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Isolation, production and *in vitro* effects of the major secondary metabolite produced by *Trichoderma* species used for the control of grapevine trunk diseases

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Antibiosis has been shown to be an important mode of action by *Trichoderma* species used in the protection of grapevine pruning wounds from infection by trunk pathogens. The major active compound from *Trichoderma* isolates known to protect grapevine pruning wounds from trunk pathogen infection was isolated and identified. The compound, a 6-pentyl-α-pyrone (6PP), was found to be the major secondary metabolite, by quantity, which accumulated in the culture filtrate of *T. harzianum* isolate T77 and the two *T. atroviride* isolates UST1 and UST2. Benzimidazole resistant mutants generated from these isolates also produced 6PP as their main secondary metabolite, except for a mutant of T77 that had lost its ability to produce 6PP. The isolates UST1 and UST2 were co-cultured with the grapevine trunk pathogens *Eutypa lata* and *Neofusicoccum parvum* in a minimal defined medium and a grapevine cane-based medium (GCBM). Co-culturing UST1 with *N. parvum* induced 6PP production in the minimal defined medium and the GCBM. The production of 6PP by UST2 was induced in the GCBM, while co-culturing with the two trunk pathogens either reduced or had no effect on 6PP production. Mycelial growth and ascospore/conidia germination of *E. lata*, *N. australe*, *N. parvum* and *Phaeomoniella chlamydospora* were inhibited by 6PP in a concentration-dependent manner. The results show that the presence of *N. parvum* and grapevine wood elicits the production of 6PP, suggesting that this metabolite is involved in *Trichoderma*–pathogen interactions on grapevine pruning wounds.

Keywords: 6-pentyl-α-pyrone, biocontrol, grapevine trunk diseases, secondary metabolites

Introduction

Grapevine trunk diseases cause decline and loss of productivity in vines at all stages of growth, and are increasingly becoming an important limiting factor to the long-term sustainability of grape and wine production (Gubler *et al.*, 2005; Larignon *et al.*, 2009). These diseases are caused by various xylem-inhabiting pathogens that include *Eutypa lata* (eutypa dieback), *Phaeomoniella chlamydospora* and *Phaeoacremonium* species (Petri disease), *Fomitiporia* spp. (esca) and Botryosphaeriaceae fungi (botryosphaeria dieback). Infection occurs through any type of wound, of which pruning wounds are the principal ports of entry (Chapuis *et al.*, 1998; Van Niekerk *et al.*, 2011).

The grapevine pruning wound is colonized by naturally occurring fungi and bacteria and these may inhibit infection of the wood tissue by trunk pathogens (Munkvold & Marois, 1993). Pruning wound protection by biological agents offers an alternative to chemical control and provides a more sustainable long term control of trunk disease pathogens on wound surfaces. Biological wound protection from *E. lata* infection by *Fusarium lateritium*, *Cladosporium herbarum*, *Bacillus subtilis* and *Trichoderma* spp. has been reported (Munkvold & Marois, 1993; John *et al.*, 2008). The biocontrol effect of *Trichoderma* spp. has also been demonstrated on a wide spectrum of grapevine trunk diseases both *in vitro* and *in vivo* (Di Marco *et al.*, 2004; Kotze *et al.*, 2011). Due to the ease of large-scale production, *Trichoderma* spp. have been developed into commercial products for biological control of numerous plant pathogens (John *et al.*, 2008; Vinale *et al.*, 2008; Woo *et al.*, 2014).

The mechanisms of action by *Trichoderma* spp. may be either a result of its antagonistic action against the pathogen or from its interaction with the plant. *Trichoderma*–pathogen interactions involve mycoparasitism and secretion of mycolytic enzymes (Howell, 2006), competition for limited resources, as well as production of antibiotics (Sivasithamparam & Ghisalberti, 1998; Harman, 2006). Central to the biocontrol activity of *Trichoderma* spp. is the production of secondary metabolites, which are natural compounds that aid the producing organism in survival and basic functions such as symbiosis, competition and differentiation (Shwab & Keller, 2008). The

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production of antibiotic secondary metabolites is often correlated to the biocontrol activity of *Trichoderma* isolates (Ghisalberti *et al.*, 1990; Worasatit *et al.*, 1994; Vinale *et al.*, 2006).

Trichoderma secondary metabolites are chemically diverse and their production varies greatly between species and between isolates of the same species (Vinale *et al.*, 2009). The huge structural and functional diversity of *Trichoderma* metabolites necessitates the continuous search for new metabolites. The generated knowledge may be important for the selection of new biocontrol agents, or the compounds themselves may be used as bioactive compounds in pesticide and antibiotic applications.

In the South African Cape wine region, three isolates of *Trichoderma* spp. were shown to be good grapevine pruning wound colonizers. One isolate of *T. harzianum* (T77) came from the roots of a grapevine nursery plant, while two isolates of *T. atroviride* (UST1 and UST2) came from grapevine wood. Field studies against grapevine trunk pathogens have shown that these isolates are effective in grapevine pruning wound protection (Kotze *et al.*, 2011; Mutawila *et al.*, 2011). *In vitro* studies showed that the volatile and culture filtrates from the two *T. atroviride* isolates significantly reduced mycelial growth and inhibited spore germination of different trunk disease pathogens (Kotze *et al.*, 2011). It is therefore important to identify the active compounds in culture filtrates. In addition, mutant isolates with high tolerance to benzimidazole fungicides, for possible integrated application, were developed from these isolates by gamma irradiation (Mutawila *et al.*, 2014). One of them lost the *in vitro* antagonistic activity, and it is therefore hypothesized that the mutation could have affected secondary metabolite production.

This study reports the isolation and identification of the major secondary metabolite from both the wild type and mutant *Trichoderma* spp. strains. The production of the isolated metabolite was evaluated under different growth conditions, particularly when grown on grapevine wood extracts and co-cultured with trunk pathogens, in order to establish its role in biocontrol. The effect of the metabolite on mycelial growth and spore germination of grapevine trunk pathogens was also tested.

Materials and methods

Fungal isolates

Trichoderma atroviride isolates UST1 and UST2 are stored at Stellenbosch University, Department of Plant Pathology's culture collection under accession numbers STE-U 6514 and 6515, respectively. Isolate T77 is the active ingredient of a registered pruning wound protection biocontrol agent, Eco 77® (Plant Health Products, South Africa). These *Trichoderma* spp. isolates were used for the isolation of secondary metabolites and the comparison of the production of the major secondary metabolite between isolates. Benzimidazole resistant mutants MT1 (STE-U 7733), MT2 (STE-U 7734) and MT77 (STE-U 7735) were developed by gamma irradiation from the wildtype isolates UST1, UST2 and T77, respectively (Mutawila *et al.*, 2014). These were included in the comparison of the production of the major secondary metabolite between the *Trichoderma* isolates.

Four grapevine trunk pathogens, *E. lata* (STE-U 5692 and 6513), *Neofusicoccum australe* (STE-U 7025 and 7029), *N. parvum* (STE-U 4439 and 4584) and *P. chlamydospora* (STE-U 6384 and 7732) were also used. The pathogens were used to determine the effect of the *Trichoderma* spp. major secondary metabolite on fungal growth, as well as the effect of co-culturing the *Trichoderma* spp. and pathogens on the production of the major secondary metabolite. All fungal isolates were maintained in tubes of sterile deionized water at 4°C. Before use, the fungi were subcultured onto freshly prepared potato dextrose agar (PDA; Biolab) and allowed to grow for 5 days at 25°C in the dark.

Extraction, purification and identification of the major secondary metabolites from culture filtrates of *Trichoderma* isolates

The secondary metabolites were produced and extracted using the method reported by Vinale *et al.* (2006). Five 5 mm diameter plugs from each of the *Trichoderma* isolates (UST1, UST2 and T77) obtained from the margins of actively growing cultures on PDA were separately inoculated into 5 L conical flasks containing 1 L potato dextrose broth (PDB; Biolab). The suspension cultures were incubated for 30 days at 25°C without shaking, after which the fungal mycelium was removed from the broth by vacuum filtration through no. 4 filter paper (Whatman).

Culture filtrates were then extracted twice with equal volumes of ethyl acetate (99·5%, Sigma). The organic fractions were combined, then dried with Na_2SO_4 and evaporated under reduced pressure at 35°C. The residue (crude extract) recovered was subjected to flash column chromatography through silica gel (50 g), eluting with a gradient of petroleum ether:acetone (9:1 to 7:3 v/v). Fractions showing similar thin-layer chromatography (TLC) profiles were combined and further purified by preparative TLC (Silica gel G, 500 μm, UNIPLATE; Analtech Inc.). The major fraction obtained had a characteristic smell of the pyrone metabolites. Fractions were run on TLC (silica gel 60; EMD Millipore) developed in hexane:acetone (7:3 v/v) alongside a standard of 6-pentyl-α-pyrone (6PP) previously isolated and characterized by Vinale *et al.* (2008). The compounds were detected using UV light (254 or 366 nm) and/or by spraying the plates with a 5% (v/v) H_2SO_4 solution in ethanol followed by baking at 110°C for 5 min.

The major fraction was further characterized to confirm identity using nuclear magnetic resonance (NMR) and mass spectroscopy (MS). The proton (^{1}H) NMR spectra were recorded with a 400 MHz Brüker Avance spectrometer, equipped with a 5 mm Bruker Broad Band Inverse probe (BBI), working at the ^{1}H frequencies of 400·13, and using residual and deuterated solvent peaks as reference standards. A high-resolution mass spectrum was obtained by a VG Autospec mass spectrometer.

Time-course production of 6PP in static and shaking cultures

Tests were carried out to determine the time course production of 6PP among the wildtype isolates. Liquid cultures of the *Trichoderma* isolates were prepared by separately inoculating 100 mL PDB in 250 mL flasks with three agar plugs (5 mm) of the respective isolates (UST1, UST2 and T77). Uninoculated negative controls were also included. There were three biological replicates for each treatment and the experiment was repeated once. The cultures were incubated at 25°C with or without

shaking at 120 rpm. Two replicates of each isolate culture were harvested at 5, 10, 15 and 20 days of incubation for metabolite extraction. Culture filtrates were harvested by vacuum filtration through a Whatman no. 1 filter paper and 2 mL of the filtrate was further filtered through a 0·20 μm RC-membrane filter (Sartorius Stedim Biotech) into glass vials for direct quantification by liquid chromatography-mass spectroscopy (LC-MS). Chromatographic separation was performed by an ultra-high performance liquid chromatography (UHPLC) apparatus equipped with two micropumps (Waters Synapt G2) and a BEH C18-column (Waters BEH C18, 2·1 × 100 mm, particle size 1·7 μm). The eluents used were A: 1% formic acid in H_2O and B: acetonitrile, and the gradient used was as follows: 95% A (0·1 min); 40% A (4 min); 100% B (5 min) and 95% A (5·1 min) eluted at a flow rate of 0·4 mL min^{-1}. Quantification was done using a standard curve constructed by standards prepared from pure 6PP (Apollo Scientific).

Comparison of the production of 6PP by *Trichoderma* isolates

The broth cultures of the wildtype isolates (UST1, UST2 and T77) and their three benzimidazole resistant mutants (MT1, MT2 and MT77) were prepared by separately inoculating 100 mL of either full strength PDB or quarter strength PDB in 250 mL flasks with five agar plugs (5 mm) of the respective isolates. Uninoculated negative controls were also included. There were three biological replicates for each treatment and the experiment was repeated once. The cultures were incubated at 25°C with or without shaking at 120 rpm for 20 days. Each treatment combination of medium (full strength or quarter PDB) and culture condition (shaking or static) was replicated twice. The cultures were filtered and the major secondary metabolite quantified from the culture filtrate as described previously.

Effect of growth medium and pathogen co-inoculation on 6PP production

Cultures were grown in defined minimum medium (Pezet's) and grapevine cane-based medium (GCBM). Pezet's medium was prepared according to Pezet (1983) without modifications, and contained 1% (w/v) glucose and 0·5% (w/v) sucrose as the carbon sources. The GCBM was prepared by sonicating 100 g of ground dormant Cabernet Sauvignon canes in 500 mL boiling deionized water (100°C). The extract was then clarified by filtration through a series of double Miracloth and Whatman no. 1 and finally Whatman no. 3 filter papers. Sucrose (10 g L^{-1}) was added to the filtrate, the pH adjusted to 5·8 using either 1 M NaOH or 1 M HCl, and the filtrate was sterilized by autoclaving.

Erlenmeyer flasks containing 100 mL of medium were co-inoculated with five mycelial disks (5 mm) of *T. atroviride*, either UST1 or UST2, and a grapevine trunk pathogen, either *E. lata* (STE-U 5700) or *N. parvum* (STE-U 4439). There were three biological replicates for each treatment and the experiment was repeated once. The flasks were incubated at 25°C with shaking at 120 rpm for 10 days, after which the cultures were filtered and 6PP quantified from the culture filtrate as described above.

Determination of the sensitivity of grapevine trunk pathogens to 6PP

The effect of 6PP on the mycelial growth and ascospore/conidia germination was tested on four fungal pathogens, *E. lata*, *N. australe*, *N. parvum* and *P. chlamydospora*. For the antifungal assays, there were three replicates for each isolate per assay and all experiments were independently repeated once. Mycelial inhibition was tested on three complex media, PDA, malt extract agar (MEA; Biolab) and GCBM, and two defined media, Pezet's medium and Vogel's medium N (Vogel). The GCBM and Pezet's medium were prepared as described previously, and agar (15 g L^{-1}; Biolab) was added before sterilization. Vogel's medium N was prepared as modified by Metzenberg (2003) and consisted of 20 mL Vogel's 50 × salts, and 1% (w/v) glucose as the only carbon source. The pH was adjusted to 5·8 using 1 M NaOH or 1 M HCl before adding agar (15 g L^{-1}) and the medium sterilized by autoclaving.

Mycelial inhibition by 6PP

Eight isolates (two of each pathogen) were used to determine the effect of 6PP on mycelial growth. Pure 6PP (Apollo Scientific) was dissolved in methanol to make a 10 g L^{-1} stock solution. Mycelial growth inhibition was tested on PDA amended with 0 (control), 50, 100, 150, 200, 250, 300 and 400 mg L^{-1} 6PP. In all cases, the final concentration of methanol in the medium was 0·1%, including in the control plates. Mycelial plugs (5 mm diameter) taken from the margins of an actively growing colony were placed in the centre (mycelium side facing down) of metabolite amended agar plates. Plates were incubated at 25°C in the dark and the radial growth of the fungal colonies was measured at 24 h intervals for all the fungi except *P. chlamydospora* where colony diameter was measured at 3 day intervals, because this fungus is slower growing. Each isolate had three replicates per concentration and the radial mycelial growth was assessed by calculating the mean diameter from two perpendicular measurements and then subtracting 5 mm from each value to account for the original plug. Percentage inhibition relative to the control was calculated from the Day 3 colony diameters for all fungi except *P. chlamydospora*, where Day 12 colony diameter was used. The percentage inhibition was used to determine the effective concentration that inhibited mycelial growth by 50% (EC_{50}).

Effect of growth medium on mycelial sensitivity to 6PP

The eight isolates of grapevine trunk pathogens were grown on complex (PDA, MEA and GCBM) and defined minimal medium (Vogel's and Pezet's) amended with 150 mg L^{-1} 6PP. Control plates of each of the media were used and were not amended with 6PP. The final concentration of methanol in the medium was 0·1%, including in the control plates. Plates were incubated at 25°C in the dark and the radial growth of the fungal colonies was measured twice perpendicularly per plate at Day 3 for all fungi except *P. chlamydospora*, where it was measured at Day 12. There were three replicates for each isolate per medium. Radial colony diameters were used to calculate percentage inhibition relative to the control.

Pathogen conidia and ascospore production

Phaeomoniella chlamydospora (STE-U 6384) conidia were produced by growing the fungus on PDA for 3 weeks at 25°C. The conidia suspension was prepared by flooding the Petri dishes with sterile water (10 mL) and the conidia dislodged from the media using a sterile needle. The suspension was collected in a sterile glass bottle. *Eutypa lata* ascospores are produced in perithecial stroma on infected old wood. For a spore suspension, stroma were collected from infected vines at the Nietvoorbij vineyards of the Agricultural Research Council of South Africa in Stellenbosch. Pieces of wood bearing stroma were immersed

in sterile water for 15 min after which the surface was lightly scraped with a scalpel to expose perithecia. Single perithecia were removed using a sterile needle, placed in a glass bottle containing sterile water (10 mL) and the bottles shaken to release ascospores from the asci. Conidia of *N. australe* (STE-U 7025) and *N. parvum* (STE-U 4439) were produced from pycnidia induced on grapevine shoots using the method of Amponsah *et al.* (2008) with some modifications. Briefly, green lignified shoots (~20 cm) of cultivar Cabernet Sauvignon were inoculated with mycelial plugs on wounds (5 mm) made in the centre of the shoots. The base of the shoots were inserted into glass bottles containing sterile water and incubated in a moist chamber. After 2 weeks, shoot pieces (5 cm) around the wound were excised, surface sterilized, air dried and placed in Petri dishes with moist filter paper and incubated until pycnidia emerged on the surface. Pycnidia were collected using a sterile scalpel, placed in glass bottles containing sterile water and crushed to release the conidia. Before the assays, all conidia and spore suspensions were filtered through sterile cheesecloth to remove mycelial fragments and the concentration adjusted to 2×10^6 conidia or spores mL^{-1}.

Inhibition of conidia/ascospore germination

Effect of the metabolite on inhibition of conidia/ascospore germination was tested on one isolate of each pathogen. Conidia or ascospore suspensions were amended with 6PP, to concentrations of 0, 50, 100, 200, 300 and 400 mg L^{-1} to a total volume of 1·5 mL in 2 mL centrifuge tubes. The final concentration of methanol in the medium was 1%, including in the control tubes. These were incubated at 25°C for 24 h, after which microscope slides were made from the suspensions and spores counted under the microscope (×400; Nikon). Spores were considered germinated when the germ tube was the size of the conidia/ascospore. The percentage germinated conidia/ascospores was determined from at least 50 conidia/ascospores per slide and there were three slides per centrifuge tube and three tubes per concentration.

Statistical analysis

All statistical analyses were carried out using SAS v. 9.2 statistical software (SAS Institute Inc.). The means from each treatment were compared for significant differences using factorial analysis of variance (ANOVA) and the means were separated by computing the Fisher's least significant difference (LSD) at $P = 0{\cdot}05$. For the antifungal assays, data from the two independent repeats was combined and the nonlinear regression model describing the inhibition × concentration interaction (used for EC_{50} determination) as well as levels of sensitivity of the pathogens between isolates were compared by ANOVA. For the effect of growth medium on 6PP inhibition of mycelial growth and the inhibition of spore/conidia germination, treatments within each pathogen were compared separately by analysis of variance followed by Fisher's LSD test at $P = 0{\cdot}05$.

Results

Isolation and identification of secondary metabolites

Five homogenous fractions were obtained from the *T. atroviride* isolates (UST1 and UST2) and seven fractions were obtained from the *T. harzianum* isolate (T77). The first fraction (fraction I) showed similar chromatographic and spectroscopic properties among all isolates and it was also extracted in the highest quantities. The metabolite content of fraction I was 280–340 mg compared to the next largest fraction (21–43 mg), for all isolates. The compound in fraction I showed chromatographic and spectroscopic properties similar to 6-pentyl-α-pyrone (6PP). The metabolite R_f value was 0·65 in hexane:acetone (7:3 v/v). The MS spectral data indicated a protonated molecular ion peak at *m/z* 167·1 and the ^1H NMR spectrum was consistent with that of 6PP reported by Cutler *et al.* (1986).

Effect of culture harvest time on concentration of 6PP

The time course production of 6PP by the wildtype *Trichoderma* isolates is shown in Figure 1. Analysis of variance found significant isolate × culture condition × time interactions ($P < 0{\cdot}001$). The *T. atroviride* isolate UST1 produced 6PP in a faster and more abundant manner under the shaking conditions used, reaching a maximum of 82 mg L^{-1} at Day 10. This was a significantly higher ($P < 0{\cdot}05$) rate than that of the rest of the isolates at that time point (Fig. 1a). In contrast, under static conditions isolate UST2 produced significantly higher ($P < 0{\cdot}05$) quantities of 6PP at all time points, except for Day 20 where the quantity was not significantly ($P > 0{\cdot}05$) different from that of UST1 (Fig. 1b). The *T. harzianum* isolate produced more 6PP in the shaken than static cultures, but in both conditions the 6PP quantities were significantly lower ($P < 0{\cdot}05$) than those of the *T. atroviride* isolates except for Day 20 under shaking conditions (no significant difference with UST1).

Comparison of the production of 6PP by *Trichoderma* isolates

A comparison of the LC-MS total ion chromatograms of the wildtype and mutant isolates is shown in Figure 2. The production of secondary metabolites was dependent on the richness of the medium (full strength or quarter PDB) and the culture conditions (shaken or static). However, 6PP was the most common and abundant metabolite from all the isolates for all media and culture conditions except for the mutant of *T. harzianum*, MT77, which could not produce 6PP. A metabolite with retention time 2·33 min and a molecular weight (M_w) of 726·3795 was found in shaking cultures of UST1 and MT1. This compound was also found in UST2 and MT2 but the peak was much less pronounced and inconsistent, indicating a lower production compared to the UST1 isolate. As *T. atroviride* isolates are also known to produce peptaibiotics (Degenkolb *et al.*, 2008), the compositional analysis data and compound fragmentation pattern was used to find similarities with known compounds belonging to this type of metabolite. A database of peptaibiotics compiled by Stoppacher *et al.* (2013) was downloaded from http://peptaibiotics-database.boku.ac.at. With the aid of the database, the closest match to the compound was found to be with members of the trichocompactin

group ($C_{33}H_{58}N_8O_{10}$; M_w 726), peptaibiotics isolated from *T. brevicompactum* (Degenkolb *et al.*, 2006).

Analysis of variance of the quantities of 6PP produced by each isolate in the different media and culture conditions revealed significant isolate × medium × culture condition interactions ($P < 0{\cdot}001$). In full strength PDB, 6PP was produced by all the isolates (except MT77) in both static and shaken cultures (Fig. 3a) while in quarter strength PDB, UST1 and MT1 could not produce 6PP (Fig. 3b). The isolate UST2 and its mutant, MT2, were the highest producers of 6PP. For the *T. atroviride* isolates 6PP production was either similar ($P > 0{\cdot}05$) or higher ($P < 0{\cdot}05$) in the static than shaken cultures, while in the *T. harzianum* isolate 6PP production was always significantly ($P < 0{\cdot}05$) higher in the shaken than static cultures (Fig. 3).

Effect of growth medium and pathogen co-inoculation on production of 6PP

Analysis of variance showed significant isolate × medium × co-culture interactions ($P = 0{\cdot}038$). The effect of medium and pathogen co-inoculation on 6PP production by the *T. atroviride* isolates is reported in Figure 4. In both isolates, 6PP production was significantly higher ($P < 0{\cdot}05$) in the GCBM compared to the defined Pezet's medium. The production of 6PP was highest when UST1 was co-cultured with *N. parvum* GCBM. Isolate UST2 generally produced more 6PP than UST1 in GCBM, except for when UST1 was co-cultured with *N. parvum*. The co-culturing of UST1 with *N. parvum* resulted in a significant increase ($P < 0{\cdot}05$) of 6PP production in both culture media. This was not observed with UST2, where only a slight increase in 6PP production was observed when it was co-cultured with *N. parvum* in Pezet's medium, but to a level not significantly higher ($P > 0{\cdot}05$) than the control culture. The pathogen *E. lata* had no effect on 6PP production when co-cultured with both *T. atroviride* isolates.

Sensitivity of grapevine trunk pathogens to 6PP

There were no differences in mycelial growth between the two independent experiments ($P > 0{\cdot}05$), so the data from both experiments were combined. The effect of 6PP on both mycelial growth and conidia/ascospore germination was highly significant at all concentrations tested ($P < 0{\cdot}001$).

Mycelial inhibition

Three nonlinear regression models (Gompertz, logistic and modified exponential) were fitted to the mycelial growth inhibition data and all gave good statistical fits ($R^2 > 0{\cdot}798$). The Gompertz (sigmoidal) model consistently gave the highest correlation for all isolates ($R^2 > 0{\cdot}95$, $P < 0{\cdot}01$). Based on the mean EC_{50} values (Table 1), the inhibition of mycelial growth by 6PP varied for the different pathogens. There were no significant differences ($P > 0{\cdot}05$) in the susceptibility of isolates of the same pathogen to 6PP, despite some noticeable differences in the EC_{50} values between the isolates of *N. parvum* ($P = 0{\cdot}057$). *Phaeomoniella chlamydospora* was the least sensitive ($EC_{50} = 91{\cdot}72$ mg L^{-1}) of the trunk pathogens. The mean mycelial growth with time on 6PP amended medium for one of each isolate of the pathogens tested is shown in Figure 5. There was significant ($P < 0{\cdot}01$) reduction in mycelial growth from the lowest concentration tested (50 mg L^{-1}) and mycelial growth was totally inhibited at 400 mg L^{-1} in all pathogens.

Effect of growth medium on mycelial sensitivity to 6PP

There were significant isolate × pathogen × medium interactions ($P < 0{\cdot}001$) for all the pathogens. Inhibition of mycelial growth was dependent on growth medium and the pathogen isolate (Table 2). All the pathogens were more sensitive to 6PP when growing on the nutrient-poor Vogel's N medium. However, there was a variation in the response of the pathogens when they were growing on the other media. Sensitivity to 6PP on Pezet's medium did not significantly differ ($P > 0{\cdot}05$) from that on the complex medium used for the *Neofusicoccum* spp. Inhibition of *P. chlamydospora* on amended MEA was significantly higher ($P < 0{\cdot}05$) than on PDA and GCBM.

Conidia/ascospore germination

Conidia/ascospore germination was significantly ($P < 0{\cdot}001$) reduced by all concentrations of 6PP tested and totally inhibited at 300 and 400 mg L^{-1}. The latter

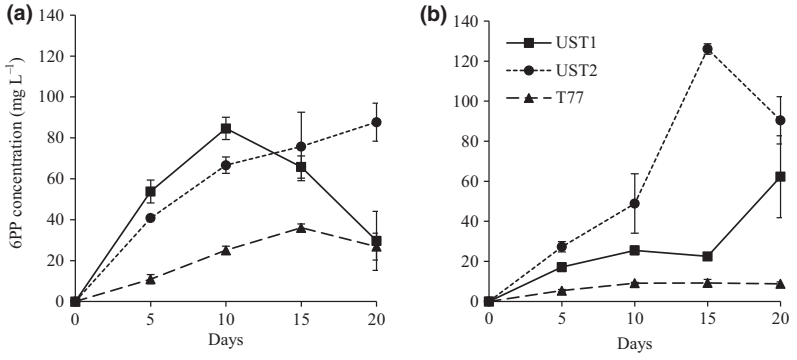

Figure 1 Time course production of 6-pentyl-α-pyrone (6PP) by isolates of *Trichoderma atroviride* (UST1 and UST2) and *Trichoderma harzianum* (T77), used in grapevine pruning wound protection, grown in full strength potato dextrose broth in shaken (a) and static (b) cultures. Each point on the line is the mean ± the standard deviation of three independent biological replicates.

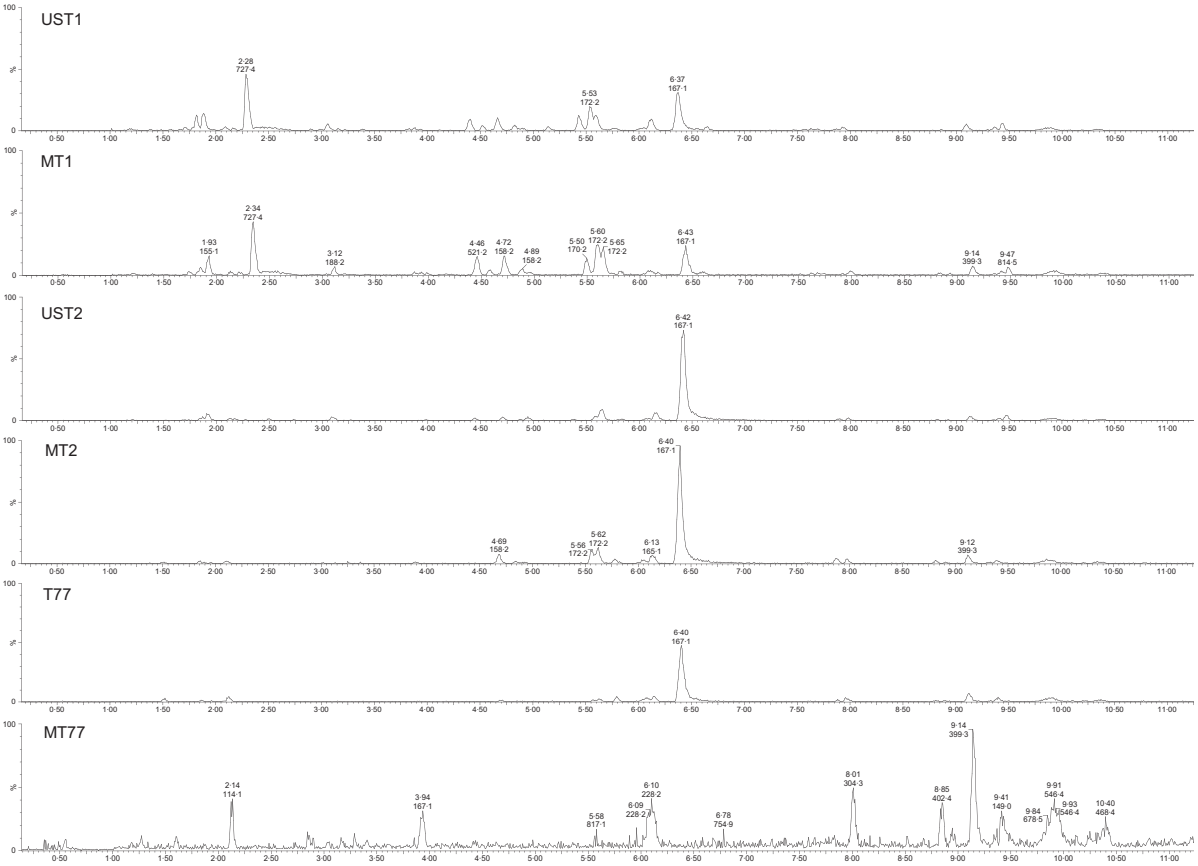

Figure 2 LC-MS chromatograms showing the major secondary metabolite peaks from wildtype *Trichoderma atroviride* (UST1, UST2) and *Trichoderma harzianum* (T77) isolates and their benzimidazole resistant mutants (MT1, MT2 and MT77) grown in full strength potato dextrose broth for 20 days with shaking at 120 rpm. The major secondary metabolite in all isolates except MT77 was 6-pentyl-α-pyrone with a molecular weight of 167·1 (M^+H). Another major peak was observed from UST1 and MT1 with a molecular weight of 727 (M^+H) which was found to be closely related to peptaibiotics of the trichocompactin group.

two concentrations tested were excluded from the analysis of variance for the effect of 6PP concentration on conidia/ascospore germination. The effect of 6PP on conidia/ascospore germination is shown in Figure 6. Germination was inhibited by more than 60% at 100 mg L^{-1} in all pathogens. Due to high variation between treatments, EC_{50} values could not be computed for the sensitivity of conidia/ascospore germination to 6PP.

Discussion

The antagonism of culture filtrates and volatiles produced by *Trichoderma* species used in grapevine pruning wounds has previously been demonstrated (John *et al.*, 2004; Kotze *et al.*, 2011). However, the secondary metabolites responsible for these properties had not been identified or characterized for *Trichoderma* species used in grapevine pruning wounds. This is the first report on the identification of secondary metabolites produced by *Trichoderma* isolates used in grapevine pruning wound protection, and on the characterization of their *in vitro* effect on mycelial growth and spore germination of grapevine trunk pathogens. The metabolite 6-pentyl-α-pyrone (6PP) was the major metabolite produced by all three isolates tested and is a well-known antimicrobial compound produced by *Trichoderma* species (Vinale *et al.*, 2006; El-Hasan *et al.*, 2007). This compound is a volatile and has a characteristic sweet coconut smell that is characteristic of *Trichoderma* species of the section Trichoderma (Dodd *et al.*, 2003). In this study, the production of 6PP was highly dependent on isolate, nutrition and culture conditions. It appears that after reaching a peak, the concentration of 6PP starts to decline. This is in agreement with other studies aimed at maximizing 6PP production *in vitro* (Prapulla *et al.*, 1992; Serrano-Carreón *et al.*, 2004). The fungus producing 6PP is also inhibited by the 6PP at concentrations of 90–110 mg L^{-1} and the addition of resin (amberlite) in the growth medium was found to reduce such inhibition (Prapulla *et al.*, 1992). The *T. atroviride* isolates (UST1 and UST2) produced higher quantities of 6PP than *T. harzianum* (T77). There was a large variation between the *T. atroviride* isolates, with UST2 producing more than UST1 when grown under static (not-shaking) conditions. These iso-

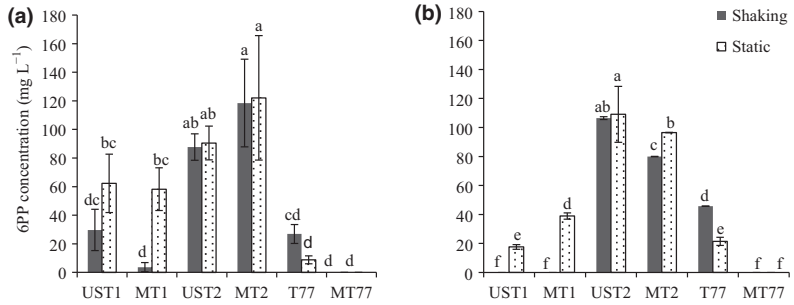

Figure 3 A comparison of 6-pentyl-α-pyrone (6PP) production between wildtype *Trichoderma atroviride* (UST1 and UST2) and *Trichoderma harzianum* (T77) used in grapevine pruning wound protection, and their mutant isolates (MT1, MT2 and MT77). The fungi were grown in full strength potato dextrose broth (PDB) (a) and quarter strength potato dextrose broth (b) for 20 days either shaking or static. The mutants were generated by gamma irradiation and are resistant to benzimidazole fungicides. Each bar is the mean ± the standard deviation of three independent biological replicates. Bars with the same letter on top show means that are not significantly different for each medium. LSD = 39·65 (PDB) and 12·39 (quarter PDB).

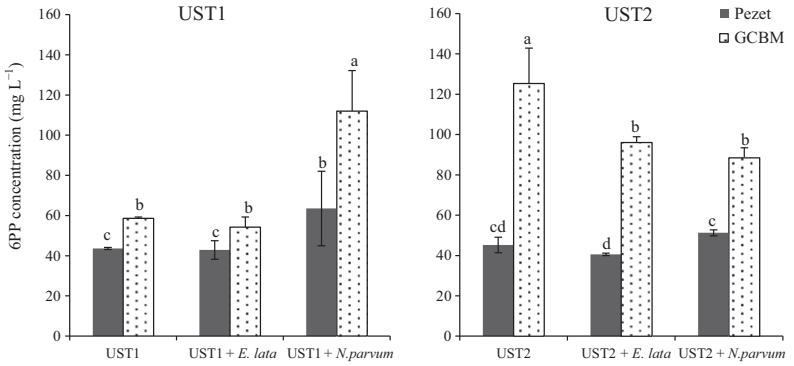

Figure 4 Effect of growth medium (Pezet and grapevine cane-based medium (GCBM)) and the co-inoculation with a pathogen (*Eutypa lata* and *Neofusicoccum parvum*) on the production of 6PP by two isolates of *Trichoderma atroviride*, UST1 and UST2. Each bar is the mean ± the standard deviation of three independent biological replicates. Bars with the same letter on top show means that do not significantly differ according to Fisher's least significant difference (LSD; UST1 = 10·22 and UST2 = 8·72: $P = 0·05$).

lates have been evaluated for their grapevine wound protective effect against infection by trunk pathogens and the *T. atroviride* isolates were found to be better than *T. harzianum* (Kotze *et al.*, 2011; Mutawila *et al.*, 2011), which is probably due to the fact that *T. atroviride* was producing more 6PP. Another metabolite from *T. atroviride* that had a fragmentation pattern similar to that of trichocompactin Ia and Ib isolated from *T. brevicompactum* (Degenkolb *et al.*, 2006) could also be involved in their biocontrol activity. Peptaibiotics from *Trichoderma* are known antibiotics, but the biological activity of the brevicompactum group has not been characterized.

To establish the likelihood of 6PP production during wound colonization, a grapevine cane extract culture medium was used as a close approximation of the natural substrate. Mahoney *et al.* (2003) demonstrated enhanced production of toxins (eutypine and eulatachromene) by *E. lata* isolates grown in a grapevine extract compared to artificial media. The more natural substrate could be representative of secondary metabolite production on the wound. Production of 6PP in the GCBM varied between the two isolates, but was higher in the GCBM compared to the defined medium for both isolates. However, what was particularly interesting was that 6PP production almost doubled in the GCBM when isolate UST1 was co-cultured with *N. parvum*. Previous reports have also demonstrated that the production of secondary metabolites is induced by fungal cell wall material of other fungi (Serrano-Carreón *et al.*, 2004; Vinale *et al.*, 2009). In the current study, the induction of 6PP production was only observed with *N. parvum* and not with *E. lata*. The difference in behaviour of *T. atroviride* towards the two fungi is difficult to explain as it was not possible to measure the growth of the *T. atroviride* isolates in liquid co-cultures. Unlike UST1, the co-cultivation of UST2 with *N. parvum* in GCBM did not result in a significant increase in 6PP production. *In vitro* assays showed that UST1 was more aggressive than UST2. UST1 overgrew and inhibited most grapevine trunk pathogens in dual plate assays (Kotze *et al.*, 2011), while chitinase assays also revealed higher activities in UST1 compared to UST2 (authors' unpublished data).

Reports by Worasatit *et al.* (1994), Ghisalberti *et al.* (1990) and Pezet *et al.* (1999) on the mechanisms of action of *Trichoderma* spp. have associated pyrone production with biocontrol activity. In the current study, the high production of 6PP in the GCBM strongly suggests the involvement of 6PP in the protection of wood from infection by wood rotting fungi. Volatile compounds from *T. harzianum* isolates used in Vinevax® had fungistatic effects on *E. lata* mycelium, while the cultural filtrates completely inhibited the pathogen mycelial growth (John *et al.*, 2004). More recently, Kotze *et al.* (2011) further demonstrated the inhibitory effects of *T. harzianum* and *T. atroviride* on more grapevine trunk pathogens *in vitro* and the wound protective effect of the *Trichoderma* spp. isolates *in vivo*. None of these reports identified the compounds that were responsible for the observed effect and the inhibitory concentrations of 6PP on grapevine trunk pathogens are comparable to those reported for *Botrytis cinerea* (Walter *et al.*, 2000) and *Fusarium verticillioides* (syn. *F. moniliforme*) (El-Hasan *et al.*, 2007). Conidia/ascospore germination was more sensitive to 6PP compared to mycelium growth on all the grapevine trunk pathogens tested. However, 6PP was not equally effective on the various fungal pathogens assayed, in terms of mycelium growth inhibition. As pruning wound infection occurs mainly through conidia/ascospores, a higher inhibitory effect on conidia/ascospore germination by 6PP is more likely to result in reduced infection of *Trichoderma* protected wounds. However, the reduced effect on mycelial growth may also suggest that 6PP-producing *Trichoderma* are less likely to eliminate pathogens already growing in the pruning wound. Better wound protection is achieved when a longer time is allowed between the application of *Trichoderma* biocontrol agents and pathogen inoculation (Munkvold & Marois, 1993; John *et al.*, 2008). This is related to the need by the *Trichoderma* biocontrol agent to grow on the

Table 1 Sensitivity of grapevine trunk pathogens to the secondary metabolite 6-pentyl-α-pyrone (6PP) of *Trichoderma* spp. based on *in vitro* inhibition of the mycelium

Pathogen	Isolate STE-U no.	EC_{50} (mg L^{-1}) of 6PP[a]	
		EC_{50}	Mean EC_{50}[b]
Eutypa lata	5692	48·41	47·41 ± 1·41
	6513	46·41	
Phaeomoniella chlamydospora	6384	90·02	91·72 ± 1·70
	7732	93·42	
Neofusicoccum australe	7025	46·99	47·96 ± 1·54
	7029	48·92	
Neofusicoccum parvum	4439	48·60	46·04 ± 3·62
	4584	43·48	

[a]The EC_{50} is the effective concentration of 6PP that inhibited radial growth by 50%. EC_{50} values are compared to the control with solvent only (0·1% methanol), computed from a Gombertz (sigmoid) function (for all isolates $R^2 > 0{\cdot}956$; $P < 0{\cdot}001$).

[b]EC_{50} ± SE of mean of each isolate from two independent experiments.

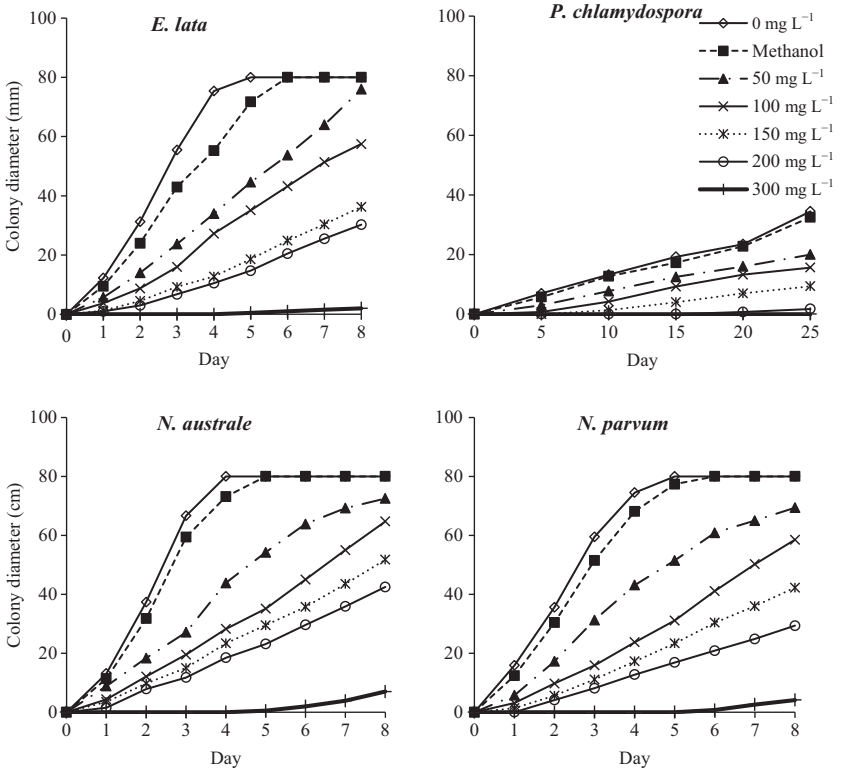

Figure 5 Mean mycelial growth inhibition with time, of grapevine trunk pathogens (*Eutypa lata*, *Phaeomoniella chlamydospora*, *Neofusicoccum australe* and *Neofusicoccum parvum*) on potato dextrose agar amended with varying concentrations (0–300 mg L^{-1}) of 6-pentyl-α-pyrone.

Table 2 Effect of growth medium on sensitivity of grapevine trunk pathogens to 6-pentyl-α-pyrone (6PP), a secondary metabolite from *Trichoderma* spp.

	Inhibition of mycelial growth of pathogen isolates (%)[b]							
	Eutypa lata		*Phaeomoniella chlamydospora*		*Neofusicoccum australe*		*Neofusicoccum parvum*	
Medium[a]	5692	6513	6384	7732	4439	4584	7025	7029
PDA	82·48 c	80·31 c	70·01 e	60·52 f	73·97 c	78·74 b	78·26 c	78·13 cd
MEA	73·87 e	77·01 d	78·07 d	87·35 bc	64·23 e	72·00 cd	75·48 def	76·80 cde
GCBM	69·45 f	76·32 d	68·50 e	62·68 f	64·87 e	69·46 d	69·88 h	74·16 efg
Pezet's	92·05 b	100 a	85·74 c	85·27 c	72·19 cd	72·96 c	71·56 gh	73·13 gf
Vogel's N	100 a	100 a	92·09 a	90·49 ab	83·75 a	81·56 ab	93·75 b	96·88 a
LSD[c]	2·26		2·98		2·70		4·62	

PDA, potato dextrose agar; MEA, malt extract agar; GCBM, grapevine cane-based medium.
[a]Pathogens were grown on medium amended with 150 mg L^{-1} 6PP.
[b]Values followed by the same letter are not significantly different for the same pathogen.
[c]Data was analysed by ANOVA followed by Fisher's LSD test at $P = 0{\cdot}05$ for each pathogen separately.

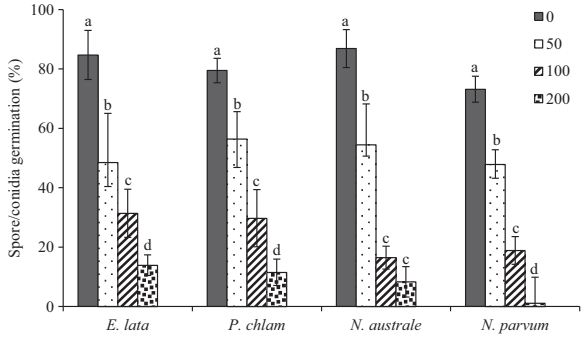

Figure 6 Inhibition of ascospore and conidia germination by varying concentration (0–200 mg L^{-1}) of 6-pentyl-α-pyrone (6PP) on grapevine trunk pathogens *Eutypa lata*, *Phaeomoniella chlamydospora*, *Neofusicoccum australe* and *Neofusicoccum parvum*. Percentages of germinated ascospores/conidia are means of three replicates of two independent experiments. Bars with the same letter on top show no significant differences in the percentage germination within a pathogen according to Fisher's least significant difference (LSD; *E. lata* = 13·82, *P. chlamydospora* = 10·74, *N. australe* = 12·42 and *N. parvum* = 9·91: $P = 0{\cdot}05$).

wound and start responding to stimuli such as the presence of competing microbes. The importance of 6PP in the *Trichoderma*–pathogen interaction is further suggested by the reduction/loss of *in vitro* antagonistic ability of the mutant MT77 that could not produce 6PP. Although there was little variation in the sensitivity of isolates of the same pathogen to 6PP, in the natural populations such variation may occur. Walter *et al.* (2000) produced mutant isolates of *B. cinerea* by UV irradiation that were tolerant to high concentrations of 6PP, but the tolerance to 6PP was lost after growth on plant material (kiwifruit slices). Some fungi that can break down 6PP to less toxic compounds have also been reported (Cooney & Lauren, 1999). However, because biological control agents have more than one mechanism of action, resistance to the biocontrol agent is not likely to develop. Testing of sensitivity of *B. cinerea* to pyrrolnitrin, an antifungal substance produced by a biocontrol strain of *Pseudomonas chlororaphis*, revealed a wide range of sensitivity to the compound, but all isolates were equally sensitive to the bacteria (Ajouz *et al.*, 2011). This indicates the involvement of other mechanisms and the importance of having more than one mode of action.

The concentrations of the 6PP *in situ* production are likely to be higher at the microzone of interaction between the competing fungi (*Trichoderma* and pathogen), and are not toxic to the plant. It is likely that 6PP will continue to be produced for as long as the *Trichoderma* persists on the plant, which suggests the importance of selecting a strong producer of 6PP for biocontrol applications on grapevine trunk pathogens.

Trichoderma species are amongst the most commercialized fungal genus of biocontrol agents, and, as far as is known, represent the only biological active principle registered so far for the control of grapevine trunk diseases. The selection of potential biocontrol agents is largely based on the ability to antagonize pathogens *in vitro*, by using, among other mechanisms, the activity of cell wall degrading enzymes (Harman *et al.*, 2004). However, the production of secondary metabolites may be equally important for some pathosystems and should also be considered in selection of 'elite' biocontrol agents. The search for bioactive compounds from biocontrol agents should continue to be an important branch of traditional biotechnology, especially in the light of a necessary reduction in the use of chemical fungicides. Secondary metabolites produced by beneficial fungi such as *Trichoderma* spp. are known to be highly biodegradable and may provide opportunities to discover novel and safer biofungicides. In addition, it would be interesting to investigate the effect of *Trichoderma* secondary metabolites on the ability of the pathogen to produce toxins that are recognized as key virulence/pathogenicity factors.

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References

Ajouz S, Walker A, Fabre F, Leroux P, Nicot P, Bardin M, 2011. Variability of *Botrytis cinerea* sensitivity to pyrrolnitrin, an antibiotic produced by biological control agents. *BioControl* **56**, 353–63.

Amponsah N, Jones E, Ridgway H, Jaspers MV, 2008. Production of *Botryosphaeria* species conidia using grapevine green shoots. *New Zealand Plant Protection* **61**, 301–5.

Chapuis L, Richard L, Dubos B, 1998. Variation in susceptibility of grapevine pruning wound to infection by *Eutypa lata* in south-western France. *Plant Pathology* **47**, 463–72.

Cooney J, Lauren D, 1999. Biotransformation of the *Trichoderma* metabolite 6-*n*-pentyl-2*H*-pyran-2-one (6PAP) by selected fungal isolates. *Journal of Natural Products* **62**, 681–3.

Cutler H, Cox R, Crumley F, Cole P, 1986. 6-Pentyl-α-pyrone from *Trichoderma harzianum*, its plant growth inhibitory and antimicrobial properties. *Agricultural Biology and Chemistry* **50**, 2943–5.

Degenkolb T, Grafenham T, Nirenberg H, Gams W, Bruckner H, 2006. *Trichoderma brevicompactum* complex: rich source of novel and recurrent plant-protective polypeptide antibiotics. *Journal of Agricultural and Food Chemistry* **54**, 7047–61.

Degenkolb T, von Döhren H, Nielsen KF, Samuels GJ, Brückner H, 2008. Recent advances and future prospects in peptaibiotics, hydrophobin and mycotoxin research, and their importance for chemotaxonomy of *Trichoderma* and *Hypocrea*. *Chemistry and Biodiversity* **5**, 671–80.

Di Marco S, Osti F, Cesari A, 2004. Experiments on the control of esca by *Trichoderma*. *Phytopathologia Mediterranea* **43**, 108–15.

Dodd S, Lieckfeldt E, Samuels G, 2003. *Hypocrea atroviridis* sp. nov., the teleomorph of *Trichoderma atroviride*. *Mycologia* **95**, 27–40.

El-Hasan F, Walker F, Schone J, Buchenauer H, 2007. Antagonistic effect of 6-pentyl-alpha-pyrone produced by *Trichoderma harzianum* toward *Fusarium moniliforme*. *Journal of Plant Diseases and Protection* **114**, 62–8.

Ghisalberti E, Narbey M, Dewan M, Sivasithamparam K, 1990. Variability among strains of *Trichoderma harzianum* in their ability to reduce take-all to produce pyrones. *Plant and Soil* **121**, 287–91.

Gubler WD, Rolshausen PE, Trouillas FP *et al*., 2005. Grapevine trunk diseases in California. *Practical Winery and Vineyard* **Feb**, 6–25.

Harman GE, 2006. Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathology* **96**, 190–4.

Harman GE, Howell CR, Viterbo A, Chet I, Lorito M, 2004. *Trichoderma* species – opportunistic, avirulent plant symbionts. *Nature Reviews, Microbiology* **2**, 43–56.

Howell CR, 2006. Understanding the mechanisms employed by *Trichoderma virens* to effect biological control of cotton diseases. *Phytopathology* **9**, 178–80.

John S, Scott ES, Wicks T, Hunt J, 2004. Interactions between *Eutypa lata* and *Trichoderma harzianum*. *Phytopathologia Mediterranea* **43**, 95–104.

John S, Wicks TJ, Hunt JS, Scott ES, 2008. Colonisation of grapevine wood by *Trichoderma harzianum* and *Eutypa lata*. *Australian Journal of Grape and Wine Research* **14**, 18–24.

Kotze C, Van Niekerk JM, Halleen F, Fourie PH, 2011. Evaluation of biocontrol agents for grapevine pruning wound protection against trunk pathogen infection. *Phytopathologia Mediterranea* **50** (Supplement), S247–63.

Larignon P, Fontaine F, Farine S, Clément C, Bertsch C, 2009. Esca et Black Dead Arm, deux acteurs majeurs des maladies du bois chez la vigne. *Vigne Comptes Rendus Biologies* **332**, 765–83.

Mahoney N, Lardner R, Molyneux R, Scott ES, Smith L, Schoch T, 2003. Phenolic and heterocyclic metabolite profiles of the grapevine pathogen *Eutypa lata*. *Phytochemistry* **64**, 475–84.

Metzenberg R, 2003. Vogel's medium N salts, avoiding the need for ammonium nitrate. *Fungal Genetics Newsletter* **50**, 2–3.

Munkvold G, Marois J, 1993. Efficacy of natural epiphytes and colonizers of grapevine pruning wounds for biological control of Eutypa dieback. *Phytopathology* **83**, 624–9.

Mutawila C, Fourie PH, Halleen F, Mostert L, 2011. Grapevine cultivar variation to pruning wound protection by *Trichoderma* species against trunk pathogens. *Phytopathologia Mediterranea* **50** (Supplement), S264–76.

Mutawila C, Halleen F, Mostert L, 2014. Development of benzimidazole resistant *Trichoderma* strains for the integration of chemical and biocontrol methods of grapevine pruning wound protection. *BioControl*. doi: 10.1007/s10526-014-9647-y

Pezet R, 1983. *Rapport interne*. Suisse: Station Fédérale de Changins.

Pezet R, Pont V, Tabacchi R, 1999. Simple analysis of 6-pentyl-alpha-pyrone, a major antifungal metabolite of *Trichoderma* spp, useful for testing the antagonistic activity of these fungi. *Phytochemical Analysis* **10**, 285–8.

Prapulla S, Karanth N, Engel K, Tressl R, 1992. Production of 6 pentyl-alpha-pyrone by *Trichoderma viride*. *Flavour Fragrance Journal* **7**, 231–4.

Serrano-Carreón L, Flores C, Rodriguez B, Galindo E, 2004. *Rhizoctonia solani*, an elicitor of 6-pentyl-alpha-pyrone production by *Trichoderma harzianum* in a two liquid phases, extractive fermentation system. *Biotechnology Letters* **26**, 1403–6.

Shwab E, Keller N, 2008. Regulation of secondary metabolite production in filamentous ascomycetes. *Mycological Research* **112**, 225–30.

Sivasithamparam K, Ghisalberti E, 1998. Secondary metabolism in *Trichoderma* and *Gliocladium*. In: Kubicek C, Harman G, eds. Trichoderma *and* Gliocladium. London, UK: Taylor and Francis, 139–91.

Stoppacher N, Neumann N, Burgstaller L *et al*., 2013. The comprehensive peptaibiotics database. *Chemistry and Biodiversity* **10**, 734–43.

Van Niekerk JM, Halleen F, Fourie PH, 2011. Temporal susceptibility of grapevine pruning wounds to trunk pathogen infection in South African grapevines. *Phytopathologia Mediterranea* **50** (Supplement), S139–50.

Vinale F, Marra R, Scala F, Ghisalberti EL, Lorito M, Sivasithamparam K, 2006. Major secondary metabolites produced by two commercial *Trichoderma* strains active against different phytopathogens. *Letters in Applied Microbiology* **43**, 143–8.

Vinale F, Sivasithamparam K, Ghisalberti EL, Marra R, Woo SL, Lorito M, 2008. *Trichoderma*–plant-pathogen interactions. *Soil Biology and Biochemistry* **40**, 1–10.

Vinale F, Ghisalberti EL, Sivasithamparam K *et al*., 2009. Factors affecting the production of *Trichoderma harzianum* secondary metabolites during the interaction with different plant pathogens. *Letters in Applied Microbiology* **48**, 705–11.

Walter M, Boyd-Wilson K, Perry JH, Hill RA, 2000. *Botrytis* tolerance to 6-pentyl-alpha-pyrone and massoialactone. *New Zealand Plant Protection* **53**, 375–81.

Woo SL, Ruocco M, Vinale F *et al*., 2014. *Trichoderma*-based products and their widespread use in agriculture. *The Open Mycology Journal* **8**, 71–126.

Worasatit N, Sivasithampram K, Ghisalberti E, Rowland C, 1994. Variation in pyrone production, lytic enzymes and control of rhizoctonia root rot of wheat among single-spore isolates of *Trichoderma koningii*. *Mycological Research* **98**, 1357–63.