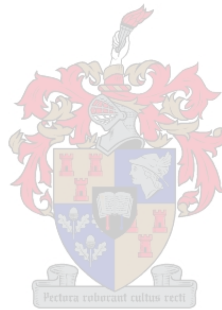
THE ROLE OF SUCKER WOUNDS AS PORTALS FOR GRAPEVINE TRUNK PATHOGEN INFECTIONS

GUGULETHU JOY MAKATINI



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Supervisor: Dr. L. Mostert

Co-supervisor: Dr. F. Halleen

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DECLARATION

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SUMMARY

Grapevine trunk diseases are responsible for reduced wine and table grape production world-wide. Trunk disease infections are caused by xylem-inhabiting pathogens which include species of Botryosphaeriaceae, Diatrypaceae, Hymenochaetales and Diaporthales, as well as *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. Winter pruning wounds are regarded as the main infection-sites for trunk disease pathogens. However, the role of sucker wounds as portals of trunk disease infections has been minimally investigated. Knowledge of the potential role of grapevine trunk pathogen infections that occur through sucker wounds is important for better wound protection strategies. The aim of this study was to determine the role of grapevine sucker wounds as portals of entry for trunk disease pathogens and to assess the use of *Trichoderma* spp. for sucker wound protection.

The susceptibility of sucker wounds to different trunk disease pathogens was assessed from natural as well as artificial infections. In addition the duration of sucker wound susceptibility in the field was also ascertained. Sucker wounds were sampled from three wine and two table grape vineyards during 2011 and 2012 in the Western Cape province of South Africa. Thereafter, fungal isolations were made from 161 sucker wounds and the cultures were identified based on cultural and morphological characteristics as well as the internal transcribed spacer regions and 5.8S ribosomal RNA gene. Sixty-two percent of the wounds were naturally infected by at least one of the trunk pathogens. *Phomopsis* (*Po.*) *viticola* (46%; 18%), *Diplodia* (*D.*) *seriata* (30%; 9%) and *Phaeomoniella* (*Ph.*) *chlamydospora* (27%; 5%) were the most predominant trunk disease pathogens isolated from sucker wounds of field wine and table grape cultivars, respectively. Lower incidences of *Phaeoacremonium aleophilum* (18%), *Eutypella* sp. (3%), *Cryptovalsa ampelina* (2%), *Diplodia* sp. (1%) and *Neofusicoccum australe* (1%) were obtained, however, only from wine grapes. Sucker wounds on 1-year-old potted grapevine plants of Chardonnay cultivar were inoculated with spore suspensions of *Eutypa lata*, *N. parvum, Pa. aleophilum*, *Ph. chlamydospora* and *Po. viticola* in the glasshouse. After 4 months all the inoculated pathogens could be re-isolated at the following incidences: *N. parvum* (85%), *Ph. chlamydospora* (75%), *Po. viticola* (65%), *Pa. aleophilum* (55%) and *E. lata* (45%). Sucker wound susceptibility was further ascertained under field conditions on 12-year-old Cabernet Sauvignon vines by artificial inoculation of

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the same pathogen species. After 5 months three pathogens could be re-isolated at the following incidences: *Po. viticola* (65%), *N. parvum* (32.5%) and *Ph. chlamydospora* (7.5%). The duration of susceptibility of field sucker wounds to *Ph. chlamydospora* was assessed for a period of 4 weeks. The wounds remained susceptible for 4 weeks with a decline in susceptibility after one week. This study showed that sucker wounds are susceptible to the major trunk disease pathogens and thus could play an important role in grapevine trunk disease epidemiology.

In the second part of this thesis a possible management strategy to prevent infections of sucker wounds was investigated. The use of *Trichoderma* (*T.*) *harzianum* against two trunk pathogens on sucker wounds was tested in the field. Additionally the sensitivity of *T. harzianum* and *T. atroviride* was tested *in vitro* against 16 fungicides that are used to control powdery mildew, downy mildew, Botrytis rot and Phomopsis cane and leaf spot. In October 2012, sucker wounds were made on 1-year-old wood of Cabernet Sauvignon and spray-treated with Eco-77® immediately after desuckering, and then inoculated with spore suspensions of either *Ph. chlamydospora* or *Po. viticola* after 24 hours. After 5 months, isolations were made from the sucker wounds to evaluate the efficacy of the *Trichoderma* treatment. *Trichoderma harzianum* reduced the incidence of *Ph. chlamydospora* by 66.65%. Although the incidence of *Po. viticola* was reduced by 15.37%, it was not significantly different from the control treatment. The inhibition of mycelial growth and conidial germination of *T. harzianum* and *T. atroviride* were screened against 16 fungicides. The fungicides were applied at 0, 0.25, 0.5, 1 and 2 times the recommended dosages. Systemic fungicides boscalid, metrafenone and trifloxystrobin, as well as contact fungicides quinoxyfen and meptyldinocap were least toxic to *Trichoderma* spp. isolates. For the conidial germination assay, boscalid, trifloxystrobin, penconazole and metrafenone (systemic) plus quinoxyfen and folpet (contact) were compatible with *Trichoderma* spp. These fungicides were regarded as being compatible with *Trichoderma* spp. isolates because they gave mean percentage inhibitions of less than 50% at all the tested dosages. Spiroxamine and pyrimethanil gave the highest mean percentage inhibitions for both mycelial inhibition and conidial germination. The findings of this study showed that *T. harzianum* can protect sucker wounds against *Ph. chlamydospora* in the field. Furthermore, some fungicides applied for the control of powdery mildew and Phomopsis cane and leaf spot can be alternatively or

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simultaneously applied with *T. harzianum* and *T. atroviride*, however, this will have to be verified with field trials.

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OPSOMMING

Wingerd stamsiektes is wêreldwyd verantwoordelik vir verminderde wyn- en tafeldruif produksie. Stamsiektes word veroorsaak deur patogene wat in die xileem voorkom, insluitend verskeie spesies in die Botryosphaeriaceae, Diatrypaceae, Hymenochaetales en Diaporthales, asook *Phaeomoniella chlamydospora* en *Phaeoacremonium* spp. Winter snoeiwonde word beskou as die hoof bron van infeksies vir stamsiekte patogene. Die rol van suierwonde as poorte van infeksie vir stamsiektes is nog nie goed bestudeer nie. Kennis van die potensiële rol van wingerd stamsiekte patogeen infeksies wat deur suierwonde plaasvind is belangrik vir die formulasie van beter wondbeskerming strategieë. Die mikpunt van hierdie studie was om die rol van suierwonde as ingangsportale vir wingerd stamsiekte patogene te bepaal en om die gebruik van *Trichoderma* spp. vir suierwond beskerming te evalueer.

Die vatbaarheid van suierwonde vir verskillende stamsiekte patogene is geëvalueer vanuit natuurlike, sowel as kunsmatige infeksies. Die duur van suierwond vatbaarheid in die veld is ook bepaal. Suierwonde is versamel vanuit drie wyn- en twee tafeldruif wingerde gedurende 2011 en 2012 in die Wes Kaap provinsie van Suid Afrika. Hierna is swam isolasies gemaak vanuit 161 suierwonde en die kulture is geïdentifiseer volgens kultuur en morfologiese kenmerke, sowel as die interne transkribeerde spasieerders en 5.8S ribosomale RNA geen. Twee-en-sestig persent van die wonde was geïnfekteer deur ten minste een van die stamsiekte patogene. *Phomopsis* (*Po.*) *viticola* (46%; 18%), *Diplodia* (*D.*) *seriata* (30%; 9%) en *Phaeomoniella* (*Ph.*) *chlamydospora* (27%; 5%) was die mees algemene stamsiekte patogene wat, respektiewelik, vanuit die wyn- en tafeldruif kultivars verky is. Laer hoeveelhede *Phaeoacremonium aleophilum* (18%), *Eutypella* sp. (3%), *Cryptovalsa ampelina* (2%), *Diplodia* sp. (1%) en *Neofusicoccum australe* (1%) is verkry, en slegs vanaf wyndruiwe. Suierwonde op 1-jaar oue Chardonnay wingerdplante in potte is in die glashuis geïnokuleer met spoorsuspensies van *Eutypa lata*, *N. parvum, Pa. aleophilum*, *Ph. chlamydospora* en *Po. viticola*. Na 4 maande kon al die geïnokuleerde patogene her-isoleer word teen die volgende hoeveelhede: *N. parvum* (85%), *Ph. chlamydospora* (75%), *Po. viticola* (65%), *Pa. aleophilum* (55%) en *E. lata* (45%). Suierwond vatbaarheid is verder geëvalueer onder veld kondisies op 12-jaar oue Cabernet Sauvignon plante deur kunsmatige inokulasie van die selfde patogeen spesies. Na 5 maande kon drie patogene her-isoleer word teen die volgende

hoeveelhede: *Po. viticola* (65%), *N. parvum* (32.5%) en *Ph. chlamydospora* (7.5%). Die duur van vatbaarheid van suierwonde teen *Ph. chlamydospora* in die veld is geevalueer oor ‘n periode van 4 weke. Die wonde het vatbaar gebly vir 4 weke met ‘n afname in vatbaarheid na ‘n week. Hierdie studie demonstreer dat suierwonde vatbaar is vir die hoof wingerd stamsiektes en dus ‘n belangrike rol in die epidemiologie van wingerd stamsiektes kan speel.

In die tweede deel van hierdie tesis is ‘n moontlike bestuurs-strategie ondersoek om infeksie van suierwonde te verhoed. Die gebruik van *Trichoderma* (*T.*) *harzianum* teen twee stampatogene op suierwonde is getoets in die veld. Verder is die *in vitro* sensitiwiteit van *T. harzianum* en *T. atroviride* getoets teen 16 fungisiedes wat gebruik word in die beheer van poeieragtige meeldou, donsskimmel, Botrytis vrot en Phomopsis streepvlek. Gedurende Oktober 2012 is suierwonde gemaak op 1-jaar oue hout van Cabernet Sauvignon en onmiddelik behandel met Eco-77® na suiering. Wonde is dan geïnokuleer met spoorsuspensies van óf *Ph. chlamydospora* óf *Po. viticola* na 24 uur. Na 5 maande is isolasies gemaak vanaf suierwonde om die doeltreffendheid van van die *Trichoderma* behandeling te evalueer. *Trichoderma harzianum* het die voorkoms van *Ph. chlamydospora* met 66.65% verminder. Alhoewel die voorkoms van *Po. viticola* verminder is met 15.37%, was dit nie ‘n beduidende verskil in vergelyking met die kontrole behandeling nie. Die inhibisie van miselium groei en konidia ontkieming van *T. harzianum* en *T. atroviride* is getoets teen 16 fungisiedes. Die fungisiedes is aangewend teen 0, 0.25, 0.5, 1 en 2 keer die aanbevole dosisse. Sistemiese fungisiedes boscalid, metrafenone en trifloxystrobin, sowel as kontak fungisiedes quinoxyfen en meptyldinocap was die minste toksies teen *Trichoderma* spp. Gedurende die konidia ontkiemingstoets was boscalid, trifloxystrobin, penconazole en metrafenone (sistemies) en quinoxyfen en folpet (kontak) versoenbaar met *Trichoderma* spp. Die fungisiedes is beskou as bruikbaar met *Trichoderma* spp. isolate omdat hulle gemiddelde persentasie inhibisies van minder as 50% teen al die getoetste dosisse gelewer het. Spiroxamine en pyrimethanil het die hoogste gemiddelde persentasie inhibisie gelewer vir beide die miselium inhibisie en konidia ontkieming. Die bevindings van hierdie studie het gewys dat *T. harzianum* suierwonde kan beskerm teen *Ph. chlamydospora* in die veld. Verder sou sommige fungisiedes wat aangewend word vir die bestuur van poeieragtige meeldou en streepvlek moontlik alternatiewelik of gelyktydig met *T. harzianum* en *T. atroviride* aangewend word, alhowel dit met veldproewe bevestig moet word.

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CHAPTER 1

AN OVERVIEW OF GRAPEVINE TRUNK DISEASES WITH SPECIFIC REFERENCE TO PRUNING WOUNDS AS PORTALS OF INFECTION

1.1 Introduction

Grapevine (*Vitis vinifera*) trunk diseases are a major threat to wine and table grape production world-wide and their occurrence has increased significantly over the last two decades (Mugnai *et al*., 1999; Graniti *et al*., 2000; Edwards *et al*., 2001a; Gubler *et al*., 2004; Alaniz *et al*., 2007; Bruno *et al*., 2007; Martin & Cobos, 2007). Trunk disease infections are caused by fungal pathogens that infect and inhabit xylem vessels of grapevines primarily through pruning wounds (Christen *et al*., 2007; Edwards *et al*., 2007; Gramaje & Armengol, 2011). The major trunk diseases include Botryosphaeria dieback (caused by species of Botryosphaeriaceae) (Castillo-Pando *et al*., 2001; Larignon *et al*., 2001; Van Niekerk *et al*., 2006; Urbez-Torres *et al*., 2006; Urbez-Torres, 2009), Eutypa dieback (caused by species of Diatrypaceae) (Moller & Kasimatis, 1978; Munkvold *et al*., 1994; Rolshausen *et al*., 2004; Pitt *et al*., 2010; Trouillas *et al*., 2011; Luque *et al*., 2012; Wayne *et al*., 2013), Petri disease [caused by *Ph. chlamydospora* (Crous & Gams, 2000; Cobos & Martin, 2008) and *Phaeoacremonium.* spp. (Dupont *et al*., 1998; Aroca *et al*., 2008; Essakhi *et al*., 2008)], esca (caused by wood rotting species of the Hymenochaetales together with Petri disease fungi) (Larignon & Dubos, 1997; Mugnai *et al*., 1999; Cortesi *et al*., 2000; Graniti *et al*., 2000; Fischer, 2002, 2006; Surico *et al*., 2008; White *et al*., 2011a, b) and Phomopsis dieback (caused by Diaporthales) (Mostert *et al*., 2001; Melanson *et al*., 2002; Rawnsley *et al*., 2004; Van Niekerk *et al*., 2005; Gramaje & Armengol, 2011; Kaliterna *et al*., 2012). Grapevine trunk disease pathogens may act in synergy or succession to produce an array of symptoms in grapevines (Graniti *et al*., 2000; Cobos & Martin, 2008; Valtaud *et al*., 2009; Spagnolo *et al*., 2011; White *et al*., 2011a).

1.2 General symptoms

Grapevine trunk diseases are more often observed on mature vines; however, Petri disease is also common in young grapevines (Scheck *et al*., 1998; Fourie & Halleen, 2004; Gramaje *et*

al., 2008). Trunk disease pathogens require time to colonise the vascular tissue, thus the expression of aerial symptoms is prolonged and only becomes apparent several years after initial infection (Carter, 1991; Gubler *et al*., 2005; Christen *et al*., 2007). Typical symptoms (Figure 1) that can be seen on diseased grapevines include dieback of spurs and arms (Van Niekerk *et al*., 2006; Epstein *et al*., 2008), black or brown discolouration or internal streaking of wood (Figure 1f) (Edwards *et al*., 2007; Van Niekerk *et al*., 2006, 2011; Kuntzmann *et al*., 2010; White *et al*., 2011a), small black-brown spots (Figure 1a, e) (Van Niekerk *et al*., 2006), wedge-shaped (Figure 1b, c) or sectorial necrosis [Figure 1g (I)] (Larignon & Dubos, 1997; Essakhi *et al*., 2008; Van Niekerk *et al*., 2011), white rot [Figure 1g (II)] (White *et al*., 2011a) and general wood decay (Larignon & Dubos, 1997). Blockage of vessels which results from the spread of pathogens in and around xylem vessels and parenchyma cells is also another symptom of diseased vines (Pascoe & Cottral, 2000; Edwards *et al*., 2007; Gramaje & Armengol, 2011). This blockage impedes water and nutrient supply, thus leading to decreased productivity in the vine. Severely infected vines are a result of vascular cankers or lack of vigour (Rooney-Latham *et al*., 2005; Amponsah *et al*., 2008), also termed 'grapevine decline syndrome' (Castillo-Pando *et al*., 2001; Larignon *et al*., 2001; Martin & Cobos, 2007). Grapevine decline can slowly lead to grapevine death (Ferreira *et al*., 1994; Edwards *et al*., 2007). The sudden wilting and dying of whole vines which occurs during hot and dry conditions in summer is called apoplexy (Figure 1i) (Mugnai *et al*., 1999; Eskalen & Gubler, 2001; Essakhi *et al*., 2008).

1.3 Economic impact

Grapevine trunk diseases are one of the main causes of shortened vineyard lifespan (Munkvold *et al*., 1994; Peros *et al*., 2008) and reduction in the quantity and quality of fruit and wine as a result of non-uniform berry maturity (Calzarano *et al*., 2001). Trunk diseases have led to severe economic losses in the grapevine industry world-wide due to increased management costs and replanting costs (Pascoe, 1999). In Australia, Eutypa dieback was calculated to have cost growers approximately $20 million annually in lost production of Shiraz (Sosnowski *et al*., 2005) whilst Eutypa dieback was responsible for an estimated loss of over $260 million in California (Siebert, 2001). In South Africa, Van Niekerk *et al*. (2003) calculated that 367 tons of Cabernet Sauvignon grapes worth approximately R1.7 million were lost to *Eutypa* dieback during the 2000 and 2001 season in the Stellenbosch region.

1.4 Trunk diseases

The predominant grapevine trunk diseases world-wide, will be reviewed, namely Botryosphaeria dieback, Eutypa dieback, Petri disease, esca as well as Phomopsis dieback. The etiology and symptomatologies of the different trunk diseases will be discussed. Furthermore, an overview of the general infection and transmission pathways of the trunk disease pathogens will be provided.

1.4.1 Botryosphaeria dieback

Botryosphaeria dieback, also known as black dead-arm (Lehoczky, 1974), is caused by various species of the Botryosphaeriaceae. It was only recently that these species were given recognition as significant pathogens in the grapevine trunk disease complex (Castillo-Pando *et al*., 2001; Larignon *et al*., 2001; Urbez-Torres & Gubler, 2009; Baskarathevan *et al*., 2011). Recent studies (Gramaje & Armengol, 2011; Spagnolo *et al*., 2011) have found 17 members of the Botryosphaeriaceae to be potential pathogens of grapevine whilst 21 species were reported by Urbez-Torres (2011). The anamorph species that occur on grapevines are currently placed in the following genera: *Diplodia*, *Dothiorella*, *Fusicoccum*, *Guignardia*, *Neofusicoccum*, *Lasiodiplodia* and *Phaeobotryosphaeria* (Amponsah *et al*., 2012b). In South Africa, Van Niekerk *et al*. (2004) reported the following species: *Diplodia seriata*, *D. mutila*, *Lasiodiplodia theobromae*, *Neofusicoccum australe*, *N. parvum* and *N. luteum*. The latter two species were found to be the most virulent. Apart from grapevines, species of the Botryosphaeriaceae have also been reported on perennial plants as well as other tree species planted next to orchards, forests and riparian areas (Farr *et al*., 1989; Slippers *et al*., 2007).

Species of the Botryosphaeriaceae have been associated with wedge-shaped necrosis (Figure 1b) (Urbez-Torres *et al*., 2006; Van Niekerk *et al*., 2006; Urbez-Torres, 2011) which occurs on trunks, cordons and spurs. Other symptoms include dieback of shoots and canes, brown streaking, delayed bud-burst and bud mortality as well as bunch-rot in some instances (Larignon *et al*., 2001; Taylor *et al*., 2005; Van Niekerk *et al*., 2006; Savocchia *et al*., 2007; Spagnolo *et al*., 2011; Urbez-Torres, 2011). Symptoms are normally seen on mature vines which are 10 years old or older (Urbez-Torres *et al*., 2008), however, cankers caused by *L. theobromae* have been observed in younger vines from 5 to 7 years old (Urbez-Torres *et al*.,

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2008). Other symptoms that have been associated with these fungi are stunted growth (Van Niekerk *et al*., 2006), bleached canes and incomplete graft unions (Amponsah *et al*., 2008).

1.4.2 Eutypa dieback

Eutypa dieback, which is also known as the 'dying arm disease' of grapevines (Moller & Kasimatis, 1978; Mahoney *et al*., 2005), is caused by *Eutypa lata* (syn. *E. armeniacae*). *Eutypa lata* (anamorph: *Libertella blepharis*) belongs to the Diatrypaceae family of which the genera *Eutypa*, *Diatrype*, *Diatrypella*, *Eutypella*, *Cryptosphaeria* and *Cryptovalsa* have been found on grapevines (Glawe & Rogers, 1982; Mostert *et al*., 2004; Pitt *et al*., 2010; Luque *et al*., 2012). To date, 14 species from the Diatrypaceae family have been reported from grapevine (Farr & Rossman, 2011) and these include *Eutypa lata*, *Eutypa leptoplaca*, *Cryptovalsa ampelina*, *Diatrype* and *Diatrypella* species (Rappaz, 1987; Mostert *et al*., 2004; Rolshausen *et al*., 2004; Trouillas *et al*., 2010). *Eutypa lata* was first diagnosed as a dieback pathogen on apricot (*Prunus armeniaca*) (Carter, 1957) and was later found to be the causal agent of dieback on grapevines. To date, this fungus has been found to infect a broad host range of more than 80 plant species world-wide in 27 families (Carter *et al*., 1983; Bolay & Carter, 1985) including economically important fruit crops such as apple (Glawe *et al*., 1983), pear (Carter, 1982), almond (Carter, 1982), pistachio (Rumbos, 1986a), sweet cherry (Rumbos, 1986b; Munkvold & Marois, 1994) and lemon (Koyeas, 1978).

One of the most characteristic symptoms of Eutypa dieback are wedge-shaped cankers (Figure 1c) that occur on the wood (Sosnowski *et al*., 2007). Symptoms usually appear in vines that are older than 6 years (Munkvold, 2001). Eutypa dieback foliar symptoms can be best observed in early spring (Moller & Kasimatis, 1974) and they include stunted shoots which occur as a result of shortened internodes (Figure 1d) (Pitt *et al*., 2010). Additionally, leaves are reduced in size, are cup-shaped with rugged margins and also exhibit necrosis and chlorosis (Petzoldt *et al*., 1981). The continual extension of wood infections eventually leads to the death of grapevines (Gubler *et al*., 2005).

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1.4.3 Petri disease

Petri disease, which was previously known as 'black goo', was first discovered in Italy in 1912 (Mugnai *et al.*, 1999). It was later discovered in South Africa, then in United States of America and then in other countries (Surico, 2001). This disease affects vines ranging from 1- to 5-years-old and has caused major losses of young vines in newly planted vineyards (Mugnai *et al.*, 1999; Pascoe & Cottral, 2000; Surico *et al.*, 2006). *Phaeomoniella chlamydospora* and several species of *Phaeoacremonium* with *Pa. aleophilum* being the most predominant, are the main causal agents of this disease (Dupont *et al.*, 2000; Mostert *et al.*, 2006; Essakhi *et al.*, 2008; Gramaje *et al.*, 2009a; Gramaje & Armengol, 2011). To date, 20 species have been isolated from grapevines (Gramaje *et al.* 2009a). *Phaeoacremonium* species have been associated with dieback of deciduous crops as well as other woody hosts (Mostert *et al.*, 2006). *Phaeomoniella chlamydospora*, on the other hand, has a very restricted host range, having only been found on grapevines and pine trees (Crous & Gams, 2000).

External symptoms of Petri disease include stunted growth, sparse and chlorotic foliage with necrotic margins, dieback and reduced vigour (Mugnai *et al.*, 1999). Internal symptoms are mainly visible in the trunk and cordons and include black spots on transversally dissected vines as well as dark brown to black streaking (Figure 1f) (Mugnai *et al.*, 1999; Bruno *et al.*, 2007). Severely infected vines exhibit discolouration of xylem vessels and often ooze black xylem sap or "black goo" (Figure 1e) as a result of gums (Mugnai *et al.*, 1999; Bruno *et al.*, 2007), tyloses and phenolic compounds that form as result host in response to fungal infection (Bruno *et al.*, 2007).

1.4.4 Esca

Esca was discovered more than 100 years ago in France, Italy and America (Ravaz, 1898; Petri, 1912; Viala, 1926; Bourdot & Galzin, 1927). In Europe it was known as apoplexy and in America as folletage (Chiarappa, 2000). This disease occurs worldwide, causing decline of both old and young vines (Bruno *et al.*, 2007). *Fomitiporia mediterranea, Ph. chlamydospora* and species of *Phaeacremonium* were seen as the main causal agents of esca (Mugnai *et al.*, 1999; Cortesi *et al.*, 2000; Fischer, 2002). Apart from these, species of Botryosphaeriaceae and *Phomopsis* have also been isolated from vines showing esca symptoms (Péros *et al.*,

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2008). Research showed that esca involved successive fungal infections by different fungi (Larignon and Dubos, 1997; Fleurat-Lessard *et al*., 2010). Although *Ph. chlamydospora*, *Pa. aleophilum* and *F. mediterranea* can occur together, their interaction is not necessary for disease development (Surico, 2009). Furthermore, these pathogens can all play a primary role, thus it is no longer valid to suggest that fungi or diseases in the complex act in synergy or succession. Surico (2009) therefore suggested that the diseases comprising of esca should be referred to individually, namely brown wood-streaking, Petri disease, grapevine leaf stripe and esca (syn. white rot).

Esca consists of an array of symptoms (Mugnai *et al*., 1999; Fischer & Kassemeyer, 2003) which can be divided into five syndromes (Graniti *et al.*, 2000). These include dark wood streaking, Petri disease, young esca (black or brown wood streaking usually occurring in vines between 8 and 10 years), white rot (which is spongy wood decay which may also result in external symptoms) and esca proper (which occurs when white rot develops in the trunk of mature vines together with or in succession to brown streaking). Foliar symptoms have been found to vary from year to year with vines showing symptoms only in some years (Mugnai *et al*., 1999; Fischer & Kassemeyer, 2003). Leaves may appear chlorotic with irregular regions between the main veins or along the leaves, referred to as 'tiger stripes' (Figure 1h) (Mugnai *et al*., 1999). More recently, Lecomte *et al*. (2012) showed that esca consists of a variety of foliar symptoms which are highly variable and are largely determined by the disease severity, age as well as cultivar type, red or white. Fruit symptoms consist of black tiny spots which are referred to as black measles (Chiarappa, 2000). The complete collapse of the grapevine, called apoplexy (Figure 1i), is regarded as the most severe symptom of esca (Bruno *et al*., 2007).

1.4.5 Phomopsis dieback

Phomopsis dieback is caused by several species of *Phomopsis*, with *Phompsis viticola* being the most prevalent (Mostert *et al*., 2001; Van Niekerk *et al*., 2005; Urbez-Torres *et al*., 2013). Van Niekerk *et al*. (2005) found 15 *Phomopsis* spp. to occur on grapevines in South Africa. *Phomopsis* species are able to infect a wide host range (Uddin *et al*., 1997; Mostert *et al*.,

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2001; Van Niekerk *et al*., 2005) such as peach and almonds (Farr *et al*., 1999), sunflowers, roses and cranberries (Van Niekerk *et al*., 2005).

Of the different trunk disease pathogens *Phomopsis* spp. has most probably been investigated the least, because these species are more commonly associated with the symptoms of Phomopsis cane and leaf spot on green shoots (Figure 1k) and leaves of grapevines. *Phomopsis* species have been isolated from internal wood symptoms (Figure 1j) and pruning wounds (Phillips, 1998; Van Niekerk *et al*., 2005). Urbez-torres *et al*. (2006, 2009) and White *et al*. (2011a) isolated *Po. viticola* from V-shaped cankers located on mature grapevine wood. Recently, *Po. viticola* and other *Phomopsis* species were isolated from canker symptoms which were similar to those caused by *E. lata* and several Botryosphaeriaceae spp. (Urbez-Torres *et al*., 2013), once again emphasising the prevalence of *Phomopsis* species in perennial cankers of declining vines.

1.5 Infection

Grapevine trunk pathogens infect vines primarily via exposed xylem vessels of pruning wounds (Munkvold & Marois, 1995; Chapuis *et al*., 1998; Chiarappa, 2000; Larignon & Dubos, 2000; Rolshausen *et al*., 2010; Urbez-Torres *et al*., 2013). During infection, fungal spores germinate on pruning wounds and colonise grapevines by growing in and around xylem vessels and parenchyma cells (Pascoe & Cottral, 2000; Edwards *et al*., 2007; Gramaje & Armengol, 2011). Each disease complex generally occurs as a result of multiple infections by different trunk pathogens on the same wound (Spagnolo *et al*., 2011). Wound infections may be enhanced by environmental conditions such as drought, frost damage, hail damage, poor nutrition, poor pruning practices and other stress factors. Trunk disease infections by *Ph. chlamydospora*, *Phaeoacremonium* species, as well as some wood decay fungi can occur very early in the life cycle of vines in mother vines (Fourie & Halleen 2002, 2004; Graniti, 2006). Apart from infecting pruning wounds, *Po. viticola* can also infect from bud-break up until shoots are 15 to 20cm in length, on leaves and shoots (Pine, 1959). New infections are important for the establishment of a disease and these are largely dependent on successful transmission of the pathogens.

1.6 Transmission of trunk pathogens

In vineyards, different fruiting structures occur on infected wood on grapevines as well as pruning debris that is often left on the vineyard floor. Species of the Botryosphaeriaceae can form both asexual and sexual fruiting structures (Gubler *et al*., 2005); however, the sexual stage is rarely found. These species overwinter as pycnidia on diseased wood or vine parts (Gubler *et al*., 2005; Van Niekerk *et al*., 2006; Urbez-Torres & Gubler, 2010). Conidia produced within pycnidia are aerially dispersed by wind and water from rain and sprinkler irrigation onto pruning surfaces (Gubler *et al*., 2005). Inoculum dispersal can be further aided by insect feeding (Moyo, 2013) and possibly contaminated pruning tools (Epstein *et al*., 2008). Species of the Diatrypaceae form perithecia on dead grapevine tissue in fungal stroma (Chapuis *et al*., 1998; Luque *et al*., 2012). Ascospores released from perithecia are then principally carried by wind and water onto infection-sites (Ramos *et al*., 1975). *Phaeomoniella chlamydospora* forms conidia via pycnidia or hyphomycete conidiophores (Crous & Gams, 2000; Edwards *et al*., 2001a). *Phaeacremonium* species can either form conidia via hyphomycete conidiophores or ascospores via perithecia (Rooney-Latham *et al*., 2005). The conidia and ascospores of *Phaeacremonium* species and *Ph. chlamydospora* are dispersed both aerially by wind and by rain splashes (Pascoe, 1999; Chiarappa, 2000; Larignon & Dubos, 2000; Fourie & Halleen, 2004; Edwards, 2006; Marchi *et al*., 2006; Retief *et al*., 2006; Serra *et al*., 2011). *Phomopsis viticola* forms pycnidia on infected wood and dormant shoots and these conidia are released in the presence of adequate rainfall during late winter and early spring onto new growth, cuttings and buds (Hewitt & Pearson, 1988). Infected propagation material is also a source of inoculum by which trunk disease pathogens are spread to newly planted vineyards (Fourie & Halleen 2002, 2004; Van Niekerk *et al*., 2006; Gramaje *et al*., 2009b).

1.7 Role of pruning wounds as portals of infection

1.7.1 Pruning wounds

Pruning is an important annual cultural practice in grapevine management. During pruning, fruiting wood is selected and excess plant material and canes are removed in order to maintain vine shape, form and to regulate the number of buds per vine (Creasy & Creasy, 2009). This practice allows for the production of an economically viable crop by ensuring a balance in fruit productivity and vegetative growth. This practice helps to optimize yields

without compromising vigour and maturity (Bordelon, 2009). Grapevines are pruned mainly during dormancy in winter which often coincides with the main rainy season. Desuckering is a form of pruning that is carried out during spring to remove suckers and excess shoots or buds (Figure 2), resulting in the formation of spring or sucker wounds. Despite the numerous benefits, pruning results in wounding and the exposure of xylem vessels and creates the possibility of trunk disease infections.

1.7.2 Susceptibility of winter pruning wounds

Grapevine pruning wound susceptibility has been under investigation for many years. Studies have shown that the duration of pruning wound susceptibility ranges between 2 to 16 weeks (Eskalen *et al*., 2007; Van Niekerk *et al*., 2011). Wounds are most susceptible immediately after pruning and susceptibility declines as the interval between pruning and infection increases (Petzoldt *et al*., 1981; Eskalen *et al*., 2007; Urbez-Torres & Gubler, 2010). The duration of susceptibility is dependent on numerous factors. One factor is physiological wound response, which causes desiccation and accumulation of phenolic compounds such as suberin, tyloses and polysaccharide gums in xylem vessels (Munkvold & Marois, 1995). Wound healing also plays a major role in a decline in susceptibility and it has been shown to occur more rapidly under warm and dry conditions (Bostock & Stermer, 1989). Some studies have suggested that late pruning towards bud-break can also minimise infections due to decreased inoculum availability and increased sap-flow (Halleen & Fourie, 2005; John *et al*., 2005). Moreover, wounds made during spring may be less susceptible due to increased competition by beneficial microflora (Munkvold & Marois, 1995) whose growth may be promoted by the release of xylem exudates that contain carbohydrates, amino and organic acids (Halleen *et al*., 2010). Susceptibility to *E. lata* was found to be the highest in early winter and it decreased in late winter and early spring (Gu *et al*., 2005; Sosnowski *et al*., 2008). In South Africa; however, late-pruning is not a guarantee for fewer infections because wounds pruned later in winter were more susceptible to infection than those made earlier (Van Niekerk *et al*., 2011; Mutawila, 2013). The majority of trunk disease pathogens infect via winter pruning wounds; however, sucker wounds made during spring time can also serve as infection portals (Epstein *et al*., 2008; Lecomte & Bailey, 2011).

1.7.3 Susceptibility of sucker wounds

An extensive investigation was conducted by Lecomte & Bailey (2011) in France to establish the susceptibility of sucker wounds to *E. lata*. In their study, a survey for natural infections and artificial inoculation studies under glasshouse and field conditions were carried out mainly on the cultivar Cabernet Sauvignon. From the survey, 2.1% of sucker wounds were found to be naturally infected, much less than the winter pruning wounds of which 13% were infected. Sucker wounds artificially inoculated with spores of *E. lata* showed that 9.6% and 36% of wounds were infected after a two week and one year incubation period, respectively. Lecomte & Bailey (2011) concluded that although sucker wounds were less susceptible than winter wounds, they may pose a significant threat to grapevine infections by *E. lata*, thus they may play a secondary role in Eutypa dieback epidemiology. In California, a study was performed during which trunks of 14 field grapevines were desuckered during spring and exposed to natural conditions (Epstein *et al*., 2008). After one year, 64% of vines were naturally infected by *Diplodia seriata* via the sucker wounds. The authors suggested that *D. seriata* infections may be aided by spring rains. Van Niekerk (2008) suggested that sucker wounds can become infected by *Phaeoacremonium* spp. following rain events during spring and early summer in the summer rainfall region, due to the presence of inoculum in vineyards. To date, two studies have shown that *D. seriata* and *E. lata*, respectively, can infect sucker wounds; however, the susceptibility of sucker wounds to other trunk pathogens remains uncertain. Since winter pruning wound infections have been associated with high rainfall which increases inoculum availability (Van Niekerk *et al*., 2010), the knowledge on inoculum sources and spore discharge during spring or the desuckering period is crucial.

1.8 Spore release during desuckering

Rain is essential for spore discharge and dissemination of most trunk disease fungi. Species of the Botryosphaeriaceae require rain for the release of conidia from pycnidia and for dispersal (Epstein *et al*., 2008; Van Niekerk *et al*., 2010). Although most spore release occurs in winter during or following high rainfall events, several studies have shown that spores of Botryosphaeriaceae species can also be released during periods of low rain fall or during spring. In Kuntzmann *et al*. (2009), spores of *D. seriata* and *D. mutila* were trapped throughout the year, although the time periods during which the release peaked varied. In Amponsah *et al*. (2009), spores of *Neofusicoccum* spp. and *Diplodia* spp. were detected in

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airborne spore traps and rainwater runoffs throughout the year. In Epstein *et al*. (2008), conidia release of *Diplodia seriata* (syn. *Botryosphaeria obtusa*) occurred throughout the pruning period and into spring. Generally *E. lata* ascopores are released when the mean rainfall is favourable for discharge (Ramos *et al*., 1975; Van Niekerk *et al*., 2010). Although *E. lata* occurs less frequently in spring (Van Niekerk *et al*., 2010), in California (Gubler *et al*., 2005), the amount of spores released also peaked following rain events in late autumn and spring. It was also suggested that *E. lata* ascospore release may peak during the first rains of spring if preceded by weeks of no rain and conducive temperature (Gubler *et al*., 2005). Esca and Petri disease fungal spores are also discharged in high numbers and at a peak in winter (Van Niekerk *et al*., 2010). However, spores of some esca and Petri disease pathogens have been trapped even outside of winter time. In Larignon & Dubos (2000), spores of *Ph. chlamydospora* were trapped throughout the whole year, while spores of *Pa. aleophilum* were mostly trapped during the vegetative period. Similarly, spores of *Ph. chlamydospora*, *Pa. inflatipes* and *Pa. aleophilum* were trapped throughout the year in California (Eskalen & Gubler, 2001) and the latter was trapped even in the absence of rain. Pycnidia of *Phomopsis viticola* occur on canes, spurs, petioles, and clusters remaining on the dormant vines (Van Niekerk *et al*., 2010). Mature pycnidia of *Po. viticola* erupt and conidia are released during wet or rainy conditions in the spring (Hewitt & Pearson, 1988). The conidia are splash dispersed by rain and infect green leaves and shoots (Pine, 1959). Although spores of the trunk disease pathogens are released more frequently and abundantly during the rainy season, it is evident that spores are also available during the desuckering period, in spring.

1.9 Wound protection

Since winter pruning wounds of grapevines are the primary portals of trunk disease infections, it is reasonable to say that wound protection is the key to minimising trunk diseases in grapevines. Apart from remedial surgery (Sosnowski *et al*., 2011), which is the removal of infected vine parts, there are currently no curative control measures available for protection against trunk disease fungi and therefore preventative measures are essential. There are a few cultural practices that are currently used to minimise infections. Sanitation, consisting of the removal of infected wood and pruning debris, is practiced for the reduction of inoculum in vineyards (Halleen *et al*., 2010; Rolshausen *et al*., 2010). Double and late pruning can also be practised to minimise infections (Weber *et al*., 2007). Other control

measures entail the application of pruning wound protectants and these can be classified as chemical, physical or biological.

1.9.1 Chemical wound protection

Chemical control is based on the use of fungicides or physical barriers such as paints and pastes for wound protection. Benzimidazole fungicides such as benomyl were greatly relied upon in the past. This fungicide was shown to have good efficacy against a broad spectrum of trunk disease pathogens but was discontinued (Sosnowski *et al*., 2004; Ramsdell, 1995; Halleen *et al*., 2010). Boron was also shown to be effective against trunk pathogens; however, it has been associated with bud failure (Rolshausen & Gubler 2005; Weber *et al*., 2007). Sodium arsenite best controlled esca by reducing disease severity and delaying expression of symptoms (Mugnai *et al.,* 1999; Surico *et al.,* 2008). Sodium arsenite was banned due to phytotoxicity and toxicity to humans (Christen *et al*., 2005; Surico *et al*., 2006; Fussler *et al*., 2008). Despite the effectiveness of some fungicides, their efficacy on pruning wounds is short-lived, lasting for approximately 7 to 14 days (Munkvold & Marois, 1993) and requiring multiple applications to protect the wounds until they have fully recovered (Weber *et al*., 2007). Since some of the effective fungicides have been discontinued, there have been numerous studies done in search of alternative fungicides. *In vitro* tests have shown the efficacy of several fungicides in inhibiting the growth of trunk pathogens (Jaspers, 2001; Bester *et al.,* 2007; Halleen *et al.,* 2010; Gramaje *et al.,* 2011; Amponsah *et al.,* 2012a; Pitt *et al.,* 2012), while some have further been shown to be effective wound protectants in the field (Halleen *et al.,* 2010; Rolshausen *et al.,* 2010; Amponsah *et al.,* 2012a; Pitt *et al.,* 2012; Diaz & Latorre, 2013). Bester *et al*. (2007) found benomyl, tebuconazole, prochloraz manganese and flusilazole to limit lesion length on one-year-old Chenin blanc vines which were inoculated with Botryosphaeriaceae. Halleen *et al*. (2010) found benomyl and flusilazole to be effective in the field against *Ph. chlamydospora*. Rolshausen *et al*. (2010) found thiophanate-methyl to be most effective against different trunk pathogen fungi on pruning wounds. Despite the numerous efforts of chemical control, there are only a few fungicides registered for grapevine pruning wound protection, while several paints and pastes are generally available.

1.9.2 Biological wound protection

Biological control is an environmentally friendly and sustainable option for pruning wound protection. Although the use of biological control agents (BCAs) as pruning wound protectants has been intensively investigated, only a few commercial products are currently registered (Weber *et al*., 2007). Some BCAs that have been researched for wound protection are *Trichoderma* spp. (Munkvold & Marois, 1993; Hunt *et al.,* 2001; John *et al*., 2005; Halleen *et al*., 2010; Kotze *et al*., 2011; Mutawila *et al*., 2011a), *Bacillus subtilis* (Ferreira *et al*., 1991; Halleen *et al*., 2010), *Fusarium lateritilium* (Carter, 1971) and *Clasdosporium herbarium* (Munkvold & Marois, 1993). Of these BCAs, *Trichoderma* spp. are the most broadly used as they have shown good efficacy against trunk disease fungi (John *et al*., 2005; Halleen *et al*., 2010; Kotze *et al*., 2011; Mutawila *et al*., 2011a, b; Mutawila, 2013).

1.9.2.1 *Trichoderma* species

Trichoderma is a soil dwelling saprophytic fungus that grows rapidly, sporulates abundantly and is competitive with other microorganisms (John *et al*., 2005). These fungi also show resistance or high tolerance to chemical pesticides and produce various antibiotics (Vinale *et al*., 2008) which makes them highly capable of surviving harsh conditions (Benítez *et al*., 2004). On grapevines, *Trichoderma* has been shown to penetrate, rapidly colonize and simultaneously inhibit grapevine trunk pathogens (Lonsdale, 1992; Hunt, 2001; John *et al*., 2005; Harvey & Hunt, 2006; Mutawila *et al*., 2011a, b). Apart from grapevine pruning wounds, *Trichoderma* spp. have also been found to inhibit infections by wood decay fungi, namely Basidiomycetes, in pruning wounds of beech trees (*Fagus sylvatica*) (Mercer & Kirk, 1984).

1.10 Scope of this study

To date, research has focussed mainly on the role of winter pruning wounds in grapevine trunk disease epidemiology. Although winter wounds are regarded as the major portals of infection, the role of sucker wounds in grapevine trunk disease epidemiology remains uncertain. Two studies revealed that sucker wounds can be infected by *E. lata* and *D. seriata* respectively. However, sucker wound susceptibility to other trunk disease fungi has not been investigated. Since spore discharge of the trunk pathogens can occur during the desuckering

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period, sucker wounds may also play an important role in the infection of grapevines. The main aims of the study were to investigate the role of sucker wounds as portals of infection for the different grapevine trunk disease fungi. The specific objectives of the study were to: i) establish the susceptibility of sucker wounds to different grapevine trunk disease pathogens in a controlled environment and in the field; ii) determine the duration of sucker wound susceptibility; iii) investigate control options by the application of *Trichoderma* suspension on sucker wounds.

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Figures

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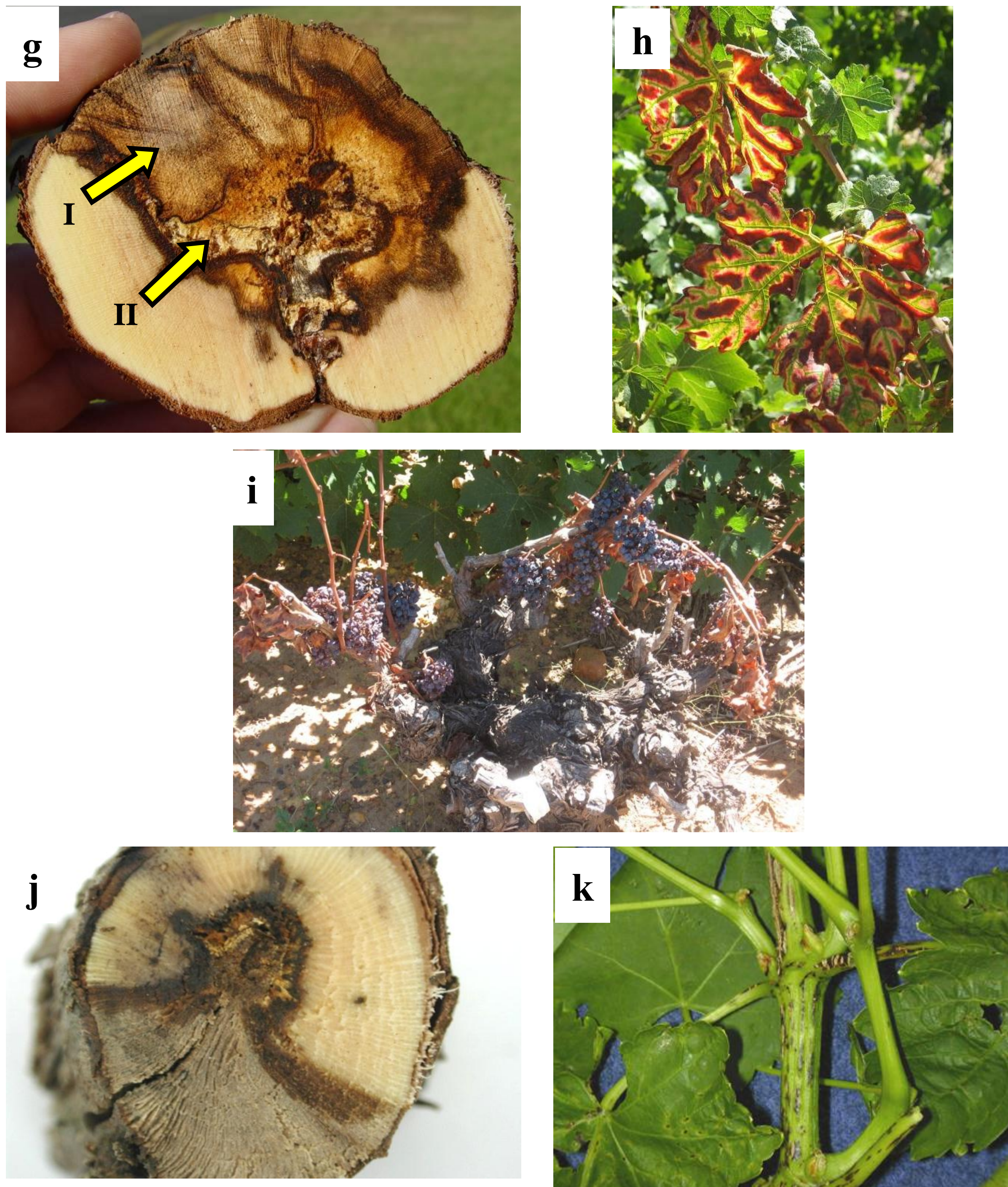

Figure 1. Typical internal [transverse (a, b, c, d, g, j) and longitudinal (f) sections] and foliar (d, h, i, k) symptoms of grapevine trunk diseases: Botryosphaeria dieback (a, b), Eutypa dieback (c, d), Petri disease (e, f), esca (g, h, i) and Phomopsis dieback (j, k). Some of the symptoms observed include black spots (a, e), brown and black streaking (f), brown internal necrosis (a), wedge-shaped necrosis (b, c), white wood rot (g), tiger-stripes (h) and apoplexy (i). (Photograph a from Van Niekerk *et al*., 2011 and b-k from Dr. F. Halleen).

Figure 2. Young green suckers or excess shoots (indicated by arrows) to be removed during desuckering in spring (a, b); desuckered shoots lying on the vineyard floor (c). (Photograph: c from Dr. L. Mostert).

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CHAPTER 2

THE SUSCEPTIBILITY OF GRAPEVINE SUCKER WOUNDS TO TRUNK DISEASE PATHOGENS

Abstract

Winter pruning wounds are regarded as the primary portals of infection for grapevine trunk disease pathogens. Sucker wounds, also known as spring wounds, can also provide openings through which trunk disease pathogens can infect; however, little is known about the range of trunk pathogens that can infect sucker wounds. The duration of sucker wound susceptibility to infection is also unknown. In this study, naturally and artificially infected grapevine sucker wounds were examined for their potential as portals of entry for trunk disease pathogens. Additionally, the duration of wound susceptibility in the field was determined. Sucker wounds were sampled from three wine and two table grape vineyards during 2011 and 2012 in the Western Cape province of South Africa. Isolations were made from 161 sucker wounds and the fungal cultures were identified on cultural and morphological characteristics as well as the internal transcribed spacer regions and 5.8S ribosomal RNA gene. Sixty-two percent of the wounds harboured at least one trunk pathogen. The following trunk pathogens were isolated from sucker wounds from field grapevines: *Phomopsis* (*Po.*) *viticola*, *Diplodia seriata*, *Phaeomoniella* (*Ph.*) *chlamydospora*, *Phaeoacremonium* (*Pa.*) *aleophilum*, *Eutypella* sp., *Cryptovalsa ampelina*, *Neofusicoccum australe* and *Diplodia* sp. Sucker wounds on 1-year-old potted grapevine plants were inoculated with *Eutypa lata*, *N. parvum, Pa. aleophilum*, *Ph. chlamydospora* and *Po. viticola*. The plants were maintained in a glasshouse. After 4 months all the inoculated pathogens could be re-isolated. Sucker wound susceptibility was further ascertained under field conditions on 12-year-old Cabernet Sauvignon vines by artificial inoculation with the same species. After 5 months, isolations were made from the sucker wounds. *Phomopsis viticola, N. parvum* and *Ph. chlamydospora* could be re-isolated. The duration of susceptibility of field sucker wounds was assessed for 4 weeks. Sucker wounds made on 12-year-old Cabernet Sauvignon vines were inoculated with conidial suspensions of *Ph. chlamydospora.* The wounds remained susceptible for 4 weeks with a decline in susceptibility after one week. This study confirms that sucker wounds are

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susceptible to the major trunk disease pathogens and thus could play an important role in grapevine trunk disease epidemiology.

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2.1 Introduction

Grapevine (*Vitis vinifera*) trunk diseases are a major threat to wine and table grape production world-wide and their occurrence has increased significantly over the last two decades (Mugnai *et al*., 1999; Mostert *et al*., 2005; Alaniz *et al*., 2007). Trunk diseases are caused by a complex of fungi that infect and inhabit the xylem vessels of grapevines and thereafter may act individually, in synergy or succession to produce symptoms. The different trunk diseases include Botryosphaeria dieback (caused by species of Botryosphaeriaceae) (Van Niekerk *et al*., 2004; Urbez-Torres *et al*., 2006; Urbez-Torres, 2009), Eutypa dieback (caused by species of the Diatrypaceae) (Petzoldt *et al*., 1981; Munkvold *et al*., 1994; Pilotti *et al*., 2005), Petri disease [caused by *Ph. chlamydospora* (Crous & Gams, 2000; Cobos & Martin, 2008) and *Phaeoacremonium.* spp. (Dupont *et al*., 1998; Aroca *et al*., 2008; Essakhi *et al*., 2008)], esca (caused by wood rotting species of the Hymenochaetales together with Petri disease fungi) (Larignon & Dubos, 1997; Mugnai *et al*., 1999; Cortesi *et al*., 2000; Graniti *et al*., 2000; Fischer, 2002, 2006; Surico *et al*., 2008; White *et al*., 2011a, b) and Phomopsis dieback (caused by various species of *Phomopsis*) (Mostert *et al*., 2001; Van Niekerk *et al*., 2005). Grapevine trunk diseases mainly affect mature vines; however, Petri disease is common in young vines. Trunk disease symptoms may include the death of spurs and cordons, internal streaking and the discolouration as well as necrosis of wood (Aroca *et al*., 2008; Van Niekerk *et al*., 2011a; White *et al*., 2011a). Vascular cankers and loss of vine vigour which can slowly lead to the death of grapevines are some of the characteristics of severely infected vines (Gubler *et al*., 2005; Edwards *et al*., 2007). Grapevine decline is frequently responsible for reduced lifespan and premature re-establishment of vineyards which consequently has severe economic implications for growers. Most trunk disease pathogens infect grapevines through exposed xylem tissue of pruning wounds or other grapevine openings such as mechanical wounds (Gramaje *et al*., 2008; Gramaje & Armengol, 2011).

Winter pruning wounds are regarded as the primary portals of entry for grapevine trunk disease pathogens (Adalat *et al*., 2000; Gubler *et al*., 2005; Van Niekerk *et al*., 2006; Rolshausen *et al*., 2010). Not only because of the multiple susceptible wounds present during the dormancy period, but also due to the high amount of spores that are released during that period (Gubler *et al*., 2005; Van Niekerk *et al*., 2010). However, although fungal spores are

released more abundantly during the pruning season, aerial spores of trunk disease pathogens are also present outside the winter pruning period. Spore release of *Po. viticola* has been associated with high rainfall during late winter and early spring (Anderson & Colby, 1943; Van Niekerk *et al*., 2010). In South Africa, *E. lata*, *Po. viticola* and Botryosphaeriaceae spp. have been trapped during spring (Van Niekerk *et al*., 2010). *Eutypa lata* spores were trapped during spring (Weber *et al*., 2007). *Phaeomoniella chlamydospora*, *Pa. inflatipes* and *Pa. aleophilum* spores were trapped throughout the year in California (Eskalen & Gubler, 2001; Eskalen *et al*., 2004), with the latter being trapped even in the absence of rain (Eskalen & Gubler, 2001). The availability of aerial inoculum during spring and in the absence of rain events in some cases indicates that it is possible for grapevines to be infected through sucker wounds, which are made during the desuckering period, in spring.

The susceptibility of winter pruning wounds to trunk disease fungal infections varies depending on the time during which the wounds are made. Late pruning has been found to be a useful tool for minimising trunk disease infections due to the reduced risk of wound infection by trunk pathogens such as *E. lata* (Moller & Kasimatis, 1980; Munkvold & Marois, 1995b; Chapuis *et al.,* 1998), *Pa. aleophilum* and *Ph. chlamydospora* (Larignon & Dubos, 2000) in late winter. The duration of vine susceptibility to *Pa. aleophilum*, *Ph. chlamydospora* (Larignon & Dubos, 2000) and *E. lata* (Munkvold & Marois, 1995b, Petzoldt *et al*., 1981; Munkvold & Marois, 1995b; Weber *et al*., 2007) has also been found to be shorter in late winter and during the vegetative season in the latter case.

The duration and decline pattern of susceptibility of winter pruning wounds can serve as an indication pattern of sucker wound susceptibility and duration. Studies have shown that there are several factors that contribute to the decline of vine susceptibility during late winter and spring. Wound healing occurs more rapidly in late winter and early spring due to the active physiological state of vines and warmer temperatures (Munkvold & Marois, 1995b). A strong positive correlation between higher mean temperatures following pruning and the rate of suberin accumulation and wound healing was observed by Munkvold & Marois (1995b). A decrease in the frequency of trunk disease infections in late winter and spring has also been attributed to colonisation of wounds by natural epiphytes (Munkvold & Marois, 1995a, b).

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Xylem sap flow has also been suggested to have the ability to reduce or prevent infections by decreasing the amount of spores that can land on wounds through washing off (Munkvold & Marois, 1995b; John *et al*., 2005). It would then appear that wounds made when vines are physiologically active during spring time would be less susceptible to infection; however, other studies have found contradicting results. Serra *et al*. (2008) observed that infection percentages could also be high in late spring. A similar observation was made by Van Niekerk *et al*. (2011b) and Mutawila (2013) who found that pruning wounds made during late winter were more susceptible than those made earlier in South Africa vineyards. These results were attributed to climatic conditions that were conducive to infection.

Previous research has mainly focused on winter pruning wounds that are made on lignified woody tissue of *V. vinifera*; however, two studies have reported on trunk pathogens that can also infect sucker wounds (Epstein *et al*., 2008; Lecomte & Bailey, 2011). In Lecomte & Bailey (2011), a study was performed in France to investigate the susceptibility of sucker wounds to *E. lata*. A survey was conducted to assess the natural infection of *E. lata* on sucker wounds versus winter pruning wounds. The authors found that 2.1% of sucker wounds in comparison to 13% of winter pruning wounds were naturally infected by *E. lata*. Furthermore, artificial inoculation of sucker wounds made by either removal of buds or suckers, confirmed sucker wound susceptibility to *E. lata*. It was concluded that although sucker wounds are not the primary portals of pathogen entry, they may pose a significant threat to the infection of grapevines by *E. lata*. In California, 14 vines from which at least one infected cordon was removed by remedial surgery, were also desuckered on the trunks and wounds were left exposed to natural infections (Epstein *et al*., 2008). After one year, the vines were removed and sucker wounds were analysed for infections by *Diplodia seriata.* Sixty-four percent (9 of 14) of the vines showed natural sucker wound infections by *D. seriata*.

There are other trunk disease pathogens that can also infect non-lignified or green tissue in addition to winter pruning wounds. *Phomopsis viticola* can infect green tissue and cause the typical lesions of the Phomopsis cane and leaf spot disease during spring (Hewitt & Pearson, 1988; Mostert *et al*., 2001; Van Niekerk *et al*., 2005). Since *Po. viticola* can overwinter in

buds, desuckering, which entails bud removal, can promote further infections via damaged healthy and wounded buds (Uddin & Stevenson, 1997). Botryosphaeriaceae spp. can also infect green tissues such as wounded green shoots, leaves, buds and berries (Van Niekerk, 2008; Gramaje & Armengol, 2011; Amponsah *et al*., 2012). Several species of Botryosphaeriaceae have been isolated from non-woody grapevine tissue including dormant buds, flowers and pea-sized and mature berries (Amponsah *et al*., 2012; Wunderlich *et al*., 2011). In California, *Pa. aleophilum* was also found in infected berries (Eskalen *et al*., 2004). Since *Pa. aleophilum*, *Po. viticola* and Botryosphaeriaceae spp. can infect green tissue, it is mostly likely that these trunk pathogens can also infect sucker wounds.

The susceptibility of grapevine sucker wounds to a broader selection of trunk pathogens as well as the duration of susceptibility to infections is unknown. The availability of trunk pathogen inoculum during spring time raises the question of the potential role of sucker wounds in trunk disease epidemiology. The objectives of this study were therefore to i) assess naturally infected sucker wounds from both wine and table grape vineyards in the Western Cape Province of South Africa; ii) assess sucker wound susceptibility towards a wider range of trunk disease pathogens in a controlled environment and in the field and iii) evaluate the duration of sucker wound susceptibility to infection in the field.

2.2 Materials and methods

2.2.1 Sucker wound survey

2.2.1.1 Sample collection

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

grapes) of 1- to 3-year-old wood with sucker wounds were sampled randomly from each vineyard and taken to the laboratory for fungal isolations.

2.2.1.2 Fungal isolations

Sucker wounds were selected (leaving approximately 2cm of cane above and below the wound) and excised, using pruning shears, from at least ten of the canes or spurs from a vineyard. Wounds that showed wood discolouration typical of trunk disease infections were analysed further. In total, fungal isolations were made from 161 wounds. Wood pieces were surface-disinfected in 70% ethanol for 30 seconds, then for 1 minute in 3.5% sodium hypochlorite solution and again in 70% ethanol for 30 seconds. Sucker wounds were aseptically dissected longitudinally across the wound. Fungal isolations were made aseptically from symptomatic (browning or streaking) (Figure 1) wood that originated from sucker wounds. Wood fragments were taken from the wound scar interphase. Additionally, if symptoms were not found, isolations were made from tissue that seemed healthy from the interphase. From each symptom type, 12 wood fragments (0.5mm × 1.0mm) were obtained from each sucker wound and plated onto 90mm Petri dishes containing Potato Dextrose Agar (PDA, Biolab, Wadeville, South Africa) amended with chloromycetin (250mg/L) (4 pieces per plate). The plates were incubated at approximately 25 ^{o}C and monitored daily for 4 weeks. Fungal colonies resembling taxa associated with grapevine trunk diseases were hyphal-tipped and grown on PDA. Pure cultures were stored in double-sterilised distilled water (dH_2O) in 14ml McCartney bottles and kept at 4 ^{o}C. Representative isolates are stored in the culture collection of the Department of Plant Pathology, Stellenbosch University, South Africa.

2.2.1.3 Fungal identification

Fungi were identified according to cultural and morphological characteristics as species of Botryosphaeriaceae (Van Niekerk *et al*., 2004; Crous *et al*., 2006; Damm *et al*., 2007; Phillips *et al*., 2008), Diatrypaceae (Glawe & Rogers, 1982), *Phaeoacremonium* (Mostert *et al*., 2006; Essakhi *et al*., 2008), Diaporthales (Mostert *et al*., 2001; Van Niekerk *et al*., 2005) and *Phaeomoniella chlamydospora* (Crous & Gams, 2000). Genomic DNA was isolated from 2-week-old fungal mycelia obtained from PDA plates. A CTAB-based

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

2.2.2 Susceptibility of sucker wounds to five trunk pathogens in the glasshouse

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

2.2.2.1 Plant cultivation

One-year-old dormant Chardonnay and Crimson Seedless canes were obtained from mother blocks. The canes were trimmed to three buds, submerged in water for 4 to 6 hours and then in the recommended dosage of dodecyl dimethyl ammonium chloride [ICA International Chemicals South (Pty), Ltd] for 5 minutes. Treated canes were stored with wetted perlite in plastic bags at 4 ^{o}C until they were required. Prior to use, dormant canes were hot-water treated at 50 ^{o}C for 30 minutes. To enhance rooting, the distal ends of the canes were dipped in Dynaroot hormone powder (Efekto, PBR Trading

International cc) and planted in plastic trays that contained perlite. Trays were drip irrigated three times a day and kept in the glasshouse at 28 ^{o}C. After 2 weeks, rooted canes were planted in plastic bags (13cm in diameter and 25cm in height) in a mixture of potting soil and perlite (3:1). The plants were maintained at 25 ^{o}C and allowed to bud in the glasshouse prior to inoculations. Dormant, grafted and rooted 1-year-old Chardonnay plants were obtained from a certified nursery and planted in individual plastic bags. These vines were also maintained in the glasshouse as described previously and also allowed to bud.

2.2.2.2 Inoculum preparation

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

2.2.2.3 Inoculations

For the first glasshouse trial, sucker wounds were created by removing the apical shoot (50 – 70mm long) from each plant (Figure 2a). For the second trial, the second shoot from the pruning wound was removed. The reason for this was that a field trial failed when the first shoot was removed due to the dieback that occurred beyond the sucker wound,

therefore the second shoot was removed in the further trials. Each sucker wound was inoculated with a 20µl droplet (1000 spores or conidia) (Figure 2b) of spore suspension. Control plants received the same volume of sterile dH_2O. The trials were laid out in a complete randomised block design with three and two blocks for trials one and two, respectively, each block consisting of ten plants per treatment.

2.2.2.4 Fungal isolation and identification

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

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

2.2.3.1 Inoculum preparation and inoculation

During October 2012 in spring, 12-year-old Cabernet Sauvignon vines, at Infruitec-Nietvoorbij (Agricultural Research Council, Stellenbosch), which were trained to bilateral cordons on a horizontally divided trellis with approximately seven spurs per cordon, were spur pruned to five buds during the dormant season. Sucker wounds were created by removing the second shoot (50 – 70 mm length) from the pruning wound on the 1-year-old cane (Figure 2 c and d). Six sucker wounds were made per vine for the different treatments. Inocula of *E. lata*, *N. parvum*, *Pa. aleophilum*, *Ph. chlamydospora* and *Po.*

viticola were prepared as previously described. Each vine received 20 µl of each of the five pathogen inocula (1000 spores or conidia) as well as sterile dH_2O as control. The trial was laid out in a complete randomised block design with four blocks that consisted of ten plants per treatment.

2.2.3.2 Fungal isolations and identification

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

2.2.4 Duration of susceptibility of sucker wounds on field grapevines

2.2.4.1 Inoculum preparation and inoculation

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

2.2.4.2 Fungal isolation and identification

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

2.2.5 Data analysis

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

2.3 Results

2.3.1 Sucker wound survey

Trunk disease symptoms that were observed from the sucker wounds included wood discolouration, browning and streaking (Figure 1). Sixty-two percent of the collected wounds were infected with at least one trunk pathogen. Multiple fungal pathogens were obtained from 19% of the wounds (Figure 1a, b and d). There was a higher incidence of infected sucker wounds from wine grapes (84%) in comparison with table grapes (16%) (Table 1). The results of the BLAST analyses are provided in Table 2. Of the different trunk disease fungi isolated, *Po. viticola* was the most common, followed by *D. seriata* and *Ph. chlamydospora* in both wine and table grape sucker wounds (Table 3). Additionally, low numbers of *Pa. aleophilum*, *Eutypella* sp, *C. ampelina*, *N. australe* and *Diplodia* sp. were also isolated, although these were only from wine grapes.

2.3.2 Glasshouse trials

Sucker wounds of Chardonnay and Crimson Seedless were susceptible to *Ph. chlamydospora* and *Eutypa lata*. The analysis of variance did not reveal a significant cultivar × treatment interaction ($P = 0.78$, Appendix A, Table 1) which indicated that both cultivars responded similarly to the two pathogens. For both Chardonnay and Crimson Seedless, significant differences were found between the two pathogen treatments ($P = 0.0009$ for Chardonnay and $P = 0.0001$ for Crimson Seedless, Appendix A, Table 2). The incidence of *Ph. chlamydospora* in inoculated sucker wounds was significantly higher than *E. lata* in Chardonnay as well as Crimson Seedless (Table 4). No trunk disease pathogens were isolated from the controls. For the second glasshouse trial, all of the inoculated fungi were re-isolated

(Table 5). Significant differences were found between the pathogen treatments ($P = 0.0018$, Appendix A, Table 3). *Neofusicoccum parvum* was isolated from 85% of the wounds, significantly higher than *Pa. aleophilum* (55%) and *E. lata* (45%). No trunk disease pathogens were re-isolated from the controls.

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

Of the five pathogens that were inoculated, only three were re-isolated namely *Po. viticola*, *N. parvum* and *Ph. chlamydospora* (Table 6). *Phomopsis viticola* (65%) was re-isolated significantly more than *N. parvum* (32.5%) and *Ph. chlamydospora* (7.5%) ($P < 0.001$; Appendix A, Table 4).

2.3.4 Duration of susceptibility of sucker wounds *in vivo*

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

2.4 Discussion

In this study, naturally infected sucker wounds as well as artificially inoculated sucker wounds made on 1-year-old grapevine canes were assessed for the presence and susceptibility to trunk disease pathogens. Sucker wounds were found to be susceptible to numerous taxonomically unrelated trunk disease pathogens including species of Botryosphaeriaceae, Diatrypaceae, *Phomopsis viticola, Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora*. In the field, sucker wounds remained susceptible to infection by *Ph. chlamydospora* for the 4 week period during which it was tested, although susceptibility declined after 1 week. Sucker wounds were made on 1-year-old canes by removing a green

shoot. These wounds could also be referred to as bud wounds, since sucker wounds are usually referred to wounds made from the removal of unwanted shoots from the trunk or cordon of the vine. Since the removal of a green shoot resembles a sucker wound, we refer to these the wounds made in the glass house and field trials as sucker wounds. This study provides new knowledge on the role of sucker wounds as portals for grapevine trunk disease pathogen infections, as well as the period of time for which these wounds can remain susceptible.

The susceptibility of sucker wounds and the potential role they may play in grapevine trunk disease epidemiology has been largely overlooked. Infrequent rainfall and spore release as well as vine physiological state during the vegetative period (Petzoldt *et al*., 1981; Chapuis *et al*., 1998; Weber *et al*., 2007) in comparison to higher rainfall and spore release during the dormancy period (Van Niekerk *et al*., 2010) has led to the assumption that sucker wounds are less important than winter pruning wounds as sites of infection. In the present study, *C. ampelina, D. seriata*, *Diplodia* sp., *Eutypella* sp., *N. australe, Pa. aleophilum*, *Ph. chlamydospora* and *Po. viticola* were isolated from naturally infected sucker wounds. *Phomopsis viticola*, *D. seriata, Ph. chlamydospora* and *Pa. aleophilum* were the most commonly isolated species. The relatively higher occurence of *Po. viticola* and *D. seriata* in naturally infected sucker wounds could be ascribed to the ability of these pathogens to infect green material. Since sucker wounds are made by the breaking off of green plant material, the wound scar that remains exposes metabolically active tissue. It is well known that *Po. viticola* infects grapevines during spring (Hewitt & Pearson, 1988). *Phomopsis viticola* can also infect grapevines via active or wounded buds (Hewitt & Pearson, 1988; Uddin & Stevenson, 1997) and wounded green shoots (Van Niekerk *et al*., 2005). Species of the Botryosphaericeae can also infect and cause lesions on green shoots (Van Niekerk *et al.*, 2004; Amponsah *et al*., 2012). Higher incidences of pathogens were found on wine grape versus table grape wounds and this may be due to different trellising and pruning styles. Wine grape canes are much shorter than table grape canes because they are usually pruned to two buds, whereas table grape canes are pruned to as many as 15 buds. Since the shorter wine grape canes are closer to older wood (spur, cordon and trunk) than table grapes, wine grape canes are in closer proximity to inoculum and are more prone to infection by fungal spores that are released and dispersed by rain from fruiting structures occurring on older wood.

The inoculum sources for most trunk pathogens have been found to be pycnidia or perithecia which occur on diseased wood of infected vines and pruning debris (Edwards & Pascoe, 2001a, b; Eskalen *et al*., 2002; Rooney-Latham *et al*., 2005; Pitt *et al*., 2010; Gramaje & Armengol, 2011). Since spores of most trunk pathogens are airborne, and their spore release is dependent on rain or relative high humidity to a large extent, infection of the surveyed sucker wounds may have occurred following such events (Van Niekerk *et al*., 2006, 2010). *Phomopsis viticola* and species of Botryosphaeriaceae have been trapped during and after periods of rainfall in spring (Van Niekerk *et al*., 2010). Spore release of *Phomopsis viticola* has also been associated with high rainfall during late winter and early spring (Anderson & Colby, 1943; Van Niekerk *et al*., 2010). Uddin *et al*. (1997) found the highest inoculum of *Po. viticola* to be released during spring. *Phaeacremonium aleophilum* and *Ph. chlamydospora* were aerially dispersed after rain events during winter and spring in California vineyards (Eskalen & Gubler, 2001; Rooney-Latham *et al*., 2005). Additionally, *Pa. aleophilum* spores were even released in the absence of rain. In French vineyards, *Ph. chlamydospora* was trapped throughout the year whilst *Pa. aleophilum* only occurred during the vegetative period (Larignon & Dubos, 2000). These studies therefore provide sufficient proof that inocula of trunk disease pathogens are available during other seasons apart from winter, and throughout the year in some cases.

The susceptibility of sucker wounds to *E. lata, N. parvum, Pa. aleophilum, Ph. chlamydospora* and *Po. viticola* was ascertained under controlled glasshouse conditions as well as in the field. The re-isolation incidence of pathogens from the glasshouse grown vines was higher than that obtained under field conditions. This was most probably due to the higher humidity and stable temperatures in the glasshouse. From the field vine inoculations *Po. viticola* and *N. parvum* infected the sucker wounds significantly more than *Ph. chlamydospora*. Additionally, *Phaeoacremonium aleophilum* and *E. lata* could not be re-isolated which indicated that the environmental conditions may have favoured infection of *Po. viticola* and *N. parvum*. The failure of *E. lata* re-isolation was unexpected as this was in contrast with a previous study in which the authors re-isolated *E. lata* after 1 year under field conditions (Lecomte & Bailey, 2011). The absence of *E. lata* and *Pa. aleophilum* and the lower incidences of *N. parvum* and *Ph. chlamydospora* from the field trial versus the glasshouse trial could be attributed to competition by naturally occurring wound colonizers.

Munkvold & Marois (1995a) found that natural wound colonizers had a high potential of reducing *E. lata*. Moreover, Petri dishes from the field trials had much higher numbers of saprophytes as compared to the glasshouse Petri dishes. Furthermore, the absence of *E. lata* and *Pa. aleophilum* from inoculated sucker wounds in the field was probably due to a strong wind that blew on the day of inoculation, which may have led to the quick evaporation of spore droplet prior to infection.

Sucker wounds made on field grapevines remained susceptible to *Ph. chlamydospora* for the 4 week period investigated in this study. Winter pruning wounds can remain susceptible for up to 16 weeks (Adalat *et al*., 2000; Larignon & Dubos, 2000; Gubler *et al*., 2001). Susceptibility of winter wounds is higher after pruning and then declines with pruning wound age (Petzoldt *et al*., 1981; Munkvold & Marois, 1995b). Wounds made earlier in the dormancy period were found to remain susceptible for longer than those made later (Munkvold & Marois, 1995b). Additionally, winter pruning wounds made in late winter or early spring had very low susceptibility, even on the day of pruning. It is therefore highly unlikely that sucker wounds which are made during spring would remain susceptible for the same duration as winter wounds, since the former are made on metabolically active tissue that would heal faster (Munkvold & Marois, 1995b). In the current study, wound susceptibility was higher during the first week and declined thereafter. The decline in susceptibility can be attributed to the healing of the sucker wounds. Pruning wounds heal due to increased suberin and lignin deposition as well as degree day accumulation (Munkvold & Marois, 1995b).

In the present study sucker wounds that were artificially inoculated were made by removing shoots from one-year-old wood, unlike Lecomte and Bailey (2011) who made ‘true’ sucker wounds by removing suckers and buds from trunks and arms. Although such wounds are more difficult to assess, they give a better reflection of the real risk of sucker wound infection that exists. In contrast, such wounds are more likely to be already infected prior to inoculation. For future research, sucker wounds can be made on both young (1-year old) and old (trunks and arms) wood. To fully assess sucker wound susceptibility a wider variety of

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cultivars should be evaluated for natural infections, as well as with artificial inoculations, against a range of trunk disease pathogens.

2.5 Conclusions

The results of the sucker wound survey showed that sucker wounds can be infected naturally by grapevine trunk disease pathogens such as *Phomopsis viticola*, Botryosphaeriaceae species (mainly *Diplodia seriata*), *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum*. The high incidence of *Po. viticola* across the surveyed locations as well as the high isolation incidences from the artificial inoculation trials indicate that sucker wounds were highly susceptible to this fungus. Similarly the high incidence of *D. seriata* in naturally infected sucker wounds as well the high incidence of *N. parvum* in the artificially inoculated trials showed that these fungi have a high potential for infecting sucker wounds. This study clearly shows that sucker wounds are susceptible to trunk disease pathogens and may therefore play a role in the epidemiology of these diseases. The potential risk of grapevine infection that exists through sucker wounds may have to be taken into consideration in wound protection strategies.

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Tables and Figures

Table 1. Results of field survey investigating the presence of trunk disease pathogens in sucker wounds sampled from two wine and table grape cultivars from different location in the Western Cape, South Africa.

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

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Table 2. BLAST (from GenBank) identification results of the species isolated from sucker wounds collected during a survey from five different grape-growing areas of the Western Cape Province of South Africa.

Fungal taxon	Representative cultures	Compared to Genbank accession	% Similarity	% Gap
Phomopsis viticola	GJM 29	HQ 288246.1	99	0
Diplodia seriata	GJM 21	GU 121890.1	100	0
Phaeoacremonium aleophilum	GJM 30	HQ 605018.1	100	0
Eutypella sp.	GJM 89	FJ 172284.1	99	0
Cryptovalsa ampelina	GJM 71	AY 920391.1	99	0
Neofusicoccum australe	GJM 61	FJ 150696.1	99	3
Diplodia sp.	GJM 42	EU860386.1	95	2

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Table 3. Incidence of trunk disease pathogens isolated from sucker wounds from the field survey.

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

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Table 4. Incidence of *Eutypa lata* and *Phaeomoniella chlamydospora* in sucker wounds made on 1-year-old own rooted Chardonnay and Crimson Seedless potted vines kept under controlled conditions in a glasshouse and assessed three months after inoculation.

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

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

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Table 5. Incidence of *Eutypa lata*, *Neofusicoccum parvum*, *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora* and *Phomopsis viticola* in sucker wounds made on 1-year-old own rooted Chardonnay potted vines kept under controlled conditions in a glasshouse and assessed four months after inoculation.

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

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

Table 6. Mean incidence of *Eutypa lata*, *Neofusicoccum parvum*, *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora* and *Phomopsis viticola* from sucker wounds made on Cabernet Sauvignon field vines and assessed after five months.

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

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

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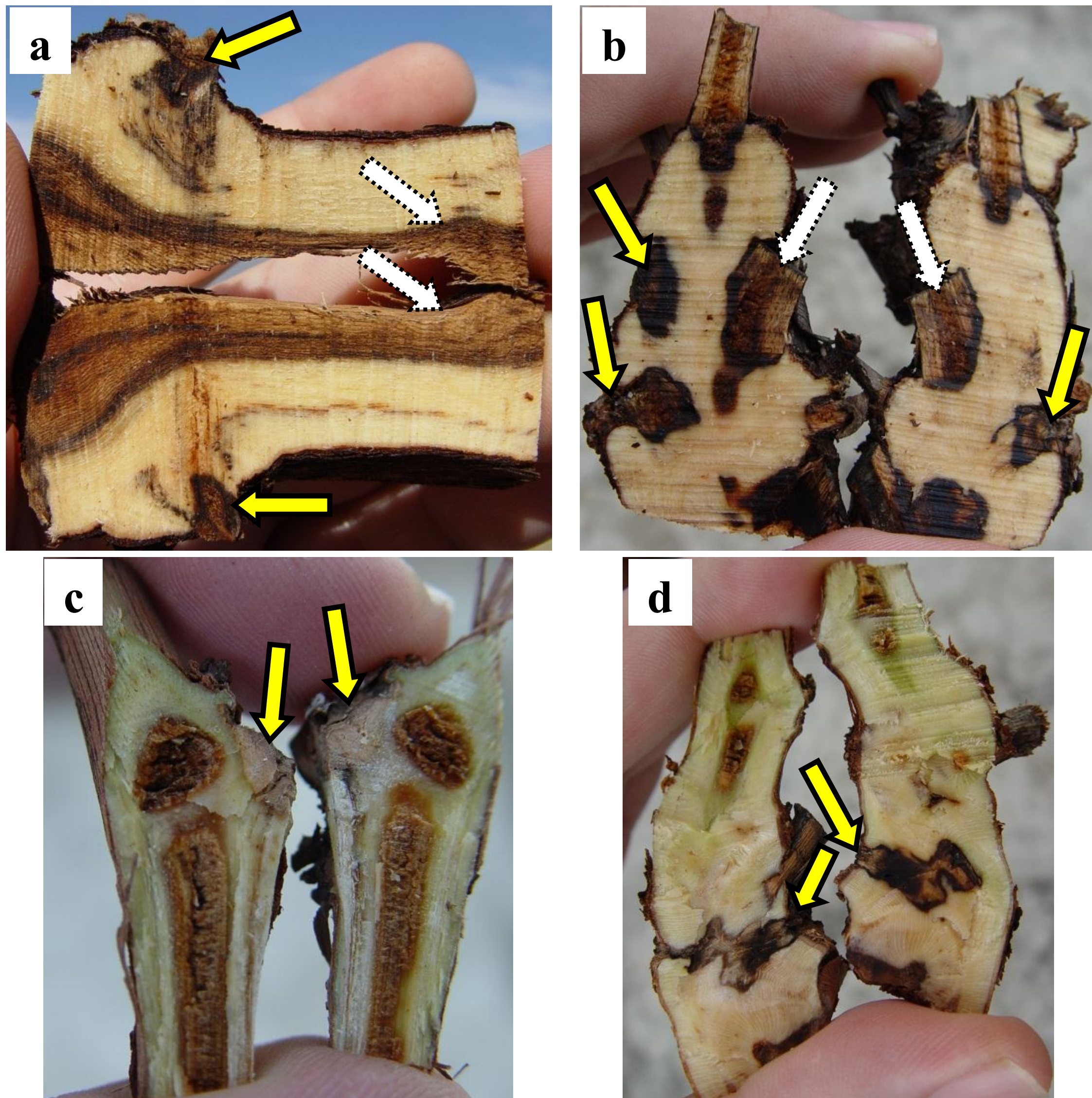

Figure 1. Vertically split grapevine wood collected during the survey showing sucker wounds (yellow solid arrows) with their respective trunk disease symptoms. Symptoms included brown discolouration (a, b, d) around the wound and streaking (a, c) from the wound. The following pathogens: *Diplodia seriata* (d), *Phaeomoniella chlamydospora* (a, b, c, d), *Phaeoacremonium aleophilum* (b), *Phomopsis viticola* (a) were obtained from the above wounds. No isolations were made from winter wounds and their symptoms (white dotted arrows).

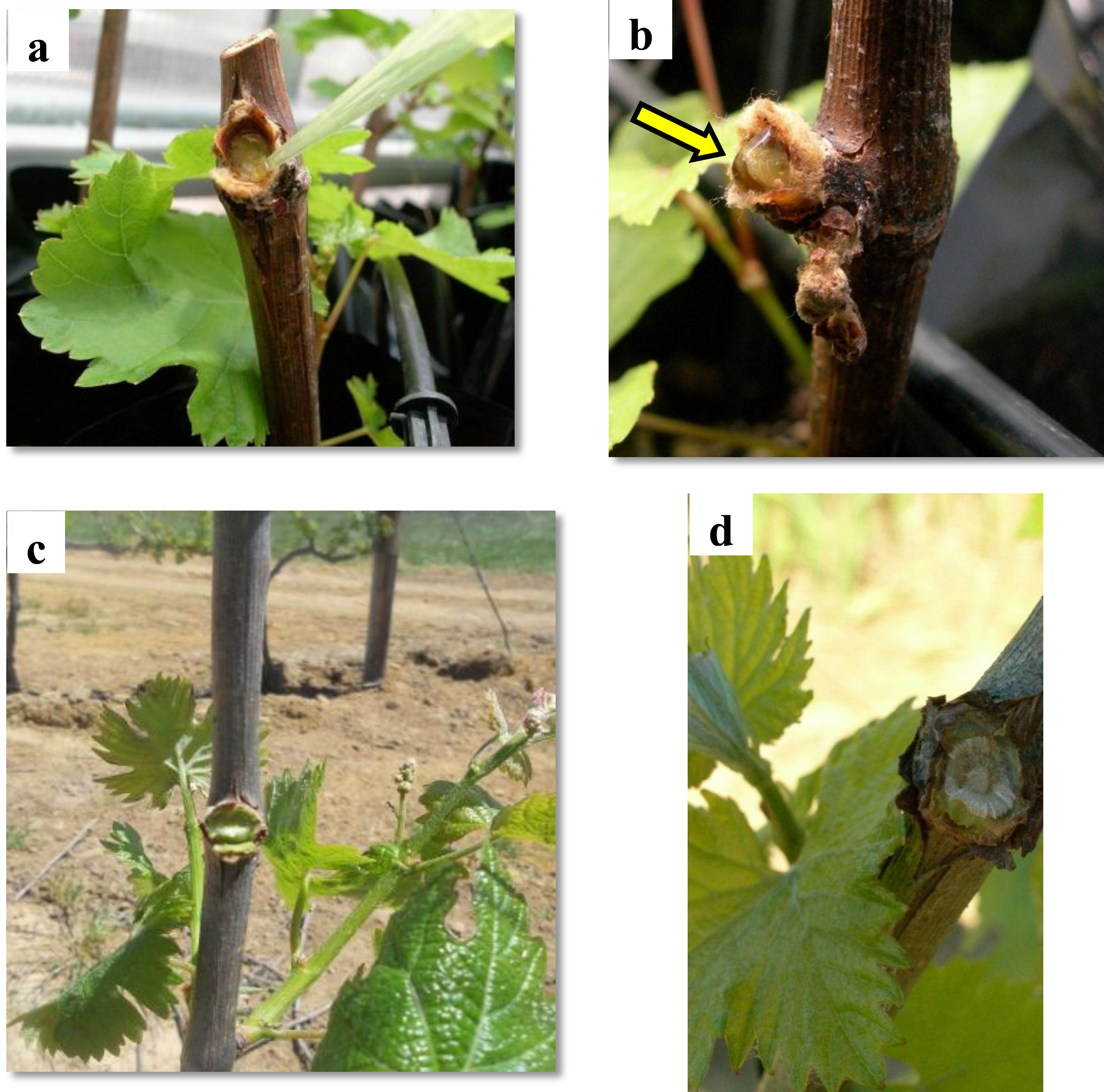

Figure 2. Examples of sucker wounds that were made on Chardonnay plants in the glass-house (a, b) and 1-year-old canes of 12-year-old Cabernet Sauvignon on field vines (c, d). The method of inoculation using a pipette is demonstrated (a) and a spore droplet (indicated by arrow) on the sucker wound (b).

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

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CHAPTER 3

EVALUATION OF *TRICHODERMA* AS A BIOLOGICAL CONTROL AGENT FOR SUCKER WOUND PROTECTION AGAINST GRAPEVINE TRUNK DISEASE PATHOGENS

Abstract

Although winter pruning wounds are the major portals of entry for trunk disease pathogens, sucker wounds are also susceptible to trunk disease pathogens and contribute to grapevine infections. In South Africa there are currently no registered fungicides for grapevine pruning wound protection and the biological control agents (BCAs) *Trichoderma* spp. are currently the only products commercially available. The efficacy of a *Trichoderma harzianum*-based commercial product, Eco-77®, was tested against the trunk pathogens *Phaeomoniella chlamydospora* and *Phomopsis viticola* on sucker wounds in the vineyard. During October 2012, sucker wounds were made on 1-year-old wood of Cabernet Sauvignon. The wounds were spray-treated with Eco-77® immediately after desuckering, and 24 hours later the sucker wounds were inoculated with spore suspensions of either *Ph. chlamydospora* and *Po. viticola*. After 5 months, isolations were made from the sucker wounds to assess the efficacy of the *Trichoderma* treatment. *Trichoderma harzianum* reduced the incidence of *Ph. chlamydospora* by 66.65%. Even though the incidence of *Po. viticola* was reduced by 15.37%, it was not significantly different from the control treatment. *In vitro* sensitivity studies were utilised to investigate the effect of fungicides, applied during spring against other diseases (downy and powdery, Botrytis rot and Phomopsis cane and leaf spot), on *Trichoderma* spp. The inhibition of mycelial growth and conidial germination of *T. harzianum* and *T. atroviride* were screened for 16 fungicides. The fungicides were applied at 0, 0.25, 0.5, 1 and 2 times the recommended dosages. *Trichoderma* isolates were the least sensitive to the systemic fungicides boscalid, metrafenone and trifloxystrobin, as well as contact fungicides quinoxyfen and meptyldinocap for the mycelial inhibition. These fungicides were regarded as being compatible with *Trichoderma* isolates because they gave mean percentage inhibitions of less than 50% inhibition at all the tested dosages. For the conidial germination assay, boscalid, penconazole, trifloxystrobin, and metrafenone (systemic) plus quinoxyfen and folpet (contact) were compatible with *Trichoderma*.

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Spiroxamine and pyrimethanil gave the highest mean percentage inhibition for both mycelial inhibition and conidial germination. The findings of this study showed that *T. harzianum* can potentially be used to protect sucker wounds against *Ph. chlamydospora*. Furthermore, some fungicides applied on grapevines against powdery mildew and Phomopsis cane and leaf spot during springtime can alternatively or simultaneously be applied with *T. harzianum* or *T. atroviride*.

3.1 Introduction

Pruning of grapevines is an annual practice that is performed during dormancy to ensure a balance between productivity and vegetative growth. Since pruning results in the exposure of xylem vessels, abundant rainfall and increased inoculum availability during dormancy leads to infections by grapevine trunk disease pathogens (Moller & Kasimatis, 1978; Chapuis *et al.,* 1998; Serra *et al.,* 2008; Van Niekerk *et al*., 2011). Winter pruning wounds are considered to be the main infection portals for xylem-inhabiting trunk pathogens which cause dieback and decline of grapevines. Grapevine trunk diseases are caused by a variety of fungal pathogens including species of Diatrypaceae [(Eutypa dieback) (Munkvold *et al*., 1994; John *et al*., 2005; Weber *et al*., 2007; Pitt *et al*., 2010; Trouillas *et al*., 2011; Luque *et al*., 2012)], *Phaeomoniella chlamydospora* and *Phaeacremonium* species [(Petri disease) (Mugnai *et al*., 1999; Crous & Gams, 2000; Edwards & Pascoe, 2004; Essakhi *et al*., 2008)], wood rotting fungi from the Hymenochaetales [(esca) (Mugnai *et al*., 1999; Fischer, 2002; Graniti *et al*., 2000)], species of the Botryosphaeriaceae [(Black dead arm or Botryosphaeria canker) (Larignon *et al*., 2001; Van Niekerk *et al*., 2006; Urbez-Torres *et al*., 2006; Urbez-Torres, 2009)] and *Phomopsis* spp. [(Phomopsis dieback) (Mostert *et al*., 2001; Rawnsley *et al*., 2004; Van Niekerk *et al*., 2005; Gramaje & Armengol, 2011; Urbez-Torez *et al*., 2012, 2013)]. Apart from infecting winter pruning wounds, it has been shown that *E. lata* (Lecomte & Bailey, 2011) and *Diplodia seriata* (Epstein *et al*., 2008) can infect sucker wounds made during spring.

To date, most research has focussed on the role of winter pruning wounds in trunk disease epidemiology and therefore the significance of sucker wounds remains uncertain. A recent study concluded that sucker wounds may provide a risk of grapevine infection by *E. lata*, although this risk is less than in the case of winter pruning wounds (Lecomte & Bailey, 2011). Epstein *et al*. (2008) also found sucker wounds to be naturally susceptible to *D. seriata* on vines in the field. Furthermore, in chapter 2 it was also demonstrated that sucker wounds are susceptible to *E. lata*, *N. parvum*, *Pa. aleophilum*, *Ph. chlamydospora* and *Po. viticola*. Winter pruning wounds can remain susceptible for up to 16 weeks with the susceptibility declining as the wounds age (Petzoldt *et al*., 1981; Munkvold & Marois, 1995; Eskalen *et al*., 2007; Van Niekerk *et al*., 2011). The susceptibility of sucker wounds to *Ph. chlamydospora* was assessed up to 4 weeks (Chapter 2) and the pathogen was re-isolated for

the whole duration, similarly with declining frequency over the 4 week period. Since sucker wounds are susceptible to trunk disease pathogens it is reasonable to suggest that they also need to be protected from trunk disease infections.

Pruning wound protection has been the focus of much research. Currently there is a lack of curative measures for wound protection (Van Niekerk *et al*., 2011) and therefore preventative control appears to be the only viable option. To date, most wound protection strategies have relied on chemical, physical and biological control. Fungicides and physical barriers including paints and pastes form the basis of chemical control for wound protection. Benomyl was one of the most effective fungicides used to control *E. lata* and other trunk disease pathogens (Munkvold & Marois, 1993; Ramsdell, 1995). This fungicide was discontinued in 2001, and is no longer available in most countries (Weber *et al*., 2007; Halleen *et al*., 2010; Rolshausen *et al*., 2010). Boron also displayed efficacy against trunk pathogens, but was linked to bud failure (Rolshausen & Gubler, 2005; Weber *et al*., 2007). Although some fungicides were effective against trunk pathogens, they protected wounds for only 14 days (Munkvold & Marois, 1993) and therefore multiple applications were required until pruning wounds had fully recovered (Halleen & Fourie, 2005; Weber *et al*., 2007; Halleen *et al*., 2010) resulting in cost implications for growers. Since then much work has been done to research alternative fungicides. *In vitro* studies have shown the ability of several fungicides to inhibit the growth of trunk pathogens (Jaspers, 2001; Bester *et al.,* 2007; Halleen *et al.,* 2010; Gramaje *et al.,* 2011; Amponsah *et al.,* 2012; Pitt *et al.,* 2012). Moreover, some fungicides have further shown effectiveness as wound protectants under field conditions (Halleen *et al.,* 2010; Rolshausen *et al.,* 2010; Amponsah *et al.,* 2012; Pitt *et al.,* 2012; Díaz & Latorre, 2013). Bester *et al*. (2007) found benomyl, flusilazole, prochloraz manganese and tebuconazole to limit lesion size on 1-year-old Chenin blanc vines inoculated with species of the Botryosphaeriaceae. Halleen *et al*. (2010) showed benomyl and flusilazole to be effective in controlling in *E. lata* and *Ph. chlamydospora* in the field. Rolshausen *et al*. (2010) found thiophanate-methyl to be the most effective against different trunk fungi on pruning wounds. Despite the extensive research that has been done on chemical control, there are a few fungicides specifically registered for grapevine pruning wound protection while several paints and pastes are generally registered for pruning wounds on all woody species.

The demand for biological control methods has increased as environmental protection has become more important in recent years. *Bacillus subtilis* (Ferreira *et al*., 1991; Kotze *et al*., 2011), *Cladosporium herbarum* (Munkvold & Marois, 1993) and *Fusarium lateritium* (Munkvold & Marois, 1993; John *et al*., 2005) were found to be effective biological control agents (BCAs) against *E. lata*. *Trichoderma* spp. have received the most attention as BCAs because this genus has shown good potential in protecting wounds. *Trichoderma* spp. have shown good efficacy when they were used in integrated control in soil amendments (Chet, 1987; Chet *et al*., 1997; Elad *et al*., 1998; Harman, 2000; Fourie *et al*., 2001; Harman *et al*., 2004; Lorito *et al*., 2004) and in seed treatments (Bardia & Rai, 2007) for protection of several other crops such as cucumber, cotton, potato, tobacco, due to be their antagonism against soil pathogens (Smith *et al*., 1990; Nemec *et al*., 1996). These species have also been found to induce resistance in several crops including grapevines (Calderon *et al*., 1993) and cucumber (De Meyer *et al*., 1998), as well as growth stimulating effects in grapevines (Fourie *et al*. 2001), lettuce, tomato and pepper (Vinale *et al*., 2004).

The potential of the genus *Trichoderma* as BCAs of plant pathogens was first recognized in the 1930s (Weindling, 1932; Schubert *et al*., 2008). A few *Trichoderma* species such as *T. harzianum*, *T. atroviride* and *T. viride* have been found to be competitive as antagonists for pruning wound protection because they provide a ‘bio-barrier’ against a broad spectrum of trunk disease pathogens (Gubler *et al*., 2005). Numerous studies have shown the efficacy of *Trichoderma* as a wound protectant against wood decay fungi on urban trees and other woody hosts (Dubos & Ricard, 1974; Pottle & Shigo, 1975; Mercer & Kirk, 1984; Lonsdale, 1992; Spiers & Brewster, 1997; Schubert *et al*., 2008). *Trichoderma* spp. provide long-term pruning wound protection in comparison with fungicides. In Schubert *et al*. (2008), *Trichoderma* spp. were re-isolated after 30 months on urban trees and even after 5 years in Lonsdale (1992). On grapevines, *Trichoderma* spp. has been shown to penetrate (Harvey & Hunt, 2006; Mclean *et al*., 2009) and protect wounds against *E. lata* (John *et al*., 2005; Halleen *et al*., 2010; Mutawila *et al*., 2011b) and other trunk disease fungi for at least 8 to 12 months (Halleen *et al*., 2010; Mutawila *et al*., 2011a). The mechanisms of control used by *Trichoderma* spp. include competitive exclusion, mycoparasitism and antibiosis that occur when *Trichoderma* spp. compete with other pathogens for space, nutrients and water (Benítez *et al*., 2004; John

et al., 2004). Their efficacy are influenced by many different factors such as pH, temperature, moisture and other microflora (Howell, 2003).

There are some challenges that limit the use of *Trichoderma* spp. as BCAs for wound protection. *Trichoderma* spp. require time to colonize wounds and therefore there is a window period of susceptibility prior to establishment (John *et al*., 2005; Mutawila, 2013). Timing of application with regard to the vine's physiological status is also crucial because propagules of fungus may be washed off by xylem sap during vine 'bleeding', which will consequently lead to poor establishment (Mutawila, 2013). *Trichoderma* spp. may also be incompatible with fungicides, making it difficult to incorporate them into integrated pest management strategies. For grapevines, many chemical sprays are applied against diseases such as powdery and downy mildew, Botrytis rot and Phomopsis cane and leaf spot during the desuckering period. However, the sensitivity of *Trichoderma* spp. towards these fungicides is unknown.

The objectives of the current study were to i) evaluate *Trichoderma* spp. against the trunk pathogens *Ph. chlamydospora* and *Po. viticola* on sucker wounds in the field and ii) determine the sensitivity of *Trichoderma* spp. (*T. atroviride* and *T. harzianum*) *in vitro* against 16 fungicides that are used to control powdery mildew, downy mildew, Botrytis rot and Phomopsis cane and leaf spot.

3.2 Materials and methods

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

3.2.1.1 Isolates used and inoculum preparation

A *Trichoderma harzianum*-based pruning wound product, Eco-77®, was kindly provided by Plant Health Products (PHP, PTY Ltd., Nottingham Road, South Africa). Eco-77® was applied at the recommended rate of 0.5g/L. *Phaeomoniella chlamydospora* and *Po. viticola* are maintained in the culture collection of the Department of Plant Pathology at the University of Stellenbosch, STE-U 6384 and STE-U 7768, respectively. *Phaemoniella*

chlamydospora conidial suspension was prepared from a 2-week-old fungal culture grown on Potato Dextrose Agar (PDA, Biolab, Wadeville, South Africa). A colonized mycelium block measuring 10mm × 10mm was placed in sterile dH_2O and shaken vigorously to suspend conidia. A conidial suspension of *Po. viticola* was made by suspending conidial droplets from pycnidia that formed on 4-weeks-old fungal cultures on PDA with sterilised pine needles.

3.2.1.2 Field inoculations

A 12-year-old Cabernet Sauvignon vineyard trained to bilateral cordons on a horizontally divided trellis situated in Stellenbosch was used for field inoculations. During July 2012 (winter), the vines were spur pruned to 5 buds. In October 2012 (spring), sucker wounds were created by removing the second shoot (50 – 70mm in length) below the pruning wound of the 1-year-old canes. Wounds were then spray-treated with Eco-77® by means of a hand-held trigger spray canister. After 2 days, sucker wounds were inoculated with 1000 spores of *Ph. chlamydospora* and *Po. viticola* conidial suspensions. Treatments included Eco-77®, *Po. viticola*, *Ph. chlamydospora*, plus a combination (Eco-77® + pathogen) and sterile dH_2O as a control. The trial was laid out in a complete randomised block design with three blocks that consisted of ten vines. Each vine received all six treatments. Five months later, sucker wounds were excised (leaving approximately 2cm above and below the sucker wound) and taken to the laboratory for fungal re-isolations and identification.

3.2.1.3 Fungal isolations from sucker wounds

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

3.2.1.4 Data analysis

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

3.2.2 *In vitro* fungicide sensitivity testing of *T. harzianum* and *T. atroviride*

3.2.2.1 Isolates and fungicides used

In vitro assays were performed using two *Trichoderma* spp. isolates T1 (*T. atroviride*) and Eco-77® (*T. harzianum*). Both isolates were used for mycelial inhibition and conidial germination tests. Isolate T1 is maintained in the culture collection of the Department of Plant Pathology at the University of Stellenbosch STE-U 6514. Sixteen commercial fungicides including contact and systemic products that are used for the control of powdery (*Uncinula necator*) and downy mildew (*Plasmopara viticola*), Botrytis rot (*Botrytis cinerea*) and Phomopsis cane and leaf spot (*Phomopsis viticola*) (Table 1) were screened.

3.2.2.2 Inhibition of mycelial growth

All 16 fungicides were screened *in vitro* for the mycelial inhibition of Eco-77® and T1. Stock solutions were made by suspending fungicides directly into 1000ml of sterile dH_2O. Fungicide solutions were then pipetted in appropriate quantities to bottles that contained molten PDA at approximately 50 ^{o}C to achieve 0.25, 0.5, 1 and 2 times the recommended dosages (Table 1). Potato dextrose agar without fungicide was used as a control treatment. Plates were inoculated within 24 hours with mycelium plugs of 5mm diameter obtained from the margins of actively growing 7-day-old cultures of Eco-77® and T1. Each fungicide concentration, as well as the controls were replicated three times. Petri dishes were incubated at 25 ^{o}C for 3 days. At 24 hours the diameter of each colony was measured twice, at perpendicular angles. The diameter measurements were recorded again at 48 hours and the trial was repeated once. For each isolate × fungicide × concentration combination, the

percentage inhibition was calculated in relation to the respective control treatment. The percentage inhibition was calculated as follows: 100 × [(colony diameter of control) – (colony diameter on fungicide-amended plate)/ (colony diameter of control)]. The fungicides were regarded as being compatible with *Trichoderma* isolates if they gave less than 50% mean percentage inhibition at all the tested dosages.

3.2.2.3 Inhibition of conidial germination

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

3.2.2.4 Data analysis

For the mycelial inhibition, the percentage inhibition of both experiments was pooled. The data was subjected to analysis of variance (ANOVA) and the Student's t-test for the least significant differences (LSD) at 5% significance level ($P < 0.05$). Significant differences in conidial inhibition between the fungicides were determined using a one-way ANOVA. The data was subjected to analysis of variance (ANOVA) and the Student's t-test for the least significant differences (LSD) at 5% significance level ($P < 0.05$). Analyses was performed

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using SAS version 9.2 (SAS, 2008) statistical software (SAS Institute Inc, SAS campus Drive, Cary, North Carolina, USA).

3.3 Results

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

The analysis of variance (Appendix B, Table 1) revealed a significant (P = 0.03) difference between *Ph. chlamydospora* treatments. *Phaeomoniella chlamydospora* mean incidence decreased by 66.65% when it was inoculated on Eco-77® treated sucker wounds (Table 2). The ANOVA (Appendix B, Table 1) revealed no significant differences between *Po. viticola* treatments (P = 0.07), the mean incidence of *Po. viticola* (Table 2) decreased by 15.37% when it was inoculated on wounds treated with Eco-77®. The ANOVA (Appendix B, Table 2) also revealed a significant difference (P = 0.0018 and P = 0.01) in Eco-77® treatments. *Trichoderma* mean incidences decreased by 85.72% and 74.99% when it was challenged with *Ph. chlamydospora* and *Po. viticola*, respectively (Table 3).

3.3.2 *In vitro* fungicide sensitivity testing of *T. harzianum* and *T. atroviride*

3.3.2.1 Mycelial inhibition

Analysis of variance revealed a significant isolate × concentration × fungicide interaction (P < 0.0001) for mycelial inhibition for both *Trichoderma* isolates at 24 and 48 hours (Appendix B, Tables 3 and 4). All 16 fungicides inhibited the growth of *Trichoderma* to some degree at all the tested concentrations after 24 and 48 hours. The mean percentage inhibition generally increased with an increase in concentration whilst the sensitivity of isolates to most fungicides generally decreased with an increase in time. At 0.25× (Figure 1), *Trichoderma* isolates were only sensitive to spiroxamine and penconazole (systemic) and to all contact fungicides except for quinoxyfen, meptyldinocap and metiram (T1) after 24 hours. At 0.5× (Figure 3), *Trichoderma* isolates were only sensitive to spiroxamine, flusilazole and fenarimol (Eco-77® only) (systemic) and to all contact fungicides except quinoxyfen and meptyldinocap after 24 hours. At the recommended dosage (Figure 5), isolates were sensitive to all systemic fungicides except boscalid, metrafenone and trifloxystrobin and to all contacts except quinoxyfen and meptyldinocap after 24 hours. At 2x (Figure 7), isolates were sensitive

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to spiroxamine, flusilazole and fenarimol (systemic) and to all contact fungicides except quinoxyfen and meptyldinocap. Similar trends were observed after 48 hours (Figures 2, 4, 6 and 8) for all the concentrations. Additionally, isolates were less sensitive after 48 hours. *Trichoderma harzianum* and *T. atroviride* is therefore compatible with the systemic fungicides boscalid, metrafenone and trifloxystrobin, as well as contact fungicides quinoxyfen and meptyldinocap.

3.3.2.2 Inhibition of conidial germination

Analysis of variance revealed significant isolate × fungicide ($P < 0.0001$), fungicide ($P < 0.0001$) as well as isolate ($P < 0.0001$) interactions (Appendix B, Table 5). *Trichoderma atroviride* (T1) was significantly less sensitive to fungicides than *T. harzianum* (Eco-77®). The following fungicides: boscalid, penconazole, metrafenone and trifloxystrobin (systemic) plus quinoxyfen and folpet (contact) inhibited less than 50% of conidial germination (Figure 9). No conidia germinated in the presence of spiroxamine (systemic) and mancozeb, propineb, metiram and diathon (contact). The fungicides with the highest inhibition were therefore all contacts (mancozeb, propineb, metiram, pyrimethanil and diathanon), except for spiroxamine which is systemic (Figure 9).

3.4 Discussion

This study demonstrated the potential use of *Trichoderma harzianum* for sucker wound protection against trunk pathogens *Phaeomoniella chlamydospora* and *Phomopsis viticola*. The application of *Trichoderma* products on sucker wounds has not been reported previously. *Trichoderma harzianum* colonised sucker wounds and could be re-isolated after 5 months. The BCA significantly decreased the incidence of *Ph. chlamydospora* in sucker wounds when it was applied as a wound treatment prior to pathogen inoculation.

Trichoderma harzianum reduced the incidence of *Ph. chlamydospora* by 66.65% in sucker wounds that were treated with Eco-77®. Various field trials have shown that *Trichoderma* spp. can inhibit the infection of winter pruning wounds by trunk disease fungi *E. lata* (Carter & Price, 1974; Carter, 1983; Munkvold & Marois, 1993; John *et al*., 2005; Halleen *et al*.,

2010, Mutawila *et al*., 2011b; Mutawila, 2013) and *Ph. chlamydospora* (Mutawila *et al*., 2011b). In Halleen *et al*. (2010), *Trichoderma* treatments also reduced the incidence of natural infections by Botryosphaeriaceae spp., *Ph. chlamydospora*, *Phaeoacremonium* spp. and *Phomopsis* spp. and, moreover, Eco-77® significantly reduced the total pathogen count in pruning wounds. In the present study, although *T. harzianum* also reduced the incidence of *Po. viticola* by 15.37%, this reduction was not statistically significant. A longer lag period between the application of *Trichoderma* and *Po. viticola* could have improved the efficacy of the *Trichoderma*. When *Po. viticola* was applied 7 days after the application of Eco-77® on winter pruning wounds, the incidence of *Po. viticola* was reduced from 34.82% to 18.48% (Kotze, 2008). John *et al*. (2005) also found that delaying the inoculation of *E. lata* by 14 days after treating wounds with *T. harzianum* and *Fusarium lateritium* increased the efficacy of BCAs and significantly reduced the re-isolation incidence of *E. lata*. It was therefore suggested that BCAs need an establishment period for colonising wound surfaces. Furthermore, Mutawila (2010), found that *Po. viticola* killed 1-year-old grapevines after 90 days despite the application of *T. harzianum* after pruning. The fact that *Trichoderma* did not strongly inhibit the infection of *Po. viticola* in sucker wounds as well as in 1-year-old grapevines might indicate that this pathogen was more competitive in infecting active xylem tissue than *Trichoderma*. *Phomopsis viticola* is well known to infect green shoots and cause Phomopsis cane and leaf spot. *In vitro* studies by Kotze *et al*. (2011) showed that of the ten *Trichoderma* spp. isolates tested only one was observed to inhibit the growth of *Po. viticola* on microscopic level. It would then seem that *Trichoderma* spp. have a limited effect on *Po. viticola*; however, this would have to be ascertained by testing multiple isolates of *Po. viticola.*

Trichoderma harzianum incidence ranged from 23% to 27% when it was applied as an individual treatment on sucker wounds. Low incidences could be ascribed to the presence of vascular 'bleeding' that most likely washed off conidia from the wound surface as has been found with pruning wounds (Munkvold & Marois, 1995; John *et al*., 2005; Halleen *et al*., 2010; Mutawila, 2013). Harvey & Hunt (2006) also obtained poor re-isolation incidences of 25% and 50% when *T. harzianum* was applied between 15 and 30 minutes, respectively, after pruning. In the present study, sap flow was observed on the day of and the day following desuckering. This may have led to poor establishment of *T. harzianum*, which was applied

immediately after desuckering. Since grapevines are physiologically active during spring, stronger plant defence could have reduced colonisation by *T. harzianum* as suggested by Lecomte and Bailey (2011) for the lower infection levels found from sucker wounds versus winter pruning wounds. Another reason for the low sucker wound colonization by *Trichoderma* may be due to cultivar differences. The inoculation of Eco-77® onto winter pruning wounds of Cabernet Sauvignon had 27.5% incidence of *T. harzianum*, lower than for other cultivars tested, when isolated eight months after inoculation in the field (Mutawila *et al.*, 2011a).

Trichoderma spp. have been isolated from winter pruning wounds 8 to 12 months after applying the BCA (Halleen *et al.*, 2010; Mutawila *et al.*, 2011a). The current study demonstrated that *Trichoderma* may provide long term protection of sucker wounds because it was re-isolated after 5 months. The exact time needed for sucker wound protection is not known, however, in chapter 2 it was shown that sucker wounds can still be infected after 4 weeks. The application of *Trichoderma* spp. on sucker wounds would then have a longer term protection advantage in comparison with fungicides. Fungicides can protect wounds for approximately 2 weeks (Creasar & Wicks, 2002; Sosnowski *et al.*, 2004, 2008). For both winter pruning and sucker wounds, the application of *Trichoderma* spp. hold the advantage of providing protection over the period of wound susceptibility.

In vitro mycelial inhibition and conidial germination tests revealed the sensitivity of *T. atroviride* (T1) and *T. harzianum* (Eco-77®) towards fungicides that are applied during spring. Isolate T1 appeared to be generally less sensitive to fungicides than Eco-77® for mycelial inhibition and conidial germination. Fungicides that inhibited *Trichoderma* isolates' mycelial growth and conidial germination with less than 50% were regarded as compatible and can possibly be combined in an application or applied shortly after each other in the field. Systemic fungicides boscalid, metrafenone and trifloxystrobin as well as contacts quinoxyfen and meptyldinocap displayed compatibility with *Trichoderma* isolates inhibiting less than 50% of the mycelial growth at all the tested concentrations. In contrast, spiroxamine and pyrimethanil inhibited *Trichoderma* spp. percentages were frequently more than 90%. For the conidial germination boscalid, penconazole and trifloxystrobin (systemic) and quinoxyfen

and folpet (contact) appeared to be compatible by inhibiting less than 50% of conidial germination.

All of the fungicides inhibited both *T. harzianum* and *T. atroviride* to a lesser or greater degree. There appeared to be an initial decline in the growth rate of *Trichoderma* mycelium in fungicide treatments after 24 hours; however, a recovery was observed after 48 hours. In the current study, the overall increase in mycelial inhibition that was observed with an increase in fungicide concentrations was also observed by Sarkar *et al*. (2010) and Tapwal *et al*. (2012).

Only a few of the fungicides used in the current study have been tested against *Trichoderma* spp. Various studies have shown that *Trichoderma* spp. are highly sensitive to mancozeb. Mclean *et al*. (2001) reported a 100% inhibition of *T. harzianum* by mancozeb, similar to the results of this study. These results are also in agreement with Gupta *et al*. (1995) who reported an inhibition of *T. viride* isolates by mancozeb *in vitro*. Bagwan (2010) however reported that copper oxychloride and mancozeb were safe to use with *T. harzianum* and *T. viride*. Figueras-Roca *et al*. (1996) reported that fenarimol had low inhibition towards five *Trichoderma* spp. (*T. hamatum*, *T. harzianum*, *T. koningii*, *T. reesei* and *T. saturnisporum*). The conidial germination results of this study also suggest that fenarimol is compatible with *Trichoderma* spp.

It is evident from the results that *Trichoderma* isolates were more sensitive to multi-site contact than systemic fungicides. This was attributed to the fact that multi-site fungicides work against multiple metabolic sites (McGrath, 2004), whereas single-site fungicides only interfere with one of the numerous metabolic pathways, enzymes or proteins that is required by the fungus. Three of the single-site mode of action fungicides for powdery mildew, namely boscalid, matrafenone and trifloxystrobin (also registered for Phomopsis cane and leaf spot), were less inhibitive towards *Trichoderma*. These fungicides together with quinoxyfen and meptyldinocap (also used for *Uncinula necator*) have the potential to be used in combination or alternately with *Trichoderma* in spring.

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3.5 Conclusions

Due to increased grapevine trunk diseases world-wide and the need to find more sustainable means of crop protection, pruning wound protection with BCAs is increasingly becoming the focus of trunk disease research. Since *Trichoderma* spp. are the only registered agents for wound protection in South Africa, the current study investigated its efficacy on sucker wounds. The results of this study demonstrated for the first time in grapevines the ability of *Trichoderma* to protect sucker wounds infected by *Ph. chlamydospora*, which is an important trunk disease pathogen in South African vineyards. Furthermore, inhibition of mycelial growth and conidial germination assays of *Trichoderma* spp. showed that the fungicides boscalid, metrafenone, trifloxystrobin, quinoxyfen and meptyldinocap could be applied in combination or alternatively with *Trichoderma* spp. during spring. This recommendation; however, would need to be ascertained with field trials.

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Tables

Table 1: Fungicides used against *Botrytis cinerea*, *Phomopsis viticola*, *Plasmopara viticola* and *Uncinula necator*, selected for screening *in vitro* compatibility with *Trichoderma harzianum* and *Trichoderma atroviride*.

No.	Active	Chemical group according to the FRAC code list (2013)	Trade name	Mode of action	Recommended against	Concentration of active	Dosage ppm = mg/L
1	spiroxamine	5 - SBI Class II	Prosper	Systemic	*U. necator*	500g/L	300
2	boscalid	7 - carboxamide	Cantus	Systemic	*U. necator*	500g/kg	400
3	penconazole	3 - DMI	Topaz	Systemic	*U. necator*	100g/L	22.5
4	flusilazole	3 - DMI	Olymp	Systemic (protective & curative)	*U. necator*	100g/L	35
5	metrafenone	U8 - benzophenone	Vivando	Locally systemic & contact	*U. necator*	500g/L	125
6	trifloxystrobin	11 - QOI	Flint	Systemic & contact	*Po. viticola* *U. necator*	500g/kg	200
7	fenarimol	3 - DMI	Rubigan	Systemic	*U. necator*	11.60%	24
8	quinoxyfen	13 - quinolene	Legend	Contact	*U. necator*	250g/L	62.5
9	meptyldinocap	29 - dinitrophenol	Karathane Star	Contact	*U. necator*	35.71%	140
10	mancozeb	M3 - dithiocarbamate	Dithane	Contact	*Po. viticola* *Pl. viticola*	750g/kg	150
11	copper hydroxide	M1 - inorganic	Virikop	Contact	*Pl. viticola*	538g/L	807
12	folpet	M4 - phthalamides	Folpan	Contact	*Po. viticola*	80% w/w	1000
13	propineb	M3 - dithiocarbamate	Antracol	Contact	*Pl. viticola*	70% ai/kg	2100
14	pyrimethanil	9 - AP	Scala	Contact	*B. cinerea*	400g/L	800
15	metiram	M3 - dithio-carbamate	Polyram	Contact	*Pl. viticola* *Po. viticola*	700g/kg	1400
16	diathanon	M9 - quinones	Delan	Contact	*Pl. viticola* *Po. viticola*	700 g/kg	525

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Table 2. Mean incidence of *Phaeomoniella chlamydopsora* and *Phomopsis viticola* re-isolated from sucker wounds of Cabernet Sauvignon 5 months after sucker wounds were inoculated with individual pathogens (*Phaeomoniella chlamydospora* or *Phomopsis viticola*) or in combination with *Trichoderma harzianum* (Eco-77®).

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

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

Table 3. Mean incidence of *Trichoderma harzianum* re-isolated from sucker wounds of Cabernet Sauvignon 5 months after sucker wounds were treated with an individual treatment Eco-77® or a combination treatment with either *Phaeomoniella chlamydospora* or *Phomopsis viticola*.

Treatment	Mean percentage isolation incidence (%)	
	Eco-77*	**Eco-77#**
Individual	23.33[a]	26.67[a]
Combined	3.33[b]	6.67[b]

*Means followed by the same letter are not significantly different ($P > 0.05$; *LSD = 9.42; #LSD = 14.89)

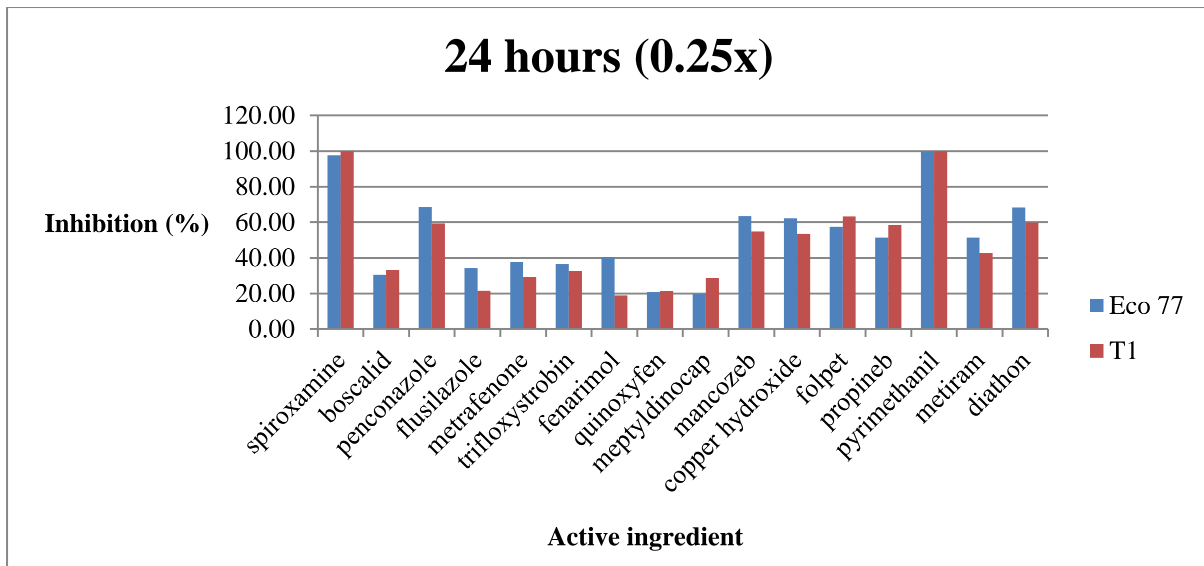

Figure 1. The percentage mycelial growth inhibition of *Trichoderma* species isolates caused by fungicides at 0.25 times the recommended dosage after 24 hours at 25 °C incubation.

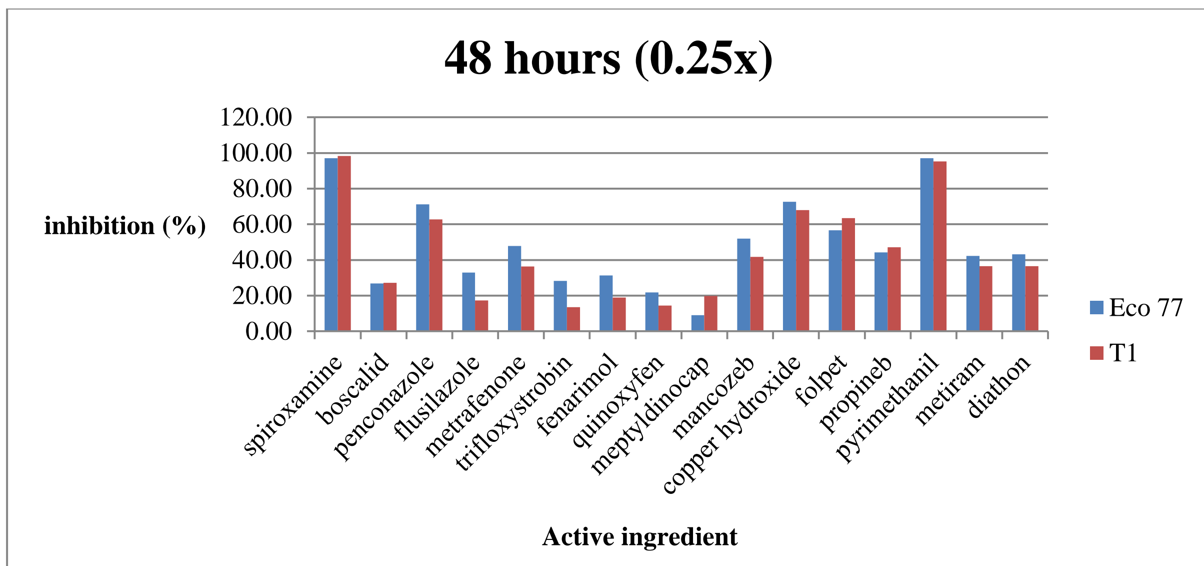

Figure 2. The percentage mycelial growth inhibition of *Trichoderma* species isolates caused by fungicides at 0.25 times the recommended dosage after 48 hours at 25 °C incubation.

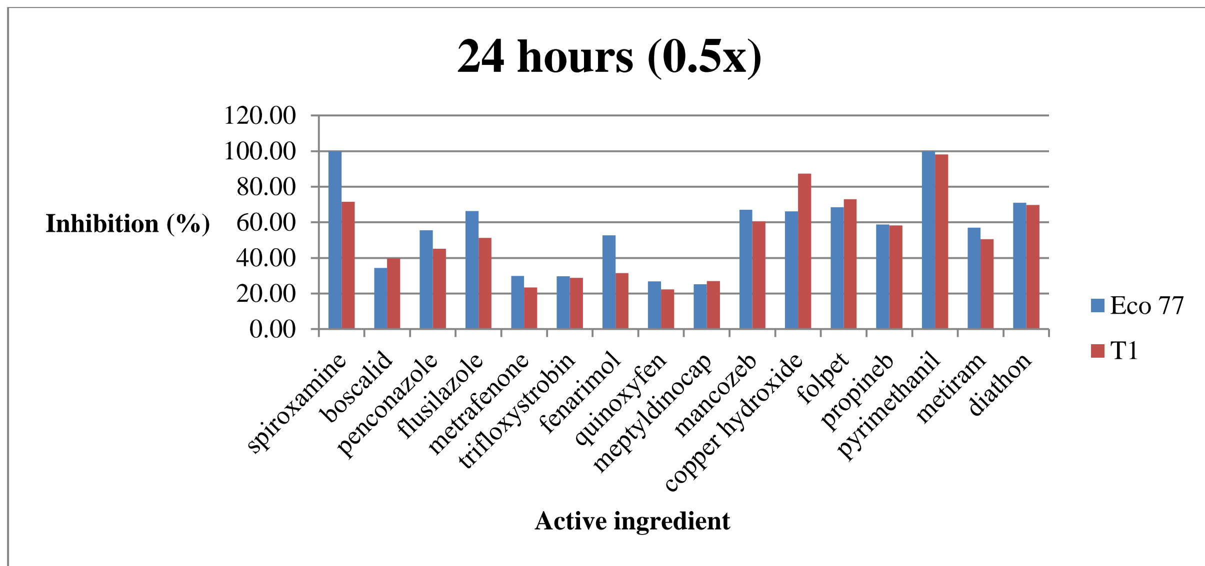

Figure 3. The percentage mycelial growth inhibition of *Trichoderma* species isolates caused by fungicides at 0.5 times the recommended dosage after 24 hours at 25 ^{o}C incubation.

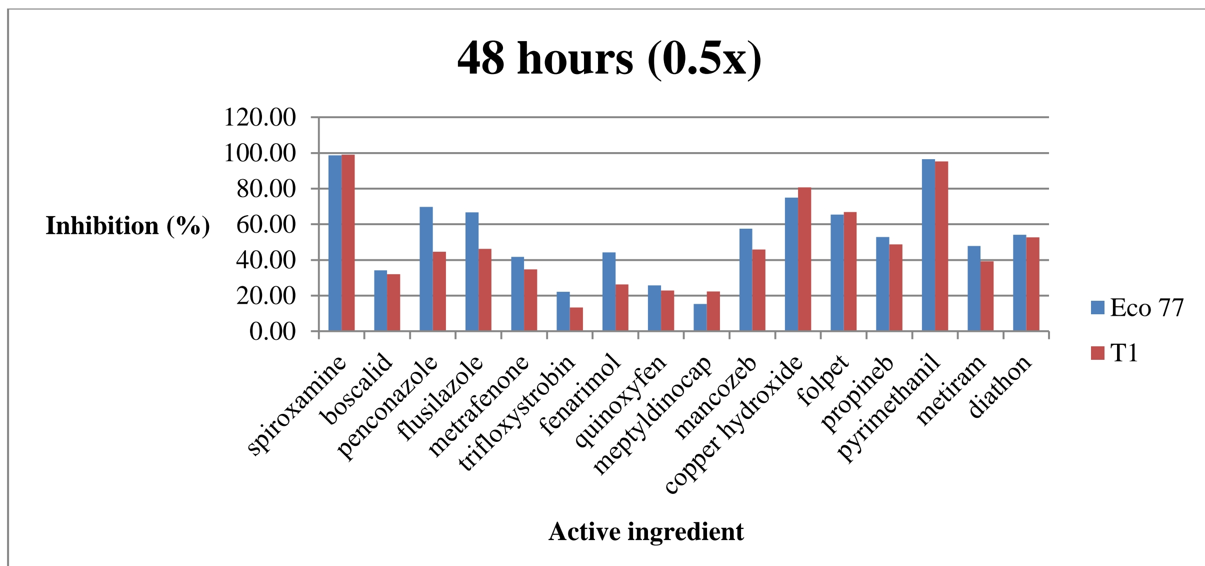

Figure 4. The percentage mycelial growth inhibition of *Trichoderma* species isolates caused by fungicides at 0.5 times the recommended dosage after 48 hours at 25 ^{o}C incubation.

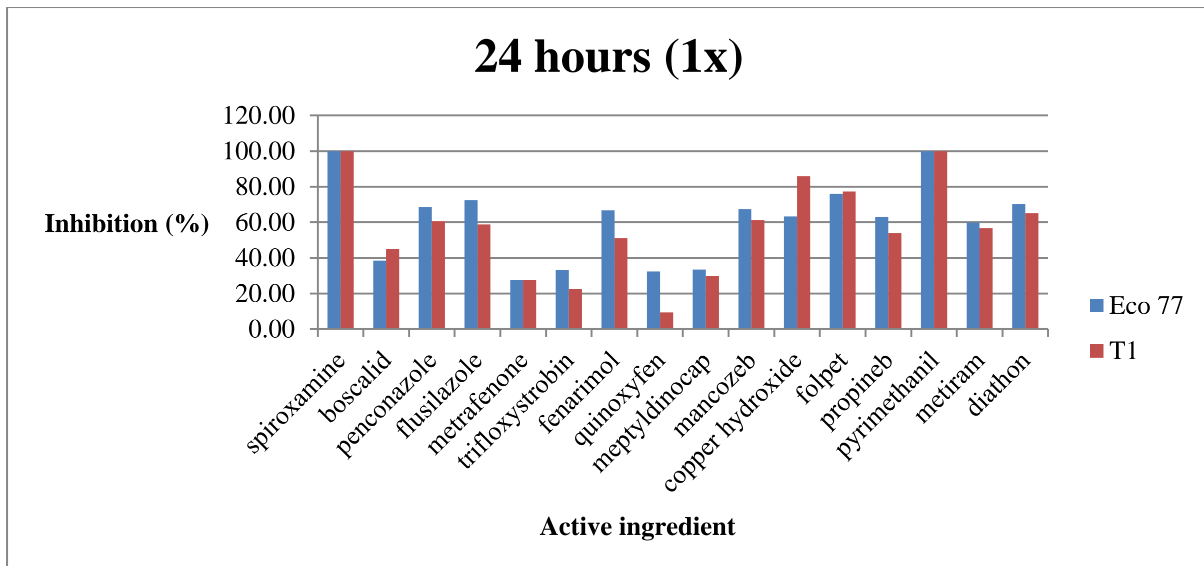

Figure 5. The percentage mycelial growth inhibition of *Trichoderma* species isolates caused by fungicides at 1 times the recommended dosage after 24 hours at 25 °C incubation.

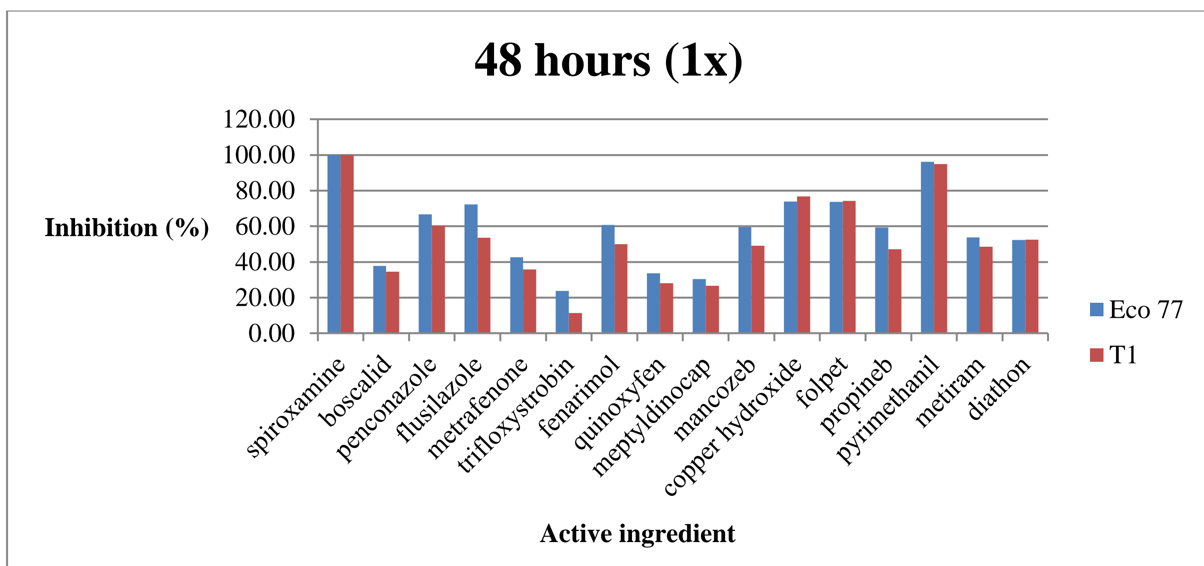

Figure 6. The percentage mycelial growth inhibition of *Trichoderma* species isolates caused by fungicides at 1 times the recommended dosage after 48 hours at 25 °C incubation.

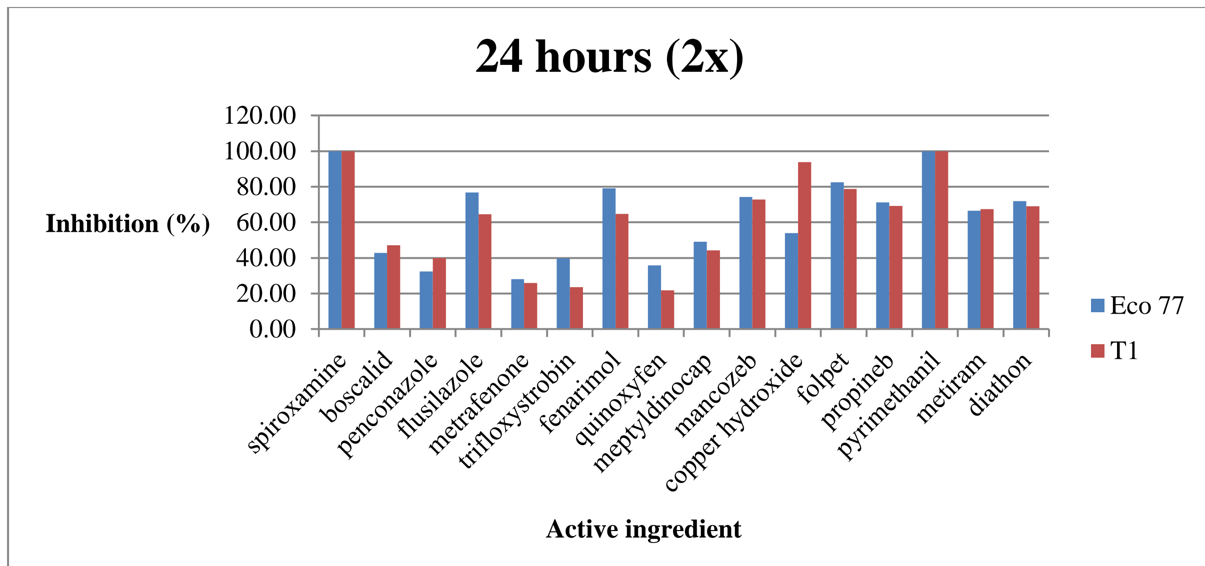

Figure 7. The percentage mycelial growth inhibition of *Trichoderma* species isolates caused by fungicides at 2 times the recommended dosage after 24 hours at approximately 25 ^{o}C incubation.

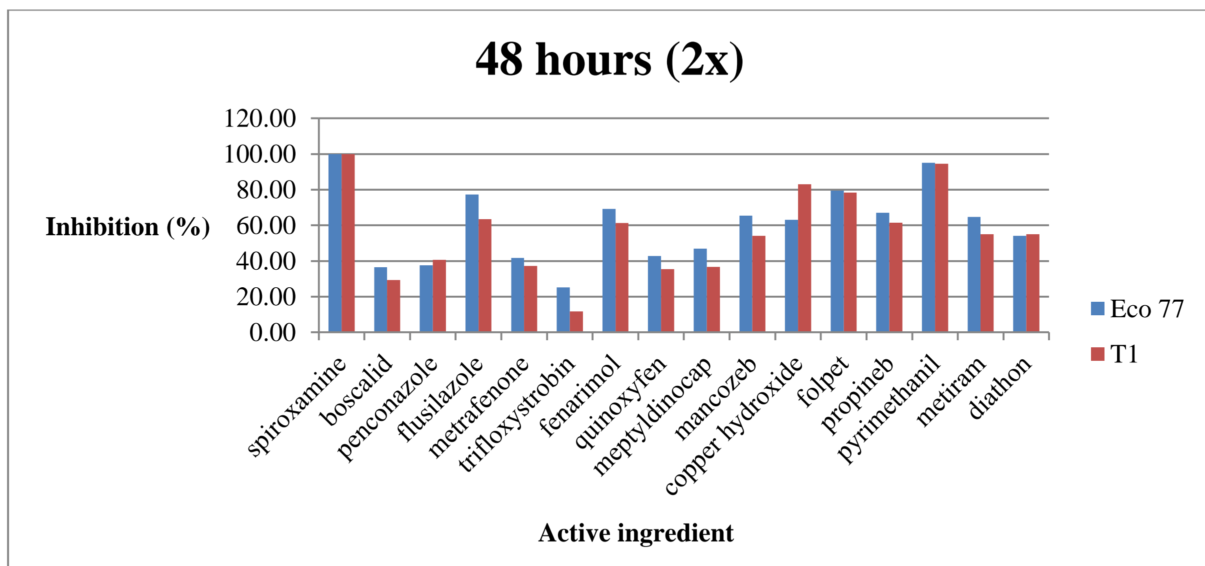

Figure 8. The percentage mycelial growth inhibition of *Trichoderma* species isolates caused by fungicides at 2 times the recommended dosage after 48 hours at 25 ^{o}C incubation.

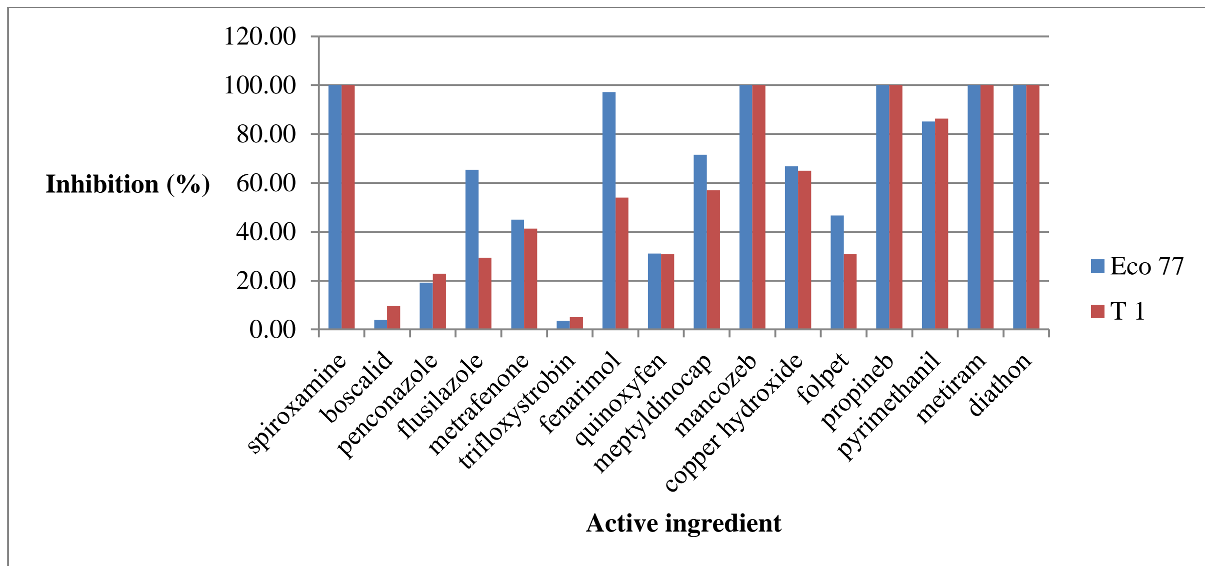

Figure 9. The percentage inhibition of spore germination of *Trichoderma* species isolates caused by fungicides at the recommended dosage after 24 hours incubated in a shaking incubator at 25 ^{o}C. [(LSD = 60.90 (Eco-77®); 54.82 (T1)].

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APPENDIX A

Table 1. Analysis of variance for the percentage incidence of *Eutypa lata* and *Phaeomoniella chlamydospora* re-isolated from sucker wounds of one-year-old Chardonnay and Crimson Seedless plants 3 months after inoculation under controlled conditions.

Source	DF	Sum of Squares	Mean Square	Probability
Cultivar	1	136.11	136.11	0.44
Block	4	244.44	61.11	0.87
Treatment	2	11405.56	5702.78	0.00
Cultivar × Treatment	2	105.56	52.78	0.78

Table 2. Analysis of variance for the percentage incidence of *Eutypa lata* and *Phaeomoniella chlamydospora* re-isolated from sucker wounds of 1-year-old Chardonnay and Crimson Seedless plants 3 months after inoculation under controlled conditions.

Source		Chardonnay			Crimson Seedless		
	DF	Sum of Squares	Mean Square	Probability	Sum of Squares	Mean Square	Probability
Pathogen treatment	2	6866.67	3433.33	0.0009	5422.22	2711.11	0.0001
Error	6	733.33	122.22		266.67	44.44	

Table 3. Analysis of variance for the percentage incidence of *Eutypa lata, Neofusicoccum parvum, Phaeacremonium aleophilum, Phaeomoniella chlamydospora* and *Phomopsis viticola* re-isolated from sucker wounds of 1-year-old Chardonnay (grafted) plants four months after inoculation under controlled conditions.

Source	DF	Sum of squares	Mean square	Probability
Treatment	5	9041.67	1808.33	0.0018
Error	6	650.00	108.33	

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Table 4. Analysis of variance for the percentage incidence of *Eutypa lata, Neofusicoccum parvum, Phaeacremonium aleophilum, Phaeomoniella chlamydospora* and *Phomopsis viticola* re-isolated from sucker wounds of 12-year-old Cabernet Sauvignon 5 months after inoculation under field conditions.

Source	DF	Sum of squares	Mean square	Probability
Block	3	75.00	25.00	0.83
Treatment	3	10325.00	3441.67	< 0.001
Error	9	775.00	86.11	

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Table 5. Analysis of variance for the percentage incidence of *Phaeomoniella chlamydospora* isolated from sucker wounds of 12-year-old Cabernet Sauvignon field vines inoculated at weekly intervals for 4 weeks and assessed after 5 months.

Source	DF	Sum of squares	Mean square	Pr > F
Block	3	200.00	66.67	0.28
Weeks	4	485.00	121.25	0.07
Treatment	1	1440.00	1440.00	<0.0001
Week×Treatment	4	485.00	121.25	0.07

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APPENDIX B

Table 1. Analysis of variance of the incidence of *Phaeomoniella chlamydospora* and *Phomopsis viticola* re-isolated from sucker wounds of Cabernet Sauvignon that were inoculated with individual treatments of the pathogens and combination treatments with Eco-77® 5 months after inoculation.

Source	***Phaeomoniella chlamydospora***				***Phomopsis viticola***			
	DF	**Sum of squares**	**Mean square**	**Probability**	**DF**	**Sum of Squares**	**Mean Square**	**Probability**
Treatment	2	622.22	311.11	0.03	2	3266.67	1633.33	0.07
Error	6	266.67	44.44		6	2333.33	388.89	

Table 2. Analysis of variance of the incidence of *Trichoderma harzianum* re-isolated from sucker wounds of Cabernet Sauvignon that were inoculated with individual treatments Eco-77® and combination treatments with pathogens 5 months after inoculation.

	Trichoderma harzianum						
Source	***Ph. chlamydospora***				***Po. viticola***		
	DF	**Sum of squares**	**Mean square**	**Probability**	**Sum of Squares**	**Mean Square**	**Probability**
Treatment	2	955.56	477.78	0.0018	1155.56	57.78	0.01
Error	6	133.33	22.22		333.33	55.56	

Table 3. Analysis of variance of mycelial growth (diameter) of *Trichoderma* species isolates (*T. harzianum* (Eco-77®) and *T. atroviride* (T1) on PDA Petri dishes amended with 16 different fungicides at four concentrations (0.25x, 0.5x, 1x and 2x) of the recommended dosages after 24 hours.

Source	**DF***	**Eco-77®**			**T1**		
		Type I SS**	**MS*****	***P*********	**Type I SS****	**MS*****	***P*********
Trial	1	980.93	980.93	<.0001	13.21	13.21	0.35
REP(Trial)	4	759.54	189.89	<.0001	205.33	51.33	0.01
Conc	4	266325.60	66581.40	<.0001	240252.45	60063.11	<.0001
Tr×C	4	1440.33	360.08	<.0001	2072.73	518.18	<.0001
Fungicide	15	138380.99	9225.40	<.0001	161567.61	10771.17	<.0001
Tr×Fun	15	19849.35	1323.29	<.0001	12569.25	837.95	<.0001
C×Fun	60	55624.92	927.08	<.0001	63798.46	1063.31	<.0001
Tr×C×Fun	60	19921.30	332.02	<.0001	19218.14	320.30	<.0001

*DF = Degrees of freedom **SS = Square root of squares ***MS = Mean sum of squares ****P = Probability

Table 4. Analysis of variance of mycelial growth (diameter) of *Trichoderma* species isolates (*T. harzianum* (Eco-77®) and *T. atroviride* (T1) on PDA Petri dishes amended with 16 different fungicides at 5 concentrations (0.25x, 0.5x, 1x and 2x) of the recommended dosages after 48 hours.

Source	DF*	Eco-77®			T1		
		Type I SS**	MS***	*P*****	Type I SS**	MS***	*P*****
Trial	1	2.05	2.05	0.63	118.44	118.44	<.0001
REP(Trial)	4	332.86	83.21	<.0001	209.61	52.40	<.0001
Conc	4	243838.38	60959.59	<.0001	201708.40	50427.11	<.0001
Tr×C	4	1271.03	317.76	<.0001	813.037	203.26	<.0001
Fungicide	15	137028.18	9135.21	<.0001	171347.50	11423.17	<.0001
Tr×Fun	15	24056.55	1603.77	<.0001	12027.36	801.82	<.0001
C×Fun	60	57058.45	950.97	<.0001	58893.64	981.56	<.0001
Tr×C×Fun	60	17852.02	297.53	<.0001	12779.57	212.99	<.0001

*DF = Degrees of freedom **SS = Square root of squares ***MS = Mean sum of squares ****P = Probability

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Table 5. Analysis of variance conidial germination of *Trichoderma* species isolates (*T. harzianum* (Eco-77®) and *T. atroviride* (T1) after treatment with 16 different fungicides at the recommended dosages after 24 hours.

Source	**DF***	**Type I SS****	**MS*****	***P*******
Isolate	1	2832.11	2832.11	<.0001
Fungicide	16	390207.23	24387.95	<.0001
Isolate×Fungicide	16	13708.69	856.79	<.0001

*DF = Degrees of freedom **SS= Square root of squares ***MS=Mean sum of squares ****P = Probability