.002

Viterbo A., Chet I., 2006. TasHyd1, a new hydrophobin gene from the biocontrol agent *Trichoderma asperellum*, is involved in plant root colonization. *Molecular Plant Pathology* 7, 249–258. https://doi.org/10.1111/j.1364-3703.2006.00335.x

Woo S.L., Scala F., Ruocco M., Lorito M., 2006. The molecular biology of the interactions between *Trichoderma* spp., phytopathogenic fungi, and plants. *Phytopathology* 96, 181–185. https://doi.org/10.1094/PHYTO-96-0181

Woo S.L., Ruocco M., Vinale F., Nigro M., Marra R., … Lorito M., 2014. *Trichoderma*-based products and their widespread use in agriculture. *The Open Mycology Journal* 8, 71–126. https://doi.org/10.2174/1874437001408010071