IMPROVING PRUNING WOUND PROTECTION AGAINST GRAPEVINE TRUNK DISEASE PATHOGENS

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Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Summary

Grapevine trunk diseases are a cause of decline and loss of productivity in grapevines at all stages of growth. These diseases are caused by a complex of wood-inhabiting fungi that infect mainly through pruning wounds. The management of these diseases relies on wound protection to prevent infection since there are no eradicative control measures to cure infected vines. There are few or no fungicides registered for grapevine pruning wound protection in most countries, while *Trichoderma* biocontrol agents are often available. This study aimed at improving grapevine wound protection by *Trichoderma* (*T*.) spp. and to gain a better understanding of the factors and mechanisms involved in biocontrol.

The effect of pruning time (early or late) and five timings of application of the biocontrol agent after pruning on pruning wound colonisation by *T. atroviride* and *T. harzianum* were determined. Chenin blanc and Cabernet Sauvignon vineyards were pruned in July (early) and August (late) of 2011 and 2012, and pruning wounds were treated with suspensions of the *Trichoderma* spp. at various times (0, 6, 24, 48 and 96 hours) after pruning. Wound colonisation was depended on the physiological state of the vine at pruning for both cultivars. However, for the 2012 season in Chenin blanc, wound colonisation was similarly high for both pruning times, which was attributed to high rainfall and humidity. Application of the biocontrol agents 6 hours after pruning consistently resulted in high wound colonisation by the *Trichoderma* spp. in both cultivars and pruning times. In both cultivars, pruning wound infection due to natural inoculum was higher in wounds made in late winter than those made earlier.

The effect of conidial formulation in nutritional (glucose, yeast extract and urea) and bio-enhancing (chitin and cell free culture filtrates) additives, on pruning wound colonisation by *T. atroviride* was also investigated. Nutritional additives increased the extent of pruning wound colonisation by *T. atroviride* compared to the un-amended conidial suspensions in a glass house study. The additives as well as Garrison, a fungicide containing pruning wound paint, and Eco77®, a registered *T. harzianum* biocontrol product, were tested in field trials for wound protection from infection by *Phaeomoniella* (*Pa.*) *chlamydospora*. In 2011, the pathogen was inoculated a day after pruning and all the *Trichoderma* spp. treatments similarly reduced *Pa. chlamydospora* infection by 75% to 90% in Thompson Seedless, while control was less in Chenin blanc and ranged from 40% to 74%. In 2012, the trial was carried out on Chenin blanc only and the pathogen was inoculated at intervals of 1, 3 and 7 days after pruning. Wound protection by the *Trichoderma* treatments was highest when wounds were inoculated with *Pa. chlamydospora* seven days after pruning. Two conidial formulations, a culture filtrate made from a chitin based medium and a combination of yeast

extract, urea and glucose, consistently enhanced biocontrol efficacy. These formulations reduced *Pa. chlamydospora* infection to levels similar to those of Garrison.

The integration of chemical and biological wound protection could provide both immediate and long term wound protection, but is limited by the sensitivity of the biocontrol agent to fungicides. Benzimidazole resistant *Trichoderma* strains were generated by gamma irradiation from the wild type isolates of *T. atroviride* (UST1 and UST2) and *T. harzianum* (T77). Mutants from UST1 and UST2 were of similar biological fitness as the wild type isolates and retained their *in vitro* antagonistic activity against grapevine trunk pathogens, while the mutant from T77 had reduced fitness and was not antagonistic to the pathogens. The wild type, UST1, and its mutant were tested alone and in combination with thiophanate methyl and carbendazim, respectively, for their ability to prevent pruning wound infection by *Pa. chlamydospora*. The combination of the UST1 mutant and carbendazim was the most effective treatment and gave the highest reduction in *Pa. chlamydospora* infection (70% to 93% control).

Grapevine cell cultures were used to compare the response of grapevines to *T. atroviride* and *Eutypa* (*E.*) *lata* as a first step to determining the importance of *Trichoderma*-grapevine interactions in pruning wound bio-protection. The expression of genes coding for enzymes of the phenylpropanoid pathway and pathogenesis related (PR) proteins was profiled over a 48-hour period using quantitative reverse transcriptase PCR. The cell cultures responded to fungal elicitors in a hypersensitive-like response that lead to a decrease in cell viability. Fungal elicitors from both fungi triggered the same genes and 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. Higher expression of PAL and CHIT IV in cell cultures treated with the *T. atroviride* elicitor led to a significantly higher ($P < 0.05$) total phenolic content and chitinolytic enzyme activity of the cell cultures compared to cell cultures treated with the *E. lata* elicitor. The response of the cell cultures to the *T. atroviride* elicitor signifies that the induction of grapevine resistance may be involved in wound bio-protection.

The role of secondary metabolites produced by *Trichoderma* spp. used in pruning wound protection was also investigated. 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. This metabolite was found to inhibit 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*. Results

therefore, indicate that 6PP is involved in the *Trichoderma*-pathogen interactions on pruning wounds.

The results of this study have provided new information in regards to the application of *Trichoderma*-based pruning wound products. The best time of application proved to be 6 hours post pruning. The formulation of conidial suspensions of *Trichoderma* spp. with nutritional additives and in protein extracts of the biocontrol agent showed potential in reducing variability of wound bio-protection. However, further research would be necessary to develop commercial products. The application of a fungicide together with *Trichoderma* spp. in the field holds promise to improve control, but would require further trials for possible commercialisation. This study is the first to report on grapevine host defence genes that are activated by the *Trichoderma* spp. used in pruning wound protection. Together with the characterisation of the major secondary metabolite produced by these *Trichoderma* spp., this information aids in understanding the mechanisms involved in the complex interaction between the biocontrol agent, the host and the pathogen.

Opsomming

Wingerdstamsiektes veroorsaak terugsterwing en verlies aan produktiwiteit in wingerdstokke gedurende alle groeifases. Hierdie siektes word veroorsaak deur ‘n verskeidenheid van hout-koloniserende swamme wat die wingerdstok meestal deur snoeiwonde infekteer. Die bestuur van hierdie siektes is afhanklik van wondbeskerming om infeksie te verhoed, omdat daar geen uitwissende beheermetodes na infeksie bestaan nie. In meeste lande is daar min of geen swamdoders geregistreer vir snoeiwond beskerming, terwyl *Trichoderma* biobeheer agente gereëld beskikbaar is. Hierdie studie poog om wingerd wondbeskerming deur *Trichoderma* (*T.*) spp. te verbeter en ‘n meer volledige begrip van die faktore en meganismes betrokke by biologiese beheer te ontwikkel.

Die effek van die tydsberekening van snoei (vroeg of laat) en vyf behandelingstye van die biobeheer agent na snoei op die kolonisering van snoeiwonde deur *T. atroviride* en *T. harzianum* is bepaal. Chenin blanc en Cabernet Sauvignon wingerde is gesnoei gedurende Julie (vroeg) en Augustus (laat) in 2011 en 2012, en snoeiwonde is behandel met *Trichoderma* spp. suspensies op verskillende tydspunte (0, 6, 24, 48 en 96 ure) na snoei. Wond-kolonisering was afhanklik van die fisiologiese toestand van die wingerdstok gedurende snoei vir albei kultivars. Gedurende die 2012 seisoen was wond-kolonisering ewe hoog vir albei snoeitye op Chenin blanc. Dit is verklaar deur hoë reënval en humiditeit gedurende daardie seisoen. Die aanwending van biobeheer agente 6 ure na snoei het konsekwent hoë kolonisering deur *Trichoderma* spp. tot gevolg gehad op albei kultivars en albei snoeitye. In albei kultivars is wondinfeksie as gevolg van natuurlike inokulum hoër gewees in wonde gemaak gedurende laat winter as in wonde wat vroeër in die seisoen gemaak is.

Die effek van konidia formulasie in voeding (glukose, gisekstrak en urea) en bioverbetering (chitien en sel-vrye kultuurfiltraat) toevoegings op snoeiwond-kolonisering deur *T. atroviride* is ook ondersoek. Voeding toevoegings het die omvangs van snoeiwond-kolonisering deur *T. atroviride* vergroot in vergelyking met ongewysigde konidia suspensies gedurende ‘n glashuis studie. Die toevoegings, sowel as Garrison, ‘n snoeiwond verf wat ‘n swamdoder bevat, en Eco77®, ‘n geregistreerde *T. harzianum* biobeheer produk, is getoets in veldproewe vir wondbeskerming teen infeksie deur *Phaeomoniella* (*Pa.*) *chlamydospora*. In 2011 is die patogeen geïnokuleer ‘n dag na snoei en al die *Trichoderma* spp. behandelings het infeksie verminder met 75% tot 90% op Thompson Seedless. Beheer was minder suksesvol op Chenin blanc, waar slegs 40% tot 74% beheer behaal is. In 2012 is die proef uitgevoer slegs op Chenin blanc en die patogeen is geïnokuleer teen intervalle van 1, 3 en 7 dae na snoei. Wondbeskerming by die *Trichoderma* behandelinge was die hoogste

wanneer wonde sewe dae na snoei geïnokuleer is met *Pa. chlamydospora*. Twee konidia formulasies, ‘n kultuurfiltraat wat bestaan het uit ‘n chitien-gebaseerde medium en ‘n kombinasie van gisekstrak, urea en glukose het deurlopend die effektiwiteit van biobeheer verbeter. Hierdie formulasies het *Pa. chlamydospora* infeksie verminder tot soortgelyke vlakke behaal deur Garrison.

Die integrasie van chemiese- en biobeheer in wondbeskerming kan onmiddelike en langtermyn wondbeskerming bied, maar is beperk deur die sensitiwiteit van die biobeheer agent teen swamdoders. Benzimidazole-weerstandbiedende *Trichoderma* isolate is ontwikkel deur gamma-bestraling van die wilde-tipe isolate van *T. atroviride* (UST1 en UST2) en *T. harzianum* (T77). Mutante van UST1 en UST2 het soortgelyke biologiese fiksheid getoon as die wilde-tipe en het hul *in vitro* antagonistiese aktiwiteit teen wingerd stampatogene behou, terwyl die mutant van T77 verminderde fiksheid getoon het en nie meer antagonisties teen patogene was nie. Die wilde-tipe, UST1, en sy mutant is apart en in kombinasie met thiofanaatmetiel en carbendazim, respektiewelik, getoets vir die vermoë om snoeiwonde te beskerm teen *Pa. chlamydospora.* Die kombinasie van die UST1 mutant met carbendazim was die mees effektiewe behandeling en het die hoogste vermindering in *Pa. chlamydospora* infeksie gelewer (70 tot 93% beheer).

As ‘n beginpunt om die belang van Trichoderma-wingerd interaksies in snoiewondbeheer te bepaal, is die invloed van *T. atroviride* en *Eutypa (E.) lata* op somatiese selkulture van wingerd vergelyk. Die effek van dié behandelings op ensieme in die fenielpropanoïedweg en patogenese-verwante (PR) proteïene is bepaal deur intydse PKR (real time PCR) van die korresponderende gene oor ‘n 48 uur tydperk. Die swam-afkomstige ontlokkers het ‘n hipersensitiewe-tipe reaksie in die selkulture ontlok, wat tot ‘n afname in sellewensvatbaarheid gelei het. Ontlokkers afkomstig van beide swamme het dieselfde gene aangeskakel en het induksie van fenielalanien ammoniak-liase (PAL), 4 kumaroïel Ko-A ligase (CCo-A), stilbeen sintase (STS), chitienase klas IV (CHIT IV), PR 3 en PR 4 veroorsaak en ‘n onderdrukking in chalkoon sintase (CHS) gene tot gevolg gehad. Hoër uitdrukking van PAL en CHIT IV in selkulture behandel met die *T. atroviride* ontlokker het gelei tot ‘n beduidende hoër ($P < 0.05$) totale fenoolinhoud en chitienolitiese aktiwiteit in selkulture in vergelyking met selkulture wat behandel is met die *E. lata* ontlokker. Die reaksie van die selkulture op die *T. atroviride* ontlokker dui daarop dat die induksie van wingerd weerstandbiedenheid betrokke mag wees in wond biobeheer.

Die rol van sekondêre metaboliete geproduseer deur *Trichoderma* spp. wat gebruik word in snoeiwond beheer is ook ondersoek. ‘n Vlugtige antimikrobiese verbinding, 6-pentiel α-pyroon (6PP) is geïsoleer en bepaal om die hoof sekondêre metaboliet afkomstig vanuit die *T. atroviride* (UST1 en UST2) en *T. harzianum* (T77) isolate te wees. Hierdie metaboliet

is betrokke by inhibisie van miselium groei, spoor en konidium ontkieming van *E. lata*, *Neofusicoccum* (*N.*) *australe, N. parvum* en *Pa. chlamydospora*. Die produksie van 6PP is geïnduseer deur die *T. atroviride* in wingerd hout ekstrak te kweek. In die geval van UST1, is die 6PP konsenstrasie verdubbel deur die isolaat met saam met *N. parvum* te kweek. Hierdie resultaat is ‘n aanduiding dat 6PP betrokke is in die *Trichoderma*-patogeen interaksie op snoeiwonde.

Die resultate van hierdie studie het nuwe inligting met betrekking tot die aanwending van *Trichoderma*-gebaseerde snoeiwond produkte verskaf. Die beste tyd vir aanwending van sulke produkte was 6 ure na snoei. Die formulasie van konidia suspensies van *Trichoderma* spp. met voeding toevoegings en in proteïen ekstrakte van die biobeheer agent het potensiaal getoon in die vermindering van variasie in wondbeskerming deur biobeheer agente. Verdere navorsing sal nodig wees om kommersiële produkte te ontwikkel. Die aanwending van ‘n swamdoder saam met *Trichoderma* spp. in die wingerd is belowend om beheer te verbeter, maar het meer proewe nodig voor kommersialisering. Hierdie studie is die eerste om wingerd beskerming gene wat deur *Trichoderma* spp. geaktiveer word aan te meld. Laasgenoemde, saam met die beskrywing van die hoof sekondêre metaboliete wat deur hierdie *Trichoderma* spp. geproduseer word, dra by tot ‘n meer volledige begrip van die meganismes betrokke by die komplekse interaksie tussen die biobeheer agent, die gasheer en die patogeen.

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Contents

CHAPTER 1

General Introduction and Project Aims

1.1 Grape production in South Africa

Grapevines (*Vitis vinifera*) are grown in temperate and cool climatic regions of the world, traditionally in Europe and the Middle-East from where they were spread to the so called 'new world' in the Americas (North and South), Australia, New Zealand and South Africa. In South Africa, viticulture can be traced back to the 17th century when the Dutch explorers arrived in the present day Cape Town. Unlike the rest of the new world where wine was produced for local consumption, by the 18th century wines from South Africa specifically from Constantia were being exported and considered among the most favoured wines of that time. According to the South African Wine Industry Information and Systems, and the South African Table Grape Industry, the land currently under grape production is just over 140 000 hectares (Anonymous, 2012 & 2013). Grapevine production in South Africa is concentrated along the coastal areas of the Western and Northern Cape provinces which have a Mediterranean climate. Minor production of table grapes also occurs in inland regions under a sub-tropical climate. According to the South African Wine Industry Information and Systems, the grapevine industry along with the associated tourism contributes more than 10% of South Africa's Gross Domestic Product (GDP).

To achieve optimum yields and high quality fruit, grapevines are annually pruned so as to maintain a balance between vegetative and reproductive growth. Winter pruning removes most of the previous season growth and aims at providing space among shoots for optimal aeration and light penetration. Pruning also reduces humidity levels in the canopy, which also results in a reduction of foliar diseases (Mullins *et al*., 1992). During the pruning process, unhealthy wood is also removed thereby ensuring that the new season's growth is produced on healthy wood. However, the wounds created by the pruning process are important infection sites for wound pathogens that cause wood diseases and grapevine decline.

1.2 Grapevine trunk diseases

Grapevine trunk diseases are caused by a broad range of wood-inhabiting fungi and symptoms are a result of one or a combination of several fungi. Trunk pathogens, either individually or collectively, are responsible for graft failure, loss of vigour and productivity in established vines, spots on berries, late ripening and altered flavour, as well as death of vines (Mugnai *et al.*, 1999; Pascoe & Cottral, 2000; Fourie & Halleen, 2004; Gubler *et al*., 2005; Lorrain *et al*., 2012). Grapevine trunk diseases include Eutypa dieback (Diatrypaceae

spp.), Petri disease (*Phaeomoniella chlamydospora* and *Phaeoacremonium* spp.), esca (Petri disease fungi and wood rot Basidiomycetes), Botryosphaeria dieback (Botryosphaeriaceae species) and Phomposis dieback (*Phomopsis*/*Diaporthe* spp.). Infection occurs through wounds and pruning wounds are regarded as the primary sites of infection (Chapuis *et al*., 1998; Larignon & Dubos, 2000; Van Niekerk *et al*., 2006).

The occurrence of grapevine decline diseases caused by fungal trunk pathogens has drastically increased causing significant yield and economic losses in all grapevine producing areas (Scheck *et al*., 1998; Rumbos & Rumbou, 2001; Van Niekerk *et al*., 2003; Sosnowski *et al*., 2005). In addition to reducing yield and quality of grapes, they also increase vineyard management costs and reduce the life of a vineyard (Munkvold *et al.*, 1994). All of the vineyards in the different grapevine production areas in South Africa have trunk diseases to varying degrees (Van Niekerk *et al*., 2011; White *et al*., 2011). Due to continual loss of vines, reduced yield and production of poor fruit, vineyards are removed and new vineyards planted much sooner than planned. These diseases are occurring in a more severe form and have become an increasingly important limiting factor threatening the sustainability of grape and wine production.

Trunk diseases are difficult to manage. This is mainly due to the complexity of the diseases as they are caused by a variety of unrelated fungi, which make it difficult to find one control method that is equally effective against all the pathogens. Cultural practices, such as sanitation, are very important in reducing the inoculum pressure and delaying establishment of the diseases. However, due to the high number of wounds made on an individual vine every year, it is virtually impossible to completely control trunk diseases through cultural practices. Preventing infection by the protection of wounds is therefore the major way of controlling trunk diseases.

1.3 Grapevine pruning wound protection

Management of the trunk pathogens involves cultural practices such as sanitation in the vineyard to reduce the amount of inoculum as well as the timing of pruning to avoid periods of high wound susceptibility. Treatment of pruning wounds with chemical fungicides, paints and pastes, and biocontrol agents has been found to protect wounds from infection and is currently the most reliable way of preventing infection. A major challenge to pruning wound protection is that the wounds remain susceptible for several weeks until they are fully healed (Munkvold & Marois, 1995; Eskalen *et al*., 2007; Van Niekerk *et al*., 2011). Wound treatment agents should be able to persist until wounds are healed and be effective against all trunk pathogens.

Several fungicides have been found to have a wound protective effect against trunk pathogens (Rolshausen & Gubler, 2005; Sosnowski *et al*., 2008; Halleen *et al*., 2010; Rolshausen *et al*., 2010). Many more fungicides have been tested *in vitro* (Jaspers, 2001, Bester *et al*., 2007; Amponsah *et al*., 2012; Gramaje *et al*., 2012) but very few are registered for pruning wound protection. Fungicide efficacy on the pruning wound declines with time and does not persist for the entire period that wounds remain susceptible (Munkvold & Marois, 1995). Some of the effective fungicides such as sodium arsenite and benomyl have also been pulled off the market in most grapevine producing regions due to human and environmental toxicity.

Grapevine pruning wounds are colonised by naturally occurring non-pathogenic fungi and bacteria, some of which have been found to prevent infection by trunk pathogens (Carter & Price, 1974; Munkvold & Marois, 1993). These saprophytes grow on the wound and can provide protection until wounds heal and are no-longer susceptible to infection. Biological control (biocontrol) agents for pruning wound protection have thus been developed as alternatives to chemical control, most of which are based on *Trichoderma* species. The biocontrol effect of *Trichoderma* spp. has been demonstrated on a wide spectrum of grapevine trunk diseases (Di Marco *et al*., 2004; John *et al*., 2005; Kotze *et al*., 2011). The advantage of using biological control pruning wound protection is in the long term protection given by the fungus growing in the pruning wound (John *et al*., 2005). The protective effect of *Trichoderma* biocontrol agents on the wound has largely been attributed to the antagonistic effect of the biocontrol agent on the pathogens which includes mycoparasitism, secretion of mycolitic enzymes, competition for limiting resources, as well as the secretion of antibiotic metabolites (Sivasithamparam & Ghisalberti, 1998; Di Marco *et al*., 2004; Kotze *et al*., 2011). However, there is a growing body of evidence that shows that *Trichoderma*-plant interactions may also be involved in biocontrol (De Meyer *et al*., 1998; Palmieri *et al*., 2012; Martínez-Medina *et al*., 2013).

Despite extensive research and increased availability, there has been limited adoption of biocontrol agents in commercial agriculture mainly due to inconsistent and unpredictable performance in the field (Harman *et al*., 2000; Ojiambo & Scherm, 2006). Reports are also available of studies that question the effectiveness of biocontrol agents in grapevine wound protection (Larignon, 2010). The causes of poor field performance are usually diverse and not well understood, but are due to both biotic and abiotic factors. The biotic factors include host susceptibility and interactions of the biocontrol agent with the host plant cultivar and non-target organisms (Ryan *et al*., 2004; Mutawila *et al*., 2011). Abiotic factors include climate, physical and chemical composition of host substrate, as well as the application method/strategy. In grapevine pruning wound protection with biocontrol agents, it

is generally acknowledged that biocontrol agents perform better when pathogen inoculation is delayed to allow better colonisation by the biocontrol agents (Munkvold & Marois, 1993; John *et al*., 2005; Kotze *et al*., 2011). Despite the challenges of biocontrol, its importance cannot be contested especially with the continued deregistration of effective fungicides.

1.4 Rationale and scope of study

Many grapevine farmers and viticulturists realise the importance of pruning wound protection for sustainability and longevity of their vineyards. It has become important to ensure that products used for wound protection are both effective and cost effective. Currently in South Africa, biocontrol agents of *Trichoderma* spp. are the only products specifically registered for grapevine pruning wound protection.

For over a decade, the grapevine trunk diseases research groups of Stellenbosch University and the Agricultural Research Council Infruitec-Nietvoorbij, South Africa have been studying the etiology, epidemiology and control of grapevine trunk diseases. Emanating from this research, two strains of *Trichoderma atroviride* (UST1 and UST2) isolated from grapevine wounds and a commercial *T. harzianum* (Eco 77®) were found to have substantial antagonistic properties against grapevine trunk pathogens (Kotze *et al*., 2011). *In vitro* and field tests against grapevine trunk pathogens showed that the strains have grapevine pruning wound protection effect (Kotze *et al*., 2011; Mutawila *et al*., 2011). *In vitro* tests on UST1 and UST2 also showed them to secrete volatile and non-volatile secondary metabolites that inhibited spore germination and reduced mycelial growth of trunk disease pathogens (Kotze *et al*., 2011). The identity of these metabolites is unknown and needed to be determined. The huge structural and functional diversity of *Trichoderma* metabolites makes it necessary for the continual search of new metabolites. These may be important in selection or screening of potential biocontrol agents or may be developed for application as bio-active compounds in pesticides and antibiotics. However, some strains of *Trichoderma* spp. have also been reported to produce trichothecene toxins (Degenkolb *et al*., 2008) such that it has become essential to test for mycotoxins in all potential bio-pesticide strains that may enter the food chain.

Field studies showed that the *Trichoderma* isolates UST1 and UST2 are effective in protecting grapevine pruning wounds from trunk diseases and can persist in the grapevine wood for at least 8 months (Kotze *et al*., 2011; Mutawila *et al*., 2011). However, variation was observed in the efficacy of the biocontrol agents depending on the grapevine cultivars (Mutawila *et al*., 2011). The factors that could explain this variation were the effect of grapevine metabolic state on wound colonisation by *Trichoderma* spp. as well as the biocontrol-grapevine interactions. Therefore, in this study an effort was made towards

improving grapevine pruning wound protection by *Trichoderma*-based biocontrol agents. In order to better understand the mechanisms of biocontrol *in vivo*, the *Trichoderma*-grapevine-pathogen interactions were also investigated.

First, the effect of grapevine pruning time and the time, after pruning, of application of the biocontrol agent on pruning wound colonisation by *Trichoderma* spp. were determined (Chapter 3). Formulations that can improve colonisation of pruning wounds and efficacy of biocontrol agents in the field will be very important in enhancing consistency in the field while integration of biocontrol agents with conventional fungicides will be invaluable. In a previous field study the addition of a sticking agent, Nu Film 17, to *T. atroviride* suspensions could not significantly enhance biocontrol efficacy in wound protection (Mutawila, 2010). In the current study nutritional amendments were tested for their effect in improving *T. atroviride* wound colonisation and wound protection (Chapter 4). Fungicide resistant *Trichoderma* isolates were also generated for integration with conventional fungicides so as to benefit from the complementary effect of the immediate protection by the fungicide and long term protection by the biocontrol agent (Chapter 5).

There are currently no studies on the molecular response of grapevine to *Trichoderma* spp. used in pruning wound protection. So as a first step to understanding these interactions, a model system (grapevine cell cultures) was used to compare response of grapevines to a trunk pathogen and the biocontrol agent (Chapter 6). Lastly, since the secondary metabolites of the *Trichoderma* spp. used in pruning wound protection are not known, the major metabolite from the biocontrol isolates was isolated, identified and its role in pruning wound protection determined (Chapter 7).

1.5 Aims of the study

The main aim of the study was to improve grapevine pruning wound protection against trunk pathogen infection with the use of *Trichoderma* spp. biocontrol agents. The study further aimed to improve the application of *Trichoderma* spp. biocontrol agents, to understand factors that affect *Trichoderma* efficacy in the field and gain insight into the biocontrol mechanisms involved. The specific objectives of the study were to:

i. Determine the effect of grapevine pruning time and application time of the biocontrol agent on pruning wound colonisation by *Trichoderma* spp.,
ii. Determine the effect of nutritional amendments on pruning wound colonisation by *T. atroviride* and wound protection,
iii. Develop benzimidazole resistant *Trichoderma* isolates for integration with fungicides in wound protection,

iv. Determine the response of grapevine to *Trichoderma* colonisation by a comparison of grapevine cell culture response to a grapevine trunk pathogen and the biocontrol agent, and

v. Isolate and identify the major secondary metabolites from the *Trichoderma* spp. used for grapevine wound protection and determine their role in biocontrol.

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CHAPTER 2

Grapevine trunk diseases: Grapevine response and disease management

2.1 Summary

Grapevine pruning is a critical viticultural practice, carried out during the dormant season so as to maintain a balance between vegetative and reproductive growth. Wounds made during this process are the primary entry sites of infection for trunk disease pathogens that cause premature grapevine decline. Grapevine trunk diseases namely, Eutypa dieback, Petri disease, esca, Botryosphaeria dieback, and Phomopsis dieback cause loss of productivity and increase production costs. These diseases have been reported worldwide in all grapevine producing areas and are an important threat to the economical sustainability of viticulture. There are no eradicative measures, except remedial pruning, to cure infected vines and so the only control strategy currently available is to protect wounds from infection. This review gives an overview of the current knowledge on grapevine response to infection by trunk pathogens and management of trunk diseases in the vineyard.

2.2 Introduction

Grapevine trunk diseases refer to a combination of several vine disorders that result from the infection of the woody perennial parts of the vine and manifest in various external and internal symptoms. They are caused by a complex of wood-inhabiting fungi and symptoms are a result of one or a combination of several pathogens. Trunk diseases are a cause of gradual grapevine decline and loss of productivity at all stages of vine growth (Munkvold *et al*., 1994; Mugnai *et al*., 1999; Pascoe & Cottral, 2000; Siebert, 2001; Van Niekerk *et al*., 2003; Gramaje & Armengol, 2011). These diseases, typically associated with older vines, were often overlooked due to their slow development and symptom expression relative to the more common seasonal foliar diseases. However, grapevine trunk diseases have become an important limitation to attaining full potential of vineyards. In the last and the first decades of the 20th and 21st centuries, respectively, the increased incidence and severity of grapevine trunk diseases has awakened both growers and scientists alike to a new threat to the long-term sustainability of grape, wine and raisin production. Grapevine trunk diseases now occur in all grapevine producing areas although severity of the specific diseases may differ among regions (Mugnai *et al*., 1999; Pascoe & Cottral, 2000; Halleen *et al*., 2003; Gubler *et al*., 2005; Kuntzmann *et al*., 2010; Pitt *et al*., 2010; Bertsch *et al*., 2012).

Trunk pathogens, either individually or collectively, are responsible for graft failure (Adalat *et al*., 2000; Fourie & Halleen, 2004), loss of vigour and productivity in established

vines, spots on berries, late ripening and altered flavour as well as death of vines (Munkvold *et al*., 1994; Mugnai *et al*., 1999; Oliveira *et al*., 2004; Gubler *et al*., 2005; Larignon *et al*., 2009; Bertsch *et al*., 2012). In addition to reducing yield and quality of grapes, they also increase costs of vineyard management and reduce the life of vineyards. The main grapevine trunk diseases are Petri disease, esca, Eutypa dieback and Botryosphaeria dieback. There are no curative measures to infected vines and due to their incremental effect by the time symptoms appear there is not much that can be done to save the vine without losing production. Maintaining infected vineyards becomes unsustainable, due to continual loss of vines and production of poor fruit, forcing growers to re-establish the vineyard.

Grapevine trunk diseases are now reported at all stages of growth, but it is in the vineyard that losses are substantial. Poor vine establishment due to young grapevine decline has resulted in replanting of parts or entire vineyards in Greece (Rumbos & Rumbou, 2001) and California (Scheck *et al*., 1998). The majority of studies on the economic losses due to grapevine trunk disease have been done on Eutypa dieback, once considered the most important trunk disease. In California, losses due to Eutypa and Botryosphaeria dieback have been estimated to be up to US$ 260 million annually (Siebert, 2001). In Australia losses due to Eutypa dieback were estimated at A$ 2, 800 (~US$ 2, 550) per hectare in Shiraz vineyards with more than 50% disease incidence (Wicks & Davies, 1999) while in South Africa yield losses in Cabernet Sauvignon were estimated at ZAR 3000 (~US$ 300) per hectare in the Stellenbosch grape-region (Van Niekerk *et al*., 2003). In the French regions of Indre and Loire damages due to grapevine trunk diseases have been valued at US$ 16-18 million (FAV 37, 2010). It is important to note that economic losses could be even higher as most of the loss estimates were computed using only yield loss and did not take into account costs associated with retraining or removal of infected vines as well as revenue lost in poor quality grapes produced from infected vines.

2.3 Grapevine trunk diseases: an overview

Grapevine trunk diseases, particularly esca have been known since ancient times in the Mediterranean regions (Surico *et al*., 2008; Surico, 2009). The pathogens that cause young grapevine decline were described in 1912 and 1964 in Italy and California, respectively (Petri, 1912; Chirappa, 1964). These diseases were considered minor, affecting mainly old vines and managed by simple cultural practices. The recent occurrence of these diseases in a more destructive manner has been attributed to many factors, mainly the extensive establishment of vineyards and changes in nursery and cultural practices. In California, the cultivation of grafted vines with Phylloxera resistant rootstocks that are susceptible to some trunk pathogens as compared to the once own-rooted grapevine

cultivars has been attributed to the increased incidence of trunk diseases (Gubler *et al*., 2004). Sodium arsenite was considered to be the most effective fungicide against grapevine trunk diseases, especially esca, and its ban is consequentially attributed to the increased disease severity (Fussler *et al*., 2008; Larignon *et al*., 2009). However, the occurrence of grapevine trunk diseases has been increasing at alarming rates even in areas where sodium arsenite was never used (Bertsch *et al*., 2012). Changes in cultural practices, particularly the reduction in sanitary care in nurseries and scion-mother vineyards, is also responsible for the low quality of planting material and the dissemination of trunk diseases. Grapevine trunk pathogens are frequently isolated from symptomless plant tissue (Halleen *et al*., 2003; Aroca *et al*., 2006 & 2010) substantiating suggestions that the fungi may exist as latent infections, becoming pathogenic or inducing plant response later when the vines are subjected to stress (Whiting *et al*., 2001; Gubler *et al*., 2004 & 2005). Therefore, climate change, particularly increases in temperature and erratic rainfall, could also have contributed to increased severity of trunk diseases by increasing water stress on the vines (Surico *et al*., 2008; Sosnowski *et al*., 2011a). Due to the intricate nature of grapevine trunk diseases, they are considered a disease complex and the diseases within this complex are briefly discussed below.

2.3.1 Petri disease and esca

Petri disease, also known as black goo is caused by *Phaeomoniella* (*Pa.*) *chlamydospora* and several species of *Phaeoacremonium* (*Pm.*) (Crous & Gams, 2000; Mostert *et al*., 2006a). The pathogens colonise the xylem vessels where they cause blockage of water and solute transport. Blockage of vessels is a result of either the presence of fungal mycelium in the vessel lumen or by tylosis and gums produced by the plant in response to vessel infection (Edwards *et al*., 2007; Mutawila *et al*., 2011). The symptoms of Petri disease include graft failure, shortened internodes, leaf chlorosis, dieback, wilting and decline of young vines. Internally, the diseased vines show black/brown spots in transverse section (Figure 1A) and streaks when longitudinally sectioned (Figure 1B) (Edwards *et al*., 2001; Fourie & Halleen, 2002; Gubler *et al*., 2004; Mostert *et al*., 2006a). Petri disease is often associated with vines below the age of eight and hence is usually associated with nursery infections (Halleen *et al*., 2003; Fourie & Halleen, 2004; Gubler *et al*., 2004; Gramaje & Armengol, 2011). However, in a study of genetic variability among *Pa. chlamydospora*, Mostert *et al*. (2006b) found single vines infected by different pathogen genotypes, showing that infection occurred from different sources and could be from both the nursery and vineyard. Petri disease pathogens produce fruiting bodies (pycnidia and/or perithecia) on infected tissue from where inoculum for pruning wound infection originates (Eskalen & Gubler, 2002; Rooney-Latham *et al*., 2005a).

Petri disease pathogens are also associated with esca in older vines. Esca, strictly means wood decay which refers to the white rot internal symptoms (Figure 1C) of the diseases caused by Basidiomycetes, of which *Fomitiporia* species are the most predominant (Mugnai *et al*., 1999; Fischer, 2006; White *et al*., 2011). White rot is often seen in vines also expressing leaf-stripe symptoms (also called “tiger-stripes”; Figure 1D) and hence, the name esca has also been used to refer to the leaf stripe symptoms (Surico *et al*., 2008). Leaf-stripe symptoms have been shown to be caused by the tracheomycosis fungi that cause Petri disease in the absence of the white rot fungi (Edwards *et al*., 2001; Romanazzi *et al*., 2009). Surico (2009) proposed that the term esca be used to refer to the wood rot symptoms and “Grapevine Phaeotracheomycosis complex,” to refer to Petri disease and leaf stripe symptoms which are caused by the same fungi. Another symptom associated with the Phaeotracheomycosis complex is that berries formed on infected vines are small, cracked and may have small black spots (also called black measles) which are believed to be due to phytotoxins produced by the fungi (Mugnai *et al*., 1999; Gubler *et al*., 2004 & 2005). External symptom expression especially of the leaf stripes is erratic and are not seen every year in diseased vines (Edwards *et al*., 2001; Marchi *et al*., 2006). The apoplectic form of esca is characterized by a sudden loss of leaf turgor, resulting in wilting of the entire plant or a branch and is regarded as acute esca (Mugnai *et al*., 1999; Letousey *et al*., 2010). It usually occurs when a wet period is followed by hot and dry weather during the summer (Mugnai *et al*., 1999).

Figure 1: Symptoms of Petri disease and esca. Black goo in transverse section (A) and wood streaking in longitudinal section originating from a pruning wound (B) in vines with Petri disease. White wood rot (C) and leaf stripe symptoms (D) caused by Basidiomycetes and Phaeotracheomycosis fungi, respectively. (Photographs: A from Dr. L. Mostert; B and C, from Dr. F. Halleen).

2.3.2 Eutypa dieback

Eutypa dieback is caused by species of Diatrypaceae of which *Eutypa* (*E*.) *lata* is the most prevalent. Eutypa dieback was once considered to be the most important grapevine trunk disease (Munkvold *et al*., 1994; Gubler *et al*., 2005). This disease is still important and remains a major grapevine trunk disease whose management has been further complicated by the increased occurrence of other trunk diseases that share a similar disease cycle. Another diatrypaceous specie, *Cryptovalsa ampelina*, has long been associated with grapevine wood, but was considered less virulent compared to *E*. *lata* (Mostert *et al*., 2004). More diatrypaceous species have now been identified and are also associated with grapevine canker and Eutypa dieback-like symptoms (Trouillas & Gubler, 2004; Trouillas *et al*., 2010).

Symptoms of Eutypa dieback are often observed early in spring when shoots on infected arms show chlorotic, distorted/cupped leaves with tattered margins and shortened

internodes (Figure 2A). Internally, the infected arms/trunks show a wedge-shaped wood necrosis (Figure 2B) (Moller *et al*., 1974; Carter, 1988). In successive seasons the number of shoots showing dieback symptoms increases until eventually the whole arm fails to initiate new growth. The whole vine subsequently dies if the infected parts are not removed. Inflorescence dry out before berries form, and if bunches form they are often small and distorted. The pathogen mycelium does not grow in the new shoots and foliar symptoms are due to phytotoxins produced by the fungi growing in the arms (Tey-Rulh *et al*., 1991; Rudelle *et al*., 2005; Andolfi *et al*., 2011).

2.3.3 Botryosphaeria dieback

Botryosphaeria dieback, is caused by fungi of the family Botryosphaeriaceae namely species of *Botryosphaeria* (*B*.), *Neofusicoccum* (*N.*), *Lasiodiplodia* (*L.*), *Diplodia* (*D.*), and *Dothiorella* (Van Niekerk *et al*., 2004; Crous *et al*., 2006; Úrbez-Torres *et al*., 2006; Larignon *et al*., 2009; Pitt *et al*., 2010). Fungi of the Botryophaeriaceae are cosmopolitan, and colonise a wide range of woody species either as saprophytic endophytes or as pathogens. It is for this reason that they were often overlooked and not considered pathogens of grapevine (Castillo-Pando *et al*., 2001; Crous *et al*., 2006). On grapevine, some of the Botryosphaeriaceae species causing cankers are also isolated from asymptomatic wood and even non-woody tissue (Halleen *et al*., 2003; Van Niekerk *et al*., 2004; Wunderlich *et al*., 2011).

Cankers caused by species in the Botryosphaeriaceae may be chronic, causing a gradual grapevine decline or acute, causing a severe and rapid defoliation and wilt of part or the whole grapevine plant. The chronic symptoms occur in vines above the age of 8 years and cause a gradual loss of vigour and yield (Phillips, 1998; Larignon & Dubos, 2001). Symptoms include dead spurs, bud necrosis, mild leaf chlorosis (Figure 2C), and shoot dieback. Sometimes the bleaching of canes, associated with Phomopsis cane and leaf spot, is also observed (Phillips, 1998 & 2000). Cankers develop mainly on trunks, cordons and also on canes and are seen on the surface as sunken darkened areas of the bark often located close to a large wound or a spur from where they extend and may cause girdling which leads to wilting of shoots on the cordon. When the bark on the canker is removed it reveals a red-brown discolouration or wood necrosis that starts from a pruning wound and has a wedge shape when viewed in cross-section (Figure 2D). When large cankers are left to develop, they may cause the sudden wilting and collapse of shoots on a vine with no prior foliar symptoms (Larignon & Dubos, 2001; Gubler *et al*., 2005). Species associated with cankers are belong to the several genera that include *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia* and *Neofusicoccum* (reviewed by Úrbez-Torres, 2011). Fruiting bodies

(pycnidia) are produced on the surface of the cankers which usually have a charcoal black appearance and serve as sources of inoculum for new infections.

Figure 1.2: Typical external and internal symptoms of Eutypa dieback (A, B) and Botryosphaeria canker (C, D). Foliar symptoms are often severe in Eutypa dieback (A) compared to Botryosphaeria canker (C). Both diseases cause wedge shaped wood necrosis (B, D). (Photographs: A from Van Niekerk *et al*., 2003; B, from Dr. F. Halleen, and D from Dr J.R. Urbez-Torres and Dr. G.M. Leavitt).

2.3.4 Phomopsis dieback

Cosmopolitan species of *Phomopsis* (*P*.) are well known saprophytes, endophytes and pathogens on both woody and non-woody species (Gomes *et al*., 2013). In grapevines *P*. *viticola* is a well-known causal agent of Phomopsis cane and leaf spot while (Phillips, 1998 & 2000; Mostert *et al*., 2001; Van Niekerk *et al*., 2005). Since the discovery of *E*. *lata* as the causal agent of dead arm in grapevines, *Phomopsis* spp. were thought to be less important as trunk pathogens (Moller & Kasimatis, 1981). However, recent studies have shown that *Phomopsis* and *Diaporthe* species are regularly isolated from cankers (Úrbez-Torres *et al*., 2012 & 2013) and pruning wounds (Van Niekerk *et al*., 2005) of grapevines. At

the Grapevine Trunk Disease workshop held in Spain in 2012, it was proposed that the trunk disease caused by *Phomopsis* spp. be referred to as Phomopsis dieback. Symptoms of Phomopsis cane and leaf spot are often seen on green canes as brown-black lens-shaped lesions surrounded with a yellow hallow at the bottom of the cane near the cordon. These lesions may coalesce and girdle the bark causing wilt or weak stems that easily break during windy weather or on working the vine (Phillips, 1998 & 2000). On leaves, brown round lesions surrounded by pale yellow areas are seen. The disease also causes loss of vine vigour, smaller bunches and sometimes rotting of grapes (Phillips, 2000; Erincik *et al*., 2001). Phomopsis dieback symptoms include lack of spring growth and bud mortality following growth (Úrbez-Torres *et al*., 2013). Internal wood symptoms have also been observed and these are similar to wood necrosis symptoms reported for other dead arm pathogens (Mostert *et al*., 2001; Van Niekerk *et al*., 2005; Úrbez-Torres *et al*., 2013).

2.4 Infection and disease cycle of grapevine trunk diseases

The infection process and symptom development of grapevine trunk diseases are very similar. For the purposes of this review, these diseases are treated as a complex rather than as separate diseases.

2.4.1 Grapevine nurseries as sources of infection

Grapevine nurseries have been found to be a major source of infected material. Infection may occur at various stages of the nursery propagation process. Hydration of rootstock and scion cuttings by drenching in hydration tanks for periods of between 1-12 hours (Fourie & Halleen, 2006) provides an opportunity for infection early in the propagation process. Retief *et al*. (2006) detected *Pa. chlamydospora* in hydration water after pre-storage hydration. Poor sanitary practices in the nursery may lead to contamination of the hydration water while infected cuttings and microorganisms carried on the surfaces of the cuttings can also contaminate the water. Infection can also occur further in the propagation process during grafting and callusing as well as the nursery fields (Halleen *et al*., 2003; Vigues *et al*., 2010)

Grapevine trunk pathogens are wound colonisers and the large number of wounds created in the grafting process exposes wood to infection. Petri disease pathogens have been detected and isolated from washings of grafting tools and machinery (Pollastro *et al*., 2009; Aroca *et al*., 2010). Grapevine trunk pathogens have also been detected and isolated from callusing material (Retief *et al*., 2006; Aroca *et al*., 2010) from where it can infect cuttings or infection can occur from the contact of infected and healthy material in the callusing boxes (Larignon *et al*., 2006). Infection could also originate from root infections after planting of the grafted material in nursery soils. Petri disease pathogens, *Pa.*

chlamydospora and *Phaeoacremonium* species, have also been shown to infect uninjured roots of tissue-cultured grapevines (Feliciano & Gubler, 2001). However, higher infection rates of the Petri disease pathogens are found on grafted grapevines compared to non-grafted vines indicating the importance of wounds made during the propagation process (Gubler *et al*., 2004; Aroca *et al*., 2006; Gramaje & Armengol, 2011).

Petri disease pathogens are the most predominant fungi associated with infection in the nursery propagation process (Halleen *et al*., 2003; Fourie & Halleen, 2004; Gramaje & Armengol, 2011). However, other trunk pathogens namely, Botryosphaeriaceae and *Phomopsis* species have also been isolated from grapevine material in the propagation process (Halleen *et al*., 2003; Vigues *et al*., 2008 & 2010). Vigues *et al*. (2008) reported higher infection rates by species of Botryosphaeriaceae than Petri disease pathogens after the hydration process of propagation material. A major source of infection to the nursery plants are contaminated shoots used in the grafting process originating from infected scion and rootstock mother plants (Fourie & Halleen 2002; Aroca *et al*., 2006 & 2010; Halleen & Mostert, 2012). These either introduce inoculum in the hydration water, callusing material and nursery soils or systemically infect healthy material it is grafted to.

2.4.2 Vineyard infection processes

In the vineyard, ascospores and/or conidiospores are released from infected vines where fungal fruiting bodies form on the surfaces of infected wood. Eutypa dieback is more common where the mean annual rainfall exceeds 250 mm, which is required for perithecia development (Carter, 1988). Diatrypaceous species have a large host range such that inoculum may not only originate from vineyards (Carter, 1991; Cloete *et al*., 2011). For the release of *E. lata* ascospores from the perithecia, rain events of at least 2 mm or an equivalent in overhead irrigation or snowmelt is required (Ramos *et al*., 1975; Pearson, 1980; Trese *et al*., 1980). Once the stroma has been wetted, ascospore release continues for as long as the perithecia remain wet. The release of *Pa. chlamydospora* conidia coincided strictly with rainfall events in Californian vineyards (Eskalen & Gubler 2002; Eskalen *et al*., 2004) while aerial spores of *Togninia* (*To*.) *minima*, (teleomoph of *Pm. aleophilum*) were associated with rainfall, but they were also occasionally trapped in the absence of precipitation (Eskalen *et al*., 2004; Rooney-Latham *et al*., 2005b). In France, rainfall was shown to play a major role in *Pa. chlamydospora* wound infection while *Pm. aleophilum* spore availability was not linked to rainfall (Larignon & Dubos, 2000). The release of conidia of Botryosphaeriaceae species has also been found to be dependent on rainfall events (Epstein *et al*., 2008; Amponsah *et al*., 2009; Van Niekerk *et al*., 2010; Úrbez-Torres *et al*., 2010). Rainfall had no effect on the release of *Fomitiporia* (*F*.) *mediterranea*

basidiospores which occurred at average daily temperatures above 10 °C and 80% relative humidity (Fischer, 2009).

Moisture together with wind is necessary for the spread of spores. Some Botryoshearіaceae species were found mostly in rain water collected below the vines, than on wind/airborne spore traps indicating the role of rain in dispersal (Epstein *et al*., 2008; Amponsah *et al*., 2009). Waterborne dispersal was also reported for basidiospores of *F. mediterranea*, the major causal organism of white wood rot (esca) in Europe (Fischer, 2009). In Mediterranean areas where grapevines are produced, rainfall events are mostly experienced during the winter season when vines are pruned which makes pruning wounds ideal infection sites for rain dispersed inoculum. However, rainfall events at any time of the year can also trigger the release of *E*. *lata* ascospores (Pearson, 1980; Trese *et al*., 1980). Although spores of *To*. *minima* and Botryosphaeriaceae species have been linked to rainfall activity they were also found to be present in the vineyard all year round (Eskalen & Gubler, 2002; Amponsah *et al*., 2009; Kuntzmann *et al*., 2009; Úrbez-Torres *et al*., 2010).

Pruning wounds are the principal infection sites of aerial inoculum of trunk pathogens (Moller & Kasimatis, 1978; Chapuis *et al*., 1998; Serra *et al*., 2008; Fischer, 2009). Recent studies have demonstrated the susceptibility of wounds made after the removal of suckers from the trunk and canes in spring or summer. These wounds were shown to be infected by Botryosphaeriaceae species in California (Epstein *et al*., 2008) and *E*. *lata* in France (Lecomte & Bailey, 2011). A survey carried out in South African vineyards also revealed trunk infections from various trunk pathogens originating from sucker wounds (Makatini *et al*., 2012). Current studies have further shown that the wounds are susceptible to most fungi involved in grapevine trunk diseases. The importance of these wounds as entries of infection is further strengthened by reports of higher inoculum availability in the vineyards in spring and summer, when suckers are removed, compared to winter for species of Botryosphaeriaceae (Amponsah *et al*., 2009; Kuntzmann *et al*., 2009; Van Niekerk *et al*., 2010).

On the wound the spores lodge in exposed vessels where they germinate and colonise the wood tissue. Pathogens grow in the vascular tissue causing wood rot and a gradual decline in the infected grapevines. There is usually a delay between infection and appearance of symptoms, making the diseases difficult to detect until extensive damage has occurred. Cankers and fruiting bodies develop on surfaces of infected parts and become sources of inoculum for further infections. Cankers caused by species of Botryosphaeriaceae and *Phomopsis* may develop within the year of infection especially on canes (Phillips, 1998; Amponsah *et al*., 2009). Cankers caused by *E*. *lata* take time to develop and are usually found further away from the site of infection (Moller & Kasimatis,

1978 & 1981). Similarly, basidiocarps also take a long time to develop and are not seen on every vine showing wood rot symptoms. Cankers may not be easily noticeable unless the vines start showing decline symptoms and wood is sectioned. Also fruiting bodies are small and not always visible with the eye in the case of the Ascomycota. Basidiocarps of the Basidiomycota form rarely and are usually underneath the arms of the vines. Appearance of foliar symptoms, depending on the pathogens involved, is intermittent, and not observed in every growing season in which case infected vines are indistinguishable from healthy vines (Mugnai *et al*., 1996; Phillips, 1998; Edwards *et al*., 2001; Marchi *et al*., 2006; Sosnowski *et al*., 2007b).

Inoculum can also originate from outside the vineyard from other woody species that are alternative hosts of the pathogens (Munkvold *et al*., 1993; Cloete *et al*., 2011). Fruiting bodies on woody hosts next to a vineyard become a continual source for future infections. There is also increasing evidence that spores may be deposited on the wounds by arthropods. Epstein *et al*. (2008) recovered rove beetles (Staphilinidae) infested with *D. seriata* on traps placed over pruning wounds and hypothesised that they could disseminate wound pathogens. A more detailed study in South Africa found several arthropods on grapevine trunks and pruning wounds to also carry Petri disease and other trunk pathogens on their exoskeletons (Moyo *et al*., 2012).

2.5 Pathogenesis of Trunk Pathogens

Once spores land on the wound and enter exposed xylem vessels, they germinate and grow into the wound through xylem pits and eventually through cell walls with the aid of cell wall degrading enzymes (English & Davis, 1978; Pascoe & Cottral, 2000; Rudelle *et al*., 2005). The interaction of each specific pathogen and the grapevine host determines the resulting symptom expression. Grapevine trunk diseases can generally be grouped, depending on wood symptom development, into tracheomycosis, soft rot and white rot. Tracheomycosis is due to the growth of fungi in the vascular system causing blockage of water and nutrient transport (Edwards *et al*., 2007; Mutawila *et al*., 2011). On wood colonisation, *Pa. chlamydospora* advances slowly from the inoculation site and is mainly restricted to the xylem vessels and does not cause wood rot (Lorena *et al*., 2001; Mutawila *et al*., 2011). The host respond to the pathogen by the production of gels (gum) and tyloses to occlude the xylem vessels and stop further pathogen colonisation. The blockage of vessels by the plant response could be the reason external symptoms of tracheomycosis resemble those of water and nutrient deficiency which include leaf chlorosis, stunted growth, dieback and wilting (Pascoe & Cottral, 2000; Edwards *et al*., 2001).

On the contrary, the other Ascomycota pathogens cause wood rot that is classified as soft rot and is characterised by extensive carbohydrate loss in the wood with little or no effect on the lignin, leaving a brown and soft appearing wood rot (English & Davis, 1978; Worrall *et al*., 1997). Most of what is known about their pathogenesis is based on studies on Eutypa dieback. Integral to colonisation of grapevine wood by *E*. *lata* is the production of cellulases, hemicellulases and xylanases that degrade the structural carbohydrates making-up the xylem secondary cell walls, especially the cellulose rich secondary wall (English & Davis, 1978; Blanchette, 1995 & 2000; Rudelle *et al*., 2005). The fungi grow and extend longitudinally within the secondary walls of the xylem and the fibre tracheid where they also penetrate and cause death of the vessel associated cells (xylem parenchyma) (English & Davis, 1978; Rudelle *et al*., 2005). The fungi do not degrade the middle lamella but can degrade lignin to a limited extent (Blanchette, 1995 & 2000; Rolshausen *et al*., 2008). Wood necrosis follows after the loss of xylem function and plant response by sealing-off the infected sites through the formation of gums and tylosis. The same enzymes in *E*. *lata* have also been found in species of Botryosphaeriaceae (Encinas & Daniel, 1995; Dekker *et al*., 2001) which may also share a similar wood colonisation pattern. The Basidiomycota fungi cause white rot through the simultaneous break down of carbohydrate and lignin in the wood often causing a bleaching discolouration of the wood. Even after extensive decay, some non-decayed wood still remain which result in the friable mass characteristic of white wood rot (Worrall *et al*., 1997; Enoki *et al*., 1998).

In addition to the cell wall degrading enzymes, trunk pathogens also secrete phytotoxic secondary metabolites that are also involved in their pathogenesis. Most of these toxins have been isolated in culture and their role *in planta* has not been well characterised except for toxins involved in Eutypa dieback. Eutypine, a toxin from *E*. *lata* was isolated from both culture and sap of diseased plants (Tey-Rulh *et al*., 1991). Eutypine was found to be a protonophoric compound, that is, it causes leakage of protons from mitochondria and cells which interferes with respiration and carbohydrate metabolism (Deswarte *et al.*, 1996). Mahoney *et al*. (2003) isolated other metabolites from *E*. *lata* that caused necrosis on grapevine leaf disks namely, eulatinol, eulatachromene and its benzofuran cyclisation product. Eulatachromene was also detected in wood tissue colonised by the pathogen (Mahoney *et al*., 2003; Rolshausen *et al*., 2008) indicating that Eutypa dieback foliar symptoms could be a result of several metabolites. Toxins from the Petri disease pathogens (*Pa. chlamydospora* and *Pm. aleophilum*), sclerone, isosclerone and exopolysaccharides, are phytotoxic and could be involved in leaf stripe symptoms (Sparapano *et al*., 2000). Interestingly, isosclerone and exopolsaccharides have also been isolated from *N*. *parvum* (Evidente *et al*., 2010) which could explain leaf stripe symptoms observed on vines with

Botryosphaeria dieback caused by Botryosphaeriaceae species in France (Larignon *et al*., 2001). The Petri disease pathogens also produce 4-hydroxybenzaldehyde, a phytotoxin, that was also detected in culture filtrates of *F. mediterranea* the causal agent of wood white rot (Tabacchi *et al*., 2000).

2.6 Response to wood pathogens

The wood tissue is critical for mechanical support, conduction of solutes and food reserve, and hence plants have evolved mechanisms to preserve its integrity. Like the rest of the plant, woody tissue is protected from pathogens through constitutive and inducible defence reactions and a clear understanding of the defence mechanisms would be critical in the development of pathogen resistant cultivars. However, for most woody species and particularly grapevines, classical breeding takes a long time as resistance to pathogens should augment other important qualities such as grape and/or wine quality. Nevertheless, in the short term an understanding of these defence mechanisms is still important for the enhancement of management practices which optimise the plant's natural disease resistance.

2.6.1 Preformed barriers to wood infection

Passive resistance is not pathogen specific and involves preformed anatomical and chemical barriers to penetration and/or colonisation. It is passive because it is not activated by the arrival of the pathogens although some of these are also inducible during or after infection. The outer surfaces of plants are in constant interaction with the environment and are responsible for the passive protection of the internal organs. The bark comprises of the periderm, cortex, phloem and cambial tissues which together form the first line of defence to the entrance of most potential pathogens. Suberized phellem (outer periderm) is waterproof and highly resistant to enzyme attack and slow to decompose (Merrill, 1992). Only a few microorganisms are able to hydrolyse suberin, a hydrophobic polyester linked to a phenolic matrix and wax lamellae (Ofong & Pearce, 1994; Pearce, 1996). The bark also contains various constitutive antimicrobial compounds that include hydrolysable and condensed tannins, and polyphenols (Shain, 1995; Pearce, 1996).

Some constitutive proteins have also been implicated in protection of the bark from wood rotting fungi and some have been further associated with resistance. A proteinacous inhibitor of polygalacturonase (PG) was extracted from chestnut bark and was shown to inhibit PG produced by chestnut blight fungus (*Cryphonectria parasitica*) (Shain, 1995). Chitinase and β-1,3-glucanase were also reported to be constitutively expressed in healthy bark on oak and sugar maple trees (Wargo, 1975; Chun *et al*., 1999; Robinson *et al*., 2000). These enzymes could be involved in the hydrolysis of fungal cell wall components. Infection

by *E*. *lata* inoculated on the bark tissue (phloem) of apricot was inhibited and resulted in death of the pathogen soon after germination of the spores (English & Davis, 1978).

Normal wood is constituted of approximately 20-35% lignin, the structural component of wood that is highly resistant to biodegradation (Barber & Mitchell, 1997). Lignin protects woody tissue against pathogens, however, white rot Basidiomycota fungi can break down lignin. Lignin is a complex polymer of phenylpropanoids (lignans) formed by the polymerisation of three monolignols (hydroxycinnamyl alcohol units), *i.e*. 4-coumaryl, coniferyl and sinapyl (Boerjan *et al*., 2003; Ralph *et al*., 2004). Lignans are synthesised from the linkage of two monolignols and also have antimicrobial properties and are presumably plant defence compounds (Saleem *et al*., 2005). Lignin is found in the plant cell wall interwoven with hemicellulose, together forming a formidable structural and chemical barrier that is resistant to cell wall degrading enzymes. During infection, cell wall lignification establishes a mechanical barrier by rendering the cell more resistant to mechanical pressure applied by the penetrating fungi as well as limit diffusion of toxins from the fungi to the host and of nutrients from the host to the pathogen (Nicholson & Hammerschmidt, 1992).

Wounds create sites for pathogen infection, that are compromised in their ability to stop fungal infection, because the physical barriers are broken and the woody tissues are exposed. To preserve its integrity, the woody tissue has to be protected by active defence.

2.6.2 Active defence to wood infection

When the pathogen circumvents the passive defence mechanisms it becomes imperative that the plant detects the presence of the pathogen. Recognition of the pathogen triggers defence systems based on chemicals and cell wall barriers that confine the parasite in the infected cells and prevent further ingress. There are two main models proposed for the response of sapwood to fungi and/or decay namely, compartmentalisation of decay in trees (CODIT) and the reaction zone formation models. The CODIT model, proposed by Shigo and Marx (1977) and further established by Shigo (1984), describes the progression of wood discolouration and decay. The model is based on barriers, termed walls 1-4, that limit the spread of fungi. The first wall (wall 1) is formed by the plugging of vessels and tracheids and thus limits the axial spread of the fungi while wall 2 is an anatomical component that limits the radial spread by cell wall thickening. Wall 3 forms a boundary to lateral spread (perpendicular to the rays) and is formed by the ray parenchyma. Wall 4, also called the barrier zone is formed by the cambium at the time of wounding and limits the spread of infection into new wood tissue formed after wounding. The barrier wall is the strongest and most durable of the CODIT walls and unlike walls 1 to 3, which are formed with wood extant at the time of injury, wall 4 is formed only after wounding.

Shain (1967, 1971 & 1979) proposed the reaction zone model which can be equated to the CODIT model (Shigo, 1984) with the exception of the barrier zone (wall 4). The reaction zone is a zone of active host response found between infected and healthy wood. The reaction zone is non-specific and is continuously retreating ahead of the pathogen colonised wood (and hence similar to the CODIT walls 1-3). Central to both models is the development of physical and chemical barriers that inhibit or seal-off sound wood from colonisation. Extracts from the reaction zones are inhibitory to fungal growth (Shortle, 1979; Pearce & Woodward, 1986; Yamada, 2001), similar to extracts from similar zones in *Botrytis cinerea* and *Plasmopara* (*Pl*.) *viticola* infected leaves (Langcake, 1981; Dercks & Creasy, 1989; Pezet *et al*., 2004). The compounds in the reaction zones are induced as they are found at lower levels in sound wood and increase in quantity after fungal infection (Pearce, 1991; Yamada, 2001). Pearce (1996) argued that none of the models mentioned above is sufficient to fully describe the interaction between living sapwood and wood colonising fungi. However, these models together give an indication of the scale of interactions between the host and the pathogens in wood tissue.

2.6.3 Grapevine response to wound infection

Several studies have shown similarities in the response of grapevines to wound infection and those described in the CODIT and reaction zone models. Grapevines respond to wound infection by producing gums and tylosis that form bio-chemical and physical barriers to pathogen colonisation (Del Rio *et al*., 2001; Troccoli *et al*., 2001; Edwards *et al*., 2007; Mutawila *et al*., 2011). These are similar to the wall 1 of the CODIT model (Shigo, 1984). Strengthening of cell walls around the infected cells occurs through the deposition of lignin and pectin observed as thickening of secondary walls (Rudelle *et al*., 2005; Mutawila *et al*., 2011), similar to walls 2 and 3 of the CODIT model. Histochemical staining of infected wood tissue has also revealed that phenolic compounds accumulate in the tyloses, gels and the cells around (equivalence of the reaction zone of the Shain model) infected vessels (Amalfitano *et al*., 2000 & 2011; Del Rio *et al*., 2001; Troccoli *et al*., 2001). These compounds are phytoalexins and have been shown to inhibit the growth of Petri disease pathogens and *E*. *lata* as well as inhibit their cell wall degrading enzymes (Del Río *et al*., 2004; Santos *et al*., 2006).

2.6.3.1 Grapevine phytoalexins: Grapevine phytoalexins, are stilbene compounds whose synthesis is induced during pathogen infection (Dercks & Creasy, 1989). They are secondary metabolites produced from the phenylpropanoid pathway which also synthesise flavonoids and phenolic structural polymers, lignin and suberin (Barber & Mitchell, 1997; Hammerschmidt, 1999). Biosynthesis of these secondary metabolites involves the coordinated regulation of the general phenylpropanoid pathway as well as the flavonoid,

stilbene and lignin branch pathways. The phenylpropanoid pathway (Figure 3) begins with deamination of phenylalanine, an amino acid product of the Shikimate biosynthetic pathway, to cinnamic acid. This step, catalysed by phenylalanine ammonia-lyase (PAL), represents a switch from primary (Shikimate pathway) to secondary (phenylpropanoid pathway) metabolism (Barber & Mitchell, 1997). Lignin biosynthesis branches-off after the formation of hydroxycinnamic acid CoA esters which are reduced to their respective monolignols by the successive action of several enzymes that include cinnamoyl CoA reductase and cinnamyl alcohol dehydrogenase (Hahlbrock & Scheel, 1989). At the end of the general phenylpropanoid pathway, condensation of 4-coumaric acid and three molecules of malonyl-coenzyme A (malonyl-CoA) by either of the enzymes chalcone synthase and stilbene synthase, marks the branching point of the flavonoid and stilbene pathways, respectively (Hahlbrock & Scheel, 1989; Vannozzi *et al*., 2012). Stilbene synthase catalyses the formation of resveratrol, the precursor of stilbenes, while chalcone synthase forms chalcones, the precursors of flavonoid compounds.

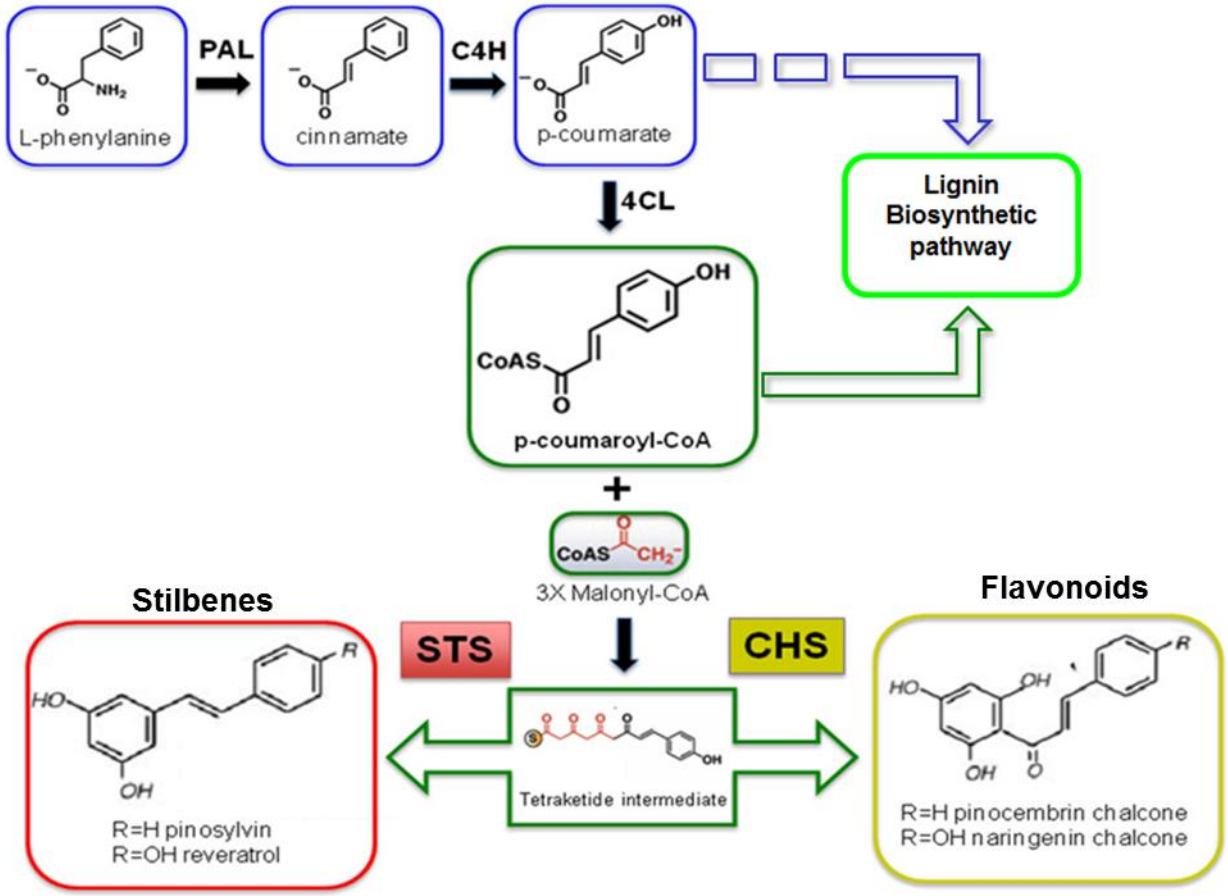

Figure 3: The general phenylpropanoid pathway showing the sub-branches to the synthesis of lignin, stilbenes and flavonoids. The enzymes catalysing each step of the general phenylpropanoid pathway are also shown: PAL, phenyl ammonium lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl CoA ligase; STS, stilbene synthase and CHS, chalcone synthase. (modified from Vannozzi *et al*., 2012).

2.6.3.2 Grapevine pathogenesis related proteins: Grapevines also respond to infection by synthesising numerous "pathogenesis-related (PR)" proteins which are low molecular weight (5-70 kDa) proteins that are characterised by their high solubility, stability at low pH, and high resistance to proteolysis (Linthorst, 1991). Most PR proteins have antimicrobial properties, through either their hydrolytic activities on the fungal cell walls or contact toxicity, or are involved in defence signalling that result in induced defence responses (Van Loon *et al*., 2006). Other defence mechanisms include enzyme inhibition as in the polygalactronase inhibiting proteins (PGIP), increased peroxidase activity and production of reactive oxygen species as well as accelerated cell death (in hypersensitive response). By their original definition, PR proteins are induced by biotic stress, however, some of them also accumulate during certain physiological processes and as a result of abiotic stress (Van Loon *et al*., 2006). Active defence can be observed at the sight of infection (localised acquired resistance) only or can also result in a systematic broad

spectrum resistance called systemic acquired resistance (SAR), observed at tissues distant from the site of infection (Van Loon *et al*., 1998; Durrant & Dong, 2004). Systemic resistance can also be induced by beneficial microorganisms or chemicals, which mimic the effect of pathogen infection or induce similar stress, through induction of a defence state known as induced systemic resistance (ISR) (Van Loon *et al*., 1998).

Several grapevine PR genes have been characterised which include, PR 1 (Bertsch *et al.*, 2003), β-1,3 glucanases (PR 2), chitinases (PR 3 and PR 4) (Kortekamp, 2006), thaumatin-like protein (PR 5) (Pocock *et al*., 2000) and a serine proteases inhibitor (PIN) belonging to the class of antifungal PR 6 proteins (Van Loon & Van Strien, 1999). Expression of PR proteins is selective and depends on the infecting pathogen or the elicitor (Glazebrook, 2005). Kortekamp (2006) inoculated grapevine cultivar Riesling with a non-host pathogen, *Pseudoperonospora* (*Ps.*) *cubensis* (downy mildew of cucumber) and found that PR 2, PR 4 and PGIP were induced and largely accumulated as compared to when inoculated the host pathogen *Pl. viticola*. When leaves were subsequently inoculated with *Pl. viticola*, after treatment with *Ps. cubensis* the severity of downy mildew was significantly reduced further demonstrating the importance of the PR genes expressed on inoculation with the non-host pathogen. The transcripts of genes encoding PR proteins accumulate in the leaves of plants infected with trunk disease pathogens. When grapevine plantlets were inoculated with *E. lata*, the expression of genes encoding PR proteins chitinases, glucanases, osmotins, thaumatins and polygalacturonase inhibitor proteins were strongly induced (Rotter *et al*., 2009). The same genes were also found to be overexpressed in leaves of grapevine plants expressing symptoms of *E. lata* compared to leaves from healthy plants (Camps *et al*., 2010). Letousey *et al.* (2010) also reported the increased expression of chitinase transcripts in leaves of esca diseased vines prior to the apoplectic symptom expression. Agüero *et al.* (2008) isolated some PR proteins from grapevine xylem sap, however, there is no information available regarding the presence of these molecules in the wood tissue harbouring trunk pathogens.

Resistance or susceptibility of a plant is determined by the speed and magnitude with which the plant activates and expresses their defence response as well as the effectiveness of the response mechanism on the pathogens. The slow development of grapevine trunk diseases, usually taking several years between infection and expression of foliar symptoms, suggests that the plant can delay the development of these pathogenic fungi (Troccoli *et al*., 2001; Larignon *et al*., 2009). There is currently limited knowledge on grapevine molecular response to trunk pathogens at the site of infection.

2.6.4 Grapevine cultivar resistance

There are no known cultivars resistant to grapevine trunk pathogens, however, many studies have shown cultivar variation in the expression of symptoms and severity of the diseases (Munkvold *et al*., 1994; Surico *et al*., 2000; Marchi, 2001; Sosnowski *et al*., 2007a). While environmental conditions, soil type and cultural practices also influence symptom expression, cultivar differences in symptom expression have been found (Mauro *et al*., 1988; Feliciano *et al*., 2004; Sosnowski *et al*., 2007b). Cultivars considered susceptible to Eutypa dieback include Cabernet Sauvignon, Cabernet Franc, Chenin blanc, Pinot noir, Riesling and Sauvignon blanc while cultivars Merlot, Sémillon and Barbera are considered tolerant (Mauro *et al*., 1988; Carter, 1991; Munkvold *et al*., 1994; Sosnowski *et al*., 2007a). The range of susceptibility to *E*. *lata* has been explained by the ability of cultivars to detoxify toxins (Mauro *et al*., 1988; Colrat *et al*., 1999). However, Rolshausen *et al.* (2008) showed that the wood tissue of Merlot, a tolerant cultivar, contained higher lignin content and hence could resist the pathogen colonisation better than susceptible Cabernet Sauvignon, which had lower lignin content. Similar data is lacking for the other trunk diseases, however, Cabernet Sauvignon, Chenin blanc and Sauvignon blanc are generally regarded as susceptible to Botryosphaeria canker while Sémillon and Merlot are considered tolerant (Larignon & Dubos, 2001). In Italy, Graniti *et al*. (2000) and Marchi (2001) reported that Sémillon, Cabernet Sauvignon and Sauvignon blanc were susceptible to esca while Merlot and Pinot noir were less susceptible.

2.7 Management of Grapevine trunk diseases

Since there are no eradicative control measures for infected vines, prophylactic treatments are the only effective way to control these diseases. There are three approaches to preventing wound infection by trunk pathogens and these are viticultural, chemical and biological control.

2.7.1 Viticultural practices

It is inevitable that wounds will be made on a grapevine as this is the only way to balance vegetative and reproductive growth of the vine. Cultural control aims at exploiting the biological knowledge of the wound and the pathogens to make viticultural decisions that will minimise infection or the impact of the trunk diseases.

When vineyards are established, pruning the minimum required will reduce the surface area exposed to possible infections. A double increase in wound diameter results in more than four times increase in the surface area of exposed wood (Figure 4). Wounds are equally susceptible to infection regardless of the age on the wood on which they are made (Trese *et al*., 1980; Munkvold & Marois, 1995; Úrbez-Torres & Gubler, 2010). However,

wounds on older wood have a larger surface area of exposed wood than the wounds on one-year-old canes. High numbers and large surface area of exposed pruning wounds have been positively correlated with severity of Eutypa dieback (Munkvold & Marois, 1995; Gu *et al*., 2005). The severity of pruning is determined by the vigour of the vine growth. Vines with more vegetative growth are pruned more severely which creates more and bigger wounds and a greater potential for pathogen infection (Gu *et al*., 2005). Excessive nutrition can result in increased vigour and more pruning wounds (Gu *et al*., 2005; Dumot *et al*., 2012). Training systems that require less extensive pruning are desirable.

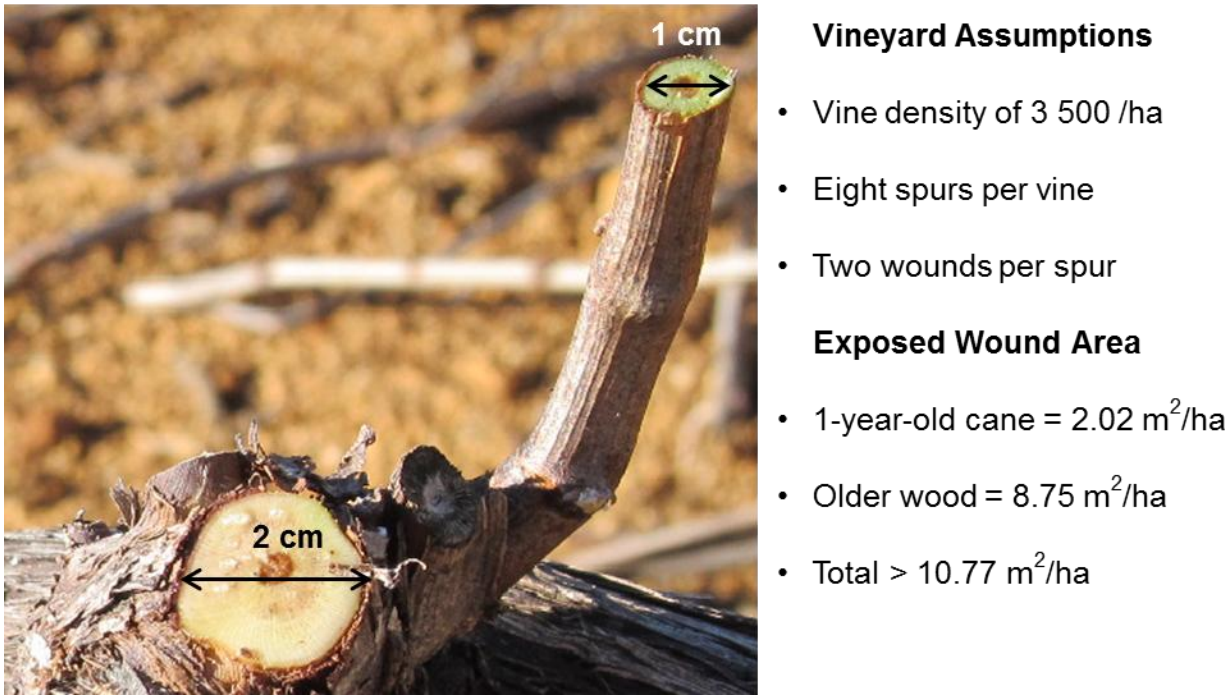

Figure 4: An estimation of the total area of exposed wood in a spur pruned vineyard with a vine density of 3 500 /ha (1.4 × 1.4 m) and a wound diameter of 1 cm and 2 cm on the 1-year-old cane and the 2-year-old cane, respectively. The surface area of exposed wood on pruning wounds increases by more than four times for every double increase in wound size.

The susceptibility of wounds to infection diminishes with time after pruning (Petzoldt *et al*., 1981; Munkvold & Marois, 1995; Eskalen *et al*., 2007; Van Niekerk *et al*., 2011). The duration of wound susceptibility varies with pruning time and is a function of the wound healing process, which involves the occlusion of exposed vessels, lignification and suberisation (Biggs, 1987; Biggs & Miles, 1988; Munkvold & Marois, 1995; Sun *et al*., 2006). The healing process has been found to be faster at higher temperatures than at low temperatures due to increased rate of suberisation and thus reduced duration of wound

susceptibility (Biggs, 1990; Munkvold & Marois, 1995). Wounds remain susceptible for up to 6 months but the critical time for infection ranges from two to eight weeks (Munkvold & Marois, 1995; Chapuis *et al*., 1998; Eskalen *et al*., 2007; Van Niekerk *et al*., 2011). Munkvold & Marois (1995) further found an increased rate of natural wound colonisation by saprophytes which protecting wounds from infection when temperatures are higher. These factors together with the reduced availability of pathogen inoculum, form the basis for the recommended practice of pruning grapevines late in winter when temperatures begin to rise as it reduces trunk pathogen infection as compared to wounds made early or in the middle of the winter season (Petzoldt *et al*., 1981; Munkvold & Marois, 1995; Chapuis *et al*., 1998). Epidemiology studies from many regions have also shown that spores of *E*. *lata* (Ramos *et al*., 1975; Pearson, 1980; Trese *et al*., 1980; Carter, 1991), Botryospheraiaceae species (Úrbez-Torres *et al*., 2010; Van Niekerk *et al*., 2010) and *Pa. chlamydospora* (Eskalen & Gubler, 2002; Rooney-Latham *et al*., 2005a) are released during or after rainfall events and hence it is recommended to avoid pruning during wet weather.

Surico *et al*. (2008) recommended that in areas that experience severe winters, pruning should be delayed as much as possible until wounds can heal faster. In regions where winters are mild, pruning should be done as early as possible so that wounds heal before infection by newly disseminated spores. Double pruning, which involves a first non-selective pruning to ~30 cm above the spur positions early in the dormant season, followed by the traditional pruning to two/three buds later in winter has been found to be effective in reducing *Eutypa lata* infection in California (Weber *et al*., 2007). This practice is recommended for large vineyards where labour costs for pruning are high and mechanical pruning or unskilled staff would carry out the first indiscriminate pruning while the skilled pruners would carry out the final pruning faster. Double pruning also holds the advantage that initial infections are pruned away. Weber *et al*. (2007) demonstrated that *E*. *lata* inoculated on wounds made in the first pruning was eliminated in the second pruning. Pathogen infection from natural inoculum was also higher in the wounds made in the first pruning (early winter) compared to the second pruning (late winter) further proving that wounds made in the late winter are less susceptible to infection. However, it is important to note that double pruning does not provide any additional advantages if the normal pruning was to be done once late in winter.

Vineyard sanitation is also important for the reduction of inoculum. Infected wood or cankers in the vineyard and adjacent orchards should be removed and destroyed. The vineyards should be monitored during the vegetative season to identify and record all diseased vines which should be eliminated as they may be sources of inoculum. In order to replace dead arms/cordons, remedial surgery (also called trunk renewal) is carried out in

which the infected cordons are removed and disease free shoots trained up, to replace the removed sections (Di Marco *et al*., 2000; Calzarano *et al*., 2004; Sosnowski *et al*., 2004 & 2011). The effectiveness of the method is dependent on removal of all the infected parts and that the shoots used for training are not infected. However, using the discolored wood to determine depth of infection is unreliable as the pathogen grows further than the stained region (Di Marco *et al*., 2000; Sosnowski *et al*., 2007a) and hence it is recommended to remove at least 10 cm of healthy wood after the stain (Sosnowski *et al*., 2011b). This technique has been reported mainly for Eutypa dieback but can equally be applied for other trunk diseases.

Cultural practices cannot guarantee wound protection and pruning wound protection is still needed. Pathogen inoculum can be reduced but cannot be completely eliminated and is always available in the vineyards. All wounds need to be protected as they are equally susceptible to infection regardless of the age of the wood on which they are made (Munkvold & Marois, 1995; Úrbez-Torres & Gubler, 2010).

2.7.2 Chemical wound protection

Chemical control is based on the use of fungicides or physical barriers (paints and pastes) to protect the wound from infection. Sodium arsenite is considered to have been the best fungicide used for the control of esca as it had wound protective effect and also reduced disease severity in infected vines or delayed symptom expression (Mugnai *et al*., 1999; Surico *et al*., 2008). Sodium arsenite was banned due its environmental and human toxicity (Fussler *et al*., 2008) and since then, there have been numerous studies carried out to find alternative fungicides. *In vitro* tests have shown the efficacy of several fungicides in inhibiting the growth of trunk pathogens (Jaspers, 2001; Bester *et al*., 2007; Halleen *et al*., 2010; Gramaje *et al*., 2011; Amponsah *et al*., 2012; Pitt *et al*., 2012) while some have further been shown to be effective wound protectants in the field (Sosnowski *et al*., 2008; Halleen *et al*., 2010; Rolshausen *et al*., 2010; Amponsah *et al*., 2012; Pitt *et al*., 2012; Díaz & Latorre, 2013). However, there are few fungicides specifically registered for grapevine pruning wound protection while several paints and pastes are generally registered for pruning wounds on all woody species.

Benomyl was found to be effective against *E*. *lata* dieback and was used in pruning wound protection in the USA until 2001 (Munkvold & Marois, 1993a; Rolshausen *et al*., 2010). The potential of benomyl in pruning wound protection against *E*. *lata* was first shown on apricot (Moller & Carter, 1969) and was later adopted on grapevine (Moller & Kasimatis, 1980) when it was shown that the same canker pathogen on apricot infects grapevine (Moller & Kasimatis, 1978). Several studies have confirmed the effectiveness of benomyl in

preventing grapevine infection by *E. lata* (Pearson, 1982; Gendloff *et al*., 1983; Munkvold & Marois, 1993a; Sosnowski *et al*., 2008; Halleen *et al*., 2010). Other benzimidazoles namely, carbendazim (Sosnowski *et al*., 2008) and thiophanate methyl (Rolshausen *et al*., 2010) were also found to be effective in pruning wound protection against *E. lata*. Thiophanate methyl and carbendazim have further been shown to be effective in wound protection against species of Botryosphaeriaceae (Rolshausen *et al*., 2010; Amponsah *et al*., 2012; Pitt *et al*., 2012; Díaz & Latorre, 2013), *Pa. chlamydospora* and *Phaeoacremonium* spp. (Rolshausen *et al*., 2010; Díaz & Latorre, 2013) and the white wood rot fungus, *Inocutis* sp. (Díaz & Latorre, 2013). Benomyl and carbendazim are no-longer available in USA, Europe and Australia (Rolshausen *et al*., 2010; Pitt *et al*., 2012), however, they remain available and are registered for the control of botrytis bunch-rot on grapevine in South Africa, New Zealand and Chile (Halleen *et al*., 2010; Amponsah *et al*., 2012; Díaz & Latorre, 2013). Other fungicides shown to also be effective in wound protection include demethylation inhibitors, flusilazole and tebuconazole (Rolshausen *et al*., 2008; Sosnowski *et al*., 2008; Pitt *et al*., 2012; Díaz & Latorre, 2013).

Pruning wound paints/pastes are a popular choice for pruning wound protection on various woody species but their efficacy is debatable (Shigo & Shortle, 1983; Spiers & Brewster, 1997; Hudler & Jensen-Tracy, 2002; Van Niekerk *et al*., 2011). Several studies have shown that some wound sealants interfere with wound cicatrisation (healing) making the wound susceptible for longer periods. Furthermore, exposure to ultraviolet (UV)-light and the shrinking of dead wood under the paint creates cracks that collect moisture and create an ideal environment for fungal growth (Shigo & Shortle, 1983; Spiers & Brewster, 1997; Hudler & Jensen-Tracy, 2002). In a study of grapevine pruning wound susceptibility to natural air-borne inoculum, wounds treated with a non-fungicidal bitumen based acrylic emulsion, Tree Seal Pruning Grade, had higher levels of pathogen infection than untreated wounds (Van Niekerk *et al*., 2011). Ideal wound sealants should therefore be breathable to allow natural wound cicatrisation, have high elasticity and resistance to UV-light to reduce likelihood of cracking and also contain fungicides (Spiers & Brewster, 1997; Epstein *et al*., 2008).

Pruning wound paints with fungicides that have been tested for grapevine wound protection are Gelseal and Greenseal which contain tebuconazole, Garrison which contains cyproconazole and iodocarb, Bioshield and Biopaste which contain boric acid (Sosnowski *et al*., 2008; Rolshausen & Gubler, 2005; Rolshausen *et al*., 2010; Pitt *et al*., 2012). Garrison, was developed for pruning wound protection of fruit trees from *Chondrostereum purpureum*, the causal agent of silver leaf. Several studies have shown Garrison to be effective in protecting grapevine pruning wounds from infection by most trunk pathogens (Sosnowski *et*

al., 2008; Rolshausen *et al*., 2010; Pitt *et al*., 2012). Boric acid combined with a pruning wound paste provided antifungal activity and reduced pruning wound infection by *E*. *lata*, however, when applied at high concentrations the boric acid was phytotoxic and caused mortality of apical bud/shoots (Rolshausen & Gubler, 2005). The efficacy of 5% boric acid has further been confirmed against *E*. *lata* (Sosnowski *et al*., 2008), Botryosphaeriaceae species, *Pa. chlamydospora* and *Phaeoacremonium* spp. (Rolshausen *et al*., 2010). The application of pruning wound paints on all wounds in a vineyard may not be economically viable due to labour costs involved and so are mainly applied on larger wounds such as those made on the trunk or cordons on re-working the vine.

2.7.3 Biological wound protection

Fresh pruning wounds are colonised by saprophytic fungi and bacteria some of which can prevent infection by pathogens (Carter & Price 1974; Petzoldt *et al*., 1981; Munkvold & Marois, 1993b & 1995; Chapuis *et al*., 1998). Several wound colonising bacteria and fungi are antagonistic to grapevine trunk pathogens *in vitro* and these include *Bacillus subtilis* (Ferreira *et al.*, 1991), *Erwinia carotovora* (Schmidt *et al.*, 2001), *Cladosporium* (*Cl*.) *herbarum* (Munkvold & Marois, 1993b), *Fusarium lateritium* (Carter, 1971) and *Trichoderma* species (Munkvold & Marois, 1993b; John *et al*., 2004). Since the saprophytes grow on the wound, they are likely to provide protection until the wound is fully healed and no-longer susceptible to infection. The protective activity of the wound saprophytes can be attributed to direct antagonism, occupation of space and/or also activation of plant defence. Antibiotic secondary metabolites were isolated from *F. lateritium* (Carter & Price, 1974; Munkvold *et al*., 1993b), *B*. *subtilis* (Ferreira *et al*., 1991) and *T*. *harzianum* (John *et al*., 2004), but none of these metabolites have been identified. Munkvold & Marois (1993b) attributed wound protection by *Cl*. *herbarum* to its fast colonisation of the wound and the production of hydrophobic conidia that provide a physical barrier to pathogen spores carried in water droplets. Of the potential wound saprophytes found to protect grapevine pruning wounds, only *Trichoderma* spp. have been commercially developed for grapevine pruning wound protection, which is most likely due to ease of large scale production of these fungi.

Trichoderma spp. are among the most studied biocontrol fungi and have been developed into commercial products for biological control of plant pathogens (Chet, 1987; Harman, 2000 & 2004; Vinale *et al*., 2008a). The mechanisms of action of *Trichoderma* spp. include mycoparasitism, secretion of mycolitic enzymes, competition for limiting resources (Harman *et al*., 1993; Haran *et al*., 1995; Howell, 2006), production of antibiotic metabolites (Sivasithamparam & Ghisalberti, 1998; Vinale *et al*., 2006) as well as induction of plant resistance (Gallou *et al*., 2008; Vinale *et al*., 2008b). *Trichoderma* species are predominantly soil inhabitants and hence, are used mostly for the control of soil-borne pathogens of root

diseases. However, *Trichoderma* spp. have also been shown to protect pruning wounds against wood rot fungi. Grosclaude *et al.* (1973) demonstrated that *T. viride* could completely protect wounds of plum trees from infection by *C. purpureum* while Woodgate-Jones & Hunter (1983) further reported the curative effect of *T. viride* on trees expressing silver leaf symptoms. Highely (1997) found that *T. virens* could only protect infection of maple and pine by various white rot and brown fungi, but could not stop decay once the pathogen was established, although it reduced the rate of wood decay. Similar results were also obtained on the protection of pruning wound on urban trees by *T. atroviride* where the biocontrol agent was more effective against soft rot (Ascomycota) fungi than white rot fungi (Basidiomycota) (Schubert *et al*., 2008).

In grapevine, *Trichoderma* spp. have been shown to protect pruning wounds from infection by *E. lata* (Munkvold & Marois, 1993b; Hunt *et al*., 2001; John *et al*., 2005 & 2008). Wound protection from other trunk pathogens has also been demonstrated in nurseries and vineyards. Di Marco *et al*. (2004) showed the protection of pruning wounds and nursery plants from infection by *Pa. chlamydospora* and reduction of wood streaking caused by the pathogen in infected canes. Similar findings were reported from nursery plants treated with *T. harzianum* in South Africa (Fourie & Halleen, 2004 & 2006). Kotze *et al*. (2011) tested several strains of *Trichoderma* spp. and found that the efficacy of two isolates of *T. atroviride,* UST1 and UST2, was either similar or superior to that of benomyl when pathogens were inoculated 7 days after pruning. However, several reports have shown variable control with *Trichoderma* spp. when the pathogens are inoculated soon after application of the biocontrol agent (John *et al*., 2005; Halleen *et al*., 2010; Pitt *et al*., 2012).

Since biocontrol efficacy is dependent on establishment of the biocontrol agent on the wound, the time that is required for the biocontrol agent to colonise the wound creates a window of infection. Carter & Price (1975) took advantage of the tolerance of *F. lateritium* to benomyl and applied a mixture of the biocontrol agent and the fungicide on pruning wounds of apricot that were immediately inoculated with *E. lata*. The combination of *F. lateritium* and benomyl resulted in the least infection although this did not significantly differ from the treatments applied separately. However, since environmental conditions on the wound are not always optimal for colonisation by the biocontrol agents, integration of chemical and bio-protection is likely to provide more consistent and long term wound protection. In California, a mixture of boric acid and *Cl. herbarum* (Bioshield) provided wound protection against most of the trunk pathogens (Rolshausen & Gubler, 2005). The mixture gave better wound protection than the biocontrol agent alone but protection did not significantly differ from the boric acid alone.

2.7.4 Management of infected vines

Several fungicides have been tested for their curative effect with varying results. Injection of triazole fungicides, propiconazole and difenoconazole, and an elicitor of plant resistance, 2-hydroxybenzoic acid, into trunks of grapevines showing Eutypa dieback and esca symptoms, did not have any effect on disease development (Darrieutort & Lecomte, 2007). Application of copper oxychloride in the vineyard after harvest, at pruning and when shoots were 8-10 cm reduced the foliar symptoms of esca but had no effect on wood colonization by *Pa. chlamydospora* (Di Marco *et al*., 2011b). *In vitro* tests with copper oxychloride inhibited the production of scytalone by *Pm. aleophilum*, which explained the reduction of foliar symptoms on grapevines (Di Marco *et al*., 2011b). When remedial surgery was used for trunk renewal of esca diseased vines, the application of fungicides cyproconazole, tetraconazole and fosetyl-Aluminium by trunk injection reduced the recurrence of esca in the new shoots (Calzarano *et al.*, 2004).

The application of Brotomax, an enhancer of phenolic compounds, as a foliar spray and drench was reported to stimulate growth and reduce Petri disease symptoms in infected nursery plants (Del Río *et al*., 2004). The injection of formulations of fosetyl-aluminium resulted in reduction of wood necrosis caused by *Pa. chlamydospora* and *Pm. aleophilum* on potted vines and reduced symptom expression and death of vines due to esca in field trials (Di Marco *et al*., 2011a).

An ancient custom still practiced today in some European countries is believed to delay the recurrence of esca foliar symptoms. This involves opening the trunks, of vines expressing symptoms, in the middle and inserting a stone so as to expose the rotted wood to the air (Surico *et al*., 2008). The real effect of the practice has not been scientifically proven.

Management of infected vines aims at maintaining vineyard productivity, however, the costs involved may be a deterrent to adoption of these practices. Applying fungicides or growth stimulants by trunk injection is expensive and may only be economically feasible in high value vineyards. Effective fungicides or biostimulants that can be applied by cheaper methods are more desirable.

2.8 Conclusions

Grapevine trunk diseases are increasingly becoming an important threat to the sustainability of viticulture and the industries that depend on it. In the past decade, there has been considerable gain in the knowledge of the etiology, symptomatology and epidemiology of grapevine trunk diseases. Knowledge on host-pathogen interactions is limited and could answer some important questions such as, the delayed and erratic expression of symptoms as well as the occurrence of latent pathogen infection. Grapevine trunk pathogens inhabit

mature wood tissue and so studies on host-pathogen interaction should target response of the wood tissue to the pathogen.

Management of grapevine trunk diseases currently relies on sanitation and the employment of biological and chemical wound protection. Neither of these methods is alone sufficient for complete and long term wound protection but their integration is likely to provide better protection. Cure of infected vines is still yet to be achieved even at experimental level. With the continued loss of effective fungicides due to environmental and/or human toxicity, wound bio-protection seems to be the only method that is currently available that is likely to remain available in the long term. There exists, therefore, a challenge to improve the efficacy of biocontrol agents while also looking at ways to enhance the plant's own defence mechanisms against the pathogens.

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Chapter 3

Optimisation of time of application of *Trichoderma* biocontrol agents for grapevine pruning wound protection

3.1 Abstract

The protection of grapevine pruning wounds is a very important practice as these are entries for wood infection by trunk pathogens that cause grapevine decline. The application of *Trichoderma* species on wound surfaces reduces wound infection and several wound protection products based on *Trichoderma* spp. have been registered. The effect of pruning time (early or late) and five timings of application of the biocontrol agent after pruning (0, 6, 24, 48 and 96 hours), on grapevine pruning wound colonisation by *Trichoderma*-based pruning wound protectants were determined. Field trials were carried out over two seasons on two wine-grape cultivars Chenin blanc and Cabernet Sauvignon which are normally pruned early and late in the winter season, respectively, in South Africa. These pruning times correspond with the time these cultivars break from the winter dormancy period. Colonisation of grapevine pruning wounds by the *Trichoderma* spp. was dependent on the physiological state of the vines as well as the weather conditions at pruning. In dormant vines colonisation remained high from immediate application (0 hours) up to 48 hours after pruning. In vines at break of dormancy colonisation was highest at 6 and 24 hours after application. Applying the biocontrol agent 6 hours after pruning consistently resulted in high incidences of *Trichoderma* spp. in both cultivars at either early or late pruning regardless of vine physiological state or the weather conditions. It was also found that, in the South African Stellenbosch wine region, wound infection from natural inoculum is higher in the late winter compared to the early winter. The implications of these findings on trunk disease management are discussed.

3.2 Introduction

The pruning of grapevines is a critical viticultural practice that is carried out every winter to maintain balance between vegetative and reproductive growth. Pruning wounds made during this process are important entry ports of infection for trunk disease pathogens that cause premature grapevine decline and loss of productivity. Grapevine trunk diseases are responsible for graft failure, loss of vigour and productivity in established vines, wood cankers, spots on berries, late ripening and altered flavour, as well as death of spurs and/or the entire vines (Mugnai *et al*., 1999; Pascoe & Cottral, 2000; Surico *et al*., 2008). These diseases are responsible for substantial economic losses and have become an important threat to the sustainability of viticulture and wine industries worldwide.

Eutypa dieback, caused by *Eutypa* (*E*.) *lata*, was once considered to be the most important grapevine trunk disease (Munkvold *et al*., 1994; Gubler *et al*., 2005). However, more fungi are currently known to cause grapevine decline and are important pathogens in the grapevine trunk disease complex. Petri disease is caused by *Phaeomoniella* (*Pa.*) *chlamydospora* and *Phaeoacremonium* species (Crous *et al*., 1996; Mostert *et al.*, 2006). Wood rotting basidiomycete fungi, of which *Fomitiporia* species are the most prevalent cause esca (Fischer, 2006). Basidiomycetes often co-occur with Petri disease fungi resulting in both wood rot and leaf-stripe (also called tiger-stripe) symptoms and hence this condition has also been referred to as esca (Surico *et al*., 2008). Botryosphaeria dieback is caused by several species within the Botryosphaeriaceae family, namely species of *Botryosphaeria*, *Neofusicoccum*, *Lasiodiplodia* and *Diplodia* (Van Niekerk *et al*., 2004; Úrbez-Torres *et al*., 2006). *Phomopsis viticola*, often associated with grapevine cane and leaf spot, and other *Phomopsis* spp. are also involved in grapevine dieback known as Phomopsis dieback (Van Niekerk *et al*., 2005). These diseases simultaneously occur in all grapevine producing areas although severity of the specific diseases may differ among regions (Mugnai *et al.*, 1999; Pascoe & Cottral, 2000; Halleen *et al*., 2003; Gubler *et al*., 2005).

Infection by trunk pathogens may occur through any wound, but pruning wounds are the principal ports of entry (Chapuis *et al*., 1998; Van Niekerk *et al*., 2011). Grapevine trunk diseases may also originate from the nursery where infection occurs through the propagation process (Fourie & Halleen, 2006; Gramaje & Armengol, 2011). Nursery infections occur mainly from the use of infected propagation material originating from mother plants infected through wounds in the vineyard (Halleen *et al*., 2003; Aroca *et al.,* 2010). The pruning season (grapevine dormant period) coincides with the period of pathogen spore release which usually originates from infected wood from previous seasons. Wounds may remain susceptible for a very long time but the most critical time for infection ranges from two to eight weeks (Eskalen *et al.*, 2007; Van Niekerk *et al*., 2011) and therefore it is inevitable that unprotected wounds will become infected. There are currently no eradicative control measures to cure infected vines except removal of infected vines or parts of infected vines (remedial surgery) (Sosnowski *et al*., 2011b). Due to the incremental effect of the diseases, by the time symptoms are observed there is not much that can be done to save the vine without losing production. Therefore the major way of managing trunk diseases in field grapevines is to prevent pathogen entry through pruning wounds.

Wound protectants should be effective against the whole range of trunk pathogens while also protecting the wound for the whole period of wound susceptibility. Wound dressings using acrylic based paints, contaiing or without fungicides are a popular practice, however, their efficacy is debatable (Van Niekerk *et al*., 2011). Several fungicides have been

found to be effective, but such protection is short lived and no one fungicide is equally effective against the whole suite of trunk pathogens (Bester *et al*., 2007; Sosnowski *et al*., 2008; Rolshausen *et al*., 2010). Sodium arsenite was considered the most effective fungicide against esca pathogens, having protective effect as well as delaying the onset of foliar symptoms (Surico *et al*., 2008), but was banned due to environmental and human toxicity. There are few or no fungicides registered for pruning wound protection in grapevine producing countries while often biological control agents are available, most of which are based on the fungal genus *Trichoderma* (*T*.). For some producers following 'biological/organic agriculture,' biological control (biocontrol) is their main option for disease control.

Pruning wounds are colonised by natural fungi and bacteria, some of which prevent trunk disease infection, particularly when pathogen infection does not occur immediately (Munkvold & Marois, 1995). *Trichoderma* species have shown success in pruning wound protection (John *et al.*, 2005; Kotze *et al*., 2011). Kotze *et al*. (2011) found fungicides to be less effective in pruning wound protection as compared to *Trichoderma* spp. treatments when wounds were challenged with the pathogen seven days after application. The main advantage of using biological control pruning wound protection is in the long term protection provided by the fungus growing in the pruning wound.

The success of bio-protection is dependent on growth and establishment of the fungi on the wound and this can be affected by several factors. Antagonism and competitive exclusion of the pathogens by the biocontrol agent, as is seen *in vitro*, are usually considered the mechanisms of action of the biocontrol agent. However, plant-biocontrol agent interactions have also been found to be more important in some pathosystems (Elad *et al*., 1996; De Meyer *et al*., 1998). The physico-chemical properties of the environment in which the biocontrol agent is to grow as well as the response of the plant to the growth of the biocontrol agent may affect their establishment and efficacy. Grapevine cultivar variation to wound protection by *Trichoderma* spp. has been reported, but cultivar differences could not be exclusively ascribed to cultivar-*Trichoderma* interactions (Mutawila *et al*., 2011a).

When grapevines are pruned, the various cultivars will be at different physiological states (active or dormant) which could have an effect on *Trichoderma* establishment and the resulting control. Cultivars are pruned early, middle or late in the dormant season according to the specific cultivar's requirement as well as to stagger the pruning work load over a reasonable period of time. Delaying pruning until just before natural bud break, results in more uniform shoot growth and also provides better protection from frost damage (Martin & Dunn, 2000; May, 2004). Wounds made in late winter also heal faster, providing only a small window of infection and hence the recommendation to prune late (Munkvold & Marois,

1995). However, delayed pruning may lead to bleeding due to sap-flow which may wash away any pruning wound protectants applied.

Studies on biocontrol of plant pathogens focus largely on the biocontrol agent-pathogen interactions and less on the effects the biocontrol-plant interactions may have on the resulting biocontrol efficacy. In grapevine wound protection, colonisation of the wound by the biocontrol agent is requisite for control. Wound colonisation may be affected by the intrinsic properties of the wound, which vary with the grapevine physiological state. The current study was carried out to determine the effect of pruning time (early or late) and the time of wound treatment after pruning (0, 6, 24, 48 and 96 hours), on wound colonisation by *Trichoderma* biocontrol agents. Trials were carried out on two cultivars, Chenin blanc (an early pruned cultivar) and Cabernet Sauvignon (a late pruned cultivar) in an effort to ascertain the best time to apply *Trichoderma* biocontrol agents.

3.3 Materials and methods

3.3.1 Fungal isolates and inoculum preparation

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.

3.3.2 Effect of pruning time and time of application on wound colonisation

***3.3.2.1 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).

***3.3.2.2 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.

***3.3.2.3 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).

3.3.2.3 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.

3.3.4 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.

3.4 Results

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%).

3.4.1 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 ($P < 0.001$; Appendix A, Table 1) 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 1. 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 2).

3.4.2 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 ($P < 0.001$; Appendix A, Tables 2 and 3) 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).

3.4.2.1 Incidence of biocontrol agent: The *Trichoderma* incidences in each cultivar and for each treatment and for the different pruning times are shown in figure 1. 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 (Figure 1). 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*).

3.4.2.2 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 ($P < 0.05$; Appendix A, Tables 4 and 5) in the isolation frequencies of *Trichoderma* spp. in both cultivars and trials. The mean isolation frequencies of *Trichoderma* spp. in each cultivar and for each treatment and for the different pruning times are shown in figure 2.

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 (Figure 2), a detail that could not be perceived from the incidence data (Figure 1). 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.

3.4.3 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 ($P = 0.01$; Appendix A, Table 6) and hence analysis of variance for each cultivar and year was carried out separately.

3.4.3.1 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 3). 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.

3.4.4 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 ($P > 0.05$; Appendix A, Table 7 and 8) 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 ($P < 0.05$; Appendix A, Tables 7 and 8) 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 (Figure 3). 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).

3.4.5 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.

3.5 Discussion

The current study confirms the protective effects of the *Trichoderma* species used in this study on grapevine pruning wound as reported by Halleen *et al*. (2010) and Kotze *et al*. (2011). The major mechanisms of action are antibiosis, mycoparasitism and competitive

exclusion and have been characterised both *in-vitro* and *in-vivo* (Kotze *et al*., 2011; Mutawila *et al*., 2011b). The efficacy of biocontrol agents at commercial level is considered moderate which has led to limited adoption despite their increased availability. One of the major setbacks to the adoption of biocontrol agents has been their inconsistency and poor disease control in the field. Many potential biocontrol agents have been dropped once they reach the field trial level of testing (Butt *et al*., 2001). These difficulties derive not so much from the lack of activity of the biocontrol agents in the field as from the existence of unknown host and abiotic factors which must concur for successful pathogen control. The present study showed that there are cultivar differences to the colonisation of grapevine pruning wounds by *Trichoderma* species in the field. These cultivar differences are compounded by seasonal variability to the colonisation of pruning wounds by the biocontrol agents. Cultivar differences could be a result of the physiological state of the vine at the time of pruning, which determines how the grapevine responds to the growth of the biocontrol agent on the wound.

Pruning time had opposite effects to the colonisation of the two cultivars by the *Trichoderma* spp., the Chenin blanc having higher incidence and colonisation extent in the late pruning while the Cabernet Sauvignon had better colonisation following early pruning. The pruning times, mid-July and mid-August, used in these trials are normal pruning times practised in the Western Cape wine region respectively, for Chenin blanc which breaks dormancy earlier, and Cabernet Sauvignon which breaks dormancy later. The grapevine responds to pruning by cicatrisation to seal off the exposed wood. This process involves suberisation, lignification and occlusion of exposed vessels by the formation of pectin gels (or gum) and tylosis (Sun *et al.*, 2008; Mutawila *et al*., 2011b). Vines in the dormant state are metabolically inactive and so these wound healing processes occur at a slower rate which could be the reason for better colonisation by *Trichoderma* spp. in the dormant Cabernet Sauvignon. On the contrary, Chenin blanc had higher colonisation in the late pruning, when the vines are metabolically active, compared to the early pruning when the vines were breaking dormancy. This difference in wound colonisation by *Trichoderma* spp. between the Chenin blanc and the Cabernet Sauvignon confirms cultivar variation to pruning wound protection by *Trichoderma* spp. (Mutawila *et al*., 2011a) and could also explain the inconsistency of *Trichoderma* wound protection in previous studies (Halleen *et al*., 2010; Larignon, 2010). The variation between cultivars can be explained by intrinsic wood properties such as nutrient content and availability and pH (Ferreira, 1999; Bates *et al*., 2002; Weyand & Schultz, 2006; Holzapfel *et al*., 2010), and the vine's defence mechanisms which differ between cultivars. These wound properties would be expected to be expressed more in metabolically active grapevines than in dormant vines and hence the higher *Trichoderma* spp. incidences in July than in August for Cabernet Sauvignon. The content of

nitrogen and non-structural carbohydrates in grapevine canes have been shown to be highest during dormancy, but get depleted to their lowest during budburst and initial shoot development (Bates *et al*., 2002; Weyand & Schultz, 2006; Holzapfel *et al*., 2010).

The colonisation of the Chenin blanc pruning wounds by the *Trichoderma* species in the first year of the trial (2011) followed a similar pattern as that reported for pathogen infection in the same cultivar and area. In a study of temporal susceptibility of grapevine pruning wounds to trunk disease pathogens, Van Niekerk *et al*. (2011), reported higher pathogen infection occurred in August inoculated wounds than in July inoculated wounds. This was attributed to more rainfall in August than July in the Stellenbosch area. High rainfall could also be the reason for similarly high incidences of *Trichoderma* spp. in the July and August pruning of 2012 in the Chenin blanc. However, in the Cabernet Sauvignon, the increased rainfall in August of 2012 had no effect on pruning wound colonisation by the *Trichoderma* spp. This shows that, while weather conditions have a definite effect on wound colonisation, pathogen spore/conidia availability and dispersal patterns in the vineyard, the wound properties also play an important role in colonisation.

Vascular bleeding could also be a contributing factor to the lower incidence of *Trichoderma* spp. in Chenin blanc in July of 2011 and Cabernet Sauvignon in August of both trial years by washing off *Trichoderma* spp. conidia from the wounds particularly when the pruning wounds received treatment immediately (Harvey & Hunt, 2006). During dormancy, vascular bleeding may occur when there is excess water in the soil for the root system. However, when pruning is carried out in late winter or early spring (before or at bud-break), bleeding occurs due to positive root pressure as water and reserves are mobilised to initiate and sustain the growth of new shoots (Winkler *et al*., 1974; Mullins *et al*., 1992). Breaking of dormancy is dependent on ambient and soil temperature, rootstock genotype as well as cultural practices such as winter pruning. Most of all, the grapevine cultivar (genotype) determines when budburst occurs irrespective of the climatic conditions. To the best of our knowledge there are currently no reports on the effect of vine physiological state on wound colonisation by biocontrol agents. However, such studies on pathogen infection have shown that wounds made late in the winter (or early spring) are less susceptible to infection by grapevine trunk pathogens due to faster healing (or cicatrizing) by the active vine as well as the faster growth of saprophytes on the wound in warmer weather (Munkvold & Marois, 1995; Chapuis *et al*., 1998; Úrbez-Torres & Gubler, 2011).

Most of the previous studies on grapevine pruning wound bio-protection applied wound treatments shortly after pruning with varying results (John *et al*., 2005; Halleen *et al*., 2010; Kotze *et al*., 2011; Mutawila *et al*., 2011a). Label instructions on most *Trichoderma* spp. based pruning wound protection products recommend application of the wound treatment

shortly after pruning. This stems from the need to protect the wound from as soon as it can be infected. However, the current study shows that immediate application of *Trichoderma* on the wounds does not necessarily result in effective wound colonisation by the biocontrol agent and hence may result in unpredictable or inconsistent wound protection. Waiting a few hours (6 and 24 hours in this study) after pruning could result in better wound colonisation by *Trichoderma* spp. and better wound protection. Harvey & Hunt (2006) also found that pruning wounds treated with *T. harzianum* (in Vinevax™) 4 hours or 24 hours after pruning had the highest incidence of the biocontrol agent.

The current study further showed that waiting several hours after pruning to apply the pruning wound bio-protectant may result in consistent wound colonisation regardless of the pruning season. Although the 6 hour application did not always have the highest *Trichoderma* incidence, it consistently resulted in high incidences and more importantly the extent of colonisation (isolation frequency) was always high. This finding is significant for achieving more predictable and less variable control in grapevine pruning wound protection using biocontrol agents based on *Trichoderma* spp. The lower incidences and wound colonisation by *Trichoderma* spp. after 24 hours is due to the wound healing process and are an indication of a short window period for the application of the biocontrol agent. Although it has been shown that the wounds may remain susceptible to pathogen infection for longer than 16 weeks (Eskalen *et al*., 2007; Van Niekerk *et al*., 2011) the biocontrol agent should be applied within 24 hours of pruning or several hours after pruning for the best and more consistent results. Furthermore, *Trichoderma* species have only been shown to have pruning wound protective effect and curative effects have not been proven or demonstrated. The longer the time between pruning and wound treatment, the more likely the wound will be infected by trunk pathogens. In the first several hours after pruning, before wound treatment, sap bleeding is also likely to wash off most pathogen propagules that land on the wound.

Decisions on the timing of pruning depend on viticultural, disease management and economic reasons. Several studies have shown that pruning late in the dormant season results in more even shoot growth along the cane or cordon due to reduced apical dominance and delayed bud break (Martin & Dunn, 2000; May, 2004). This compounded by reports that wounds made late in winter are less susceptible to infection due to faster wound healing and lower levels of pathogen inoculum (Munkvold & Marois, 1995; Chapuis *et al*., 1998; Surico *et al*., 2008; Úrbez-Torres *et al*., 2010; Úrbez-Torres & Gubler, 2011) make late pruning a recommended practice. Findings from the current study show that pruning wounds made late in winter had more pathogen infections than those made earlier for both trial years contrary to reports from California (Munkvold & Marois, 1995; Úrbez-Torres & Gubler, 2011) and Europe (Larignon & Dubos, 2000). This is an indication that the levels of inoculum are

lower in July as compared to August in the trial area. The results obtained in this study confirm spore trapping (Van Niekerk *et al*., 2010) and wound susceptibility (Van Niekerk *et al*., 2011) results from the same region. These studies reported on a two year monitoring period in which more pathogen aerial spores were found in August as compared to July of the same season as well as higher wound infection in August compared to July. This finding has serious implications on the practice of late pruning as a disease management tool for reducing pruning wound infection. Pruning early in the dormant season could result in reduced wound infections as the wounds would heal before the major spore dissemination events late in winter. However, complete escape from infection, by timing pruning, is virtually impossible as inoculum is always available in the vineyards such that unprotected wounds will be infected. Moreover, the availability of labour also influences the time of pruning and so protection of wounds is essential whenever pruning is carried out. This finding cannot be generalised for all Cape grapevine producing areas as the current study and both studies of Van Niekerk *et al*. (2010 & 2011) were carried out in the Stellenbosch wine region.

This study showed the importance of understanding the field environment and cultural practices in the agro-system to which a biocontrol agent is to be introduced. Biocontrol agents, being living organisms, are not likely to be equally effective in all agricultural situations and hence the current study unravels factors that may enhance pruning wound protection by *Trichoderma* spp. Pruning time had opposite effects on the colonisation of the two cultivars by the biocontrol agent indicating either cultivar differences or the effect of the grapevine's physiological state. However, wound treatment 6 hours after pruning consistently resulted in the highest wound colonisation regardless of the pruning time. It is important to note that these findings are only applicable to spur pruned grapevines and treatments could perform different in cane end pruned vines as well as on larger wounds. It was also found that the pruning of grapevines late in winter could result in higher wound infection by trunk disease pathogens in the Stellenbosch wine region of South Africa. The recommendation to prune earlier in the dormant season would need to be validated by conducting similar studies on a wider selection of cultivars and in other grapevine producing regions in South Africa.

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Tables and Figures

Table 1: 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).

Table 2: The extent of wound colonisation as determined by the percentage isolation frequency of *Trichoderma* species 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 mean isolation frequencies per wound, 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	22.17^{EFG}	35.67^{D}	51.75^{B}	60.33^{A}	43.33^{C}	18.00^{GHI}	33.83^{D}	25.92^{E}
Eco77	14.08^{I}	24.00^{EF}	37.42^{D}	44.75^{C}	34.00^{D}	15.67^{I}	20.67^{FGH}	16.58^{HI}

Values followed by the same letter are not significantly different from each other (P > 0.05; LSD 4.18).

Table 3: 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.

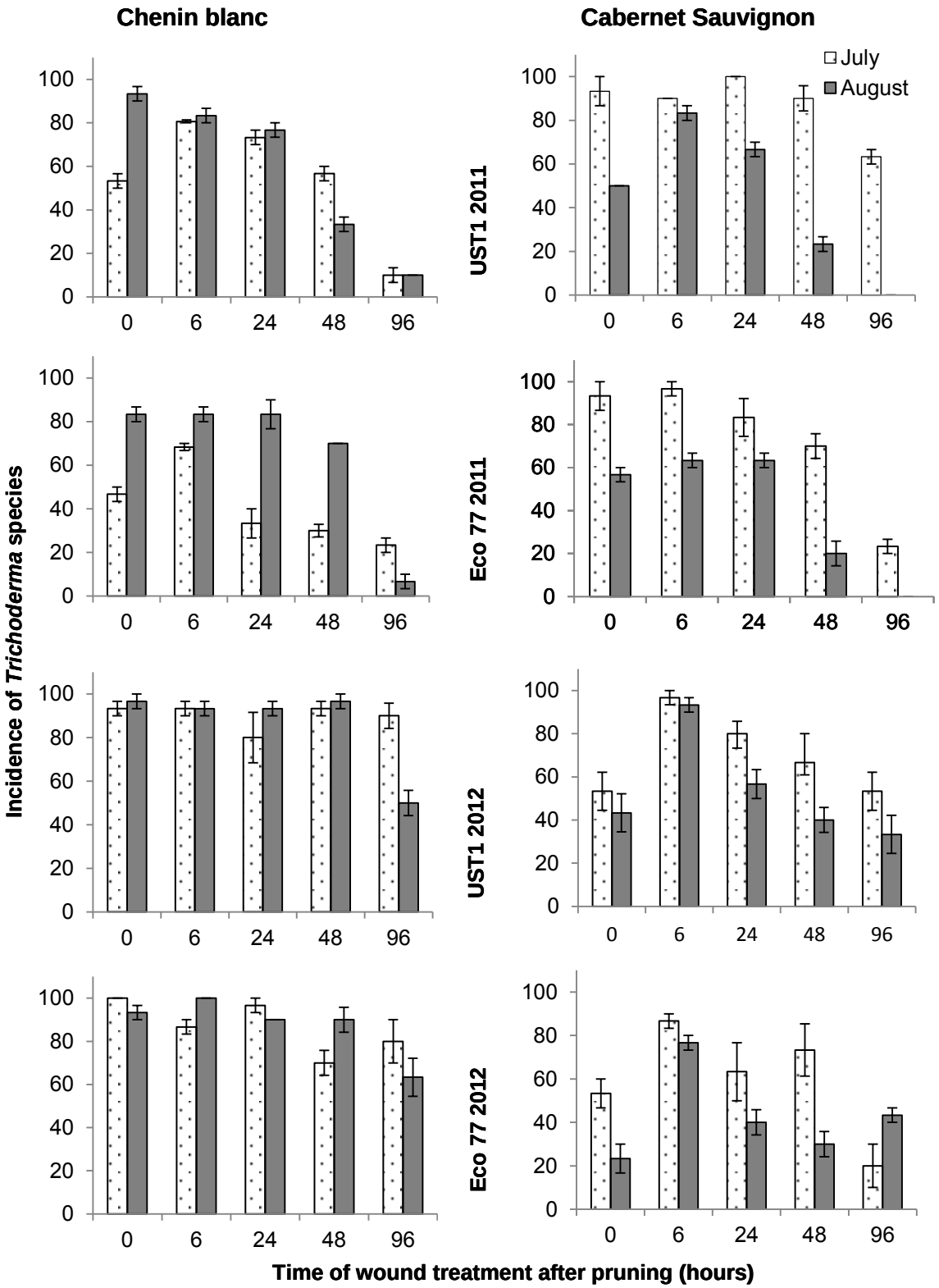

Figure 1: Percentage of wounds from where *Trichoderma* species were isolated in Chenin blanc and Cabernet Sauvignon pruning wounds treated with *Trichoderma* spp. suspensions, UST1 and Eco 77, at different times after pruning (0 – 96 hours)in the 2011 and 2012 seasons. Bars represent standard error of the mean.

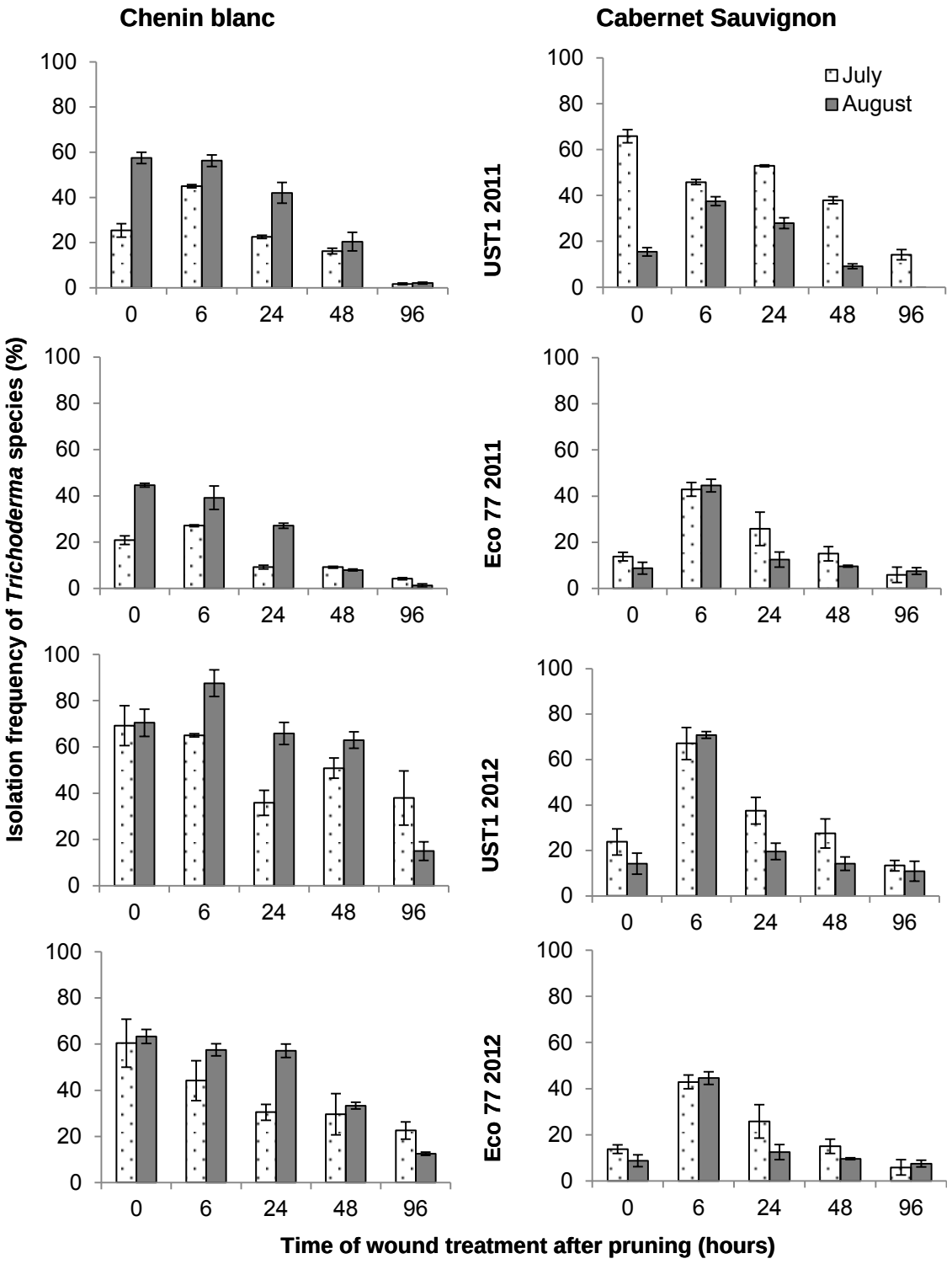

Figure 2: The extent of pruning wound colonisation as estimated by the isolation frequency of *Trichoderma* species from wounds of Chenin blanc and Cabernet Sauvignon treated with *Trichoderma* suspensions, UST1 and Eco 77, at different times (0 – 96 hours) after pruning in the 2011 and 2012 seasons. The isolation frequency is ratio of wood pieces from which *Trichoderma* sp. grew to the total number of wood pieces plated for isolation per wound. Bars represent standard error of the mean.

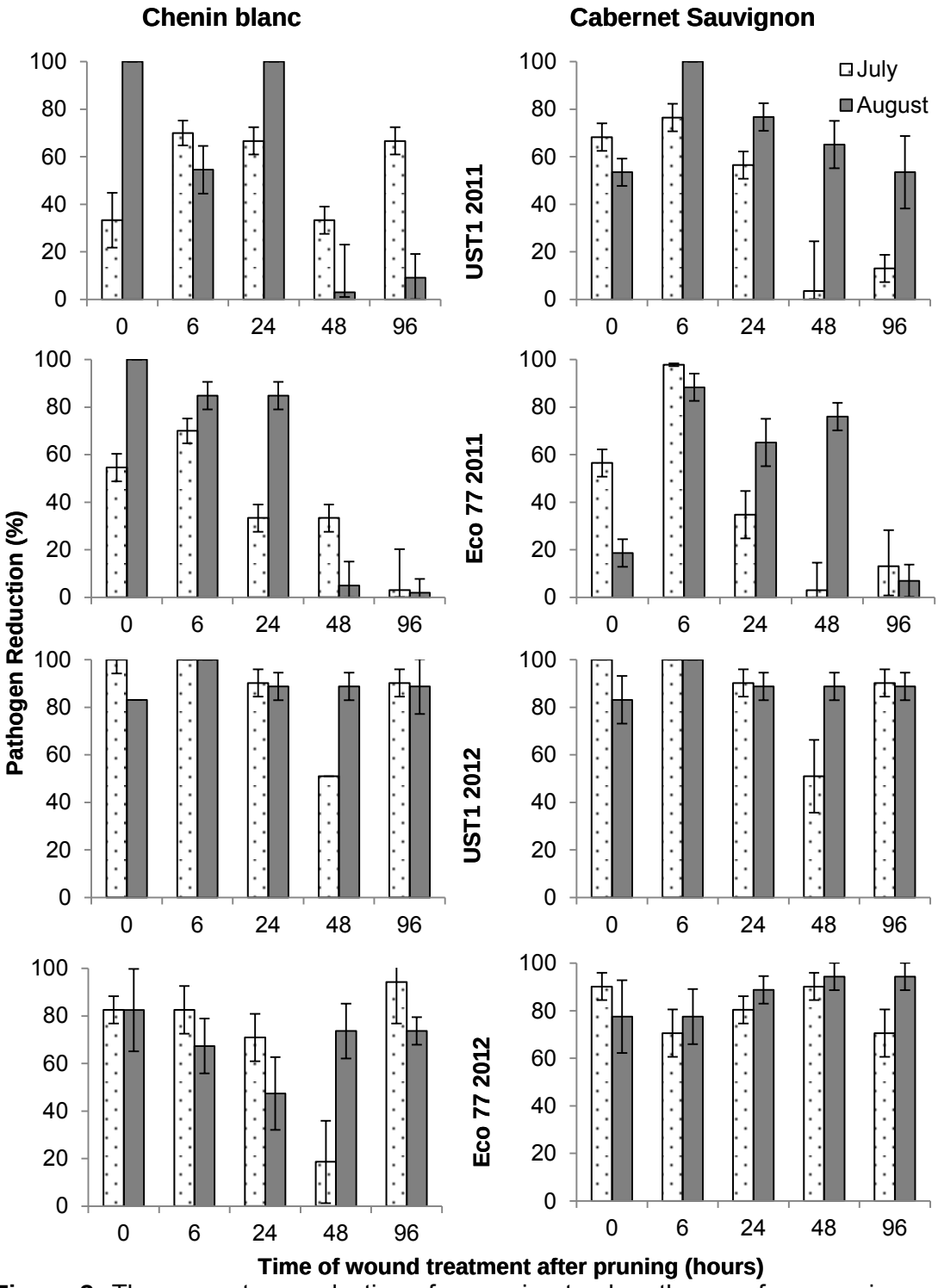

Figure 3: The percentage reduction of grapevine trunk pathogens from pruning wounds of Chenin blanc and Cabernet Sauvignon treated with *Trichoderma* suspensions, UST1 and Eco 77, at different times (0 – 96 hours) after pruning in the 2011 and 2012 seasons. Pathogen reduction was calculated as a percentage of the difference between the mean pathogen incidence in the water control treatments and the Trichoderma treatments. Bars represent standard deviation from the mean.

Chapter 4

The effect of biocontrol enhancers on *Trichoderma atroviride* efficacy in grapevine pruning wound protection from infection by *Phaeomoniella chlamydospora*

4.1 Abstract

The effect of biocontrol enhancers on the colonisation of grapevine pruning wounds by *Trichoderma atroviride* and wound protection from infection by *Phaeomoniella chlamydospora* was determined. Nutritional additives (glucose, yeast extract and urea), a humectant (water absorbing gel) and colloidal chitin were added to *T. atroviride* suspensions and tested either separately or in combination. Biocontrol enhancers that increased the colonisation extent of pruning wounds by the *T. atroviride* were further tested in field trials where a fungicide containing pruning wound paint, Garrison, and a registered *T. harzianum* biocontrol agent, Eco 77, were also included. In 2011, the field trials were carried out in Chenin blanc and Thompson Seedless vineyards. Pruning wounds were treated immideately after pruning and the pathogen was inoculated after a day. All the *Trichoderma* spp. treatments similarly reduced *Pa. chlamydospora* infection by 75% to 90% in the Thompson Seedless while in the Chenin blanc, control ranged from 40% to 74%. In 2012 the trial was carried out on Chenin blanc and the pathogen inoculated at intervals of one, three and seven days after pruning. Wound protection by the *Trichoderma* treatments was highest when wounds were inoculated with *Pa. chlamydospora* seven days after pruning. Garrison was always amongst the best treatments in reducing *Pa. chlamydospora* but its efficacy was lower on wounds infected by natural inoculum. Two nutritional amendments, a culture filtrate made from a chitin based medium and a combination of yeast extract, urea and glucose consistently enhanced efficacy in comparison with un-amended *T. atroviride* and they reduced *Pa. chlamydospora* infection to levels similar to those of Garrison.

4.2 Introduction

Phaeomoniella (*Pa.*) *chlamydospora* along with several species of *Phaeoacremonium* causes Petri disease also known as black goo (Crous & Gams, 2000). The pathogen is also associated with grapevine leaf-stripe symptoms (also called tiger-stripes) and spots on fruits (black measles) (Gubler *et al*., 2005; Surico *et al*., 2008; Surico, 2009). Due to the association of Petri disease with young vines, *Pa. chlamydospora* infections have largely been linked to the nursery propagation process (Mugnai *et al*., 1999; Gramaje & Armengol, 2011). However, nursery infections have been shown to mainly originate from infected scion and rootstock mother-vines (Fourie & Halleen, 2002; Halleen *et*

al., 2003; Aroca *et al*., 2006 & 2010). Additionally, pruning wounds have been established as the primary sites of infection by *Pa. chlamydospora* in vineyards (Larignon & Dubos, 2000; Eskalen *et al*., 2007; Rolshausen *et al*., 2010). The pathogen produces fruiting bodies (pycnidia) on wood surfaces and inside cracks of infected vines from where inoculum is produced usually during or following rainfall events (Larignon & Dubos, 2000; Edwards *et al*., 2001; Eskalen & Gubler, 2001). The pruning season, end of winter to early spring, coincides with periods of wet weather in most grapevine producing regions and hence wounds made during this period are at high risk of getting infected.

When pruning is carried out in warm temperatures, the rapid growth of non-pathogenic microorganisms on the pruning wound inhibits pathogens by competing for space and nutrients which also contributes to lower infection levels (Munkvold & Marois, 1993; Chapuis *et al*., 1998). Saprophytic wound colonisers such as *Cladosporium* (*Cl*.) *herbarum*, *Fusarium* (*F*.) *lateritium* and *Trichoderma* (*T*.) species have been isolated from pruning wounds and have been shown to be antagonistic to trunk pathogens and provide wound protection *in vivo* (Munkvold & Marois, 1993; Kotze *et al*., 2011). The advantage of biocontrol agents is they provide long term wound protection, which is needed since grapevine pruning wounds can remain susceptible for a period of 4 to 16 weeks (Eskalen *et al.*, 2007; Van Niekerk *et al*., 2011). Due to the ease of large scale production, biocontrol agents based on *Trichoderma* spp. have been commercialised.

Trichoderma spp. have shown success in the biological control of wood pathogens through pruning wound protection (John *et al*., 2005 & 2008; Schubert *et al*., 2008; Kotze *et al*., 2011). The ability of *Trichoderma* spp. to inhibit the growth of pathogens is due to the combined action of fungal cell wall degrading enzymes and the production of antimicrobial secondary metabolites that aid the biocontrol agent in competing for limited resources on the pruning wound (Lorito, 1998; Vinale *et al*., 2006 & 2008). Wound protection is thus dependent on the colonisation and establishment of the biocontrol agent on the wound. Abiotic factors such as availability of water and nutrients can determine how fast and deep the biocontrol agent colonises the wound.

Nutritional enhancement of the biocontrol agent formulae can improve establishment of the biocontrol agent, prior to infection, resulting in better efficacy of wound bio-protection. The enhancement of biocontrol efficacy by nutritional amendments has been demonstrated for both bacterial and fungal biocontrol agents (Hjeljord *et al*., 2001; Schmidt *et al*., 2001; Schubert *et al*, 2008). Hjeljord *et al*. (2000) reported delayed conidial germination and the loss of biocontrol activity by *Trichoderma* spp. against *Botrytis cinerea* and *Mucor piriformis* at low temperatures and nutrient-poor conditions. The addition of nutrients (carbon and nitrogen sources) resulted in an increase in conidia viability and germination rate resulting in

improved biocontrol efficacy (Hjeljord *et al*., 2001). On the contrary, nutrient addition alone had no effect on colonisation of pruning wounds on urban trees by *Trichoderma* sp., but the addition of a water storing gel increased wound colonisation by the biocontrol agent (Schubert *et al*., 2008).

The choice of nutritional additives is very important. Simple sugars support rapid growth of *Trichoderma* but can also suppress the production of inducible hydrolytic enzymes involved in fungal mycoparasitism (Mach *et al*., 1999; El-Katatny *et al*., 2000). Nutritional addition can also increase pathogen growth and disease severity as the pathogen may also utilise the available nutrients (Guestsky *et al*., 2002). However, nutrients that are more efficiently used by the biocontrol agent than the pathogens may provide an advantage. *Trichoderma* spp. are known to break down chitin and can use it as a carbon source while the same chitinolytic enzymes are also involved in the antagonism against pathogenic fungi in biocontrol (Lorito, 1998; Rey *et al*., 2001; Sandhya *et al*., 2004). In addition, protein extracts from some *Trichoderma* biocontrol agents exhibit antifungal properties as they contain lytic enzymes such as chitinases, glucanases and proteases (Lorito, 1998; Monte, 2001). Another strategy to enhance biocontrol efficacy would therefore be to use the protein extracts in the formulation.

Trichoderma atroviride (UST1) was isolated from grapevines, tested against grapevine trunk pathogens and shown to be effective both *in vitro* and in field studies in South Africa (Kotze *et al*., 2011; Mutawila *et al*., 2011). *In vitro*, UST1 is antagonistic to grapevine trunk pathogens exhibiting both mycoparasitism and antibiosis. Wound protection by UST1 was statistically similar or better than the fungicide, benomyl, when pathogens were inoculated seven days after pruning (Kotze *et al*., 2011). However, when pathogens are inoculated shortly after pruning, wound bio-protection has been reported to be poor and inconsistent (Munkvold & Marois, 1993; Halleen *et al*., 2010). Field efficacy of the biocontrol agents seems to be dependent on the time that the *Trichoderma* spp. take to establish on the wound before exposure to the pathogen.

In a previous study the addition of a sticking agent, Nu Film 17, to *T. atroviride* suspensions could not significantly enhance biocontrol efficacy in wound protection (Mutawila, 2010). The enhancement of biocontrol efficacy by nutritional additives on this isolate has never been tested. The objective of this study was therefore to determine the effect of nutritional amendments and bio-enhancers on the efficacy of *T. atroviride* (UST1) in grapevine pruning wound protection against infection by *Pa. chlamydospora*.

4.3 Materials and Methods

4.3.1 Fungal isolates and inoculum preparation

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).

4.3.2 *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.

4.3.3 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*.

4.3.4 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.

4.3.4.1. 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.

4.3.4.2. 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.

4.3.4.3. 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.

4.3.4.4. 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).

4.3.5 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.

4.4 Results

4.4.1 *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.

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

The incidence of *T. atroviride* and *Pa. chlamydospora* and pathogen reduction are shown in Table 1. Analysis of variance on the incidence of the biocontrol agent and pathogen found highly significant differences ($P < 0.001$; Appendix B, Table 1) 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 ($P < 0.001$: Appendix B, Table 2) 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 ($P = 0.007$; Appendix B, Table 2). *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 (Appendix B, Figure 1.). The regression coefficient (r^2) for the curve of the model ranged from 0.74 to 0.98 (Table 2).

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% (Figure 1). 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 (Figure 1A). 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 (Figure 1B). Therefore, the nutritional amendments Yeast extract, Yeast extract + Urea and Yeast extract + Urea + Glucose were chosen for further field testing.

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

4.4.3.1. Optimisation of broth formulation: The addition of an organic nitrogen source (peptone) significantly ($P < 0.001$; Appendix B, Table 1) 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.

4.4.3.2. Field evaluation 2011: Analysis of variance found significant cultivar × treatment interactions ($P < 0.001$; Appendix B, Table 3) in the incidence of *Trichoderma* spp. and *Pa. chlamydospora* and thus analysis for the cultivars was done separately (Appendix B, Table 4). The incidence of *Trichoderma* spp. was higher in the Thompson Seedless compared to the Chenin blanc (Table 3). 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.

Pathogen incidence in the two cultivars is summarised in table 4. 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 3). Normally after pruning the wood below, the wound dies back down to the first shoot (apical node).

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 4). 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 4). 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 (Table 3).

4.4.3.3. 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 ($P < 0.001$; Appendix B, Table 5) 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 5). 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 5).

In pathogen-inoculated wounds, analysis of variance found significant treatment × inoculation day interactions ($P < 0.001$; Appendix B, Table 5) 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 6). 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 6). 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.

4.5 Discussion

The formulation of biocontrol agents in regards to additives or composition of formulae is held proprietary by the producers and is neither published nor patented as it is difficult to enforce such patents. One of the key steps in maximising the potential of biocontrol agents is to enhance their survival and bioactivity after delivery. Nutritional additives may increase efficacy by either increasing growth of the biocontrol agent or enhancing its antagonistic activity on the target pathogen. Nutritional amendments of *T. atroviride* increased the colonisation extent of grapevine pruning wounds in the glasshouse trial as shown by the higher isolation frequency of the biocontrol agent with increasing depth from the wound surface in comparison with the unamended *T. atroviride* suspension. Better wound colonisation at greater depth would result in better wound protection. On pruning wounds, *Trichoderma* spp. wound protection is currently believed to be due to the antagonistic properties of the biocontrol agent against the pathogen and occupation of space and making it unavailable for pathogen growth (John *et al*., 2005; Mutawila *et al*., 2011b). These mechanisms depend on the colonisation and establishment of the biocontrol agent on the wound. Another possible mode of action on the wound is the activation of grapevine defence by the *Trichoderma* spp. which still requires the colonisation of the wound during which the biocontrol agent produces elicitors that trigger the defence reaction.

The use of moisture retaining polymers (humectants) in biocontrol agent preparations has shown potential in improving performance and consistency as they make free water available for the growth of the biocontrol agent (Chittick & Auld, 2001; Sanogo *et al*., 2002; Schubert *et al*., 2008 & 2009). In the glasshouse trial, on detached grapevine canes, the humectant treatments, polyacrylate alone and in combination with the yeast extract and urea resulted in better *T. atroviride* wound colonisation compared to the UST1 in water suspension. These treatments could not be tested further because the polyacrylate paste was difficult to apply on the small pruning wounds due to its granular-gel form. In the field, the polyacrylate may also rehydrate after any rainfall activity and form a wet ‘sponge’ on the pruning wound. Schubert *et al*. (2008) reported enhanced viability, adhesion and establishment of *T. atroviride* on pruning wounds of urban trees when the biocontrol agent was applied in a polyacrylate formula. On the urban trees, the pruning wounds were larger (mean diameter of 6.4 cm), which made it easier to apply the polyacrylate gel relative to the

grapevine pruning wounds on one-year-old grapevine canes (of less than 2 cm diameter) used in the current study. Polyacrylate formulae might be applicable for larger wounds made on the trunk or cordons. However, it would have to be tested in the field.

Addition of a nitrogen source, peptone, to minimal nutrient medium significantly increased the chitinolytic activity of the *T. atroviride* culture filtrate which was then used in combination with conidia of *T. atroviride* in the Broth treatment. *Trichoderma* spp. are well known producers of chitinolytic enzymes that breakdown chitin and are involved in the degradation of fungal cell walls and their biocontrol activity (Lorito, 1998; Rey *et al*., 2001; Sandhya *et al*., 2004). Chitinases are part of the antagonistic arsenal of *Trichoderma* spp. against other fungi which also includes other hydrolytic enzymes, such as glucanases and proteases (Schirmböck *et al*., 1994; Shakeri & Foster, 2007) and antibiotic metabolites (Vinale *et al*., 2006 & 2008). Although only chitinolytic activity was used for selecting the optimal harvest time of the culture filtrate, the filtrate also contained other *Trichoderma* hydrolytic enzymes. Culture filtrates of antagonistic *Trichoderma* spp. containing proteins have been shown to have antifungal properties (Lorito *et al*., 1994; Schirmböck *et al*., 1994; Lorito, 1998).

In the detached grapevine cane assay all the *T. atroviride* treatments significantly reduced wound infection by *Pa. chlamydospora* at higher levels than in the field trials. Although some of the treatments tested further in field trials could significantly reduce wound infection compared to the control treatments, most were not significantly better than the un-amended treatment (UST1 in water). In the 2011 trials there was a clear difference in reduction of *Pa. chlamydospora* between the two cultivars with better control in the Thompson Seedless by all the treatments. This is not likely to be due to cultivar differences in susceptibility to *Pa. chlamydospora* since the infection levels in the wounds that received the control treatment are quite comparable for the two cultivars. The incidence of *Trichoderma* spp. was higher in the Thompson Seedless compared to the Chenin blanc and the poor colonisation of the biocontrol agents in the Chenin blanc is the most likely reason for the poor control. A previous study on grapevine cultivar variation to pruning wound protection by *Trichoderma* spp. showed that high incidence of *Trichoderma* spp. was strongly correlated to pathogen reduction in both Chenin blanc and Thompson Seedless (Mutawila *et al*., 2011b).

One major difference between the 2011 and 2012 trials was the low *Trichoderma* incidences found in the 2011 trials. In both trials, pruning was carried out late in winter (August) but in the 2011 trials the wounds were treated immediately after pruning (within 30 minutes). Sap flow was observed after pruning in both vineyards and could have washed-off the *Trichoderma* conidia from the pruning wounds resulting in poor establishment of the

biocontrol agent. In the 2012 trial, pruning wound treatment was carried out 6 hours after pruning. This was adopted following results from Chapter 3 that showed that applying *Trichoderma* biocontrol agents 6 hours after pruning consistently resulted in higher wound colonisation. The importance of applying wound protectants several hours after pruning is further shown by the dripping-off of the paint treatment, Garrison due to sap-flow in the Thompson Seedless. In addition to fungicidal activity, Garrison also seals the wound creating a physical barrier to pathogens that fall on the wound surface. In cases where the paint drips off the wound is left even more susceptible for infection because the fungicidal activity of the paint would have exterminated saprophytes that could grow on the wound some of which provide protection (Munkvold & Marois, 1993). This further demonstrates the importance of optimising the time of application of both chemical and biocontrol agents for wound protection.

Garrison was always amongst the best treatments in reducing *Pa. chlamydospora* infection, but failed to reduce natural pathogen infection in both cultivars in the 2011 trials. 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%). When *Pa. chlamydospora* was inoculated, the fungicidal activity of the Garrison was still active and when the fungicide activity diminishes the physical barrier of the paint should continue to provide wound protection. However, it was also observed that about 20% of Garrison painted wounds did not heal eight months after pruning. Infection from natural inoculum can occur at any time as long as the wound is still susceptible and if the integrity of the sealant on the wound surface is compromised then the wound below is likely be infected. This could explain the poor control, by Garrison, of infections due to natural inoculum. Garrison has been reported to be effective in grapevine pruning wound protection (Sosnowski *et al*., 2008; Rolshausen *et al*., 2010; Pitt *et al*., 2012) and the current study also supports these findings. However, from the current study it appears that Garrison may interfere with the normal pruning wound healing process and thus the host resistance but further research is required. In most studies on pruning wound protection, efficacy is assessed from inoculations carried out shortly after pruning. With pruning wound sealants, it may be also important to consider their effect on wound healing and inoculate the pathogen at different time intervals.

In the Chenin blanc, *Pa. chlamydospora* was isolated at a higher incidence in 2012 than 2011 in the control treatments that were inoculated within a day of pruning. In the wounds that did not receive the biocontrol treatments, in the 2012 trial, *Pa. chlamydospora* was isolated less frequently from wounds that were inoculated after three and seven days most likely due to the reduction in susceptibility as the wounds healed (Serra *et al*., 2008;

Van Niekerk *et al*., 2011). Despite higher *Pa. chlamydospora* infection in the 2012 than the 2011 trial, the Broth and the Yeast extract + Urea + Glucose, treatments reduced pathogen infection to levels not significantly different from the fungicidal paint, Garrison, at all pathogen inoculation intervals. The reduction of *Pa. chlamydospora* infection by these *T. atroviride* treatments (of 59% to 90%) in all trials is comparable to pathogen reduction reported with fungicides (of 52% to 85%) in California (Rolshausen *et al*., 2010). Wound protection by the other *Trichoderma* treatments was generally better when wounds were inoculated with *P. chalmydospora* after seven days.

Many studies have shown that biocontrol agents need time to germinate and colonise the wound before they can provide protection. Munkvold & Marois (1993) achieved better control of *Eutypa lata* using *Cl. herbarum* and *F. lateritium* when the pathogen was inoculated 14 days after the biocontrol agents. Kotze *et al*. (2011) also reported effective wound protection from *E. lata*, *Pa. chlamydospora* and species of the Botryospheriaceae when the pathogens were inoculated after seven days. John *et al*. (2005) proposed that 2 days was sufficient for *T. harzianum* to colonise the wound and impair wound infection by *E. lata*. A way to address the need for time between wound treatment and pathogen infection is to apply the biocontrol agent in a formulation that is antifungal and can protect the wound until the biocontrol agent has germinated and colonised the wound. The *T. atroviride* culture filtrate (Broth treatment) used in the current study contains antifungal hydrolytic enzymes that can inhibit growth of pathogen propagules on the wound immediately after application. Since the culture filtrate was made using chitin based medium it therefore contained chitin oligosaccharides which are known to stimulate the production of chitinases in *Trichoderma* spp. and also elicit plant defence (Hahn *et al*., 1993; Brunner *et al*., 2003; Falcón-Rodríguez *et al*., 2012). The expression of chitinases by *Trichoderma* spp. is induced by chitin and its oligosaccharides (Brunner *et al*., 2003) and their presence on the pruning wound is therefore likely to result in earlier expression of cell wall degrading enzymes by the biocontrol agent. Chitin oligosaccharides have been shown to induce faster wound healing through the deposition of lignin and other phytoalexins (Barber *et al*., 1989; Hahn *et al*., 1993; Falcón-Rodríguez *et al*., 2012). This could explain the relatively better control by the Broth treatment in the 2012 trial. Similar control by the yeast extract + urea + glucose treatment could be due to faster growth of the biocontrol agent due to provision of both a nitrogen and carbon source since the nitrogen sources alone (Yeast extract or Yeast extract + Urea) did not result in similar control.

It has been shown that the co-inoculation of *Trichoderma* spp. and *Pa. chlamydospora* on pruning wounds reduces the wood streaking, black goo symptoms and the extent of pathogen colonisation of the xylem vessels (Di Marco *et al*., 2004; Mutawila *et*

al., 2011b). In the current study, *Pa. chlamydospora* was co-isolated with the biocontrol agent from the same wound. Although these wounds were considered infected, it is not known how extensive the pathogen would grow and if disease would develop in the presence of the biocontrol agent. Long term studies would be required to answer this question.

The use of nutritional amendments on biocontrol agent applications has been shown to increase pathogen control efficacy in some pathosystems that include soil (Hjeljord *et al*., 2001), leaves and fruits (Schisler *et al*., 2004) as well as wood (Ferreira *et al*., 1991; Schmidt *et al*., 2001). On the contrary, John *et al*. (2005) found that the nutrient base in a registered grapevine pruning wound bio-protectant Trichoseal had no enhancement effect on the efficacy of its active agent, *T*. *harzianum*. Similarly, Schubert *et al*. (2008) also found nutritional additives to have no effect on the ability of *T*. *atroviride* to protect wounds on urban trees, but efficacy was enhanced by adding a water retaining polymer. 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. The culture filtrate can further be improved by increasing the concentration of the *Trichoderma* proteins (hydrolytic enzymes). Commercially, the *T*. *atroviride* conidia and protein extract concentrate of the culture filtrates could be provided separately for mixing just before application. However, more work would need to be done to optimise the production of the protein extract. Valuable knowledge has been gained on the potential of bio-enhancers on *Trichoderma* efficacy on grapevine pruning wounds. Field trials against other grapevine trunk pathogens, together with testing on a wider selection of grapevine cultivars will aid in the development of an effective *Trichoderma* pruning wound formulation.

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Tables and Figures

Table 1: The mean incidence of the biocontrol agent, *Trichoderma atroviride*, grapevine trunk pathogen *Phaeomoniella chlamydospora,* and pathogen reduction from pruning wounds of one-year-old grapevine canes treated with *T. atroviride* spore suspension amended with different additives (formulae). Treated wounds were inoculated with *Pa. chlamydospora* one-day after treatment.

Nutritional amendment	**Percentage Incidence (%)***		**Pathogen reduction (%)**
	T. atroviride	*Pa. chlamydospora*	
UST1 in water	72.72^{BC}	9.00^{B}	85.86
UST1 + Yeast extract (Y.E)	88.23^{ABC}	11.76^{B}	81.52
UST1 + Urea	88.23^{ABC}	5.88^{B}	90.76
UST1 + Yeast extract + Urea	94.12^{AB}	11.76^{B}	81.52
UST1 + Y.E + Urea + Glucose	100^{A}	0^{B}	100
UST1 + Polyacrylate	94.12^{AB}	0^{B}	100
UST1 + Polyacrylate + Yeast extract	76.47^{ABC}	6.25^{B}	90.18
UST1 + Polyacrylate + Urea	68.82^{BC}	0^{B}	100
UST1 + Polyacrylate + Y.E + Urea	94.12^{AB}	0^{B}	100
UST1 + Polyacrylate + chitin	76.47^{ABC}	0^{B}	100
Sterile water (control)	0^{D}	63.63^{A}	-
LSD (P = 0.05)	22.76	13.20	-

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

Table 2: Coefficients for non-linear regression equations ($y = a + b^{-x}$) for prediction of the isolation frequency (y) of *T. atroviride* with depth (x) from the surface of grapevine pruning wounds. The *T. atroviride* was applied as conidial suspensions amended with various nutrients. The frequency of colonisation is a relative measure of the extent of wood colonisation by the biocontrol agent.

Treatment	Intercept (a)	Slope (b)	R^2-value	*P*-value
UST1 water suspensiom	1.946	38.35	0.96	0.003
UST1 + Yeast extract (Y.E)	5.738	101.49	0.74	0.061
UST1 + Urea	-3.538	107.51	0.88	0.013
UST1 + Y.E + Urea	-1.103	138.18	0.96	0.003
UST1 + Y.E + Urea + Glucose	0.450	144.98	0.92	0.011
UST1 + Polyacrylate (Pacr)	-1.676	154.58	0.88	0.018
UST1 + Pacr + Y.E	-4.454	102.34	0.94	0.006
UST1 + Pacr + Urea	1.538	35	0.96	0.004
UST1 + Pacr + Y.E + Ur	5.976	70.27	0.85	0.031
UST1 + Pacr + chitin	-0.135	67.81	0.98	<0.001

Table 3: 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.

Table 4: 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.

Table 5: 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 6: 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}	25.00^{B}	5.90^{BCD}
	(68)[1]	(54)	(85)
UST1 + Broth	18.81^{CDE}	5.56^{B}	9.03^{BC}
	(74)	(90)	(78)
UST1 + Yeast extract (Y.E)	21.35^{BCD}	22.98^{B}	9.58^{BC}
	(71)	(58)	(76)
UST1 + Y.E+Urea	39.43^{B}	10.83^{B}	0^{D}
	(47)	(80)	(100)
UST1 + Y.E+Urea+Glucose	10.76^{DE}	6.25^{B}	10.62^{BC}
	(85)	(89)	(74)
Eco 77	36.61^{BC}	19.44^{B}	12.22^{B}
	(50)	(64)	(70)
Garrison	0^{E}	11.65^{B}	2.78^{CD}
	(100)	(79)	(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.

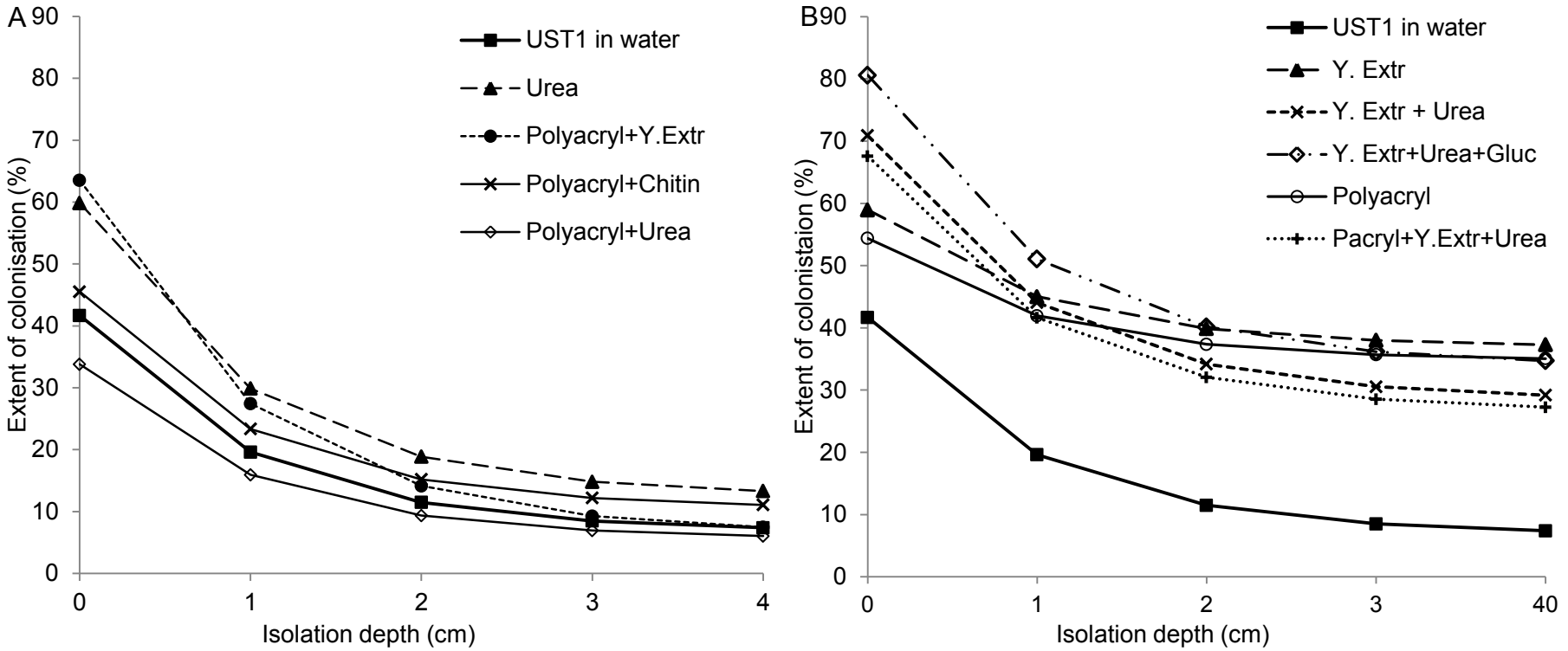

Figure 1: The effect of different nutritional additives on the extent of grapevine pruning wound colonisation by *Trichoderma atroviride* UST1. The extent of colonisation at 2 cm depth was used to determine treatments that either did not enhance colonisation (A) or enhanced *T. atroviride* colonisation (B) significantly (P = 0.05) compared to the treatment where conidia were suspended in water (UST1 in water). Isolation frequencies of the biocontrol agent at various depths from the pruning wound surface which were used to generate a none-linear regression model ($y = a + b^{-x}$; where x is the depth) to estimate the colonisation extent with depth from the wound surface. The *T. atroviride* UST1, was applied as spore suspensions with various nutritional amendments as compared to application with water only. Y. Extr = Yeast extract; Gluc = glucose and Pacryl = Polyacrylate.

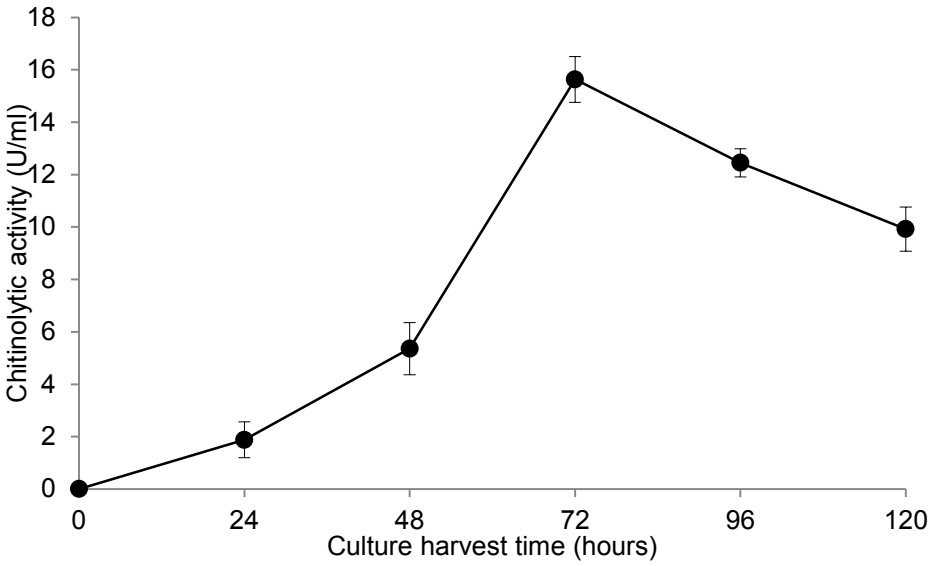

Figure 2: The time course chitinolytic activity of culture filtrates of *Trichoderma atroviride* UST1. This was used to determine the optimal harvest time (72 hours) for the broth which was used as a treatment in the evaluation of the effect of different nutritional amendments on *T. atroviride* pruning wound protection. Each value on the line represents mean chitinolytic activity ± the standard deviation of three independent biological replicates.

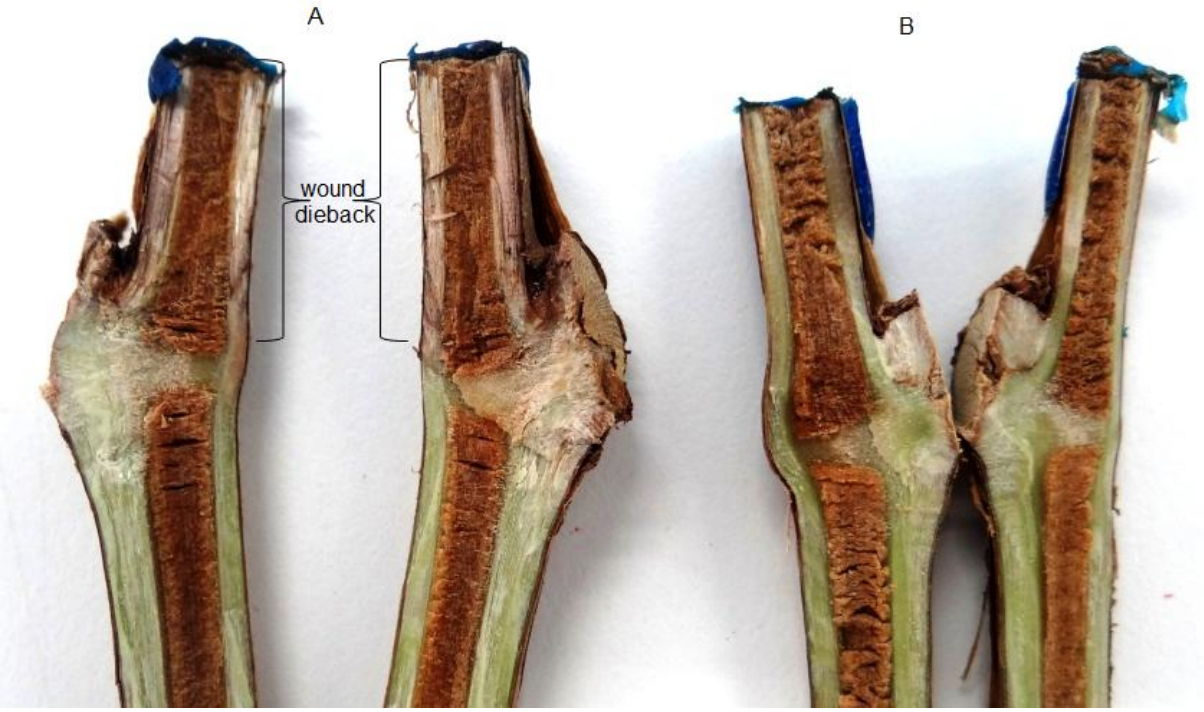

Figure 3: Vertical section through grapevine canes eight months after pruning wound treatment with a wound sealant, Garrison, showing normal wound healing (A) with wood dieback down to the apical node and an unhealed wound (B).

Chapter 5

Development of benzimidazole resistant Trichoderma strains for the integration of chemical and biocontrol methods of grapevine pruning wound protection

5.1 Abstract

In the protection of grapevine pruning wounds from trunk pathogen infection, fungicides provide mainly short term protection while biocontrol agents provide mainly long term protection. The integration of fungicide and biological wound protection could provide better wound protection, but is limited by the susceptibility of the biocontrol agents to the fungicides. The susceptibility of three *Trichoderma* isolates (UST1, UST2 and T77) to benzimidazole fungicides was tested and resistant mutants were developed by gamma irradiation (250 Gy). All the *Trichoderma* isolates were found to be naturally resistant to thiophanate methyl while mycelial growth was completely inhibited by 2.5 µg/mL of benomyl and carbendazim. Stable benzimidazole resistant mutants that could grow at 100 µg/mL were developed from each wild type *Trichoderma* isolate. There was no reduction in biological fitness and *in vitro* antagonist activity for mutants generated from UST1 and UST2 while the mutant from T77 had reduced fitness and antagonistic activity compared to its wild type. The wild type and the mutant from UST1 were tested in the field, alone and in combination with carbendazim and thiophanate methyl, to assess their ability to prevent pruning wounds from infection by *Phaeomoniella* (*Pa*.) *chlamydospora*. The wild type and the mutant of UST1 reduced the infection of *Pa. chlamydospora* significantly when applied alone and in combination with the fungicides. The mutant of UST1 applied with carbendazim gave the best reduction of infection when *Pa. chlamydospora* was applied 24 hours after pruning. The *Trichoderma* transformants generated in this study can be applied in combination with benzimidazole fungicides for a more effective and sustainable wound protection.

5.2 Introduction

Grapevine decline caused by fungal trunk pathogens has become increasingly important in all grapevine producing regions of the world. Grapevine trunk diseases are collectively responsible for graft failure, decline and death of young vines, loss of vigour and productivity in established vines, spots on berries, late ripening and altered flavour, as well as death of vines (Mugnai *et al*., 1999; Fourie & Halleen, 2004; Gubler *et al*., 2005). These diseases are caused by a variety of taxonomically unrelated fungi that colonise wood tissue where they interfere with water and solute transport and cause degradation of conducting

cells (Pascoe & Cottral, 2000; Mutawila *et al*., 2011). The diseases include Petri disease and esca, caused by *Phaeomoniella* (*Pa.*) *chlamydospora* and *Phaeoacremonium* species as well as wood rotting basidiomycetes, Eutypa dieback caused by species of Diatrypaceae of which *Eutypa* (*E*.) *lata* is the most common and Botryosphaeria cankers caused by species of the Botryosphaeriaceae.

Despite their diversity, trunk pathogens follow an almost similar disease cycle infecting vines through wounds, of which pruning wounds are the most important ports of entry (Petzoldt *et al.*, 1981; Chapuis *et al*., 1998; Van Niekerk *et al*., 2011). Additionally the high pathogen diversity makes it very difficult to find one control method that is equally effective against the whole suite of trunk pathogens. One of the major control methods is to protect pruning wounds by chemical fungicides or biological control agents. Sodium arsenite was once considered the most effective fungicide for the control of esca as it also delayed the expression of foliar symptoms and reduced its severity in diseased vines (Mugnai *et al*., 1999; Surico *et al*., 2008). This fungicide is no longer available due to environmental and human toxicity (Surico *et al*., 2008). Other fungicides such as sterol demethylation inhibitors (flusilazole and myclobutanil) and boron (applied as boric acid) have also been found to be effective. However, the fungicides were not equally effective against all trunk pathogens while boron was further shown to inhibit growth of the apical bud (Rolshausen & Gubler, 2005; Rolshausen *et al*., 2010). Broad spectrum benzimidazole fungicides namely benomyl, carbendazim and thiophanate methyl are among the most effective available wound protectants (Moller & Kasimatis, 1980; Rolshausen *et al*., 2010; Díaz & Latorre, 2013).

Wounds remain susceptible until they are fully healed which takes a period of at least 4 to 16 weeks depending on the time of pruning (Larignon & Dubos, 2000; Van Niekerk *et al*., 2011). Fungicide efficacy on the pruning wound is short-lived and does not last until the wound is no-longer susceptible to infection (Carter & Price, 1975; Munkvold & Marois, 1993a). Multiple applications of fungicides would be needed to achieve a longer period of control (Munkvold & Marois, 1993a), however, this would be detrimental to the environment and more costly. On the other hand biocontrol agents that colonise the wound may provide long term protection. *Fusarium* (*F*.) *lateriteum* and *Trichoderma* (*T*.) species have been shown to protect pruning wounds from infection by several trunk pathogens (Carter & Price, 1975; Munkvold & Marois, 1993b; John *et al*., 2005; Kotze *et al*., 2011). Munkvold & Marois (1993b) showed that protection by biocontrol agents was always better when pathogen inoculation was carried out several days after application of the biocontrol agent. However, despite increased availability of registered biocontrol products adoption, has been limited due to an inherent belief in the farming community that biocontrol agents are less effective than conventional pesticides (Harman, 2000). There are also reports of poor and

inconsistent wound protection by biocontrol agents (Gendloff *et al*., 1983; Creaser & Wicks, 2002; Halleen *et al*., 2010). Poor control is largely attributed to the time needed for wound colonisation, a period which creates a window of infection by trunk pathogens. Since there are currently no curative control measures for infected vines, long-term strategies for wound protection are needed.

Integration of chemical and biocontrol agents can provide both short and long term wound protection. The major limitation to the integration of biocontrol agents into conventional disease management systems is their sensitivity to fungicides. The breeding/production of fungicide resistant biocontrol agents is a pre-requisite to such integration. Fungicide resistant biological strains have a further advantage that they can be applied together with a reduced concentration of fungicide and hence reducing environmental impact. Mutagenesis by exposure to chemicals and physical mutagens as well as protoplast fusion have been employed to improve efficacy of biocontrol agents (Hanson & Howell, 2002; Hatvani *et al*., 2006). Ethidium bromide and 1-methyl-3-nitro-1-nitrosoguanidine are the main chemical mutagens used while ultra-violet light is the major physical mutagen. In several biocontrol and phytopathogenic fungi the genes responsible for tolerance/resistance to benomyl have been isolated (Yan & Dickman, 1996; Ma *et al*., 2003) and so genetic engineering has also been used to generate benomyl resistant biocontrol agents for use in integrated disease management systems (Ossanna & Mischke, 1990; Brunner *et al*., 2005). However, chemical and physical mutagenesis have an advantage over protoplast fusion and genetic engineering as it is much easier to get authorisation from regulatory authorities to apply the mutants in the field.

Gamma irradiation and other ionising radiation are widely recognised methods for the decontamination of cereal grains and other food stuffs from microorganisms, worms and insects that degrade the quality of stored products (World Health Organisation, 1994; Braghini *et al*., 2009). Exposure of cells to gamma rays at non-lethal doses induces DNA damage (single or double strand breaks) which on repair may result in gene mutation. This has been used to generate variability in plant breeding (Ahloowalia & Maluszynski, 2001; Kovács & Keresztes, 2002). Ionising radiation, particularly gamma radiation cause deleterious single or double-strand breaks and DNA crosslinks which on repair result in chromosomal rearrangements and gene disruption (Dadachova & Casadevall, 2008). This has been used successfully to generate genetic variation in crop breeding while in microbiology, gamma radiation has been used to sterilise food and medical supplies. However, despite the potential of gamma radiation to generate novel fungal mutants there are only a few reports on such studies.

In this study we report the development of benzimidazole resistant mutants of *T. atroviride* and *T. harzianum* using gamma irradiation. The mutants and the wild type isolates were tested *in vitro* for their ability to inhibit the growth of trunk disease pathogens namely, *E. lata*, *Diplodia* (*D.*) *seriata*, *Neofusicoccum* (*N.*) *parvum* and *Pa. chlamydospora*. One of the wild type and mutant isolates was also tested in the field for their efficacy in protecting grapevine pruning wounds against infection by the trunk pathogen *Pa. chlamydospora*.

5.3 Materials and Methods

5.3.1 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.

5.3.2 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.

5.3.3 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.

5.3.4 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.

5.3.5 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.

5.3.6 *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.

5.3.6.1. 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.

5.3.6.2. 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.

5.4 Results

5.4.1 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 (Figure 1A). 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.

5.4.2 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.

5.4.3 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.

5.4.4 Mutants' *in vitro* antagonism against grapevine trunk pathogens

The micro- and macroscopic interactions observed between the mutant *Trichoderma* strains and the pathogens in dual cultures are summarised in Table 1. 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* (Figure 1C) and *Pa. chlamydospora*. Disintegration of pathogen hyphae was observed on interaction of both MT1 and MT2 with *D. seriata* (Figure 1D). The adhesion of mutants MT1 and MT2 hyphae to pathogen hyphae was also observed on all pathogens. No microscopic interactions were observed with MT77.

5.4.5 *In vivo* evaluation of grapevine wound protection

5.4.5.1. 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; Appendix C, Table 1) nor inoculation time differences (P = 0.124; Appendix C, Table 1), but significant treatment differences (P < 0.001; Appendix C, Table 1) 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%).

***5.4.5.2. Incidence of* Pa. chlamydospora:** Significant treatment × inoculation day interactions (P < 0.001; Appendix C, Table 2) 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 2. 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 2).

5.4.5.3. Pathogen incidence under natural inoculum: The incidence of grapevine trunk pathogens in the non-inoculated wounds is also shown in table 2. 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 2).

5.5 Discussion

Gamma irradiation of biocontrol *T. atroviride* and *T. harzianum* isolates generated stable benzimidazole resistant mutant strains. The mutants from the *T. atroviride* isolates were of similar fitness as the wild type isolates while the mutant MT77 was of reduced fitness compared to the wild type *T. harzianum* T77. The mutant MT1 was tested in the field and was able to colonise and protect pruning wounds from infection alone and in combination with carbendazim.

To develop fungicide mutant *Trichoderma* strains by gamma irradiation, the sensitivity of the wild type isolates to radiation had to be determined. The sensitivity of the wild type isolates as estimated by the D_{10} values (211-216 Gy) is slightly lower than reported for mycotoxin producing species of *Alternaria* and *Aspergillus* (>240 Gy; Braghini *et al*., 2009; Blank & Corrigan, 1995). While there are several studies on the irradiation of

Trichoderma spp., none of these has looked at their sensitivity to gamma irradiation or determined their D_{10} values.

The sensitivity of the wild type isolates to the thiophanate methyl differed from their sensitivity to benomyl and carbendazim. All the wild type isolates were resistant to thiophanate methyl while susceptible to benomyl and carbendazim. Mutants isolated from benomyl amended media were cross resistant to carbendazim and *vice versa* and also maintained their resistance towards thiophanate methyl. Although the chemical structures of benomyl and thiophanate methyl are not similar, they are both metabolised to a common active compound, methyl 2-benzimidazole carbamate (carbendazim), and hence cross-resistance between the fungicides is expected. Keinath & Zitter (1998) found isolates of *Didymella bryoniae* that were resistant to thiophanate methyl, but susceptible to benomyl, though all isolates resistant to benomyl were also resistant to thiophanate methyl. It appears that resistance to thiophanate methyl does not necessarily result in cross resistance to benomyl, while alleles conferring resistance to benomyl also confer resistance to thiophanate methyl. The anti-fungal effects of benzimidazoles come from their binding to β-tubulin, the main protein in microtubules, which leads to inhibition of the microtubule assembly (Leroux *et al*., 2000). Benzimidazole resistance results from changes in the binding sites on the β-tubulin protein. Studies on a wide variety of fungi have identified several mutations in the β-tubulin gene that confer resistance to benzimidazoles (Faretra & Pollastro, 1991; Yan & Dickman, 1996; Leroux *et al*., 2000). These mutations have not been linked to specific benzimidazole fungicides, but it is clear from the current and the Keinath & Zitter (1998) study that there are some factors (natural or due to mutation) that confer resistance to thiophanate methyl only and not the other benzimidazoles.

Most benzimidazole resistant fungi have been generated by ultraviolet light mutagenesis (Lewis & Papavizas, 1991; Hatvani *et al*., 2006; Paparu *et al*., 2009) while resistance has also been generated by genetic engineering. Ossanna & Mischke (1990) generated benomyl resistant *Gliocladium virens* by transformation with a resistant *Neurospora crassa* β-tubulin gene. Compared to the current study, ultraviolet light and transformations generated more resistant strains for each mutagenesis attempt than gamma irradiation. However, the number of mutants generated by physical mutagenesis (ultra violet light and gamma radiation) could be a function of the radiation dosage since radiation is also detrimental to the survival of conidia. The mutants generated in the current study retained their resistance after sub-culturing for ten cycles and after storage at 4 °C for more than a year. Some benomyl resistant *F. oxysporum* isolates generated by ultraviolet light mutagenesis lost their resistance after storage on 4 °C (Paparu *et al*., 2009).

The mutants MT1 and MT2 showed similar *in vitro* antagonism to grapevine trunk pathogens as the wild type isolates from which they were generated. Similar antagonistic reactions were also reported for the wild type isolates by Kotze *et al*. (2011). The *in vivo* wound protection effect of MT1 alone and in combination with carbendazim was further shown in the field trial. Mutant MT77 had reduced fitness and showed no antagonistic effect *in vitro* against most trunk pathogens compared to the wild type, a result of detrimental mutations by random mutagenesis. However, this is also an indication of the potential of gamma radiation to create novel phenotypes in fungi which can be used in functional genomics studies especially when such mutants can be selected for easily. For example, it can be speculated that if MT77 loss of fungal antagonism compromises its biocontrol action, then a full phenotypic and genetic characterisation of the isolate may reveal the exact mode of action of the wild type *T*. *harzianum* T77 and the genes responsible for its wound protective effect.

The protection of grapevine pruning wounds is currently the best way to prevent the infection of trunk pathogens and there are no post-infection eradication methods for diseased vines without the loss of production. Since wounds remain susceptible to infection for a longer period, and wound protection by fungicides does not persist until the wound is no longer susceptible, some researchers have therefore suggested multiple fungicide applications (Munkvold & Marois, 1993a). Biological control agents such as *F. lateritium* and *Trichoderma* spp. grow on the wound and provide long term protection but lack the instant protective effect. Carter & Price (1975) took advantage of the natural tolerance of *F*. *lateritium* to benomyl and applied the combination of *F. lateritium* and a reduced concentration of benomyl for pruning wound protection against *E*. *lata*. The combination treatment was more effective than the biocontrol agent alone, but not significantly better than benomyl at high concentrations. In the current study, all the treatments reduced wound infection and there was no clear distinction of the superiority of the fungicides only treatments over the biocontrol agents when *Pa. chlamydospora* was inoculated a day after pruning. This highlights the difficulty of pruning wound protection with either chemical or biocontrol agents. Interestingly, the combination of MT1 and carbendazim gave the best control when wounds were inoculated a day after pruning and reduced infection by 70%. Wound protection by all treatments was better when *Pa. chlamydospora* was inoculated seven days after pruning. Kotze *et al*. (2011) reported 64% and 77% reduction in *Pa. chlamydospora* infection by benomyl and UST1, respectively, when the pathogen was inoculated seven days after pruning. In the current study, better control was achieved with the combination treatments which gave at least 85% reduction in *Pa. chlamydospora* infection when the pathogen was inoculated seven days after pruning. Efficacy of the

treatments when the pathogen was inoculated seven days after pruning is increased by the reduced susceptibility as the wounds heal (Munkvold & Marois 1993b; Van Niekerk *et al*., 2011).

Pathogen inoculum pressure had an influence on wound protection in the field. The infection levels of *Pa. chlamydospora* at both inoculation times (90% and 71% in the inoculated controls) was relatively higher than previously reported. Inoculation of spur pruning wounds with *Pa. chlamydospora* resulted in a maximum of 51% infection in Italy (Serra *et al*., 2008), 58% in California (Rolshausen *et al*., 2010), 35% (Kotze *et al*., 2011) and 25% (Van Niekerk *et al.,* 2011) in South Africa. Rolshausen *et al*. (2010) reported a 52% reduction in *Pa. chlamydospora* infection when pruning wounds were treated with thiophanate methyl (Topsin M). It would be expected that under conditions of less inoculum, as was found in the un-inoculated wounds in the *in vivo* evaluation, wound protection should be very effective. The pathogen control found in the un-inoculated wounds is a closer representation of the efficacy of the wound treatments under natural conditions. Wounds that received a combination of the fungicides and biocontrol agent had the lowest incidence of natural infections (although not significantly lower than the rest of the treatments) highlighting the potential for the integration of chemical and biological wound protection.

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.

In conclusion, it was found that all the wild type *Trichoderma* isolates were naturally resistant to thiophanate methyl while strains resistant to carbendazim and benomyl were generated using gamma irradiation. While phenotypic characterisation of the mutant strains revealed that two of the strains were of similar biological fitness as their wild type isolates,

further molecular characterisation may reveal the basis of their resistance, if it differs between the mutants and from that reported in other fungi. The study further highlights a potential use of gamma radiation in plant pathology or mycology generating novel phenotypes that can be used in gene annotation studies.

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Tables and Figures

Table 1: Macro- and microscopic interactions observed between dual cultures of benomyl and carbendazim resistant *Trichoderma* mutant strains and grapevine trunk pathogens.

Specie	**Strain**	**Interactions between *Trichoderma* and pathogen hyphae**							
		Diplodia seriata		*Eutypa lata*		*Neofusicoccum parvum*		*Phaeomoniella chlamydospora*	
		Macro[1]	Micro[2]	Macro	Micro	Macro	Micro	Macro	Micro
T. atroviride	USPP-T1	OG	HA, HD	OG	HA, HC	IZ, OG	HA, HC	OG	HA, HC
	MT1	OG	HA, HD	OG	HA, HC	IZ, OG	HA, HC	OG	HA, HC
T. atroviride	USPP-T2	OG	HA, HD	OG	HA	IZ, OG	HA	OG	HA
	MT2	OG	HA, HD	OG	HA	IZ, OG	HA	OG	HA
T. harzianum	T77	OG	HD	OG	HA, HD	IZ,OG	HA	OG	N
	MT77	N	N	N	N	N	N	OG	N

[1]Macroscopic interactions: OV – overgrowth of the pathogen by the mutant *Trichoderma* strain; IZ – inhibition zone between the mycelium of the biocontrol agent and pathogen before hyphal interaction.

[2]Microscopic interactions: HA – adhesion of biocontrol agent hyphae to pathogen hyphae; HC – coiling of biocontrol agent hyphae around the pathogen hyphae; HD – disintegration of pathogen hyphae when the biocontrol agent hyphae is adhering to the pathogen hyphae; N – No interactions observed.

Table 2: 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}	16.67^{DE}	12.50^{B}
	(42)[3]	(76)	(79)
Thiophanate methyl	41.67^{C}	16.67^{DE}	6.25^{B}
	(53)	(76)	(89)
UST1	43.75^{C}	18.75^{DE}	10.42^{B}
	(51)	(74)	(82)
MT1	43.75^{C}	10.42^{E}	14.58^{B}
	(51)	(85)	(75)
UST1+Thiophanate	45.83^{C}	6.25^{E}	4.17^{B}
methyl	(49)	(91)	(93)
MT1+Carbendazim	27.08^{D}	10.42^{E}	4.17^{B}
	(70)	(85)	(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.

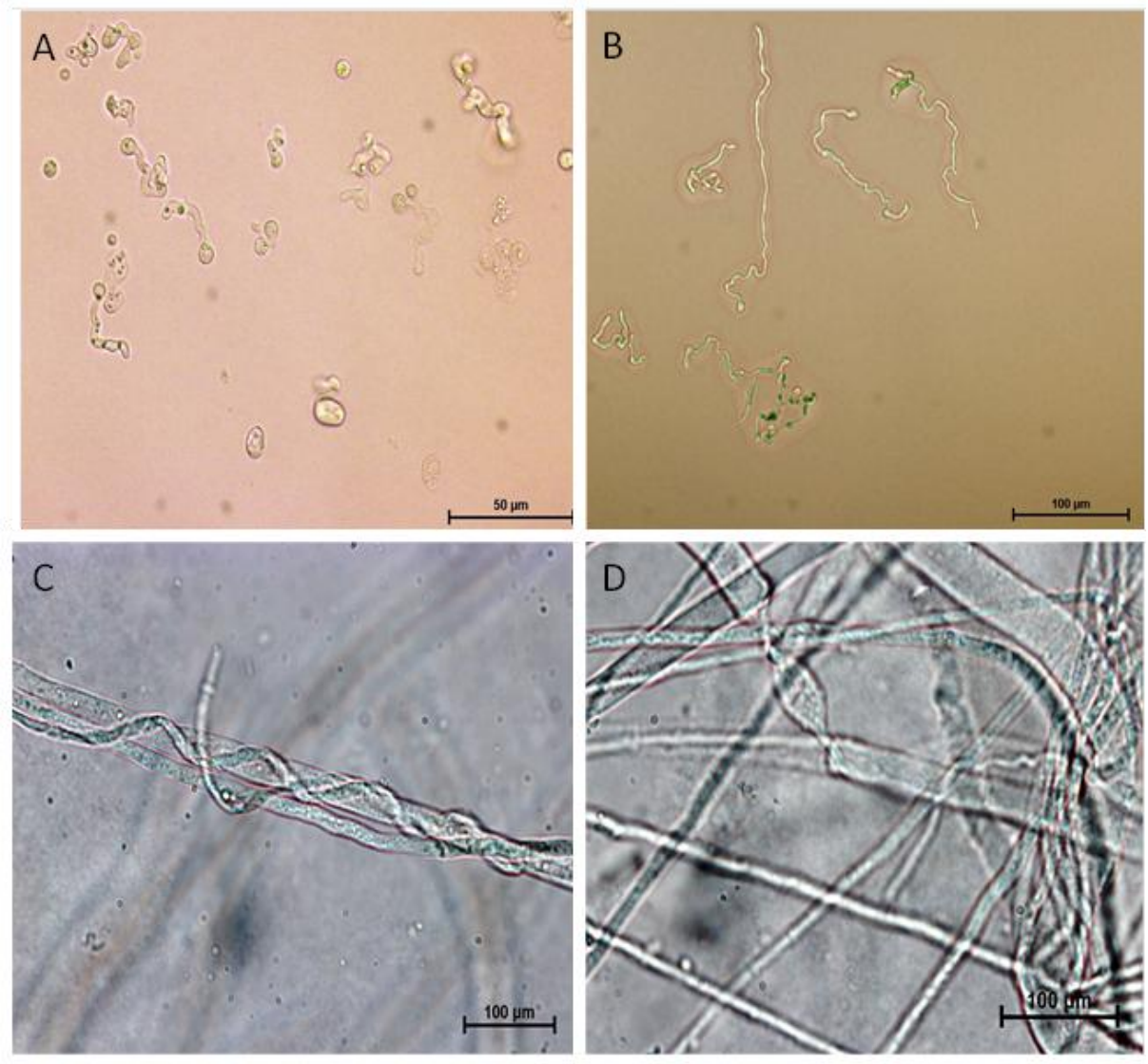

Figure 1: Effect of benomly (10 µg/mL) on conidia germination and germ tube extension of wild type (A) and resistant (B) *Trichoderma atroviride* UST1 and mutant MT1, respectively. Antagonistic action of mutant MT1 on *Neofusicoccum parvum*, coiling around pathogen hyphae (C) and *Diplodia seriata* disintegration of pathogen hyphae (D), both characteristics of mycoparasitism. The resistant mutant was generated by gamma irradiation of the wild type conidia.

Chapter 6

Response of *Vitis vinifera* cell cultures to *Eutypa lata* and *Trichoderma atroviride* culture filtrates: Expression of defence related genes and phenotypes.

6.1 Abstract

Cell suspension cultures of *Vitis vinifera* cv. Dauphine were used in a comparative study of the early response of grapevine to the vascular pathogen, *Eutypa lata*, and the biological control agent *Trichoderma atroviride*, which was used in pruning wound protection. The expression of genes coding for enzymes of the phenylpropanoid pathway and pathogenesis related (PR) proteins was profiled over a 48-hour period using quantitative reverse transcriptase PCR. The cell cultures responded to elicitors of both fungi with a hypersensitive-like response that lead to a decrease in cell viability. Similar genes were triggered by both the pathogen and biocontrol agent but the patterns and magnitude of expression was dependent on the specific fungal elicitor. Culture filtrates of both fungi caused up-regulation of phenylalanine ammonia-lyase (PAL), 4 coumaroyl Co-A ligase (CCo-A) and stilbene synthase (STS), and a down regulation of chalcone synthase (CHS) genes. The pathogen filtrate caused a biphasic pattern in the up-regulation of PAL and STS genes which was not observed in cells treated with filtrates of the biocontrol agent. Phenotypic assays showed significantly higher total phenolic content and chitinolytic enzyme activity in the cell cultures treated with the *T*. *atroviride* filtrate than the pathogen filtrate which corresponded to the higher expression of PAL and chitinase class IV genes. The response of the cell cultures to *T*. *atroviride* filtrate putatively signifies that the induction of grapevine resistance contributes to wound protection by the biocontrol agent.

6.2 Introduction

Eutypa dieback, also called eutyposis, is caused by *Eutypa* (*E*.) *lata* and is an important grapevine trunk disease reported from all major grapevine growing regions worldwide (Pascoe & Cottral, 2000; Rolshausen & Gubler, 2005; Halleen *et al.*, 2010). Eutypa dieback is characterised by stunted new growth, usually with cupped leaves and marginal necrosis, withered inflorescences, wedge shaped wood necrosis, dead arms/trunks and, if infected parts are not removed, the whole vine eventually dies (Munkvold *et al.*, 1994; Gubler *et al.*, 2005). Infection usually occurs through pruning wounds where the fungus colonises exposed xylem vessels and cause necrosis by producing cell wall degrading enzymes (Schmidt *et al.*, 1999; Rolshausen *et al.*, 2008) and toxins (Molyneux *et al.*, 2002; Andolfi *et al.*, 2011). Economic losses due to the disease are primarily a result of reduced

grape yields, increased vineyard management costs and reduced vineyard life (Munkvold *et al.*, 1994).

There are no eradicants to cure Eutypa dieback infected vines except to remove the infected parts and retrain shoots from uninfected trunks, also known as remedial surgery (Sosnowski *et al.*, 2011). Management of Eutypa dieback is primarily dependent on preventing wound infection using cultural, chemical and biological methods. Cultural methods are aimed at avoiding periods when wounds would be highly susceptible while chemical and biological methods are aimed at protecting wounds through their anti-fungal effects. There are limited effective fungicides available in most countries due to the banning and withdrawal of fungicides detrimental to the environmental and toxic to human health (Surico *et al.*, 2008; Rolshausen *et al.*, 2010).

The protection of wounds by non-pathogenic saprophytic bacteria and fungi is well documented (Munkvold & Marois, 1995; John *et al.*, 2005; Kotze *et al.*, 2011). Several biocontrol agents have been registered and most of these use *Trichoderma* (*T.*) species as protective agents. The wound protective effect of these agents is believed to be due to the specific modes-of-interaction between the biocontrol agent and the pathogen, of which mycoparasitism (John *et al.*, 2004) and competitive exclusion (Mutawila *et al.*, 2011a) have been demonstrated in grapevine wood.

Woody species have developed mechanisms to protect themselves against wood pathogens through their continued interaction with pathogens. They respond to wood infection by compartmentalisation of the infected area through the production of cell wall strengthening poly-phenolic and poly-aliphatic compounds in an attempt to impede further fungal ingress (Shigo, 1984). Grapevines respond by plugging the xylem vessels with gums and tyloses to stop further colonisation of the vessels (Amalfitano *et al.*, 2000; Mutawila *et al.*, 2011a), while chemical barriers in the form of phenolic antifungal phytoalexins accumulate in and around the infected areas (Schnee *et al.*, 2008). Grapevine phenolic compounds are products of secondary metabolism and are synthesised by the phenylpropanoid pathway. These phenolic compounds can act directly in defence (phytoalexins) or make up polymers, such as lignin and suberin, which render the cell walls more resistant to pathogens and prevent water loss (Shigo, 1984). Production of stilbenes has been demonstrated in grapevine wood in response to infection by trunk pathogens (Amalfitano *et al.*, 2000 & 2011) and these compounds have been shown to limit the growth of fungi and inhibit activity of their cell wall degrading enzymes (Coutos-Thévenot *et al.*, 2001; Del Río *et al.*, 2004; Lygin *et al.*, 2009; Srivastavaa *et al.*, 2013).

Biosynthesis of the secondary metabolites involves the coordinated regulation of the phenylpropanoid pathway and its flavonoid (anthocyanin), stilbene (phytoalexins) and lignin branch pathways (Figure 1). The deamination of phenylalanine, an amino acid product of the Shikimate biosynthetic pathway, to cinnamic acid is the first committed step of the general phenylpropanoid pathway. This step, catalysed by phenylalanine ammonia-lyase (PAL), represents a switch from primary (Shikimate pathway) to secondary metabolism. The general phenylpropanoid pathway ends by branching to either the synthesis of flavonoids through chalcone synthase (CHS) or the synthesis of stilbenes by stilbene synthase (STS).

Plants also respond to infection by synthesising numerous pathogenesis-related (PR) proteins which may act directly against the pathogen or its infection structures, or indirectly by inducing the production of elicitors that trigger defence mechanisms (Kitajima & Sato, 1999; Van Loon & Van Strien, 1999; Van Loon *et al.*, 2006; Fung *et al.*, 2008; Gomès & Coutos-Thévenot, 2009). Several grapevine PR genes have been characterised and these include, signalling PR 1 (Wielgoss & Kortekamp, 2006), hydrolytic enzymes PR 2 (β-1,3 glucanases), PR 3, PR 4 (chitinases) and chitinase class IV (CHIT IV), PR 5 (osmotin) (Jacobs *et al.*, 1999), anti-microbial PR 6 (serine proteases inhibitor) and polygalacturonase inhibiting proteins (PGIP) (De Lorenzo & Ferrari, 2002). Expression of PR proteins is selective and depends on the infecting pathogen or the elicitor (Glazebrook, 2005). A state of enhanced defence capacity by mobilisation of cellular responses (phytoalexins and PR proteins) before or after pathogen attack can be induced by micro-organisms or products of the hydrolysis of plant cell polymers (Bishop & Ryan, 1987; Aziz *et al.*, 2007; Hématy *et al.*, 2009; Seifert & Blaukopf, 2010). Induced response is regulated by signalling molecules such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) which act as messengers resulting in the expression of different downstream signal-dependent defence pathways (de Wit, 2007). Studies in *Arabidopsis thaliana* have revealed that SA mediates systemic acquired resistance (SAR) and resistance to biotrophic pathogens. On the contrary, JA and ET mediate induced systemic resistance (ISR) and resistance to necrotrophic pathogens (Glazebrook, 2005).

There are currently only a few studies on the response of grapevines to *E. lata* (Rotter *et al.*, 2009; Camps *et al.*, 2010). Most of what is known on grapevine response to fungal pathogens derives from studies on foliar and fruit pathogens while little is known about response to wood pathogens. The lack of information on grapevine-trunk pathogen interactions derives from the difficulty of carrying out gene expression studies on potted or field vines. This is mainly due to the impracticality of isolating RNA from lignified vascular tissue and the difficulty of separating biotic from abiotic response. Even when RNA is isolated from the wood, there is high likelihood of dilution of transcripts from the site of

infection by those from healthy parts since infection is rarely uniformly spread. For these reasons, the available studies on grapevine-*E*. *lata* interactions have been carried out on grapevine plantlets (Rotter *et al.*, 2009) and leaves expressing symptoms (Camps *et al.*, 2010) rather than wood. Moreover, these studies investigated the response during symptom expression (7 weeks after infection) and did not consider the early response of the grapevine to the pathogen. An understanding of these early defence responses would be critical for understanding disease progression and be useful in cultivar comparative studies aimed at the development of pathogen resistant or tolerant cultivars. Cell suspension cultures provide a useful and reproducible model system for the study of early grapevine response to infection, as demonstrated by Lima *et al.* (2012). These authors showed the usefulness of cell suspension cultures in evaluating the response of grapevine to *Phaeomoniella* (*Pa.*) *chlamydospora*, which is another grapevine trunk pathogen.

In the current study grapevine cell cultures were used in a comparative study profiling their response to culture filtrates of *E*. *lata* and *T. atroviride*. Grapevine cell suspensions, derived from green (pea size) berries of *Vitis* (*V*.) *vinifera* cv. Dauphine, were used. These suspensions were previously characterised (Sharathchandra *et al.*, 2011) and shown to constitutively produce low levels of defence related proteins. In this study the cell suspension cultures were treated with extracts from the two fungi in a time-course experiment of molecular gene expression (qRT-PCR) and metabolite profiling (enzyme activities and polyphenol content). The results obtained are discussed in context of grapevine pruning wound susceptibility to *E*. *lata* infection and putative involvement of *Trichoderma*-grapevine interactions in pruning wound protection by the biocontrol agent.

6.3 Materials and Methods

6.3.1 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.

6.3.2 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.

6.3.3 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 6th 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.

6.3.4 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:

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

6.3.5 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.

6.3.6 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.

6.3.7 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.

6.3.8 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.

6.3.9 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.

6.3.10 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.

6.3.11 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.

6.4 Results

6.4.1 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 (Figure 2). Elicitation of cells resulted in browning of cell suspension cultures and reduction of cell viability in an elicitor concentration dependent manner (Figure 3). 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 (Figure 3). 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.

6.4.2 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.

Figures 4 and 5 show relative gene expression levels over time for the enzymes of the phenylpropanoid pathway in response *E. lata* and *T. atroviride* filtrates. 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 (Figure 4). 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 (Figure 5). 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 (Figure 5).

Figures 6 and 7 show the time-course expression levels of pathogenesis related proteins after elicitation by fungal filtrates of *E. lata* and *T. atroviride*. 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) (Figure 6). 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 (Figure 6). 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 (Figure 7). 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 (Figure 7). 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 (Figure 7). 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.

6.4.3 Total phenol content of cell cultures

Treatment of cell cultures with cell free fungal broth elicitors resulted in a significant ($P < 0.001$; Appendix B, Table 2) increase in the content of phenolic compounds (Figure 8). 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.

6.4.4 β-1, 3-glucanase activity

The β-1, 3-glucanase activity of the cell cultures at 24 and 48 hours after treatment with elicitors from the *T. atroviride* and *E. lata* are shown in figure 9. Elicitation resulted in significant ($P < 0.001$; Appendix B, Table 2) 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.

6.4.5 Chitinolytic activity

The chitinolytic activity of cell cultures harvested at 24 and 48 hours after treatment with fungal elicitors are shown in figure 10. The untreated cells exhibited some chitinolytic activity indicating constitutive activity, but elicitation resulted in a significant ($P = 0.0014$; Appendix B, Table 2) 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.

6.5 Discussion

Treatment of the cell cultures with the fungal culture filtrates induced changes in the gene expression of grapevine cell cultures typical of response to fungal infection. Elicitors in the fungal filtrates may include remnants of fungal cell walls and proteins secreted by the fungi into the medium. These elicitors were heat stable since autoclaved fungal filtrates retained their plant cell eliciting activity even though the levels of expression in some cases differed from those triggered by the fresh filtrates. Cellulases and proteinases from some biocontrol strains of *T. virens* have been shown to elicit hypersensitive responses in cell cultures (Calderón *et al.*, 1993; Hanson & Howell, 2004). *Eutypa lata* secretes plant cell wall degrading enzymes (Rolshausen *et al.*, 2008) which would also trigger plant responses. The current study adds to the growing knowledge of molecular interactions between grapevine and pathogenic and non-pathogenic fungi. Previous studies reporting on the response of grapevines to *E. lata* used diseased or infected grapevine plants in the vineyard and glasshouse (Camps *et al.*, 2010), infected grapevine plantlets (Rotter *et al.*, 2009) and cell cultures elicited with phytotoxic secondary metabolites of the fungi (Afifi *et al.*, 2003).

The berry suspension cultures used in the current study were derived from the pea size hard and green pre-véraison berries. Proteomic analysis of these cells showed low expression levels of PR proteins (Sharathchandra *et al.*, 2011), making them ideal for elicitation studies. Gene and phenotypic expression by the cell cultures was dependent on the elicitor, further confirming the suitability of the cell cultures.

Fungal cultural filtrates have been successfully used for *in vitro* screening of cultivar susceptibility to fungal pathogens (Thakur *et al.*, 2002; Esmaiel *et al.*, 2012). In the current study, grapevine cell cultures treated with the fungal filtrates exhibited a hypersensitive-like response. This was more pronounced in the cells treated with the biocontrol agent elicitors as shown by the decline in cell viability. This was accompanied by changes in gene expression of defence related genes of the phenylpropanoid pathway as well as PR proteins. The synthesis of phenyl-propanoids in plants is considered an adaptive mechanism for defence against any kind of stress as their synthesis is elicited by both biotic and abiotic stimuli (Langcake, 1981). Grapevine phytoalexins belong to the stilbene family and are derivatives of resveratrol, a polyphenolic compound synthesised via the phenypropanoid pathway. The first reaction to the pathway is catalysed by phenylalanine ammonia-lyase (PAL) and the last step to resveratrol synthesis is catalysed by stilbene synthase (STS). A previous study on the response of grapevine plantlets to *E. lata* reported an up-regulation of PAL and other genes coding for enzymes of the flavonoid pathway but did not evaluate the expression of STS genes (Rotter *et al.*, 2009). In the current study, the cell cultures responded to the culture filtrates by the up-regulation of both PAL and STS genes. There was a bi-phasic pattern in the up-regulation of PAL and STS transcripts in the cell cultures treated with the pathogen elicitor indicating a coordinated expression of genes of the phenylpropanoid pathway. A bi-phasic pattern in the expression of PAL and STS was also reported in grapevine cell cultures in response to cell wall extracts of another grapevine trunk pathogen, *Pa. chlamydospora* (Lima *et al.*, 2012). However, the bi-phasic response could be due to activity of two or more elicitors, all present simultaneously, since in both the current and the Lima *et al.* (2012) studies, crude extracts (culture filtrate and fungal biomass, respectively) were used for the elicitation of cell cultures. A characterisation of the fungal extracts could clarify if this is due to a single or multiple elicitors.

In the current study, up-regulation of the PAL gene was much higher in the cells treated with the *T. atroviride* elicitors compared to those treated with *E. lata* elicitors. It would be expected that when triggered, genes for all the enzymes involved in a specific pathway are up-regulated to similar levels for significant increase in metabolic products of that pathway (in this case stilbenes). It is therefore, not unexpected that the cells treated with elicitors from the biocontrol agent had significantly higher total phenolic compounds than the

cells treated with the pathogen filtrates (Figure 8). The cell cultures used in the current study are from a white grapevine cultivar, Dauphine, which lack anthocyanins (Boss *et al.*, 1996) and hence the increase in phenolic content of the cells is only likely to be due to the accumulation of stilbenic compounds. However, the STS gene expression results without the phenotypic characterisation should be interpreted with caution stemming from the recent annotation of STS genes in the *V. vinifera* cv. Pinot noir genome that has revealed an unusually large STS gene family (Vannozzi *et al.*, 2012).

The efficacy of the increase in phenolic compounds in stopping *E. lata* infection in grapevine wood tissue is equivocal. Coutos-Thévenot *et al.* (2001) have shown that resveratrol limits the growth of *E. lata in vitro* while Del Río *et al.* (2004) showed that phenolic compounds inhibited activity of its cell wall degrading enzymes suggesting that phenolic compounds play a role in limiting wood colonisation *in vivo*. The accumulation of phenolic compounds in response to either *E. lata* or *Trichoderma* species has not been shown in grapevine wood despite extensive knowledge of the wound protective effect of pruning wound saprophytes, some of which have no direct antagonistic effect on the pathogens (Munkvold & Marois, 1995; Chapuis *et al.*, 1998). However, the accumulation of phenolic compounds has been demonstrated in grapevine response to *Pa. chlamydospora* (Amalfitano *et al.*, 2000). This response to *Pa. chlamydospora* was shown to be insufficient to stop the pathogen growing in the wood tissue as the pathogen was observed actively growing in xylem vessels clogged with phenolic deposits (Mutawila *et al.*, 2011a). Comparative studies of the response of cell lines of susceptible *V. vinifera* and generally resistant *V. rupestris* to a bacterial effector, Harpin elicitor, also alluded to similar conclusions. Both *Vitis* species responded with an increased STS transcription, but levels were lower in susceptible *V. vinifera* compared to *V. rupestris* and resveratrol accumulation in the cells followed a similar pattern as the transcription (Qiao *et al.*, 2010; Chang *et al.*, 2011). It could be concluded from the current study that grapevines exhibit a basal response to *E. lata*, an indication of plant recognition of the pathogen but the response may not be sufficient to stop infection. Induction of the accumulation of phenolic compounds by a biocontrol agent could aid wound protection from pathogen infection. The synthesis of stilbenes is induced through the induced systemic resistance pathway involving signalling molecules jasmonic acid and ethylene (Belhadj *et al.*, 2008; Lijavetzky *et al.*, 2008). Recently, Lima *et al.* (2012) have shown that grapevine cell cultures respond to methyl jasmonate (methyl ester of jasmonic acid) in a similar pattern as they respond to *Pa. chlamydospora*. The application of *T. harzianum* to pruning wounds of kiwi fruit (*Actinidia deliciosa*) has been demonstrated to increase the content of wood phenolic compounds

which aids wound protection against wood pathogens *Phaeacremonium aleophilum* and *Fomitiporia mediterranea* (Neri *et al.*, 2008).

There are no known Eutypa-dieback resistant cultivars, but the cultivar Merlot is considered more tolerant to the disease. Tolerance in Merlot has largely been attributed to its ability to break down phytotoxins, secreted by the fungi (Colrat *et al.*, 1999), as well as a relatively higher wood lignin content compared to susceptible cultivars (Rolshausen *et al.*, 2008). In addition to antifungal effects, grapevine stilbenes could also be involved in cell wall strengthening. Adrian *et al.* (2012) observed the accumulation of resveratrol on to cell walls from culture medium after treatment of grapevine cell suspension cultures with resveratrol. In the same study, absorption of resveratrol by leaf petioles resulted in its localisation in cell walls of non-lignified (parenchyma and collenchyma) and lignified (xylem and sclerenchyma) tissue. Localised cell wall thickening and increased fluorescence of the thickened walls, which is likely due to stilbene accumulation, was also observed in mature grapevine wood infected with *E. lata* (Mutawila *et al.*, 2011a).

The up-regulation of STS transcripts was at the expense of the flavonoid pathway which branches from the phenylpropanoid pathway. Chalcone synthase (CHS) catalyses the first committed step in the synthesis of flavonoids using/competing for the same substrates, coumaroyl CoA and malonyl CoA, as STS. A switch from flavonoid synthesis to stilbene synthesis signifies a shift to defence metabolism. Similar results were also obtained with grapevine cell culture elicited by the bacterial effector Harpin (Qiao *et al.*, 2010) and in grapevine leaves elicited by *Plasmopara* (*Pl.*) *viticola* (causal organism of downy mildew) (Vannozzi *et al.*, 2012). However, this is in contrast to the results obtained from grapevine plantlets infected with *E. lata*, where an overexpression of genes of the flavonoid pathway (chalcone synthase, chalcone isomerase, dihydroflavonol 4-reductase and anthocyanidin reductase) was reported (Rotter *et al.*, 2009). This discrepancy can be explained by the use of transcripts from the whole plantlet (leaves and stems), in the Rotter *et al.* (2009) study, rather than the infected parts where response is likely to be localised. In cell suspension cultures, all cells are in contact with the elicitor and are likely to respond similarly. Furthermore, in the Rotter *et al.* (2009) study, the plantlets were harvested seven weeks after infection and hence the response observed may not represent the early events in the interaction of the pathogen and the plant. In another study, treatment of grapevine cell suspension cultures with *E. lata* phytotoxin, eutypine, had no effect on the expression of both CHS and leucoanthocyanidin dioxygenase (LDOX) but UDP glucose-flavonoid glucosyltransferase (UFGT) (Afifi *et al.*, 2003). This may show that grapevine cells respond differently to the pathogen proteins/enzymes compared to the toxin. The suppression of the flavonoid pathway following exposure to fungal pathogens has also been shown in other

plant species namely parsley (Lozoya *et al.*, 1991), onion (Mclusky *et al.*, 1999) and sorghum (Lo & Nicholson, 1998). It is important to note however, that STS genes are known to occur as a family of closely related highly conserved genes and it is difficult to clearly discriminate between individual members (Vannozzi *et al.*, 2012) and in the current study, only two STS genes were tracked. Unlike STS which belongs to a large gene family, CHS is coded by three genes in the grapevine (Vannozzi *et al.*, 2012). It can therefore be concluded from the current study, that flavonoid synthesis is repressed in the early response of grapevines experiencing biotic stress.

The cell cultures also responded to the fungal filtrates by activating some of the genes coding for pathogenesis related proteins. Significant down-regulation of PR 1 was observed in cells treated with elicitors from the biocontrol agent (at 6, 12 and 48 hours) while there were no significant changes in the cells treated with the pathogen elicitor. This is contrary to the study of Camps *et al.* (2010) where PR 1 overexpression was found in leaves of diseased grapevines. Wielgoss & Kortekamp (2006) found that PR 1 was constitutively expressed in grapevine callus and there was no further induction after treatment with elicitors from the biotrophic pathogen *Pl. viticola*. Constitutive expression in callus cells was attributed to induction by sugars in the culture medium. However, PR 1 was induced in leaves of grapevine plants inoculated with *Pl. viticola* (Wielgoss & Kortekamp, 2006). The down regulation of PR 1, in the current study, could hint to an alternative pathway to grapevine response to a pathogen/non-pathogen compared to that of the biotrophic pathogen *Pl. viticola*. However, this cannot be concluded from this study as there were no significant changes in PR 1 expression in response to the necrotroph *E. lata*.

A coordinated expression of PR 2 and PR 5 was observed. These genes exhibited a single peak at similar time for each specific elicitor, 12 and 24 hours post treatment for the pathogen and biocontrol elicitors, respectively. A coordinated over expression of PR 2 and PR 5 has also been observed in *Arabidopsis thaliana* inoculated by the necrotrophic bacteria *Erwinia carotovora* sbsp. *carotovora* (Li *et al.*, 2004). PR 1, together with PR 2 and PR 5 are considered markers for systemic acquired resistance (SAR). In most herbaceous species the activation of these genes is classically believed to be regulated by the signalling molecule salicylic acid (Van Loon & Van Strien, 1999; Van Loon *et al.*, 2006a; Loake & Grant, 2007). Transcription factors of the WRKY family have been shown to be responsible for the regulation of expression of PR 2 and PR 5 in grapevine (Marchive *et al.*, 2013) and *A. thaliana* (Li *et al.*, 2004). A slightly higher relative expression of PR 2 in the fresh pathogen elicitor treated cells after 12 hours also resulted in a significantly higher β-1, 3-glucanase activity in the pathogen treated cells at 24 hours compared to cells treated with the fresh elicitor of *T. atroviride*. The antifungal effect of β-1, 3-glucanase has been demonstrated on

the grapevine powdery mildew fungus, *Erysiphe nector* (syn. *Uncinula nector*), where β-1, 3-glucanase activity of leaves was correlated with resistance (Giannakis *et al.*, 1998). The same study also found a synergy between the β-1, 3-glucanase and chitinase enzymes in limiting fungal growth.

Chitinase pathogenesis proteins (CHIT IV, PR 3 and 4) were also triggered by culture filtrates of both the pathogen and the biocontrol agent, although the expression patterns were different. Peak expression of CHIT IV occurred earlier, before peak expression of PR 3 and 4, which may imply a role by CHIT IV in the regulation or induction of other chitinases. Chitinase class IV proteins are found in the apoplast (Anand *et al.*, 2004; Pechanova *et al.*, 2010) and these apoplastic chitinases are believed to be part of the early defence response as they act directly on the invading fungal hyphae (Gerhardt *et al.*, 1997). The action of apoplastic chitinases on hyphae releases fungal elicitors that penetrate the plant cells and further trigger other defence genes (Kasprzewska, 2003; Grover, 2012). Expression levels of CHIT IV and PR 3 were much higher in cells treated with the culture filtrate of the biocontrol agent as compared to the pathogen and likewise chitinolytic activity was higher in the cells treated with the biocontrol agent filtrate than those elicited with the pathogen filtrate.

Also interesting was the much higher overexpression of PR 6, the protease inhibitor, in cells treated with the *T. atroviride* compared to those treated with the pathogen culture filtrates. Proteases are essential for the breakdown of plant proteins for nutrition of the invading pathogens. PR 6 is induced by the jasmonic acid signalling pathway and which is also associated with induced systemic resistance (ISR) (as reviewed by Koiwa *et al.*, 1997 and Haq *et al.*, 2004). A cysteine protease inhibitor from pearl millet was shown to have potent antifungal activity against several plant pathogenic fungi and a wood saprophyte, *T. reesei* (Joshi *et al.*, 1998). It is possible that during the grapevine-*Trichoderma* interactions, the host regulates growth of the saprophyte by producing inhibitors to the *Trichoderma* proteases. These inhibitors are also likely to inhibit pathogen proteases if infection was to occur after the host response to *Trichoderma* spp. Pathogenesis related proteins have been found in xylem sap (Buhtz *et al.*, 2004; Kehr *et al.*, 2005) where their antifungal activity may inhibit xylem pathogens while products of such activity may trigger downstream defence pathways through pathogen associated molecular patterns.

The protection of grapevine pruning wounds by *Trichoderma* spp. is largely attributed to the antagonistic effect of the biocontrol agent against trunk pathogens. The response of the cell cultures to the *Trichoderma* filtrates by increasing the phenolic content and activity of fungal cell wall hydrolytic enzymes, suggests a more complex involvement in the grapevine-*Trichoderma* interactions to afford pruning wound protection. *Trichoderma* colonisation could prime the wound for a quicker and more intensive response to trunk pathogen infection,

which warrants further study. In a study of grapevine cultivar variability to pruning wound protection by *Trichoderma* spp., some cultivars were identified where extensive wound colonisation by *Trichoderma* spp. could not reduce pruning wound infection (Mutawila *et al.*, 2011b). This could have been a result of *Trichoderma*-grapevine interactions that negatively influence the grapevine-pathogen interactions. Further studies could also compare defence response of grapevine cultivars to colonisation by the biocontrol agent and how it may affect infection by trunk pathogens.

In conclusion, the grapevine cell cultures responded to elicitors from the trunk pathogen *E. lata* and a wound protectant biocontrol agent *T. atroviride* by increasing expression of defence related genes on the phenylpropanoid pathway and some PR proteins. Response to *T. atroviride* elicitors was, for several of the defence genes and phenotypes, more pronounced compared to response to the pathogen elicitors. This may suggest the involvement of *Trichoderma*-grapevine interaction in the protection of pruning wounds and/or a priming effect of the grapevine against pathogen infection, but this needs further investigation *in planta*. Response of grapevine cell cultures to elicitation by culture filtrates of *E. lata* and *T. atroviride*, in the current study, triggered genes associated with both the salicylic acid and jasmonic acid signalling pathways. Induced resistance in plants can be obtained by SAR mediated by salicylic acid or through ISR mediated by jasmonic acid and/or ethylene (Van Loon *et al.*, 2006 a,b). These pathways are not mutually exclusive such that some pathogens can trigger both SAR and ISR (Kariola *et al.*, 2003; Li *et al.*, 2004). It is not known what kind of synergistic or antagonistic interactions occur between these pathways. The cell culture model that was developed in this study can provide a simple but reliable system for gene expression studies. The different patterns of expression of the defence related genes could be an indication of the participation of transcription factors in the response of grapevine to pathogenic and non-pathogenic fungi which later determine whether interactions will result in disease, resistance or symbiosis.

6.6 References

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Tables and Figures

Table 1: Primers used in quantitative reverse transcriptase-PCR and GenBank accession of the sequences on which primer design was based.

Gene	Accession No.	Primer	Sequence (5'-3')	Amplicon size	Annealing Temp (°C)
PR1	XM002273752	Sense	GCAACTATATCGGACAACGTCCTT	80	56
		Antisense	TCACCATGCTCTAACAGTACCCA		
PR2	XM002277475	Sense	TTCAAGCCTGAAGTCACGTCC	89	56
		Antisense	TAAGGGTACAGGTTAACAAGCAGT		
PR3	XM002281734	Sense	GGTAGACCTGGTAAACAACCCT	85	56
		Antisense	GGGTGTCATCCAGAACCAGAAG		
PR4	XM002264684	Sense	GCCCAGAGCGCCAGCAATGT	125	56
		Antisense	CGCCATGCCAAGGGCTTGCT		
PR5	XM002282928	Sense	TGGGCACATTTTCGTGGTCATGT	138	58
		Antisense	ACTTGGACGGGACCATAGAGGTTAG		
PR6	XM002284411	Sense	AACCATTAAGAGGGAGAATCCTCA	95	56
		Antisense	CACGGACCCTAGTGCAGTAAA		
CHIT IV	XM002275480	Sense	GTGTGTCCGGGAAGGATTACT	99	54
		Antisense	TCAAGCCATCAAACCCAATGC		
PAL	XM002281763	Sense	GGTGAGCTTCACCCCTCCAGGT	96	56
		Antisense	GGAGCTGCAGGGGTCATCAATGT		
CHS1	AF020709	Sense	CATTGGTGCAGACCCAGATAC	94	54
		Antisense	GATTGCACCCTCGGAGTCG		
CHS3	XM002263983	Sense	CCGCTGTTATAGTTGGTTCCG	81	56
		Antisense	GGATTGTCTGGGCTGCTGA		
STS	XM002268806	Sense	AAGGGTCCGGCCACCATCCT	115	58
		Antisense	ACGCAGTCATGTGCTCGCTCT		
STS2	XM003634020	Sense	TCGAAGCAACGAGGCATGTGCTAA	118	58
		Antisense	TCACCTGTGGTGGCCCTCTCC		
4CL	XM002273418	Sense	ATTGTTACGGAAAGGCGGT	113	56
		Antisense	GGATTGAAGCAATGGTCCTAGC		
Actin	AF369525	Sense	TGGTCGTACAACTGGTATTGTGCTG	116	58
		Antisense	CACGTCCAGCAAGGTCAAGACGA		
60SRP	XM002270599	Sense	ATCTACCTCAAGCTCCTAGTC	165	50
		Antisense	CAATCTTGTCCTTTCCT		
VATP16	XM002269086	Sense	CTTCTCCTGTATGGGAGCTG	112	50
		Antisense	CCATAACAACTGGTACAATCGAC		

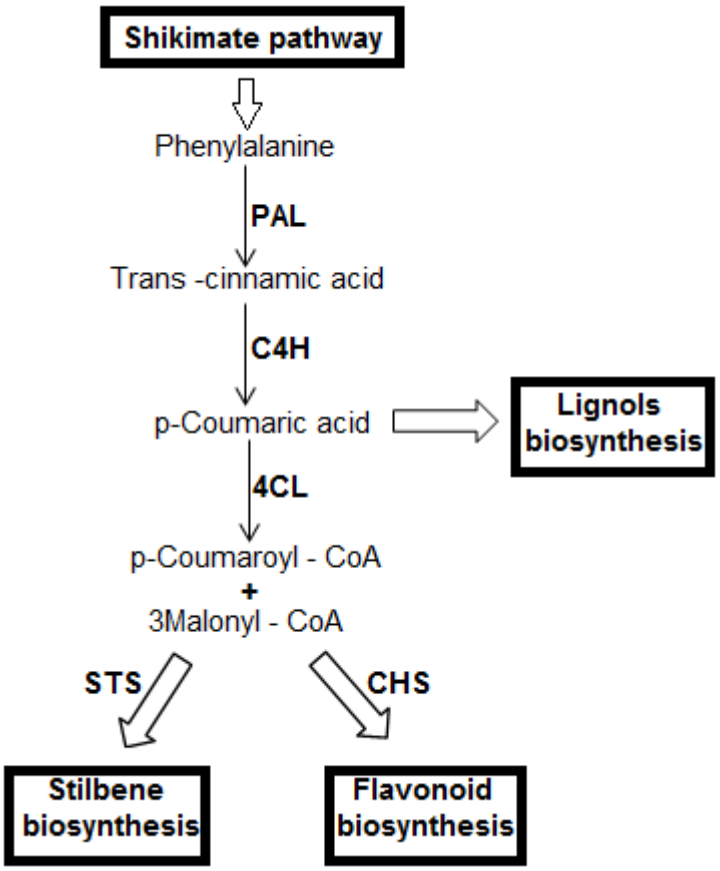

Figure 1: A simplified representation of the general phenylpropanoid pathway showing the switch from primary metabolism (shikimate pathway) and the branches leading to the sub-pathways for the synthesis of stilbenes (phytoalexins), flavonoids (anthocyanins and tannins) and ligninols (monomers of lignin). Enzymes catalysing each step are: PAL, phenylalanine lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl CoA ligase; STS, stilbene synthase and CHS, chalcone synthase.

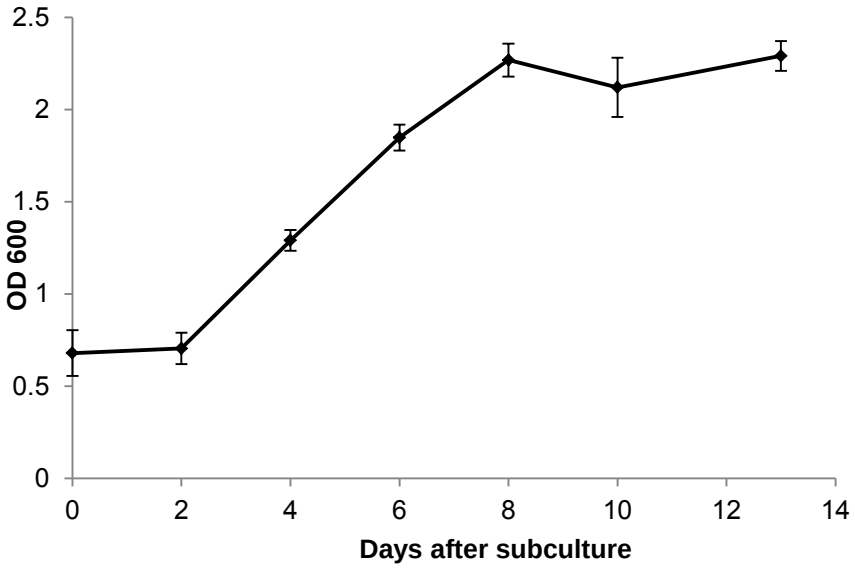

Figure 2: Growth curve of cell suspension culture of *Vitis vinifera* cv. Dauphine derived from green berry explants. Suspension elicitation was carried out at day six (log phase). Each point on the line is the mean ± the standard deviation of two independent biological replicates.

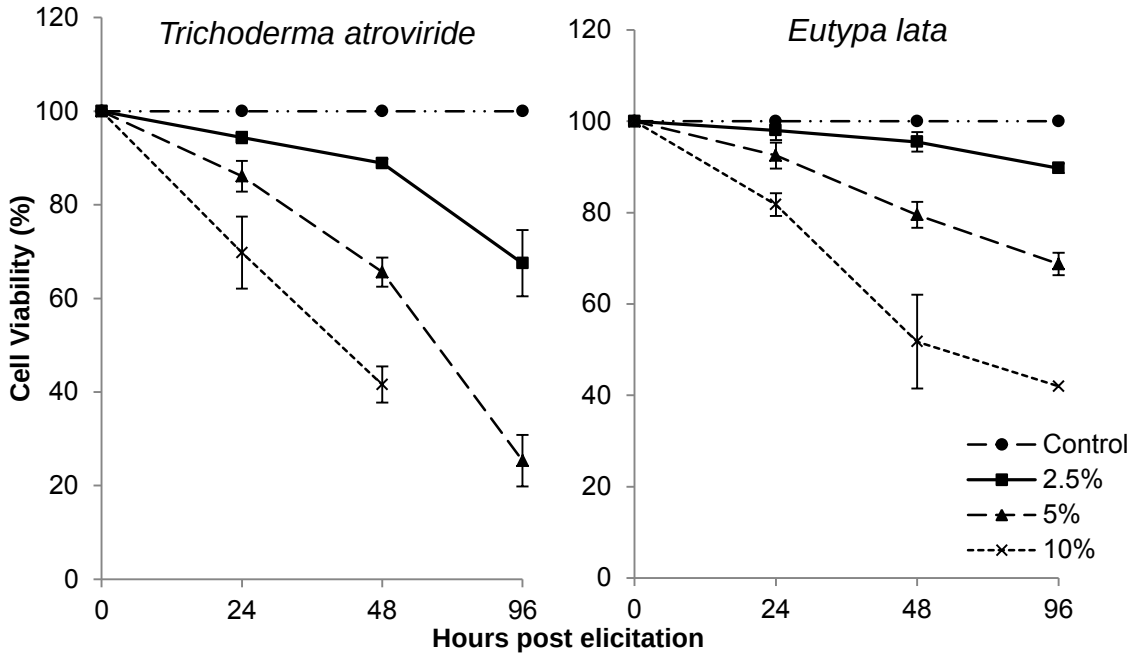

Figure 3: Concentration depended, decrease in the viability of grapevine cell suspensions after elicitation with fresh cell free culture filtrates of *T. atroviride* and *E. lata*. Cell suspension cultures were treated with varying concentrations (2.5, 5 and 10% v/v) of the elicitor on day six after sub-culturing and cell viability measured at 24 hour intervals using the TTC (2, 3, 5-triphenyl tetrazolium) test. Reduction of TTC was not detected in cultures treated with 10% filtrate of *T. atroviride* after 96 hours. Values are means ± the standard deviation of two independent biological replicates.

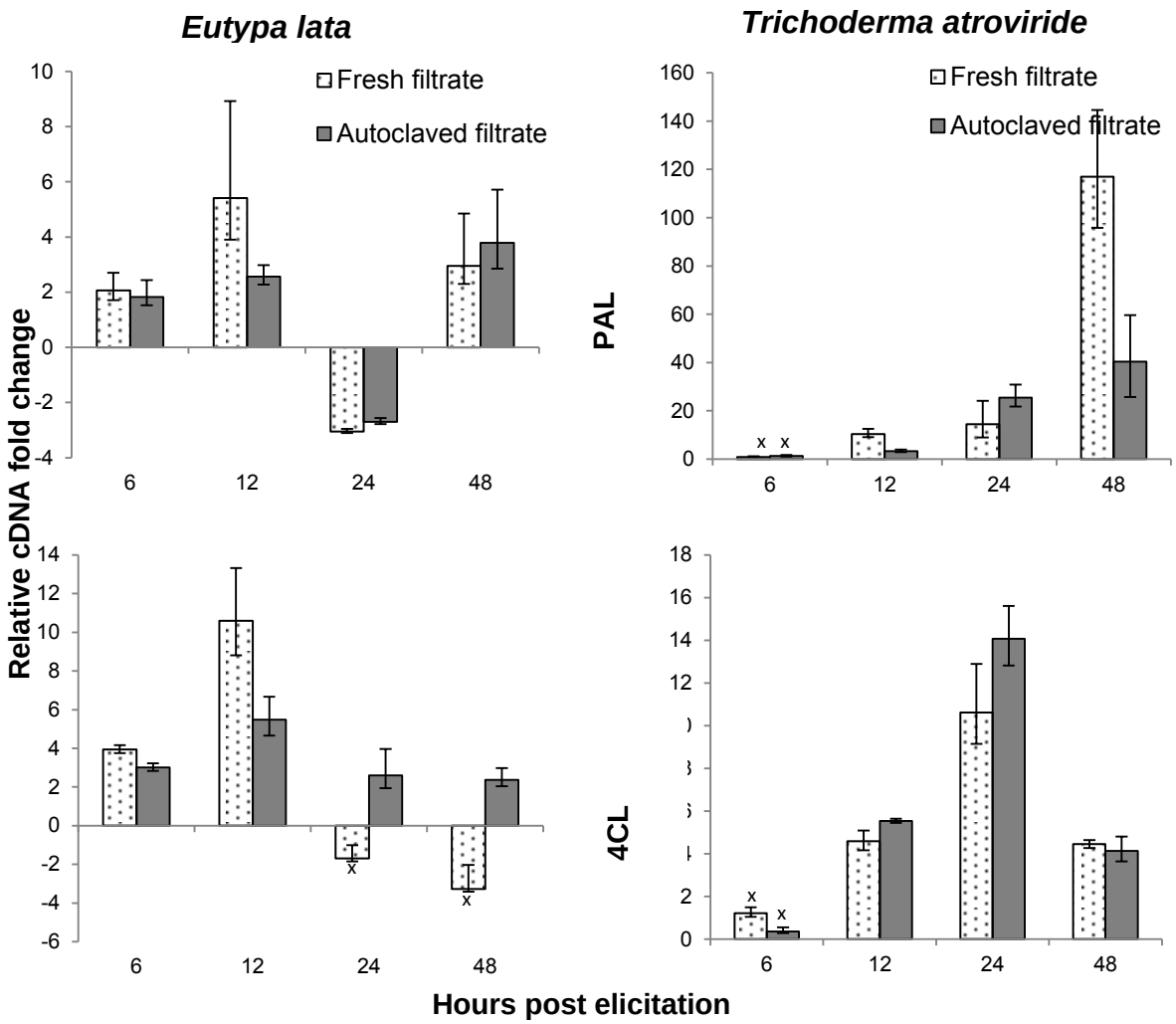

Figure 4: Time-course changes in relative expression of genes coding for enzymes of the general phenylpropanoid pathway, phenylammonium lyase (PAL) and 4-coumaryl Co-A ligase (4CL), in *V. vinifera* cv. Dauphine cell cultures treated with freshly prepared or autoclaved fungal culture filtrates of trunk pathogen *E. lata* and bio-control agent *T. atroviride*. Expression was measured relative to untreated controls for each time point. Bars are mean ± standard deviation of three independent biological replicates. Where expression was not significantly different ($P > 0.05$) from the untreated control the bars are labelled with an 'x'.

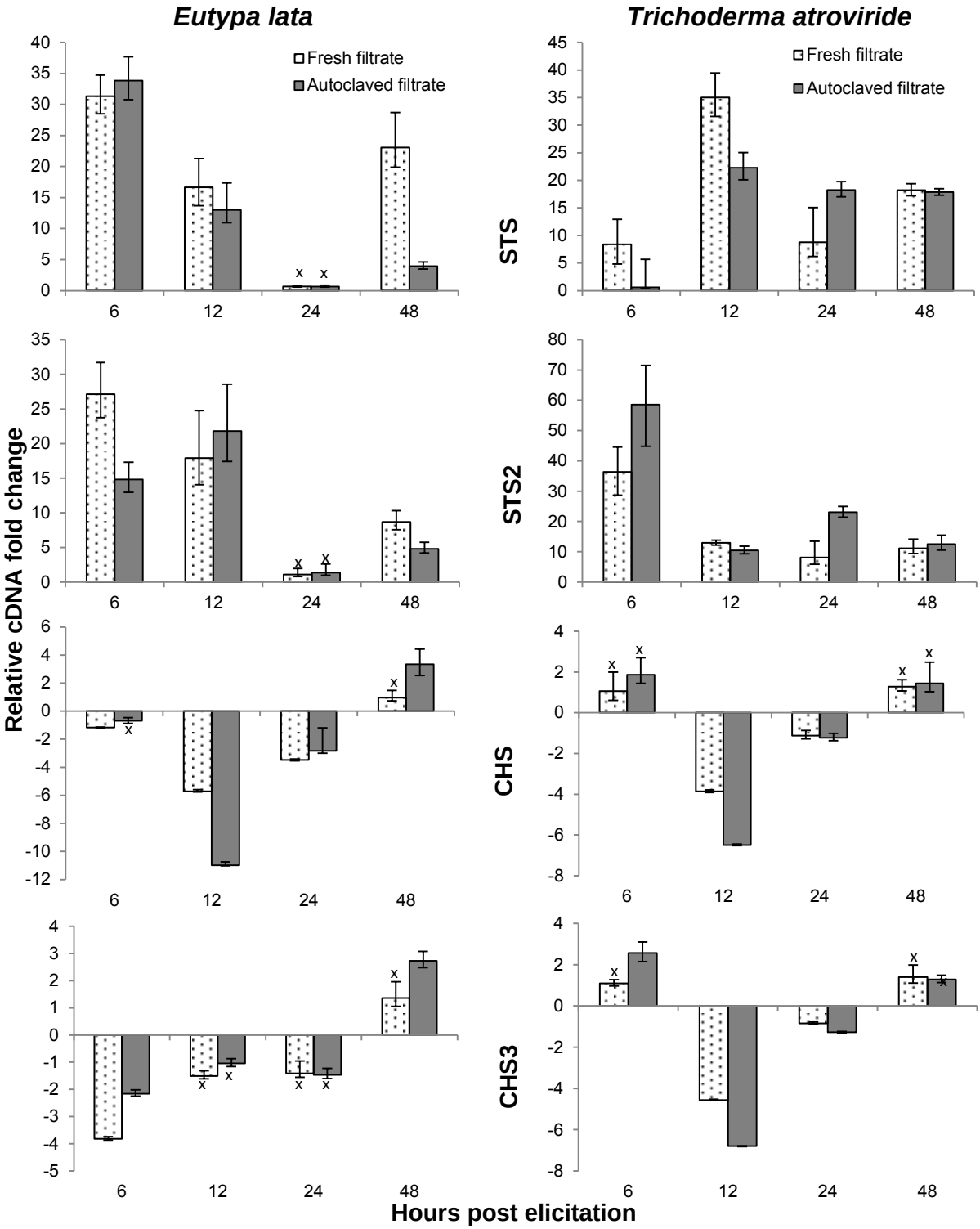

Figure 5: Relative gene expression in *V. vinifera* cv. Dauphine cell cultures treated with freshly prepared or autoclaved fungal culture filtrates of trunk pathogen *E. lata* and bio-control agent *T. atroviride* showing the up-regulation of stilbene sysnthase (STS and STS2) and down-regulation of chalcone synthase (CHS and CHS3) enzymes of the phenylpropanoid pathway. Expression was measured relative to untreated controls for each time point. Bars are mean ± standard deviation of three independent biological replicates. Where expression was not significantly different ($P > 0.05$) from the untreated control the bars are labelled with an 'x'.

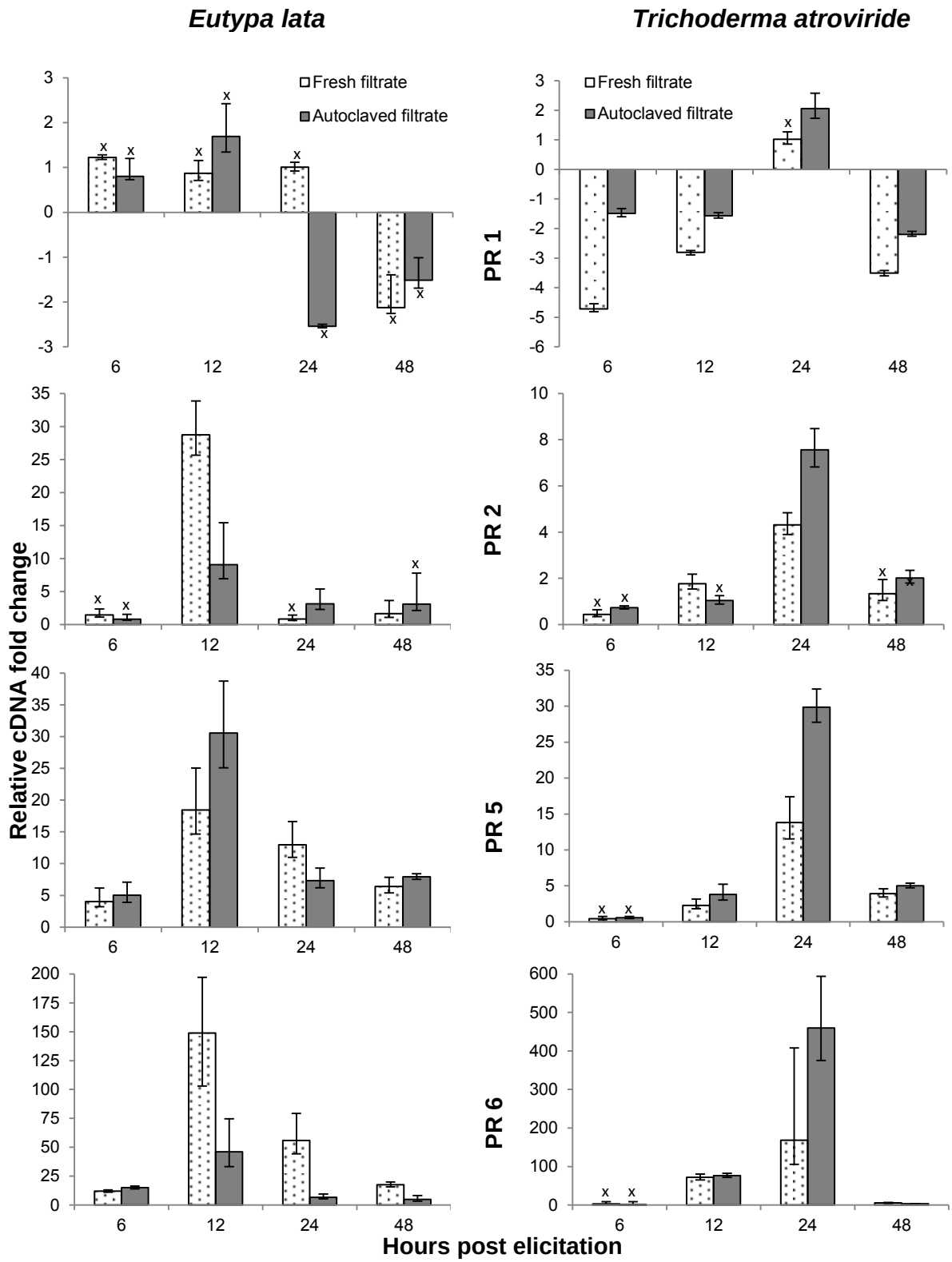

Figure 6: Relative expression of genes coding for pathogenesis related (PR 1, 2, 5 and 6) protein in *V. vinifera* cv. Dauphine cell suspension cultures at 6, 12, 24 and 48 hours after treatment with freshly prepared or autoclaved culture filtrates of either *E. lata* or *T. atroviride*. Expression was measured relative to untreated controls of each time point. Bars are mean ± standard deviation of three independent biological replicates. Where expression was not significantly different ($P > 0.05$) from the untreated control the bars are labelled with an 'x'.

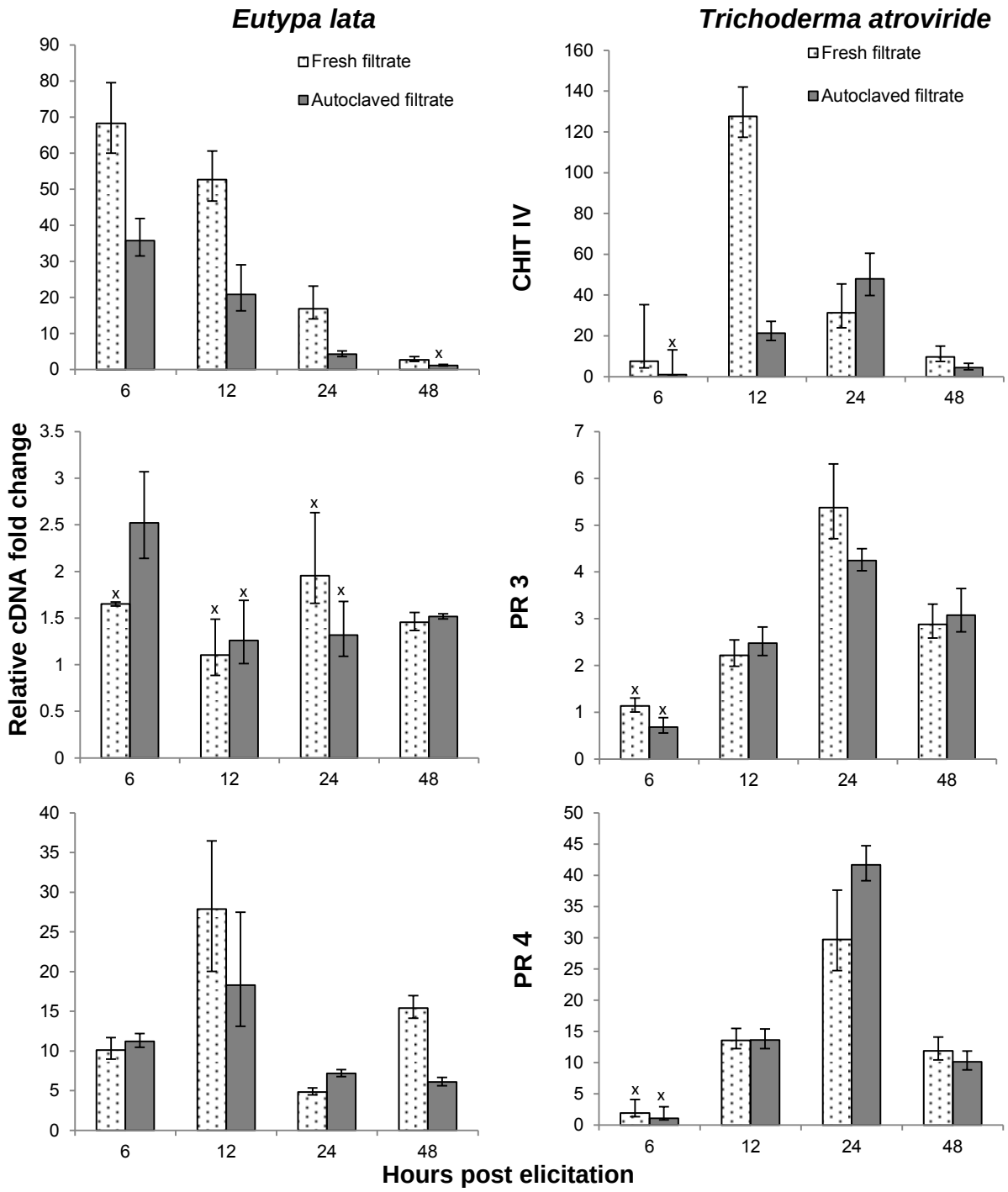

Figure 7: Relative expression with time of genes coding for chitinase pathogenesis related proteins (CHIT IV (Chitinase IV), PR 3 and 4) in *V. vinifera* cv. Dauphine cell suspension after treatment with freshly prepared (fresh) or autoclaved cell free culture filtrates of trunk pathogen *E. lata* and bio-control agent *T. atroviride*. Expression was measured relative to untreated controls of each time point. Bars are mean ± standard deviation of three independent biological replicates. Where expression was not significantly different ($P > 0.05$) from the untreated control the bars are labelled with an 'x'.

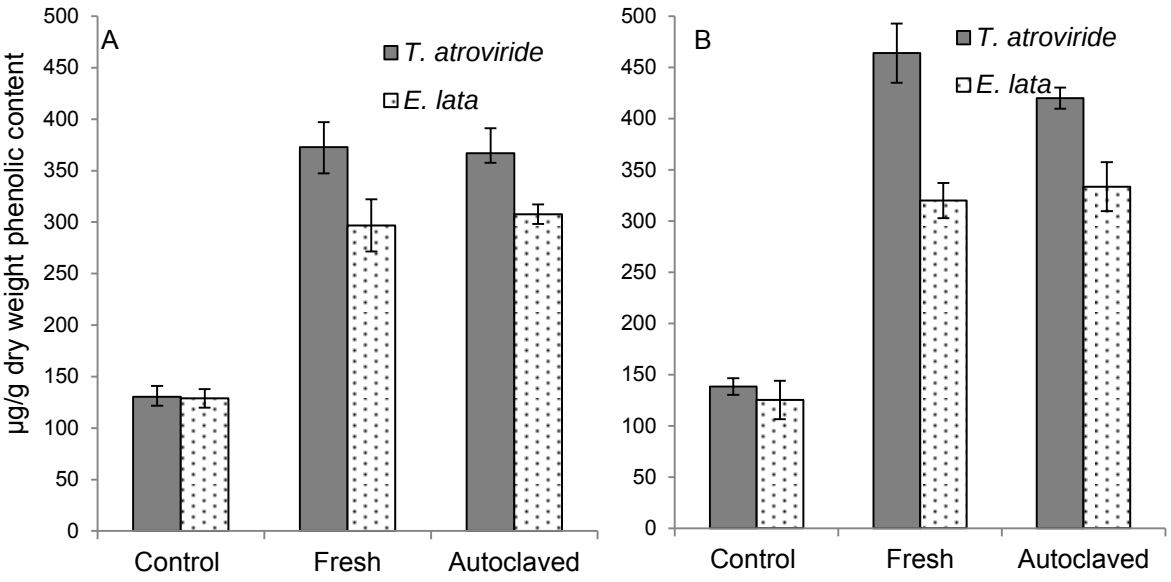

Figure 8: Total phenolic content of *V. vinifera* cv. Dauphine cell suspension cultures showing a significant ($P < 0.001$) increase in phenolic content of the cells 24 hours (A) and 48 hours (B) post elicitation with cell free culture filtrate elicitors (freshly prepared (fresh) or autoclaved) from either pathogen (*E. lata*) or bio-control agent (*T. atroviride*). Phenolics were extracted from freeze dried cells and quantities determined by the Folin-Ciocalteu reagent as gallic acid equivalents. Bars are mean ± standard deviation of three independent biological replicates.

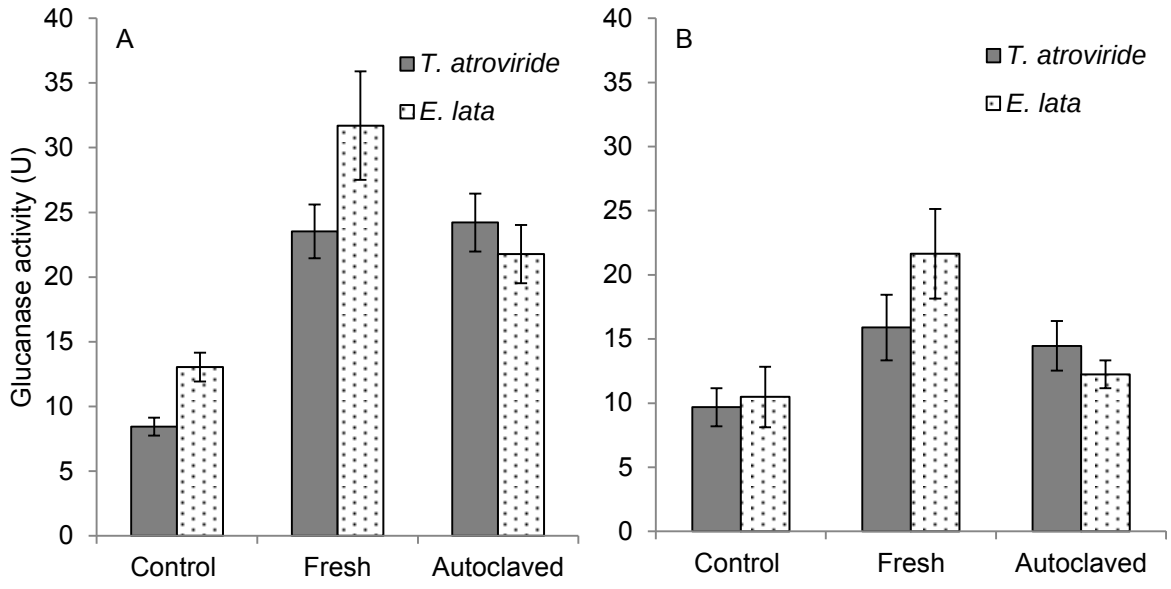

Figure 9: Activity of β-1, 3-glucanase enzymes from grapevine cell suspension cultures, at 24 hours (A) and 48 hours (B) after treatment with freshly prepared (fresh) or autoclaved cell free culture filtrate of either *E. lata* or *T. atroviride*. Enzyme activity was measured as released reducing sugars from laminarin, as substrate. Bars are mean ± standard deviation of three biological replicates. A unit (U) of activity was defined as the quantity of protein that released of one µ Mole of reducing sugar per millilitre per minute.

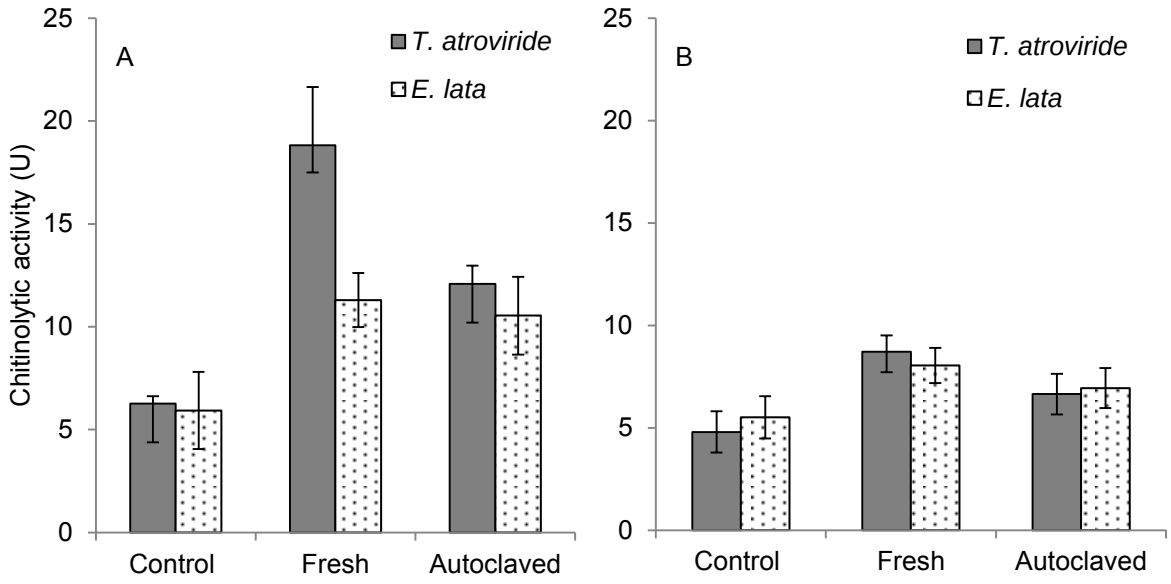

Figure 10: Activity of chitinolytic enzymes from grapevine cell suspension cultures, at 24 hours (A) and 48 hours (B) after treatment with freshly prepared (fresh) or autoclaved cell free culture filtrates of either *E. lata* or *T. atroviride*, showing much higher activity at 24 hours with the *T. atroviride* fresh elicitor. Enzyme activity was measured as reduction in turbidity of a chitin (1%) substrate suspension. Bars are mean ± standard deviation with of three independent biological replicates. A unit (U) of activity was defined as the quantity of protein that reduced the turbidity of chitin substrate by 5% per minute.

Chapter 7

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

7.1 Abstract

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

7.2 Introduction

Grapevine trunk diseases are a silent and often hidden cause of decline and loss of productivity in vines at all stages of growth and are increasingly becoming an important limiting factor to the long-term sustainability of grape and wine production (Van Niekerk *et al*., 2003; Gubler *et al*., 2005; Larignon *et al*., 2009). These diseases are caused by various xylem-inhabiting pathogens that include *Eutypa* (*E*.) *lata* (eutypa dieback), *Phaeomoniella* (*Pa.*) *chlamydospora* and *Phaeoacremonium* species (Petri disease), *Fomitiporia* spp. (esca) and Botryosphaeriaceae fungi (Botryosphaeria dieback). Infection occurs through any type of wound, of which pruning wounds are the principal ports of entry (Chapuis *et al*., 1998; Serra *et al*., 2008; Rolshausen *et al*., 2010; Van Niekerk *et al*., 2011).

The grapevine pruning wound is colonised by naturally occurring fungi and bacteria and these may inhibit infection of the wood tissue by trunk pathogens. Pruning wound protection by biological agents offers an alternative and more sustainable long-term control of trunk disease pathogens on wound surfaces. Biological wound protection from *Eutypa lata* infection by *Fusarium* (*F.*) *lateritium*, *Cladosporium herbarum*, *Bucillus subtilis* and *Trichoderma* (*T.*) spp. has been reported (Ferreira *et al*., 1991; Munkvold & Marois, 1993; John *et al*., 2008). The biocontrol effect of *Trichoderma* spp. has also been demonstrated on a wide spectrum of grapevine trunk diseases both *in vitro* and *in vivo* (Fourie & Halleen, 2004; Di Marco *et al*., 2004; Kotze *et al*., 2011). Due to the ease of large scale production, *Trichoderma* spp. have been developed into commercial products for biological control of numerous plant pathogens (John *et al*., 2008; Vinale *et al*., 2008).

The mechanisms of action by *Trichoderma* spp. may either be a result of its antagonistic action against the pathogen or from its interaction with the plant. *Trichoderma*-pathogen interactions involve mycoparasitism and secretion of mycolytic enzymes (Howell, 2006; Woo *et al*., 2006), competition for limited resources, as well as production of antibiotics (Sivasithamparam & Ghisalberti, 1998; Harman, 2006). Central to the biocontrol activity of *Trichoderma* spp. is the production of secondary metabolites, which are natural compounds that aid the producing organism in survival and basic functions such as symbiosis, competition and differentiation (Shwab & Keller, 2008). The production of antibiotic secondary metabolites is often correlated to the biocontrol activity of *Trichoderma* strains (Ghisalberti *et al*., 1990; Worasatit *et al*., 1994; Vinale *et al*., 2006).

Trichoderma secondary metabolites are chemically diverse and their production varies greatly between species and strains of the same species. The huge structural and functional diversity of *Trichoderma* metabolites makes it necessary for the continual search of new metabolites. These may be important in selection or screening of potential biocontrol agents or may be developed for application as bio-active compounds in pesticides and antibiotics.

A strain of *T. harzianum* (T77 now commercialised as Eco 77®) was isolated from grapevine nursery rhizosphere and two strains of *T. atroviride* (UST1 and UST2) isolated from grapevine wood, have shown to be good pruning wound colonisers. Field studies against grapevine trunk pathogens have shown that these strains are consistent in grapevine pruning wound protection (Kotze *et al*., 2011; Mutawila *et al*., 2011). *In vitro* studies showed that the volatile and culture filtrates from the two *T. atroviride* strains significantly reduced mycelial growth and inhibited spore germination of different trunk disease pathogens (Kotze *et al*., 2011). The identity of the active compounds in culture filtrates needed to be determined. Mutant isolates with high tolerances to benzimidazole fungicides, for possible

integrated application, were developed from these strains by gamma irradiation (Chapter 5). One of the mutants lost its *in vitro* antagonistic activity and therefore, it was important to determine the effect of mutation on secondary metabolite production.

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

7.3 Materials and Methods

7.3.1 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.

7.3.2 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 (^{1}H) NMR spectra were recorded with a 400 MHz Brüker Avance spectrometer, equipped with a 5mm Bruker Broad Band Inverse probe (BBI), working at the ^{1}H frequencies of 400.13, and using residual and deuterated solvent peaks as reference standards. A high resolution mass spectrum was obtained by a VG Autospec mass spectrometer.

7.3.3 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.

7.3.4 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).

7.3.5 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.

7.3.6 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.

7.3.6.1 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.

7.3.6.2 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.

7.3.6.3 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 *P. chlamydospora* where day-12 colony diameter was used. The percentage inhibition was used to determine the effective concentration that inhibited mycelial growth by 50% (EC_{50}).

7.3.6.4 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 *P. chlamydospora* where it was measured at day 12. There were three replicates for each isolate per medium. Radial colony diameters were used to calculate percentage inhibition relative to the control.

7.3.6.5 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.

7.3.7 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).

7.4 Results

7.4.1 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).

7.4.2 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 ($P < 0.001$; Appendix E, Table 1). 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 (Figure 1A). 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 (Figure 1B). 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.

7.4.3 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 2. 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).

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 ($P < 0.001$; Appendix E, Table 2). In full strength PDB, 6PP was produced by all the isolates (except MT77) in both static and shake cultures (Figure 3A) while in quarter strength PDB, UST1 and MT1 could not produce 6PP (Figure 3B). 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 (Figure 3).

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

Analysis of variance showed significant isolate × medium × co-culture interactions ($P = 0.038$; Appendix E, Table 3). The effect of medium and pathogen co-inoculation on 6PP production by the *T*. *atroviride* isolates is shown in figure 4. 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.

7.4.5 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 ($P < 0.001$).

7.4.5.1 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; Appendix E, Table 4). Based on the mean EC_{50} values (Table 1), the inhibition of mycelial growth by 6PP varied for the different pathogens. There were no significant differences (P > 0.05) 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; Appendix E, Table 5). *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.

7.4.5.2 Effect of growth medium on mycelial sensitivity to 6PP: There were significant isolate × pathogen × medium interactions (P < 0.001; Appendix E, Table 6) for all the pathogens. Inhibition of mycelial growth was dependent on growth medium and the pathogen isolate (Table 2). All the pathogens were more sensitive to 6PP when growing on 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.

7.4.5.3 Conidia/spore germination: Conidia/spore germination was significantly (P < 0.001; Appendix E, Table 7) 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.

7.5 Discussion

The antagonism of culture filtrates and volatiles produced by *Trichoderma* species used in grapevine pruning wounds has previously been demonstrated (John *et al*., 2004; Kotze *et al*., 2011). However, the secondary metabolites responsible for these properties had not been identified or characterised. This is the first report on the identification of secondary metabolites produced by *Trichoderma* isolates used in grapevine pruning wound protection

and a characterisation of their *in vitro* effect on mycelial growth and spore germination of grapevine trunk pathogens. The metabolite 6-pentyl α-pyrone (6PP), was the major metabolite produced by all the three isolates tested and it is a well-known antimicrobial compound produced by *Trichoderma* species (Ghisalberti & Sivasithamparam, 1991; Vinale *et al*., 2006; El-Hasan *et al*., 2007). This compound is a volatile and has a characteristic sweet coconut smell which is characteristic of *Trichoderma* species of the section Trichoderma (Dodd *et al*., 2003). However, other *Trichoderma* species, not included in the section Trichoderma such as *T*. *harzianum*, can also produce this metabolite (Rey *et al*., 2001; Vinale *et al*., 2008), despite some suggestion that this could be a result of miss-identification (Dodd *et al*., 2003).

The production of 6PP was highly dependent on strain, nutrition and culture conditions. It appears that after reaching a peak the concentration of 6PP starts to decline. This is in agreement with other studies that aimed at maximising 6PP production *in vitro* (Prapulla *et al*., 1992; Sarhy-Bagnon *et al*., 2000; Serrano-Carreón *et al*., 2004). The producing fungus is also inhibited by the 6PP at concentrations of 90-110 mg/L and the addition of resin (amberlite) in the medium reduces inhibition (Prapulla *et al*., 1992). The *T*. *atroviride* strains (UST1 and UST2) produced more quantities of 6PP than the *T*. *harzianum* (T77). There was a large variation between the *T*. *atroviride* strains with strain UST2 producing more than UST1 when grown under static (none-shaking) conditions. These isolates have been evaluated for their grapevine wound protective effect against infection by trunk pathogens and the *T*. *atroviride* isolates were found to be better than the *T*. *harzianum* (Kotze *et al*., 2011; Mutawila *et al*., 2011) which is likely due to the *T*. *atroviride* producing more 6PP. Another metabolite from *T*. *atroviride* that had a fragmentation pattern almost similar to that of trichocompactum Ia and Ib isolated from *T*. *brevicompactum* (Degenkolb *et al*., 2006) could also be involved in their biocontrol activity. Peptaibiotics from *Trichoderma* are known antibiotics, however, the biological activity of the brevicompactum group has not been characterised.

To establish the likelihood of 6PP production during wound colonisation, a grapevine cane extract culture medium was used as a close approximation of the natural substrate. Mahoney *et al*. (2003) demonstrated enhanced production of toxins (eutypine and eulatachromene) by *E*. *lata* isolates grown in a grapevine extract compared to artificial media. The more natural substrate could be representative of secondary metabolite production on the wound. Production of 6PP in the GCBM varied between the two isolates, but was higher in the GCBM compared to the defined medium for both isolates. However, what was particularly interesting was that 6PP production almost doubled in the GCBM when isolate UST1 was co-cultured with *N*. *parvum*. Previous reports have also demonstrated that

the production of secondary metabolites is induced by fungal cell wall material (Serrano-Carreón *et al*., 2004; Vinale *et al*., 2009). In the current study, the induction of 6PP production was only observed with *N. parvum* and not with *E. lata*. The difference in behaviour of *T*. *atroviride* towards the two fungi is difficult to explain since it was not possible to measure the growth of the *T*. *atroviride* isolates in co-cultures. However, it can be speculated that the slow growing *E. lata* was inhibited by the *T*. *atroviride* before the competing fungus could elicit the production of the antifungal 6PP in the biocontrol agent. Again it is difficult to explain why the co-cultivation of UST2 with *N*. *parvum* in GCBM did not result in significant 6PP production. A possible explanation is that the interaction of UST2 and *N*. *parvum* could have resulted in the reduction of growth of UST2 as the biocontrol agent is also likely to have responded by producing hydrolytic enzymes (chitinases and glucanases) which also consume metabolic resources (Serrano-Carreón *et al*., 2004; Rocha-Valadez *et al*., 2005). *In vitro* assays showed that UST1 was more aggressive than UST2. UST1 overgrew and inhibited most grapevine trunk pathogens in dual plate assays (Kotze *et al*., 2011), while chitinase assays also revealed higher activities in UST1 compared to UST2 (unpublished data). It is therefore a possibility that the growth of *N*. *parvum* was inhibited by UST1 before the interactions between the two fungi could have an effect on the growth of UST1. However, the initial interactions could have induced a competitive response from UST1 and hence the higher level of 6PP production.

Some reports on the mechanisms of action of *Trichoderma* spp. have associated pyrone production with biocontrol activity. Worasatit *et al*. (1994) found no correlation between the production of fungal cell wall hydrolytic enzymes (chitinase, glucanase and xylanase) by *T*. *koningii* and their protection of wheat against Rhizoctonia root rot, but could associate efficacy with pyrone production. More specifically, the production of 6PP has been correlated with biocontrol activity of *T*. *harzianum* on Petri dish assays and *in vivo* for the soil-borne pathogen *Gaeumanomyces graminis* (Ghisalberti *et al*., 1990) and the post-harvest pathogen *Botrytis* (*B*.) *cinerea* (Pezet *et al*., 1999). In the current study, the high production of 6PP in the GCBM strongly suggests the involvement of 6PP in the protection of wood from infection by wood rotting fungi.

Volatile compounds from *T*. *harzianum* isolates used in Vinevax® had fungistatic effects on *E. lata* mycelium, while the cultural filtrates completely inhibited the pathogen mycelial growth (John *et al*., 2004). More recently, Kotze *et al*. (2011) further demonstrated the inhibitory effects of *T*. *harzianum* and *T*. *atroviride*, on more grapevine trunk pathogens *in vitro* and the wound protective effect of the *Trichoderma* spp. isolates *in vivo*. None of these reports identified the compounds that were responsible for this, although John *et al*. (2004) speculated on the involvement of 6PP due to the coconut smell that was produced by the

isolates that had mycelial inhibitory activity. The fungal inhibitory concentrations of 6PP on grapevine trunk pathogens are comparable to those reported for *B. cinerea* (Walter *et al*., 2000) and *Fusarium verticillioides* (syn. *F. moniliforme*) (El-Hasan *et al*., 2007). Conidia/spore germination was more sensitive to 6PP compared to mycelial growth on all the grapevine trunk pathogens tested. Mycelial inhibition also varied with the pathogen, indicating that 6PP is not equally effective on all fungal pathogens. Since pruning wound infection occurs mainly through conidia/spores, a higher inhibitory effect on conidia/spore germination by 6PP is more likely to result in reduced infection of *Trichoderma* protected wounds. However, the reduced effect on mycelial growth may also suggest that 6PP producing *Trichoderma* are less likely to eliminate pathogens already growing in the pruning wound. Currently, there are no reports of *Trichoderma* spp. eliminating pathogen (curative effect) infected wounds, but only wound protective action when the pathogen is inoculated after the biocontrol agent. Better wound protection is achieved when a longer time is allowed between the application of *Trichoderma* biocontrol agents and pathogen inoculation (Munkvold & Marois 1993; John *et al*., 2005 & 2008). This is related to the need by the *Trichoderma* biocontrol agent to grow on the wound and start responding to stimuli such as the presence of competitors on the wound. The importance of 6PP in the *Trichoderma*-pathogen interaction is further demonstrated by the reduction/loss of *in vitro* antagonistic ability of the mutant MT77 which could not produce 6PP. The loss of 6PP production by the mutant was also accompanied by poor conidia production indicating a possible link between genetic control of 6PP production and asexual reproduction. This has also been reported in other fungal species such as the aflatoxin producing *Aspergillus* spp., where conidia production has been shown to be regulated by the same genes as aflatoxin production (Fox & Howlett, 2008).

Although there was less/little variation in the sensitivity of isolates of the same pathogen to 6PP, in the natural populations such variation may occur. Walter *et al*. (2000) produced mutant *B. cinerea* strains that were tolerant to high concentrations of 6PP by UV irradiation, but the tolerance to 6PP was lost after growth on plant material (kiwifruit slices). Some fungi that can breakdown 6PP to less toxic compounds have also been reported (Cooney & Lauren, 1999). Therefore, there is a possibility that 6PP tolerance/resistance may develop in natural populations if 6PP was to be applied as a fungicide. However, since biological control agents have more than one mechanism of action, resistance to the biocontrol agent is not likely to develop. Testing of sensitivity of *B. cinerea* to pyrrolnitrin, an antifungal substance produced by a biocontrol strain of *Pseudomonas chlororaphis*, revealed a wide range of sensitivity to the compound, but all isolates were equally sensitive to the

bacteria (Ajouz *et al*., 2011). This indicates the involvement of other mechanisms and the importance of having more than one mode of action.

Applying 6PP as a fungicide has several disadvantages which include the facts that it is volatile and therefore, it is not likely to persist on the site of application and that it is also phytotoxic at high concentrations. The phytotoxic concentrations of 6PP, are much less than those reported to be antimicrobial (Cutler *et al*., 1986; Parker *et al*., 1997). When produced by the biocontrol agent *in situ* the 6PP concentrations are likely to be higher at the micro-level of interaction between the competing fungi (*Trichoderma* and pathogen) and are not toxic to the plant. The 6PP will continue to be produced for as long as the *Trichoderma* persists in the plant, which makes it important to choose a strain that is a high producer of the secondary metabolite if it is the major mode of action.

Trichoderma species are amongst the most commercialised genus of biocontrol agents and for grapevine trunk diseases they are the only registered (or commercialised) biocontrol agents. The selection of potential biocontrol agents is largely based on their ability to antagonise pathogens *in vitro* and activity of their cell wall degrading enzymes (Elad *et al*., 1996; Harman *et al*., 1993 & 2004). For current and potential agents for wound protection the same selection methods have been used. However, the production of secondary metabolites may be important for some patho-systems and should also be considered in selection of potential biocontrol agents. The search for bio-active compounds from biocontrol agents should continue to be an important branch of traditional biotechnology, especially in the light of continued reduction in chemical fungicides. Secondary metabolites, being natural products, are bio-degradable and should attract attention for the development of safe fungicides. It would be interesting to also investigate the effect of *Trichoderma* secondary metabolites on pathogen toxin production since the pathogenicity/virulence of some grapevine trunk pathogens is attributed to their toxin production.

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Tables and Figures

Table 1: Sensitivity of grapevine trunk pathogens to the secondary metabolite, 6-pentyl α-pyrone (6PP), of *Trichoderma* spp. based on *in vitro* inhibition of 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	6384	90.02	91.72 ± 1.70
chlamydospora	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.

Table 2: Effect of growth medium on sensitivity of grapevine trunk pathogens to 6-pentyl α-pyrone (6PP), a secondary metabolite from *Trichoderma* spp. Pathogens were grown on medium amended with 150 mg/L of 6PP.

Medium[1]	**Inhibition of mycelial growth of pathogen isolates (%)**[2]							
	Eutypa lata		*Phaeomoniella chlamydospora*		*Neofusicoccum australe*		*Neofusicoccum parvum*	
	5692	6513	6384	7732	4439	4584	7025	7029
PDA	82.48^{C}	80.31^{C}	70.01^{E}	60.52^{F}	73.97^{C}	78.74^{B}	78.26^{C}	78.13^{CD}
MEA	73.87^{E}	77.01^{D}	78.07^{D}	87.35^{BC}	64.23^{E}	72.00^{CD}	75.48^{DEF}	76.80^{CDE}
GCBM	69.45^{F}	76.32^{D}	68.50^{E}	62.68^{F}	64.87^{E}	69.46^{D}	69.88^{H}	74.16^{EFG}
Pezet's	92.05^{B}	100^{A}	85.74^{C}	85.27^{C}	72.19^{CD}	72.96^{C}	71.56^{GH}	73.13^{GF}
Vogel's N	100^{A}	100^{A}	92.09^{A}	90.49^{AB}	83.75^{A}	81.56^{AB}	93.75^{B}	96.88^{A}
LSD[3]	2.26		2.98		2.70		4.62	

[1]PDA – potato dextrose agar; MEA – malt extract agar; GCBM – grape cane based medium

[2]Values followed by the same letter are not significantly different for the same pathogen.

[3]Data was analysed by ANOVA followed by Fischer's LSD test at P = 0.05 for each pathogen separately.

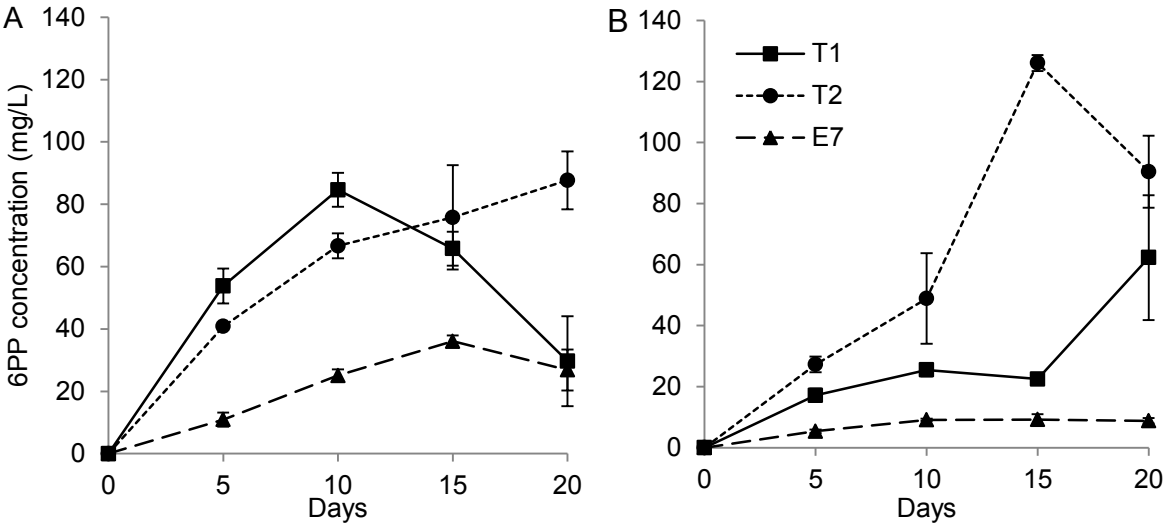

Figure 1: Time course production of 6-pentyl-α-pyrone (6PP) by *T. atroviride* (UST1 and UST2) and *T. harzianum* (E77), used in grapevine pruning wound protection, grown in full strength PDB in shake (A) and static (B) cultures. Each point on the line is the mean ± the standard deviation of three independent biological replicates.

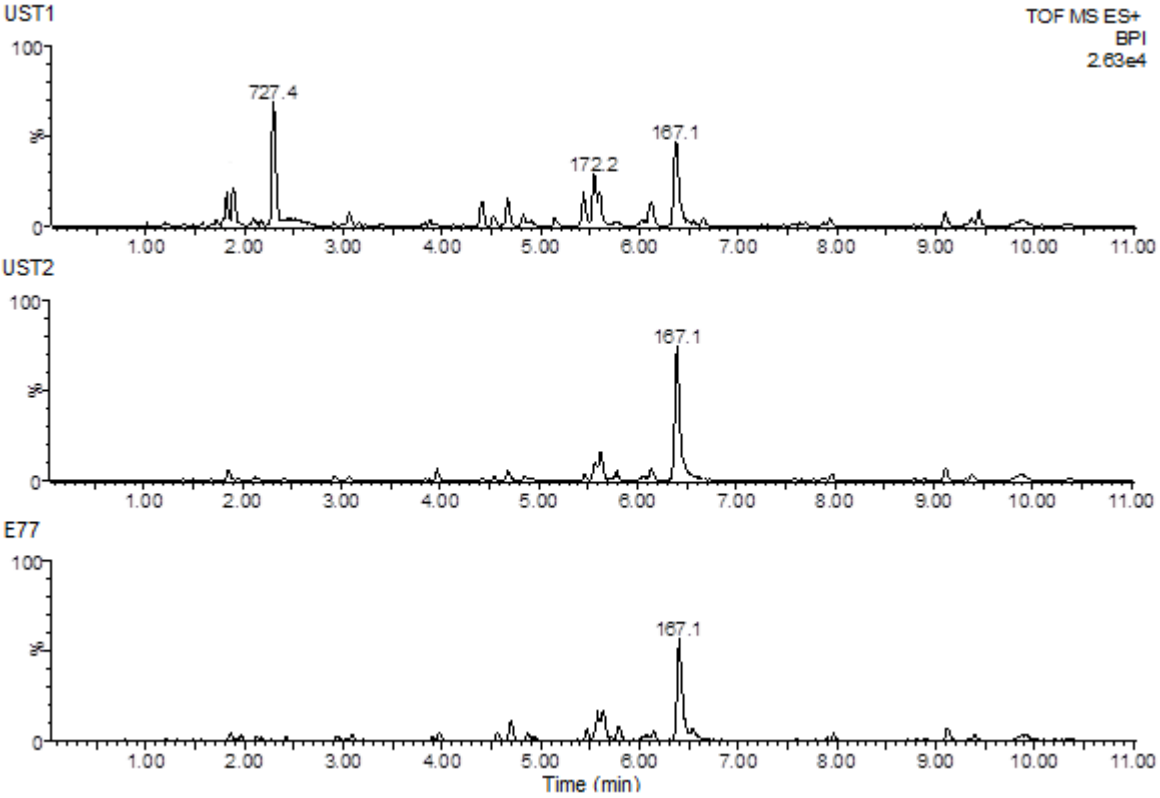

Figure 2: LC-MS chromatograms showing the major secondary metabolite peaks from *T. atroviride* (UST1 and UST2) and *T. harzianum* (E77) isolates grown in full strength potato dextrose broth for 20 days with shaking at 120 rpm. The major secondary metabolite in all isolates was 6-pentyl α-pyrone with 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.

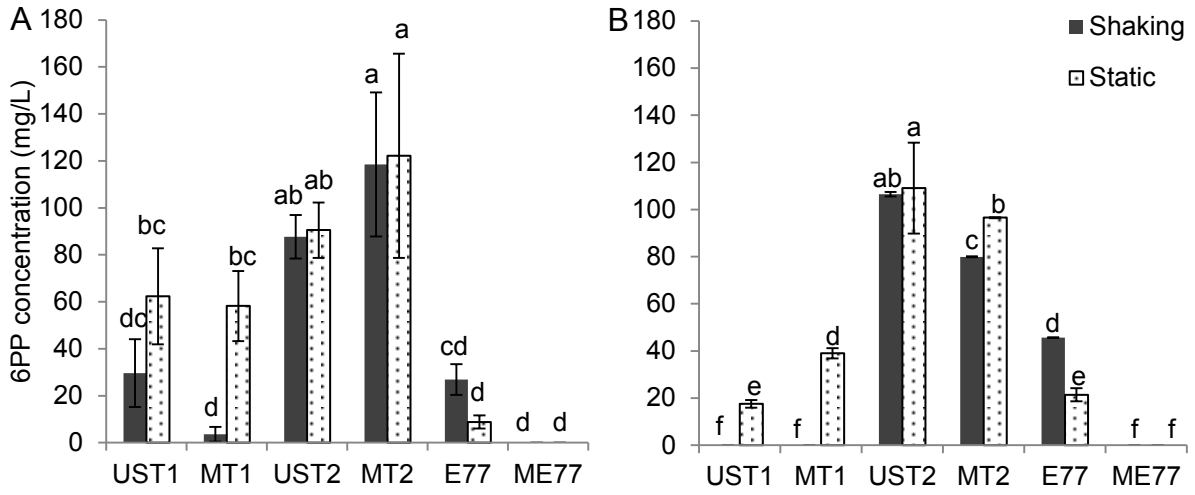

Figure 3: A comparison of 6-pentyl α-pyrone production between wild type *T*. *atroviride* (UST1 and UST2) and *T*. *harzianum* (E77) used in grapevine pruning wound protection, and their mutant strains (MT1, MT2 and ME77). The fungi were grown in full strength potato dextrose broth (A) and quarter strength potato dextrose broth (B) for 20 days either shaking or static. The mutants were generated by gamma irradiation and are resistant to benzimidazole fungicides. Each bar is the mean ± the standard deviation of three independent biological replicates. Bars with the same letter on top show means that are not significantly different for each medium. LSD = 39.65 (PDB) and 12.39 (quarter PDB).

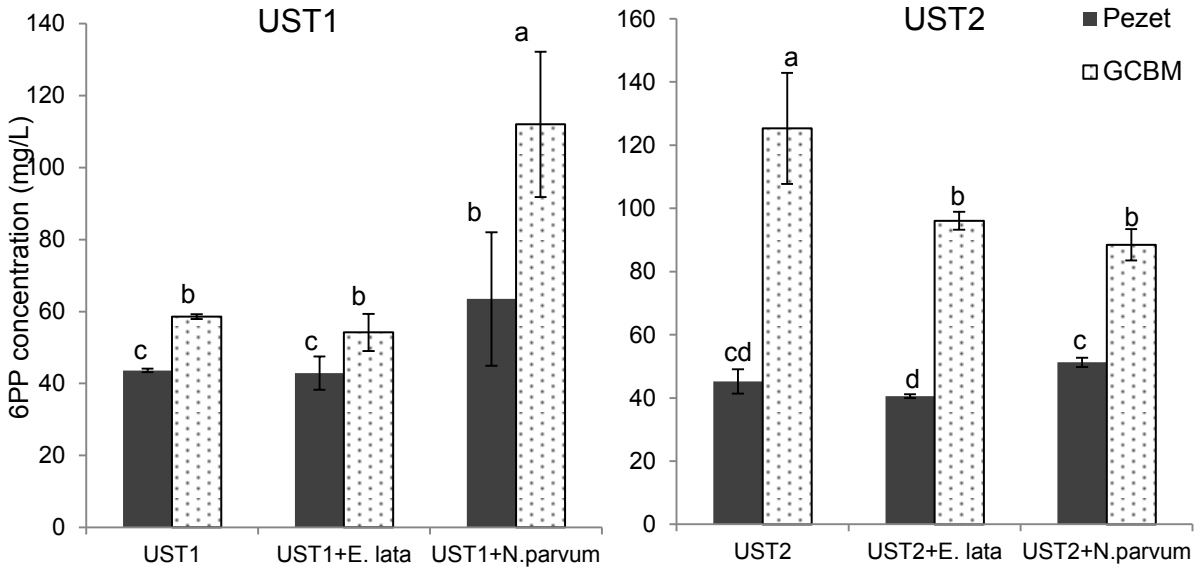

Figure 4: The effect of growth medium (Pezet and grapevine cane based medium (GCBM)) and the co-inoculation with a pathogen (*E. lata* and *N. parvum*) on the production of 6PP by two isolates of *T. atroviride*, UST1 and UST2. Each bar is the mean ± the standard deviation of three independent biological replicates. Bars with the same letter on top show means that do not significantly differ according to Fischer's least significant difference (LSD; UST1 = 10.22 and UST2 = 8.72: *P* = 0.05).

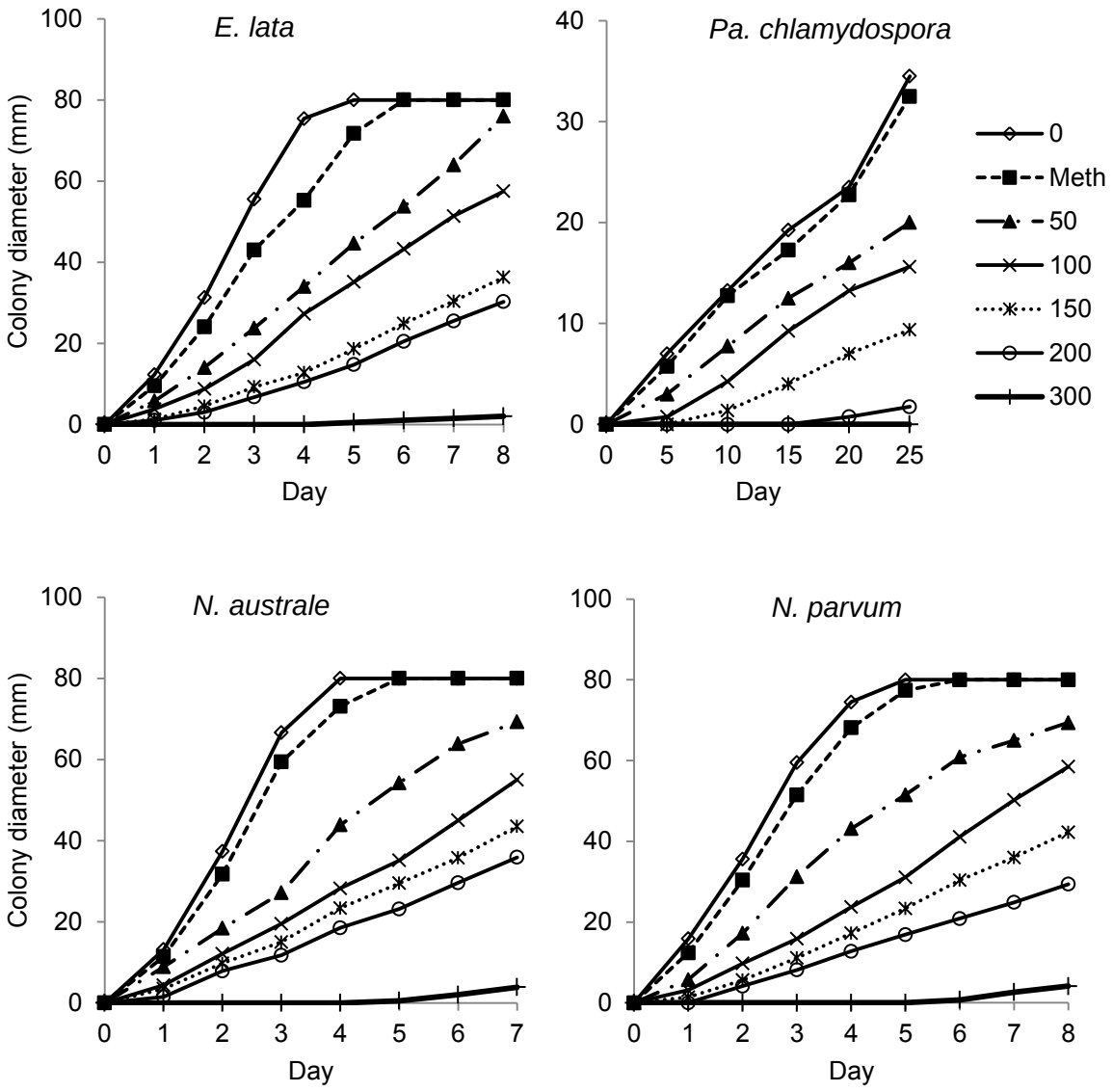

Figure 5: Mean mycelial growth inhibition with time, of grapevine trunk pathogens (*E. lata, Pa. chlamydospora*; *N. australe* and *N. parvum*) on potato dextrose agar amended with varying concentrations (0 – 300 mg/L) of 6-pently α-pyrone (6PP). Radial mycelial growth was assessed by calculating the 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.

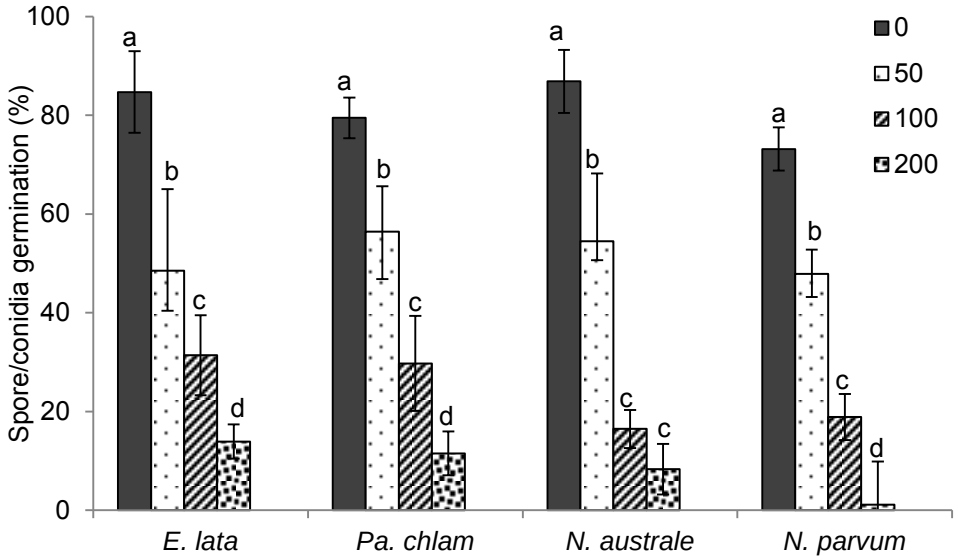

Figure 6: Inhibition of spore and conidia germination by varying concentration (0-200 mg/L) of 6-pentyl α-pyrone (6PP) on grapevine trunk pathogens *E. lata*, *Pa. chlamydospora* (*Pa. chlam*), *N. australe* and *N. parvum*. Percentages of germinated spores/conidia are means of three replicates of two 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).

Chapter 8

Concluding remarks and future perspectives

The general aim of this study was to improve grapevine wound protection with *Trichoderma* (*T*.) spp. biocontrol agents. A better understanding of the factors that influence control efficacy from the plant and the biocontrol agent were obtained. The effect of the time of pruning and the time of wound treatment after pruning, on the colonisation of grapevine wounds by *Trichoderma* spp. was investigated in an effort to come up with a recommendation on the best time to apply *Trichoderma* products. Several nutritional amendments were also tested for their effect on enhancing wound protection efficacy by *T*. *atroviride*. Benzimidazole resistant mutants of *T*. *atroviride* and *T*. *harzianum* were also developed for possible integration of chemical and biological control. A cell culture model system was employed to compare the response of grapevines to a biocontrol agent, *T*. *atroviride*, and a trunk pathogen, *Eutypa* (*E*.) *lata*, as a first step to determining the importance of *Trichoderma*-grapevine interactions in pruning wound bio-protection. Lastly the major secondary metabolite produced by the *T*. *atroviride* and *T*. *harzianum* isolates used in the present study was identified and its role in pruning wound protection investigated.

8.1 Improving wound protection

8.1.1 Time of application of *Trichoderma* biocontrol agents

The present study has shown that grapevine pruning time has an effect on colonisation of pruning wounds by the *Trichoderma* spp. Budburst is associated with wound sap bleeding and a reduction in the carbon and nitrogen content of grapevine canes. In addition, wound healing and activation of defence is much faster in an active than dormant grapevine. The effect these factors had on wound colonisation is distinctly observed in the Cabernet Sauvignon which had higher *Trichoderma* colonisation in July when it is dormant than in August at bud burst. In the Chenin blanc this effect of the physiological state on wound colonisation was observed only in 2011. In 2012 the high rainfall and relative humidity was associated with higher levels of wound colonisation at both pruning times than in 2011.

There are no reports on the effect of grapevine physiological state on grapevine pruning wound colonisation by *Trichoderma* spp. Sap-flow or bleeding, the wound healing process and defence response, all of which are associated with physiological status of the grapevine, could be factors that affect wound colonisation by *Trichoderma* spp. This can explain the variation in pruning wound colonisation and protection by *Trichoderma* spp. observed between grapevine cultivars when pruning was carried out at the same time on all

cultivars (Mutawila *et al*., 2011). While the effect of sap-flow is obvious, the effect of grapevine wound healing and response cannot be concluded from the current study and should be studied further.

The application of the biocontrol agents 6 hours after pruning resulted in consistently high incidences and wound colonisation extent by the *Trichoderma* spp. regardless of the cultivar, pruning time or weather. This is in agreement with a study from New Zealand (Harvey & Hunt, 2006) which found the highest wound colonisation by *T*. *harzianum* when it was applied 4 hours after pruning. Further evidence of the importance of applying the biocontrol agent after 6 hours is shown by the field trials carried out and described in Chapter 4. In the 2011 trial, the biocontrol agent treatments were applied immediately after pruning and low *Trichoderma* incidences were found. In the follow-up trial, in 2012, the treatments were applied six hours after pruning, resulting in *Trichoderma* incidences that were significantly higher.

It was also found that in both cultivars and trial years, pruning wound infection due to natural inoculum was higher in wounds made in late winter (August) than those made earlier (in July), regardless of the cultivar's physiological state. This confirms the results obtained by Van Niekerk *et al*. (2011), from artificial inoculation of a Chenin blanc vineyard from the same area. However, it differs from several studies that have shown that wounds made later in winter or in spring are less susceptible to infection compared to wounds made earlier in the dormant period (Petzoldt *et al*., 1981; Larignon & Dubos, 2000; Munkvold & Marois, 1995; Úrbez-Torres & Gubler, 2011). From the current and the Van Niekerk *et al*. (2011) studies it can be concluded that in the Stellenbosch area pruning wounds made earlier in winter are at less risk of getting infected than those made late. This could be due to the high rainfall received in the area in late winter which makes more inoculum available for wound infection. However, this cannot be generalised for the whole South African Cape region and future studies should validate the risk of infection of wounds made in early and late winter in other grapevine producing regions.

8.1.2 Use of nutritional amendments

Several nutritional amendments were shown to increase the extent of pruning wound colonisation by *T*. *atroviride* in a glasshouse trial. However, in field trials none of the nutritional amendments gave significant increase in wound colonisation by *T. atroviride* compared to the non-amended suspension. In the first field trial pathogen control was better in the Thompson Seedless compared to the Chenin blanc. This was likely due to the higher incidences of *Trichoderma* spp. in the Thompson Seedless compared to the Chenin blanc. Garrison, a fungicide containing pruning wound paste was the best treatment in the Chenin

blanc while in the Thompson Seedless, Garrison efficacy did not differ from the *Trichoderma* spp. treatments. The lower efficacy of Garrison in the Thompson Seedless was more likely due to sap drip-off from the almost vertically pruned canes which aided wash-off on painted wounds.

This study also confirmed results from previous reports that biocontrol agents need time to colonise the wound before they can provide protection (Munkvold & Marois, 1993; John *et al*., 2005). After three days of applying the biocontrol agent, it had colonised the wound sufficiently to provide similar protection the wound sealant.

Two amendments namely, the broth and the yeast extract + urea + glucose treatments were consistent in their efficacy in the field and provided protection almost similar to the wound sealant (Garrison) even when the pathogen was inoculated within a day of pruning. The effect of yeast extract + urea + glucose can be attributed to mainly, the faster wound colonisation by *T. atroviride* while for the broth treatment there are several possible mechanisms of action. In addition to faster growth of the *T. atroviride*, the broth also contains hydrolytic enzymes (chitinase and glucanase) and chitin oligomers. The hydrolytic enzymes are antifungal and hence provide instant wound protection while some proteins are involved in activation of defence as shown in Chapter 6. Chitin oligomers are known to induce chitinase production in *Trichoderma* spp. (Brunner *et al*., 2003; Djonovic *et al*., 2007; Wasli *et al*., 2010) and also activate plant defence (Barber *et al*., 1989; Kasprzewska, 2003; Zipfel & Robatzek, 2010). Further studies should elucidate the exact mode of action of the broth formulae and its possible application in other systems such as in the grapevine nursery where it can be used to prime (immunise) nursery plants.

8.1.3 Integration of biological and chemical control

The wild type isolates were found to be naturally resistant to thiophanate methyl. However, this fungicide is currently not available in South Africa as it is not registered for any crop. Benomyl and carbendazim are available and are registered for the control of Botrytis bunch rot on grapevines. It would be easier to extend the label of benomyl and carbendazim to include pruning wound protection than to register thiophanate methyl on grapevine.

Gamma irradiation was able to generate stable benzimidazole resistant *Trichoderma* strains. Mutants from *T. atroviride* (MT1 & MT2) retained their *in vitro* antagonism against trunk pathogens while the mutant from *T. harzianum* (MT77) did not. The growth of MT77 was also different from the wild type (T77) and could not produce the major antifungal secondary metabolite secreted by T77 (Chapter 7). Molecular characterisation of MT77 could reveal genes that are responsible for the disrupted functions and aid in the annotation of important biocontrol genes. The current study shows the potential use of gamma irradiation

to generate novel fungal traits in the same way as used in plant breeding to generate plant variability.

There was no difference in wound protection efficacy between the benzimidazole fungicides and biocontrol agents when applied separately, but the combination of the mutant MT1 and carbendazim was the best treatment when *Phaeomoniella* (*Pa*.) *chlamydospora* was inoculated within a day of pruning. The integration of biological and chemical control could provide better wound protection than either method separately. Benomyl and carbendazim are only available in a few grapevine producing countries and hence the scope of integrating the mutants generated here is limited. However, the mutants can also be used in ecological studies such as determining the fate of *Trichoderma* spp. in the environment since their benzimidazole resistance provides a selectable marker.

8.2 Grapevine response to *E. lata* and *T. atroviride*.

Pruning wound protection by *Trichoderma* spp. is currently thought to be only due to the antagonism of the biocontrol agent on the pathogens or competition for space. While the *Trichoderma*-pathogen interactions are well documented, the *Trichoderma*-grapevine interactions have received little attention. The response of grapevine cell suspension cultures to cell free culture filtrates of *T. atroviride* and *Eutypa lata* showed that the grapevine responds to the biocontrol agent by activating the same defence genes as activated by the pathogen. Cell cultures responded to the biocontrol and pathogen culture filtrates in a hypersensitive-like response which caused a browning and decline of viability of cell cultures and is associated with oxidative burst. This response was more pronounced in the cells treated with the biocontrol agent than the pathogen. Elicitors from both fungi caused an up-regulation of genes involved in the synthesis of phytoalexins and a down regulation of genes involved in the flavonoid synthesis indicating a switch to defence metabolism. The pathogen elicitors caused a biphasic pattern in the up-regulation of phenylalanine ammonia-lyase (PAL) and stilbene synthase (STS) an indication of coordinated expression or metabolic channelling. Biphasic gene expression of PAL and STS has also been shown on response of grapevine cell cultures to cell extracts of *Pa. chlamydospora* (Lima *et al*., 2012). Elicitors from *T. atroviride* caused a slow but higher expression of PAL and STS compared to the pathogen elicited cells.

No prior studies have reported on the initial response of grapevine to infection by *E. lata*. Rotter *et al*. (2009) reported the over-expression of PAL and CHS seven weeks after grapevine plantlets were inoculated with *E. lata*. The over-expression of CHS is contrary to the current study where expression of CHS was down regulated when measured after 6 to 48 hours. However, the down-regulation of CHS and over-expression of STS in response to

infection has been reported in grapevine leaves infected with *Plasmopara viticola* after 6 to 48 hours of infection (Vannozzi *et al*., 2012) similar to results from the current study. *Eutypa lata* infection can therefore trigger a basal defence response from the grapevine which is not necessarily sufficient to stop colonisation. The *T*. *atroviride* and *E*. *lata* elicitors also caused an up-regulation of antifungal pathogenesis related proteins (PR 2, 3, 4, 5, 6 and chitinase IV (CHIT IV)). The biocontrol elicitors caused down regulation of PR 1 while the pathogen elicitor had no effect on PR 1 expression. It cannot be ascertained which defence mechanism is triggered by the biocontrol and pathogen elicitors between systemic acquired resistance and induced systemic resistance as genes involved in both mechanisms were triggered by elicitors from both fungi.

The gene expression patterns were further confirmed by metabolic profiling of products of the defence genes. The higher levels of PAL and STS expression in the cells treated with biocontrol elicitor also resulted in a significantly higher increase in total phenolic content of these cells compared to the cells treated with the pathogen elicitor. The chitinolytic (CHIT IV, PR 3 and 4) and β-1, 3-glucanase activities also increased in the elicitor treated cells in a similar pattern as the expression of the genes coding for the enzymes.

The current study gives first evidence of the role of *Trichoderma*-grapevine interactions in pruning wound protection by *Trichoderma* species. It has been shown that grapevines respond to trunk pathogen infection by production of phenolic compounds that limit fungal growth and activity of their hydrolytic enzymes (Del Río *et al*., 2004; Amalfitano *et al*., 2011). The antifungal activity of chitinase and β-1, 3-glucanase enzymes is well-established. From Chapter 3, it was shown that the grapevine defence response to wound colonisation by *Trichoderma* spp. may affect establishment of the biocontrol agent on the wound. In the same way, the response of the vine to the growth of the biocontrol agent on the wound could limit pathogen infection. This could be through induction of defence genes, though further research is necessary to confirm if findings on cell cultures are the same as in pruning wounds.

8.3 The role of secondary metabolites in wound protection

The present study showed that 6-pentyl α-pyrone (6PP) a volatile antimicrobial is the major secondary metabolite from the *T. atroviride* and *T*. *harzianum* isolates used in this study. Prior studies on *Trichoderma* spp. used in grapevine pruning wound protection had demonstrated that the different isolates produce volatile antimicrobial compounds (John *et al*., 2004; Kotze *et al*., 2011), but the identity of the metabolites was not known. Production levels of 6PP were dependent on the *Trichoderma* isolate, growth medium and culture conditions. Higher concentrations of 6PP were obtained when the *T. atroviride* isolates were

grown in a grapevine cane extract based medium while for UST1 the concentration further doubled when it was co-cultured with *Neofusicoccum* (*N*.) *parvum*. This indicates that 6PP production is enhanced by wood tissue as has been shown with secondary metabolite (toxin) production in *E*. *lata* (Mahoney *et al*., 2003). The stimulation of 6PP production in the presence *N*. *parvum* further shows that the metabolite is involved in the *Trichoderma*-pathogen interactions *in vivo*.

The secondary metabolite, 6PP, reduced mycelial growth, spore and conidia germination of grapevine trunk pathogens. The germination of spores and conidia was more susceptible than mycelial growth, however, mycelial growth was more susceptible on nutrient poor culture media. The presence of 6PP on wounds can, therefore, reduce infection of pruning wounds from spores or conidia and inhibit wood colonisation. Wood is a nutrient poor substrate and it would be expected that 6PP would inhibit pathogens in this environment. Future studies should also look at the effect of 6PP on the production of toxins by pathogens as it has been shown to inhibit the production of fusaric acid, a phytotoxic pathogenicity factor of maize pathogen *Fusarium verticillioides* (El-Hasan *et al*., 2008). The metabolite, 6PP, has also been reported to have plant growth promotion activity (Vinale *et al*., 2008). It can be speculated that its production on the wound can result in faster wound healing and thus decreasing the risk of wound infection by reducing the time it remains susceptible. This will need to be explored as it may provide another mechanism of wound protection by the biocontrol agent or for possible application of the secondary metabolites in combination with fungicides.

Other secondary metabolites were also found in the *Trichoderma* spp. culture filtrates. These could not be identified due to the low levels secreted. Peptibiotic metabolites from the *T*. *atroviride* isolates were detected. These compounds are interesting because they appear to be novel. Peptibiotic metabolites are known to be potent antibacterial, antifungal, antiviral as well as elicitation of plant defence (Szekeres *et al*. 2005). Further studies should identify these metabolites and characterise their biological activity.

8.4 Conclusion

Pathogen inoculum is always available in vineyards at pruning and so it is inevitable that unprotected wounds are likely to be infected. It is clear from this study that pruning wound colonisation by *Trichoderma* spp. will differ between cultivars depending on when they are pruned and the time they are applied on the wound after pruning. From a practical point of view, *Trichoderma* pruning wound protection agents should be applied six hours after pruning. This is more important when sap-flow is observed from pruning wounds. Understanding the complex mechanisms involved in the three way interaction between

grapevine host, pathogen and *Trichoderma* biocontrol agent will aid in obtaining more consistent results with biocontrol. Further research is necessary to fully understand grapevine response as well as cultivar variability to *Trichoderma* biocontrol of trunk diseases.

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Appendix A

Table 1: Analysis of variance for the effects of year, cultivar and pruning time on the incidence and isolation frequency of *Trichoderma* species from pruning wounds of Chenin blanc and Cabernet Sauvignon treated with the bio-control agents after pruning in July and August over two trial seasons, 2011 and 2012.

Factor	Degrees of freedom	Incidence		Isolation frequency	
		F-value	*P*-value	F-value	*P*-value
Year	1	339.78	< 0.001	72.05	< 0.001
Cultivar (Cult)	1	36.65	< 0.001	1.63	0.021
Pruning time (Pt)	1	47.90	< 0.001	16.40	< 0.001
Treatment (Trt)	2	3086.21	< 0.001	1703.88	< 0.001
Year × Cult	1	255.12	< 0.001	238.58	< 0.001
Year × Pt	1	3.82	0.053	21.19	< 0.001
Year × Cult × Pt	1	63.39	< 0.001	29.65	< 0.001
Year × Cult × Pt × Trt	2	16.13	< 0.001	7.11	< 0.001

Table 2: Analysis of variance for the effects of pruning time, time of wound treatment after pruning and the wound treatment agent on the incidence of *Trichoderma* species in pruning wounds of Chenin blanc treated with the bio-control agents, *T. atroviride* (UST1) and *T. harzianum* (Eco 77), at different times after pruning (0, 6, 24, 48 and 96 hours) in July and August over two trial seasons, 2011 and 2012.

Factor	Degrees of Freedom	2011		2012	
		F-value	*P*-value	F-value	*P*-value
Pruning time (Pt)	1	115.35	< 0.001	0.61	0.444
Time of wound treatment (Wt)	4	316.68	< 0.001	8.53	0.001
Treatment agent (Trt)	2	888.32	< 0.001	1421.89	< 0.001
Pt × Wt	4	40.87	< 0.001	6.07	0.003
Pt × Tmt	2	19.74	< 0.001	0.79	0.462
Wt × Trt	8	51.87	< 0.001	5.91	< 0.001
Pt ×Wt × Trt	8	8.61	< 0.001	4.59	0.001

Table 3: Analysis of variance for the effects of pruning time, time of wound treatment after pruning and the wound treatment agent on the incidence of *Trichoderma* species in pruning wounds of Cabernet Sauvignon treated with the bio-control agents, *T. atroviride* (UST1) and *T. harzianum* (Eco 77), at different times after pruning (0, 6, 24, 48 and 96 hours) in July and August over two trial seasons, 2011 and 2012.

Factor	Degrees of Freedom	2011		2012	
		F-value	*P*-value	F-value	*P*-value
Pruning time (Pt)	1	377.27	< 0.001	44.49	< 0.001
Time of wound treatment (Wt)	4	133.22	< 0.001	48.09	< 0.001
Treatment agent (Trt)	2	893.04	< 0.001	226.54	< 0.001
Pt × Wt	4	11.59	< 0.001	6.36	0.002
Pt × Trt	2	80.83	< 0.001	4.18	0.022
Wt × Trt	8	32.91	< 0.001	5.62	< 0.001
Pt ×Wt × Trt	8	7.83	< 0.001	2.43	0.0303

Table 4: Analysis of variance for the effects of pruning time, time of wound treatment after pruning and the wound treatment agent on the isolation frequency of *Trichoderma* species in pruning wounds of Chenin blanc treated with the bio-control agents, *T. atroviride* (UST1) and *T. harzianum* (Eco 77), at different times after pruning (0, 6, 24, 48 and 96 hours) in July and August over two trial seasons, 2011 and 2012.

Factor	Degrees of Freedom	2011		2012	
		F-value	*P*-value	F-value	*P*-value
Pruning time (Pt)	1	207.71	< 0.001	6.81	0.018
Time of wound treatment (Wt)	4	339.61	< 0.001	28.19	< 0.001
Treatment agent (Trt)	2	592.73	< 0.001	399.68	0.002
Pt × Wt	4	46.94	< 0.001	6.67	< 0.001
Pt × Trt	2	37.78	< 0.001	2.91	0.066
Wt × Trt	8	58.30	< 0.001	12.54	< 0.001
Pt ×Wt × Trt	8	7.26	< 0.001	2.71	0.017

Table 5: Analysis of variance for the effects of pruning time, time of wound treatment after pruning and the wound treatment agent on the isolation frequency of *Trichoderma* species in pruning wounds of Cabernet Sauvignon treated with the bio-control agents, *T. atroviride* (UST1) and *T. harzianum* (Eco 77), at different times after pruning (0, 6, 24, 48 and 96 hours) in July and August over two trial seasons, 2011 and 2012.

Factor	Degrees of Freedom	2011		2012	
		F-value	*P*-value	F-value	*P*-value
Pruning time (Pt)	1	54.65	< 0.001	11.33	0.003
Time of wound treatment (Wt)	4	220.31	< 0.001	82.51	< 0.001
Treatment agent (Trt)	2	557.77	< 0.001	192.60	< 0.001
Pt × Wt	4	33.22	< 0.001	3.09	0.042
Pt × Trt	2	84.71	< 0.001	3.02	0.060
Wt × Trt	8	39.04	< 0.001	22.43	< 0.001
Pt ×Wt × Trt	8	9.55	< 0.001	2.20	0.048

Table 6: Analysis of variance for the effects of year, cultivar and pruning time on the incidence of grapevine trunk pathogens in pruning wounds of Chenin blanc and Cabernet Sauvignon treated with the bio-control agent after pruning in July and August over two trial seasons, 2011 and 2012.

Factor	Degrees of freedom	F-value	*P*-value
Year	1	41.91	< 0.001
Cultivar (Cult)	1	0.01	0.907
Pruning time (Pt)	1	52.70	< 0.001
Year × Cult	1	6.77	0.0104
Year × Pt	1	2.17	0.1431
Year × Cult × Pt	1	0.57	0.4528

Table 7: Analysis of variance for the effects of pruning time, time of wound treatment after pruning and the wound treatment agent on the incidence of grapevine trunk pathogens in pruning wounds of Chenin blanc treated with the bio-control agents, *T. atroviride* (UST1) and *T. harzianum* (Eco 77), at different times after pruning (0, 6, 24, 48 and 96 hours) in July and August over two trial seasons, 2011 and 2012.

Factor	Degrees of Freedom	2011		2012	
		F-value	*P*-value	F-value	*P*-value
Pruning time (Pt)	1	14.60	0.001	30.94	< 0.001
Time of wound treatment (Wt)	4	2.84	0.055	14.21	< 0.001
Treatment agent (Trt)	2	4.86	0.013	168.39	< 0.001
Pt × Wt	4	5.56	0.004	4.97	0.007
Pt × Trt	2	0.92	0.405	5.77	0.006
Wt × Trt	8	2.36	0.035	3.17	0.007
Pt ×Wt × Trt	8	1.13	0.365	3.03	0.009

Table 8: Analysis of variance for the effects of pruning time, time of wound treatment after pruning and the wound treatment agent on the incidence of grapevine trunk pathogens in pruning wounds of Cabernet Sauvignon treated with the bio-control agents, *T. atroviride* (UST1) and *T. harzianum* (Eco 77), at different times after pruning (0, 6, 24, 48 and 96 hours) in July and August over two trial seasons, 2011 and 2012.

Factor	Degrees of Freedom	2011		2012	
		F-value	*P*-value	F-value	*P*-value
Pruning time (Pt)	1	3.98	0.061	18.70	0.004
Time of wound treatment (Wt)	4	6.62	0.002	0.68	0.616
Treatment agent (Trt)	2	5.05	0.011	115.5	< 0.001
Pt × Wt	4	5.89	0.003	0.63	0.650
Pt × Trt	2	2.86	0.069	10.20	< 0.001
Wt × Trt	8	0.58	0.785	0.81	0.597
Pt ×Wt × Trt	8	0.52	0.838	0.50	0.849

Appendix B

Table 1: Analysis of variance for the incidence of *Trichoderma atroviride* and *Phaeomoniella* (*Pa.*) *chlamydospora* in detached cane assay, and the chitinolytic activity of culture filtrates from *T. atroviride* grown in suspension with or without peptone.

Factor	Degrees of Freedom	F-value	*P*-value
T. atroviride	10	12.46	<0.0001
Pa. chlamydospora	10	17.06	<0.0001
Chitinolytic activity	1	23.61	<0.0001

Table 2: Analysis of variance for the isolation frequency of *Trichoderma atroviride* and *Phaeomoniella* (*Pa.*) *chlamydospora* in detached cane assay.

Factor	Degrees of Freedom	*T. atroviride*		*Pa. chlamydospora*	
		F-value	*P*-value	F-value	*P*-value
Treatment (trt)	10	3.27	.0008	5.51	<0.0001
Isolation zone (Iso Z)	4	63.40	<0.0001	3.31	0.0107
Trt × Iso Z	40	1.66	0.0071	4.13	<0.0001

Table 3: Analysis of variance on the incidence of *Trichoderma* spp. and *Phaeomoniella* (*Pa.*) *chlamydospora* in the Chenin blanc and Thompson Seedless.

Factor	Degrees of Freedom	*Trichoderma spp.*		*Pa. chlamydospora*	
		F-value	*P*-value	F-value	*P*-value
Cultivar (Cult)	1	29.57	< 0.001	21.61	< 0.001
Treatment (Trt)	7	14.02	< 0.001	11.32	< 0.001
Cult × Trt	7	16.63	< 0.001	7.14	< 0.001

Table 4: Analysis of variance on the incidence of *Trichoderma* spp. and *Phaeomoniella* (*Pa.*) *chlamydospora* for the cultivars separately.

Factor	Degrees of Freedom	Chenin blanc		Thomson Seedless	
		F-value	*P*-value	F-value	*P*-value
Natural Inoculum	7	3.19	0.018	3.39	0.007
Trichoderma spp.	7	5.81	< 0.001	10.20	< 0.001
Pa. chlamydospora	7	9.64	< 0.001	6.41	< 0.001

Table 5: Analysis of variance for incidence of *Trichoderma* spp., *Phaeomoniella* (*Pa.*) *chlamydospora* and natural pathogens infection in 2012.

Factor	Degrees of Freedom	F-value	*P*-value
Natural Inoculum	7	18.27	< 0.001
Trichoderma spp. (Trich)	7	54.15	< 0.001
Pa. chlamydospora (Pch)	7	22.69	< 0.001
Day	3	32.02	< 0.001
Trich × Day	21	0.95	0.527
Pch × Day	21	3.86	< 0.001

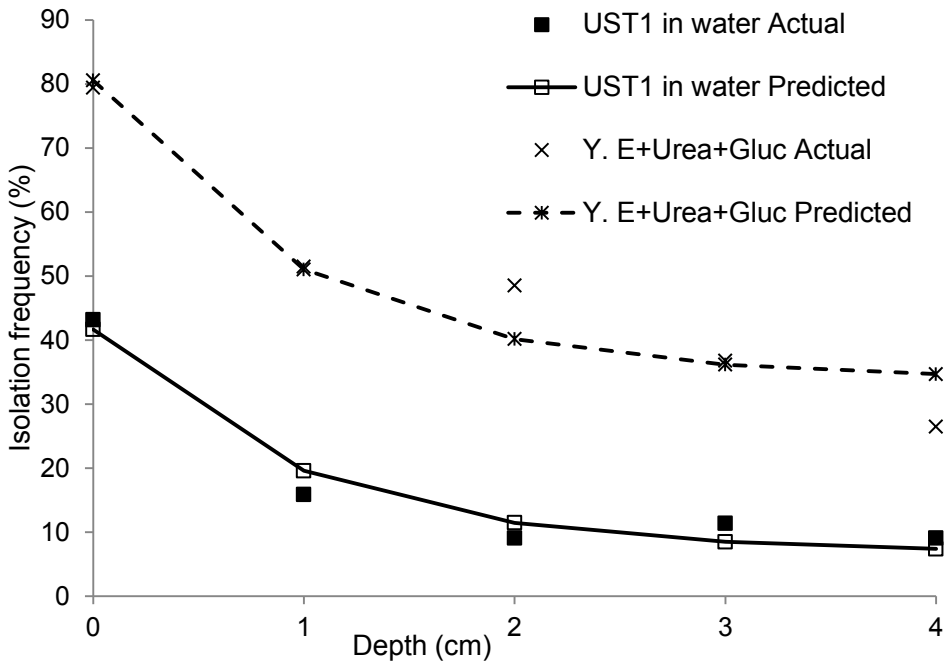

Figure 1: Graph of the actual versus the predicted isolation frequencies of *T. atroviride* for the treatments, UST1 water suspension (UST1 in water) and yeast extract + urea + glucose (Y.E+Urea+Gluc).

Appendix C

Table 1: The analysis of variance for the differences in D_{10} values for the susceptibility the wild type *Trichoderma* isolates to gamma irradiation. Also shown is the analysis of variance for the incidence of *Trichoderma atroviride* in grapevine pruning wounds treated with the wild type or carbendazim resistant mutant strains of biocontrol agent alone or in combination with a fungicide.

Factor	Degrees of Freedom	F-value	*P*-value
D_{10}-values	2	1.39	0.3310
Treatment (trt)	4	76.34	< 0.0001
Inoculation day (Inoc D)	2	2.19	0.1244
Trt × Inoc D	8	1.05	0.4163

Table 2: Analysis of variance for the incidence of *Phaeomoniella chlamydospora* in grapevine pruning wounds inoculated with the pathogen a day or seven days after pruning and treatment with *Trichoderma atroviride* and fungicides separately or in combination. The analysis of variance for the incidence of grapevine trunk pathogens in un-inoculated wounds is also shown.

Factor	Degrees of Freedom	F-value	*P*-value
Treatment (trt)	6	25.08	< 0.0001
Inoculation day (Inoc D)	2	152.06	< 0.0001
Trt × Inoc D	12	6.04	< 0.0001
Natural Inoculum	6	13.38	< 0.0001

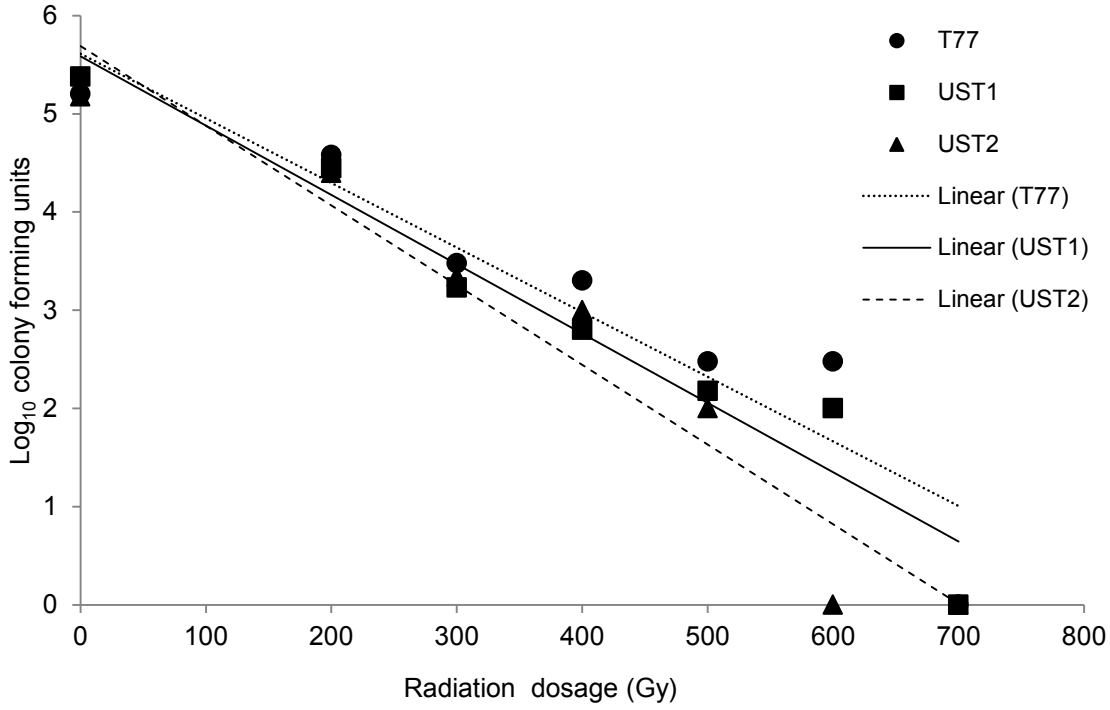

Figure 1: Sensitivity of wild type isolates of *Trichoderma harzianum* (T77) and *T. atroviride* (UST1 and UST2) to gamma irradiation. Correlation analysis revealed strong negative relationship between gamma irradiation dosage and conidial colony forming units. Correlation coefficients (r^2) for the isolates were 0.938, 0.879 and 0.944 for T77, UST1 and UST2, respectively.

Appendix D

Table 1: Quantitative PCR (qPCR) parameters, after validation, of the amplification of defence related and reference genes used in the gene expression experiment.

Gene	qPCR efficiency	Slope (M-value)	R^2
PR1	0.950	-3.343	0.985
PR2	0.976	-3.380	0.994
PR3	1.092	-3.119	0.985
PR4	0.953	-3.475	0.998
PR5	0.993	-3.337	0.986
PR6	1.041	-3.210	0.995
CHIT IV	0.993	-3.477	0.999
PAL	0.992	-3.342	0.949
CHS1	0.974	-3.385	0.984
CHS3	1.041	-3.209	0.996
STS	0.986	-3.353	0.997
STS2	1.039	-3.210	0.994
4CL	1.030	-3.249	0.996
Actin	0.959	-3.412	0.998
60SRP	0.983	-3.492	0.998
VATP16	0.973	-3.389	0.992

Table 2: Analysis of variance for the differences total phenolic content, chitinolytic and 1, 3-glucanase activity in cell suspension cultures elicited with cell free culture filtrates.

Factor	**Degrees of Freedom**	**F-value**	***P*-value**
Phenol content	3	118.08	< 0.001
Glucanase activity	3	113.32	< 0.001
Chitinolytic activity	3	18.51	0.0014

Appendix E

Table 1: Analysis of variance on the effect of culture conditions on the time course production of 6-pentyl α-pyrone.

Factor	Degrees of Freedom	F-value	*P*-value
Isolate (I)	2	16.12	< 0.001
Condition (C)	1	20.02	< 0.001
Time (T)	4	24.42	< 0.001
I × C	2	11.47	< 0.001
I × T	8	18.23	< 0.001
T × C	4	10.63	< 0.001
I × C × T	8	14.62	< 0.001

Table 2: Analysis of variance for the differences in 6-pentyl α-pyrone production in different media and culture conditions.

Factor	Degrees of Freedom	F-value	*P*-value
Isolate (I)	5	17.15	< 0.001
Medium (M)	1	9.63	0.005
Condition (C)	1	18.32	< 0.001
I × M	5	21.34	< 0.001
I × C	5	28.11	< 0.001
M × C	1	4.69	0.041
I × M × C	5	42.54	< 0.001

Table 3: Analysis of variance for the effect of medium and co-culturing with a pathogen on 6PP production.

Factor	Degrees of Freedom	F-value	*P*-value
Isolate (I)	1	7.07	0.014
Medium (M)	1	5.13	0.033
Co-culture(Cc)(Medium)	1	2.23	0.149
I × M	1	0.39	0.540
Cc (I × M)	1	4.85	0.038

Table 4: Regression coefficient and probability of fit for the Gombertz (sigmoidal) function used to determine the LD_{50} for the sensitivity of grapevine trunk pathogens to 6PP.

Pathogen	Isolate	R^2	*P*-value
Eutypa lata	5692	0.97	0.001
	6513	0.96	0.002
Phaeomoniella chlamydospora	6384	0.98	< 0.001
	7732	0.98	< 0.001
Neofusicoccum australe	7025	0.96	0.002
	7029	0.95	0.003
Neofusicoccum parvum	4439	0.98	0.003
	4584	0.98	0.002

Table 5: Analysis of variance on the difference in susceptibility to 6PP between isolates of grapevine trunk pathogens as measured by their LD_{50}.

Pathogen	Degrees of Freedom	F-value	*P*-value
E. lata	1	0.90	0.518
Pa. chlamydospora	1	1.86	0.402
N. australe	1	0.69	0.558
N. parvum	1	123.16	0.057

Table 6: Analysis of variance on the effect of growth medium on isolates of grapevine trunk pathogens sensitivity to 6PP.

Factor	Degrees of Freedom	F-value	*P*-value
Pathogen (P)	2	40233.50	< 0.001
Isolate (Pathogen)	3	48.10	< 0.001
Medium (M)	14	10647.00	< 0.001
M × P	28	388.55	< 0.001
Isolate (P × M)	42	19.22	< 0.001

Table 7: Analysis of variance on the effect of different concentrations of 6PP on the germination of spores and conidia of grapevine trunk pathogens.

Pathogen	Degrees of Freedom	F-value	*P*-value
E. lata	4	53.31	< 0.001
Pa. chlamydospora	4	91.31	< 0.001
N. australe	4	85.89	< 0.001
N. parvum	4	94.29	< 0.001