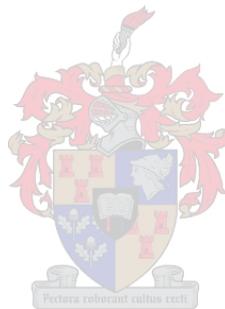


# **BIOLOGICAL CONTROL OF THE GRAPEVINE TRUNK DISEASE PATHOGENS: PRUNING WOUND PROTECTION**

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## SUMMARY

### BIOLOGICAL CONTROL OF THE GRAPEVINE TRUNK DISEASE PATHOGENS: PRUNING WOUND PROTECTION

In recent years, several studies have conclusively shown that numerous pathogens, including several species in the Botryosphaeriaceae, *Phomopsis*, *Phaeoacremonium*, as well as *Phaeomoniella chlamydospora* and *Eutypa lata*, contribute to premature decline and dieback of grapevines. These pathogens have the ability to infect grapevines through pruning wounds, which leads to a wide range of symptoms developing that includes stunted growth, cankers and several types of wood necrosis. Pruning wounds stay susceptible for 2 to 16 weeks after pruning and sustained levels of pruning wound protection is therefore required. The aims of this study were to (i) evaluate the ability of several biological agents to protect pruning wounds, (ii) characterise unknown *Trichoderma* strains and identify their modes of action and (iii) determine the optimal time of season for biological agent application.

Several biological agents were initially evaluated in a laboratory for their antagonism against trunk disease pathogens. The best performing control agents were tested in a field trial conducted on Merlot and Chenin blanc vines in the Stellenbosch region. Spurs were pruned to three buds and the fresh pruning wounds were treated with benomyl as a control treatment, *Trichoderma*-based commercial products, Vinevax® and Eco77®, *Bacillus subtilis*, and *Trichoderma* isolates, USPP-T1 and -T2. Seven days after treatment the pruning wounds were spray inoculated with spore suspensions of four *Botryosphaeriaceae* spp. (*Neofusicoccum australe*, *N. parvum*, *Diplodia seriata* and *Lasiodiplodia theobromae*), *Eutypa lata*, *Phaeomoniella chlamydospora* and *Phomopsis viticola*. After a period of 8 months the treatments were evaluated by isolations onto potato dextrose agar. *Trichoderma*-based products and isolates in most cases showed equal or better efficacy than benomyl, especially USPP-T1 and -T2. Moreover, these isolates demonstrated a very good ability to colonise the wound tissue.

The two uncharacterised *Trichoderma* isolates (USPP-T1 and USPP-T2), which were shown to be highly antagonistic toward the grapevine trunk disease pathogens, were identified by means of DNA comparison, and their ability to inhibit the mycelium growth of the trunk disease pathogens by means of volatile and non-volatile metabolite production studied. The two gene areas that were used include the internal transcribed spacers (ITS 1 and 2) and the 5.8S ribosomal RNA gene and the translation elongation factor 1 $\alpha$  (EF). The

ITS and EF sequences were aligned to published *Trichoderma* sequences and the percentage similarity determined and the two *Trichoderma* isolates were identified as *Trichoderma atroviride*. The volatile production of *T. atroviride* isolates was determined by placing an inverted Petri dish with *Trichoderma* on top of a dish with a pathogen isolate and then sealed with parafilm. *Trichoderma* isolates were grown for 2 days on PDA where after they were inverted over PDA plates containing mycelial plugs. The inhibition ranged from 23.6% for *L. theobromae* to 72.4% for *P. viticola*. Inhibition by non-volatile products was less than for the volatile inhibition. Inhibition ranged from 7.5% for *N. parvum* to 20.6% for *L. theobromae*. In the non-volatile inhibition USPP-T1 caused significantly more mycelial inhibition than USPP-T2.

The timing of pruning wound treatment and subsequent penetration and colonisation of the wound site was also determined. One-year-old canes of the Shiraz and Chenin blanc cultivars were grown in a hydroponic system, pruned and spray treated with a spore suspension of *Trichoderma atroviride* (USPP-T1) as well as a fluorescent pigment. On intervals 1, 3, 5 and 7 days after treatment, the distal nodes were removed and dissected longitudinally. From the one half, isolations were made at various distances from the pruning surface, while the other half was observed under ultra-violet light to determine the depth of fluorescent pigment penetration. Shortly after spray-inoculation of a fresh pruning wound, *Trichoderma* was isolated only from the wound surface and shallow depths into the wound (2 to 5 mm). One week after inoculation, *Trichoderma* was isolated at 10 mm depths, and after 2 weeks, at 15 mm depths. Fluorescent pigment particles were observed to a mean depth of 6 mm, which suggests that initial isolation of *Trichoderma* at these depths was resultant of the physical deposition of conidia deeper into the pruning wound tissue, whereas the isolation of *Trichoderma* from deeper depths might be attributed to colonisation of grapevine tissue. In a vineyard trial, fluorescent pigment was spray-applied to pruning wounds of Shiraz and Chenin blanc grapevines during July and September at intervals 0, 1, 3, 7 and 14 days after pruning. One week after treatment, the distal nodes were removed and dissected longitudinally. Each half was observed under UV light and the pigment penetration measured. For Chenin blanc and Shiraz, July pruning wounds showed significant deeper penetration of the pigment than pruning wounds treated in September. Moreover, pruning wounds made in September showed pigment particles in longitudinal sections up to 1 day after pruning, whereas wounds made in July showed pigment particles up to 3 days in the xylem vessels. These findings suggest that the best time for application of a biological control agent should be within the first 24 hours after pruning.

## OPSOMMING

### BIOLOGIESE BEHEER VAN WINGERD STAMSIEKTE PATOGENE: SNOEIWOND BESKERMING

Dit is wel bekend dat stamsiektes veroorsaak word deur 'n kompleks van patogene wat die vermoë besit om wingerdplante deur snoeiwonde te infekteer en sodoende terugsterwing te veroorsaak. Verskeie swamspesies behoort tot hierdie kompleks, wat *Botryosphaeriaceae*, *Phomopsis*, *Phaeoacremonium*, *Phaeomoniella* and *Eutypa lata* insluit. Snoeiwonde bly vatbaar vir infeksie tot 16 weke na snoei, en daarom is dit van kardinale belang dat die wonde vir hierdie periode beskerm word. Die doel van hierdie studie was om (i) verskeie biologiese agente te evalueer en elk se vermoë om snoeiwonde te beskerm, te bepaal; (ii) die identifikasie van ongeïdentifiseerde *Trichoderma* isolate en die meganisme wat hulle gebruik om patogene te inhibeer, te bepaal; en (iii) die bepaling van die beste tyd gedurende die seisoen om behandelings toe te dien.

Verskeie potensiële biologiese agente is eerstens in 'n laboratoriumproef geëvalueer waarna die isolate wat die meeste potensiaal getoon het verder in 'n veldproef geëvalueer is. Die veldproef is in die Stellenbosch-distrik van Suid-Afrika op twee verskillende kultivars, naamlik Shiraz en Chenin blanc, uitgevoer. Dit het behels dat die draers tot 3 ogies gesnoei is, waarna die vars snoeiwonde met spoorsuspensies van die ongeïdentifiseerde *Trichoderma* isolate, USPP-T1 en USPP-T2, sowel as die geregistreerde konsentrasies van die kommersiële produkte, Eco 77, Vinevax, *Bacillus subtilis* en Biotricho, behandel is. Benomyl is aangewend as die behandelde kontrole en water as die onbehandelde kontrole. Sewe dae nadat die snoeiwonde behandel is, is hulle met 'n spoorsuspensie van *Botryosphaeriaceae* spp. (*Neofusicoccum australe*, *N. parvum*, *Diplodia seriata* en *Lasidiopodia theobromae*), *Eutypa lata*, *Phaeomoniella chlamydospora* en *Phomopsis viticola* geïnkuleer. Na 8 maande in die veld is die proef geëvalueer. Die *Trichoderma* produkte en isolate het in verskeie gevalle net so goed of selfs beter as die behandelde kontrole benomyl gevaar het. Dit was veral noemenswaardig vir die USPP-T1 en USPP-T2 isolate, terwyl hierdie isolate ook die vermoë getoon het om die snoeiwonde suksesvol te koloniseer.

Die ongeïdentifiseerde *Trichoderma* isolate, USPP-T1 en USPP-T2, is identifiseer deur twee geen-areas ITS en EF te vergelyk met gepubliseerde *Trichoderma* nukleotied volgordes. Die onbekende isolate is daarvolgens as *Trichoderma atroviride* geïdentifiseer. Weens die

isolate se vermoë om hoogs antagonisties op te tree teenoor die stamsiekte patogene, is die meganismes wat daarby betrokke is verder bestudeer. Om te bepaal of hierdie isolate vlugtige metaboliete produseer, is petri bakkies bevattende die *Trichoderma* isolate vir 2 dae geïnkubeer, waarna petri bakkies bevattend die patogene onderstebo op die petri bakkies bevattende die *Trichoderma* geplaas is. Die mate van inhibisie het gewissel tussen 23.6% vir *L. theobromae* en 72.4% vir *P. viticola*. Alhoewel bevredigende inhibisie tydens die produksie van vlugtige metaboliete waargeneem is, het die produksie van nie-vlugtige metaboliete 'n drastiese kleiner effek op inhibisie van die patogene gehad. Inhibisie deur die produksie van nie-vlugtige metaboliete het variëer tussen 7.5% vir *N. parvum* en 20.6% vir *L. theobromae*. Dit is egter ook waargeneem dat USPP-T1 statisties meer patogeen groei inhibeer het weens nie-vlugtige metabolite produksie as USPP-T2.

Die vermoë van die *Trichoderma* isolate om snoeiwonde te koloniseer sowel, as die beste tyd vir aanwending van hierdie behandelings is ook bepaal. Een-jaar-oue lote is in 'n hidroponiese sisteem gegroei, waarna dit gesnoei is en die vars wonde met *T. atroviride* spore en fluoresserende pigment behandel is. Op 1, 3, 7 en 14 dae na behandeling is die boonste node verwyder en in die lengte gedissekteer. Een helfte is onder UV lig bestudeer om die mate van penetrasie van die pigment in die snoeiwond te bepaal, terwyl isolasies op verskillende dieptes vanuit die ander helfte gemaak is. Direk na behandeling is *Trichoderma* vanuit die oppervlak van die wond tot op 'n diepte van 5 mm geïsoleer. Een week na behandeling is dit isoleer tot op 10 mm en twee weke na behandeling tot op 15 mm geïsoleer. Aanvanklik is die pigment waargeneem tot op 6 mm wat ooreenstem met die aanvanklike penetrasie van die *Trichoderma* spore. Tydens 'n veldproef is fluoresserende pigment aangewend op snoeiwonde van Shiraz en Chenin blanc op 0, 1, 3, 7 en 14 dae na snoei, tydens Julie en September. Een week na behandeling is die boonste node verwyder en in die lengte gedissekteer. Beide helftes is onder UV lig bestudeer en die penetrasie diepte gemeet. Snoeiwonde in Julie het statisties dieper penetrasie getoon as snoeiwonde in September. Waar snoeiwonde in September slegs penetrasie getoon het tot en met 1 dag na snoei, het snoeiwonde in Julie penetrasie getoon tot en met 3 dae na snoei. Dus wil dit voorkom of die kritiese tyd vir aanwending die eerste 24 uur na snoei is.

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# 1. INTEGRATED MANAGEMENT OF GRAPEVINE TRUNK DISEASES WITH SPECIAL REFERENCE TO BIOLOGICAL CONTROL WITH *TRICHODERMA* AND *BACILLUS* SPECIES

## INTRODUCTION

In recent years, several studies have conclusively shown that numerous pathogens contribute to premature decline and dieback of grapevines (Larignon and Dubos, 1997; Mugnai *et al.*, 1999; Peros *et al.*, 1999; Halleen *et al.*, 2003). These pathogens have the ability to infect grapevines through pruning wounds (Lehoczky, 1974; Ferreira *et al.*, 1989; Adalat *et al.*, 2000; van Niekerk *et al.*, 2005), and lead to the development of a wide range of symptoms developing that includes stunted growth (Munkvold *et al.*, 1994; Mahoney *et al.*, 2003), cankers and several types of wood and leaf necrosis (Van Niekerk *et al.*, 2006; Sosnowski *et al.*, 2005). These diseases lead to a reduction in both the quantity and quality of grapes, causing severe economic losses (Calzarano *et al.*, 2001, 2004; Siebert, 2001; van Niekerk *et al.*, 2003). Extensive research has identified the pathogens in this complex to be several species in the Botryosphaeriaceae (Crous *et al.*, 2006; van Niekerk *et al.*, 2004), *Phomopsis* (van Niekerk *et al.*, 2005), *Phaeoacremonium* (Crous and Gams, 2000; Mostert *et al.*, 2006), as well as *Phaeomoniella chlamydospora* (Mugnai *et al.*, 1999) and *Eutypa lata* (Munkvold *et al.*, 1994). Historically, management of trunk diseases relied on sanitary practices as well as the protection of the surface area of pruning wounds (Carter and Price, 1974; Moller and Kasimatis, 1980; Ferreira *et al.*, 1991; Munkvold and Marois, 1993a, b; John *et al.*, 2001; Halleen and Fourie, 2005; Rolshausen *et al.*, 2005; Sosnowski *et al.*, 2005; Bester *et al.*, 2007). In this chapter, the symptoms, disease cycle and management of abovementioned pathogens of grapevines are discussed as well as the life cycle and modes of action of biological control agents used in the protection of pruning wounds against infection by these pathogens.

## EUTYPA DIEBACK OF GRAPEVINES

*Eutypa lata* (Pers.) Tul. & C. Tul. (syn. *E. armeniaca* Hansf. & Carter) (Anamorph: *Libertella blepharis* A.L. Smith) has been identified as the causal agent of Eutypa dieback also known as eutypiose dead arm disease (Carter 1991; Munkvold *et al.*, 1994; Péros and Larignon, 1998). *E. lata* is an ascomyceteous, canker forming pathogen that penetrates

woody hosts with ascospores through exposed xylem tissue at wound sites; the fungus colonises the xylem tissue, then the cambium followed by the phloem tissue (Deswarte *et al.*, 1996a). This pathogen was first described from apricot trees showing dieback symptoms (Carter *et al.*, 1985), but several reports from Australia and North America have revealed that it has an additional 80 host species (Carter *et al.*, 1985), including lemon (Kouyeas, 1978), apples (Glawe *et al.*, 1983), pears (Carter, 1982), almonds (Carter, 1982) and grapevines (Chapuis *et al.*, 1998). It was first reported from grapevines in California in 1967 when Moller *et al.* (1968) found mature and developing stromata of the pathogen on dead grapevine wood in a vineyard situated near an infected apricot orchard. Since then it has been recognised as an aggressive pathogen of grapevines wherever they are grown, causing decline and dieback, which results in economic losses due to lower yields of poorer quality grapes (Munkvold *et al.*, 1991, 1994; Wicks and Davies, 1999; Siebert, 2001; van Niekerk *et al.*, 2003).

### **Disease cycle**

Eutypa dieback of grapevines does not occur in arid areas as a mean annual rainfall of at least 300 mm is needed to produce ascospore forming perithecia on trunks or arms in a layer of stromatic tissue (Carter, 1957; Ramos *et al.*, 1975; Magarey and Carter, 1986). Pycnidiospores produced by the anamorph stage of the pathogen (*Libertella blepharis* (syn. *Cytosporina* spp.) A.L. Smith) are believed to play a lesser role in the disease cycle of the pathogen most likely due to its low germination rates (Ju *et al.*, 1991). Environmental conditions conducive to ascospore release from perithecia have been reported as being 2 mm of rain at temperatures above 0°C (Pearson, 1980; Trese *et al.*, 1980; Magarey and Carter, 1986). Furthermore, recent a report by van Niekerk *et al.* (2007) has shown aerial ascospore levels to peak after spring and autumn rain suggesting that stromal exposure to moisture is needed for ascospore release. These airborne ascospores, originating from perithecial stroma are the most important inoculum source (Chapuis *et al.*, 1998). After release, these airborne ascospores infect the vine through several types of wounds, mostly pruning wounds (Ramos *et al.*, 1975; Ferreira *et al.*, 1989) but also smaller wounds, such as desuckering wounds (Lecomte *et al.*, 2005). Due to pruning wounds being the main infection portal for *E. lata*, it gains access to the vascular system through which it then spreads at an average of 10-20cm per year (Carter, 1957; Ferreira *et al.*, 1989; Carter 1991).

## Symptoms

The first symptoms of Eutypa dieback usually become visible at least 2–3 years after the first infection took place (Munkvold and Marois, 1995), and severe dieback is often observed in grapevines that are older than 10 years (Munkvold *et al.*, 1994). In early spring, when the new growth cycle starts, the first foliar symptoms become visible when new, healthy shoots are 25–30 cm long (Carter, 1988). Affected shoots are stunted and malformed with small chlorotic cup-shaped leaves with necrotic margins and underdeveloped fruit clusters (Petzoldt *et al.*, 1981; Magarey and Carter, 1986; Carter, 1988; Munkvold *et al.*, 1994; Munkvold and Marois, 1995). Desiccation of flowers from these shoots leads to fewer clusters developing (Magarey and Carter, 1986; Munkvold *et al.*, 1994) and along with the factors previously mentioned, contributes to a reduction in yield and wine quality and consequently economical loss (Munkvold *et al.*, 1994; Wicks and Davies, 1999; Siebert *et al.*, 2001; Mahoney *et al.*, 2003).

Abovementioned shoot and foliar symptoms are normally associated with faint vascular streaking that leads to a canker surrounding an old pruning wound where the initial infection took place. These cankers often penetrate deep into the xylem of grapevines, causing dieback or total death of the grapevine cordon (Petzoldt *et al.*, 1981; Munkvold and Marois, 1995). Internally, necrosis of xylem tissue that develops as result of infection can be observed as a wedge-shaped discoloured sector in cross-sections of infected trunks and cordons (Moller and Kasimatis, 1978; Carter, 1988; Chapuis *et al.*, 1998). In some cases, foliar symptoms develop on parts of the vine without any signs of internal wood necrosis. This was found to be the effect of a phytotoxin, eutypine, produced by the pathogen in older, infected wood (Tey-Rulh *et al.*, 1991).

Eutypine (4-hydroxy -3-(3-methyl -3-butane-1-ynyl) is a toxic compound believed to be transported to the herbaceous parts of the plant, spreading throughout leaves and inflorescences (Deswarte *et al.*, 1996b; Amborabe *et al.*, 2001). However, more recently Mollyneux *et al.* (2002) and Mahoney *et al.* (2005) isolated the corresponding phenol of eutypine, eutypinol, more readily from infected grapevine wood than eutypine. The abovementioned authors suggested that symptom expression could therefore be the result of a suite of metabolites produced by *E. lata*. Studies conducted by Deswarte *et al.* (1996b) into the effect of eutypine on grapevine cells showed that the toxin behaves as a weak lypophylic acid and induces drastic changes in the respiration and energy balance of grapevine cells as well as alterations of the cellular ultrastructure, which could explain the symptoms expressed

in the herbaceous parts of the grapevine. It was furthermore found to also have an effect on the colour development in berries. The red and black colours of grapes result from the accumulation of anthocyanins in the skin of the berry (Boss *et al.*, 1996). Eutypine affect the accumulation of the anthocyanins in the grapevine cell cultures by inhibiting the expression of the UDP glucose-flavonoid 3-*O*-glucosyl transferase (UFGT) gene (Afifi *et al.*, 2002).

## **Management**

### ***Chemical***

The fungicide benomyl has been shown to be an effective protectant of pruning wounds against *E. lata* infection (Munkvold and Marois, 1993b, Creaser and Wicks, 2002). However, use of benomyl was discontinued in 2001 (Sosnowski *et al.*, 2004; Malone, 2006). Therefore, multiple studies were conducted to find an acceptable replacement. However, alternative chemicals have been tested in the past as shown in the study by Munkvold and Marois (1993b) who found that not only did fluzilazole have the ability to inhibit the ascospore germination and mycelium growth of *E. lata*, but also had the ability to protect pruning wounds artificially inoculated with the pathogen. Moreover, Malone (2006) stated that products consisting of the active ingredients; benomyl and fluzilazole as well as fluazanim had potential as pruning wound protectants. Furthermore, Sosnowski *et al.* (2004), reported that carbendazim and pyrimethanil reduced infection in pruning wounds artificially inoculated with *E. lata* up to 14 and 7 days after inoculation, respectively.

Several application methods have been tested. Airblast sprays of benomyl were found to lead to a reduction of 48.5% in infection by *E. lata* over a five year period (Ramsdell, 1995). Pearson *et al.* (1982) suggested hand painting chemicals onto pruning wounds after studies found that painting benomyl at 10 mg/mL onto fresh pruning wounds provided between 84% and 100% control of *E. lata*. Moreover, studies conducted by Rolshausen and Gubler (2005), who painted boric acid onto pruning wounds in the form of Biopaste (mixture of 5% boric acid and *Cladosporium herbarum* spores) and Bioshield (mixture of 5% boric acid and latex paint), conclusively showed that chemicals can be hand painted in paste form or sprayed as a water based solution onto pruning wounds to provide good protection against *E. lata* infection. Due to the fact that ascospores are released by perithecia after as little as 2 mm of rainfall, it is recommended that wounds should be treated in abovementioned manner directly after pruning, prior to any rainfall taking place (Chapuis *et al.*, 1998).

### **Biological**

Several biological control agents have already been evaluated *in vitro* and *in vivo* against *E. lata* with varying results. Carter and Price (1974) used a benomyl resistant strain of *Fusarium lateritium* as it was believed that the benomyl would provide good short term protection of pruning wounds until the *F. lateritium* has thoroughly colonised the wounds, thereby providing good long term protection against *E. lata* infection. Other biological agents that have been tested include several strains of *Trichoderma* spp. (Hunt *et al.*, 2001; John *et al.*, 2004; John *et al.*, 2005), *Bacillus subtilis* (Ferreira *et al.*, 1991) and *Erwinia herbicola* (Schmidt *et al.*, 2001).

According to the abovementioned studies, *Trichoderma* isolates produce volatile as well as non volatile products *in vitro* that inhibit the growth of *E. lata*, giving an indication of the mechanisms involved with *in vivo* protection of pruning wounds. When pruning wounds were treated with *Trichoderma*-based products 1 day prior to being inoculated with *E. lata*, the infection was reduced by almost 67% in comparison with untreated controls (John *et al.*, 2005). These findings led to the development of several *Trichoderma*-based products for the protection of pruning wounds (Agrimm Technologies Ltd., Christchurch, New Zealand). These products are based on a mixture of seven *T. harzianum* and *T. atroviride* strains in liquid formulations for injection into vines, dressings and spray formulations for application to wounds.

Studies conducted by Ferreira *et al.* (1991) found that *B. subtilis* inhibits mycelial growth of *E. lata in vitro* by 88%, and when used as a pruning wound treatment, it reduced the incidence of the pathogen in pruning wounds by 100%. A later study by Schmidt *et al.* (2001), showed that a transposon mutant of *E. herbicola* had a 100% efficiency rate against *E. lata* on autoclaved grape wood.

### **Cultural practices**

It is well documented that dead wood and old tree trunks are the primary inoculum source of *E. lata* (Carter, 1957; Ramos *et al.*, 1975; Magarey and Carter, 1986; Sosnowski *et al.*, 2004b). Therefore, sanitation of vineyards, by removing debris, dead wood and old trunks, is of utmost importance in controlling the disease.

Wound age and date of pruning have been shown to affect susceptibility to *E. lata* infection in apricot trees (Carter and Moller, 1967). This is also noticeable in grapevines where the pruning wounds are more susceptible to *E. lata* infection after pruning early in the

dormant season compared to later (Petzoldt *et al.*, 1981; Munkvold and Marois, 1995; Chapuis *et al.*, 1998). Initial studies indicated that pruning wounds made on 1-year-old wood were more resistant to infection compared to wounds made in older wood (Moller and Kasimatis, 1980; Trese *et al.*, 1980). However, Munkvold and Marois (1995) found in a later, more comprehensive study that the age of wood at the time of pruning did not significantly affect the susceptibility of the wounds. Pruning later in the dormant season could also have an added benefit. At this time pruning wounds normally exudes large amounts of plant sap, which might flush any spores from the vascular tissue (Munkvold and Marois, 1995). It is also important not to prune during or shortly after periods of rainfall as *E. lata* inoculum levels have been shown to be higher during these periods that could lead to more infections taking place (Welgemoed, 1989).

Grapevine cultivars differ significantly in their susceptibility to *E. lata* infection (Munkvold and Marois, 1995; Chapuis *et al.*, 1998), especially premium red cultivars (Sosnowski *et al.*, 2004b). Studies have shown Grenache to be highly susceptible to *E. lata* infection, especially in comparison to Cabernet Sauvignon and Merlot. Foliar symptom expression of the Grenache was more severe than the other cultivars after *E. lata* inoculation compared to latter mentioned cultivars (Sosnowski *et al.*, 2007), suggesting that by growing less susceptible hosts the effect of the disease might be reduced.

Remedial pruning is a practice widely used by growers in the management of *E. lata* infected grapevines (Sosnowski *et al.*, 2004a). This entails cutting the infected arm or trunk of a vine away up to the point where no internal necrosis, indicating infected wood, is visible and then cutting a further 10–20cm of healthy wood. The wound where the arm or trunk was cut must then be treated with a biological- or chemical sealant or both (Moller and Kasimatis, 1980; Welgemoed, 1989).

## **BOTRYOSPHAERIACEAE DISEASES OF GRAPEVINE**

This disease of grapevines, also known as Botryosphaeria canker, bot canker or black dead arm have been shown to be caused by a number of species residing in the Botryosphaeriaceae, including *Botryosphaeria* and aggregate genera *Lasiodiplodia* and *Neofusicoccum*, that are cosmopolitan in nature and known to have wide host ranges (Denman *et al.*, 2000; van Niekerk *et al.*, 2004; Crous *et al.*, 2006). On grapevines they are associated with symptoms such as bud mortality, dieback, internal wood necrosis, vascular

streaking and various other symptoms attributed to the infection of the vascular tissue (van Niekerk *et al.*, 2004; Luque *et al.*, 2005; Taylor *et al.*, 2005; Wood and Wood, 2005; van Niekerk *et al.*, 2006).

Correct field diagnosis of the disease is further hampered due to the overlap of symptoms between different species and certain symptoms that closely resemble other diseases such as Phomopsis dead arm caused by *Phomopsis viticola* (Sacc.) Sacc. and Eutypa dieback caused by *E. lata* (Chamberlain *et al.*, 1964; Magarey and Carter, 1986; Castillo-Pando *et al.*, 2001; Hewitt and Pearson, 1988; Phillips, 1998; Larignon and Dubos, 2001; Leavitt, 2004; van Niekerk *et al.*, 2006). Due to this confusing scenario, the involvement of species in the Botryosphaeriaceae needs to be confirmed by isolations onto artificial media. Species identification is difficult using teleomorphs, which are seldom encountered in nature and do not readily form in culture (Denman *et al.*, 2000). Consequently, morphological species identification have relied on the characteristics of the associated anamorph genera that showed a greater range of morphological characteristics than the teleomorph, making species identification easier (Denman *et al.*, 2000; Luque *et al.*, 2005).

Initially these anamorph genera in the Botryosphaeriaceae were assigned to 18 coelomycete genera (Phillips *et al.*, 2005). This number was later reduced to only four namely *Fusicoccum* Corda, *Diplodia* Fr. *Dothiorella* Sacc. and *Lasiodiplodia* Ellis & Everh (Crous and Palm, 1999; Denman *et al.*, 2000; Phillips *et al.*, 2005). Recent revision of the species in the Botryosphaeriaceae using morphological characteristics combined with DNA sequence data, identified 10 different lineages within the Botryosphaeriaceae, including the *Diplodia* / *Lasiodiplodia* / *Tiarosporella* group with unknown teleomorphs, *Botryosphaeria sensu stricto* with *Fusicoccum* anamorphs and *Neofusicoccum*, with *Botryosphaeria*-like teleomorphs and *Dichomera*-like synanamorphs, which also includes the different species associated with diseases of grapevines (Crous *et al.*, 2006). Currently, four species in the Botryosphaeriaceae are regarded as being the most important pathogenic species on grapevine in South Africa (van Niekerk *et al.*, 2004; 2006). These species include *Neofusicoccum australe* (previously *Botryosphaeria australis*), *Diplodia seriata* (previously *Botryosphaeria obtusa*), *Lasiodiplodia theobromae* (previously *Botryosphaeria rhodina*) and *Neofusicoccum parvum* (previously *Botryosphaeria parva*) (van Niekerk *et al.*, 2004, Crous *et al.*, 2006). Species in the Botryosphaeriaceae could have a large economical impact on the grape and wine industry as seen by the fact that *Diplodia seriata* alone have been reported as

being responsible for losses of up to 20% in the Bordeaux wine region of France (Larignon *et al.*, 2001).

### **Symptoms**

As stated previously, the symptoms associated with infection of grapevines by species in the Botryosphaeriaceae can easily be confused with symptoms of other trunk disease pathogens such as Phomopsis cane and leaf blight (Phillips, 2002) caused by *P. viticola* or Eutypa dieback caused by *E. lata*. Eutypa dieback can be distinguished from the symptoms caused by species in the Botryosphaeriaceae by the stunted and chlorotic spring growth that is typical of *E. lata* infection (Gubler *et al.*, 2005). Symptoms of Botryosphaeriaceae infection develop slowly and can only be visibly noticed in infected vineyards older than 8 years (Larignon and Dubos, 2001; Van Niekerk *et al.*, 2004). Due to the fact that this group of pathogens can occur endophytically in host plants, severe symptom expression usually occurs when the vine has been subjected to predisposing stress conditions such as water stress (Smith *et al.*, 1996; Stanosz *et al.*, 2001; Burgess *et al.*, 2005).

Within cordons and trunks, a wide variety of symptoms is often visible and includes wedge shaped necrotic lesions combined with brown vascular streaking (Castillo-Pando, 2001; van Niekerk *et al.*, 2004; Gubler *et al.*, 2005). Similar to abovementioned symptom is an arched shaped necrotic lesion in the vascular tissue that is clearly visible in cross sections of the trunks and cordons (van Niekerk *et al.*, 2006). In the field, vascular streaking in the xylem tends to originate at pruning wounds or where structural damage has occurred, extending in both directions up or down the vascular tissue.

External symptoms include dieback of canes and shoots, delayed bud burst and death of spur positions (Gubler *et al.*, 2005; Van Niekerk *et al.*, 2004; Taylor *et al.*, 2005), while berry rot symptoms have also been reported (Hewitt and Pearson, 1988; Larignon and Dubos, 2001; Leavitt, 2004). Cankers formed below infected pruning wounds or other infection points are usually small but can extend up to half of the length of the cordon (Castillo-Pando, 2001). These symptoms are characteristic of grapevine decline (Taylor *et al.*, 2005) that leads to a reduction in yield due to the loss of cordon production as well as an increase in production cost due to the implementation of control measures to protect the vineyard (Gubler *et al.*, 2005).

## **Disease cycle**

In dry arid conditions, or in the absence of a host plant, Botryosphaeriaceae spp. can survive saprophytically on dead wood and bark (Castillo-Pando *et al.*, 2001). Dead wood and pruning debris serve as the main inoculum sources (Hewitt and Pearson, 1988). Large numbers of conidia are produced in pycnidia underneath the bark of cordons, spurs and dead wood (Gubler *et al.*, 2005). Moreover, Ahimera *et al.* (2004) stated that *Botryosphaeria dothidea* sporulated on dead wood left in trees and on the floor of pistachio orchards. These pycnidia erupt after rain or prolonged periods of wetness releasing large numbers of conidia that can easily be spread by wind or splashed in water drops from rain or sprinkler irrigation (Holmes and Rich, 1970; Creswell and Milholland, 1988; Pusey, 1989; Gubler *et al.*, 2005). Therefore, periods of wetness are compulsory for high levels of infection, as described by Pusey and Bertrand (1993) who found that long periods of wetness along with high inoculum levels contributed to substantial infections of host plants. Studies have shown that, not only can these conidia infect the host plants through pruning wounds or structural damage (Lehoczky 1974; 1988), but they also have the ability to infect the host plant through lenticels and stomata (Brown and Hendrix, 1981; Kim *et al.*, 2001). Furthermore, it has been reported that Botryosphaeriaceae has the ability to survive endophytically in healthy tissue of *Eucalyptus* spp. Given this ability, it is possible to have an infected host that does not display any symptoms (Smith *et al.*, 1996). This paves the way for the pathogen to be introduced to new areas through propagation material (Burgess *et al.*, 2005).

## **Management**

### ***Chemical***

Historically, sodium arsenite was applied in France to control Botryosphaeria canker using the same recommendations as specified Esca disease (Larignon and Dubos, 2001). The recommended application method for sodium arsenite included foliar sprays as well as painting of the treatment directly onto infected vines as a preventative measure (Mugnai *et al.*, 1999). Due to the environmental impact of sodium-arsenite the product was banned from further use in several countries (Lyubun *et al.*, 2002).

Infection portals of the disease were shown to be natural openings such as lenticels or stomata as well as wounds caused by pruning, and so management consequently focused on the protection of these openings (Taylor *et al.*, 2005). An *in vitro* study testing the efficacy of 10 different fungicides against four different species of Botryosphaeriaceae; *N. australe*, *D.*

*seriata*, *L. theobromae* and *N. parvum* indicated that benomyl and tebuconazole gave the best results with regards to mycelial inhibition, with *D. seriata* being the most sensitive to these two fungicides (Bester and Fourie, 2005). Bioassays done by Bester *et al.* (2007) indicated that benomyl, tebuconazole and prochloraz were the most effective in protecting pruning wounds against infection by the four abovementioned four Botryosphaeriaceae species. Furthermore, Brown-Rytlewski *et al.* (2000) reported that wounds painted with benomyl, trifloxystrobin and kresoxim-methyl reduced canker size of apple trees after inoculation with *B. dothidea* and *D. seriata* compared to control treatments, while Leavitt (2003) showed that captan, benomyl, iprodione and penconazole reduced the infection of grapevine pruning wounds inoculated with *L. theobromae* after treatment. Boron based fungicides were also tested and shown to be effective against the entire complex of wood decay fungi associated with grapevines (Gubler *et al.*, 2005).

### **Biological**

Harvey and Hunt (2005) assessed the antagonistic ability of eight strains of *Trichoderma* consisting of *T. atroviride* and *T. harzianum* towards five Botryosphaeriaceae isolates, with promising results such as mycoparasitism at microscopic level. However, no studies were conducted to investigate these results further. Studies by Kexiang *et al.* (2002) on *Botryosphaeria berengariana* f. sp. *piricola*, the casual agent of apple ring rot showed that two isolates of *Trichoderma* (*Trichoderma harzianum* T88 and *T. atroviride* T95) produced volatile and non-volatile antibiotics that inhibited the mycelial growth of the pathogen. The authors also reported that spore suspensions of isolates T88 and T95 controlled the canker forming pathogen on stems and shoots of apples. These findings suggest that there is merit in studying the ability of *Trichoderma* isolates to biologically control Botryosphaeriaceae infection in grapevines along with the fact that there is little previous research.

## **PETRI AND ESCA DISEASES OF GRAPEVINES**

Petri disease, previously known as “black goo”, “slow dieback”, “slow decline” or “Phaeoacremonium grapevine decline” (Ferreira *et al.*, 1994; Ferreira, 1998; Scheck *et al.*, 1998b; Morton, 2000) is a problem in most areas where grapevines are grown (Mostert *et al.*, 2003). Larignon and Dubos (1997) stated it to be one of the most destructive diseases in Europe and California. It occurs asymptotically in propagation material (Halleen *et al.*, 2003) and infected vines only express symptoms when under stress (Borie *et al.*, 2002).

Vineyards between the ages of 1 and 5 years seem to be the most susceptible to this disease (Fourie and Halleen, 2004). It is caused by a combination of *Phaeoconiella chlamydospora* and several *Phaeoacremonium* species (Scheck *et al.*, 1998a; Mugnai *et al.*, 1999; Morton, 2000; Mostert *et al.*, 2006). Although *Phaeoacremonium aleophilum* is the most prominently isolated species from grapevine, several other species have been known to infect grapewood to a lesser extent. These include *Pm. angustius*, *Pm. australiensis*, *Pm. inflatipes*, *Pm. parsiticum* and *Pm. scolyti*, *Pm. subulatum* and *Pm. viticola*. (Mostert *et al.*, 2006) Affected vines show stunted and chlorotic growth, while older vines tend to display increasing decline over several years before eventually dying (Ferreira, 1998; Jaspers, 2001). It has caused large losses of young vineyards in newly established vineyards (Bertelli *et al.*, 1998; Scheck *et al.*, 1998a; Ferreira *et al.*, 1999; Mugnai *et al.*, 1999; Pascoe and Cottral, 2000), while wine made from grapes harvested from infected vines are also of a poorer quality (Calzarano *et al.*, 2001; 2004).

Esca disease of grapevines is related to Petri disease and has been known for a very long time in grape producing areas of the world (Mugnai *et al.*, 1999). It is regarded as a complex disease with several fungal pathogens involved in its etiology (Surico, 2000). It is distinguished from Petri disease by the white rot observed in the older vines affected by esca (Graniti *et al.*, 2000; Surico, 2001). This characteristic symptom has been shown to be caused by several basidiomycete species in the genus *Fomitiporia*, including *F. mediterranea*, *F. polymorpha* and *F. australiensis* (Fischer *et al.*, 2005; Fischer, 2006; 2007). Apart from the several basidiomycetes associated with Esca disease, *Pa. chlamydospora* and several known *Phaeoacremonium* species have also been shown to be associated with the internal wood decay symptoms in affected grapevines (Larignon and Dubos, 1997; Fischer and Kassemeyer, 2003).

## **Symptoms**

### ***Petri disease***

Grapevines affected by Petri disease exhibits stunted growth and signs of decline and dieback (Ferreira, 1998; Fourie and Halleen, 2004a). The shoots have shortened internodes, small leaves displaying interveinal chlorosis that develops into necrosis that eventually causes leaf drop (Scheck *et al.*, 1998b; Fourie and Halleen, 2004a). In cross sections of diseased vines black to brown streaking can be observed combined with black to brown spots in the xylem tissue and an unusually dark pith (Ferreira *et al.*, 1994; Sparapano *et al.*, 2000a;

Laukart *et al.*, 2001. A black exudate, reminiscent of honey or yeast extract oozes from these spots (Crous and Gams, 2000; Morton, 2000; Chicau *et al.*, 2000; Sparapano *et al.*, 2000a).

Usually these symptoms occur in the rootstock, the graft union, the scion trunk, the scion arms or cordons and to a lesser extent the roots (Morton, 2000). The exudate is a host response to infection of the vascular tissue by the pathogen (Ferreira *et al.*, 1994). Production of tyloses, gums and phenolic compounds are reactions by the grapevine to keep the pathogen out of the xylem, which also leads to blockage of the host plant xylem (Mugnai *et al.*, 1999; Lorena *et al.*, 2001). Studies have revealed that for every 1% increment of the xylem blocked by these materials, the functional xylem decreased with 16% (Edwards *et al.*, 2007). During water stress the host is predisposed to the pathogenic phase of the pathogen, which leads to the vine displaying more severe symptoms (Mugnai *et al.*, 1999).

### ***Esca disease***

Typical of a disease caused by a complex of pathogens, Esca disease is characterised by a variety of foliar and internal symptoms. Diseased vines can in some cases be identified by delayed budding in spring when the new growth cycle starts (Mugnai *et al.*, 1999). Leaves of vines affected by esca infection are observed as having light green to chlorotic spots that enlarge between the veins, coalescing and turning the leaves of red cultivars dark red, with the chlorotic spots finally becoming rectangular in shape causing the classical “tiger–striped” leaf symptom (Mugnai *et al.*, 1999; Surico *et al.*, 2000). In severe cases, the canes of the vine wilt suddenly, causing a symptom usually described as apoplexy (Mugnai *et al.*, 1999; Surico *et al.*, 2000). Berry symptoms include spotting that can vary from small brown spots to violet and purple spots, from there the name “Black measles.” Berries on infected vines ripen later in the season than normal, with lower sugar content and, also shows cracks and transverse splits on the berry surface (Mugnai *et al.*, 1999).

When examining plants displaying abovementioned foliar symptoms, a wide range of internal symptoms can be observed. The most characteristic is white rot of the xylem tissue that gradually converts the wood into a spongy white mass, which is separated from the healthy wood by a dark brown margin (Mugnai *et al.*, 1999; Ari, 2000; Köklü, 2000). In cross sections of the trunk and cordons, other symptoms such as black spots in the xylem can be observed. These spots sometimes occur in clusters (Mugnai *et al.*, 1999; Surico *et al.*, 2000) and in longitudinal sections of infected trunks and cordons these spots are visible as black streaking of the vascular tissue (Mugnai *et al.*, 1999; Surico *et al.*, 2000). The streaking usually originates at wounds and extends from there in both directions through the

rest of the xylem (Mugnai *et al.*, 1999). In some cases, pink to dark red brown regions are visible in the region of decayed or necrotic tissue (Surico *et al.*, 2000).

## **Disease cycle**

### ***Petri disease***

Recent studies into the epidemiology of Petri disease have shown that the main sources of inoculum of *Phaeoacremonium* spp. and *Pa. chlamydospora* in vineyards include infected plant material, soil and wind dispersed propagules (Bertelli *et al.*, 1998; Larignon *et al.*, 2001; Rooney *et al.*, 2001).

The pathogens were detected in the soil and water beneath infected grapevines, presumably in the form of chlamydospores, which can survive for long periods (Rooney *et al.*, 2001; Whiteman *et al.*, 2002; Damm and Fourie, 2005; Retief *et al.*, 2006). Pycnidia of *Pa. chlamydospora* and *Phaeoacremonium* spp. are formed on excoriated wood from where spores are released after periods of rainfall (Larignon and Dubos, 2000; Edwards 2001). Given these sources of inoculum, new infections by the pathogens might occur through roots and pruning wounds, although root symptoms are not always visible in diseased vines (Morton, 2000; Borie *et al.*, 2002).

Spores of *Pa. chlamydospora* and *Pm. aleophilum* have the ability to penetrate the vine through wounds made during or by other physical damage (Adalat *et al.*, 2000; Larignon and Dubos, 2000; Borie *et al.*, 2002). Pruning wound infections were found to be more severe when pruning occurred later in the dormant season; with wounds remaining susceptible to infection for between 1 and 16 weeks (Larignon and Dubos, 2000; Gubler *et al.*, 2001; Eskalen *et al.*, 2007; Serra *et al.*, 2007). After penetration, the pathogens grow readily inside the xylem of the host plant (Larignon and Dubos, 1997; Sparapano *et al.*, 2000a; Lorena *et al.*, 2001). Initially, *Pa. chlamydospora* grows in the xylem parenchyma, after which it moves radially into the xylem vessels where tyloses are formed by the host in reaction to the infection (Lorena *et al.*, 2001). The movement of the pathogen is usually with the sap flow upwards in the stem (Lorena *et al.*, 2001).

Exopolysaccharides are produced in the infected xylem and transported through the functional xylem to the foliar parts of the plant (Sparapano *et al.*, 2000b). Studies have shown that these exopolysaccharides are a mixture of  $\alpha$ -glucans, called pallulans (Sparapano *et al.*, 2000b). Research found that pallulans are capable of causing the foliar symptoms associated with Petri disease due to its ability to reduce the chlorophyll content of the plant,

hence causing chlorosis of the leaves, leading to a decrease in photosynthesis and therefore stunted growth (Sparapano *et al.*, 2000b; Santos *et al.*, 2004).

*Pa. chlamydospora* and *Pm. aleophilum* can also colonise plants endophytically without causing symptoms, which results in propagation material being a potential inoculum source (Bertelli *et al.*, 1998). It has been shown in several studies that mother vines as well as young nursery vines can be infected by the various pathogens associated with Petri disease of grapevines (Mugnai *et al.*, 1999; Pascoe and Cottral, 2000; Rego *et al.*, 2000; Halleen *et al.*, 2003; Edwards *et al.*, 2004; Fourie and Halleen, 2002; Fourie and Halleen, 2004b; Retief *et al.*, 2006). Apart from nursery vines being made from infected propagation material, studies have shown that inoculum of *Pa. chlamydospora* is present in the hydration tanks and callusing media in nurseries (Whiteman *et al.*, 2003; Retief *et al.*, 2006). This indicates that young vines can potentially also become infected during the nursery handling process. Infected plant material is consequently regarded as the means of long distance spread of this disease.

### ***Esca disease***

As stated previously, Esca is regarded as a complex disease with many associated pathogens. Research has led to the view that Esca is not a single disease but rather a complex of diseases (Surico, 2001). Diseases included in this complex are Petri disease, young Esca, white rot and ultimately acute Esca, which is seen as the last step of a series of pathological events beginning in the nursery and ending with the death of the grapevine in the field (Surico, 2001).

Young vines are often already infected by the pathogens causing Petri disease and these infections are transferred to new plantings where they could lead to the development of Petri disease and young esca. Young Esca can be distinguished from Petri disease by the development of “tiger stripe” foliar symptoms in certain years, depending on the prevailing weather conditions (Marchi *et al.*, 2006).

In cases where vines do not die at a young age from Petri disease or young Esca, they may be infected in the vineyard by *F. mediterranea* or other pathogenic basidiomycete species that are able to infect vines through pruning wounds or other mechanical damage (Mugnai *et al.*, 1999; Sparapano *et al.*, 2000a). Development of Esca is a slow process and Reizenzein *et al.* (2000) found that esca foliar symptom expression increased dramatically with increasing grapevine age. However, the expression of foliar symptoms has been

observed to be highly erratic between years (Reisenzein *et al.*, 2000; Surico *et al.*, 2000a). This erratic symptom expression was proposed to be the result of external factors such as drought stress, rainfall and temperatures in summer during the period of symptom development or waterlogged conditions in the vineyard (Mugnai *et al.*, 1999; Surico *et al.*, 2000a; Marchi *et al.*, 2006). However, the exact role of water supply in symptom development is not yet fully understood.

Analyses of the distribution of Esca affected grapevines in a vineyard found that in some vineyards the affected vines were randomly distributed (Surico *et al.*, 2000b; Redondo *et al.*, 2001; Sofia *et al.*, 2006) while in others they seemed to aggregate in groups (Pollastro *et al.*, 2000b; Stefanini *et al.*, 2000). These observations led to the conclusion that the disease either spreads by airborne inoculum in the vineyard, hence the random occurrence, or by infected plant material that causes affected plants to aggregate (Surico *et al.*, 2000b; Marchi *et al.*, 2006).

## **Management**

### ***Chemical***

Sodium arsenite was previously used as a preventative application with great effect. It was applied as a foliar spray or painted onto the trunk and arms of infected vines at a concentration of 12.5 g/L. However, in recent years have been banned due to its toxicity towards humans and animals (Mugnai *et al.*, 1999; Di Marco *et al.*, 2000).

Other chemicals tested without great results include fenaril, furmetemide, tenodarid and fosetyl-Al (Mugnai *et al.*, 1999). Systemic fungicides were found to inhibit mycelial growth of *Pa. chlamydospora in vitro* (Jaspers, 2001). These include benomyl, which is registered as pruning wound treatment of *E. lata* but has been removed from the market since 2001, and carbendazim with EC<sub>50</sub> values of less than 0.08 mg l<sup>-1</sup>, while pyrimethanil and cyprodinil/fludioxonil had EC<sub>50</sub> values of less than 0.02 mg l<sup>-1</sup>. These were, however, not as effective as the contact fungicides when it came to inhibition of spore germination (Jaspers, 2001). These findings concluded that systemic fungicides may have the ability to inhibit the spread of *Pa. chlamydospora* inside the vine, while hydroxyquinoline sulphate has the ability to protect the propagation material during the nursery processes. Phosphonate has been found to work synergistically with resveratrol (Laukart *et al.*, 2001), a compound associated with resistance of vines against infection by *Pa. chlamydospora* (Laukart *et al.*, 2001; Lorena *et al.*, 2001).

As previous studies have reported that *Pa. chlamydospora* and *Pm. aleophilum* infect vines during the propagation stages (Feliciano and Gubler, 2001; Fourie and Halleen, 2002, Retief *et al.*, 2006), preventative measures should be applied at these stages. Currently, nurseries rely on drenching propagation material with broad spectrum of fungicides that include captan, iprodione and 8 hydroxyquinoline sulfate with the aim to lower the inoculum levels present on the material (Fourie and Halleen, 2004a). These methods have been found to be ineffective against mycelial growth and germination of *Pa. chlamydospora*. (Jaspers, 2001). However benomyl, captan and Sporekill have been tested and recommended by Fourie and Halleen (2004b, 2006).

### **Biological**

Studies conducted by Di Marco *et al.* (2004) showed that *Trichoderma* is capable of protecting small pruning wounds, by limiting black goo in the vessels of the vines artificially inoculated with *Pa. chlamydospora*. Several studies on the protection of pruning wounds against *E. lata* infection have identified some *Trichoderma* isolates, *B. subtilis* and *F. lateritium* (Ferreira *et al.*, 1991; John *et al.*, 2004; John *et al.*, 2005). Therefore, these biocontrol agents might have the same ability towards the causal pathogens of Petri and Esca disease. Fourie and Halleen (2004a) suggested that *Trichoderma* drenches of rootstock could be implemented commercially. Although their findings showed *Trichoderma* efficiency to be variable, in some cases it did reduce *Pa. chlamydospora* and *Phaeoacremonium* infection of rootstock compared to water treatments. By drenching rootstock and scion cuttings in several treatments as well as a *T. harzianum* product (Trichoflow-T, Agrimm Technologies) prior to cold storage and once again after grafting, *Pa. chlamydospora* incidence in basal ends was reduced, especially in a machine grafting regime (Fourie and Halleen, 2006). The ability of *Trichoderma* to increase root hair volume as well decrease *Pa. chlamydospora* infection was illustrated by Di Marco and Osti (2007), by treating propagation material with Rootshield® (Intrachem Bio Italia S. p. A., Bergamo, Italy) at several stages. Similar results were reported by Fourie *et al.* (2001).

### **Cultural**

The control of Esca disease relies mainly on cultural practices (Mugnai *et al.*, 1999). Di Marco *et al.* (2000) listed several strategies for pre- and post-foliar expression of symptoms. These included protecting large pruning wounds by sealing wounds with a dressing or healing varnish. Furthermore, the author suggested the removal of pruning residues as well as the cutting of infected parts of the vine below the rotted wood.

However as *Phaeoconiella* and *Phaoacremonium* have been found to be present in rootstock cuttings (Fourie and Halleen, 2002), preventative measures prior to grafting are needed. Several researchers have found that treating propagation material with hot water for 30 minutes at 50°C followed by 30 minutes in cold water (Crous *et al.*, 2001; Fourie and Halleen, 2004a), lowered or eradicated the occurrence of the pathogen in the propagation material. These findings were in contrast with those of Rooney and Gubler (2001) who found that a treatment of 51°C for 30 minutes followed by 23°C for 30 minutes did not decrease sporulation or mycelium growth of *Pa. chlamydospora* and *Pm. inflatipes* at all.

### PHOMOPSIS DISEASES OF GRAPEVINES

The genus *Phomopsis* (Sacc.) Bubák is a very diverse genus containing pathogenic and saprophytic species (Uecker, 1988). Initially, species of *Phomopsis* and its teleomorph *Diaporthe* (Nitschke) were identified using cultural and morphological characteristics. However, these characteristics were found to overlap between species, which led to species being identified based on host (Rehner and Uecker, 1994). This concept was disproven when it was observed in several studies that some *Phomopsis* species were able to infect a wide range of hosts (Rehner and Uecker, 1994; Uddin *et al.*, 1997, 1998; Mostert *et al.*, 2001; van Niekerk *et al.*, 2005).

Due to the shortcomings of the abovementioned techniques, DNA sequence data combined with morphological and pathological data have been used to distinguish between *Phomopsis* species (Farr *et al.*, 1999; Moleleki *et al.*, 2002; van Niekerk *et al.*, 2005; Schilder *et al.*, 2005). Using these techniques van Niekerk *et al.* (2005) identified 15 different *Phomopsis* species from grapevines. These included *P. viticola*, *P. vitimegaspora* (teleomorph: *D. kyushuensis*) and *D. perijuncta* that were known pathogens of grapevines, with *P. viticola* and *P. vitimegaspora* being the most virulent (Mostert and Crous, 2000; van Niekerk *et al.*, 2005). Nonetheless, *P. viticola* is most frequently isolated from grapevine pruning wounds. *Phomopsis amygdali*, a known pathogen from peaches and almonds (Farr *et al.*, 1999), was also shown in this study to occur on grapevines. It was furthermore identified by artificial inoculation studies to be just as virulent as *P. viticola* and therefore a potentially important grapevine pathogen (van Niekerk *et al.*, 2005).

Economically, the diseases caused by *Phomopsis* spp. can have a significant impact on the industry, but mostly in older vineyards with losses of between 30% and 50% being

recorded in vineyards where Phomopsis cane and leaf spot, caused by *P. viticola*, occurred (Swart and De Kock, 1994; Mostert and Crous, 2000; Erincik *et al.*, 2001; Schilder *et al.*, 2005).

### Symptoms

Symptoms of Phomopsis cane and leaf spot usually develop within 30 days after infection (Cucuzza and Sall, 1982; Hashim, 2003). These symptoms are visible as dark round lesions with a surrounding pale yellow halo on the internodes of young shoots (Mostert and Crous, 2000; Schilder *et al.*, 2005). As the disease develops, brown to black lens-shaped lesions develop on the basal 6 internodes of green shoots (Pine, 1959; Hewitt and Pearson, 1988). These lesions form near the base of the shoot and can lead to weakening of shoots, which could lead to the shoots breaking off in strong wind or under the weight of grapes (Pine, 1959; Hewitt and Pearson, 1988; Scheper *et al.*, 1995; Rawnsley *et al.*, 2004). When infected shoots become dormant, pycnidia develop on the lesions. These pycnidia lift up the shoot epidermis, admitting air underneath, which gives the shoots a bleached appearance (Hewitt and Pearson, 1988b).

Infection of bunch rachis can also occur that causes breakage of the clusters and therefore loss of fruit (Hewitt and Pearson, 1988). Furthermore found that the rachis remains susceptible to infection throughout the growing season, with symptoms developing 3 to 4 weeks after infection (Erincik *et al.*, 2001). In the field, fruit rots have also been observed with diseased berries turning brown, while gradually shrivelling (Hewitt and Pearson, 1988). These infections are believed to originate from latent infection that occurred during or shortly after bloom (Pscheidt and Pearson, 1989; Erincik *et al.*, 2001). Young, green berries are also regarded as being more susceptible to infection compared to more mature berries with infection that can take place at either the pedicel or stylar end (Pscheidt and Pearson, 1989).

Apart from causing the characteristic cane and leaf spot symptoms, *P. viticola* was also isolated from pruning wound stubs as well as internal wood decay (Fischer and Kassemeyer 2003; Bester, 2006; van Niekerk, 2008). It has especially been associated with an internal necrosis that looks very similar to the wedge-shaped necrosis characteristic of *E. lata* infection that often leads to incorrect identification of the associated causal organism (Mostert and Crous, 2000; Castillo-Pando *et al.*, 2001).

Symptoms associated with swelling arm disease of grapevines occur on all vine parts (Kuo and Leu, 1998; Kajitani and Kanematsu, 2000). This disease is caused by *P. vitimegaspora* and was first described in Japan and later also Taiwan (Kajitani and Kanematsu, 2000). The first symptoms of this disease develop as small black spots at the base of new, green shoots. These spots become larger and ultimately form a blackened zone that girdles the shoot, leading to shoot blight. On other parts of green shoots oblong or elliptical shaped lesions can also develop. A number of years after infection, hypertrophied nodes can appear on canes with cankers developing on the cordon (Kuo and Leu, 1998; Kajitani and Kanematsu, 2000).

### Disease cycle

*P. viticola* overwinters in the form of dark pycnidia and mycelium, in the lower nodes and internodes of one year old canes and the bark of older, infected canes or shoots. It can also occur on propagation material originating from nurseries, which can therefore be, along with abovementioned plant parts, seen as the primary source of inoculum (Pine, 1959; Cucuzza and Sall, 1982; Jailloux *et al.*, 1987; Hewitt and Pearson, 1988; Castillo-Pando *et al.*, 1997; Mostert and Crous, 2000; Schilder *et al.*, 2005, Król, 2005; Clarke *et al.*, 2004).

After prolonged periods of wetness and rain the pycnidia releases two different types of conidia, namely  $\alpha$ -conidia and  $\beta$ -conidia (Pine, 1959; Cucuzza and Sall, 1982; Sergeeva *et al.*, 2003). The  $\alpha$ -conidia have an average diameter of  $9.8 - 12.6 \times 3.1 - 4.5 \mu\text{m}$  (Schilder *et al.*, 2005) and are dispersed through irrigation water and rain splash to susceptible plant parts (Pine, 1959; Hewitt and Pearson, 1988; Swart and De Kock, 1994; Merrin *et al.*, 1995). Natural openings such as lenticels and stomata seems to be the infection portals of the disease (Pine, 1959), however *Phomopsis* species have been isolated from pruning wounds (Fourie and Halleen, 2004b; Van Niekerk *et al.*, 2005), which contributed to speculation that the pathogen can cause internal wood decay as observed by Fischer and Kassemeyer (2003). From these initial infections the pathogen can further infect any green parts of the vine (Schilder *et al.*, 2005) including canes, leaves, rachis and berries with the berry, infections leading to the highest yield loss (Erincik *et al.*, 2001).

It was found that the optimum temperature for growth and spore germination of *P. viticola* is between 25°C and 26°C (Schilder *et al.*, 2005), while the optimum conditions for lesion development is when the temperatures are at 15 – 25°C for a period of 20 hours (Erincik *et al.*, 2003). These conditions are especially prevalent during spring when budburst

and vegetative shoot growth of grapevines occur that could lead to severe infection of this new growth if sufficient control is not applied (Sergeeva *et al.*, 2003). Apart from the optimal infection conditions, Schilder *et al.* (2005) also found that different isolates of *P. viticola* have different affinities to different organs of the vine and therefore infect either bunches or the shoots.

## **Management**

### ***Chemical***

Control of this disease relies primarily on the application of fungicides before and during the vegetative growth stages of grapevines. It was shown that when fungicides were applied during grapevine dormancy, the inoculum carried over was drastically reduced (Król, 2005; Castillo-Pando *et al.*, 1997). Fungicides that were found to be effective for these dormant applications include copper oxychloride, copper oxychloride/sulphur, copper sulphate/lime, folpet, phostyl-Al + mancozeb, probineb and sulphur (Mostert and Crous, 2000). Studies done with strobilurins, which has a wide range of activity, showed that it could inhibit spore germination and mycelium growth of *P. viticola* (Mostert and Crous, 2000). *In vitro* studies testing chlorothalonil, flusilazol and tiophanate-methyl showed satisfactory results by inhibiting mycelium growth and prevented spore germination (Król, 2005).

### ***Hot water treatment***

Several studies of treating propagation material with hot water at 50°C for 30 minutes have shown that it eradicates or decreases inoculum of fungi (Clarke *et al.*, 2004; Fourie and Halleen, 2004a). Treating canes in this manner drastically reduced *P. viticola* levels, although it was not entirely eliminated (Clarke *et al.*, 2004).

## **BIOLOGICAL CONTROL AGENTS**

It is well known that grapevine trunk disease pathogens infect grapevines mainly through pruning wounds (Lehoczky, 1974; Ferreira *et al.*, 1989; Adalat *et al.*, 2000; van Niekerk *et al.*, 2005). Control of trunk diseases therefore relies on pruning wound protection, which has focused mainly on chemical applications in the past (Munkvold and Marois, 1993b; Ramsdell, 1995; Carter and Price, 1974; Pearson, 1982). However, recent studies have shown pruning wounds stay susceptible to infection for up to 16 weeks after pruning

(Munkvold and Marois, 1995; Larignon and Dubos, 2000; Gubler *et al.*, 2001, van Niekerk, 2008), combined with findings of Munkvold and Marois (1993a) that chemicals such as benomyl showed a decrease in efficiency 2 weeks after treatment, have indicated that it is becoming increasingly important to find alternative methods of pruning wound protection. Reports by several researchers on the potential of *Trichoderma* spp. and *Bacillus subtilis* to protect pruning wounds against *E. lata* infection, suggests that biological control could be an alternative measure of treatment that could provide the needed long term protection of pruning wounds (Ferreira *et al.*, 1989; Ferreira *et al.*, 1991; John *et al.*, 2004; John *et al.*, 2005). These potential biological agents are discussed with respect to their life cycles and different modes of action.

### ***TRICHODERMA* SPP.**

*Trichoderma* is an asexually reproducing genus that occurs saprophytically in soils, and endophytically in the sapwood of several species of *Cola* and *Theobroma* (Danielsen and Davey, 1973a; Samuels 2006). Being saprophytic, the genus has the ability utilise to several compounds as nitrogen and carbon sources (Danielsen and Davey, 1973a), are fast growing photosensitive fungi that produce masses of powdery green conidia (Gressel and Hartman 1968; Samuels, 2006). The teleomorph (Genus: *Hypocera*) of the genus seem to be the most abundantly isolated from woody hosts, while the strains known to be biological control agents seem to have no known sexual stages, therefore it seems as if they have evolved independently (Harman *et al.*, 2004a).

Several species of *Trichoderma* are well known biological control agents of plant pathogens (Elad and Kapat, 1999). These species include *T. harzianum* and *T. atroviride* (Brunner *et al.*, 2003). According to literature, these species employ numerous mechanisms in the control of pathogenic fungi, including mycoparasitism (Almeida *et al.*, 2007), antibiosis (Bélanger *et al.*, 1995; Calistru *et al.*, 1997) and competition (Sivan and Chet, 1989).

Another reason for the genus being such a successful biological agent candidate is due to the ease at which large quantities of the propagules can be produced at a very low cost (Woo *et al.*, 2005). Latest reports conclude that *Trichoderma* based products make up one third of the total biological control preparations sold worldwide for the control of horticultural diseases (Steyaert *et al.*, 2003).

## Life cycle

*Trichoderma* spp. reproduces asexually by producing masses of powdery green single-celled conidia on many substrates (Papavizas, 1985). Recent studies have, however, shown that some species have the ability to produce intercellular chlamydospores as well (Harman, 2001; Samuels, 2006). Although conidia are released in large numbers, their germination is affected by external factors such as nutrients, temperature and light (Danielsen and Davey, 1973b; Papavizas 1985). As *Trichoderma* are commonly found in soils and on moist wood in forests (Chaverri and Samuels, 2003), it is probable that these conidia are spread through wind or water.

## Modes of action

### *Systemic resistance*

Findings by several researchers have suggested that several strains of *Trichoderma* spp. are capable of inducing systemic resistance in a variety of commercial crops such as beans, lettuce, peppers, tomato, cucumber, maize and cotton (De Meyer *et al.*, 1998; Ahmed *et al.*, 2004; Benitez *et al.*, 2004; Harman *et al.*, 2004a, b; Shoresh *et al.*, 2005). Defence responses by plants rely mainly on the ability of their ability to recognise elicitors and pathogenic fungi (Benitez *et al.*, 2004). *Trichoderma* spp. are capable of producing several elicitors such as the Small protein 1 (Sm1) that is capable of inducing systemic resistance to *Colletotrichum* spp. in cotton plants (Djonovic *et al.*, 2006). Sm1 reduced the expression of cotton plant infection by *Colletotrichum* spp. Moreover, Shoresh *et al.* (2005) reported that cucumber plants pre-inoculated with *Trichoderma asperellum* showed an increased expression of peroxidase,  $\beta$ -1, 3-glucanase and chitinase 1. These reactions occur in the jasmonate/ethylene signaling pathways and resulted in an increased resistance to leaf infection by *Pseudomonas syringae* pv. *lachrymans* in cucumber plants to infection by. These findings are supported by Yidida *et al.* (1999) who reported that the hyphae of *Trichoderma* spp. penetrate and colonise the roots of cucumber plants inducing an increased level of peroxidase and chitinase activity, as well as the deposition of callose on the inside of plant cell walls. The first indication of the ability of *Trichoderma* spp. to induce systemic resistance was observed when soils were treated with the T-39 strain of *T. harzianum* and although the strain only colonised the roots of the bean plants planted in the soil, it lead to the bean plants being resistant to infections by foliar pathogens, such as *Botrytis cinerea* and *Colletotrichum lindemuthianum* (Bigirimana *et al.*, 1997)

### ***Antibiotic production***

Toxic metabolites produced by *Trichoderma* spp., have been grouped into three distinct groups of antibiotics, namely gliotoxin (Weindling, 1941), viridin (Brian and McGowan, 1945) and glioviren (Howell and Stipanovic, 1983). The latter have been known to be highly effective against several *Pythium* spp. (Papaivizas, 1985). Gupta *et al.* (1999) reported that certain species of *Trichoderma* such as *T. hamatum* and *T. pseudokoningii* caused the loss of turgor and hyphal collapse of pathogenic fungi without hyphal contact and suggested that this could be due to diffusible substances. Findings by John *et al.* (2004) concurred that certain *Trichoderma* strains produced volatile and non-volatile antibiotics capable of inhibiting mycelium growth as well as spore germination of *E. lata*. These reports are supported by the findings of several other researches conducted on the ability of *Trichoderma* to produce antibiotics and their toxicity towards pathogenic fungi (Calistru *et al.*, 1997; Kucuk *et al.*, 2004). In culture, some *Trichoderma* isolates produce a coconut-like aroma that has been identified as 6-n-pentyl-2H-pyran-2-one. This metabolite has been reported as having inhibitory capabilities towards pathogenic fungi (Luis and Reino *et al.*, 2008).

### ***Mycoparasitism and enzyme production***

Almeida *et al.* (2007) reported observing that the hyphae of an antagonistic strain of *T. harzianum* attached itself to the hyphae of *Rolstonia solani*, where coiling and appressoria formation occurred. Inbar *et al.* (1996) and Benhamou and Chet (1993) observed coiling and appressorium-like structures on the hyphae of *Sclerotinia sclerotiorum* and *Pythium ultimum* respectively. Only after physical contact, was cell-wall degrading enzymes produced. These findings suggest, as stated by Benhamou and Chet (1993), that physical contact lead to the process of hyphal disintegration. The process of enzyme production leads to hyphal collapse (Steyert *et al.*, 2003).

Although it is well known that *Trichoderma* strains produce lytic enzymes, not all of them have been identified. These lytic enzymes include  $\alpha$ -1, 3-glucanase, found to be secreted into medium when *T. harzianum* were grown on cell walls (Ait-Lahsen *et al.*, 2001), *N*-acetyl- $\beta$ -D-glucosaminidase and 42-kDa endochitinase known to be associated with hydrolysis of pathogenic fungi cell walls (Kubicek *et al.*, 2001; Grinyer *et al.*, 2005) and  $\beta$ -1, 6-glucanase (Omero *et al.*, 2004). These lytic enzymes assist the *Trichoderma* in penetrating the hyphae of the pathogen and utilise its cellular content as a nutrient source (Omero *et al.*, 2003). Strains of *T. harzianum* have also been observed to produce a protolactic enzyme that is able to inactivate a hydrolytic enzyme formed by *B. cinerea* on bean leaves (Howell,

2003). This protolactic enzyme producing *Trichoderma* also has the ability to penetrate the eggs of the root knot nematode, *Meloidogyne javanica*, significantly reducing root galling (Howell, 2003).

### **Competition**

Studies have shown that *Trichoderma* spp. can compete in the rhizosphere with pathogens for space and nutrients. This is, however, only possible if the *Trichoderma* spp. can colonise and grow in the rhizosphere (Howell, 2003). Findings by Elad and Baker (1985) suggested that certain carbon sources as well as several other nutrients contribute to *Fusarium oxysporum* chlamydospore germination. However, studies by Sivan and Chet (1989) revealed that *T. harzianum* has the ability to compete for these nutrients and carbon sources in the rhizosphere and then reduce the chlamydospore germination. Filamentous fungi such as *Aspergillus fumigatus* and *A. nidulans* are highly reliant on iron uptake for survival. Therefore, in circumstances of low iron levels, they have the ability to produce low-molecular-weight ferric iron specific chelators, known as siderophores, to mobilise free iron (Eisendle *et al.*, 2004). *Trichoderma* spp., however, have the ability to produce more effective siderophores than the *Aspergillus* spp., and thus compete efficiently for iron (Chet and Inbar, 1994).

### **Growth stimulation**

Although not of direct relevance to pruning wound protection, increase in root mass, plant growth and yield is an effect brought about by root colonisation by *Trichoderma* spp. Ousely *et al.* (1992) found that when lettuce seedlings were treated with *Trichoderma* spp., the dried mass increased by 26%. Similarly, Harman *et al.* (2001) reported that the roots of maize plants treated with a *Trichoderma* strain were larger and more robust, with the plants requiring 40% less nitrogen than untreated plants. Other studies by Harman *et al.* (2004b) showed an increase in secondary root formation as well as an increase in root hair formation after *Trichoderma* treatment. Apart from increasing yield and biomass, *Trichoderma* spp. can also degrade toxic metabolites such as cyanide, which are produced by some microflora to protect their niche (Harman *et al.*, 2004a). Along with this capability *Trichoderma* spp. has the ability to increase the uptake of an array of nutrients which include copper, iron, manganese phosphorus and sodium. Several plant nutrients such as rock phosphate,  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Mn}^{4+}$  and  $\text{Zn}^0$  is made soluble by *Trichoderma* spp. and are thus more accessible to the plant (Altomare *et al.*, 1999).

## **BACILLUS SUBTILIS**

*B. subtilis* is an aerobic, Gram positive bacterium capable of producing endospores and consist of vegetative rod shaped cells (Errington, 1993, Branda *et al.*, 2001). Commonly, the bacterium occurs in soils, water sources and plants and has the natural ability to compete for nutrients and space (Irtwange, 2006). Although the species has several antimicrobial characteristics (Whips, 2001) and the potential of being a grapevine pruning wound protectant (Ferreira *et al.*, 1991; Schmidt *et al.*, 1999), biological control using *B. subtilis* has mainly focused on post harvest protection of tropical fruit (Korsten *et al.*, 1991; Leibinger *et al.*, 1997; Korsten, 2006).

### **Life cycle**

Motile strains of bacteria swim using flagella that is positioned around their oblong shaped bodies but can also move along a concentration gradient through diffusion (Ott, 2001a, b). These actions rely on the nutrient composition of the area (Ott, 2001a); the higher the nutrient contents the faster their movement and reproduction. Under favourable conditions *B. subtilis* has the ability to reproduce vegetatively (Pontastico-Caldas *et al.*, 1992); the motile rod shaped cells double in their cell length and divide in two matching sister cells (Errington, 1993). However, if *Bacillus* occurs under conditions of nutritional starvation, the vegetative cells have the ability to sporulate (Errington, 1993; Sharpe *et al.*, 1997; Branda *et al.*, 2001). Sporulation consists of production of a smaller preospore inside a mother cell. The preospore has a protective outer cell wall, which must protect it during the period of stress (Endospore formation). During the stress period it remains dormant and relies on the endospore until the conditions turn favourable (Pontastico-Caldas *et al.*, 1992; Errington, 1993).

### **Modes of actions**

Several modes of action are employed by bacterial species in their quest for rhizospheric competence. These include parasitism by extracellular enzymes antibiosis, competition, and, induced resistance and plant growth promotion (Whips, 2001). However, according to literature, antibiosis (Ferreira *et al.*, 1989; Chan *et al.*, 2003), production of extra cellular enzymes (Manjula, 2004), induced resistance and plant growth promotion (Amer and Utkhede, 2000) seem to be the methods employed by *B. subtilis*.

### ***Extra-cellular enzymes***

Hydrolytic enzymes form an integral part of mechanisms used during antagonistic activity. *B. subtilis* has been known to produce chitinase as reported by Manjula *et al.* (2004) who purified 4 proteins from a *B. subtilis* strain AF1 using affinity chromatography. However, only one of these proteins,  $\beta$ -1, 4-TV-acetylglucosaminidase (NAGase), had chitinolytic capabilities. Subsequently, Sakai *et al.* (1998) purified three thermostable endochitinase from an uncharacterised *Bacillus* sp MH-1 strain. Manjula *et al.* (2004) reported that NAGase had an inhibitory effect on *Puccinia arachidis* (groundnut rust) and *Aspergillus niger* (soft rot of lemons). However, the 4 proteins isolated by Manjula *et al.* (2004), on their own had a lesser effect on the pathogens than all the compounds combined. This is due to the synergy between antibiotics produced by *B. subtilis* produce and the different biocontrol mechanisms of the *B. subtilis* (Manjula *et al.*, 2004).

### ***Antibiosis***

It has been reported that *B. subtilis* produce an array of extra cellular metabolites. Three of these are produced through ribosomal mechanisms: TasA (Stover and Driks, 1999), subtilosin (Babasaki *et al.*, 1985) and sublancin (Paik *et al.*, 1998), while three are produced through non-ribosomal mechanisms: surfactin (Nakano *et al.*, 1988), bacilysin (Walker and Abraham, 1970) and plipastatin (Tsuge *et al.*, 1999). Bacilysin has been shown to have some antifungal properties as reported by Asaka and Shoda (1996) who observed its ability to suppress *Rhizoctonia solani*, the causal pathogen of tomato damping off. Although the production pathway of several antibiotics' is still unknown, their ability to suppress pathogens is already known. One of these is fengycin, a known antifungal lipopeptide (Vanittanakom *et al.*, 1986), while several other uncharacterised antibiotics have been reported as having an antagonistic effect towards *Monilinia fructicola* and *A. flavus* (Moita, 2005). Findings by Chan *et al.* (2003) and Ferreira *et al.* (1991) suggest that antibiotic substances produced by *B. subtilis* initiated the swelling of the hyphae of *Fusarium graminearum* and *E. lata*, which is supported by findings of Baker and Cook (1982) that antibiotics can penetrate hyphae and cause malformations and swellings. Production of the antifungal substances coincides with the beginning of sporulation, reaching a peak at the end of the exponential growth phase where after it abruptly comes to a stop (Ochi and Ohsawa, 1984; Moita, 2005).

### ***Induced resistance or plant growth promotion***

Although induced resistance and plant growth promotion is well known bacterial effects in soils, it is still fairly uncommon for *B. subtilis* (Whips, 2001). However, findings by Amer and Utkhede (2000) suggested otherwise. After inoculating soil with *B. subtilis*, lettuce showed not only an increase in fresh root and shoot mass, but also a remarkable reduction in infection by *Pythium aphanidermatum*. This is supported by reports that *B. subtilis* increased the yield of cucumber plants with *P. aphanidermatum* being present or absent (Utkhede *et al.*, 1999). These authors conclude that it is possible for the species to contribute as a growth-promoting rhizobacterium or stimulate induced resistance of a host plant.

## **CONCLUSION**

Research has proven pruning wounds to be the main infection portals of the trunk disease pathogens, although chemicals are well known for their ability to protect the pruning wounds, they seem to only be effective for up to 2 weeks. This maybe insufficient protection as research has shown spores from trunk disease pathogens to be present for the entire season and pruning wounds remained susceptible for up to 16 weeks after pruning. As mentioned, several pathogens contribute to the trunk disease complex and very little research has been conducted on alternative control measures. Biological control is well documented as a means to protect pruning wounds against infection by *E. lata*. Further evaluation of this control option is therefore needed to assess the potential of biological control in providing sustained pruning wound protection against the complex of grapevine trunk disease pathogens.

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## 2. EVALUATION OF BIOCONTROL AGENTS FOR GRAPEVINE PRUNING WOUND PROTECTION AGAINST TRUNK PATHOGEN INFECTION

### ABSTRACT

Trunk diseases lead to premature decline and dieback of grapevine and are caused by a complex of pathogens, including *Eutypa lata*, *Phaeomoniella chlamydospora*, and species of Botryosphaeriaceae (incl. *Botryosphaeria* and aggregate genera), *Phomopsis* spp. and *Phaeoacremonium* spp. These pathogens infect the grapevine mainly through pruning wounds, thereby making pruning wound protection an essential management strategy. Research has shown that pruning wounds stay susceptible for up to 16 weeks after pruning and spores of trunk pathogens were trapped throughout the pruning season. These findings suggest that long-term pruning wound protection is required for prevention of infection. To address the problem several potential antagonists were evaluated against most trunk disease pathogens in a laboratory trial by incubating the antagonist and pathogen simultaneously on potato dextrose agar to determine macroscopic interactions, as well as synthetic nutrient agar medium to observe microscopic interactions. The best performing control agents were there after tested in a field trial. The field trial was conducted on a  $\pm 10$ -year-old Merlot and  $\pm 18$ -year-old Chenin blanc vineyard in the Stellenbosch region. The spurs were pruned to three buds and the fresh pruning wounds were treated with benomyl as control treatment, *Trichoderma*-based commercial products, Vinevax® and Eco77®, *Bacillus subtilis*, and *Trichoderma* isolates, USPP-T1 and -T2. Seven days after treatment the pruning wounds were spray inoculated with spore suspensions of four *Botryosphaeriaceae* spp. (*Neofusicoccum australe*, *N. parvum*, *Diplodia seriata* and *Lasiodiplodia theobromae*), *Eutypa lata*, *Phaeomoniella chlamydospora* and *Phomopsis viticola*. After a period of 8 months the treatments were evaluated by isolation onto PDA. *Trichoderma*-based products and isolates in most cases showed equal or better efficacy than benomyl, especially USPP-T1 and -T2. Moreover, these isolates demonstrated a very good ability to colonise the wound tissue.

## INTRODUCTION

Trunk diseases lead to premature decline and dieback of grapevine and are caused by a complex of pathogens, including *Eutypa lata* (Moller *et al.*, 1968) and *Phaeoconiella chlamydospora* (Mugnai *et al.*, 1999), species of Botryosphaeriaceae (incl. *Botryosphaeria*, *Neofusicoccum* and *Lasiodiplodia*) (van Niekerk *et al.*, 2004; Crous *et al.*, 2006), *Phomopsis* (van Niekerk *et al.*, 2005) and *Phaeoacremonium* spp. (Mostert *et al.*, 2006). *Eutypa* dieback, Black dead arm, Esca, Petri and *Phomopsis* dead arm are grapevine trunk diseases of great economic importance in the wine and grape industries across the world, primarily due to premature decline and dieback (Munkvold *et al.*, 1994; Mahoney *et al.*, 2003; Gubler *et al.*, 2005). Growers also suffer yield loss and poor wine quality as a result of uneven ripening of berries, as caused by *E. lata* (Wicks and Davies, 1999), or delayed ripening lower sugar content of grapes on vines affected by Esca disease (Mugnai *et al.*, 1999). Economical losses in the Stellenbosch region of South Africa due to *Eutypa* dieback alone has been estimated at R1570 / ha in Cabernet Sauvignon vineyards (van Niekerk *et al.*, 2003).

As pruning wounds were shown to be the main infection portals (Lehoczky, 1974; Ferreira *et al.*, 1989; Adalat *et al.*, 2000; van Niekerk *et al.*, 2005), it is therefore of utmost importance to protect pruning wounds against the whole pathogen complex. However, researchers have commonly focussed mainly on protecting pruning wounds against *E. lata* infection by using various fungicides and/or biological control agents (Carter and Price, 1975; Moller and Kasimatis, 1980; Ferreira *et al.*, 1991; Munkvold and Marois, 1993a, b; John *et al.*, 2001; Halleen and Fourie, 2005). Chemicals shown by *in vitro* and *in vivo* studies to be effective against *E. lata* include benomyl (Munkvold and Marois, 1993b; Ramsdell, 1995; Carter and Price, 1974; Pearson, 1982), carbendazim (Sosnowski *et al.*, 2004) and fluzilazole (Munkvold and Marois, 1993b), as well as the application of boric acid in a paste form to pruning wounds (Rolshausen., 2005). Several species of *Botryosphaeriaceae* have been found to be most sensitive to benomyl and tebuconazole (Bester *et al.*, 2007). Esca on the other hand has traditionally been controlled with whole-vine sprays with sodium arsenite (Mugnai *et al.*, 1999). Recent studies have recommended drenching of propagation material with a wide-spectrum fungicide including captan, iprodione and 8-hydroxyquinaline sulfate to pro-actively control Petridisease (Mugnai *et al.*, 1999; Fourie and Halleen, 2004). In the case of *Phomopsis* dead arm disease, early-season sprays with copper oxychloride, copper

oxychloride/sulphur, copper sulphate/lime, folpet, fosetyl-Al + mankozeb, probineb, sulphur and strobilurin are used (Mostert and Crous, 2000).

Research has shown that pruning wounds stay susceptible to infection for 2–16 weeks after pruning (Munkvold and Marois, 1995; Larignon and Dubos, 2000; Gubler *et al.*, 2001, van Niekerk, 2008). Moreover, Moller *et al.* (1977) demonstrated a reduction in chemical protection of apricot pruning wounds over time, and Munkvold and Marois (1993a) observed a decrease in efficiency of benomyl when grapevine pruning wounds were inoculated with *E. lata* 2 weeks after treatment. Chemicals should also not be applied during or before rains as it was hypothesised that rain washed away the chemical residue from pruning wounds (Munkvold and Marois, 1993a).

The abovementioned findings put doubt in the ability of fungicides to provide the required long-term protection of pruning wounds. Biological control agents offer the potential to provide long-term protection of pruning wounds. This can be supported by studies that have shown that *Fusarium lateritium* (Carter and Price, 1974) and *Trichoderma* (Hunt *et al.*, 2001) were isolated from pruning wounds 15 weeks and 8 months, respectively, after treating the wounds. According to these studies and others such as John *et al.* (2004), *Trichoderma* isolates produce volatile as well as non volatile products *in vitro* that inhibit *E. lata* leading to *in vitro* and *in vivo* inhibition. *Trichoderma* based treatments have protected pruning wounds against *E. lata* with a 67% reduction of the pathogen in pruning wounds, when the pathogen was inoculated 1 day after *Trichoderma* treatment (John *et al.*, 2005a). *F. lateritium*, including a benomyl resistant strain, has also been tested extensively in field and laboratory trials to reduce *E. lata* infections of grapevine wood (Carter and Price, 1974, 1975; McMahan *et al.*, 2001; John *et al.*, 2005a). While it demonstrated a moderate level of protection, these results were not as convincing as those with *Trichoderma*.

Several species of bacteria have been shown to have antagonistic activity towards other fungi. A *Bacillus subtilis* isolate, taken from the pruning wound of a grapevine with symptoms of Eutypa dieback, inhibited mycelial growth of *E. lata* by 88% and suppressed the incidence of the pathogen by 100% in pruning wounds (Ferreira *et al.*, 1991). A later study by Schmidt *et al.* (2001), showed that a transposon mutant of *Erwinia herbicola* had a 100% efficiency rate against *E. lata* on autoclaved grape wood.

Collectively, these studies have demonstrated the potential of antagonistic organisms to protect grapevine pruning wounds. However, most of these studies focussed almost

exclusively on *E. lata*, and to a large extent ignored the other pathogens in the grapevine trunk disease complex. The aim of this study was to identify, through *in vitro* and *in vivo* trials, biological control agents that provide protection of pruning wounds against infection the most important pathogens contributing to the trunk disease complex.

## MATERIALS AND METHODS

### *In vitro* evaluation

*In vitro* evaluation of potential biological agents against the most important trunk disease pathogens involved the observation of antagonistic interaction between cultures on dual inoculated plates. The biological agents used were *Bacillus subtilis* (Ferreira *et al.*, 1991), two *Trichoderma*, six uncharacterised *Penicillium* and one *Alternaria* isolate that were isolated from grapevine pruning wounds in South Africa and seven commercially available *Trichoderma* spp. (Table 1). The unidentified *Trichoderma*, *Penicillium* and *Alternaria* isolates were co-isolated with trunk disease pathogens from Chenin blanc grapevine pruning wounds (van Niekerk, 2008) and demonstrated some antagonism against the pathogens (J.M. van Niekerk, unpublished results).

Mycelium plugs of the fungal biocontrol agents and antagonists were made with a sterile glass tube from 7-day-old cultures in a laminar flow cabinet and placed on the opposite sides a 90-mm Petri dish with potato dextrose agar (PDA; Biolab, Wadeville, South Africa) *B. subtilis* was streaked out on the one side of the dish using a sterilised needle eye while mycelium plugs of the pathogen were placed on the opposite side. All of the biological agents and pathogens were placed on the dishes simultaneously, except for *Pm. aleophilum* and *Pa. chlamydospora* that were cultured on the dishes for 14 days prior to inoculation with the biological control agents due to their slow mycelium growth rate (Larignon and Dubos 1997; Edwards and Pascoe, 2004). However, the dishes with *Penicillium* spp. were inoculated simultaneously with *Pm. aleophilum* and *Pa. chlamydospora* due to their slow growth rate in culture. Plates were incubated in dark conditions at 22-25°C for 7 to 21 days before interaction between cultures were observed for macro and microscopic interactions and digitally photographed.

To investigate the interaction on a microscopic level, the pathogens and biological agents were co-incubated as described above, but on a nutrient deficient medium, synthetic nutrient agar medium (SNA, Nirenberg, 1976) for 10 – 14 days at 22-25°C in dark conditions.

The three slower growing organisms, *Penicillium*, *Pm. aleophilum* and *Pa. chlamydospora*, were incubated for 35 days. The nutrient deficient medium was used to inhibit sporulation and to reduce the amount of hyphal growth. Slides were made from interaction zones at 5 different positions along the interaction zone and were investigated using a microscope at different levels of magnification (20×, 40×, and 100× in oil emersion). As substantial interactions could be observed from the PDA medium, dual culture evaluations were not conducted on SNA medium with *B. subtilis*. Photomicrographs were taken of the microscopic interactions between hyphae using a Nikon DMX 1200 microscope.

### ***In vivo* evaluation**

Grapevines of cultivars ±10-year-old Merlot and ±18-year-old Chenin Blanc were spur-pruned to three buds in August 2006. Immediately after pruning, the fresh pruning wounds were treated by spray inoculating with 2 ml of a suspension of either *Trichoderma* isolates USPP-T1 or USPP-T2 ( $10^6$  spores/ml), *B. subtilis* ( $10^8$  cells/ml), ECO 77® (0.5 g/L), Biotricho® (2 g/500 ml with 0.2% sucrose) or Vinevax® (10 g/L). As control treatments, wounds were treated by spraying with 2 ml benomyl (Benlate 500 WP, DowAgro Sciences; 10 g/L) or sterile deionised water. Inoculum of USPP-T1 and T2 was prepared from 7-day-old cultures on PDA, which were grown at 25°C. Plugs of mycelium were placed into 10 ml of sterile deionised water and shaken to suspend the spores, where after the concentration of spores were adjusted using a haemocytometer. Mycelium plugs remained in the spore suspension for the remainder of the treatments. The *B. subtilis* suspension was produced according to the protocol described by Ferreira *et al.* (1989). Seven days after pruning, treated pruning wounds were spray inoculated with 2 ml of  $10^4$  spores/ml spore suspensions of *E. lata*, *Pa. chlamydospora* and species of *Botryosphaeriaceae* (*Neofusicoccum australe*, *N. parvum*, *Diplodia seriata* and *Lasiodiplodia theobromae*), *Phomopsis viticola*, or 2 ml sterile water as control inoculation. Inocula for species of *Botryosphaeriaceae* were produced using the method by Van Niekerk *et al.* (2005), while the inoculum of *Pa. chlamydospora* and *P. viticola* was prepared from 7- and 14-day-old PDA cultures, respectively. *E. lata* inoculum was acquired from *E. lata* perithecia embedded in dead, decayed grapevine trunks as described by Ferreira *et al.* (1989).

Eight months after treatment, the top internode below each treated pruning wound was removed and taken to the laboratory for analysis. Pruning wound stubs were surface sterilised by immersion in 70% ethanol for 30 s, 1 min in 3.5% NaOCl and again for 30 s in

70% ethanol, before being split longitudinally under sterile conditions in a laminar flow cabinet. The incidence of the inoculated pathogens and *Trichoderma* in the xylem tissue beneath the pruning wound scar was determined by means of isolations onto potato dextrose agar (PDA) amended with 0.04 g/L streptomycin sulphate. Streptomycin sulphate was included to reduce the incidence of bacterial contamination from pruning wounds, therefore *B. subtilis* could not be re-isolated and its incidence calculated. A total of 8 tissue pieces ( $\pm 0.5 \times 1$  mm) were aseptically removed from the xylem tissue and plated onto PDA, four pieces per 90 mm PDA dish. Petri dishes were incubated at 25°C for 2–4 weeks before morphological identification of the isolated fungi.

For each cultivar, the trial layout was a completely randomised block design with three blocks. The treatment design was an  $8 \times 8$  factorial design (7 pathogen + control treatment  $\times$  6 antagonist + untreated and treated control treatment), with 5 random replicates of each treatment combination within each block. Each treated pruning wound was regarded as an experimental unit. Approximately 8 pruning wounds were treated on each vine.

The pathogen and *Trichoderma* incidences and extent of colonisation in pruning wounds were recorded as percentages of the 8 tissue pieces from each wound that were colonised. Data were subjected to analysis of variance and Student's t-test for least significant difference at the 95% confidence level ( $P < 0.05$ ) by means of SAS v8.2 statistical software (SAS Institute Inc, SAS Campus Drive, Cary, North Carolina 27513, USA).

## RESULTS

### *In vitro* evaluation

Several different interactions were macro- and microscopically observed (Table 2). The macroscopic interactions typically included the formation of inhibition zones (Fig. 1A), instances where the biological agent grew over and sporulated on the mycelium of the pathogen (as described in Antal *et al.*, 2000)(Fig. 1D). Other interactions, which were observed to a lesser extent, were defined as “growth inhibition” and “stopped growth”. Growth inhibition of the pathogenic mycelium occurred when the pathogen's mycelium growth was inhibited in dual culture without coming in contact with the biological agent's mycelium, which was not inhibited and in most cases led to overgrowth of the pathogen colony (Fig. 1C). In instances where the interaction was termed “stopped growth”, both

biological agent and antagonist kept on growing until they came in contact with one another, where after growth of both colonies seized (Fig. 1B).

Microscopic interactions were observed at 20, 40 and 100× magnification and mostly included “coiling” (i.e. the ability of the biological *Trichoderma* isolate to coil its hyphae around that of the pathogens (Fig. 2G) and “hyphal disintegration”. In the latter interaction, holes appeared in the hyphae of the pathogen (as described in Benhamou and Chet, 1997), as well as the hyphae becoming flaccid or shrivelled (as described in John *et al.*, 2004) (Fig. 2H). Observations where hyphae of the biological agent attached itself to hyphae of the pathogen were defined as “hyphal adhesion” (Fig. 2F). “Hyphal swelling” (Fig. 2E) was only found with the interactions between the pathogens and *B. subtilis*, similar to the observations by Ferreira *et al.* (1991).

The six *Penicillium* isolates only had a limited antibiotic effect against the pathogens. Inhibition zones were observed in certain cases after 14 days, but they disappeared after prolonged incubation. The *Alternaria* isolate showed promising results against *P. viticola* only (growth inhibition and mycoparasitism by means of coiling). Given these limited results, these isolates were therefore not considered for further evaluation in the field.

All of the *T. harzianum* isolates; Ag 2, Ag11, AgSS 28, Eco 77 and Biotricho predominantly grew over the complex of trunk disease pathogens with the exception of *Pm. chlamydospora* and *Pa. aleophilum*, growth of these cultures was stopped where after they were later overgrown. Although isolates Ag 11 and AgSS28 overgrew most pathogen cultures, growth of *L. theobromae* was stopped and Ag11 inhibited the growth of *N. australis* by means of an inhibition zone. Microscopically, predominantly physical interactions such as coiling of the pathogens hyphae (Fig. 2G), adhesion of the hyphae (Fig. 2F) as well as hyphal disintegration were associated with over growth. Among the reactions caused by the *T. harzianum* strain of the commercial product, ECO 77, were that the hyphal cells lost their form and appeared to have a crinkly texture (Fig. 1H). At a magnification of 100×, this abnormality could be seen as a hyphal disintegration of the pathogen’s hyphae

The interactions observed for the *T. atroviride* isolates, Ag 3, Ag 5 and Ag 8, were similar to those observed for the *T. harzianum* strains, Ag 2 and Biotricho, i.e. overgrowth of all the pathogen isolates, except for the Ag 8 and *L. theobromae* combination, which had an inhibitory effect on each other in dual culture. A wide range of microscopic interactions were

observed on more than one occasion, and included adhesion of hyphae, coiling, and hyphal disintegration

The two unidentified *Trichoderma* isolates, USPP-T1 and USPP-T2, had a substantial effect on all trunk disease pathogens, with a wide range of macroscopic and microscopic interactions observed. Macroscopically, inhibition zones were mostly observed, which were later followed by overgrowth of the pathogen by the biological agent, or stopped growth of both organisms. At microscopic level, coiling was often observed the most with both isolates, while hyphal disintegration was observed with both isolates in combination with *D. seriata*.

In dual cultures with *B. subtilis*, all the pathogens were inhibited and showed little mycelium growth and clear inhibition zones (Fig. 1A) were observed. At microscopic level, malformations of the hyphae occurred; more specifically, it was as swelling of the hyphae (Fig. 2E).

These findings lead to the elimination of the six uncharacterised *Penicillium* and the *Alternaria* isolate from further evaluation. Therefore the *Trichoderma* based products, as well as the unidentified *Trichoderma* isolates USPP-T1 and USPP-T2 and the *B. subtilis* were further evaluated.

### ***In vivo* evaluation**

A significant interaction was observed between the treatment and pathogen data from the analysis of variance ANOVA ( $P \leq 0.001$ ; Table 3).

The mean pathogen incidence in the pruning wounds treated with one of the six different biocontrol agents, water or benomyl before inoculation with the different pathogens, are given in Tables 4-8. Each of these tables shows the incidence of a specific pathogen in wounds that were treated and subsequently inoculated with that specific pathogen, as well as the other pathogens. For the sake of brevity, the results for each pathogen's incidence in control wounds (natural infection) and wounds in which it was inoculated will be discussed.

Very low natural infection levels of *E. lata* were recorded in pruning wounds of treated with biological agents or benomyl (0 to 3.45%; Table 4), while only 0.45% was isolated from unprotected (water treated) wounds. The incidence levels in the *E. lata* inoculated pruning wounds were significantly higher compared to the non-inoculated pruning wounds (8.17 to 37.50%). All pruning wound treatments, biological as well as the benomyl treatment, significantly reduced the incidence level of *E. lata* in the inoculated pruning wounds

compared to the water treated control wounds (37.50%). However, the most effective treatments in reducing the *E. lata* incidence in inoculated wounds were Vinevax (8.17%), USPP-T1 (9.13%), USPP-T2 (11.00%), Eco-77 (10.65%) and *B. subtilis* (10.71%). Biotricho also gave good results, although not statistically as good as abovementioned treatments. The worst results in terms of reducing pathogen incidence were obtained with the benomyl treatment (19.58%), which yielded significantly higher *Eutypa* levels than the biocontrol treatments. *E. lata* was entirely absent or occurred at significantly lower levels (0 to 2.23%) in wounds that were inoculated with the other pathogens. These levels did, however, not differ statistically from the natural *E. lata* infection levels.

The Botryosphaeriaceae species were only identified to genus level from the isolations made from the treated pruning stubs. Natural infection by Botryosphaeriaceae species in unprotected pruning wounds was markedly higher (10.71%; Table 5) than that observed for *E. lata*. Biotricho (0.48%) and USPP-T1 (0.00%) reduced the natural infection significantly, while the other treatments also effected a marked reduction in isolated from treated wounds (2.17 to 4.46%). Isolations from unprotected pruning wounds that were inoculated with *Diplodia seriata*, *Lasiodiplodia theobromae*, *Neofusicoccum australe* and *N. parvum* yielded 37.50, 32.59, 44.71 and 22.08% incidence of Botryosphaeriaceae species, respectively. Wounds inoculated with *N. parvum* yielded significantly lower incidences than the other species. Botryosphaeriaceae incidences in most pruning wound protection treatments were significantly lower (4.50 to 23.56%). However, in the case of *N. parvum* inoculated wounds, *B. subtilis* (16.50%), benomyl (15.52%), USPP-T2 (16.20%) and Vinevax (13.89%) treated wounds yielded statistically similar levels than unprotected wounds. The most effective treatment against all Botryosphaeriaceae species was USPP-T1 (2.50 to 9.62%). Markedly lower levels of Botryosphaeriaceae isolates were obtained from wounds that were inoculated with *E. lata*, *P. chlamydospora* and *P. viticola* (4.33, 2.40 and 5.80%, respectively) compared with natural infection levels in unprotected wounds (10.71%).

*P. viticola* (Table 6) occurred naturally in the non-inoculated pruning wounds at levels (9.82%) markedly higher than the natural infection of the *Pa. chlamydospora* and *E. lata*. Several of the treatments reduced the natural infection to significantly lower levels: Biotricho (5.77%), USPP-T2 (5.77%), USPP-T1 (6.02%) and benomyl (6.70%). Inoculated unprotected pruning wounds yielded 34.82% incidence of *P. viticola*. In this case, benomyl proved to be the best treatment by reducing *P. viticola* levels to 8.19%. However, it was not statistically more effective than Biotricho (17.79%), USPP-T1 (10.87%), USPP-T2 (17.86%)

and Vinevax (13.02%). USPP-T1, similar to *E. lata* and Botryosphaeriaceae was again the most effective biocontrol agent, although not significantly better than Biotricho, USPP-T2 and Vinevax. Relatively high (6.25 to 12.08%) levels of natural infection by *P. viticola* were observed in unprotected pruning wounds that were inoculated with the other pathogens. These levels did, however, not differ statistically from the natural *P. viticola* infection levels.

*Pa. chlamydospora* occurred at relatively low levels in the non-inoculated pruning wounds (4.91%; Table 7). Although none of the treatments reduced the natural infection statistically, the Vinevax treated wounds yielded no *Pa. chlamydospora* while the USPP-T2 treated wounds yielded a 0.48% incidence. The incidence in the unprotected (water) inoculated pruning wounds was statistically higher (34.62%) than the levels in the non-inoculated wounds. All treatments statistically reduced the incidence levels in the inoculated pruning wounds compared to the water treated controls. USPP-T1 (7.81%) again fell into the group of most effective treatments and gave results statistically similar to Vinevax (7.21%) and benomyl (12.50%). Low levels (0 to 3.75%) of co-infection by *Pa. chlamydospora*, which did not differ statistically from its natural infection levels, were observed in wounds inoculated with the other pathogens.

The mean incidence of *Trichoderma* spp. in Merlot and Chenin Blanc was fairly low in wounds that were not treated with *Trichoderma*-based biocontrol products: *B. subtilis* (Table 8; 0.13 and 0.17%, respectively), benomyl (0.12 and 0%, respectively) and water (0.56 and 0.47%, respectively). All of the *Trichoderma* products and isolates had higher incidences in the Chenin blanc wounds, except for Vinevax® which had an higher incidence in the Merlot cultivar (16.16% compared with 24.64%). USPP-T1 (33.41 and 28.44%, respectively) was furthermore constantly isolated at the highest incidence from the pruning wounds treated prior to inoculation, although not always significantly higher compared to the other *Trichoderma* based biocontrol agents.

## DISCUSSION

This study clearly demonstrated the potential of biological control agents to provide sustained protection of pruning wounds against a complex of grapevine trunk disease pathogens when infection occurred 7 days after pruning and treatment. Initially, *in vitro* dual culture evaluations were conducted to screen the potential biocontrol agents (isolates) against aggregate species of Botryosphaeriaceae (*D. seriata*, *N. australis*, *N. parvum* and

*Lasiodiplodia theobromae*), *Eutypa lata*, *Phaeoacremonium aleophilum*, *Pa. chlamydospora* and *P. viticola*.

The results of the dual culture tests demonstrated that all of isolates from the *Trichoderma* based products, Vinevax, Biotricho and Eco77, as well as the unidentified *Trichoderma* isolates, USPP-T1 and -T2, showed antagonism towards all or most of the pathogens. Various antagonistic mechanisms were observed. Macroscopically, inhibition zones, most likely indicative of antibiotic production, were most commonly observed and hyphal disintegration was observed microscopically. In cases where *Trichoderma* cultures grew over that of the pathogen, signs of mycoparasitism, as seen from coiling or hyphal adhesion, were observed microscopically. The ability of *Trichoderma* spp. to grow fast and compete for space contributes to its ability to inhibit the growth of the pathogens in dual culture (Kucuk and Kivanc, 2004). However, the formation of inhibition zones and the inhibition of the pathogens mycelium growth without contact is most likely due to the ability of *Trichoderma* spp. to produce volatile (John *et al.*, 2004; Kucuk and Kivanc, 2004) and non-volatile (John *et al.*, 2004) substances. The mycoparasitic reactions such as coiling, adhesion and penetration of pathogenic hyphae (Almeida *et al.*, 2007), predominantly coincided with the macroscopic physical contact interactions; overgrowth and stopped growth. With the ability of *Trichoderma* spp. to produce enzymes and antibiotics (Calistru *et al.*, 1997), it is capable of causing hyphal disintegration (Benhamou and Chet., 1997). However, close contact of the pathogen and *Trichoderma* isolates coincide or lead up to the processes of hyphal disintegration (Benhamou and Chet, 1993), which suggests that contact is needed for the secretion of enzymes or antibiotics. In contrast with these findings, hyphal malformations and disintegration were often observed without the *Trichoderma* isolates being in contact with hyphae of the pathogen's. This raises the question whether contact is necessary for the production of antibiotics or enzymes. Therefore, more research is needed to further identify the different antagonistic mechanisms employed by USPP-T1 and USPP-T2 against the trunk disease pathogens.

*B. subtilis* caused hyphal malformations to all of the pathogens, which can possibly be attributed to an antibiotic substance. Ferreira *et al.* (1989) identified two antibiotic products produced by this particular *B. subtilis* isolate, which were capable of inhibiting mycelial growth of *E. lata*. According to Baker and Cook (1982), the antibiotics from *B. subtilis* can penetrate the hyphae and cause the malformation of hyphae.

Given the results of the *in vitro* evaluation, Vinevax, Biotricho, Eco 77, USPP-T1 and T2 (which contain *Trichoderma* spp.) as well as the *B. subtilis* isolate were selected for further evaluation in field trials. Fresh pruning wounds of Chenin blanc and Merlot were treated with the biocontrol agents/products and individually challenged by the grapevine pruning wound invading pathogens 7 days later. The 7-day-period gave the biocontrol agents the ability to establish in the pruning wounds and therefore contributed to their competitiveness. Previous studies (Halleen and Fourie, 2005) have typically challenged treated pruning wounds shortly after treatment with the biocontrol agents since the pathogens' inocula are also present on the day of pruning. These studies concluded that chemical control of pruning wounds were superior to biological control options. Findings from our study, however, reveal the opposite. Benomyl at 10 g/L (at 20× the recommended dosage for foliar disease management in South Africa; Nel *et al.*, 2003), which was used as the chemical control standard in this study, was proven by previous studies to be the most effective option for pruning wound protection (Pearson *et al.*, 1982; Munkvold and Marois, 1993b). Moreover, Bester *et al.* (2007) demonstrated that benomyl was effective against trunk pathogens caused by Botryosphaeriaceae. However, when benomyl-treated wounds were challenged by the pathogens 1 week after treatment, its efficacy was remarkably poorer as was observed previously by Halleen and Fourie (2005). In most cases, the efficacy of some of the biocontrol agents was similar or superior to that observed for benomyl. The reduced efficacy of benomyl observed in this study might be attributed to residual breakdown of the fungicide in the wound site, as was observed by Price and Carter (1975) who found that the extractable amount of methyl benzimidazole-2-yl carbamate in the sapwood diminished in 2 weeks after application to pruning wounds. However, the dosage of benomyl used in this study (5 000 µg/mL) was lower than the 12 500 µg/mL as recommended by Munkvold and Marois (1993b) and could have contributed to its lack of efficacy. These findings and the report by Halleen and Fourie (2005), gives a clear indication that although biocontrol gives long term protection, a period of establishment is still required. With benomyl protecting the wounds when challenged directly after treatment with the wound invading fungi, the potential of treating wounds with a combination of a chemical and a biological control agent simultaneously should be investigated. This statement is supported by Carter and Price (1974) who suggested that benzimidazole-resistant *Fusarium lateritium* be integrated in mixture with benzimidazoles to treat pruning wounds. Isolations made from treated pruning wounds that were challenged with the trunk disease pathogens 1 week after pruning, yielded a particularly high incidence of the pathogens from the untreated pruning

wounds. This finding suggests that unprotected pruning wounds stay susceptible at least 1 week after pruning, which supports previous reports that pruning wounds are susceptible to trunk pathogen infection for up to 16 weeks after pruning (Munkvold and Marois, 1995; Larignon and Dubos, 2000; Gubler *et al.*, 2001, van Niekerk, 2007). These results suggest that sustained protection of the pruning wounds is therefore required for extended periods after pruning. Therefore, it can be anticipated that natural infection could have contributed to the observed infection levels, but at no instance did natural infection levels, as observed in un-inoculated wounds, yield similar levels to inoculated wounds.

Relatively high levels of secondary infections were isolated from inoculated pruning wounds, i.e. other natural infection by trunk disease pathogens in wounds that were inoculated with a specific pathogen. It is known that *E. lata* causes primary infection (Larignon and Dubos, 1997), as was also reported for *Pm. chlamydospora* and *Phaeoacremonium* spp. (Sparapano *et al.*, 2000). Ferreira *et al.* (1989) suggested that there is a succession of fungal colonisation in pruning wounds after infection by primary colonisers. However, findings of the current study suggests otherwise, with pruning wounds inoculated with *E. lata* having little or no secondary infection and to a lesser extent *Pm. chlamydospora*, and are supported by the reports of Larignon and Dubos (1997) and Sparapano *et al.* (2000) respectively. Moreover, although very low secondary infections were observed for the abovementioned pathogens, higher infections were found in pruning wounds inoculated with the aggregate species of *Botryosphaeriaceae* and *P. viticola*. This suggests that the pathogens used during the current study may have the ability to predispose wood for secondary infection. This corresponds with the suggestion by Van Niekerk (2008) that succession could follow the initial infection of *Botryosphaeriaceae*, especially basidiomycete infection. Little is known about the ability of *P. viticola* to predispose wood for secondary infections. Another reason for co-isolation of pathogens from a single pruning wound might be due to the period between treatment and inoculation of the pruning wounds; infection by other pathogens could have occurred before inoculation took place. This might also explain the high levels of *E. lata* in inoculated pruning wounds, especially as Ferreira *et al.* (1989) proposed that early wood infecting fungi need to predispose wood for *E. lata* infection. However, with reference to the high secondary infection, Fourie and Halleen (2002) have reported that several of the trunk disease pathogens have been isolated from rootstock mother plants, suggesting that some infection could have originated from the rootstocks.

Findings in the current study support previous research on protection of pruning wounds by means of *Trichoderma* or other biological control agents (Ferreira *et al.*, 1999; Di Marco *et al.*, 2004; John *et al.*, 2005a, b). *B. subtilis*, the *Trichoderma* products, Vinevax, Biotricho, Eco 77, and isolates USPP-T1 and -T2 generally reduced the incidence of the entire complex of grapevine trunk disease pathogens compared to the untreated control. Moreover, *Trichoderma* isolates were isolated 8 months after treatment from treated pruning wounds at varying levels. However, these levels are not comparable with those observed in other studies. Di Marco *et al.* (2004) recovered *Trichoderma* from 90% of treated pruning wounds after 7 days, while John *et al.* (2005b) reisolated *Trichoderma* from 50% of vines 20 months after insertion of wooden dowels impregnated with *Trichoderma*. Di Marco *et al.* (2004), nonetheless, found that there was a significant reduction in recovery percentage the longer the period after treatment. Small amounts of *Trichoderma* were recovered from untreated pruning wounds indicating low levels of natural *Trichoderma* infection. Alternatively, this could possibly be due to the fungus being present in the pruning wounds endophytically as *Trichoderma* are known endophytes in the sapwood of woody plants. (Samuels *et al.*, 2006) or cross contamination taking place during treatment of the wounds. *B. subtilis* was never isolated from pruning wounds due to the PDA being amended with 0.04 g/L streptomycin sulphate. However, pruning wounds treated with *B. subtilis* showed significantly lower incidences of all the pathogens 8 months after inoculation. Ferreira *et al.* (1991) reported that pruning wounds treated with the same strain of *B. subtilis* significantly suppressed *E. lata* 9 months after inoculation over two separate seasons. Schmidt *et al.* (2001) showed that *B. subtilis* strain B1 $\alpha$  reduced mycelium growth of *E. lata* on autoclaved grape wood, further suggesting the potential of the bacterium as a pruning wounds protectant.

The current study found that a higher incidence of *Trichoderma* was recovered from Chenin blanc for all of the *Trichoderma*-based treatments except for Vinevax. Several factors could contribute to the imbalance, as the merlot was under regular drip irrigation, stress of the Chenin blanc could have lead to the narrowing of the xylem vessels. As the findings of Lovisollo and Schubert (1998) suggested, that not only did the transactional areas of the xylem vessels decrease under water stress, but so too did the hydraulic conductivity. Another contributing factor could be nutrient value as higher conidial germination was observed when spore suspensions were amended with glucose by Schubert *et al.* (2008). This suggests that Chenin blanc could have a higher nutrient intake and availability. Unfortunately this does not explain the higher incidence of the Vinevax product in Merlot, however reports have

suggested that certain *Trichoderma* strains have higher affinities for certain woody hosts (Samuels *et al.*, 2006). However, further research is required to determine the exact cause of the cultivar impact.

Considering the ability of *Trichoderma* to colonise the pruning wound and sustain its presence, long term protection of pruning wounds with *Trichoderma* based products is an effective tool in the management of pruning wound pathogens. The USPP-T1 and -T2 isolates were especially effective and protected the pruning wounds against the entire complex of pathogens. With these isolates being isolated from grapevine pruning wounds from the same vineyard as the current study in previous trials, it could be possible that the natural origin of the strains may be grapevines. Therefore, the potential of these isolates as pruning wound protectants should be studied further, especially with regard to mechanism of control, cultivar susceptibility and application methods.

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**Table 1.** Biological agents and pathogens used in *in vitro* evaluation of potential biological agents against the most important trunk disease pathogens

<b>Organism</b>	<b>Isolate number</b>	<b>Code (Table 2)</b>	<b>Origin</b>
<b>BIOCONTROL AGENTS</b>			
<i>Trichoderma harzianum</i>	AG 2	AG 2	Vinevax, Agrimm Technologies Ltd, NZ
<i>T. harzianum</i>	AG 11	AG 11	Vinevax, Agrimm Technologies Ltd, NZ
<i>T. harzianum</i>	AGSS 28	AGSS 28	Vinevax, Agrimm Technologies Ltd, NZ
<i>T. harzianum</i>	Biotricho	Biotricho	Agro-Organics (PTY) Ltd., RSA
<i>T. harzianum</i>	ECO 77	ECO 77	Eco 77, Plant Health Products (PTY) Ltd., RSA
<i>T. atroviride</i>	AG 3	AG 3	Vinevax, Agrimm Technologies Ltd, NZ
<i>T. atroviride</i>	AG 5	AG 5	Vinevax, Agrimm Technologies Ltd, NZ
<i>T. atroviride</i>	AG 8	AG 8	Vinevax, Agrimm Technologies Ltd, NZ
<i>Trichoderma</i> spp. 1	STE-U 6514	USPP-T1	Department of Plant Pathology, Stellenbosch University, RSA
<i>Trichoderma</i> spp. 2	STE-U 6515	USPP-T2	Department of Plant Pathology, Stellenbosch University, RSA
<i>Penicillium</i> spp. 1	STE-U 6507	USPP-Pen 1	Department of Plant Pathology, Stellenbosch University, RSA
<i>Penicillium</i> spp. 2	STE-U 6508	USPP-Pen 2	Department of Plant Pathology, Stellenbosch University, RSA
<i>Penicillium</i> spp. 3	STE-U 6509	USPP-Pen 3	Department of Plant Pathology, Stellenbosch University, RSA
<i>Penicillium</i> spp. 4	STE-U 6510	USPP-Pen 4	Department of Plant Pathology, Stellenbosch University, RSA
<i>Penicillium</i> spp. 5	STE-U 6511	USPP-Pen 5	Department of Plant Pathology, Stellenbosch University, RSA

<i>Penicillium</i> spp. 6	STE-U 6512	USPP-Pen 6	Department of Plant Pathology, Stellenbosch University, RSA
<i>Alternaria</i> sp.	STE-U 6505	USPP-Alt	Department of Plant Pathology, Stellenbosch University, RSA
<i>Bacillus subtilis</i>	EE 1/10	B.subt.	Nietvoorbij, Stellenbosch, RSA
<b>PATHOGENS</b>			
<i>Neofusicoccum australe</i>	STE-U 4416		Department of Plant Pathology, Stellenbosch University, RSA
<i>Diplodia seriata</i>	STE-U 4440		Department of Plant Pathology, Stellenbosch University, RSA
<i>N. parvum</i>	STE-U 4589		Department of Plant Pathology, Stellenbosch University, RSA
<i>Lasiodiplodia theobromae</i>	STE-U 4419		Department of Plant Pathology, Stellenbosch University, RSA
<i>Eutypa lata</i>	STE-U 6513		Department of Plant Pathology, Stellenbosch University, RSA
<i>Phaeoacremonium aleophilum</i>	STE-U 5939		Department of Plant Pathology, Stellenbosch University, RSA
<i>Phaeomoniella chlamydospora</i>	STE-U 6505		Department of Plant Pathology, Stellenbosch University, RSA
<i>Phomopsis viticola</i>	STE-U 5602		Department of Plant Pathology, Stellenbosch University, RSA

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**Table 2.** Macro<sup>X</sup>- and microscopic<sup>Y</sup> interactions observed between dual cultures of selected trunk disease pathogens and biological agents

Biological agent	<i>N. australis</i>		<i>D. seriata</i>		<i>N. parvum</i>		<i>L.theobromae</i>		<i>Eutypa lata</i>		<i>Pm. aleophilum</i>		<i>Pa. chlam.</i>		<i>Phomopsis viticola</i>	
	Macroscopic	Microscopic	Macroscopic	Microscopic	Macroscopic	Microscopic	Macroscopic	Microscopic	Macroscopic	Microscopic	Macroscopic	Microscopic	Macroscopic	Microscopic	Macroscopic	Microscopic
<b>Ag 2</b>	OG	PC	OG	N	OG	HD	OG	AH	OG	N	GI,OG	PC	GI,OG	PC	OG	N
<b>Ag 3</b>	OG	HD	OG	N	OG	AH	OG	HD	OG	N	GI,OG	PC	GI,OG	N	OG	N
<b>Ag 5</b>	OG	AH	OG	AH	OG	PC	OG	HD	OG	PC	GI,OG	HD	GI,OG	C	OG	N
<b>Ag 8</b>	OG	PC	OG	AH	OG	HD	SG	N	OG	N	GI,OG	N	GI,OG	N	OG	N
<b>Ag 11</b>	IZ	N	OG	PC	OG	PC	SG	N	OG	HD	GI,OG	N	GI,OG	PC	OG	N
<b>Agss 28</b>	OG	N	OG	PC	OG	HD	SG	PC	OG	AH	GI,OG	N	GI,OG	N	OG	N
<b>USPP-T1</b>	IZ,OG	AH	IZ	HD	OG	PC	IZ	PC	IZ,OG	PC	GI,OG	PC	GI,OG	PC	OG	N
<b>USPP-T2</b>	IZ,OG	N	SG,IZ	HD	OG	PC	OG	PC	SG,IZ	N	GI,OG	N	GI,OG	N	OG	PC,HD
<b>B.subt.</b>	IZ	SM	IZ	SM	IZ	SM	IZ	SM	IZ	SM	IZ	SM	IZ	SM	IZ	SM
<b>Biotricho</b>	OG	N	OG	AH	OG	HD	OG	HD	OG	HD	GI,OG	AH	GI,OG	N	OG	N
<b>ECO 77</b>	SG	HD	IZ	N	OG	HD	OG	PC	OG	PC	GI,OG	N	GI,OG	N	OG	N

<sup>X</sup> OG = Overgrowth of the pathogen by the biological agent; IZ = Inhibition zone formed between the two cultures; SG = Growth of the pathogen stopped when they come in contact with the agent; GI = Growth of pathogen stopped after incubation with the agent.

<sup>Y</sup> AH = adhesion of the biological agent's hyphae to that of the pathogen's; HD = Hyphal disintegration; PC = Coiling by the hypha of the agent around hyphae of the pathogen; SM = Swelling and malformation of the pathogen's hyphae; N = No interactions have been observed

**Table 3.** Analysis of variance of the incidence of *Trichoderma*, *Eutypa lata*, *Phaeomoniella chlamydospora*, *Phomopsis viticola*, *Diplodia seriata*, *Lasidiplodia theobromae*, *Neofusicoccum australe* and *N. parvum* and isolated from the pruning wounds of Merlot and Chenin blanc cultivars.

Source of variation	DF	SS	MS	SL
Model	383	76715.394	200.3013	<0.0001
Cultivar	1	15.734	15.734	0.6167
Block (Cultivar)	4	514.997	128.749	0.0851
Treatment	7	1905.579	272.226	<0.0001
Pathogen	7	41582.096	5940.299	<0.0001
Treatment × Pathogen	49	15765.047	321.736	<0.0001
Cultivar × Treatment	7	327.0669	46.724	0.6344
Cultivar × Pathogen	7	535.463	76.495	0.2891
Cultivar × Treatment × Pathogen	49	1564.726	31.933	0.9982
Block (Cult × Treat × Path)	252	14504.686	57.558	0.8050
Error	1289	80916.667	62.775	
Corrected Total	1672	157632.061		

**Table 4.** Mean incidence of *Eutypa lata* that was isolated from pruning wounds 8 months after the fresh pruning wounds were treated with benomyl, *Bacillus subtilis*, two *Trichoderma* spp. (USPP-T1 and -T2), three commercial *Trichoderma*-based biocontrol products or water as pruning wound protectants, and challenged 7 days later by spray-inoculation with *Eutypa lata*, *Phaeomoniella chlamydospora*, *Phomopsis viticola*, *Diplodia seriata*, *Lasiodiplodia theobromae*, *Neofusicoccum australe* and *N. parvum* spore suspensions, or sprayed with sterile water to evaluate natural infection.

Wound treatment	<i>Eutypa lata</i> incidence (%) <sup>x</sup> in treated wounds that were challenged with							
	Natural infection	<i>Eutypa lata</i>	<i>Phaeomoniella chlamydospora</i>	<i>Phomopsis viticola</i>	<i>Diplodia seriata</i>	<i>Lasiodiplodia theobromae</i>	<i>Neofusicoccum australe</i>	<i>Neofusicoccum parvum</i>
<i>B. subtilis</i>	0.54a	10.71b	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
Benomyl	0.45a	19.58d	0.48a	0.00a	0.00a	0.00a	0.00a	0.00a
Biotricho	0.00a	15.00c	0.00a	0.00a	2.23a	0.00a	0.93a	0.00a
Eco77	0.00a	10.65b	0.48a	0.00a	0.00a	0.00a	1.56a	1.39a
USPP-T1	0.00a	9.13b	0.00a	0.00a	0.00a	0.00a	0.48a	0.00a
USPP-T2	0.00a	11.00bc	0.00a	0.00a	1.14a	0.00a	0.89a	0.00a
Vinevax	3.45a	8.17b	0.00a	1.56a	0.00a	0.00a	0.93a	0.46a
Water	0.45a	37.50e	0.00a	0.45a	0.00a	0.00a	1.44a	0.00a

<sup>x</sup>Means followed by the same letter do not differ significantly ( $P < 0.05$ ; LSD = 4.147).

**Table 5.** Mean combined incidence of species of *Botryosphaeriaceae* that were isolated from pruning wounds 8 months after the fresh pruning wounds were treated with benomyl, *Bacillus subtilis*, two *Trichoderma* spp. (USPP-T1 and -T2), three commercial *Trichoderma*-based biocontrol products or water as pruning wound protectants, and challenged 7 days later by spray-inoculation with *Eutypa lata*, *Phaeoconiella chlamydospora*, *Phomopsis viticola*, *Diplodia seriata*, *Lasiodiplodia theobromae*, *Neofusicoccum australe* and *N. parvum* spore suspensions, or sprayed with sterile water to evaluate natural infection.

Wound treatment	<i>Botryosphaeriaceae</i> incidence (%) <sup>x</sup> in treated wounds that were challenged with							
	Natural infection	<i>Diplodia seriata</i>	<i>Lasiodiplodia theobromae</i>	<i>Neofusicoccum australe</i>	<i>Neofusicoccum parvum</i>	<i>Eutypa lata</i>	<i>Phaeoconiella chlamydospora</i>	<i>Phomopsis viticola</i>
<i>B. subtilis</i>	2.17a-d	13.50g-o	15.50j-p	15.18i-p	16.50l-p	4.46a-g	2.78a-d	8.33a-l
Benomyl	4.46a-g	23.56pq	14.29h-o	12.05e-n	15.52j-p	2.50a-d	0.96ab	0.43a
Biotricho	0.48a	20.09n-p	15.48j-p	10.65c-m	12.50f-n	3.00a-e	3.70a-f	1.92a-c
Eco77	2.84a-d	14.29h-o	13.46g-o	18.23m-p	7.41a-k	2.31a-d	0.00a	1.63a-c
USPP-T1	0.00a	5.77a-h	2.50a-d	9.62b-m	4.50a-g	6.73a-j	6.25a-i	3.26a-e
USPP-T2	2.40a-d	15.34j-p	8.13a-l	10.27c-m	16.20k-p	4.00a-f	4.63a-g	2.68a-d
Vinevax	3.45a-f	15.76j-p	13.79h-o	11.11d-n	13.89h-o	1.44a-c	3.85a-f	1.56a-c
Water	10.71c-m	37.50rs	32.59qr	44.71s	22.08op	4.33a-f	2.40a-d	5.80a-h

<sup>x</sup>Means followed by the same letter do not differ significantly ( $P < 0.05$ ; LSD = 9.06).

**Table 6.** Mean incidence of *Phomopsis viticola* that was isolated from pruning wounds 8 months after the fresh pruning wounds were treated with benomyl, *Bacillus subtilis*, two *Trichoderma* spp. (USPP-T1 and -T2), three commercial *Trichoderma*-based biocontrol products or water as pruning wound protectants, and challenged 7 days later by spray-inoculation with *Eutypa lata*, *Phaeomoniella chlamydospora*, *Phomopsis viticola*, *Diplodia seriata*, *Lasiodiplodia theobromae*, *Neofusicoccum australe* and *N. parvum* spore suspensions, or sprayed with sterile water to evaluate natural infection.

Wound treatment	<i>Phomopsis viticola</i> incidence (%) <sup>x</sup> in treated wounds that were challenged with							
	Natural infection	<i>Phomopsis viticola</i>	<i>Eutypa lata</i>	<i>Phaeomoniella chlamydospora</i>	<i>Diplodia seriata</i>	<i>Lasiodiplodia theobromae</i>	<i>Neofusicoccum australe</i>	<i>Neofusicoccum parvum</i>
<i>B. subtilis</i>	16.85b-f	21.35b	6.25h-n	11.11c-m	4.50j-n	12.00b-l	7.14f-n	16.50b-g
Benomyl	6.70g-n	8.19e-m	3.75k-n	4.33j-n	4.33j-n	6.25h-n	4.46j-n	1.72mn
Biotricho	5.77i-n	17.79b-e	9.00d-n	10.19c-n	19.64bc	14.29b-j	3.24k-n	16.07b-h
Eco77	13.07b-k	18.48b-d	9.26d-n	15.38b-i	2.68l-n	5.29i-n	4.17j-n	5.09j-n
USPP-T1	6.02h-n	10.87c-l	0.48n	11.46b-m	3.37k-n	4.50j-n	3.85k-n	9.00d-n
USPP-T2	5.77i-n	17.86b-e	7.50f-n	12.96b-k	9.66c-n	7.50f-n	8.93d-n	5.56i-n
Vinevax	10.78c-m	13.02b-j	3.85k-n	1.92l-n	7.07f-n	8.62d-n	7.87e-n	6.48g-n
Water	9.82c-n	34.82a	6.25h-n	10.10c-n	8.00e-n	10.27c-n	6.25h-n	12.08b-l

<sup>x</sup>Means followed by the same letter do not differ significantly ( $P < 0.05$ ; LSD = 10.135).

**Table 7.** Mean incidence of *Phaeomoniella chlamydospora* that was isolated from pruning wounds 8 months after the fresh pruning wounds were treated with benomyl, *Bacillus subtilis*, two *Trichoderma* spp. (USPP-T1 and -T2), three commercial *Trichoderma*-based biocontrol products or water as pruning wound protectants, and challenged 7 days later by spray-inoculation with *Eutypa lata*, *Phaeomoniella chlamydospora*, *Phomopsis viticola*, *Diplodia seriata*, *Lasiodiplodia theobromae*, *Neofusicoccum australe* and *N. parvum* spore suspensions, or sprayed with sterile water to evaluate natural infection.

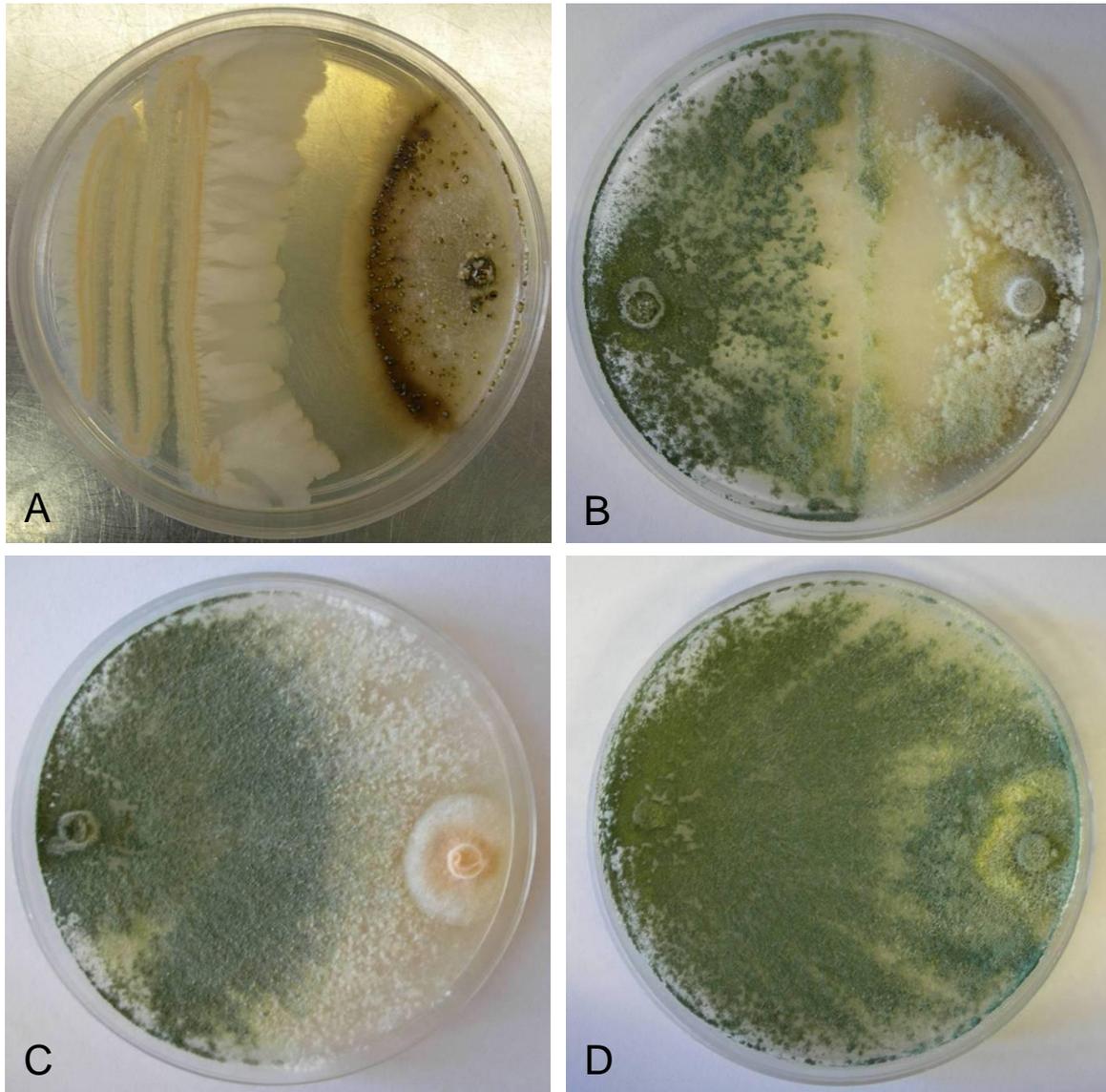
Wound treatment	<i>Phaeomoniella chlamydospora</i> incidence (%) <sup>x</sup> in treated wounds that were challenged with							
	Natural infection	<i>Phaeomoniella chlamydospora</i>	<i>Eutypa lata</i>	<i>Phomopsis viticola</i>	<i>Diplodia seriata</i>	<i>Lasiodiplodia theobromae</i>	<i>Neofusicoccum australe</i>	<i>Neofusicoccum parvum</i>
<i>B. subtilis</i>	2.17a-c	21.76fg	0.45a	3.13a-c	1.00a	1.00a	0.89a	1.50ab
Benomyl	5.36a-c	12.50de	0.42a	0.00a	1.44ab	0.89a	1.34ab	0.00a
Biotricho	5.29a-c	23.61g	0.00a	1.44ab	0.45a	0.00a	3.24a-c	2.68a-c
Eco77	1.70ab	14.90e	0.00a	1.63ab	0.45a	0.48a	0.00a	3.24a-c
USPP-T1	1.85ab	7.81cd	2.40a-c	3.80a-c	0.00a	0.00a	3.85a-c	0.00a
USPP-T2	0.48a	15.74e	2.50a-c	1.34ab	1.14a	0.63a	0.00a	2.78a-c
Vinevax	0.00a	7.21b-d	0.00a	4.17a-c	2.72a-c	1.29a	0.93a	0.93a
Water	4.91a-c	34.62h	0.00a	1.34ab	2.00a-c	1.79ab	0.48a	3.75a-c

<sup>x</sup>Means followed by the same letter do not differ significantly ( $P < 0.05$ ; LSD = 5.896).

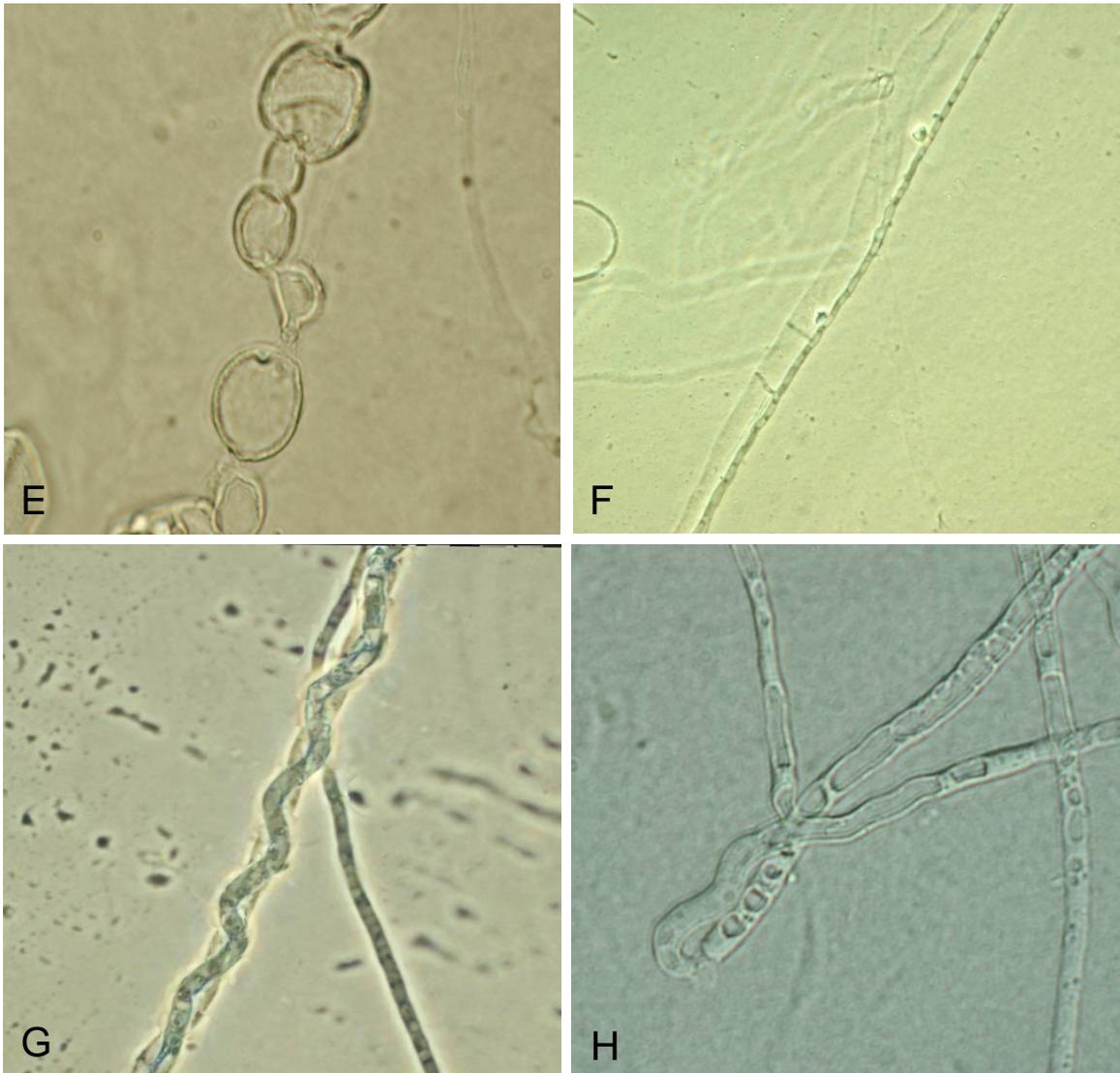
**Table 8.** The mean colonisation incidence of *Trichoderma* isolated from pruning wounds of Merlot and Chenin blanc cultivars 8 months fresh pruning wounds were treated with benomyl, *Bacillus subtilis*, two *Trichoderma* spp. (USPP-T1 and -T2), three commercial *Trichoderma*-based biocontrol products or water as pruning wound protectants, and challenged 7 days later by spray-inoculation with *Eutypa lata*, *Phaeoconiella chlamydospora*, *Phomopsis viticola*, *Diplodia seriata*, *Lasidiplodia theobromae*, *Neofusicoccum australe* and *N. parvum* spore suspensions, or sprayed with sterile water to evaluate natural infection.

Wound treatment	<i>Trichoderma</i> spp. incidence (%) <sup>x</sup> in treated wounds	
	Chenin blanc	Merlot
<i>B. subtilis</i>	0.13c	0.71c
Benomyl	0.11c	0.00c
Biotricho	10.75bc	6.37c
Eco77	22.12ab	12.50bc
USPP-T1	33.41a	28.44a
USPP-T2	20.71ab	11.74bc
Vinevax	16.16b	24.64ab
Water	0.56c	0.47c

<sup>x</sup>Means followed by the same letter do not differ significantly ( $P < 0.05$ ; LSD = 14.449)



**Figure 1.** Macroscopic interactions observed on PDA-medium. A) Inhibition zone (*B. subtilis* and *P. viticola*); B) Stopped growth (Ag 8 and *L. theobromae*); C) Growth inhibition (USPP-T1 and *Pm. aleophilum*); D) Overgrowth (*Biotricho* and *E. lata*).



**Figure 2.** Microscopic interactions as observed at 20×, 40× and 100× magnification. E) Hyhal swelling as observed in the interaction between *B. subtilis* and *P. viticola* (100×); F) Hyphal adhesion observed between USPP-T1 and *N. austral* (40×); G) Coiling of *Trichoderma* hyphae around pathogenic hyphae as observed between USPP-T1 and *L. theobromae* (100×); I) Hyphal disintegration of *D. seriata* hyphae (100×).

### 3. IDENTIFICATION OF *TRICHODERMA* SPECIES AND MODE OF INHIBITION OF GRAPEVINE TRUNK DISEASE PATHOGENS

#### ABSTRACT

Two *Trichoderma* isolates (USPP-T1 and USPP-T2), which were shown to be antagonistic toward grapevine trunk disease pathogens, were identified by means of DNA comparison, and their ability to inhibit the mycelium growth of the trunk disease pathogens by means of volatile and non-volatile metabolite production. The two gene areas that were used include the internal transcribed spacers (ITS 1 and 2) and the 5.8S ribosomal RNA gene and the translation elongation factor 1 $\alpha$  (EF). The ITS and EF sequences were aligned to published *Trichoderma* sequences and the percentage similarity determined. A 100% similarity was found with the ITS sequences of *T. atroviride* and 94%-100% similarity with EF sequences of *T. atroviride*. The two *Trichoderma* isolates were therefore identified as *Trichoderma atroviride*. The production of volatile metabolites by two *T. atroviride* isolates were determined by placing an inverted Petri dish with *Trichoderma* on top of a dish with a pathogen isolate and then sealed with parafilm. *Trichoderma* isolates were grown for 2 days on PDA where after they were inverted over PDA plates containing mycelial plugs of species in the Botryosphaeriaceae (incl. *Diplodia seriata*, *Lasiodiplodia theobromae*, *Neofusicoccum australis* and *Neofusicoccum parvum*), *Phomopsis viticola*, *Eutypa lata* or 5-day-old colonies of *Phaeoconiella chlamydospora* and *Phaeoacremonium aleophilum*. The combined plates were incubated at 25°C for 7 days. The mycelial growth of all the pathogens was inhibited by volatile metabolites produced by the *Trichoderma* isolates. The inhibition ranged from 23.6% for *L. theobromae* to 72.4% for *P. viticola*. The inhibition by non-volatiles was determined by the increase of mycelial growth of the pathogens when growing on potato dextrose broth amended with *T. atroviride* filtrate. Inhibition by non-volatile products was less than for the volatile inhibition. Inhibition ranged from 7.5% for *N. parvum* to 20.6% for *L. theobromae*. In the non-volatile inhibition USPP-T1 caused significantly more mycelial inhibition than USPP-T2.

## INTRODUCTION

*Trichoderma* is an asexual reproducing genus that occurs saprophytically in the rhizosphere as well as an endophytic coloniser of the sapwood of herbaceous plants (Samuels, 2006). The fungus is considered to be an opportunistic, avirulent plant symbiont, being a fast growing fungus that produces masses of powdery green conidia (Samuels, 2006). *Trichoderma* species, such as *Trichoderma harzianum* and *Trichoderma atroviride*, are well known biological control agents of plant pathogens (Elad *et al.*, 1993; Elad and Kapat, 1999; Brunner *et al.*, 2003). According to literature, these fungi rely on various mechanisms for the control of pathogenic fungi, namely mycoparasitism (Almeida *et al.*, 2007), antibiosis (Bélanger *et al.*, 1995; Calistru *et al.*, 1997) and competition (Sivan and Chet, 1989).

For antibiosis, *Trichoderma* produces an array of substances that include chitinase, glucanase and antibiotics such as gliotoxin and gliovirin (Howell, 2003). Some of these enzymes are known, such as  $\beta$ -1,3-glucanase (Noronha and Ulhoa, 1999). Another enzyme produced by *Trichoderma* is 42-kDa endochitinase, known to be associated with hydrolysis of pathogenic fungi cell walls (Kubicek *et al.*, 2001). Researchers have stated that antibiosis form a part of mycoparasitism and that the secretion of enzymes and antibiotics only occur in close proximity with pathogen hyphae (Benhamou and Chet, 1993). However, John *et al.* (2004) found that certain *Trichoderma* strains can produce volatile and non-volatile substances in the absence of pathogenic fungi. The volatiles produced by *T. atroviride* include derivatives of pyrone dioxolane and esters; the main component of these derivatives being 5,5'-dimethyl-2H-pyran-2-on (Kezzler *et al.*, 2000).

Papavizas (1985) stated that for biological control to be effective, accurate identification of the biological agents is necessary. Correct identification of *Trichoderma* rely on sequence comparisons since an overlap occur in morphological characters amongst different species (Freeman *et al.*, 2004; Samuels, 2006). The aim of this study was to identify two *Trichoderma* isolates (USPP-T1 and USPP-T2) to species level and to assess their potential mechanisms of antibiosis towards fungi causing trunk diseases of grapevines.

## MATERIALS AND METHODS

### DNA isolation and sequencing

Genomic DNA was extracted from the USPP-T1 and USPP-T2 isolates using the Wizard SV Genomic DNA Purification System (Promega Cooperation, Madison, WI, USA) according to the manufacturer's instructions. The internal transcribed spacers (ITS) 1 and 2 and 5.8S rRNA gene and the translation elongation factor 1 $\alpha$  (EF) gene was amplified. A fragment of approximately 900 bp of the 5' end of the EF gene was amplified using primers EF1 (O'Donnel *et al.*, 1998) and TEF1 (Samuels *et al.*, 2002). Approximately 600 bp of the 5' end of the ITS gene were amplified using primers ITS1 and ITS4 (White *et al.*, 1990). The reaction mixture for PCR contained 1  $\mu$ L of DNA sample, 1 $\times$  PCR buffer (Bioline GmbH, Luckenwalde, Germany), 0.2 pmol of the EF1 and TEF1 primers where as 0.4 pmol per primer was used of ITS1 and ITS4, 200  $\mu$ M of each of the dNTP's, 0.5 U of *Taq* DNA polymerase (Bioline GmbH), MgCl<sub>2</sub> was at 2.5 mM for EF and 1.5 mM for ITS, each reaction was made up to a final volume of 25  $\mu$ L with sterile water. The following PCR amplification cycles were run on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA): 94°C (ITS for 5 min and EF for 2 min), followed by 36 cycles of denaturation 94°C (ITS for 45 s and EF for 30 s), annealing for 30 s (at 52°C for ITS and 55°C for EF) and elongation (72°C for 60 s), and a final (6 min for ITS and 10 min for EF) extension step at 72°C. PCR products were analysed by electrophoresis at 85 V for 30 min in a 0.8 % (w/v) agarose gel in 0.5  $\times$  TAE buffer (0.4 M Tris, 0.05 M glacial acetic acid and 0.01 M ethylenediamine tetraacetic acid [EDTA], pH 7.85) and visualised under UV light following ethidium bromide staining. PCR products were purified according to the manufacturer's instructions using a commercial kit (GFX PCR DNA and Gel Band Purification, Amersham Biosciences, Roosendal, the Netherlands). Sequencing reactions were carried out with the PCR primers using a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences) according to the manufacturer's recommendations, and the resulting products were analysed on an ABI Prism 3130XL DNA Sequencer (Perkin-Elmer, Norwalk, Foster City, CA). A consensus sequence was computed from the forward and reverse sequences with Geneious Pro 3.0.6. Sequences were deposited in GenBank (Accession numbers for ITS: FJ232696, FJ232697; EF: FJ232698, FJ232699). Sequences were blasted in GenBank to obtain the most similar matches and were then manually aligned with the closest hits. *Trichoderma* sequences published by Samuels (2002) were also included in the alignment.

Sequences were also blasted on TrichoBlast (<http://www.isth.info/tools/>), an online *Trichoderma* identification database.

### ***In vitro* antibiosis**

#### ***Volatile inhibition***

The two *Trichoderma* isolates, USPP-T1 and USPP-T2, were investigated to assess the ability of the strains to inhibit pathogenic fungi by volatile antibiosis. Mycelial plugs (8 mm in diameter) of USPP-T1 and USPP-T2 were placed on 90mm Petri dishes containing Potato Dextrose Agar (PDA; Biolab, Wadeville, South Africa). These plates were incubated in the dark at 22-25°C, by covering the dishes with aluminium foil for 2 days. *Phaeoconiella chlamydospora* and *Phaeoacremonium aleophilum* have slow growing colonies and were incubated for 5 days prior to incubation with the *Trichoderma* isolates. Mycelial plugs (8 mm in diam) of *Eutypa lata*, *Phomopsis viticola*, *Lasidiopodia theobromae*, *Diplodia seriata*, *N. australis* and *N. parvum* were incubated simultaneously with those of USPP-T1 and USPP-T2. The plates containing the pathogens were inverted over the plates containing USPP-T1 and USPP-T2, sealed with Parafilm® (Pechiney plastic packaging, Menasha, WI 54925) and incubated for 7 days in the dark at 22-25°C. The *Pa. chlamydospora* and *Pm. aleophilum* plates were incubated for 14 days. The control treatments consisted of plates incubated with the pathogens inverted over plates containing only PDA. After the allocated incubation period, the diameters of the pathogen colonies were measured twice perpendicularly and the average calculated. The percentage inhibition was calculated relative to the untreated control colony diameters. Each treatment consisted of five replicates and one control.

#### ***Non-volatile inhibition***

The isolates, USPP-T1 and USPP-T2, were incubated separately in Potato Dextrose Broth (PDB; Biolab, Wadeville, South Africa) for 14 days in the dark in a rotary shaker at 25°C. After 14 days of incubation, the broth culture was filtered through Mira cloth and supplemented with PDB (70% filtrate: 30% PDB) and autoclaved. Thirty millilitres of the filtrate was aseptically dispensed into 65 mm Petri dishes. Mycelium plugs of the pathogens were placed separately in the Petri dishes with filtrate. The Botryosphaeriaceae cultures were incubated for 7 days and the other cultures for 2 weeks. Control treatments were uninoculated PDB. After incubation mycelial growth was filtered, dried for 3 days at 35°C

and weighed. The percentage inhibition was determined by comparing the weights of the pathogens incubated on the treated broth to the untreated control weights of the pathogens mycelium. Each of the treatments was replicated five times with two control treatments.

## RESULTS

### DNA isolation and sequencing

The percentage similarity to the ITS sequences (517 nucleotides) of *T. atroviride* was 100% with the following sequences; AF055212 (GT95-10), AT230666 (GT509134) and AY380906 (CBS 142.95). Further comparisons with the EF sequences (583 nucleotides) revealed 100% similarity to the *T. atroviride* sequence AF348114 (GT95-10), however, a 94% similarity was obtained with the sequences identified by Samuels (2002) as *T. atroviride* [AF348112 (GT 590-134) and AF 376051 (CBS 142.95)].

### *In vitro* antibiosis

#### *Volatile inhibition*

Statistically, there was no *Trichoderma* isolate  $\times$  pathogen interaction ( $P = 0.9964$ ; Table 1), nor was there any difference between the *Trichoderma* isolates USPP-T1 and USPP-T2 ( $P = 0.7664$ ). There was, however, a statistical difference in the degree of inhibition of the different pathogens ( $P < 0.0001$ ; Table 1). The closed system rendered a coconut odour when opened. *P. viticola* had the highest degree of inhibition (means of 72.38%; Table 2) compared to the control treatments, but not statistically more than *N. parvum* (66.87%), *E. lata* (62.58%) and *N. australis* (54.10%). *D. seriata* (45.17%), *Pa. chlamydospora* (41.59%) and *Pm. aleophilum* (32.86%) were inhibited significantly less compared with *P. viticola*. *L. theobromae* was least affected by the volatile production of the USPP-T1 and USPP-T2 being inhibited by 23.61% only, significantly less than all other pathogens besides *Pm. aleophilum*.

#### *Non-volatile inhibition*

The biological control agents showed statistical differences ( $P = 0.0038$ ; Table 3) with USPP-T1 reducing the mycelium growth by an average of 16.00% compared to 11.23% reduction by USPP-T2 (Table 4). There were also statistical differences among pathogens with regard to non-volatile production ( $P = 0.0004$ ; Table 2), with *N. parvum* being least

inhibited by only 7.46% (Table 5), although not statistically less than *N. australis* (8.24%), *Pa. chlamydospora* (10.75%) and *Pm. aleophilum* (12.27%). The mycelium growth of *L. theobromae* was reduced by 20.63%, being the most sensitive, statistically more than all the pathogens except *E. lata* (19.02%), *P. viticola* (15.33%) and *D. seriata* (15.22%). A coconut odour was detected from the broth before being autoclaved.

## DISCUSSION

The identification of the 89 known *Trichoderma* species relies on DNA sequence data due to the morphological overlap amongst different species (Samuels, 2006). Sequence data are available for comparison on GenBank for 83 taxa of *Trichoderma* (Samuels, 2006). ITS can be used in several genera to correctly identify fungal species (Kindermann *et al.*, 1998; Hermosa *et al.*, 2004; Siddiquee *et al.*, 2007). High sequence similarity of the ITS and EF sequences of USPP-T1 and USPP-T2 with sequences published by Samuels (2002), allowed these isolates to be identified as *T. atroviride*. The variation found among the EF sequences indicates some variability in this gene region of *T. atroviride*.

It is well known that arrays of substances are produced by *Trichoderma* species in the event of mycoparasitism (Benhamou and Chet, 1993) and antibiosis (Howell, 2003) of fungal pathogens. These substances include antibiotics, enzymes (Calistru *et al.*, 1997) and several other uncharacterised metabolites. Confusion still exists over the substances produced and their mode of action. The two *Trichoderma* isolates USPP-T1 and USPP-T2, which were identified as *T. atroviride*, reduced mycelium growth of all the trunk disease pathogens tested by means of the production of volatile metabolites. Both isolates rendered a coconut odour during non-volatile and volatile evaluation, this component is known as pyrone-penthy-2H-2-one (Reino *et al.*, 2006) and is characteristic of the *viride*-clade of *Trichoderma* and is commonly produced by *T. atroviride* (Samuels, 2006). Whether this particular metabolite is responsible for the volatile inhibition is still unknown. The average inhibition of trunk disease pathogens by volatiles was between 24% for *L. theobromae* and 72% for *P. viticola*. Volatile metabolites produced by *T. atroviride* have been identified by Kezzler *et al.* (2000) as derivatives of pyrone dioxolane and esters with the main component of these derivatives being 5,5'-dimethyl-2H-pyran-2-on. These volatile metabolites have been shown to inhibit the mycelium growth of several wood decay fungi (Bruce *et al.*, 2000; Humphris *et al.*, 2001),

including *E. lata* (John *et al.*, 2004). The current study confirmed that the volatile metabolites here had a fungistatic rather than a fungicidal effect.

Although a distinct coconut odour was detected in the non-volatile assay, indicating the presence of fungistatic compounds, the inhibition of mycelium growth was less than expected, as previous studies by John *et al.* (2004) showed 100% inhibition in some instances. Initial studies with this method resulted in contamination problems with filtration, which necessitated the broth being autoclaved, potentially reducing the fungistatic effect of the metabolites produced by the *Trichoderma* into the broth. However, there was a statistical difference between the ability of USPP-T1 and USPP-T2 to inhibit the growth of the pathogens with USPP-T1 being more effective. John *et al.* (2004) used the method of placing *Trichoderma* PDA plugs on sterile cellophane in a Petri dish with PDA for 2 days, where after it was replaced with *E. lata* PDA plugs, and found that non-volatile metabolites had a fungicidal as well as a fungistatic effect on the mycelium growth of several *E. lata* isolates. Further studies are required to optimise a non-destructive, sterile method to measure the effect of non-volatile metabolites on pathogenic fungal mycelium.

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**Table 1.** Analysis of variance of the percentage inhibition of mycelial growth of trunk disease pathogens due to the production of volatile metabolites by *Trichoderma atroviride* isolates USPP-T1 and USPP-T2 (Bio).

Source	DF	SS	MS	SL
Model	31	55293.115	178.649	<0.0001
Block	1	6269.640	6269.640	0.0032
Bio	1	46.818	46.818	0.7664
Pathogen	7	40896.800	5842.400	<0.0001
Bio × Pathogen	7	408.895	58.414	0.9962
Block(Bio × Pathogen)	7	7670.987	511.399	<0.0001
Error	128	11816.675	92.3178	
Corrected Total	159	67109.790		

**Table 2.** Mean percentage of mycelial growth inhibition of selected trunk disease pathogens by volatiles produced by *Trichoderma atroviride* isolates USPP-T1 and USPP-T2 after an incubation period of between 7 and 14 days.

Pathogen	Mean percentage inhibition <sup>x</sup>
<i>P. viticola</i>	72.383a
<i>N. parvum</i>	66.871ab
<i>E. lata</i>	62.580ab
<i>N. australis</i>	54.107bc
<i>D. seriata</i>	45.175cd
<i>Pa. chlamydospora</i>	41.595cd
<i>Pm. aleophilum</i>	32.863de
<i>L. theobromae</i>	23.607e

<sup>x</sup>Means followed by the same letter do not differ significantly ( $P < 0.05$ ; LSD = 15.242).

**Table 3.** Analysis of variance of the percentage inhibition of mycelial growth of trunk disease pathogens due to the production of non-volatile metabolites by *Trichoderma atroviride* isolates USPP-T1 and USPP-T2.

Source of variation	DF	SS	MS	SL
Model	15	2347.047	156.470	0.0085
Bio	1	456.623	456.623	0.0038
Path	7	1607.088	229.584	0.0004
Bio × Path	7	283.336	40.477	0.5908
Error	64	3241.107	50.642	
Corrected Total	79	5588.154		

**Table 4.** Differences in mean percentage inhibition between the two isolates of *Trichoderma atroviride*, in their ability to inhibit mycelium growth of the trunk disease pathogens by non-volatile.

Biological agent	Mean inhibition <sup>x</sup>
USPP-T1	16.003a
USPP-T2	11.225b

<sup>x</sup>Means followed by the same letter do not significantly differ (Fisher (LSD) / Analysis of the differences between the categories with a confidence interval of 95%).

**Table 5.** Mean mycelial growth inhibition of selected trunk disease pathogens due to the ability of *Trichoderma atroviride* to produce non-volatile metabolites in PDB after 14 days of incubation in a rotary shaker.

Pathogen	Mean percentage inhibition <sup>x</sup>
<i>L. theobromae</i>	20.625a
<i>E. lata</i>	19.022a
<i>P. viticola</i>	15.329ab
<i>D. seriata</i>	15.222ab
<i>Pm. aleophilum</i>	12.273bc
<i>Pa. chlamydospora</i>	10.749bc
<i>N. australis</i>	8.235c
<i>N. parvum</i>	7.461c

<sup>x</sup>Means followed by the same letter do not significantly differ (Fisher (LSD) / Analysis of the differences between the categories with a confidence interval of 95%).

#### 4. PRELIMINARY STUDIES ON PENETRATION AND COLONISATION OF PRUNING WOUNDS BY *TRICHODERMA*

##### ABSTRACT

Several *Trichoderma* spp. have been identified as potential biological pruning wound protectants against grapevine trunk disease pathogens. However, the timing of pruning wound treatment and subsequent penetration and colonisation of the wound site remained to be determined. One-year-old canes of the Shiraz and Chenin blanc cultivars were grown in a hydroponic system. At bud break, they were pruned and spray treated with a spore suspension of *Trichoderma atroviride* (USPP-T1) as well as a fluorescent pigment. At intervals 1, 3, 5 and 7 days after treatment, the distal nodes were removed and dissected longitudinally. From one half, isolations were made at various distances from the pruning surface, while the other half was observed under ultra violet light to determine the depth of fluorescent pigment penetration. Shortly after spray-inoculation of a fresh pruning wound (1 to 3 days), *Trichoderma* was isolated from the wound surface to a depth of 5 mm, and at a ratio of 3:2:1 (0, 2 and 5 mm depths). One week after inoculation, *T. atroviride* was also isolated up to a depth of 10 mm depths, and after 2 weeks *T. atroviride* was isolated up to a depth of 15 mm. Fluorescent pigment particles were observed to a mean depth of 6.0 mm, which suggests that initial isolation of *T. atroviride* at these depths was resultant of the physical deposition of conidia deeper into the pruning wound tissue, whereas the isolation from greater depths within the tissue might be attributed to growth of mycelium in grapevine tissue. In a vineyard trial, fluorescent pigment was spray-applied to pruning wounds of Shiraz and Chenin blanc grapevines during July and September at intervals 0, 1, 3, 7 and 14 days after pruning. One week after treatment, the distal nodes were removed and dissected longitudinally. Each half was observed under UV light and the pigment penetration measured. For Chenin blanc and Shiraz, July pruning wounds showed significantly deeper penetration of the pigment (ranging between 3.23 and 12.70) than pruning wounds treated in September (ranging between 2.33 and 9.62). Moreover, pruning wounds made in September were observed with pigment particles in longitudinal sections when sprayed up to 1 day after pruning, whereas wounds made in July were observed with pigment particles in the xylem vessels up to the 3<sup>rd</sup> day after pruning. These findings conclusively showed that maximum penetration of treatments into pruning wounds occur directly after pruning.

## INTRODUCTION

Protection of pruning wounds is of utmost importance in the control of grapevine trunk disease pathogens. To date, biological agents and fungicides have been tested extensively for the protection of pruning wounds against *Eutypa lata* infection (Carter and Price, 1975; Moller and Kasimatis, 1980; Ferreira *et al.*, 1991; Munkvold and Marois, 1993a, b; John *et al.*, 2001; Halleen and Fourie, 2005). However, it is known that *Phaeoconiella chlamydospora* (Mugnai *et al.*, 1999), species in the Botryosphaeriaceae (incl. *Botryosphaeria*, *Neofusicoccum* and *Lasiodiplodia*) (van Niekerk *et al.*, 2004; Crous *et al.*, 2006), *Phomopsis* spp. (van Niekerk *et al.*, 2005) and *Phaeoacremonium* spp. (Mostert *et al.*, 2006) infect grapevine through pruning wounds (Lehoczky, 1974; Ferreira *et al.*, 1989; Adalat *et al.*, 2000). Bester *et al.* (2007) evaluated several fungicides against the grapevine pathogenic species in the Botryosphaeriaceae and found that benomyl was the most effective fungicide. This finding supported those of previous reports on the chemical control of *E. lata* (Munkvold and Marois, 1993; Halleen *et al.*, 2001; Creaser and Wicks, 2002; Sosnowski *et al.*, 2004; Halleen and Fourie, 2005), *Pa. chlamydospora* (Groenewald *et al.*, 2000; Jaspers, 2001) and *Phomopsis viticola* (Mostert *et al.*, 2000; Król, 2005).

Recent findings have shown that grapevine pruning wounds stay susceptible to infection for long periods of time (Munkvold and Marois, 1995; Larignon and Dubos, 2000; Gubler *et al.*, 2001, van Niekerk, 2008) and that inocula from the various grapevine trunk disease pathogens are available throughout the pruning period (van Niekerk, 2008). *Trichoderma*-based products have the ability to inhibit pruning wound pathogens and to protect the pruning wounds against *E. lata* (John *et al.*, 2004, 2005). Moreover, *Trichoderma*-based products have the ability to provide protection of pruning wounds against the most of the trunk disease pathogens belonging to the complex (Chapter 2) and it was clearly shown that they can inhabit and colonise the pruning wound site as well (Di Marco *et al.*, 2004; Chapter 2). Field evaluations demonstrated that *Trichoderma*-based products provided similar or superior protection of pruning wounds when the treated pruning wounds were challenged with a range of trunk disease pathogens 1 week after treatment (Chapter 2). *Trichoderma* antagonised the pathogenic species through a variety of mechanisms, which included mycoparasitism, competition for nutrients and production of volatile and non-volatile antibiotics (John *et al.*, 2004, Chapter 2; Chapter 3).

Research on the best time after pruning to apply biological control agents and the period that pruning wounds stays susceptible to the treatments is scant. Several studies concluded that pruning wound susceptibility to trunk disease pathogens is lower towards the end of the dormant season (Petzold *et al.*, 1981; Munkvold and Marois, 1995). This was attributed to the faster healing ability of pruning wounds under warmer conditions later in the season (Munkvold and Marois, 1995; Van Niekerk, 2008). Munkvold and Marois (1995) suggested that the higher amount of sap flow from pruning wounds later in the dormant season could contribute to flushing out spores. However, van Niekerk (2008) observed profuse exudation during the end of the dormant season, which had little obvious influence on pathogen infection. The question still remains whether biological agents will react in the same way as pathogens in terms of pruning wound colonisation, as well as penetration of the pruning wounds following treatment. Deeper penetration of spores into the xylem vessels in pruning wounds would be advantageous for its survival, as it is well known that biological control agents survive for very short periods due to their susceptibility to UV light, desiccation and extreme temperatures (Pertrot and Gessler, 2006).

The aim of this study was to investigate the initial penetration ability of *T. atroviride* spores into the xylem of pruning wounds using a fluorescent pigment and subsequent colonisation of *T. atroviride* in these wounds.

## MATERIALS AND METHODS

### Hydroponic evaluation

One-year-old dormant cane cuttings of the cultivars Shiraz and Chenin blanc were soaked in Sporekill® (150 mL/100 L) followed by a hot water treatment (50°C for 30min), prior to being grown in a hydroponic system at  $\pm 25^{\circ}\text{C}$  in a laboratory for approximately 1 month until budding of the canes occurred. Thereafter, the distal nodes of the original canes were removed to expose fresh xylem tissue to the treatments. Exposed xylem was sprayed immediately after pruning with 2 mL of SARDI Yellow Fluorescent pigment (400 g/L, EC; South Australian Research and Development Institute, Loxton SA 5333 Australia) at 0.2 L/100 L (Furness *et al.* 2000; Brink *et al.* 2004) and a  $10^6$  spores/ml suspension of *T. atroviride* (USPP-T1; Chapter 2). Isolations were made at 1, 3, 7 and 14 days after treatment. This fluorescent pigment did not have any effect on *Botrytis cinerea*, and its particle size ranges from 2 to 10  $\mu\text{m}$  (J.C. Brink, unpublished results), which corresponds with that of

*Trichoderma* conidia ( $3.0 - 4.5 \times 3.0 - 4.5 \mu\text{m}$  (Chaverri and Samuels, 2003). One day after treatment, the distal nodes of the treated canes were removed and the stubs were surface sterilised by immersion in 70% ethanol for 30 s, 1 min in 3.5% NaOCl and again for 30 s in 70% ethanol, before being split longitudinally under sterile conditions in a laminar flow cabinet. From the one half of the stub, *Trichoderma* colonisation incidence was determined by isolating four wood chips ( $0.5 \times 1 \text{ mm}$ ); each from four different zones: directly beneath the surface of the pruning wound and at a depth of 2, 5, 10 and 15 mm from the wound surface. The wood chips were placed onto potato dextrose agar (PDA; Biolab, Wadeville, South Africa) in a 90 mm Petri dish amended with 0.04 g/L streptomycin sulphate, and plates were incubated for 2 days at  $\pm 25^\circ\text{C}$  where after the plates were evaluated for cultures of *T. atroviride*. The other halves of the dissected cane stubs were illuminated with black light (UV-B) and the maximum depth at which fluorescent pigment particles were visible at  $10\times$  magnification was measured using a stereo microscope.

Trial layout consisted of 9 cuttings for each treatment combination, 6 treated with *T. atroviride* and all 9 with fluorescent pigment. The trial was repeated on two cultivars. The *T. atroviride* incidence for each depth was recorded as percentages of the 4 tissue pieces from each depth that was isolated from. This data was subjected to analysis of variance and Student's t-test for least significant difference at the 95% confidence level. The depths at which pigment particles were observed were subjected to analysis of variance and Student's t-test for least significant difference at the 95% confidence level ( $P < 0.05$ ) by means of SAS v8.2 statistical software (SAS Institute Inc, SAS Campus Drive, Cary, North Carolina 27513, USA).

### **Field evaluation**

In order to study the suitability of using the fluorescent pigment to determine the potential penetration ability of a *Trichoderma* spore suspension into the xylem of the pruning wounds at selected intervals after pruning and at different pruning periods during the dormant season, grapevines of cultivars Shiraz and Chenin blanc were spur-pruned to three buds in July and September 2007. Thereafter, pruning wounds were sprayed with 2 mL of fluorescent pigment, at a distance of 30 cm from the pruning wound, at several intervals after pruning: directly after pruning, 1, 3, 7 and 14 days after pruning. One week after treatment of the pruning wounds, the distal nodes of spurs were removed, and dissected longitudinally. Both halves were viewed under black light illumination (UV-B) using a stereo microscope

and a measurement of the maximum depth at which pigments were observed inside xylem vessels was recorded.

Statistical design was a complete randomised design with 10 repeats (grapevine plants) with treatments randomly allocated to individual pruning wounds on each plant. The depths at which of fluorescent pigment particles were observed were subjected to an analysis of variance and a Student's t-test for least significant difference at the 95% confidence level ( $P < 0.05$ ) by means of SAS v8.2 statistical software (SAS Institute Inc, SAS Campus Drive, Cary, North Carolina 27513, USA).

## RESULTS

### Hydroponic evaluation

Fluorescent pigment was clearly visible as yellow particles in the xylem vessels of Shiraz and Chenin blanc cultivars when inspected under a stereo-microscope (Fig 1A and 1C). Pigment particles were also observed on the pruning surface 7 days after application (Fig 1B and 1D). Although auto-fluorescence occurred to some extent in the xylem tissue of the grape wood, the pigment particles were clearly distinguishable. Pigment particles were observed at a mean depth of 6.6 mm from the pruning wound surface in Chenin blanc canes (maximum depth of 8.56 mm) and at a mean depth of 5.46 mm in Shiraz canes (max 8.36 mm). The difference between cultivars was not significant ( $P = 0.3664$ ; ANOVA table not shown).

Analysis of variance of the percentage of cane stubs from which *T. atroviride* was isolated from at several depths indicated that no significant effect was observed for cultivar ( $P > 0.05$ ). A significant interaction was observed for day after treatment  $\times$  depth interaction ( $P = 0.0245$ ; Table 1). A bivariate polynomial regression analysis ( $R^2 = 0.8831$ ) was conducted to describe interaction (Fig. 2). Surface colonisation remained high, with no significant difference between day 1 (62.50%; Table 2) and day 14 (52.08%). At days 1 and 3, no *Trichoderma* was isolated up to 5 mm and at a ratio of 3:2:1 (0, 2 and 5 mm depths) from the pruning wound surface, with day 7 yielding *Trichoderma* at a depth of 10 mm (22.92%; ratio of 5:4:4:2 for 0, 2, 5 and 10 mm depths) and day 14 at 15 mm from the pruning surface (10.42%; ratio of 5:3:3:1:1 for 0, 2, 5, 10 and 15 mm).

## Field evaluation

Analysis of variance of depth of penetration by the pigment into the xylem measured in the treated pruning wounds indicated a near significant cultivar  $\times$  time  $\times$  day after pruning interaction ( $P = 0.0619$ ; Table 3). Table 4 shows the means for the cultivar  $\times$  time  $\times$  day after pruning interactions. The Shiraz  $\times$  July  $\times$  Day 0 treatment combination had the highest penetration (12.70 mm), but not statistically more than the Chenin blanc  $\times$  July  $\times$  Day 0 treatment combination (12.39 mm). For Chenin blanc and Shiraz pruning wounds that were made in July, pigment was observed only from wounds treated on days 0, 1 and 3 days after pruning; no penetration occurred for day 7 and 14. Pruning wounds made in September, however, revealed pigment only from wounds treated on days 0 and 1 after pruning. Pruning wounds treated directly after pruning in September revealed pigment penetration at markedly shallower depths (9.62 and 4.17 mm for Chenin blanc and Shiraz, respectively) compared with July wounds. Similarly, September wounds treated 1 day after pruning (3.82 and 2.33 mm for Chenin blanc and Shiraz, respectively) demonstrated significantly less penetration than July wounds (8.07 and 8.86 mm for Chenin blanc and Shiraz, respectively), or September wounds treated directly after pruning.

## DISCUSSION

The findings from the hydroponic trial clearly demonstrate the colonisation ability of *T. atroviride* isolate USPP-T1 and confirm field observations (Chapter 2). It also supports the findings of previous reports of grapevine pruning wound colonisation of *Trichoderma* spp. (Di Marco *et al.*, 2004; John *et al.*, 2004). The present study, however, provides insight into the temporal colonisation of pruning wounds shortly after treatment. Shortly after spray-inoculation of a fresh pruning wound (1 to 3 days), *T. atroviride* was only isolated from the wound surface and shallow depths into the wound (2 to 5 mm). One week after inoculation, *T. atroviride* was isolated at 10 mm depth, and after 2 weeks at 15 mm depth. When one correlates the incidence of *T. atroviride* with penetration depth of the fluorescent pigment into the pruning wounds (mean 6 mm), it suggests that initial isolation of *Trichoderma* at these depths was resultant of the physical deposition of conidia deeper into the pruning wound tissue. As the isolation ratio at various depths did not change from 1 to 3 days after pruning until 7 days after pruning, one would logically surmise that the effective colonisation of the deeper wound tissue is subject to a lag phase of at least 3 days. Moreover, mean *Trichoderma* incidence at 5 mm declined from 31% at day 1 to 10% at day 3. This 3-fold

decline was obvious for Chenin blanc and Shiraz cuttings, and might suggest an initial dieback of conidia and/or germlings of *Trichoderma*. Schubert *et al.* (2008) suggested that *Trichoderma* conidia have low environmental viability in woody tissue due to adverse micro-climatic conditions and specific wood substrates. They therefore recommended amendment of conidial suspensions with urea and glucose. Moreover with reference to wood substrate, Shigo and Hillis (1973) found heartwood of certain plant species to have fungastatic properties towards micro-organisms. In the present study, relatively little decline in the *Trichoderma* incidence was observed at the wound surface and directly below it (2 mm depth), which suggest the inhibiting factor occurred deeper into the pruning wounds. Munkvold and Marois (1995) have suggested that exudates from the pruning wounds could flush conidia from pruning wounds. This could have contributed to the initial higher incidence at the surface compared to deeper in the wound. Nonetheless, isolations 1 and 2 weeks after inoculation clearly indicated progressive colonisation of the pruning wounds at depths up to 15 mm, while isolation percentages at shallower depths remained fairly constant.

Distinct differences in penetration depth of fluorescent pigment into pruning wounds were observed for the two different pruning times, July and September of the two cultivars Shiraz and Chenin blanc. Pruning time influenced the depth of penetration directly after pruning, and also 1 and 3 days after pruning. For Chenin blanc and Shiraz, July pruning wounds showed significant deeper penetration of the pigment than pruning wounds treated in September. Moreover, pigment particles were observed in longitudinal sections when sprayed up to 1 day after pruning in September, whereas pigment particles were observed in xylem vessels up until the 3 days after pruning in July. In accordance with these observations with the fluorescent pigment, Harvey and John (2006) reported that not only were the highest percentages of *Trichoderma* isolated, but the deepest penetration occurred from pruning wounds treated between 4 and 24 hours after pruning. This suggests that the critical application time is the first 24 hours after pruning for optimum penetration.

An explanation for the difference in penetration in pruning time could be differences in the rate of wound healing. Van Niekerk (2008) found that there was variation in the susceptibility of pruning wounds to pathogen infection over two seasons and identified several factors possibly contributing to the wound healing process; such as the production of tyloses and the effects of temperature. Sun *et al.* (2006) found that tyloses are formed in the exposed xylem of pruning wounds from the day of pruning up until 7 days after, where 50% to 85% of the vessels have tyloses. Moreover, a rapid increase in the formation of tyloses

was observed from day 2 onwards, supporting the findings of decreasing penetration. While temperature has an indefinite effect on the rate of wound healing as suggested by Biggs *et al.* (1990), the author found that a higher average temperature contributed to faster wound healing of peach bark. It is probable that grapevine pruning wounds could ultimately respond in the same way, and the findings by Van Niekerk (2008) support this deduction. However, as previously mentioned, profuse exudation of the xylem could also have contributed to flushing of conidia from the pruning wounds. Therefore, considering the findings of Campbell (1993) that higher ambient and soil temperature correlate with higher exudation, the higher temperature of September could have caused the pigment to be flushed from the pruning wounds.

In summary, grapevine pruning wounds should be treated in the first 24 h after pruning, in which case the time of pruning had no effect on the penetration ability. Further studies are required to determine the colonisation rate of *Trichoderma* spp. in grapevine wood as well as the depth the *Trichoderma* spores could penetrate at different intervals after pruning, and whether this is directly correlated with the observations of the fluorescent pigment. This methodology might also prove useful to determine various means of application.

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**Table 1.** Analysis of variance of the percentage *Trichoderma* isolated at 0, 2, 5, 10 and 15 mm depths from pruning wounds of hydroponically grown Shiraz and Chenin blanc shoots.

Source of variation	DF	SS	MS	P
Model	79	142455.729	1803.237	<0.0001
Cultivar	1	752.604	752.604	0.2123
Day after treatment (DAT)	3	2028.646	676.215	0.2422
Cultivar × DAT	3	1861.979	620.659	0.2787
Rep (Cultivar × DAT)	40	36854.167	921.354	0.0025
Depth	4	84625.000	21156.250	<0.0001
Cultivar × Depth	4	1812.500	453.125	0.4401
DAT × Depth	12	11708.333	975.694	0.0245
Cultivar × DAT × Depth	12	2812.500	234.375	0.9194
Error	160	76791.667	479.948	
Corrected total	239			

**Table 2.** Mean percentage of *Trichoderma* isolated at 0, 2, 5, 10 and 15mm depths from pruning wounds hydroponically grown Shiraz and Chenin blanc shoots.

Pruning depth (mm)	Days after pruning			
	1 <sup>x</sup>	3	7	14
0	62.50a	56.25ab	47.92a-d	52.08a-c
2	41.66b-e	39.58b-f	35.42c-g	27.08e-i
5	31.25d-h	10.42i-l	39.58b-f	25.00e-j
10	0.00l	0.00l	22.92f-k	12.50i-l
15	0.00l	0.00l	0.00l	10.42i-l

<sup>x</sup>Means followed by the same letter do not significantly differ ( $P < 0.05$ ; LSD = 17.663).

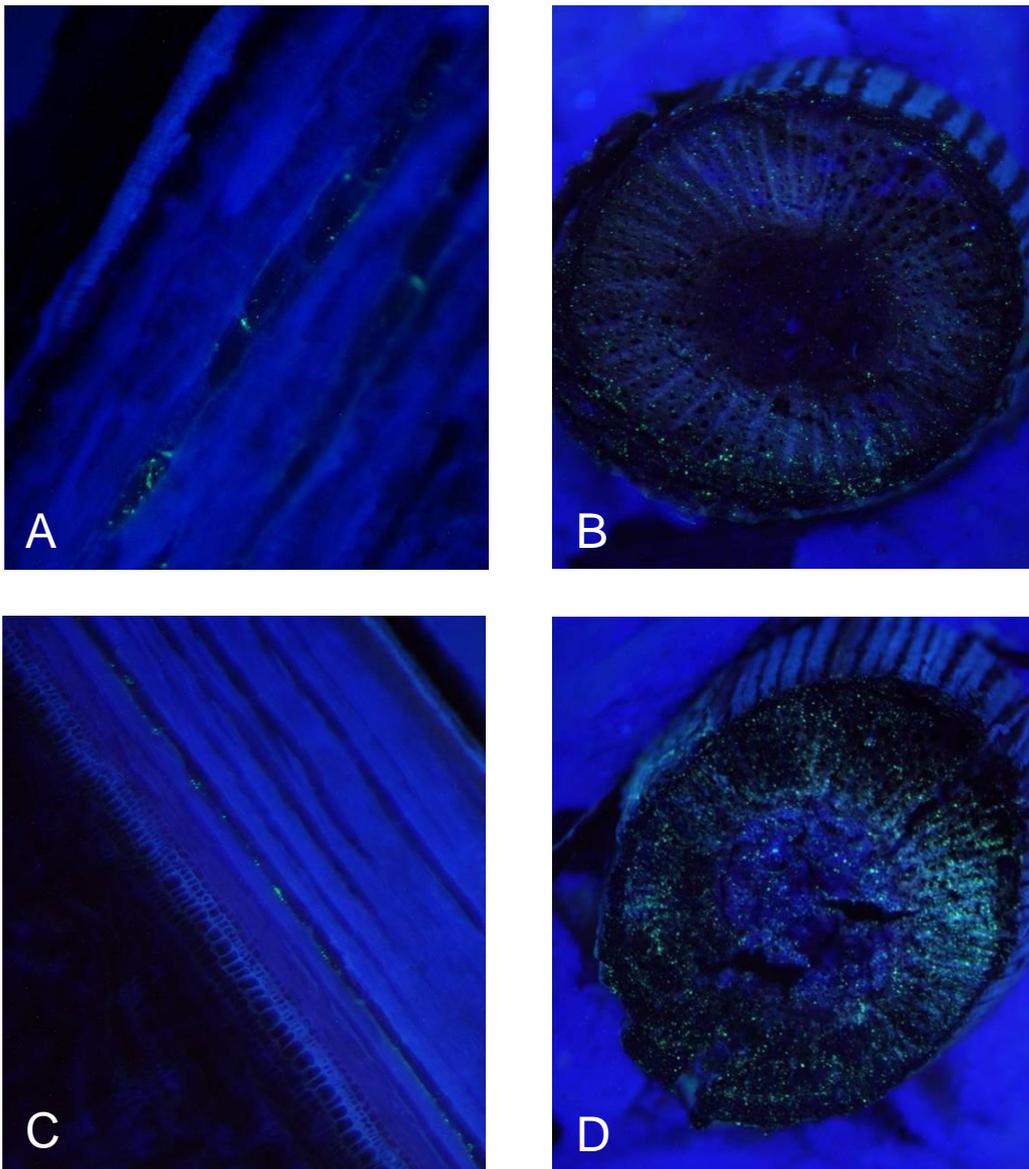
**Table 3.** Analysis of variance of the pigment penetration depth measured in the xylem tissue of Shiraz and Chenin blanc pruning wounds made during the July and September pruning times of 2007 and applied 1, 3, 7 and 14 days after pruning.

Source of variation	DF	SS	MS	P
Model	19	3816.101	200.847	<0.0001
Cultivar (C)	1	37.411	37.411	0.0687
Time (T)	1	491.411	491.411	<0.0001
Day after pruning (D)	4	2788.828	697.207	<0.0001
C × T	1	13.676	13.676	0.2696
C × D	4	49.603	12.401	0.3525
T × D	4	333.167	83.292	<0.0001
C × T × D	4	102.004	24.501	0.0619
Error	180	2007.693	11.154	
Corrected total	199	5823.793		

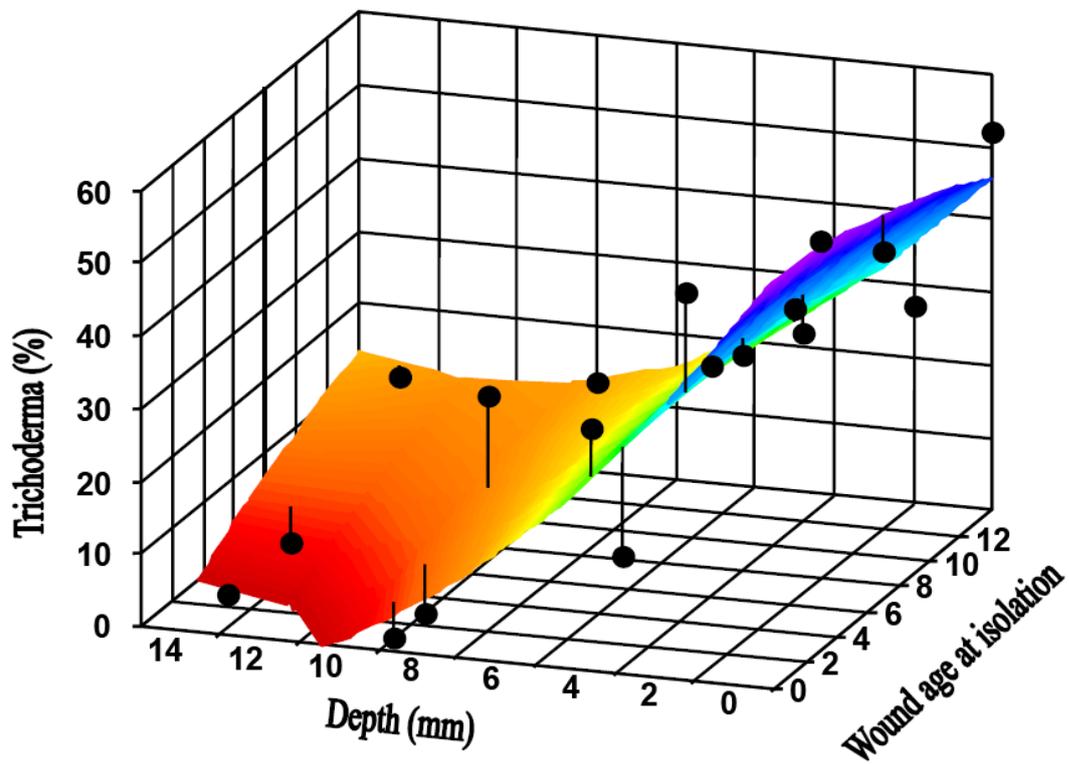
**Table 4.** Mean penetration depth of pigment into pruning wounds treated with a fluorescent pigment 0, 1, 3, 5 and 7 days after pruning Shiraz and Chenin blanc vineyards during July and September 2007.

Pruning time	Days after pruning				
	0 <sup>x</sup>	1	3	7	14
<b>Chenin blanc</b>					
July	12.39ab	8.07cd	6.04de	0.00g	0.00g
September	9.62bc	3.82ef	0.00g	0.00g	0.00g
<b>Shiraz</b>					
July	12.70a	8.86cd	3.23ef	0.00g	0.00g
September	4.17ef	2.33fg	0.00g	0.00g	0.00g

<sup>x</sup>Means followed by the same letter do not significantly differ ( $P < 0.05$ ; LSD = 2.947).



**Figure 1.** Microscopic images at 10 $\times$  magnification of the deposition of fluorescent pigment particles in xylem vessels (longitudinal cane sections; images A and C) and on the pruning wound surface (Images B and D).



**Figure 2.** Bivariate polynomial regression curve ( $z=a+bx+cy+dx^2+ey^2+fx$ ;  $a= 56.057$ ,  $b= -0.1740$ ,  $c= -7.4580$ ,  $d=-0.0405$ ,  $e = 0.2204$ ,  $f = 0.1367$ ;  $R^2 = 0.8831$ ) demonstrating the mean percentage Trichoderma isolated at various depths from pruning wounds on Shiraz and Chenin blanc cuttings 0 to 14 days after pruning.