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FINAL REPORT
2014

Programme & Project Leader Information

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Project Information

Research Organisation Project number	USPP 01/2009
Project title	Improving pruning wound protection against trunk disease pathogens

Fruit kind(s)	Wine grapes, Table grapes		
Start date (mm/yyyy)	01/2010	**End date** (mm/yyyy)	12/2013

Project keywords	Pruning wounds, *Trichoderma*, sucker wounds, trunk diseases

Approved by Research Organisation Programme leader (tick box) ☐

THIS REPORT MUST INCLUDE INFORMATION FROM THE **ENTIRE** PROJECT

Executive Summary

Trichoderma products can be used to prevent trunk disease infections of grapevine pruning wounds. This project aimed to improve the efficacy of *Trichoderma* based products and to better understand the mechanisms of control. Field trials showed that the best time to apply *Trichoderma* was six hours after pruning. Application with a back pack sprayer and paint brush gave the best results in comparison with a gator application. Several additives were tested to improve the formulation of the *Trichoderma* product, but were not significantly better than the *Trichoderma* and water mixture. Benzimidazole resistant *Trichoderma* isolates were generated by gamma irradiation from the wild type isolates. A field trial proved the concept of combining both carbendazim and *Trichoderma* MT1 on a pruning wound. Both T1 and MT1 *Trichoderma atroviride* isolates hold the potential to be used for grapevine pruning wound protection and could be commercialised.

The role of sucker wounds in trunk disease epidemiology was investigated. Glass house as well as field trials showed that sucker wounds are susceptible to trunk disease pathogens. A survey of sucker wounds from both wine and table grape cultivars in different regions in the Western Cape also showed that the sucker wounds were naturally infected by trunk disease pathogens. A field trial showed the potential for the use of *Trichoderma* products for the protection of sucker wounds from infection of trunk disease pathogens. *In vitro* assays to assess the sensitivity of *Trichoderma* towards fungicides used during spring in vineyards showed that seven of the fungicides inhibited *Trichoderma* less than 50%. However, the alternate or combined application of these fungicides with *Trichoderma* based products should first be tested in the field.

Several novel findings were made in understanding the mechanisms of *Trichoderma* spp. used for pruning wound protection. The main secondary metabolite produced by our *Trichoderma* isolates was 6-pentyl-α-pyrone. This compound successfully inhibited trunk disease pathogens with *in vitro* tests. The grapevine host reaction towards *Trichoderma* and a pathogen (*Eutypa lata*) was tested on grapevine cell cultures with real-time PCR. *Trichoderma* activated several of the defence related genes that was also activated by the pathogen, showing that the biocontol agent can prime the plant prior to pathogen infection which in turn will aid the plant to prevent infection from the pathogen.

Problem identification and objectives

Grapevine trunk diseases compromise the sustainability of viticulture. It has also been proven that pruning wounds serve as the primary ports of infection of grapevines by trunk pathogens. The role of sucker wounds in spring time as portals for trunk disease pathogens is uncertain. Although some chemicals can protect wounds it has also been shown that such protection is short-lived. Biological control offers more sustainable protection of pruning wounds. Two *Trichoderma atroviride* isolates USPP-T1 and USPP-T2, which were obtained and evaluated in the Winetech- and DFPT-funded research project USPP 04/2004, proved to be very effective wound protectants. In order to make USPP-T1 and USPP-T2 available to grape producers to effectively protect pruning wounds, further research to understand the mechanism of control, formulation of the product and best application practices is required.

Objectives of this study included:

A. Improve the practical application of *Trichoderma*

1. To determine the best formulation of the *Trichoderma* product.
2. To determine the best time and method of application.
3. Development of fungicide resistant *Trichoderma* isolates.

4. Investigate the influence of cultivar towards *Trichoderma* efficacy.

B. Establish the role of sucker wounds as portals for trunk disease infections
1. Survey sucker wounds of table and wine grapes.
2. Assess susceptibility of sucker wounds with artificial inoculation in a controlled environment (glasshouse) and in the field.
3. Assess the duration of sucker wound susceptibility in the field.
4. Determine the sensitivity of *Trichoderma* isolates towards fungicides used during spring time.
5. Field application of *Trichoderma* to assess efficacy in protecting sucker wounds.

C. Investigate the host-pathogen-*Trichoderma* interactions
1. To investigate the secondary metabolites secreted by *T. atroviride* and *T. harzianum* and their effect on trunk disease pathogens.
2. To determine grapevine host resistance response to secondary metabolites and *Trichoderma* species.
3. The histological wound response in grapevine following infection by *T. atroviride* and *T. harzianum*.

Workplan (materials and methods)

A. Improve the practical application of *Trichoderma*

1. To determine the best formulation of the *Trichoderma* product.

Fungal isolates and inoculum preparation
Of the two *Trichoderma* strains that were tested in the project USPP 04/2004, T1 was chosen to work with further since it performed better in the field trials of Kotze et al. (2008). *Trichoderma atroviride* (UST1) and *Pa. chlamydospora* isolates are stored at the University of Stellenbosch, Department of Plant Pathology culture collection under accessions STE-U 6514 and 6384, respectively. A registered pruning wound protection biocontrol agent Eco 77® was kindly provided by Plant Health Products, South Africa. The fungal isolates were maintained in tubes of sterile deionised water at 4 °C. The fungi were sub-cultured onto freshly prepared potato dextrose agar (PDA; Biolab, Wadeville, South Africa) and allowed to grow for 5 days at 25 °C in the dark.

Conidial suspensions of the *Trichoderma* isolate were prepared from 7-day-old cultures growing on PDA by adding sterile distilled water (10 mL) to each culture and scraping the surface to dislodge conidia with a sterile loop. *Phaeomoniella chlamydospora* conidia were produced by growing the fungus on PDA for 3 weeks at 25 °C. Conidial suspensions were prepared by flooding the Petri dishes with sterile water (10 mL) and the conidia dislodged using sterile loop and the suspension collected in sterile glass bottles. Conidial suspensions were filtered through sterile double cheesecloth to remove mycelial fragments. The concentrations were determined with a haemocytometer and adjusted to 5×10^4 conidia/mL for *Pa. chlamydospora* and 10^8 conidia/mL for UST1. The suspension for Eco 77® was prepared according to label instructions (0.5 g/L).

In vitro* effect of nutrients on the growth of *T. atroviride
The effect of nutrient sources on hyphal growth of *T. atroviride* UST1 was determined in Petri dish assays. A basal medium containing 0.2% KH_2PO_4, 0.2% $MgSO_4$. $7H_2O$ and 10 g/L agar (Biolab) was amended with variable concentrations of urea (0.1, 0.2, 0.3, 0.4 g/L), yeast extract (2, 3, 5 g/L) and glucose (1, 2, 3, 5, 10 g/L). Hyphal growth on all possible combinations of the nutrient supplements was tested at pH 5.5. Petri dishes were inoculated with a 5 mm diameter

mycelial plug taken from the margins of an actively growing 48-hour-old culture. Colony diameter was measured daily for 3 days. Each amendment and concentration was tested in triplicate. The colony growth data was used to select the concentrations to use for nutritional amendment of UST1 suspensions for the glasshouse trials.

Detached grapevine cane assay: Effect of biocontrol enhancers on *T. atroviride* wound colonisation and wound protection

Dormant 1-year-old canes (10-15 mm-diameter) of Chenin blanc were obtained from a certified nursery. The four-node-length canes were hydrated by soaking in water for 4 hours after which they were surface sterilised by dipping in a quaternary ammonium compound (Sporekill™, ICA International Chemicals (Pty) Ltd, Stellenbosch, South Africa) at 150 mL/100 L for 5 minutes and dried at room temperature. The canes were subjected to a hot water treatment at 50 °C for 30 minutes and then grown in a hydroponic system at ± 25 °C until budding had occurred. The hydrponic system consisted of PVC pipes that had slots (~1.5 cm diameter) that held the canes in an upright postion. The water in the hydroponic system was changed twice weekly and a hydroponic fertilizer Chemicult® (Chemicult Products (Pty) Ltd, Camp's Bay, South Africa) was added once a week at the recommended rate. After budding, the distal nodes were removed by pruning approximately 10 mm above the third node. Each wound was separately treated with *T. atroviride* UST1 conidial suspension made in sterile water with or without biocontrol enhancers. The nutritional amendments were yeast extract (3 g/L), urea (0.4 g/L) and combinations of yeast extract and urea with or without glucose (2 g/L). The effect of a humectant (water storing gel) and colloidal chitin was also tested. The humactant, (Luquasorb® FP 800, BASF SE Ludwigshafen, Germany) was added to the UST1 suspension at 4 g/L, with or without the nutritional amendments to make a thick paste. A combination of the humectant and colloidal chitin (preparation detailed below) was also included as a treatment. Control treatments received sterile water only. In all treatments with UST1, the biocontrol agent was applied at a concentration of 10^8 conidia/mL. All treatments except the pastes were sprayed as a single application using a hand held 500 mL trigger spray bottle while the humectant containing pastes were painted on to the wound using a 10 mm paint brush. Approximately 1000 conidia of the trunk pathogen, *Pa. chlamydospora* (20 µL of 5×10^4 conida/mL), were inoculated on the pruning wounds 1 day after treatment. The trial layout was a randomised block design with three blocks. Each treatment was applied to wounds on 15 canes (five per hydroponic pipe) and was randomly assigned to canes. Canes were maintained for 90 days after which fungal isolation was carried out.

Canes were surface sterilised by immersion in 70% ethanol for 30 seconds, then in 3.5% sodium hypochlorite for 1 minute and in 70% ethanol for 30 seconds and aseptically split longitudinally into two. Four wood tissue sections, one from either side of the pith of each half of the split cane, were aseptically removed and placed onto a 90 mm Petri dish containing PDA. Isolations were made from the wound interface of the live and dead tissue and at 10 mm intervals up to 40 mm below the interface. Petri dishes were incubated at 25 °C for 8 hour under white light and 16 hours in darkness for 2-4 weeks with sub-culturing to prevent overgrowth of emerging colonies. The fungi were identified and their incidence and frequency of isolation at each isolation point recorded. The frequency of isolation estimates the extent to which the wound/cane is colonised by *T. atroviride* or *Pa. chlamydospora*.

Field evaluation: Effect of biocontrol enhancers on *T. atroviride* wound colonisation and wound protection

Treatments, from the detached cane assay, that gave better *Trichoderma* wound colonization were tested further in field trials conducted in 2011 and 2012. Two additional treatments not tested in the detached cane assay were included, one containing the *T. atroviride* conidial suspension in a culture filtrate and a pruning wound paint. The culture filtrate used was prepared in minimal broth medium with colloidal chitin as the only carbon source and was termed broth. The harvest time of the culture filtrate was optimised using chitinolytic activity of the culture filtrates.

Optimisation of the broth formula: First colloidal chitin was prepared from crab-shell chitin (Sigma). A 20 g sample of crab-shell chitin was dissolved in cold concentrated HCl (350 mL) and placed at 4 °C for 24 hours with stirring. The mixture was filtered through glass into 2 L ethanol (95%) at -20 °C with rapid stirring. The resulting chitin suspension was centrifuged at 10 000 rpm for 15 minutes at 4 °C. The colloidal chitin pellets were washed repeatedly with water until the pH of the supernatant was neutral. Colloidal chitin was autoclaved and kept at 4 °C until it was used.

Secondly, the effect of adding a nitrogen source (peptone) on the chitinolytic activity of the *T. atroviride* UST1 culture filtrate was determined. The fungus was grown in 100-mL Erlenmeyer-flasks containing 100 mL of synthetic medium with colloidal chitin as the only carbon source. The medium was composed of, per litre: KH_2PO_4, 0.68 g; K_2HPO_4, 0.87 g; KCl, 0.20 g; NH_4NO_3, 1 g; $CaCl_3$, 0.20 g; $MgSO_4$, $7H_2O$, 0.20 g; $FeSO_4$, 0.002 g; $ZnSO_4$, 0.002 g; $MnSO_4$, 0.002 g and 15 g colloidal chitin with or without peptone (4.20 g/L). Flasks were inoculated with 1-mL conidial suspension of UST1 (10^6 conidia/mL) freshly prepared from 7-day-old cultures on PDA. Flasks were incubated for 48 hours on a rotary shaker at 120 rpm after which the culture filtrate was assayed for chitinolytic enzyme activity. The cultures were vacuum filtered through Whatman No. 1 filter paper (Whatman, Brentford, UK), then centrifuged at 10 000 rpm for 10 minutes and the clear supernatant was used as enzyme extract.

Chitinolytic activity was determined using colloidal chitin as substrate. The reaction mixture contained 0.5 mL of enzyme extract, 0.5 mL of 0.5% colloidal chitin and 1 mL of 50 mM potassium phosphate buffer pH 5.5. The mixture was kept in a water bath at 40 °C for 1 hour and the reactions were stopped by the addition of 3 mL dinitrosalicylic acid (DNS) reagent followed by heating at 100 °C for 10 minutes with 40% Rochelle's salt solution. The reducing sugars released were measured by the DNS method (Miller, 1959) at 530 nm using N-acetyl glucosamine as a standard. One unit (U) of chitinolytic activity was defined as the amount of enzyme which catalyzed the release of 1 µg of reducing sugar per millilitre per minute under the reaction conditions.

Lastly, the optimal harvest time for the culture filtrates was determined by growing *T. atroviride* UST1 in 1.5% colloidal chitin medium with peptone as described above. Cultures were incubated at 25 °C on a shaker at 120 rpm in the dark. Three replicates were harvested at 24-hour intervals for 120 hours. Chitinolytic activity was assayed for each day and the time with highest enzyme activity was established as the optimal broth harvest time for culture filtrates. The culture filtrate was harvested by vacuum filtration through Whatman No. 1 filter paper and the filtrate was used for suspending UST1 conidia. The conidial formulation in the chitin culture filtrate was termed the broth.

Field evaluation 2011: Field trials were conducted in two commercial vineyards, a wine grape cultivar, Chenin blanc, and table grape cultivar, Thompson Seedless, situated in Stellenbosch and Wellington in the Western Cape Province of South Africa, respectively. Both vineyards were 7 year-old when the field trials were established. The Chenin blanc was spur pruned while the Thompson Seedless was cane pruned. Wounds were treated with *T. atroviride* UST1 conidia suspensions made in sterile water with or without amendment. The nutritional amendments were yeast extract (3 g/L) and combinations of yeast extract and urea (0.4 g/L), and yeast extract, urea and glucose (2 g/L). A treatment comprising of UST1 conidial suspension made in a culture filtrate was included and was termed the broth treatment described above. Treatments with Eco 77 and a fungicide based pruning wound paste, Garrison (2.5 g/L cyproconazole + 1 g/L iodocarb, Chemcolour Industries, Christchurch, New Zealand) as well as two sterile water controls were also included. Treatments were applied to fresh pruning wounds within 30 minutes of pruning by spraying with 500 mL spray bottles for *Trichoderma* suspensions while

the paste was painted onto the wounds with a brush applicator supplied by the manufacturer. All treated wounds were inoculated with approximately 1000 spores of the *Pa. chlamydospora* after 24 hours of pruning except for one sterile water control which was not inoculated.

Field evaluation 2012: To assess if the nutritional amendments can shorten the time needed for wound colonisation and protection after *Trichoderma* application, a trial was established in which *Pa. chlamydospora* was inoculated at different times after pruning. The trial was carried out on Chenin blanc and contained the same wound treatments as in the 2011 trial. However, the treatments were applied on the wounds 6 hours after pruning as this was found to provide better pruning wound colonisation by *Trichoderma* spp. (Chapter 3). The pathogen, *Pa. chlamydospora*, was inoculated at three time intervals, 1-, 3- and 7-days, after pruning. Eight months after application, the wounds were pruned below the apical shoot and taken to the laboratory for fungal isolation. Fungal isolation was carried out after surface sterilisation as described above (4.3.3) except that wood pieces were removed from two positions, the interface between the dead and the live tissue, and 10 mm below the interface.

Identification of fungi isolated from pruning wounds: Fungal cultures were identified on cultural and morphological characters as species of the Botryosphaeriaceae (Van Niekerk *et al*., 2004), Diatrypaceae (Trouillas *et al*., 2010), *Phomopsis* species (Van Niekerk *et al*., 2005), *Pa. chlamydospora* (Crous & Gams, 2000) and *Phaeoacremonium* spp. (Mostert *et al*., 2006).

Experimental design and data analysis

All field trials were laid out as a randomised block design with four blocks. The experimental unit was a pruning wound. Each treatment combination (treatment and pathogen) was replicated on four vines per block with four wounds receiving the same treatment per vine to make a total of 16 wounds per treatment combination per block.

The incidences of *Trichoderma* and the pathogens were recorded as percentages of the total number of pruning wounds inoculated with each specific treatment. For wounds that were not artificially inoculated with the pathogen a general pathogen incidence was recorded for natural infection of the trunk pathogens. The incidence and frequency of isolation data were subjected to analysis of variance (ANOVA) and the means compared by Fischer's least significant difference (LSD) at $P = 0.05$. Analysis was performed with the SAS version 9.2 statistical software (SAS Institute Inc, Cary, North Carolina, USA). Pathogen reduction (Pr) was calculated as: $Pr = 100 ((Pc - Pt) / Pc)$, in which Pc is the mean pathogen incidence in the water control and Pt is the mean pathogen incidence in the given treatment.

2. To determine the best time and method of application.

Best time of application

Trichoderma atroviride isolate UST1 was isolated from grapevine pruning wounds in Stellenbosch, South Africa and has been shown to have pruning wound protective effect (Kotze *et al*., 2011). The isolate is stored at Stellenbosch University, Department of Plant Pathology culture collection accession STE-U 6514. Conidial suspensions were prepared from 7-day-old cultures growing on potato dextrose agar (PDA) (Biolab, Wadeville, South Africa) by dislodging conidia with a sterile loop and filtered through sterile cheese cloth to remove mycelium fragments. Conidia were counted using a haemocytometer and the concentration adjusted to 10^8 conidia/mL. A registered pruning wound protection biocontrol agent Eco-77® based on *T. harzianum* was kindly provided by Plant Health Product (South Africa) and was applied at the recommended rate of 0.5 g/L. For each application conidial suspensions were freshly prepared

and after the application, 20 µL of left over suspension were spread plated on PDA and incubated for 24 hours to determine conidia viability.

Effect of pruning time and time of application on wound colonisation

Pruning time: Field trials were conducted twice in consecutive years (2011 and 2012) on two 8-year-old commercial vineyards (Cabernet Sauvignon and Chenin blanc) in the Stellenbosch area, South Africa. Pruning was carried out on the same day in both vineyards at two separate times, an early pruning in July (recommended for Chenin blanc) and a late pruning in August (recommended for Cabernet Sauvignon). In 2011 pruning was carried out on the 7th of July and 10th of August, while it was carried out on the 17th of July and 23rd of August in 2012. At each pruning time all the vines to be treated were pruned at the same time and the wound treatment applied at the designated time thereafter. The vines were spur pruned to three buds after which the wounds were treated with either UST1, Eco-77 or sterile water. Natural inoculum was relied upon for wound infection and no pathogens were artificially inoculated. Due care was taken to prune the grapevines when there was no rain for the whole duration of pruning and wound treatment (4 days).

Time of wound treatment after pruning: The wounds of spur pruned vines at each pruning time interval were sprayed at the following intervals after pruning: immediately (within 15 minutes of pruning); 6; 24; 48 or 96 hours later using a 500 mL hand sprayer. The nozzle of the spray bottle had a plastic shield to minimise spray drift.

Assessment of wound colonisation by *Trichoderma* spp. and pathogens: Four months after wound treatment, the treated wounds were pruned off just above the second bud and brought to the lab for fungal re-isolation. The canes (3-5 cm) were surface sterilised by immersion in 70% ethanol for 30 seconds, one minute in 3.5% sodium hypochlorite and finally in 70% ethanol for 30 seconds. Shoots were then aseptically split longitudinally. For one position four wood tissue sections (~1 × 1 mm) two from either side of the pith of both pieces were plated onto one 90 mm PDA Petri dish. For each wound, isolations were made from two positions, just below the wound scar interface and about 10 mm below the first isolation. In total, eight wood pieces were placed on two plates per wound. This isolation method allowed for assessment of the extent to which the wound/cane is colonised by *Trichoderma* species or trunk pathogens (equivalent to pathogen severity) by computing the freqency of isolation of the fungi from the total wood pieces used for isolation, as reported by Mutawila *et al*. (2011b). Petri dishes were incubated at 25 °C for 4 weeks with sub-culturing when a fast growing fungus would overgrow other wood pieces. Fungal cultures were identified on cultural and morphological characters as species of the Botryosphaeriaceae (Van Niekerk *et al*., 2004), Diatrypaceae (Trouillas *et al*., 2010), *Phomopsis* species (Van Niekerk *et al*., 2005), *Pa. chlamydospora* (Crous & Gams, 2000) and *Phaeoacremonium* species (Mostert *et al*., 2006).

Weather data: Data of the weather conditions prevailing at the trial sites in both years was obtained from meteorologists at the Agricultural Research Council Infrutec-Nietvoorbij (Nietvoorbij Campus) who have a weather station located close to the trial sites.

Experimental design and data analysis

The trials were laid out as a randomised complete block design with three blocks per vineyard (cultivar). Treatments were arranged as a split plot design with the main plot a 2 × 5 factorial. The factors were two pruning times (July and August) and five wound treatment times (0, 6, 24, 48 and 96 hours). The subplot treatments were the wound treatment agents (UST1, Eco 77 and water). An experimental unit was a single pruning wound and each treatment combination (time of pruning, wound treatment time and wound treatment agent) was replicated 10 times per block. Each vine (for a specific pruning time and time of wound treatment) contained three wounds for each specific wound treatment agent (UST1, Eco 77 and water) and the wound position, along the cordon, of each treatment was independently randomised. The data for the

incidence of *Trichoderma* spp. and grapevine trunk pathogens were expressed as percentages of wounds from which the fungus was isolated from the total number of wounds. The extent of wound colonisation was determined by the isolation frequency and expressed as a percentage of the wood pieces from which the fungus was isolated from the total number of wood pieces plated per wound.

Data were subjected to factorial analysis of variance to determine significant effects and interactions of cultivars, pruning time and time of wound treatment application. Significant differences among treatments were separated using Fisher's least significant differences (LSD) at 5% significance level. SAS version 8.2 statistical software (SAS institute Inc., Cary, North Carolina, USA) was used for analysis. The efficacy of the treatments in reducing wound infection by trunk pathogens was assessed by computing percenatge pathogen reduction. Pathogen reduction (Pr) was calculated as: Pr = 100 ((Pc - Pt) / Pc), where Pc is the mean pathogen incidence in the water control and Pt is the mean pathogen incidence in the given treatment.

Best method of application

Fungal isolates and inoculum preparation

Trichoderma atroviride isolate UST1 (accessions No. STE-U 6514), shown to have pruning wound protective effect (Kotze *et al*., 2011) and trunk pathogen *Phaeomoniella chlamydospora* (accessions No. STE-U 6384) were used for this experiment. The fungal isolates were maintained in tubes of sterile deionised water at 4 °C. The fungi were sub-cultured onto freshly prepared potato dextrose agar (PDA; Biolab, Wadeville, South Africa) and allowed to grow for 5 days at 25 °C in the dark.

Conidial suspensions of UST1 were prepared from 7-day-old cultures growing on potato dextrose agar (PDA) by adding sterile distilled water (10 mL) to each culture and dislodging conidia with a sterile loop. *Phaeomoniella chlamydospora* conidia were produced by growing the fungus on PDA for 3 weeks at 25 °C and the conidial suspensions prepared as described above. Conidial suspensions were then filtered through sterile double cheesecloth to remove mycelial fragments. The concentrations were determined with a haemocytometer and adjusted to 5×10^4 conidia/mL for *P. chlamydospora* and 10^8 conidia/mL for UST1. For each application conidial suspensions were freshly prepared and after the application, 20 µL of left over suspension were spread plated on PDA and incubated for 24 hours to determine conidia viability.

Field Evaluation

The trails were carried out on two commercial Cabernet Sauvignon vineyards (6 and 8 years for vineyard 1 and 2, respectively) in the Stellenbosch area. The vines were spur pruned to three buds in mid-August. About 6-hours after pruning the wounds were treated with *T. atroviride* spore suspensions (10^8 conidia/mL) by either painting with a 19 mm paint brush, sprayed using 'knapsack' (backpack) sprayer or a gator (quad bike). The spray equipment (knapsack and gator) were fitted with a broad spectrum nozzle (Albuz AVI) and the gator was driven at a speed of 4.5 km/hour. Wounds for the control treatments were sprayed with sterile water. Three days after pruning some wounds were inoculated with approximately 1000 conidia of *P. chlamydospora* (20 µl of 5×10^4 conidia/ml).

Eight months after treatment the treated wounds were harvested and taken to the lab for fungal isolation. These were surface sterilised by immersion in 70% ethanol for 30 seconds, then 1 minute in 3.5% sodium hypochlorite and in 70% ethanol for 30 seconds and aseptically split in two. Four wood tissue sections, one from either side of the pith of each half of the split cane, were aseptically removed and placed onto a 90 mm Petri dish containing PDA. Isolations were made from the wound interface of the live and dead tissue and about 10 mm below the

interface. Petri dishes were incubated at 25 °C under 8 hour white light and 16 hours darkness for 2-4 weeks with sub-culturing to prevent overgrowth of emerging colonies. The fungi were identified and their incidence and frequency of isolation at each isolation point recorded. The wood pieces were surface sterilised and placed onto PDA dishes. Petri dishes will be incubated at 25 °C for 4 weeks. Isolated fungi were identified based on cultural and morphological characteristics and their incidence recorded.

Experimental design

The field trials were laid out as a randomised block design with four blocks. The experimental unit was a pruning wound. Each treatment combination (treatment and pathogen) was replicated on four vines per block with three wounds receiving the same treatment per vine to make a total of 12 wounds per treatment combination per block.

The incidences of *T. atroviride* and *P. chlamydospora* were recorded as percentages of the total number of pruning wounds inoculated with each specific treatment. For wounds that were not artificially inoculated with the pathogen a general pathogen incidence was recorded for natural infection of the trunk pathogens. The incidence and frequency of isolation data were subjected to analysis of variance (ANOVA) and the means compared by Fischer's least significant difference (LSD) at $P = 0.05$. Analysis was performed with the SAS version 9.2 statistical software (SAS Institute Inc, Cary, North Carolina, USA). Pathogen reduction (Pr) was calculated as: Pr = 100 ((Pc - Pt) / Pc), in which Pc is the mean pathogen incidence in the water control and Pt is the mean pathogen incidence in the given treatment.

3. Development of fungicide resistant *Trichoderma* isolates

Source of isolates and culture conditions

Two *T. atroviride* isolates UST1 and UST2, and an isolate of *T. harzianum* T77, all shown to have pruning wound protective effect (Kotze *et al*., 2011), were used for the mutation study. *Trichoderma atroviride* isolates UST1 and UST2 are stored under accession numbers STE-U 6514 and 6515 for UST1 and UST2, respectively, at the University of Stellenbosch, Department of Plant Pathology culture collection while isolate T77 was kindly provided by Plant Health Products, Pietermaritzburg, South Africa. Grapevine trunk pathogens namely, *E. lata* (STE-U 5692), *D. seriata* (STE-U 4440), *N*. *parvum* (STE-U 4439) and *Pa. chlamydospora* (6384) were also obtained from the culture collection of the Department of Plant Pathology at Stellenbosch University.

All fungal isolates were maintained in tubes of sterile deionised water at 4 °C. Before use, the fungi were sub-cultured onto freshly prepared potato dextrose agar (PDA; Biolab, Wadeville, South Africa). Conidial suspensions of the *Trichoderma* isolates were prepared from 7-day-old cultures growing on PDA by flooding the Petri dishes with sterile distilled water or buffer (10 mL) to each culture and scrapping the surface to dislodge conidia with a sterile loop. The suspensions were then filtered through sterilised cheese cloth to remove mycelium fragments, conidia counted with a haemocytometer and adjusted to the desired concentration.

Determination of sensitivity of wild type *Trichoderma* strains to fungicides

The sensitivity of *Trichoderma* wild type isolates UST1, UST2 and T77 to benzimidazole fungicides used in pruning wound protection was tested on conidial germination and mycelial growth. Sensitivity was determined towards the fungicides carbendazim (technical grade 99.40%, UAP Crop Care, Paarl, South Africa), thiophanate methyl (technical grade 97.45%, Sinochem, Shanghai, China) and Benomyl (500 WP a.i. benomyl 500 g/kg; Villa Crop Protection, Kempton Park, South Africa). Stock solutions for all fungicides were prepared in acetone to make 1 mg/mL concentration of active ingredient. Potato dextrose agar was then amended with 0, 1, 2.5, 5, 10, 30 and 50 µg/mL of each fungicide. For thiophanate methyl only,

sensitivity of the wild type isolates was also tested at 100 µg/mL. In all cases the final concentration of acetone in the medium was 0.1% including the control plates without fungicide.

For *Trichoderma* spp. mycelium inhibition, mycelial plugs (5 mm diameter) taken from the margins of an actively growing colony were placed (mycelium facing downward) in the centre of each Petri dish with amended medium. Conidia germination was tested by spread-plating 100 µL (~10^4 conidia) of conidial suspension on amended medium. There were two replicates of each *Trichoderma* wild type isolate per fungicide per test (mycelial or conidial inhibition). Plates were incubated at 25 °C in the dark for 4 days. The minimal inhibitory concentration for each fungicide was determined as the lowest concentration of the fungicide where no mycelial growth or conidia germination could be observed after 4 days of incubation. The screening concentration for fungicide resistant mutants was set at four times the minimum inhibitory concentration for mycelial growth.

Irradiation and isolation of fungicide resistant *Trichoderma* strains

Conidial suspensions of 10^5 conidia/mL (5 mL) of the wild type *Trichoderma* isolates were prepared in 0.05 M acetate buffer (pH 5.4) from 7-day-old cultures growing on PDA. Suspensions were dispensed into 15 mL conical centrifuge tubes and irradiated in air with a ^{60}Co gamma radiation source at room temperature and atmospheric pressure (10 000 Curie; Insect Sterile Technique Africa (Pty) Ltd, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa) at doses of 0, 200, 300, 400, 500, 600 and 700 Gy at a dose rate of 15 Gy/min. Dosage validation was carried out by chemical dosimetry using Fricke solution (Matthews, 1982). The survival level of conidial suspension was determined by dual spread plating (100 µL) of serial dilutions of the irradiated suspensions onto acidified PDA (pH 3.8). The plates were inverted and incubated at 25 °C for 3 days, after which colonies were counted and survival curves constructed. Two irradiations were performed, each with triplicate samples per dose. The mean plate counts were used to obtain survival curves for each isolate in response to gamma irradiation. Data was reported as D_{10} values, which is the dose that caused 90% (1 $\log_{10}$) reduction in conidia survival (Moeller *et al*., 2007). The D_{10} values were compared between the isolates by analysis of variance, using SAS version 9.2 (SAS Institute Inc., Cary, North Carolina, USA), and used to determine the dosage to be used for the generation of mutants.

Mutagenesis was carried out by irradiation of conidia suspensions at dosage 250 Gy (determined from above) and the conidia were separately spread plated on PDA amended with the appropriate screening concentration for each fungicides. Petri dishes were inverted and incubated at 25 °C in the dark and checked for the emergence of resistant colonies between 5 and 10 days. Mutants were sub-cultured on PDA amended with the screening concentration of the fungicide, single spored and stored in tubes of sterile deionised water at 4 °C. Mutants were also tested for resistance to higher concentrations (20-100 µg/mL) of fungicides.

Testing of mutant stability, fitness and cross resistance

Stability of mutants was tested on PDA amended with the screening concentration of fungicide after ten cycles of sub-culturing on fungicide-free medium. The growth pattern of the mutants on fungicide-free PDA was compared to that of the wild type isolates at 5 to 40 °C (at 5 °C intervals). Mutants were also tested for fungicide cross resistance, as described above, for both inhibition of mycelial growth and conidia germination.

Test of *in vitro* antagonism against grapevine trunk pathogens

The antagonism of mutants and wild type isolates was compared by observing their interactions in dual inoculated plates with grapevine trunk pathogens namely; *D. seriata*, *E. lata*, *N. parvum* and *Pa. chlamydospora*. Mycelial disks (5 mm) cut from the growing edges of the colonies of the *Trichoderma* strains, and the pathogens were placed at opposite sides of the same Petri dish containing PDA, simultaneously. Due to the slower growth of *Pa. chlamydospora* relative to the other pathogens, it was inoculated onto the PDA 10 days prior to inoculation of the *Trichoderma* strains. The plates were incubated at 25 °C in the dark for 5 to 10 days after which

interactions between the fungi were observed both macro- and microscopically. For microscopy, mycelial plugs (±5 mm^2) from the mycelium interaction zones from different random positions were placed on a glass slide with sterile deionised water. Hyphal interactions were observed using a Nikon Eclipse E600 microscope fitted with a Nikon digital camera DXM1200 with Automatic Camera Tamer (ACT-1) software.

***In vivo* evaluation of grapevine wound protection**

The benzimidazole resistant mutant from isolate UST1 (henceforth termed MT1) and the wild type UST1, which was naturally resistant to thiophanate methyl, were further tested *in vivo* for pruning wound protection.

Field trial: A Cabernet Sauvignon (9-year-old) vineyard situated in the Stellenbosch area was spur pruned to three buds in August 2012. About 6 hours after pruning, each pruning wound received a treatment of either the *Trichoderma* suspensions (UST1 or MT1) alone or in combination with fungicides thiophanate methyl (7 g a.i/L) and carbendazim (0.5 g a.i/L), respectively. The two fungicides were also applied alone. A water control treatment was also included. All treatments were sprayed as a single application using a hand held 500 mL trigger spray bottle. For the combination treatments the *Trichoderma* suspensions were made up in water, the fungicide added to the conidia suspension and mixed. Treated wounds were either left to natural inoculum or artificially inoculated with the Petri disease pathogen, *Pa. chlamydospora* (~1000 conidia/wound) at 24 hours or 7 days after pruning. There were a total of 21 treatment combinations (i.e. wound treatment × natural or artificial inoculation × time of artificial inoculation) and all pruning wounds on a vine received the same treatment.

Seven months after treatment, the spurs were pruned off just above the second node and the stubs with the treated wounds were taken to the laboratory for fungal isolation. The wood stubs were first surface sterilised by immersion in 70% ethanol for 30 seconds, then 3.5% sodium hypochlorite for 1 minute and finally in 70% ethanol for 30 seconds. Shoots were then aseptically split longitudinally and four wood tissue sections (~1 mm^3), two from either side of the pith, were plated onto PDA in one Petri dish. For each wound, isolations were made from two positions, at the wound scar interface (four wood pieces) and about 10 mm below the first isolation (four wood pieces) to make a total of eight wood pieces plated per wound. Plates were incubated at 25 °C for 4 weeks. Fungal colonies were sub-cultured when a fast growing fungus would overgrow other wood pieces in the same Petri dish. Fungal cultures were identified on colony and microscopic morphological characteristics.

Experimental design and data analysis: The field trial was laid out as a randomised block design with four blocks of 63 vines each and three vines per treatment combination. Each pruning wound was an experimental unit and isolation was carried out from five wounds per vine. The incidence of fungi present in the pruning wounds was expressed as a percentage of the total number of pruning wounds per treatment combination. The incidence data were subjected to analysis of variance and the means were compared using Fischer's least significant difference value (LSD) at P = 0.05. Analysis was done using SAS version 9.2 statistical software (SAS Institute Inc, Cary, North Carolina, USA). Pathogen reduction (Pr) was calculated as: Pr = 100 ((Pc - Pt) / Pc), in which Pc is the mean pathogen incidence in the water control and Pt is the mean pathogen incidence in the given treatment.

4. Investigate the influence of cultivar towards *Trichoderma* efficacy

The cultivar evaluation trial was carried out to confirm variations in grapevine cultivars to *Trichoderma* species pruning wound protection. Four node grapevine canes of three cultivars, Chenin blanc, Cabernet Sauvignon and Pinotage with no visible trunk disease symptoms were provided by Vititec. The canes were subjected to warm water treatment at 50 ˚C for 30 minutes.

The shoots were treated with a root stimulating hormone 4-indole-3-butryric acid (Dynaroot® No. 3) before planting in a mist bed for rooting. After rooting (about 50 days) the canes were planted in pots in the glass house at 23-27˚C and at least 75% relative humidity. When all canes had initiated shoots from the second node apical node, the canes were pruned below the apical node and the fresh pruning wound treated with one of nine treatments. The treatments were: *Trichoderma atroviride* (USPP-T1), *T*. *harzianum* (E7), *Phaeomoniella chlamydospora*, *Phomopsis viticola*, USPP-T1 + *P*. *chlamydospora*, USPP-T1 + *Ph*. *viticola*, E7 + *P*. *chlamydospora* and E7 + *Ph*. *viticola*. The experiment was laid out in a randomised complete block design (RCBD), with three blocks. Each block had 5 shoots (or pruning wounds) for each treatment and cultivar. After 90 days the shoots were harvested and took to the lab for fungal isolation. The canes were surface sterilised by immersion in 70% ethanol for 30 seconds, then 1 minute in 3.5% sodium hypochlorite and in 70% ethanol for 30 seconds. The canes were then split longitudinally with a flame-sterilised pruning shear. Isolations were made from the wood tissue at two positions along the split shoot namely, the interface between the dead and the live tissue and at ~1 cm below the interface. At each isolation position four tissue sections approximately 1 mm $\times$ 0.5 mm (0.5 mm^3) were aseptically removed from either side of the pith (two from each half) and plated out onto 90-mm Petri dishes containing PDA amended with 40 µg/mL streptomycin. Petri dishes were incubated at 25°C under 8 hour white light and 16 hours darkness for 2-4 weeks after which the growing fungi were identified. The differences between the treatments and cultivars, in the incidence and isolation frequency of the biocontrol agent and the pathogens were determined by analysis of variance and Fischer' test for the least significant difference at the 95% confidence level ($P < 0.05$) using Statistica statistical package.

B. Establish the role of sucker wounds as portals for trunk disease infections

1. Survey sucker wounds of table and wine grapes.

Sample collection

Sucker wounds were sampled from the two wine grape cultivars, Chenin blanc and Cabernet Sauvignon, as well as two table grape cultivars, Thompson Seedless and Crimson Seedless. The ages of the vineyards ranged from 10- to 15-years-old. The wine grape vines were sampled from Darling, Robertson and Stellenbosch and the table grape vines from Paarl and Piketberg, all situated in the Western Cape Province of South Africa. Sampling was carried out from April to June in 2011 (Darling, Robertson and Paarl) and 2012 (Stellenbosch and Piketbeg) with each vineyard sampled once only. Fifteen canes (table grapes) or spurs (wine grapes) of 1- to 3-year-old wood with sucker wounds were sampled randomly from each vineyard and taken to the laboratory for fungal isolations.

Fungal isolations

Sucker wounds were selected (leaving approximately 2cm of cane above and below the wound) and excised, using pruning shears, from at least ten of the canes or spurs from a vineyard. Wounds that showed wood discolouration typical of trunk disease infections were analysed further. In total, fungal isolations were made from 161 wounds. Wood pieces were surface-disinfected in 70% ethanol for 30 seconds, then for 1 minute in 3.5% sodium hypochlorite solution and again in 70% ethanol for 30 seconds. Sucker wounds were aseptically dissected longitudinally across the wound. Fungal isolations were made aseptically from symptomatic (browning or streaking) (Figure 1) wood that originated from sucker wounds. Wood fragments were taken from the wound scar interphase. Additionally, if symptoms were not found, isolations were made from tissue that seemed healthy from the interphase. From each symptom type, 12 wood fragments (0.5mm $\times$ 1.0mm) were obtained from each sucker wound and plated onto 90mm Petri dishes containing Potato Dextrose Agar (PDA, Biolab, Wadeville, South Africa) amended with chloromycetin (250mg/L) (4 pieces per plate). The plates were incubated at approximately 25 ^{o}C and monitored daily for 4 weeks. Fungal colonies resembling taxa associated with grapevine trunk diseases were hyphal-tipped and grown on PDA. Pure cultures

were stored in double-sterilised distilled water (dH_2O) in 14ml McCartney bottles and kept at 4 ^oC. Representative isolates are stored in the culture collection of the Department of Plant Pathology, Stellenbosch University, South Africa.

Fungal identification

Fungi were identified according to cultural and morphological characteristics as species of Botryosphaeriaceae (Van Niekerk *et al*., 2004; Crous *et al*., 2006; Damm *et al*., 2007; Phillips *et al*., 2008), Diatrypaceae (Glawe & Rogers, 1982), *Phaeoacremonium* (Mostert *et al*., 2006; Essakhi *et al*., 2008), Diaporthales (Mostert *et al*., 2001; Van Niekerk *et al*., 2005) and *Phaeomoniella chlamydospora* (Crous & Gams, 2000). Genomic DNA was isolated from 2-week-old fungal mycelia obtained from PDA plates. A CTAB-based DNA extraction method was used as described by Damm *et al*. (2008). For the species of the Botryosphaeriaceae, Diatrypaceae and *Phomosis viticola*, the internal transcribed spacers (ITS1 and ITS2) and the 5.8S ribosomal RNA gene were amplified with the primers ITS1 and ITS4 (White *et al*., 1990). The PCR conditions were the same as reported by Van Niekerk *et al*. (2004). The partial β-tubulin gene (TUB) was amplified for the *Phaeacremonium* isolates using primers T1 (O'Donnel & Cigelnik, 1997) and Bt2b (Glass & Donaldson, 1995). The PCR conditions for the TUB gene were the same as described by Mostert *et al*. (2006). PCR products were purified using a commercial kit (MSB® Spin PCRapace 250, Invitek, Berlin, Germany) according to the manufacturer's instructions. DNA sequence analysis was carried out using the Big Dye system (version 3.1 dye terminators, Applied Biosystems, California, USA) on an ABI 3130XL Genetic Analyzer. Sequences obtained for both directions were evaluated using Geneious 3.5.6 (Biomatters Ltd, New Zealand) and manually edited using Sequence Alignment Editor v. 2.0a11. The identities of the sequences were compared by the Megablast function of the NCBI's GenBank nucleotide database.

2. Assess susceptibility of sucker wounds with artificial inoculation in a controlled environment (glasshouse) and in the field.

Susceptibility of sucker wounds to five trunk pathogens in the glasshouse

Two glasshouse trials were conducted to investigate the susceptibility of sucker wounds to trunk disease pathogens. In the first trial, wine and table grape cultivars of own-rooted Chardonnay and Crimson Seedless plants were inoculated with *E. lata* and *Pa. chlamydospora*. In the second trial, the pathogens *E. lata*, *N. parvum*, *Pa. aleophilum*, *Pa. chlamydospora* and *Po. viticola* were tested on grafted Chardonnay plants.

Plant cultivation

One-year-old dormant Chardonnay and Crimson Seedless canes were obtained from mother blocks. The canes were trimmed to three buds, submerged in water for 4 to 6 hours and then in the recommended dosage of dodecyl dimethyl ammonium chloride [ICA International Chemicals South (Pty), Ltd] for 5 minutes. Treated canes were stored with wetted perlite in plastic bags at 4 ^oC until they were required. Prior to use, dormant canes were hot-water treated at 50 ^oC for 30 minutes. To enhance rooting, the distal ends of the canes were dipped in Dynaroot hormone powder (Efekto, PBR Trading International cc) and planted in plastic trays that contained perlite. Trays were drip irrigated three times a day and kept in the glasshouse at 28 ^oC. After 2 weeks, rooted canes were planted in plastic bags (13cm in diameter and 25cm in height) in a mixture of potting soil and perlite (3:1). The plants were maintained at 25 ^oC and allowed to bud in the glasshouse prior to inoculations. Dormant, grafted and rooted 1-year-old Chardonnay plants were obtained from a certified nursery and planted in individual plastic bags. These vines were also maintained in the glasshouse as described previously and also allowed to bud.

Inoculum preparation

Inocula of trunk pathogens were prepared as follows: perithecia of *E. lata* were obtained from a wood piece with visible stroma. The wood piece was moistened with water and left for 30 minutes. Under a dissecting microscope, the top layer of the stroma was scraped off to reveal active perithecia that contained elongated asci (40 – 60µm in length) with eight allantoid ascospores (6 – 12µm long). A suspension was made by adding spore droplets into dH_2O and the concentration was adjusted to 5×10^4 spores/ml using a haeomocytometer. Conidial suspensions of *Neofusicoccum parvum* (STE-U 4439) and *Po. viticola* (STE-U 7768) were made from pycnidia, with conidial droplets, that formed on water agar plates containing sterilised pine needles after 4 weeks at 25 ^oC. Pycnidia were crushed in dH_2O to release conidia and the concentration was adjusted as previously described. Conidial suspensions of *Phaeoacremonium aleophilum* (STE-U 6996) and *Phaeomoniella chlamydospora* (STE-U 6384) were made from 2-week-old cultures on PDA. Mycelium blocks measuring 10mm $\times$ 10mm were placed in sterile dH_2O and shaken vigorously to suspend the conidia and the concentrations were adjusted as previously described.

Inoculations

For the first glasshouse trial, sucker wounds were created by removing the apical shoot (50 – 70mm long) from each plant (Figure 2a). For the second trial, the second shoot from the pruning wound was removed. The reason for this was that a field trial failed when the first shoot was removed due to the dieback that occurred beyond the sucker wound, therefore the second shoot was removed in the further trials. Each sucker wound was inoculated with a 20µl droplet (1000 spores or conidia) (Figure 2b) of spore suspension. Control plants received the same volume of sterile dH_2O. The trials were laid out in a complete randomised block design with three and two blocks for trials one and two, respectively, each block consisting of ten plants per treatment.

Fungal isolation and identification

After 3 months for trial 1 and 4 months for trial 2, sucker wounds were harvested and taken to the laboratory for fungal re-isolations. Wounds were surface sterilised and aseptically dissected as previously described. Fungal isolations were performed by obtaining wood fragments from the wound scar interphase (top isolation zone) and from 5mm below or away from the first isolation point (bottom isolation zone). From each isolation position, four wood fragments (0.5mm $\times$ 1.0mm) were obtained from each half of the sucker wound and plated onto 90mm Petri dishes containing PDA amended with chloromycetin (250mg/L) (8 pieces in total, 4 pieces per plate). The plates were incubated at approximately 25 ^oC and monitored daily for 4 weeks. Inoculated fungi were identified using cultural and morphological characteristics (Glawe & Rogers, 1982; Crous & Gams, 2000). Representative cultures were sub-cultured, DNA was isolated from the cultures and PCR products were sequenced. Wound susceptibility or infection was evaluated by calculating the percentage mean pathogen incidence.

Susceptibility of sucker wounds to five trunk pathogens on field grapevines

Inoculum preparation and inoculation

During October 2012 in spring, 12-year-old Cabernet Sauvignon vines, at Infruitec-Nietvoorbij (Agricultural Research Council, Stellenbosch), which were trained to bilateral cordons on a horizontally divided trellis with approximately seven spurs per cordon, were spur pruned to five buds during the dormant season. Sucker wounds were created by removing the second shoot (50 – 70 mm length) from the pruning wound on the 1-year-old cane (Figure 2 c and d). Six sucker wounds were made per vine for the different treatments. Inocula of *E. lata*, *N. parvum*, *Pa. aleophilum*, *Pa. chlamydospora* and *Po. viticola* were prepared as previously described. Each vine received 20 µl of each of the five pathogen inocula (1000 spores or conidia) as well as sterile dH_2O as control. The trial was laid out in a complete randomised block design with four blocks that consisted of ten plants per treatment.

Fungal isolations and identification

After 5 months, sucker wounds were harvested and taken to the laboratory for re-isolations. Fungi were re-isolated and identified as previously described for the glasshouse experiments.

3. Assess the duration of sucker wound susceptibility in the field.

Inoculum preparation and inoculation

The trial was carried out in the Cabernet Sauvignon vineyard used for the sucker wound susceptibility trial, though on different vines. Sucker wounds were made on the spurs in the same manner as for the field susceptibility trial. Each of the wounds were inoculated with 20µl of spore suspension (1000 spores) of *Pa. chlamydospora*. A control treatment was applied at each time point and consisted of the same volume of dH_2O. The treatments consisted of five inoculations of *Pa. chlamydospora* spore suspensions on sucker wounds over a four week period. The first inoculation was made immediately after desuckering (week 0), after which four inoculations were made at 7 day intervals (weeks 1 to 4). The trial was laid out in a complete randomised block design in four blocks with ten replicates per treatment.

Fungal isolation and identification

After 5 months, sucker wounds were harvested and taken to the laboratory for fungal re-isolations. Fungi were identified as previously described for the glasshouse trials.

Data analysis

The incidences of the fungal isolations were calculated by the presence or absence of a positive (infected) wood fragment per wound. The data from the different trials were analysed using analysis of variance (ANOVA) and the Student's t-test to determine least significant differences (LSD) at 5% significance level ($P < 0.05$). All data analyses were performed using SAS version 9.2 (SAS Institute Inc, SAS campus Drive, Cary, North Carolina, USA).

5. Determine the sensitivity of *Trichoderma* isolates towards fungicides used during spring time

Isolates and fungicides used

In vitro assays were performed using two *Trichoderma* spp. isolates T1 (*T. atroviride*) and Eco-77® (*T. harzianum*). Both isolates were used for mycelial inhibition and conidial germination tests. Isolate T1 is maintained in the culture collection of the Department of Plant Pathology at the University of Stellenbosch STE-U 6514. Sixteen commercial fungicides including contact and systemic products that are used for the control of powdery (*Uncinula necator*) and downy mildew (*Plasmopara viticola*), Botrytis rot (*Botrytis cinerea*) and Phomopsis cane and leaf spot (*Phomopsis viticola*) were screened.

Inhibition of mycelial growth

All 16 fungicides were screened *in vitro* for the mycelial inhibition of Eco-77® and T1. Stock solutions were made by suspending fungicides directly into 1000ml of sterile dH_2O. Fungicide solutions were then pipetted in appropriate quantities to bottles that contained molten PDA at approximately 50 ^oC to achieve 0.25, 0.5, 1 and 2 times the recommended dosages (Table 1). Potato dextrose agar without fungicide was used as a control treatment. Plates were inoculated within 24 hours with mycelium plugs of 5mm diameter obtained from the margins of actively growing 7-day-old cultures of Eco-77® and T1. Each fungicide concentration, as well as the controls were replicated three times. Petri dishes were incubated at 25 ^oC for 3 days. At 24 hours the diameter of each colony was measured twice, at perpendicular angles. The diameter

measurements were recorded again at 48 hours and the trial was repeated once. For each isolate × fungicide × concentration combination, the percentage inhibition was calculated in relation to the respective control treatment. The percentage inhibition was calculated as follows: 100 × [(colony diameter of control) − (colony diameter on fungicide-amended plate)/ (colony diameter of control)]. The fungicides were regarded as being compatible with *Trichoderma* isolates if they gave less than 50% mean percentage inhibition at all the tested dosages.

Inhibition of conidial germination

The inhibition of conidial germination of Eco-77® and T1 were tested against all 16 fungicides at the recommended dosages (Table 1). Spore suspensions were prepared by flooding 7-day-old PDA cultures of Eco-77® and T1 with 5ml sterile water. The concentrations were adjusted to 1 x 10^5 spores per ml in potato dextrose broth (PDB, Biolab, Wadeville, South Africa), using a haeomocytometer. Aliqouts (0.5ml) of spore suspension and fungicide (0.5ml) were added to 1.5ml eppendorf tubes. Sterile dH_2O was used as a negative control treatment. Each spore-fungicide mix was replicated three times. Tubes were placed at 25 ^oC in a shaker incubator (100rpm). After 24h, three droplets were taken separately from each tube and viewed under a light microscope (× 400, Zeiss, West Germany). Spores were considered to have germinated if the germ tube length equalled the spore diameter. The percentage inhibition was recorded for 50 spores per sample and the mean percentage inhibition relative to the control was calculated per fungicide. The percentage inhibition was calculated as follows: 100 × [(number of germinated spores in control tubes) − (number of germinated spores in fungicide-amended tube)/ (number of germinated spores in control tubes)]

Data analysis

For the mycelial inhibition, the percentage inhibition of both experiments was pooled. The data was subjected to analysis of variance (ANOVA) and the Student's t-test for the least significant differences (LSD) at 5% significance level ($P < 0.05$). Significant differences in conidial inhibition between the fungicides were determined using a one-way ANOVA. The data was subjected to analysis of variance (ANOVA) and the Student's t-test for the least significant differences (LSD) at 5% significance level ($P < 0.05$). Analyses was performed using SAS version 9.2 (SAS, 2008) statistical software (SAS Institute Inc, SAS campus Drive, Cary, North Carolina, USA).

5. Field application of *Trichoderma* to assess efficacy in protecting sucker wounds

Isolates used and inoculum preparation

A *Trichoderma harzianum*-based pruning wound product, Eco-77®, was kindly provided by Plant Health Products (PHP, PTY Ltd., Nottingham Road, South Africa). Eco-77® was applied at the recommended rate of 0.5g/L. *Phaeomoniella chlamydospora* and *Po. viticola* are maintained in the culture collection of the Department of Plant Pathology at the University of Stellenbosch, STE-U 6384 and STE-U 7768, respectively. *Phaemoniella chlamydospora* conidial suspension was prepared from a 2-week-old fungal culture grown on Potato Dextrose Agar (PDA, Biolab, Wadeville, South Africa). A colonized mycelium block measuring 10mm × 10mm was placed in sterile dH_2O and shaken vigorously to suspend conidia. A conidial suspension of *Po. viticola* was made by suspending conidial droplets from pycnidia that formed on 4-weeks-old fungal cultures on PDA with sterilised pine needles.

Field inoculations

A 12-year-old Cabernet Sauvignon vineyard trained to bilateral cordons on a horizontally divided trellis situated in Stellenbosch was used for field inoculations. During July 2012 (winter), the vines were spur pruned to 5 buds. In October 2012 (spring), sucker wounds were created by removing the second shoot (50 – 70mm in length) below the pruning wound of the 1-year-old

canes. Wounds were then spray-treated with Eco-77® by means of a hand-held trigger spray canister. After 2 days, sucker wounds were inoculated with 1000 spores of *Pa. chlamydospora* and *Po. viticola* conidial suspensions. Treatments included Eco-77®, *Po. viticola*, *Pa. chlamydospora*, plus a combination (Eco-77® + pathogen) and sterile dH_2O as a control. The trial was laid out in a complete randomised block design with three blocks that consisted of ten vines. Each vine received all six treatments. Five months later, sucker wounds were excised (leaving approximately 2cm above and below the sucker wound) and taken to the laboratory for fungal re-isolations and identification.

Fungal isolations from sucker wounds

Sucker wounds were surface disinfected by dipping into 70% ethanol for 30 seconds, 1 minute in 3.5% sodium hypochlorite solution and again in 70% ethanol for 30 seconds. Wounds were aseptically dissected longitudinally and wood fragments were taken from the wound scar interphase and 5mm away from the first isolation. In total, 8 wood pieces were excised; four wood fragments (5mm x 1mm) from each isolation position, on each half. The wood fragments were plated onto 90mm Petri dishes containing PDA amended with chloromycetin (250mg/L). Petri dishes were incubated at approximately 25 ^{o}C and monitored for 4 weeks. Fungal cultures were identified based on cultural and morphological characters as *Pa. chlamydospora* (Crous & Gams, 2000), *Po. viticola* (Mostert *et al*., 2001; Van Niekerk *et al*., 2005) and *Trichoderma* (Gams & Bisset, 1998).

Data analysis

The incidence of *T. harzianum, Pa. chlamydospora* and *Po. viticola* were calculated by the presence or absence of these fungi per sucker wound for each treatment. The data was subjected to analysis of variance (ANOVA) and the Student's t-test for the least significant differences (LSD) at 5% significance level ($P < 0.05$). The differences in the pathogen incidences of individual and combined treatments were sought by ANOVA. Analysis was performed using SAS version 9.2 (SAS, 2008) statistical software (SAS Institute Inc, SAS campus Drive, Cary, North Carolina, USA).

C. Investigate the host-pathogen-*Trichoderma* interactions

1. To investigate the secondary metabolites secreted by *T. atroviride* and *T. harzianum* and their effect on trunk disease pathogens.

Fungal isolates

Trichoderma atroviride isolates, UST1 and UST2, are stored at the Stellenbosch University, Department of Plant Pathology 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®, and was kindly provided by Plant Health Products (South Africa). Four grapevine trunk pathogens namely, *E*. *lata* (STE-U 5692 and 6513), *Neofusicoccum* (*N*.) *australe* (STE-U 7025 and 7029), *N*. *parvum* (STE-U 4439 and 4584) and *Pa. chlamydospora* (STE-U 6384 and 7732) were also used. All fungal isolates were maintained in tubes of sterile deionised water at 4 °C. Before use, the fungi were sub-cultured onto freshly prepared potato dextrose agar (PDA; Biolab, Wadeville, South Africa) 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, Wadeville,

South Africa). 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 Whatman No. 4 filter paper (Whatman, Brentford, UK).

Culture filtrates were then extracted twice with equal volumes of ethyl acetate (99.5%, Sigma). The organic fractions were combined, then dried with sodium sulphate (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, Delaware, USA). The major fraction obtained had a characteristic smell of a known *Trichoderma* metabolite, 6-pentyl α-pyrone (6PP). Fractions were run on TLC (silica gel 60; EMD Millipore, Darmstadt, Germany) developed in hexane : acetone (7:3 v/v) alongside a standard of 6PP. The standard for 6PP was previously isolated and characterised 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 characterised to confirm identity using nuclear magnetic resonance (NMR) and mass spectroscopy (MS). The proton (^1H) NMR spectra were recorded with a 400 MHz Brüker Avance spectrometer, equipped with a 5mm Bruker Broad Band Inverse probe (BBI), working at the ^1H 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

A single metabolite, 6PP was found to be the major secondary metabolite produced by all the isolates of *Trichoderma* spp. tested. Tests were carried out to determine the time course production of this metabolite among the wild type isolates. Liquid cultures of the *Trichoderma* isolates were prepared by separately inoculating 100 mL of PDB in 250 mL flasks with three agar plugs (5 mm) of the respective isolates (UST1, UST2 and T77). 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. The cultures were filtered and the major secondary metabolite quantified from the culture filtrate as described above.

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

Three additional strains, MT1, MT2 and MT77, which are mutant progeny developed from the wild type isolates UST1, UST2 and T77, respectively were also included for these tests. The mutants were developed by gamma irradiation and are resistant to benzimidazole fungicides (Chapter 5). Broth cultures of the *Trichoderma* isolates 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. 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. 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, Goettingen, Germany) into glass vials for direct quantification by liquid chromatography-mass spectroscopy (LC-MS). Chromatographic separation was performed by an ultra-high performance liquid chromatography (HPLC) 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 acetonitrile) 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. Quantification was done using a standard curve constructed by standards prepared from pure 6PP (Apollo Scientific, Manchester, UK).

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 as by Pezet (1983) without modifications and contained 1% (m/v) glucose and 0.5% (m/v) sucrose as the carbon sources. The GCBM was prepared by sonifying 100 g of ground dormant Cabernet Sauvignon canes in 500 mL boiling deionised 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) was added to the filtrate, the pH adjusted to 5.8 using either 1 M NaOH or 1 M HCL and sterilised by autoclaving. Erlenmeyer flasks containing 100 mL of medium were co-inoculated with 5 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). 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 sensitivity of grapevine trunk pathogens to 6PP was determined on mycelium and conidia/spores of four fungal pathogens namely, *E*. *lata*, *N*. *australe*, *N*. *parvum* and *Pa. chlamydospora*. Mycelial inhibition was tested on five different artificial growth media. Three complex media and two defined media were used, so as to determine the effect of growth medium on sensitivity of fungi to 6PP.

Preparation of fungal growth medium: Complex media comprised of PDA, Malt extract agar (MEA, Biolab) and GCBM. Defined media were Vogel's medium N (Vogel) and Pezet's. The grapevine cane based medium extract was prepared as described above and agar (15 g/L, Biolab) was added before sterilisation. Vogel medium N was prepared as modified by Metzenberg (2003) and consisted of 20 mL Vogel's 50× salts, 1% (m/v) glucose as the only carbon source. Pezet's medium was prepared as previously described. The pH for both Vogel's and Pezet's media was adjusted to 5.8 using 1 M NaOH or 1 M HCl before adding agar (15 g) and sterilised by autoclaving.

Pathogen conidia and spore 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 needles. 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, pieces of wood bearing stroma were collected from infected vines at the Nietvoorbij vineyards of the Agricultural Research Council of South Africa in Stellenbosch. The wood pieces were immersed in sterile water for 15 minutes after which the surface was lightly scrapped with a scapel 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 two weeks, shoot pieces (5 cm) around the wound were excised, surface sterilised, 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 scapel, 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.

Mycelial inhibition by 6PP: Eight isolates (two of each pathogen) were used to determine the effect of 6PP on mycelial growth. The metabolite was dissolved in methanol to make a 10 g/L stock solution. Mycelial growth inhibition was tested on PDA amended with 0 (control), 50, 100, 150, 200, 250, 300 and 400 mg/L of 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 hour intervals for all the fungi except *Pa. chlamydospora* where colony diameter was measured at 3 day intervals. Each isolate had three replicates per concentration and the colony diameter was measured twice perpendicularly per plate. Percentage inhibition relative to the control was calculated from the day-three colony diameters for all fungi except *Pa. 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 of 6PP. 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 *Pa. 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.

Inhibition of conidia/spore germination: Effect of the metabolite on inhibition of conidia/spore germination was tested on one isolate of each pathogen (listed above in 7.3.6.2). Conidia or spore suspensions were amended with 6PP to concentrations of 0, 50, 100, 200, 300 and 400 mg/L to a total volume of 1.5 mL in 2 mL centrifuge tubes. These were incubated at 25 °C for 24 hours after which microscope slides were made from the suspensions and spores counted under the microscope (× 400, Nikon, Japan). Spores were considered germinated when the germ tube was the size of the conidia/spore. The percentage germinated conidia/spores was determined from at least 50 conidia/spores per slide and there were three slides per centrifuge tube and three tubes per concentration.

Statistical analysis

For the quantification of the secondary metabolites, there were three biological replicates for each treatment and assays were carried out on two technical replicates. The means from each treatment were compared for significant differences using factorial analysis of variance (ANOVA) and the means were separated by computing the Fischer's least significant difference (LSD) at $P = 0.05$. For the anti-fungal assays, there were three replicates for each isolate per assay and all experiments were independently repeated once. Data from the two independent repeats was combined and the non-linear 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 Fischer's LSD test at $P = 0.05$. All statistical analysis were carried out using SAS version 9.2 statistical software (SAS institute Inc., Cary, North Carolina, USA).

2. To determine grapevine host resistance response to secondary metabolites and *Trichoderma* species

Grapevine cell suspension cultures

Cell suspension cultures of *V. vinifera* cv. Dauphine were established from callus derived from pea size green berries as previously described by Sharathchandra *et al.* (2011). The

suspension cultures were grown in 250 mL Erlenmeyer flasks containing 100 mL of Calderon medium (Calderon *et al.*, 1994). Calderon medium contained Murashige and Skoog basal medium (4.4 g/L) supplemented with sucrose (20 g/L), casein hydrolysate (250 mg/L), kinetin (1 µM) and α-naphthalene acetic acid (0.5 µM) and the medium was adjusted to pH 5.8 before sterilisation by autoclaving. The cell cultures were maintained in the same medium and sub-cultured weekly by transferring 40 mL of suspension to 60 mL of fresh medium and agitated at 100 rpm at room temperature in darkness. The growth curve of the suspension culture was characterised by measuring the turbidity (OD_{600}) of the suspension culture over a 14-day period to determine the growth curve and optimal time for elicitation.

Fungal isolates and elicitor preparation

The grapevine trunk pathogen *Eutypa lata*, isolate STE-U 5692, was isolated from symptomatic *V. vinifera* wood. *Trichoderma atroviride* isolate STE-U 6514 was also isolated from grapevine wood and has a grapevine pruning-wound-protective effect against trunk pathogens (Kotze *et al.*, 2011). Both fungi were maintained on solid potato dextrose agar (PDA) (Biolab) at room temperature. To obtain elicitors, five disks (5 mm) from the margins of actively growing colonies of *E. lata* and *T. atroviride* were separately inoculated into 250 mL Erlenmeyer flasks containing 100 mL Calderon medium on a shaker (100 rpm) at room temperature for 5 and 10 days, respectively. The fungal mycelium was removed by vacuum filtration through Whatman No.1 filter paper (Whatman, Brentford, UK). The filtrate was then filter sterilised through a 0.22 µm pore filter to obtain a cell free, extracellular fungal filtrate that was used as the elicitor. The protein content of the fungal filtrate was estimated by the dye binding method of Bradford (Bio-Rad Protein Assay Kit, California, USA) using bovine serum albumin as the standard. The elicitor preparations were diluted to a protein concentration of 40 µg/mL using freshly prepared Calderon medium. Some of the filter sterilised elicitor preparations was further autoclaved at 121 °C and 15 psi pressure for 15 minutes to obtain a heat inactivated cell free culture filtrate. Both the fresh and autoclaved filtrates were used as elicitors. The elicitors were either used immediately or stored at -20 °C for use within 24 hours.

Elicitation of cell cultures

A preliminary trial was carried out to determine the volume to use for elicitation. In the test cell suspension cultures were separately treated with, 2.5 mL (2.5% v/v), 5 mL (5% v/v) or 10 mL (10% v/v) of fungal filtrate. The viability of the cell suspension cultures was then monitored for 96 hours at 24-hour intervals.

The 2.5% (v/v) elicitor concentration which resulted in the least mortality of the cell suspension cultures was subsequently used for further experiments. Cell suspension cultures were divided into five sets; four elicited groups treated with 2.5% (v/v) of fresh or autoclaved culture broth of *E. lata* or *T. atroviride* and one control group treated with 2.5% (v/v) fresh Calderon medium. Cell suspensions were elicited on the 6^{th} day (in the logarithmic phase of growth) and the cells were harvested at 6, 12, 24 and 48 hours post-elicitation. The cells were recovered by gentle vacuum filtration and immediately frozen in liquid nitrogen, and stored at -80 °C. The harvested cell biomass was used for RNA extraction and assayed for phenolic content and enzyme activity. There were three independent biological replicates for each elicitor per time point. From each biological replicate, two technical replicates were used for each test.

Determination of cell viability

Cell viability was measured using the TTC (2, 3, 5-triphenyl tetrazolium) test (Steponkus & Lanphear, 1967). One gram (fresh weight) of cells, recovered by gentle vacuum filtration, were suspended in 2 mL of TTC (10 g/L, in phosphate buffer pH 5.8) and incubated overnight in the dark at room temperature. The suspensions were then centrifuged at 12 000 rpm for 10 minutes, the supernatant discarded after which formazan, the product of TTC reduction in viable cells, was extracted from the cells in 2 mL of absolute alcohol at 70 °C for 30 minutes. Formazan concentration was then determined by reading absorbance at 485 nm and viability was measured as:

$$Viability\ (\%) = \frac{A485\ for\ treated\ cells}{A485\ for\ control\ cells} \times 100.$$

RNA extraction and quality check

Total RNA was extracted from 5 g (fresh weight) of the frozen biomass using a modified cetyltrimethylammoniunm bromide (CTAB) method of White *et al.* (2008). The frozen cells were transferred to pre-warmed CTAB extraction buffer (20 mL) in a 50-mL polypropylene tube and placed in a 60 °C water bath for 30 minutes with vortexing every 5 minutes. The tubes were then centrifuged at 13 000 rpm for 10 minutes at 4 °C and the supernatant transferred to a new tube. An equal volume of chloroform-isoamylalcohol (Chl:Ia) (24:1 (v/v)) was added, and to the tube was vortexed and centrifuged at 13,000 rpm for 10 minutes at 4 °C. The supernatant was transferred to a new tube and re-extracted with an equal volume of Chl:Ia and centrifuged at 13 000 rpm for 10 minutes at 4 °C. The supernatant (1.5 mL) was then transferred to 2 mL microfuge tubes to which 7.5 M LiCl was added to each tube mixed and stored at 4 °C overnight. Tubes were centrifuged at 13 000 rpm for 60 minutes at 4 °C and the supernatant discarded. The pellet was washed in 70% ethanol, air dried, dissolved in 30 µl nuclease free water and the RNA from the same samples pooled.

The quantity and quality of RNA extracted was assessed spectrophotometrically using the NanoDrop 1000 (NanoDrop Technologies Inc, Wilmington, Delaware, USA) at wavelength 230, 260, and 280 nm. The RNA integrity was verified by evaluating the 28S and 18S ribosomal bands after denaturing agarose (1%) gel electrophoresis.

Synthesis of cDNA and gene expression analysis

Total RNA (5 µg) was treated with DNase I (RQ1 RNase-Free DNase, Promega Corporation, Madison, USA) according to manufacturer's protocol before cDNA synthesis. First strand cDNA was synthesised using the GoScript™ Reverse Transcription System (Promega Corporation, Madison, USA) employing oligo-dT primers according to supplier's instructions. The quantity and quality of cDNA were determined spectrophotometrically using the NanoDrop 1000 at wavelength 230, 260 and 280 nm. Transcript levels of defence-related genes were determined by quantitative real time PCR. Genes involved in secondary metabolism (phenylpropanoid pathway) namely, phenylalanine ammonia-lyase (PAL), 4 coumaroyl Co-A ligase (4CL) and stilbene synthase (STS) and chalcone synthase (CHS), as well pathogenesis related proteins PR 1, 2, 3, 4, 5, 6 and chitinase IV (CHIT IV) were assayed. Transcript relative gene expression was normalised using three reference genes Actin, 60SRP and VATP16, which were found to be stably expressed in treated and non-treated cells. All primer pairs were designed using Primer3 and primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) except for the primers for the genes 60SRP and VATP16 (Gamm *et al.*, 2011). The GenBank accession numbers of the sequences on which primer design was based, the primer pairs, as well as the annealing temperatures are given in table 1. Amplification conditions for all primer pairs were optimised and validated for the Rotor-Gene 6000 (Corbett Research, Mortlake, New South Wales, Australia) thermal cycler for amplification efficiency of 95-100% and regression coefficient (r^2) of 0.95-1.00 (Appendix B; Table 1). The KAPA™ SYBR® FAST qPCR kit; Master Mix (2X) Universal (Kapa Biosystems, Boston, Massachusetts, USA) was used for cDNA quantification according to the manufacturer's protocol. Thermal cycling conditions used were 95 °C hold for 3 minutes followed by 45 cycles of: 95 °C for 3 seconds, annealing temperature for 20 seconds and 72 °C for 10 seconds followed by melt cycle from 72 to 95 °C with 1 °C increments. Melt curve analysis was used to confirm specificity of amplification and gel electrophoresis was performed on randomly selected PCR products of each primer pair. Transcript expression levels were determined and statistically analysed using Relative Expression Software Tool (REST®, developed by Pfaffl *et al.*, 2002 and freely available from Qiagen at: www.qiagen.com/REST). The gene expression levels in the control samples of each time point were defined as the 1× expression. However, when down regulation (i.e. expression ratio < 1) was significant ($P < 0.05$) the equivalent negative fold change is presented.

Determination of phenolic content of cell cultures

Phenolics were assayed for the 24 and 48 hour time points using a modified Folin-Ciocalteu (FC) reagent method of Shaver *et al.* (2011). Cell biomass harvested from elicited and control cell suspension cultures were lyophilised for 48 hours and the freeze dried cells stored at -20 °C

until extraction of phenolic compounds. Total phenolics were extracted from freeze dried cell biomass (200 mg) with 5 mL of 70% ethanol in 50-mL polypropylene tubes. The cell biomass-solvent mixture was homogenised with an ultrasonic homogeniser for 1 minute and left in the dark overnight after which the tubes were centrifuged at 13 000 rpm for 5 minutes and the supernatant further filtered through a 0.22 µm filter. One millilitre of the filtrate was transferred to new tubes to which 2 mL of the FC reagent was added followed by 1.5 mL of sodium carbonate mixed and placed in a water bath at 60 °C for 10 minutes. The tubes were quenched in an ice bath and the absorbance read at 765 nm. A blank of 70% (v/v) ethanol was used as a control and gallic acid was used as phenolic standard. A gallic acid standard curve was constructed ranging from 0 to 200 µg/mL (r^2 = 0.975). The assay was carried out on two technical replicates for each biological replicate used in the gene expression experiment.

Preparation of enzyme extract

Crude cell extracts were obtained for all cell culture treatments by homogenising frozen cell suspension biomass (5 g) in 10 mL of 0.1 M sodium-phosphate buffer (pH 6). The homogenate was centrifuged at 12 000 rpm at 4 °C for 20 minutes and the supernatant was used as enzyme extract, immediately or stored at -20 °C until assayed for activity (within 24 hours). Total protein of the crude extract was determined using the Bradford method (Bio-Rad Protein Assay Kit, Hercules, California, USA) using bovine serum albumin as the protein standard. For all protein and enzyme assays there were two technical replicates for each biological replicate.

Determination of chitinolytic activity

Chitinolytic activity was measured as a reduction in the turbidity of colloidal chitin (Harman *et al.*, 1993). The assay mixtures containing 1-mL colloidal chitin (0.5%) in potassium acetate buffer (pH 6) and 1-mL crude enzyme extract were incubated for 24 hours at 25 °C in a shaker incubator (100 rpm). The suspension was then diluted by adding 2 mL of distilled water and the absorbance measured at 510 nm. Controls for each assay were run parallel with boiled enzyme extract. One unit (U) of chitinolytic activity was defined as the amount of enzyme which resulted in 5% reduction in turbidity of the colloidal chitin suspension relative to the control under the reaction conditions. Colloidal chitin was prepared by dissolving 20 g of crab-shell chitin (Sigma) in cold concentrated hydrochloric acid (350 mL) and placed at 4 °C for 24 hours with stirring. The mixture was filtered through wool into 2 L ethanol (95%) at -20 °C with stirring. The resulting chitin suspension was centrifuged at 10 000 rpm for 15 minutes at 4 °C. The colloidal chitin pellets were washed repeatedly with water until the supernatant pH was neutral. Colloidal chitin was autoclaved and kept at 4 °C until it was used.

β-1, 3-Glucanase assay

β-1,3-glucanase was assayed colorimetrically by determining the amount of reducing sugar released from laminarin using the dinitrosalisylic acid (DNS) reagent method (Miller, 1959). The assay mixtures containing 250 µl of laminarin (2.5 mg/mL) in potassium acetate buffer (pH 6) with 100 µl enzyme extract (50% extract diluted in assay buffer) were incubated for 2 hours at 40 °C. The reaction was stopped by adding 500 µl DNS reagent and boiling on a heat block at 100 °C for 5 minutes. After cooling, 2 mL of deionised water were added and the absorbance was measured at 575 nm. For each assay, controls were run in parallel with inactivated enzyme extract (boiled enzyme extract). The quantity of reducing sugar released was calculated from a glucose standard curve (ranging from 0 to 63 µM; r^2 = 0.99). One unit (U) of β-1, 3-glucanase activity was defined as the amount of protein which catalysed the release of 1 µM of reducing sugar per millilitre per minute under the reaction conditions.

Statistical analysis

For gene expression, result means from technical replicates of each biological replicate were combined and used for the calculation of relative gene expression and the statistical significance thereof using REST analysis. Data for total phenolic content and enzyme assays for the two independent experiments was also combined before performing one way analysis of variance (ANOVA). Significant differences among treatments were separated using Fisher's least significant differences (LSD) at 5% significance level ($P < 0.05$). SAS version 8.2 statistical software (SAS institute Inc.) was used for analysis.

Results and discussion

A. Improve the practical application of *Trichoderma*

1. To determine the best formulation of the *Trichoderma* product

In vitro* effect of nutrients on the growth of *T. atroviride

There was no difference in the growth rate of the *T. atroviride* strain on the media tested with glucose, urea and yeast extract additives. However, despite the similar growth rate, at low glucose concentrations (1-3 g) sparse mycelial growth and earlier sporulation was observed. At high glucose concentrations (5 and 10 g) conidia were formed after 98 hours while at lower glucose concentration (1-3 g) conidia formed after 48 hours. Urea and yeast extract were neither toxic nor growth stimulating at all the concentrations tested. Since there were no toxic concentrations found, the following were then selected for amending the conidial suspensions: 0.4 g/L urea and 3 g/L yeast extract. For the glucose, a concentration of 2 g/L was chosen to prevent reduction in water activity of the *T. atroviride* suspensions and possible growth advantage for the pathogens.

Detached grapevine cane assay: Effect of biocontrol enhancers on *T. atroviride* wound colonisation and wound protection

Analysis of variance on the incidence of the biocontrol agent and pathogen found highly significant differences ($P < 0.001$) between the treatments. *Trichoderma atroviride* was not isolated from wounds treated with sterile distilled water. The incidence of *T. atroviride* ranged from 68.82% to 100% in inoculated wounds. All *Trichoderma* treatments had significantly lower *Pa. chlamydospora* incidence compared to the control and reduced the pathogen incidence by at least 81% (Table 1). This was also true for the isolation frequency (a relative measure of the extent of wound colonisation) of *Pa. chlamydospora* which revealed significant treatment × isolation zone interactions ($P < 0.001$) and reduction of the pathogen isolation in all *Trichoderma* treatments. Neither the incidence of *T. atroviride* or *Pa. chlamydospora*, nor the isolation frequencies of *Pa. chlamydospora* was sufficient to discern the best treatment among the nutritional amendments, therefore, the isolation extent of *Trichoderma* wound colonisation with depth as estimated by the isolation frequency was used.

Analysis of variance on the isolation frequency of *Trichoderma* revealed a treatment × isolation zone interaction ($P = 0.007$). *Trichoderma* isolation frequency decreased with depth from the pruning wound surface in all treatments. A non-linear regression model was derived that could reliably estimate the isolation frequency of *Trichoderma* with depth from the pruning wound surface. The regression model; $y = a + b^{-x}$, where x is the isolation depth from wound surface, was significant ($P < 0.031$) for all *Trichoderma* treatments except for the Yeast extract ($P = 0.061$) treatment. The regression coefficient (r^2) for the curve of the model ranged from 0.74 to 0.98.

The *Trichoderma* isolation frequency at 2 cm below the pruning wound surface was chosen to determine which treatments had significantly higher *Trichoderma* isolation frequency than the water suspension treatment. After pruning, the wood tissue naturally dies-back as the wound heals. Two centimetres below the wound surface is likely to be well below the dieback zone and a good indicator of colonisation in the live wood tissue. Using the regression model the *T. atroviride* isolation frequency at the 2 cm position in the water suspension would be 11.49%. The Fischer's least significance difference was 16.83 ($P = 0.05$). All treatments that had a *T. atroviride* isolation frequency below 28.32% at the 2 cm position did not differ significantly ($P > 0.05$) from the water suspension treatment. Similarly, nutritional amendments that had an

isolation frequency greater than 28.32% at 20 mm depth had significantly ($P < 0.05$) higher *T. atroviride* colonisation extent than the water suspension. Therefore, the nutritional amendments Yeast extract, Yeast extract + Urea and Yeast extract + Urea + Glucose were chosen for further field testing.

Field evaluation: Effect of biocontrol enhancers on *T. atroviride* wound colonisation and wound protection

Optimisation of broth formulation: The addition of an organic nitrogen source (peptone) significantly ($P < 0.001$) increased the chitinolytic activity of the culture filtrate from 3.20 U/mL to 5.36 U/mL. The nitrogen source (peptone) was thus included in the preparation of the broth cultures. The chitinolytic activities of the culture filtrate at different times after culture initiation are shown in Figure 2. The highest activity (15.63 U/mL) was reached after 72 hours after which the activity declined gradually at 96 (12.45 U/mL) and 120 hours (9.92 U/mL). The 72-hour time point was thus selected for harvesting the broth.

Field evaluation 2011: Analysis of variance found significant cultivar × treatment interactions ($P < 0.001$) in the incidence of *Trichoderma* spp. and *Pa. chlamydospora* and thus analysis for the cultivars was done separately. The incidence of *Trichoderma* spp. was higher in the Thompson Seedless compared to the Chenin blanc (Table 1). In the spur pruned Chenin blanc, the Yeast extract + Urea and the Broth treatments gave high incidences of *Trichoderma*, but were not significantly higher than the un-amended UST1 and yeast extract suspensions ($P > 0.05$). In the cane pruned cultivar, Thompson Seedless, the broth treatment gave the highest incidence of *Trichoderma* significantly higher ($P < 0.05$) than the rest of the treatments.

Table 1: The incidence of *Trichoderma* species isolated from pruning wounds of Chenin blanc and Thompson Seedless grapevines treated with *Trichoderma atroviride* UST1 with or without nutritional ammendments, Garrison (a fungicidal paint) and Eco 77 (a registered biocontrol product) for pruning wound protection in 2011 trials.

Nutritional amendment or Treatment	***Trichoderma* incidence (%)***	
	Chenin blanc	**Thompson Seedless**
UST1 in water	11.98^{AB}	20.84^{B}
UST1 + Broth	16.15^{A}	42.71^{A}
UST1 + Y. extract (Y.E)	10.41^{AB}	31.25^{B}
UST1 + Y.E + Urea	17.71^{A}	28.13^{B}
UST1 + Y.E + Urea + Glucose	6.77^{BCD}	25.52^{B}
Eco 77	8.33^{BC}	23.96^{B}
Garrison	1.04^{CD}	2.60^{C}
Water	0.00^{D}	4.17^{C}
LSD ($P = 0.05$)	7.66	11.32

*Values within a column followed by the same letter are not significantly different.

Pathogen incidence in the two cultivars is summarised in table 2. The pruning wound treatments were able to reduce the incidence of *Pa. chlamydospora* but with varying efficacy between the two cultivars. Garrison, the fungicidal paint, was the best treatment in reducing *Pa. chlamydospora* infection in the Chenin blanc while in the Thompson Seedless, Garrison efficacy was comparable to the biocontrol formulae. Extensive wound sap flow was observed after pruning and wound treatment in the Thompson Seedless such that for some of the wounds the Garrison paint was observed dripping off to the ground leaving the wounds exposed. Although wound sap was also observed in the Chenin blanc, it did not lead to wash-off of the Garrison paint, which could be attributed to the wound position on the spur compared to the wound on a

6-8 nodes length hanging cane. However, Garrison did not reduce natural infection in both cultivars. It was also observed that about 20% of the wounds treated with Garrison had no wound dieback below the paint (Figure 1). Normally after pruning the wood below, the wound dies back down to the first shoot (apical node).

Table 2: The incidence of *Phaeomoniella chlamydospora* and grapevine trunk pathogens in pruning wounds of Chenin blanc and Thompson Seedless grapevines treated with *Trichoderma atroviride* UST1 suspensions with or without nutritional ammendments and formulated products, Garrison (a fungicide containing paint) and Eco 77 (a registered biocontrol product), for pruning wound protection in 2011 trials. Treated pruning wounds were either inoculated with *Pa. chlamydospora* a day after pruning or left to infection by natural inoculum.

Treatment	**Pathogen incidence (%)***			
	Chenin blanc		**Thompson Seedless**	
	Pa. chlamydospora	Natural inoculum[1]	*Pa. chlamydospora*	Natural inoculum
UST1 in water	27.08^{B} (40)[1]	14.58^{AB} (46)	4.17^{B} (90)	12.50^{AB} (57)
UST1 + Broth	18.75^{B} (59)	4.17^{B} (85)	8.33^{B} (80)	8.33^{B} (71)
UST1 + Y. extract (Y.E)	25.00^{B} (45)	14.58^{AB} (46)	10.42^{B} (90)	6.25^{B} (78)
UST1 + Y.E + Urea	27.08^{B} (41)	18.75^{AB} (30)	4.17^{B} (90)	12.50^{AB} (57)
UST1 + Y.E+Urea+Glucose	12.50^{BC} (74)	6.25^{B} (77)	6.25^{B} (85)	12.50^{AB} (57)
Eco 77	12.50^{BC} (74)	12.50^{AB} (53)	10.42^{B} (75)	12.50^{AB} (57)
Garrison	2.08^{C} (95)	10.42^{AB} (61)	8.33^{B} (80)	18.75^{AB} (35)
Water (control)	45.83^{A}	27.08^{A}	41.67^{A}	29.17^{A}
LSD (P = 0.05)	16.09	18.66	12.25	19.31

*Values within a column followed by the same letter are not significantly different.
[1]Pathogen reduction (Pr) calculated as: Pr (%) = 100 ((Pc - Pt) / Pc), where Pc and Pt are the pathogen incidence in the control and treatment respectively.

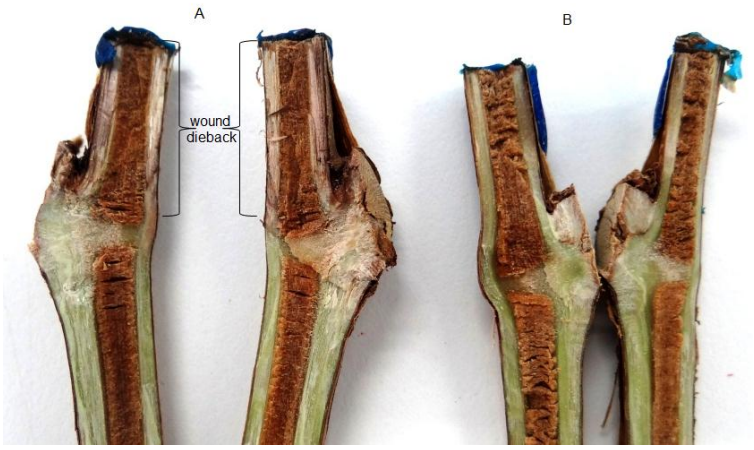

Figure 1: Vertical section through grapevine canes eight months after pruning wound treatment with a wound sealant, Garrison, showing normal wound healing (A) with wood dieback down to the apical node and an unhealed wound (B).

Wound treatment resulted in reduction of infection in both the pathogen inoculated and un-inoculated wounds. The major pathogens isolated from the pruning wounds were species of Botryospheriaceae, Diatrypaceae and *Phomopsis*. In the Chenin blanc, the treatments Yeast

Extract + Urea + Glucose and Yeast extract + Urea had highest pathogen reduction (74%) in the inoculated wounds although this was not significantly ($P > 0.05$) different from the rest the *Trichoderma* treatments. Under natural inoculum, the Broth and the Yeast extract + Urea + Glucose treatments had high pathogen control, reducing infection by 85% and 77%, respectively (Table 2). However, this did not differ significantly ($P > 0.05$) from the rest of the *Trichoderma* treatments. In the Thompson Seedless, all treatments similarly reduced *Pa. chlamydospora* infection by at least 75% (Table 2). Under natural inoculum the treatments Yeast extract and Broth were the only treatments that significantly reduced infection. It was also interestingly noted that the Broth treatment also had a comparatively high incidence of *Trichoderma* in both cultivars.

Field evaluation 2012: The incidences of *Trichoderma* spp. in the Chenin blanc were much higher in the 2012 trial (62-78%) compared to the 2011 trial (7-18%). Analysis of variance found significant differences ($P < 0.001$) in the incidence of *Trichoderma* among the treatments with the highest incidence in the Yeast extract + Urea treatment (77.80%) though this was not significantly different from treatments Yeast Extract + Urea + Glucose (76.74%), Broth (70.44%) and the un-amended UST1 (71.01%) (Table 3). The major non-inoculated pathogens that were isolated from the wounds were species of Botryospheriaceae and Diatrypaceae. In wounds not inoculated with the pathogen, all treatments except Eco 77 significantly reduced wound infection. The treatment, Yeast Extract + Urea + Glucose reduced natural pathogen infection the most (84% reduction) although this was not significantly different ($P > 0.05$) from the other nutritional amendments (Table 3).

In pathogen-inoculated wounds, analysis of variance found significant treatment × inoculation day interactions ($P < 0.001$) in the incidence of *Pa. chlamydospora*. For better comparison between treatments the pathogen incidence was then analysed separately for each inoculation day. There were significant differences ($P < 0.001$) in the incidence of *Pa. chlamydospora* among treatments (Table 4). The pruning wounds were more susceptible when inoculated a day after pruning (mean incidence 73.78%) and became less susceptible with time as shown by the reduction in the pathogen incidence in the water control treatment (Table 4). For most of the treatments there was a decline in *Pa. chlamydospora* incidence over the seven day period of inoculation. Garrison, completely inhibited infection by *Pa. chlamydospora* in wounds inoculated with the pathogen a day after pruning. In wounds inoculated at day three and seven, *Pa. chlamydospora* incidence did not significantly differ ($P > 0.05$) between the Garrison and the *Trichoderma* treatments. All the *Trichoderma* treatments significantly ($P < 0.05$) reduced pruning wound infection by *Pa. chlamydospora*. The treatments, Yeast extract + Urea + Glucose and the Broth were able to reduce infection in day-1 inoculated wounds to levels statistically similar ($P > 0.05$) to that of Garrison.

Table 3: The effect of nutritional amendments on the incidence of *Trichoderma* spp. and grapevine trunk pathogens (in non-inoculated wounds) in pruning wounds of Chenin blanc in 2012 field trial. Wounds were treated with *T. atroviride* (UST1) conidia suspensions with various nutritional amendments. A registered ready formulated biocontrol agent, Eco 77, based on *T. harzianum* and pruning wound paint with fungicide, Garrison, were also included.

Treatment	**Pruning wound incidence (%) of ***	
	Trichoderma spp.	Pathogens (Natural inoculum)
UST1 in water	71.01^{AB}	13.20^{C} (72)[1]
UST1 + Broth	70.44^{AB}	25.00^{BC} (48)
UST1 + Yeast extract (Y.E)	63.46^{B}	15.84^{C} (67)
UST1 + Y.E+Urea	77.80^{A}	21.46^{BC} (55)
UST1 + Y.E+Urea+Glucose	76.74^{A}	7.71^{C} (84)
Eco 77	61.64^{B}	34.74^{AB} (27)
Garrison	4.79^{C}	19.19^{BC} (60)
Water (control)	1.04^{C}	47.57^{A}
LSD (P = 0.05)	12.08	21.05

*All values within a column followed by the same letter are not significantly different according to Fischer's least significant difference (LSD) test at P = 0.05.
[1]Pathogen reduction (Pr) calculated as: Pr (%) = 100 ((Pc - Pt) / Pc), where Pc and Pt are the pathogen incidence in the control and treatment respectively.

Table 4: The effect of nutritional amendments of *T. atroviride* (USPP-T1) conidia suspensions on the infection on Chenin blanc pruning wounds by *Phaeomoniella chlamydospora* in 2012 field trial. Treated wounds were inoculated with *Pa. chlamydospora* 1, 3 or 7 days after pruning. A registered ready formulated biocontrol agent, Eco 77, based on *T. harzianum* and pruning wound paint with fungicide, Garrison, were also included.

Treatment	**Incidence of *Pa. chlamydospora* in wounds inoculated on**		
	Day 1	Day 3	Day 7
UST1 in water	23.26^{BCD} (68)[1]	25.00^{B} (54)	5.90^{BCD} (85)
UST1 + Broth	18.81^{CDE} (74)	5.56^{B} (90)	9.03^{BC} (78)
UST1 + Yeast extract (Y.E)	21.35^{BCD} (71)	22.98^{B} (58)	9.58^{BC} (76)
UST1 + Y.E+Urea	39.43^{B} (47)	10.83^{B} (80)	0^{D} (100)
UST1 + Y.E+Urea+Glucose	10.76^{DE} (85)	6.25^{B} (89)	10.62^{BC} (74)
Eco 77	36.61^{BC} (50)	19.44^{B} (64)	12.22^{B} (70)
Garrison	0^{E} (100)	11.65^{B} (79)	2.78^{CD} (93)
Water (control)	73.78^{A}	54.93^{A}	40.63^{A}
LSD (P =0.05)	20.32	19.55	8.60

All values within a column followed by the same letter are not significantly different.
[1]Pathogen reduction (in brackets) calculated as: Pr (%) = 100 ((Pc - Pt) / Pc), where Pc and Pt are the pathogen incidence in the control and treatment respectively.

Our current study shows none or marginal improvement of grapevine pruning wound protection by *T. atroviride* bio-enhancers. However, a chitin based culture filtrate and a combination of

yeast extract, urea and glucose showed potential in enhancing *T. atroviride* pruning wound protection. None of the formulations proved to be significantly better, therefore there is no unique patent. The commercialisation of T1 will be done in collaboration with Innovus.

Garrison was always amongst the best treatments in reducing *Pa. chlamydospora* infection. The reduction in *Pa. chlamydospora* wound infection by Garrison (of at least 79%) in the current study is slightly higher than that reported by Rolshausen *et al*. (2010) from California (63%). Garrison definitely holds potential to be used for pruning wound protection. Chempac (Pty) Ltd is interested to register Garrison for grapevine pruning wound protection. The field trials conducted will possibly be used by Chempac (Pty) Ltd for registration. An additional trial would be needed to fulfil the number of trials required for registrations.

2. To determine the best time and method of application.

Best time of application

When pruning was carried out it was apparent that cultivars were in different physiological states. In the early pruning, July, the Cabernet Sauvignon was still dormant with little or no sap bleeding was observed from the wounds. In the late pruning, August, the vines had become active and wound sap was observed. Contrary, in the early cultivar Chenin blanc, pruning wound sap was observed from almost all wounds at both pruning times, however, the bleeding was observed to be less in the August pruning.

Pruning wounds were successfully colonised by *Trichoderma* spp. applied onto the wounds. Germination percentages of all suspensions were between 98% and 100%. Control wounds were naturally infected by grapevine trunk pathogens. When both *Trichoderma* sp. and a pathogen were isolated from the same wound, such pruning wounds were regarded as infected. *Trichoderma* species were erratically isolated from a few water control treatments albeit at extremely low incidences (< 7%).

Effect of pruning time on wound colonisation by *Trichoderma* spp.

To meet the assumptions of ANOVA the full data set was transformed (weighted) by the reciprocal of the experimental error of the incidence or isolation frequency and thus the final analysis was a weighted ANOVA (John & Quenouille, 1977). Analysis of variance revealed highly significant ($P < 0.001$) year × cultivar × pruning time × treatment interactions for the incidence and isolation frequencies of *Trichoderma* spp. The mean incidences of *Trichoderma* spp. for each pruning time and for each cultivar are shown in Table 5. The incidences of *Trichoderma* spp. were highest in 2012 in Chenin blanc for both pruning times. In Cabernet Sauvignon, significantly higher ($P < 0.05$) incidencies of *Trichoderma* spp. were observed in the early pruning when the vines were dormant as compared to the late pruning when the vines were becoming active. The time of breaking dormancy often associated with pruning wound sap-flow, had low incidences of *Trichoderma* spp., that is, July 2011 for Chenin blanc and August 2011 and 2012 for Cabernet Sauvignon. The isolation frequencies of *Trichoderma* species followed a similar pattern as the incidences but revealed some further detail for Chenin blanc in 2012. Although the incidences of *Trichoderma* spp. in the July and August pruning were not significantly different for 2012, the extent of wound colonisation by *Trichoderma* spp. was significantly higher ($P < 0.05$) in the August pruning compared to the July pruning.

Table 5: Percentage incidence of *Trichoderma* species re-isolated from pruning wounds of Chenin blanc and Cabernet Sauvignon treated with *Trichoderma* suspensions at different pruning times (July and August) over two seasons. Values are means of all wounds that received *Trichoderma* treatments (UST1 and Eco 77) per pruning time.

Treatment	**Incidence of *Trichoderma* species (%)**							
	Chenin blanc				Cabernet Sauvignon			
	2011		2012		2011		2012	
	July	August	July	August	July	August	July	August
UST1	54.80^{CD}	68.00^{B}	90.00^{A}	86.67^{A}	87.33^{A}	44.67^{E}	70.00^{B}	53.33^{D}
Eco77	40.33^{E}	56.67^{CD}	87.00^{A}	86.00^{A}	73.33^{A}	40.67^{E}	59.33^{C}	42.67^{E}

Values followed by the same letter are not significantly different from each other (P > 0.05; LSD 5.42).

Effect of time of wound treatment on colonisation by *Trichoderma* spp.

Analysis of variance for the sub-plot effects (pruning time, wound treatment (UST1, Eco 77 and water) and time of wound treatment) were carried out separately for each cultivar and year since comparison of the full data set had revealed year × cultivar interactions. Significant pruning time × wound treatment × time of wound treatment interactions ($P < 0.001$) were found for the incidence and the frequency of isolation of *Trichoderma* spp. in both Chenin blanc and Cabernet Sauvignon for both field trials (2011 and 2012).

Incidence of biocontrol agent*:* In Chenin blanc, for UST1 in 2011, the incidence was significantly higher ($P < 0.05$) at the 6- and 24-hour application times (80.67% and 73.33% respectively) than the rest of the application times,in the July pruning. In the late pruning, *T. atroviride* incidence was high for the immediate application (0 hours; 93.33%) and remained relatively high for the 6 hours (83.33%) and 24 hours (76.67%) application times which did not differ significantly ($P > 0.05$) but were significantly higher ($P < 0.05$) than the 48- and 96- hour application times. In Cabernet Sauvignon the incidence of *T. atroviride* was high from the immediate up to the 48 hours application times (ranging from 90 – 100%) in the July pruning. In the late pruning of the 2011 trial the *Trichoderma atroviride* incidence in UST1 treatment was highest for the 6 hour application time (83.33%) which was significantly higher ($P < 0.05$) than the rest of the application times (< 67%). Similar patterns were observed with the *T. harzianum* treatment, in the Eco 77, where the 6 hours application time had the highest *Trichoderma* sp. incidence (68.33%), significantly higher than the rest of the application times (< 47%), in the early pruning of the Chenin blanc in 2011. In Cabernet Sauvignon, the July pruning had significantly ($P < 0.05$) higher *T. atroviride* incidence than the late pruning for each specific application time.

In 2012, there was less variation in the incidence of *Trichoderma atroviride* between the early and late pruning times in Chenin blanc for the UST1 treatment except for the significantly low ($P < 0.05$) incidence (50%) at the 96 hours application time in the August pruning. Variations were still observed between the early and late pruning times in Cabernet Sauvignon with the early pruning having relatively higher *Trichoderma* sp. incidences compared to the late pruning for most application times. The 6-hour application time resulted in significantly higher incidence of *T. atroviride* incidence in both the early (96.67%) and late (93.33%) pruning times in the Cabernet Sauvignon. The Eco 77 treatment followed similar patterns as the UST1 treatment in both cultivars except for the relatively lower *Trichoderma* sp. incidences in the Cabernet Sauvignon. Overall, the 6-hour application time had consistently high incidences of *Trichoderma* spp. regardless of the year, cultivar or *Trichoderma* treatment (*T. atroviride* or *T. harzianum*).

Frequency of isolation of biocontrol agent*:* While the incidence measured the number of pruning wounds from where *Trichoderma* spp. was isolated, the frequency of isolation measured how many times the biocontrol agent was isolated from each pruning wound. This gives a relative measure of the extent (quality) of wound colonisation by the biocontrol agent since high wound colonisation is likely to result in better wound protection. Analysis of variance revealed significant pruning time × wound treatment × time of wound treatment interactions ($P < 0.05$) in the isolation frequencies of *Trichoderma* spp. in both cultivars and trials. In 2011, the isolation frequencies for both treatments of *Trichoderma* spp., UST1 and Eco 77, followed a similar pattern as the incidence with minor exceptions. The late pruning in Chenin blanc resulted in better wound colonisation for the immediate, 6- and 24-hour application times as revealed by the significantly high ($P < 0.05$) isolation frequencies than the early pruning for the same application times. The 6-hour application had the highest isolation frequency, significantly higher ($P < 0.05$) than all the other application times in the early pruning for both *Trichoderma* spp. treatments. Cabernet Sauvignon in 2011 generally had higher isolation frequencies in the early pruning compared to the late pruning as was observed with the incidence.

In the 2012 trial, the isolation frequency of *T. atroviride*, from the UST1 treatment, in Chenin blanc showed significant differences between the early and late pruning times, a detail that could not be perceived from the incidence data. The extent of wound colonisation was significantly higher ($P < 0.05$) in the late pruning compared to the early pruning for all application times except for the 96-hour application. For the July pruning, the isolation frequencies of *T*. *atroviride* for the immediate (69.17%) and 6 hours (65%) applications, did not differ significantly ($P > 0.05$), but were significantly higher ($P < 0.05$) than the rest of the application times (< 51%). In Cabernet Sauvignon UST1 treatment, the 6 hours application for both pruning times had the highest *Trichoderma* sp. isolation frequencies of the application times. The isolation frequencies in the Eco 77 treated wounds in the 2012 trial followed similar patterns as observed with UST1 in both cultivars except that the percentage frequencies were relatively lower.

Prevalence of grapevine trunk pathogens in pruning wounds

Several grapevine trunk pathogens were isolated namely, *Pa. chlamydospora*, species of *Phaeoacremonium* and *Phomopsis* as well as of the families Botryosphaeriaceae and Diatrypaceae. Due to the high variability in the isolation of each specific pathogen between the cultivars and the trial years, wound infection was best analysed for all pathogens collectively. Comparison of the 2011 and 2012 trial data sets revealed significant year × cultivar interactions ($P = 0.01$) and hence analysis of variance for each cultivar and year was carried out separately.

Effect of pruning time on natural wound infection by trunk pathogens: Due to the high variability of pathogen infection in the *Trichoderma* spp. treated wounds, only the water control wounds were used to determine the effect of the time of pruning on wound infection. Analysis of pathogen incidence in the water control treatments revealed interesting results that have implications on the cultural practices aimed at grapevine trunk disease management in the trial area. Generally, pathogen incidence was higher in 2012 than 2011 for both pruning times in the control treatments (Table 6). Late pruning resulted in significantly ($P < 0.05$) more wound infections compared to the early pruning for both field trials and both years. In Chenin blanc, *Pa. chlamydospora* and *Phomopsis* species were the major pathogens isolated from the water treated wounds in 2011 while in Cabernet Sauvignon it was species of Botryosphaeriaceae and *Phomopsis*. In 2012, Botryosphaeriaceae species were the main pathogens isolated from both cultivars.

Table 6: Pathogen incidence in Chenin blanc and Cabernet Sauvignon water treated (control) wounds infected by grapevine trunk disease pathogens at early and late pruning times (July and August) over two seasons.

Year	Pathogen Incidence (%)			
	Chenin blanc		Cabernet Sauvignon	
	2011	2012	2011	2012
July	10.00[B]	38.00[B]	15.33[B]	34.00[B]
August	22.00[A]	57.33[A]	28.67[A]	59.33[A]
LSD[2]	8.53	10.88	12.76	11.00

Values in the same column followed by the same letter are not significantly different from each other ($p > 0.05$). [2]LSD – least significant difference.

Effect of *Trichoderma* spp. treatments on pruning wound infection

Pathogen incidence was low and highly variable in the wounds treated with *Trichoderma* spp. wounds due to the wound protective effect of the biocontrol agent as well as variability of natural inoculum. Variability of natural inoculum could also be the reason for the lack of significant ($P > 0.05$) pruning time × wound treatment × time of wound treatment interactions found in Cabernet Sauvignon for both years and Chenin blanc in 2011. The *Trichoderma* treatments resulted in significant ($P < 0.05$) reduction in wound infection in both cultivars. Among the wounds treated with *Trichoderma* spp., pathogen incidence was lower for the immediate, 6- and 24-hour wound treatment times whilst it was relatively higher for the 48- and 96-hour application times.

Due to the lack of pruning time × wound treatment × time of wound treatment interactions for all trials, the effect of the *Trichoderma* spp. treatments and time of application on wound protection was assessed by computing pathogen reduction. The isolation of grapevine trunk pathogens was reduced by the *Trichoderma* treatments and pathogen reduction was higher for almost all application times (after pruning) in the 2012 trial. In the 2011 trial, pathogen reduction was highest at the immediate, 6 and 24 hour applications, gradually declining with time. The 6-hour application time was more consistent in its pathogen reduction compared to the immediate and 24-hour application times regardless of the pruning time (July or August).

Weather data

The average temperature in the week from the day of first pruning in July and August 2011 was 15.6 °C and 12.9 °C, respectively, while the average relative humidity was 59.48% and 64.80%, respectively in 2011. In comparison with the average temperatures of 12.0 °C and 13.4 °C, and average relative humidity of 69.14% and 73.91%, respectively, were found for the same period of pruning in July and August of 2012. The total rainfall received in the whole month was 37.59 mm and 85.34 mm in July and August of 2011, respectively, in comparison to 131.60 mm and 173.70 mm in the respective months in 2012. There were more rain events in 2012 than in 2011, a factor that has been correlated more to spore availability in the vineyard (Van Niekerk *et al*., 2010). In July and August of 2011 there were three and four rain events (of > 3 mm) while there were ten and twelve in the respective months of 2012.

Best method of application

There were significant differences ($P < 0.001$) in the incidence of *Trichoderma* and *P. chlamydospora* between the *Trichoderma* application methods in the pathogen inoculated wounds. In both vineyards the gator sprayer had significantly less ($P < 0.05$) *Trichoderma* incidence than the knapsack sprayer and painted wounds which did not significantly differ ($P > 0.05$; Table 7). This was similar to what was observed in the wounds that were not inoculated with the pathogens (Table 8). Treatments that had high *Trichoderma* incidence in the pathogen inoculated wounds also markedly reduced *P. chlamydospora* wound infection compared to the water controls in both vineyards although this may have not been statistically significant (Table

7).The incidence of *P. chlamydospora* in the gator sprayed wounds in vineyard 2 was statistically similar lower ($P > 0.05$) than the control treatment. The poor control or lack of clear distinction between treatments in reducing *P. chlamydospora* incidence is likely due to the short time allowed between wound treatment and inoculation. *Trichoderma* pruning wound agents need time to germinate and colonise the wound before they can provide wound protection which creates a window of infection between application time and wound colonisation by bio-control agent. This can be addressed by formulation with nutritional amendments that enhance conidia germination and wound colonisation or through the integration of fungicides and the bio-control agent. Under natural pathogen inoculum all the *Trichoderma* treatments regardless of their *Trichoderma* incidence reduced pathogen infection in both vineyards (Table 8). Pathogen control under natural inoculum was higher in vineyard 2 which surprisingly had high pathogen incidence in pathogen inoculated wounds. From these results, it can be concluded that wound painting or spraying with a knapsack sprayer will result in better wound colonisation and protection than using a gator sprayer. This is mainly due to the human factor, targeted application is easier when painting or spraying with a hand held sprayer, manoeuvres of which are not possible with a fixed sprayer on a gator.

Table 7: Effect of *Trichoderma* application method on the incidence of *Trichoderma atroviride* and *Phaeomoniella chlamydospora* from grapevine pruning wounds of Cabernet Sauvignon inoculated with the Petri disease pathogen *P. chlamydospora* three days after treatment. The trials were carried out in two vineyards on different farms situated in the Stellenbosch.

Method of application	**Incidence (%)***			
	Vineyard 1		**Vineyard 2**	
	T. atroviride	*P. chlamydospora*	*T. atroviride*	*P. chlamydospora*
Painting	55.56^{A}	20.83^{BC} (64)[1]	52.78^{A}	47.22^{B} (44)
Back-pack sprayer	54.17^{A}	11.11^{C} (81)	45.83^{A}	48.61^{B} (43)
Gator sprayer	15.28^{B}	33.33^{B} (43)	8.33^{B}	66.67^{AB} (21)
Water	0^{C}	58.33^{A}	0^{B}	84.72^{A}
LSD	6.79	15.27	12.06	19.48

*****Values followed by the same letter are not significantly different according to Fischer's least significant difference (LSD) test at $P = 0.05$
[1]Percentage pathogen reduction

Table 8: Effect of *Trichoderma* application method on the incidence of *Trichoderma atroviride* and grapevine trunk pathogens in pruning wounds of Cabernet Sauvignon under natural inoculum. The trials were carried out in two vineyards on different farms situated in the Stellenbosch.

Method of application	**Incidence (%)***			
	Vineyard 1		**Vineyard 2**	
	T. atroviride	Pathogens	*T. atroviride*	Pathogens
Painting	62.50^{A}	9.73^{B} (73)[1]	56.95^{A}	5.56^{B} (85)
Back-pack sprayer	54.17^{A}	12.50^{B} (65)	51.39^{A}	8.33^{B} (77)
Gator sprayer	20.84^{B}	11.11^{B} (69)	8.33^{B}	6.95^{B} (81)
Water	0^{C}	36.11^{A}	0^{B}	36.11^{A}
LSD	12.06	10.37	9.82	11.69

*****Values followed by the same letter are not significantly different according to Fischer's least significant difference (LSD) test at $P = 0.05$
[1]Percentage pathogen reduction calculated

3. Development of fungicide resistant *Trichoderma* isolates.

Benzimidazole sensitivity of wild type *Trichoderma* strains

The minimum inhibitory concentrations of the fungicides were tested so as to determine the concentration to use for screening resistant mutants. All the wild type *Trichoderma* isolates were found to be naturally resistant to thiophanate methyl. Both mycelium and conidia could not be inhibited by thiophanate methyl even at the highest concentration tested (100 µg/mL). Thiophanate methyl was thus not used further for mutant screening. The other fungicides, benomyl and carbendazim, completely inhibited mycelium growth of all the wild type *Trichoderma* isolates at 2.5 µg/mL, while conidial germination could not completely be inhibited even at the highest concentration tested (50 µg/mL). Conidia plated on benomyl and carbendazim amended medium could germinate (produce germ tube size of conidia), but the fungicides prevented further germ tube growth. However, at concentrations above 10 µg/mL conidia germination was very low (< 20%). The concentration for mutant screening was therefore set at 10 µg/mL for benomyl and carbendazim.

Sensitivity of wild type *Trichoderma* strains

There was a strong negative correlation (R^2 = 0.88-0.94; Appendix C, Figure 1) between gamma irradiation dosage and conidia survival in the wild type *Trichoderma* isolates. There were no significant differences (P > 0.05) in the D_{10} values between *T. atroviride* [UST1 (213 Gy) and UST2 (211 Gy)] and *T. harzianum* [T77 (216 Gy)]. A slightly higher dose than the D_{10}, 250 Gy was used for mutagenesis.

Benzimidazole resistant mutants and mutant fitness

Only one resistant colony of *Trichoderma* developed on benomyl and carbendazim amended medium from the wild type isolates UST1 and T77, respectively. Two colonies developed, one on each of the benomyl and carbendazim amended medium from UST2. When sub-cultured on fungicide amended medium, colonies of one of the mutants from UST2 could not grow beyond 10 mm in diameter and was not tested further. Conidia of the three remaining mutants MT1 (from UST1), MT2 (from UST2) and MT77 (from T77) could germinate on amended medium without inhibition of germ tube extension (Figure 1B) even at the highest concentration tested (100 µg/mL). Mycelium of the mutants could also grow on the amended PDA, however, growth was slower than on fungicide free medium. Mutants isolated from benomyl amended medium were cross-resistant to carbendazim and vice versa. They also maintained their resistance to thiophanate methyl. Resistance was stable even after ten cycles of sub-culturing on benzimidazole free medium and after storage at 4 °C for more than a year. These mutants are now stored at the University of Stellenbosch culture collection under the accession numbers STE-U 7733, 7734 and 7735 for MT1, MT2 and MT77, respectively.

Growth of the mutants on fungicide free PDA at different temperatures was compared to that of the wild type isolates so as to assess the fitness of the mutants. All mutants, like the wild types grew at 5 to 30 °C with the highest growth rate at 25 °C. There was no growth observed at 35 and 40 °C, but when mycelium previously incubated at 35 °C for 7 days was transferred to 25 °C, growth resumed while there was no growth from dishes previously incubated at 40 °C. Mutants MT1 and MT2 had similar growth patterns as the wild type isolates, UST1 and UST2 respectively, at all temperatures tested but MT2 produced conidia after 6-7 days which was 2-3 days later than UST2. The growth of the mutant MT77 was much slower (~36-40% less) compared to the wild type T77 at all temperatures where the wild type could grow. No growth was observed for T77 at 35 and 40 °C.

Mutants' *in vitro* antagonism against grapevine trunk pathogens

The *T. atroviride* mutants MT1 and MT2, showed antagonistic action towards the grapevine trunk pathogens tested. The mutants overgrew all the pathogens tested and produced conidia profusely above the overgrown fungus. On plates dual inoculated with *E. lata*, both the mutant and the pathogen would stop growing just before the point of hyphae interactions with a small

inhibition zone between the different hyphae. Later *Trichoderma* mutants would grow over the pathogen. The mutant MT77 only overgrew *Pa. chlamydospora* while with the other fungi, it would stop to grow at the point of hyphal interaction. This was different from the wild type T77 which overgrew all pathogens in dual plates. Microscopically, antagonistic interactions between hyphae of the mutants and pathogens were readily observed with mutant MT1 compared to the other mutants. The hypha of MT1 was observed coiling around the pathogen hyphae on interaction with *N. parvum* and *Pa. chlamydospora*. Disintegration of pathogen hyphae was observed on interaction of both MT1 and MT2 with *D*. *seriata*. The adhesion of mutants MT1 and MT2 hyphae to pathogen hyphae was also observed on all pathogens. No microscopic interactions were observed with MT77.

***In vivo* evaluation of grapevine wound protection**

Incidence of *T. atroviride*: *Trichoderma atroviride* was not isolated from pruning wounds that received the water and fungicide only (carbendazim and thiophanate methyl) treatments. The fungicide only treatments were thus excluded from the analysis on the incidence of *T. atroviride* from the pruning wounds. Mutant strain MT1 was able to colonise pruning wounds in the presence and absence of the fungicide carbendazim. Analysis of variance did not find significant treatment × inoculation time interactions ($P = 0.416$) nor inoculation time differences ($P = 0.124$), but significant treatment differences ($P < 0.001$) on the incidence of *T. atroviride* in the pruning wounds. The mutant and carbendazim combination treatment (MT1+Carbendazim) had the highest *T*. *atroviride* incidence (59.03%) which was not significantly ($P > 0.05$) higher than that of the other combination treatments, UST1+Thiophanate methyl (54.16%) and treatment MT1 (53.17%). All these treatments had significantly higher ($P < 0.05$) incidence of *T. atroviride* than treatment UST1 (36.86%).

Incidence of *Pa. chlamydospora*: Significant treatment × inoculation day interactions ($P < 0.001$) were found on the incidence of *Pa. chlamydospora* in the pathogen inoculated pruning wounds. The mean incidence of *Pa. chlamydospora* in the pruning wounds are shown in table 9. The pathogen was able to infect the grapevine pruning wounds at both inoculation times (one and seven days after pruning). Pruning wound treatments reduced infection by the pathogen and the reduction in infection was higher when the pathogen was inoculated seven days after pruning. The combination treatment MT1+Cabendazim resulted in significantly ($P < 0.05$) lower *Pa. chlamydospora* incidence of all the other treatments when the pathogen was inoculated a day after pruning, reducing wound infection by 70%. When the pathogen was inoculated after seven days, all the treatments did not significantly ($P > 0.05$) differ in the pathogen incidence and reduced the infection by 74% to 91% (Table 9).

Pathogen incidence under natural inoculum: The incidence of grapevine trunk pathogens in the non-inoculated wounds is also shown in table 9. The major trunk pathogens isolated were species of the families Botryosphearіaceae (*Neofusicoccum* and *Diplodia* spp.), Diatrypaceae and *Phomopsis* spp. The Petri disease pathogen *Pa. chlamydospora* and *Phaeoacremonium* spp. were isolated at a maximum of 6.25% in the water control treatment. Although the *Trichoderma*-fungicide combination treatments had the lowest pathogen incidences (93% pathogen reduction), this was not significantly ($P > 0.05$) different from the rest of the treatments except the water control (Table 9).

Table 9: The incidence (mean percentage) of *Phaeomoniella chlamydospora* in inoculated wounds and grapevine trunk pathogens in pathogen un-inoculated wounds of Cabernet Sauvignon pruning wounds treated with wild type (UST1) and mutant (MT1) *Trichoderma atroviride* suspensions and fungicides and their combination.

Treatment	*Pa. chlamydospora* incidence (and percentage control) in inoculated wounds[1]		Pathogen incidence in un-inoculated wounds[1,2]
	1 day	7 days	
Carbendazim	52.09^{C} (42)[3]	16.67^{DE} (76)	12.50^{B} (79)
Thiophanate methyl	41.67^{C} (53)	16.67^{DE} (76)	6.25^{B} (89)
UST1	43.75^{C} (51)	18.75^{DE} (74)	10.42^{B} (82)
MT1	43.75^{C} (51)	10.42^{E} (85)	14.58^{B} (75)
UST1+Thiophanate methyl	45.83^{C} (49)	6.25^{E} (91)	4.17^{B} (93)
MT1+Carbendazim	27.08^{D} (70)	10.42^{E} (85)	4.17^{B} (93)
Control	89.59^{A}	70.84^{B}	58.33^{A}
LSD	13.76		15.60

[1]Mean values followed by the same letter are not significantly different according to Fischer's least significant difference (LSD) test at $P = 0.05$

[2]The percentage wounds infected by at least one grapevine trunk pathogens in wounds that were not inoculated with the pathogen but received wound treatment

[3]Percenatge pathogen reduction, calculated from the difference between the pathogen incidence in the control and the treatment as percentage of the incidence in the control.

In most grapevine producing areas, benomyl and carbendazim have been removed from the market (Halleen *et al*., 2010; Gramaje *et al*., 2012) while thiophanate methyl remains available and has been shown to be effective in wound protection (Rolshausen *et al*., 2010; Díaz & Latorre, 2013). The combination of the wild type isolates with thiophanate methyl could easily be recommended for pruning wound protection in South Africa, however, thiophanate methyl is not registered for grapevines in South Africa. Carbendazim and benomyl are registered for the control of Botrytis rot, but not for pruning wound protection of grapevines. It is much faster and more inexpensive for manufacturers to get authorisation to extend use of these fungicides for pruning wound protection than register new fungicides on a crop. These fungicides could then be applied in combination with the resistant mutants for effective and sustainable wound protection. Benzimidazole fungicides have a single mode of action and thus are at high risk for resistance development in the pathogens (FRAC code 1: www.frac.info). Applying the fungicides in combination with the biocontrol agent would reduce the risk of resistance development as the biocontrol agent will provide an alternative control mechanism to fungicide resistant pathogen strains.

4. Investigate the influence of cultivar towards *Trichoderma* efficacy

The incidence of *Trichoderma* spp. was dependent on cultivar as revealed by the statistical cultivar × treatment interaction ($P < 0.001$). The *T. atroviride* and *T. harzianum* did not follow the same trend in incidence on the three cultivars. In the *Trichoderma* only treatments (USPP-T1 and E7), *T. atroviride* (USPP-T1) had relatively higher incidence in Cabernet Sauvignon and lower in Pinotage while, *T. harzianum* (E7) was highest in Pinotage and lowest in Cabernet Sauvignon. Higher incidences of both *Trichoderma* species were found when the biocontrol agents were inoculated together with pathogens than when the *Trichoderma* spp. were inoculated alone except for the E7 treatment in Pinotage. Inoculation of *Phomopsis viticola* on *Trichoderma* treated wounds resulted in higher *Trichoderma* incidence as compared to the other pathogen *Phaeomoniella chlamydospora*.

Even though there were significant cultivar × treatment interactions (P < 0.001), were no significant differences found among the incidences of *P. chlamydospora* among the cultivars when treated with *Trichoderma*. When only *P. chlamydospora* was inoculated, Cabernet Sauvignon (66.67%) had a lower *P. chlamydospora* incidence compared to the other cultivars Chenin blanc (93.33%) and Pinotage (80%). Pathogen reduction computed for each cultivar gave a better appreciation of the effect of the *Trichoderma* treatments in the cultivars. *Trichoderma atroviride* (USPP-T1) treatment reduced the *P*. *chlamydopsora* incidence more than *T. harzianum* (E7) in Chenin blanc and Pinotage, and was similar in Cabernet Sauvignon.

There were significant cultivar × treatment interaction (P < 0.001) in the incidence of *Ph*. *viticola*. Cabernet Sauvignon (73.33%) had comparatively higher *Ph*. *viticola* incidence than Chenin blanc (40%) and Pinotage (46.67%) in the pathogen only treated shoots. *Trichoderma* treatments reduced the incidence of *Ph*. *viticola* to 6.67% for both the *T*. *atroviride* and *T*. *harzianum* treatments on Cabernet Sauvignon, which was not significantly different from the 13.33% of *T*. *atroviride* on Chenin blanc and *T*. *harzianum* on Pinotage.

The trends that were expected from the field trial which was conducted on eight wine and four table grape cultivars (in 2008), were not found again in the glass house trial. Chenin blanc was not the cultivar that had the highest incidence of *Trichoderma* species and highest pathogen reduction, as was found in the previous field trial. Pinotage did not have the lowest pathogen reduction of the three cultivars tested, as expected from the field trial. These inconsistencies could be attributed to the variation that was in the field trial due to differences in cultivar age, natural inoculum pressure and the environment. In the glass house trial, reactions were also not always similar between *T. atroviride* and *T. harzianum*, which can be explained by the differences in metabolite composition and enzymes secreted by the two species. The pathogen, showed variation according to cultivar and this also influenced the efficacy of the *Trichoderma*. To obtain a clearer answer to the influence of cultivar on *Trichoderma* species efficacy, a wider collection of cultivars and trunk pathogens need to be inoculated on plants in a controlled environment. This trial was conducted to verify cultivar susceptibility to *Trichoderma* growth and pathogen reduction, however, the effect is not simple and is influenced by the pathogen and *Trichoderma* species used.

B. Establish the role of sucker wounds as portals for trunk disease infections

1. Survey sucker wounds of table and wine grapes.

Trunk disease symptoms that were observed from the sucker wounds included wood discolouration, browning and streaking (Figure 2). Sixty-two percent of the collected wounds were infected with at least one trunk pathogen. Multiple fungal pathogens were obtained from 19% of the wounds (Figure 2a, b and d). There was a higher incidence of infected sucker wounds from wine grapes (84%) in comparison with table grapes (16%) (Table 10). Of the different trunk disease fungi isolated, *Po. viticola* was the most common, followed by *D. seriata* and *Pa. chlamydospora* in both wine and table grape sucker wounds (Table 11). Additionally, low numbers of *Pa. aleophilum*, *Eutypella* sp, *C. ampelina*, *N. australe* and *Diplodia* sp. were also isolated, although these were only from wine grapes.

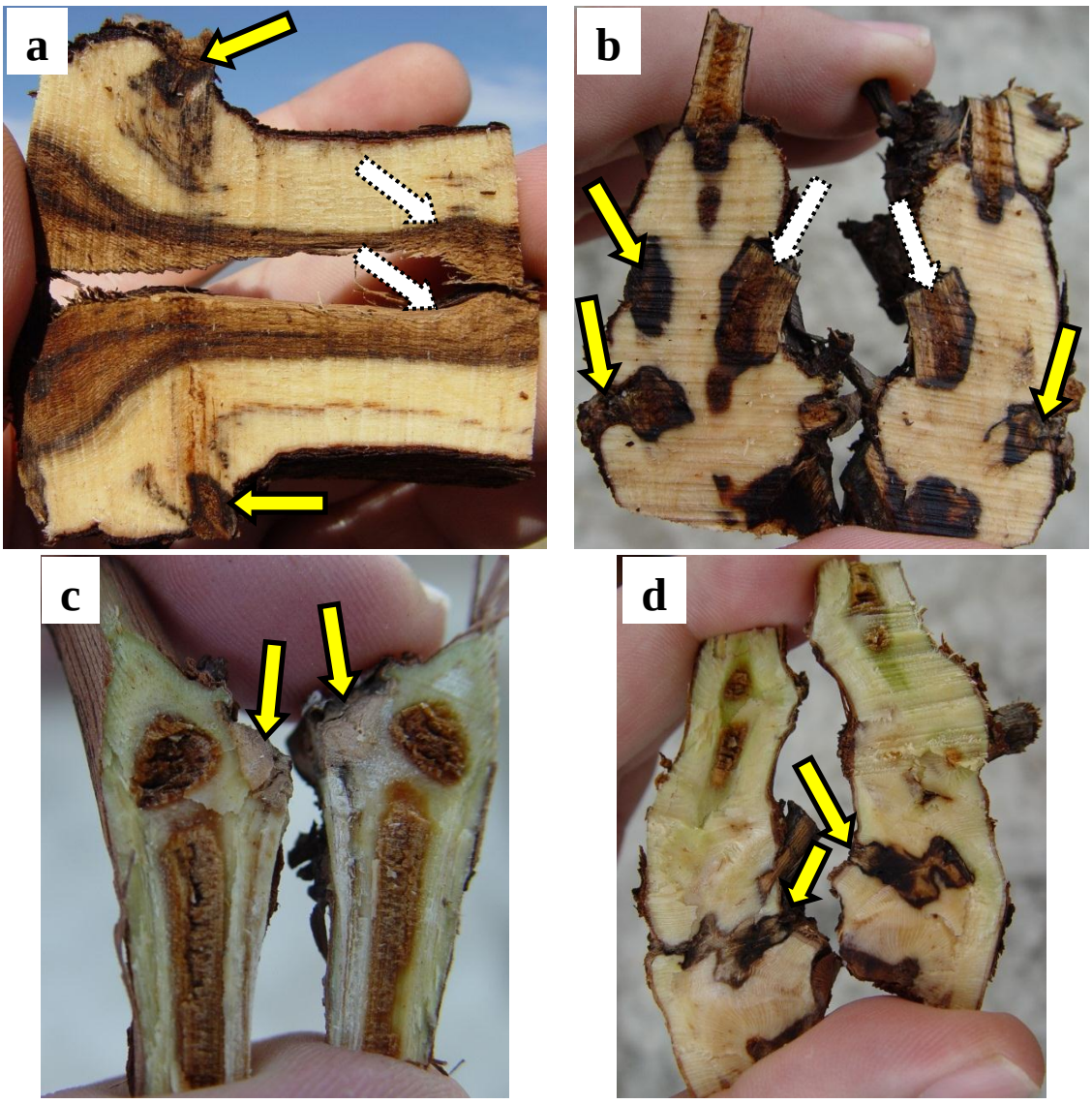

Figure 2. Vertically split grapevine wood collected during the survey showing sucker wounds (yellow solid arrows) with their respective trunk disease symptoms. Symptoms included brown discolouration (a, b, d) around the wound and streaking (a, c) from the wound. The following pathogens: *Diplodia seriata* (d), *Phaeomoniella chlamydospora* (a, b, c, d), *Phaeoacremonium aleophilum* (b), *Phomopsis viticola* (a) were obtained from the above wounds. No isolations were made from winter wounds and their symptoms (white dotted arrows).

Table 10: Results of field survey investigating the presence of trunk disease pathogens in sucker wounds sampled from two wine and table grape cultivars from different location in the Western Cape, South Africa.

Locations	**Total number of sucker wounds analysed**		**Total positive wounds (%)**	
Wine grape	**Cabernet Sauvignon**	**Chenin blanc**	**Cabernet Sauvignon**	**Chenin blanc**
Darling	28	19	89	95
Robertson	16	17	75	59
Stellenbosch	16	9	92	77
Table grape	**Crimson Seedless**	**Thompson Seedless**	**Crimson Seedless**	**Thompson Seedless**
Paarl	12	25	32	42
Piketberg	9	10	10	22

Table 11: Incidence of trunk disease pathogens isolated from sucker wounds from the field survey.

Fungal species	Incidence of pathogens (%)	
	Wine grape	Table grape
Phomopsis viticola	46	18
Diplodia seriata	30	9
Phaeomoniella chlamydospora	27	5
Phaeoacremonium aleophilum	18	0
Eutypella sp.	3	0
Cryptovalsa ampelina	2	0
Neofusicoccum australe	1	0
Diplodia sp.	1	0

2. Assess susceptibility of sucker wounds with artificial inoculation in a controlled environment (glasshouse) and in the field.

Glasshouse trials

Sucker wounds of Chardonnay and Crimson Seedless were susceptible to *Pa. chlamydospora* and *Eutypa lata*. The analysis of variance did not reveal a significant cultivar × treatment interaction (P = 0.78) which indicated that both cultivars responded similarly to the two pathogens. For both Chardonnay and Crimson Seedless, significant differences were found between the two pathogen treatments (P = 0.0009 for Chardonnay and P = 0.0001 for Crimson Seedless). The incidence of *Pa. chlamydospora* in inoculated sucker wounds was significantly higher than *E. lata* in Chardonnay as well as Crimson Seedless (Table 12). No trunk disease pathogens were isolated from the controls. For the second glasshouse trial, all of the inoculated fungi were re-isolated (Table 13). Significant differences were found between the pathogen treatments (P = 0.0018). *Neofusicoccum parvum* was isolated from 85% of the wounds, significantly higher than *Pa. aleophilum* (55%) and *E. lata* (45%). No trunk disease pathogens were re-isolated from the controls.

Table 12: Incidence of *Eutypa lata* and *Phaeomoniella chlamydospora* in sucker wounds made on 1-year-old own rooted Chardonnay and Crimson Seedless potted vines kept under controlled conditions in a glasshouse and assessed three months after inoculation.

Treatment	Mean percentage incidence of pathogens in cultivars (%)	
	Chardonnay*	Crimson Seedless#
Phaeomoniella chlamydospora	67.67[a]	60.00[a]
Eutypa lata	43.33[b]	33.33[b]

Means followed by the same letter in the same column are not significantly different (P > 0.05; *LSD = 22.09; #LSD = 13.32).

Table 13: Incidence of *Eutypa lata*, *Neofusicoccum parvum*, *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora* and *Phomopsis viticola* in sucker wounds made on 1-year-old own rooted Chardonnay potted vines kept under controlled conditions in a glasshouse and assessed four months after inoculation.

Treatment	*Mean percentage incidence of pathogen (%)
Neofusicoccum parvum	85[a]
Phaeomoniella chlamydospora	75[ab]
Phomopsis viticola	65[ab]
Phaeoacremonium aleophilum	55[bc]
Eutypa lata	45[c]

*Means followed by the same letter are not significantly different (P > 0.05; LSD = 25.468)

Susceptibility of sucker wounds to five trunk disease pathogens on field grapevines

Of the five pathogens that were inoculated, only three were re-isolated namely *Po. viticola*, *N. parvum* and *Pa. chlamydospora* (Table 14). *Phomopsis viticola* (65%) was re-isolated significantly more than *N. parvum* (32.5%) and *Pa. chlamydospora* (7.5%) ($P < 0.001$).

Table 14: Mean incidence of *Eutypa lata*, *Neofusicoccum parvum*, *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora* and *Phomopsis viticola* from sucker wounds made on Cabernet Sauvignon field vines and assessed after five months.

Treatment	*Mean percentage re-isolation incidence of pathogen (%)
Phomopsis viticola	65.00[a]
Neofusicoccum parvum	32.50[b]
Phaeomoniella chlamydospora	7.50[c]
Phaeoacremonium aleophilum	0[c]
Eutypa lata	0[c]

*Means followed by the same letter are not significantly different ($P > 0.05$; LSD = 14.844)

3. Assess the duration of sucker wound susceptibility in the field.

Sucker wounds remained susceptible to *Phaeomoniella chlamydospora* over the whole 4 week period. The analysis of variance revealed a significant treatment interaction ($P < 0.0001$, Appendix A, Table 5). Due to the relatively low re-isolation incidences, no significant differences were found between the different weekly applications ($P = 0.07$, Appendix A, Table 5). Wound susceptibility was higher directly after pruning and after one week and declined thereafter (Figure 3). *Phaeomoniella chlamydospora* was not isolated from the control wounds.

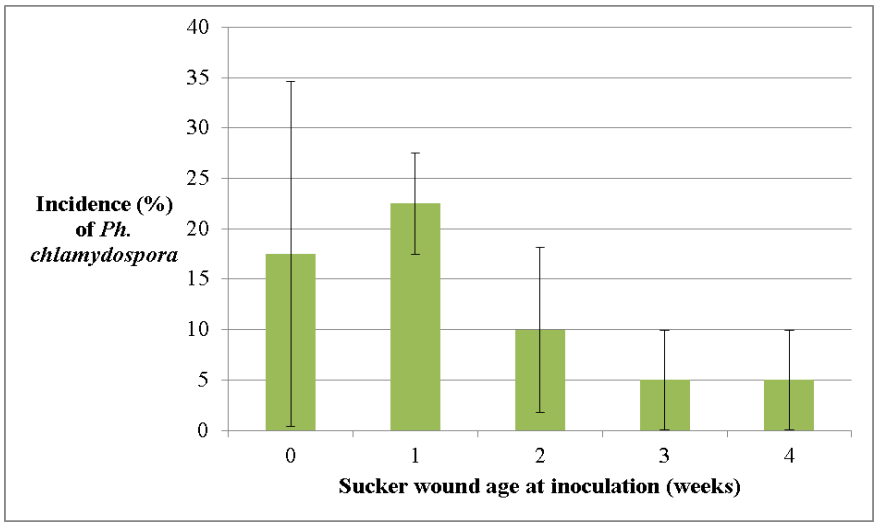

Figure 3. Duration of susceptibility of Cabernet Sauvignon sucker wounds to *Phaeomoniella chlamydospora* inoculated at weekly intervals for 4 weeks and evaluated after 5 months under field conditions.

4. Determine the sensitivity of *Trichoderma* isolates towards fungicides used during spring time.

Mycelial inhibition

Analysis of variance revealed a significant isolate × concentration × fungicide interaction ($P < 0.0001$) for mycelial inhibition for both *Trichoderma* isolates at 24 and 48 hours. All 16 fungicides inhibited the growth of *Trichoderma* to some degree at all the tested concentrations after 24 and 48 hours. The mean percentage inhibition generally increased with an increase in concentration whilst the sensitivity of isolates to most fungicides generally decreased with an increase in time. At 0.25×, *Trichoderma* isolates were only sensitive to spiroxamine and penconazole (systemic) and to all contact fungicides except for quinoxyfen, meptyldinocap and metiram (T1) after 24 hours. At 0.5×, *Trichoderma* isolates were only sensitive to spiroxamine, flusilazole and fenarimol (Eco-77® only) (systemic) and to all contact fungicides except quinoxyfen and meptyldinocap after 24 hours. At the recommended dosage, isolates were sensitive to all systemic fungicides except boscalid, metrafenone and trifloxystrobin and to all contacts except quinoxyfen and meptyldinocap after 24 hours. At 2x, isolates were sensitive to spiroxamine, flusilazole and fenarimol (systemic) and to all contact fungicides except quinoxyfen and meptyldinocap. Similar trends were observed after 48 hours for all the concentrations. Additionally, isolates were less sensitive after 48 hours. *Trichoderma harzianum* and *T. atroviride* is therefore compatible with the systemic fungicides boscalid, metrafenone and trifloxystrobin, as well as contact fungicides quinoxyfen and meptyldinocap.

Inhibition of conidial germination

Analysis of variance revealed significant isolate × fungicide ($P < 0.0001$), fungicide ($P < 0.0001$) as well as isolate ($P < 0.0001$) interactions. *Trichoderma atroviride* (T1) was significantly less sensitive to fungicides than *T. harzianum* (Eco-77®). The following fungicides: boscalid, penconazole, metrafenone and trifloxystrobin (systemic) plus quinoxyfen and folpet (contact) inhibited less than 50% of conidial germination. No conidia germinated in the presence of spiroxamine (systemic) and mancozeb, propineb, metiram and diathon (contact). The fungicides with the highest inhibition were therefore all contacts (mancozeb, propineb, metiram, pyrimethanil and diathanon), except for spiroxamine which is systemic.

5. Field application of *Trichoderma* to assess efficacy in protecting sucker wounds.

Application of *Trichoderma harzianum* on sucker wounds in the field

The analysis of variance revealed a significant ($P = 0.03$) difference between *Pa. chlamydospora* treatments. *Phaeomoniella chlamydospora* mean incidence decreased by 66.65% when it was inoculated on Eco-77® treated sucker wounds (Table 15). The ANOVA revealed no significant differences between *Po. viticola* treatments ($P = 0.07$), the mean incidence of *Po. viticola* decreased by 15.37% when it was inoculated on wounds treated with Eco-77®. The ANOVA also revealed a significant difference ($P = 0.0018$ and $P = 0.01$) in Eco-77® treatments. *Trichoderma* mean incidences decreased by 85.72% and 74.99% when it was challenged with *Pa. chlamydospora* and *Po. viticola*, respectively.

Table 15: Mean incidence of *Phaeomoniella chlamydopsora* and *Phomopsis viticola* re-isolated from sucker wounds of Cabernet Sauvignon 5 months after sucker wounds were inoculated with individual pathogens (*Phaeomoniella chlamydospora* or *Phomopsis viticola*) or in combination with *Trichoderma harzianum* (Eco-77®).

Treatment	Mean percentage isolation incidence (%)	
	Phaeomoniella chlamydospora [*]	***Phomopsis viticola*** [#]
Individual	20.00[a]	43.33[a]
Combined	6.67[b]	36.67[a]

Means followed by the same letter are not significantly different ($P > 0.05$; [*]LSD = 13.32; [#]LSD = 39.40).

When natural infection of sucker wounds (2.1%) was compared to winter pruning wounds (13%), the latter were found to be of higher importance (Lecomte and Bailey, 2011). Even though the sucker wounds might be infected less than winter pruning wounds, does the availability of aerial inoculum indicate that protection of sucker wounds would aid in preventing trunk disease infections. Protecting sucker wounds in spring time would require another spray application. Further research would be needed to verify the use of *Trichoderma* or possible fungicides that could be applied.

C. Investigate the host-pathogen-*Trichoderma* interactions

1. To investigate the secondary metabolites secreted by *T. atroviride* and *T. harzianum* and their effect on trunk disease pathogens.

Isolation and identification of secondary metabolites

Five homogenous fractions were obtained from the *T. atroviride* isolates and seven fractions were obtained from the *T. harzianum* isolate. The first fraction (fraction I) from all the isolates showed similar chromatographic and spectroscopic properties and was also isolated in the highest quantities. It was extracted at 280-340 mg compared to 21- 43 mg for the next highest fraction for all isolates, making it the major secondary metabolite by quantity. Fraction I showed similar chromatographic and spectroscopic properties as the standard sample of 6-pentyl α-pyrone (6PP). The metabolite's *Rf* 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 ^{1}H 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 wild type *Trichoderma* isolates is shown in figure 1. Analysis of variance found significant isolate × culture condition × time interactions ($P < 0.001$). The *T. atroviride* isolate UST1, produced more 6PP and faster under the shake conditions reaching a maximum of 82 mg/L at day 10 which was significantly higher ($P < 0.05$) than the rest of the isolates at that time point. Contrarily, under static conditions, isolate UST2 produced significantly higher ($P < 0.05$) quantities of 6PP on all days except for day 20 where the quantity was not significantly ($P > 0.05$) different from that of UST1. The *T. harzinum* produced more 6PP in the shake than static cultures but in both conditions the 6PP quantities were significantly lower ($P < 0.05$) than in the *T. atroviride* isolates except for the 20-day shaking conditions where it was not significantly different from that of UST1.

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

A comparison of the LC-MS total ion chromatograms (TIC) of the wild type isolates is shown in figure 4. The production of secondary metabolites was dependent on the richness of the medium (full strength or quarter PDB) and the culture conditions (shake 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 (Mw) 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 minor production compared to the UST1 strains. Since *T*. *atroviride* 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 peptaibiotics. A database of peptaibiotics compiled by Stoppacher *et al*. (2013) was downloaded from http://peptaibiotics-database.boku.ac.at. With aid of the database the closest match to the compound was found to be members of the trichocompactin group ($C_{33}H_{58}N_8O_{10}$; Mw 726) of peptabiotics isolated from *T*. *brevicompactum* (Degenkolb *et al*., 2006).

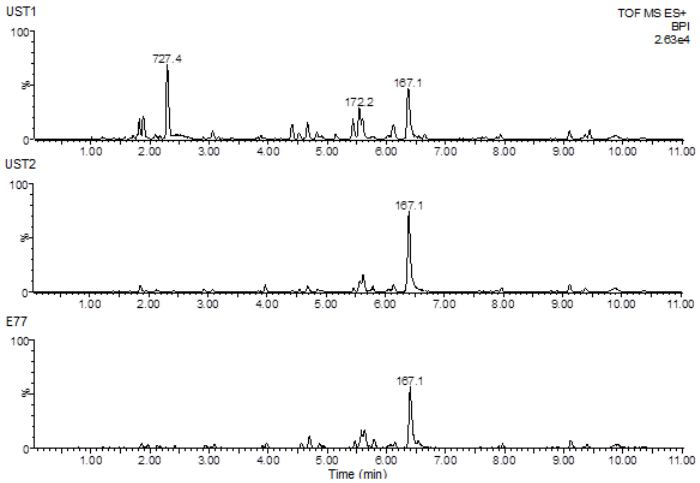

Figure 4: LC-MS chromatograms showing the major secondary metabolite peaks from *T. atroviride* (UST1 and UST2) and *T. harzianum* (E77) isolates grown in full strength potato dextrose broth for 20 days with shaking at 120 rpm. The major secondary metabolite in all isolates was 6-pentyl α-pyrone with a molecular weight of 167.1 (M^+H). Another major peak was observed from UST1 with a molecular weight of 727 (M^+H) which was found to be closely related to peptaibiotics of the trichocombactum group.

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 < 0.001). In full strength PDB, 6PP was produced by all the isolates (except MT77) in both static and shake cultures while in quarter strength PDB, UST1 and MT1 could not produce 6PP. The isolate UST2 and its mutant, MT2, were the highest producers of 6PP. For the *T*. *atroviride* strains 6PP production was either similar (P > 0.05) or higher (P < 0.05) in the static than shake cultures, while in the *T*. *harzianum* isolate 6PP production was always significantly (P < 0.05) higher in the shake than static cultures.

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

Analysis of variance showed significant isolate × medium × co-culture interactions (P = 0.038). In both isolates, 6PP production was significantly higher (P < 0.05) in the GCBM compared to the defined Pezet's medium except for UST1 when it was co-cultured with *N*. *parvum*. Isolate UST2 generally produced more 6PP than UST1 in GCBM except when UST1 was co-cultured with *N*. *parvum*. The co-culturing of UST1 with *N*. *parvum* resulted in significant increases (P < 0.05) 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 the increase was not significantly higher (P > 0.05) than in 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 > 0.05), so the data from both experiments were combined. The effect of 6PP on both mycelial

growth and conidia/spore germination was highly significant at all concentrations tested ($P < 0.001$).

Mycelial inhibition: Three non-linear regression models (Gompertz, Logistic and Modified Exponential) were fitted to the mycelial growth inhibition data and all gave good statistical fits ($R^2 > 0.798$). The Gompertz (sigmoidal) model consistently gave the highest correlation for all isolates ($R^2 > 0.95$, $P < 0.01$). Based on the mean EC_{50} values (Table 16), the inhibition of mycelial growth by 6PP varied for the different pathogens. There were no significant differences ($P > 0.05$) 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* ($P = 0.057$). *Phaeomoniella chlamydospora* was the least sensitive (EC_{50} = 91.72 mg/L) 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 ($P < 0.01$) reduction in mycelial growth from the lowest concentration tested (50 mg/L) and mycelial growth was totally inhibited at 400 mg/L in all pathogens.

Table 16: Sensitivity of grapevine trunk pathogens to the secondary metabolite, 6-pentyl α-pyrone (6PP), of *Trichoderma* spp. based on *in vitro* inhibition of mycelial growth. The EC_{50}, is the effective concentration of 6PP (in mg/L) that inhibited radial mycelial growth by 50%.

Pathogen	**Isolate STE-U No.**	**EC_{50} (mg/L) of 6PP***	
		EC_{50}	**Mean EC_{50}[1]**
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	

*EC_{50} values compared to the control with solvent only (0.1% methanol), computed from a Gombertz (sigmoid) function (for all isolates $R^2 > 0.956$; $P < 0.001$).
[1]EC_{50} ± standard error of mean of each isolate from two independent experiments.

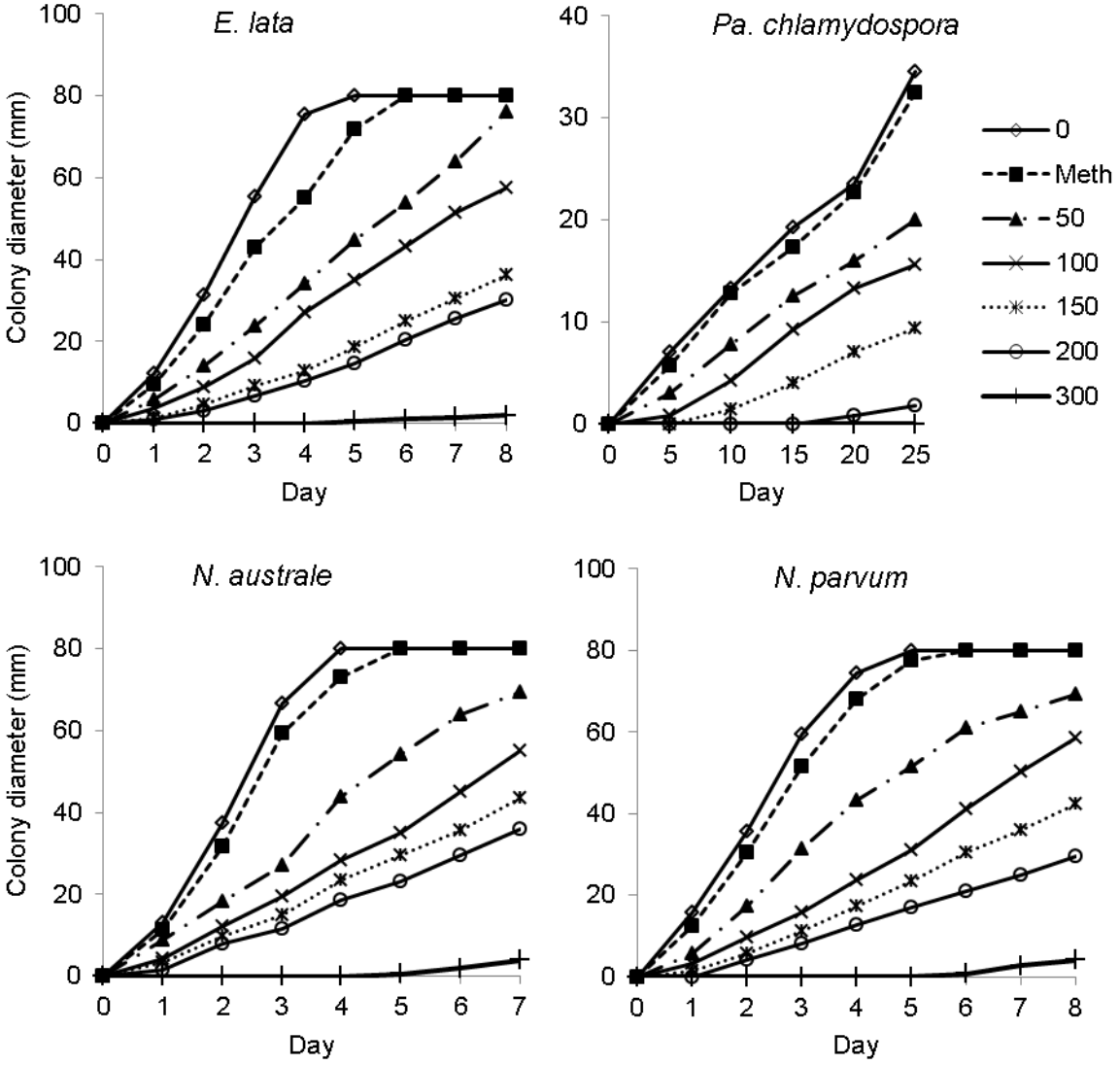

Figure 5: Mean mycelial growth inhibition with time, of grapevine trunk pathogens (*E. lata, Pa. chlamydospora*; *N. australe* and *N. parvum*) on potato dextrose agar amended with varying concentrations (0 – 300 mg/L) of 6-pently α-pyrone (6PP). Radial mycelial growth was assessed by calculating the mean diameter from two perpendicular measurements and then subtracting 5 mm from each value to account for the original plug. All amended medium contained 0.1% of methanol (solvent for 6PP) and hence methanol only amended medium (Meth) was included as a control.

Effect of growth medium on mycelial sensitivity to 6PP: There were significant isolate × pathogen × medium interactions ($P < 0.001$) for all the pathogens. Inhibition of mycelial growth was dependent on growth medium and the pathogen isolate. All the pathogens were more sensitive to 6PP when growing on nutrient poor, defined medium (Vogel's N). However, there was variation in the sensitivity of the pathogens when growing on the other media. Sensitivity to 6PP on Pezet's medium did not significantly differ ($P > 0.05$) from that on the complex medium for the *Neofusicoccum* spp. Inhibition of *Pa. chlamydospora* on amended malt extract agar was significantly higher ($P < 0.05$) than on PDA and GCBM.

Conidia/spore germination: Conidia/spore germination was significantly ($P < 0.001$) reduced by all concentrations tested and totally inhibited at 300 and 400 mg/L. The latter two concentrations tested were excluded from the analysis of variance for the effect of 6PP concentration on conidia/spore germination. The effect of 6PP on conidia/spore germination is shown in figure 6. Germination was inhibited by more than 60% at 100 mg/L in all pathogens. Due to high variation between treatments EC_{50} values could not be computed for the sensitivity of conidia/spore germination to 6PP.

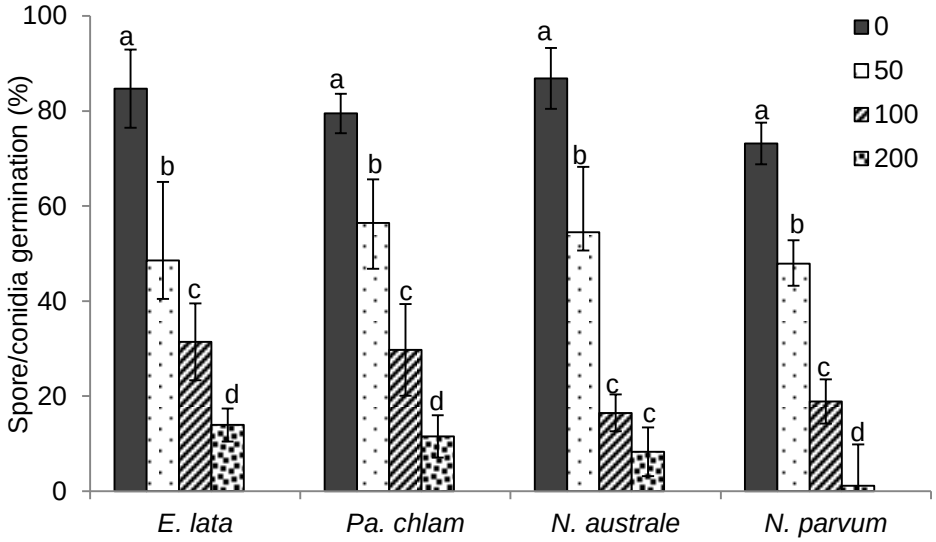

Figure 6: Inhibition of spore and conidia germination by varying concentration (0-200 mg/L) of 6-pentyl α-pyrone (6PP) on grapevine trunk pathogens *E. lata*, *Pa. chlamydospora* (*Pa*. *chlam*), *N. australe* and *N*. *parvum*. Percentages of germinated spores/conidia are means of three replicates of two independent experiments. Bars with the same letter on top show no significant differences in the percentage germination within a pathogen according to Fischer's least significant difference (LSD; *E*. *lata* = 13.82, *Pa. chlamydospora* = 10.74, *N*. *australe* = 12.42, and *N*. *parvum* = 9.91: *P* = 0.05).

2. To determine grapevine host resistance response to secondary metabolites and *Trichoderma* species

Grapevine cell suspension cultures

Cell suspension cultures were monitored over 13 days and proved to be stable and homogenous with weekly sub-culturing. The cultures had a sigmoidal growth curve with an exponential growth phase between the 3rd and 8th day and hence day 6 (exponential phase) was chosen for elicitation. Elicitation of cells resulted in browning of cell suspension cultures and reduction of cell viability in an elicitor concentration dependent manner. Cell suspension cultures browned within 24 hours when treated with 5% and 10% of the *T. atroviride* elicitor preparation, whereas the pathogen elicitor preparation caused browning by 48 hours post treatment. In both cases browning was darker in cultures that received 10% elicitor concentration where cell viability decreased by more than 50% after 48 hours. The 2.5% concentration was chosen for elicitation, as cells remained viable (> 90%) for the whole duration of the experiment, however, slight browning was observed in the elicitor treated cultures 48 hours post elicitation.

Expression of genes involved in response to pathogenesis

Analysis of relative gene expression in the cell suspension cultures indicated differential expression patterns to the *E. lata* and *T. atroviride* elicitors. Defence related genes of both the phenylpropanoid pathway and the pathogenesis related proteins were overexpressed indicating that the Dauphine cell cultures were able to recognise the fungal elicitors. However, the time and levels of expression were dependent on the elicitor and whether it was fresh or autoclaved. The pattern of expression was similar for the elicitors regardless of whether it was fresh or autoclaved.

For genes of the phenyl-propanoid pathway, PAL, 4CL and STS, their over-expression was observed earlier (at 6 hours) with the pathogen elicitors and later (12 hours) with the biocontrol agent elicitor. The levels of the PAL gene up-regulation were much higher in the *T. atroviride* elicitor (10 to 117 folds in the fresh elicitor) than in the *E. lata* elicitor (2 to 5 folds in the fresh elicitor) (Figure 4). PAL was down-regulated (-3.03 and -2.70 folds for the fresh and autoclaved

elicitors, respectively) at 24 hours post elicitation in the *E. lata* elicitor treated cells. Although there was a down-regulation of 4CL at 24 and 48 hours post elicitation with fresh filtrate, this was not significantly lower than the untreated controls. Two primer sets were used to trace the expression profile of stilbene synthase and they showed a similar pattern with minor differences in the pathogen elicited cell cultures. The expression of both STS genes in the pathogen elicited cells also showed a biphasic expression pattern, as was observed with the PAL time course expression. Expression of STS was higher at 6 and 12 hours, declined at 24 hours (not significantly different from the non-elicited control, $P > 0.05$) and increased again at 48 hours. Two sets of primer pairs were used to trace CHS and both showed similar expression patterns for each specific elicitor. The upregulation of the STS gene expression was at the expense of the CHS gene expression, whose expression either remained the same as in the controls, or was down-regulated in the cells treated by the fresh filtrate of both the *E. lata* and *T*. *atroviride*. Slight up-regulation of chalcone synthase was observed in the cells treated with autoclaved elicitors at 6 hours for *T. atroviride* (CHS3) and at 48 hours for the *E. lata* elicitors.

There were no significant changes in the expression of the PR 1 gene in the cells treated with the pathogen elicitors. The culture filtrate from the biocontrol agent caused significant ($P < 0.05$) down-regulation of the PR1 gene at all time-points except 24 hours (where expression did not differ significantly ($P > 0.05$) from the non-treated control). The expression of the other pathogenesis related proteins also showed an earlier response to the *E. lata* elicitors and a later response to the *T. atroviride* elicitors. At peak expression, 12 and 24 hours post elicitation in *E*. *lata* and *T. atroviride* treated cells, respectively, the PR 2 (β-1, 3-glucanase) gene was at least three times more over-expressed in cells treated with the fresh pathogen elicitor compared to cells treated with the biocontrol elicitors. PR 5 (osmotin like protein) and PR 6 (protease inhibitor) gene expression peaked 12 hours post elicitation in cells treated with the pathogen elicitor with a slower response in cells treated with the biocontrol agent elicitors where expression peaked at 24 hours. However, at peak expression, PR 6 was over-expressed more than three times in the cells treated with the autoclaved elicitor of the biocontrol agent as compared to cells treated with the pathogen elicitors.

The chitinases (CHIT IV, PR 3 and PR 4) showed differential expression patterns. In cells treated with the elicitor from the biocontrol agent, peak expression of CHIT IV was at 12 hours while the peak for the other chitinase genes was at 24 hours post elicitation. A similar pattern was also observed with cells that received the pathogen elicitor where peak expression for CHIT IV and PR 4 chitinase gene was observed at 6 and 12 hours, respectively. At peak expression of CHIT IV, in cells that received the fresh *T. atroviride* elicitor expression was approximately double that of cells treated with the fresh pathogen elicitor. Over-expression of the PR 3 gene was lower in the cells that received the pathogen elicitors as compared to those that were treated by *T. atroviride*. In the cells treated by *E. lata*, over-expression of PR 3 was only significant 48 hours post elicitation with the fresh elicitor and at 6 and 48 hours post elicitation with the autoclaved pathogen elicitor. The expression of PR 4 gene after elicitation was also similar to the other chitinases where the biocontrol agent elicitors triggered slightly higher expression level than the pathogen elicitors at peak expression.

Total phenol content of cell cultures

Treatment of cell cultures with cell free fungal broth elicitors resulted in a significant ($P < 0.001$) increase in the content of phenolic compounds. For both fungal elicitors, the phenolic content was higher at 48 hours compared to 24 hours, obviously due to the accumulation of the compounds over time. There were no significant ($P > 0.05$) differences in the phenolic content of cell cultures treated with the fresh or autoclaved elicitors of the same fungi for each assay time (24 and 48 hours). The total phenolic content was significantly higher in cell cultures treated with elicitors from the biocontrol agent (*T*. *atroviride*) than the pathogen (*E*. *lata*) for both assay times.

β-1, 3-glucanase activity
Elicitation resulted in significant ($P < 0.001$) increase in the β-1, 3-glucanase activity and these were higher 24 hours after treatment than at 48 hours. The fresh elicitor of *E. lata* resulted in the highest activity at both assay times (24 and 48 hours) which were significantly ($P < 0.05$) higher than the rest of the treatments. There were no significant differences in the β-1, 3-glucanase activity of cells treated with the fresh and autoclaved elicitors of the biocontrol agent (*T. atroviride*) for both assay times.

Chitinolytic activity
The untreated cells exhibited some chitinolytic activity indicating constitutive activity, but elicitation resulted in a significant ($P = 0.0014$) increase in chitinolytic activity. Activity was highest at 24 hours post elicitation. At 24 hours, the cells treated with fresh elicitor of *T. atroviride* had the highest activity (18.81 U) which was significantly higher ($P < 0.05$) than the rest of the elicited treatments. Chitinolytic activities of the other elicited treatments were significantly higher than the control treatments but not significantly different from each other. At 48 hours the chitinolytic activity was low and only the fresh broth treatments had significantly higher activity than the non-treated controls.

Milestone	Target Date	Extension Date	Date Completed	Achievement
A1. To determine the best formulation of the *Trichoderma* product.	Dec 2012	Dec 2013	June 2013	A culture filtrate made from a chitin based medium and a combination of yeast extract, urea and glucose together with *Trichoderma* conidial suspensions consistently enhanced biocontrol efficacy, however, not statistically better than the *Trichoderma* with water combination. The process of commercialisation for the isolate T1 will be initiated in 2014 with the aid of Inovus (US).
A2. To determine the best time of application.	Dec 2013		June 2013	Application of the biocontrol agents 6 hours after pruning consistently resulted in high wound colonisation by *Trichoderma* spp. Pruning wound infection due to natural inoculum was higher in wounds made in late winter (August) than those made earlier (July).
A3. To determine the best method of application.	Dec 2013		June 2013	Painting or spraying with a knapsack sprayer will result in better pruning wound colonisation and protection than using a gator sprayer.
A4. Generate fungicide resistant strains of *Trichoderma* and test in field.	Dec 2013		Dec 2013	Benzimidazole resistant *Trichoderma* strains were generated by gamma irradiation from the wild type isolates of *T. atroviride* (UST1 and UST2) and *T. harzianum* (T77). In the field trial the combination of the *Trichoderma* UST1 mutant and carbendazim was the most effective treatment and gave the highest reduction in *Pa. chlamydospora* infection (70% to 93% control).

A5. Investigate the influence of cultivar towards *Trichoderma* efficacy.	Dec 2011		Dec 2011	To verify cultivar susceptibility to *Trichoderma* growth and pathogen reduction three cultivars and two pathogens were tested on potted vines. Results were not similar to a field trial done in USPP 04/2004. The influence of cultivar is not simple and would require a wider selection of cultivars as well as more pathogens.
B1. Survey of wine and table grape sucker wounds for presence of trunk disease pathogens.	Dec 2011		Dec 2012	Sixty-two percent of the sucker wounds were naturally infected by at least one of the trunk pathogens. *Phomopsis viticola* (46%; 18%), *Diplodia seriata* (30%; 9%) and *Phaeomoniella chlamydospora* (27%; 5%) were the most predominant trunk disease pathogens isolated from sucker wounds of wine and table grape cultivars, respectively.
B2. Artificial inoculation of sucker wounds in a controlled environment (glasshouse) and in the field.	Dec 2012	Dec 2013	May 2013	Sucker wounds on 1-year-old potted grapevine plants of Chardonnay cultivar were artificially inoculated and then isolates four months later. The re-isolations constituted of *N. parvum* (85%), *Pa. chlamydospora* (75%), *Po. viticola* (65%), *Pa. aleophilum* (55%) and *E. lata* (45%). Re-isolations after five months from sucker wounds made on 12-year-old Cabernet Sauvignon vines had the following incidences: *Po. viticola* (65%), *N. parvum* (32.5%) and *Pa. chlamydospora* (7.5%).
B3. Assess the duration of sucker wound susceptibility in the field	Dec 2012	Dec 2013	May 2013	Sucker wounds remained susceptible for the 4 weeks it was investigated with a decline in susceptibility after one week.
B4. Inoculation of sucker wounds with a DsRed marked *Phaeomoniella chlamydospora* to observe the specific growth in a sucker wound.	Dec 2012		Dec 2012	No visual observations could be made from sucker wounds inoculated with DsRed marked *Phaeomoniella chlamydospora*. The fungus could be re-isolated, but not seen under fluorescence microscopy.
B5. *In vitro* evaluation of fungicide (used during sucker time) sensitivity of *Trichoderma*.	Dec 2012	Dec 2013	Oct 2013	From the mycelial inhibition tests the systemic fungicides boscalid, metrafenone and trifloxystrobin, as well as contact fungicides quinoxyfen and meptyldinocap gave mean percentage inhibition of less than 50% of *Trichoderma* spp. isolates. For the conidial germination assay, boscalid, trifloxystrobin, penconazole and metrafenone (systemic) plus quinoxyfen and folpet (contact) gave mean percentage inhibitions of less than 50% of *Trichoderma* spp. isolates.

				These fungicides could be applied alternatively or simultaneously with *T. harzianum* and *T. atroviride*, however, this will have to be verified with field trials.
B6. Field application of *Trichoderma* to assess efficacy in protecting sucker wounds.	March 2013		May 2013	*Trichoderma harzianum* reduced the incidence of *Pa. chlamydospora* by 66.65%. Although the incidence of *Po. viticola* was reduced by 15.37%, it was not significantly different from the control treatment.
C1. To investigate the secondary metabolites secreted by *T. atroviride* and *T*. *harzianum* and their effect on trunk disease pathogens.	Dec 2011	June 2013	July 2013	A volatile antimicrobial compound, 6-pentyl α-pyrone (6PP), was isolated and found to be the major secondary metabolite from the *T. atroviride* (UST1 and UST2) and *T*. *harzianum* (T77) isolates. 6PP inhibited mycelial growth, spore and conidia germination of *E*. *lata*, *Neofussicocum* (*N*.) *australe*, *N*. *parvum* and *Pa. chlamydospora*. The production of 6PP was induced when the *T*. *atroviride* isolates were grown in a grapevine wood extract medium while for UST1, the 6PP concentration was further doubled when it was co-cultured with *N. parvum*.
C2. To determine host resistance response of grapevine cell suspensions to secondary metabolites of *Trichoderma* species.	Dec 2012		Dec 2012	*Trichoderma* fungal elicitors caused up-regulation of phenylalanine ammonia-lyase (PAL), 4 coumaroyl Co-A ligase (CCo-A), stilbene synthase (STS), chitinase class IV (CHIT IV), PR 3 and PR 4, and a down regulation of chalcone synthase (CHS) genes. Similar responses were found with *E. lata* elicitor. Higher expression of PAL and CHIT IV in cell cultures treated with the *T*. *atroviride* elicitor led to a significantly higher total phenolic content and chitinolytic enzyme activity of the cell cultures compared to cell cultures treated with the *E*. *lata* elicitor. The induction of grapevine resistance may be involved in wound bio-protection.
C3. To determine whole grapevine plant host resistance response to secondary metabolites and infection by *T. atroviride* and *T*. *harzianum*.	Dec 2012			Milestone was not achieved. This objective was too ambitious for the time span of this project. The optimisation of gene expression analyses of whole plants will require another student to work on it.

C4. The histological wound response in grapevine following infection by *T. atroviride* and *T. harzianum*.	Dec 2012			Milestone was not achieved. The knowledge gained in terms of the grapevine resistance genes that was triggered by *Trichoderma* was sufficient.
D. Journal publication/s – final milestone	Dec 2014			Six articles are in preparation that will be submitted to ISI rated journals during the course of 2014. One popular article will also be written in 2014 and submitted to the Winelands and the Fruit Journal.

Accumulated outputs

Three popular publications were published in the Winelands (2011 and 2012). These publications have also been submitted to the Fruit Journal (2013).

Thirteen presentations (including both oral and poster presentations) have been made of which four was at international conferences and nine at national conferences (dates indicated under presentations).

Two presentations were made at the VinPro/Winetech information day held 18 April 2013. The PhD student gave three presentations at the VinPro information days (during August, September 2013).

One MSc and one PhD student will graduate in April 2014.

Six articles are in preparation to be submitted to international journals.

A short radio interview was done with Chris Viljoen from Radio Elsenburg (RSG) which was broadcasted 28 March 2014. The outcomes of how to apply *Trichoderma* pruning wound products was discussed.

Conclusions

The best time to apply *Trichoderma* is six hours after pruning.

Trichoderma should best be applied with either back pack sprayer or paint brush.

Carbendazim could be combined with *Trichoderma* MT1 on a pruning wounds, however, further trials would be needed for commercialisation.

Sucker wounds were naturally infected by trunk disease pathogens.

Trichoderma products could be used for the protection of sucker wounds from infection of trunk disease pathogens. Further field trials would be needed to test a wider range of pathogens and the influence of fungicides sprayed for the control of Phomopsis cane and leaf blight and powdery mildew.

The main secondary metabolite produced by our *Trichoderma* isolates was 6-pentyl-α-pyrone. This compound successfully inhibited trunk disease pathogens with *in vitro* tests.

Trichoderma activated several of the defence related genes that was also activated by the *Eutypa lata*, showing that the biocontol agent can prime the plant prior to pathogen infection which in turn will aid the plant to prevent infection from the pathogen.

Technology development, products and patents

Two of the *Trichoderma* isolates (T1 and MT1) can be developed into commercial products. The right commercialisation partner is needed to be able to do this. Choosing the company will be done in collaboration with Innovus. All of the contributing parties will be taken into account.

Suggestions for technology transfer

Another popular publication in the Winelands and Fruit Journal is necessary to disseminate the outcomes of the application of *Trichoderma* as well as the sucker wound findings to producers.

Human resources development/training

Student level (BSc, MSc, PhD, Post doc)	Cost to Project
1. Gugulethu Makatini, MSc (graduate April 2014)	R64 000
2. Cheusi Mutawila, PhD (graduate April 2014)	R175 000
3. Palesa Lesuthu (technical assistance)	None
4. Julia Marais (technical assistance)	None
5. Carien Vermeulen (technical assistance)	None

Publications (popular, press releases, semi-scientific, scientific)

Mutawila C, Mostert L, Halleen F, Fourie PH. 2011. What is Trichoderma? Winelands July: 93-94.

Mutawila C, Mostert L, Halleen F, Fourie PH. 2011. An overview of grapevine pruning wound protection in South Africa. Winelands June: 90-92.

Mutawila, C., Fourie, P., Halleen, F. & Mostert, L. 2012. Kolonisering van Cabernet Sauvignon- en Sauvignon blanc-snoeiwonde deur *Eutypa lata* en *Phaeomoniella chlamydospora* in die teenwoordigheid en afwesigheid van die bio-beheeragent *Trichoderma harzianum*. Wynland Augustus: 88-91.

Presentations/papers delivered

2011

Mutawila C, Halleen F, Fourie PH, Mostert L. 2011. Biological control of grapevine trunk disease pathogen infections of pruning wounds with *Trichoderma* species. First symposium on biocontrol of grapevine diseases. May 26-27, Toulouse, France (Oral presentation). L. Mostert received the award for the best presentation at the symposium.

Mutawila C, Slabbert K, Halleen F, Mostert L. 2011. E Production of fungicide resistant *Trichoderma* strains by mutagenesis. 47th Congress of the South African Society of Plant Pathology, Berg-en-dal, South Africa (Poster).

2012

Mutawila C, Halleen F, Mostert L. 2012. Optimisation of time of application of *Trichoderma* bio-control agents for grapevine pruning wound protection from trunk pathogen infection. 34th Congress of the South African Society for Enology and Viticulture, 14-16 November 2012, Simondium, South Africa (Oral presentation).

Makatini G, Mutawila, Halleen F and Mostert L. 2012 Sucker wounds are susceptible to pathogen infection. 34th Congress of the South African Society for Enology and Viticulture, 14-16 November 2012, Simondium, South Africa (Oral presentation).

Mutawila C, Halleen F, Mostert L. 2012. Optimisation of the time of application of *Trichoderma* bio-control agents for grapevine pruning wound protection. XII Meeting of the Working Group, Biological control of fungal and bacterial plant pathogens. Reims, France. 25-27 June 2012 (Oral presentation).

Mutawila C, Stander C, De Beer A, Vivier M, Halleen F, Mostert L. 2012. Response of *Vitis vinifera* cell cultures by culture filtrate of *Eutypa lata* and *Trichoderma atroviride*: Expression of defence-related genes. 8th International Workshop of Grapevine Trunk Diseases, 18-21 June, Valencia, Spain (Poster).

Makatini G, Mutawila C, Halleen F, Mostert L. 2012. Grapevine trunk disease pathogens associated with sucker or spring wounds in South African vineyards. 8th International Workshop of Grapevine Trunk Diseases, 18-21 June, Valencia, Spain (Poster).

2013

Mutawila C, Stander C, Vivier M, Halleen F, Mostert L. 2013. Expression analysis of defence-related genes in *Vitis vinifera* cell cultures after elicitation by culture filtrate of *Eutypa lata* and *Trichoderma atroviride*. 48th Congress of the South African Society of Plant Pathology, Warmbad, South Africa.

Makatini G, Mutawila, Halleen F and Mostert L. 2013. Grapevine trunk disease pathogens associated with sucker or spring wounds in South African vineyards. 48th Congress of the South African Society of Plant Pathology, Warmbad, South Africa (Oral presentation).

Mutawila C, Halleen F, Mostert L. 2013. The application of *Trichoderma* on grapevine pruning wounds. Vinpro/Winetech information day, 18 April 2013, Bienn Donne, Simondium (Oral presentation).

Makatini G, Mutawila, Halleen F and Mostert L. 2013. Grapevine sucker wounds are susceptible to grapevine trunk disease pathogen infections. Vinpro/Winetech information day, 18 April 2013, Bienn Donne, Simondium (Oral presentation).

Mostert L, Mutawila C, Halleen F. 2013. Improving pruning wound protection against trunk disease pathogens. SASEV/WINETECH International Conference, 13-15 November, Somerset West, South Africa (Oral presentation).

Makatini G, Halleen F, Mostert L. 2013. Susceptibility of grapevine sucker wounds to trunk diseases. SASEV/WINETECH International Conference, 13-15 November, Somerset West, South Africa (Oral presentation).

Total cost summary of the project

TOTAL COST IN REAL TERMS	COST	CFPA	DFTS	Deciduous	SATI	Winetech	THRIP	OTHER	TOTAL
YEAR 1	**273 050**				96 525	96 525	80 000		**273 050**
YEAR 2	**244 824**				88 275	88 275	68 274		**244 824**
YEAR 3	**270 908**				94 454	94 454	82 000		**270 908**
YEAR 4	**257 200**				98 600	98 600	60 000		**257 200**
YEAR 5									
TOTAL	**1 045 982**				**377 854**	**377 854**	**290 274**		**1 045 982**