PRUNING WOUND PROTECTION OF ROOTSTOCK MOTHER VINES

by

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DECLARATION

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SUMMARY

One of the factors contributing to the decline in grapevine production across the world is diseases commonly known as grapevine trunk diseases (GTD's). These diseases are typically triggered by xylem-inhabiting pathogens which causes a variety of symptoms and an overall reduction in grapevine production worldwide. To date there is a great concern on the manifestation of these fungal trunk pathogens in rootstock mother vines and their accompanied propagation material. However, there are limited and inadequate information available of the vulnerability of South African rootstocks to these pathogens. Therefore, the objectives of this study were to determine the temporal susceptibility of pruning wounds against *Phaeomoniella chlamydospora* and to assess the protection of pruning wounds of commercial rootstocks with fungicides and biological agents against *Pa. chlamydospora* in South Africa.

Pruning wound susceptibility was determined in certified mother vine blocks with two rootstock varieties most commonly grafted in South Africa, namely Ramsey and US 8-7. This was achieved by inoculations of spore suspensions directly after pruning, as well as 1, 7, 21, and 42 days after pruning. The trials were evaluated after nine months by determining *Pa. chlamydospora* incidences from inoculated wounds. A general decline in *Pa. chlamydospora* incidence was recorded up to 42 days after pruning with wounds challenged 24 hours after pruning being the most susceptible.

In vitro mycelial and germination inhibition studies were conducted in order to determine whether selected chemicals which include chemical fungicide groups such as; benzimidazole, triazole, pyridine-carboxamide and strobilurin are effective against *Pa. chlamydospora*. Mycelial growth inhibition was assessed for six fungicides. The results obtained were used to determine the EC_{50} values for *Pa. chlamydospora* (LM310) and it was observed that all the tested fungicides were effective against *Pa. chlamydospora*. Furthermore, cell viability was assessed using three of the fungicides known to be effective against germination inhibition via a microtiter assay. EC_{50} values for *Pa. chlamydospora* was determined and found to be effective. Chemical fungicides that shown mycelial and germination inhibition, including other control agents, were evaluated in a detached shoot assay by pruning, treating and challenging these shoots (Ramsey, US 8-7 and 101-14 Mgt). After a 4-week incubation period, *Pa. chlamydospora* incidence was recorded and it was found that several of the chemicals were highly effective in lowering *Pa. chlamydospora* incidence, hence protecting these wounds from infection. Consequently, these control agents were further evaluated in field trials conducted in a rootstock mother vine nursery. Pruning wounds were treated with the selected control agents immediately after pruning and challenged with a *Pa. chlamydospora* spore suspension at 1 and 7 days after pruning. The trials were evaluated after nine months by determining the incidence of *Pa. chlamydospora* from inoculated wounds.

The study concludes that integrated control treatments where the biological control agent *Trichoderma atroviride* are applied together with benzimidazole fungicides such as carbendazim or thiophanate-methyl showed to be the most effective in reducing *Pa. chlamydospora* incidence in rootstock mother vine pruning wounds.

Results from this study have provided new information regarding the protection of pruning wounds via the integration of biological and chemical control techniques applied to rootstock pruning wounds at the most susceptible time period. Considering the results obtained in this study it is suggested to prune as early in the dormant season as possible, before the end of May, and the best fungicide chemical group to provide protection for rootstock pruning wounds was benzimidazole in combination with *T. atroviride* applied within 24 hours after pruning occurred. However, further research would be necessary to develop a better understanding to produce protocols and commercial products. The application of these fungicide together with *Trichoderma* spp. in the field holds promise to improve control but would require further trials for possible commercialisation. For successful grapevine propagation and subsequent longevity of new established vineyards, the quality of the planted material plays a crucial role. Therefore, integrating existing information of GTD management in the grapevine propagation process with the knowledge attained from this thesis, can the quality of grapevine material be improved ensuring the success of the South African grape industry.

OPSOMMING

Een van die faktore wat bydra tot die afname in wingerdproduksie regoor die wêreld is siektes wat algemeen bekend staan as wingerdstamsiektes. Hierdie siektes word tipies veroorsaak deur xileem-bewoonde patogene wat 'n verskeidenheid simptome en 'n algehele vermindering in wingerdproduksie wêreldwyd veroorsaak. Tot dusver is daar groot kommer oor die manifestasie van hierdie patogene in wingerde met onderstamme en hul gepaardgaande voortplantingsmateriaal. Daar is egter beperkte en onvoldoende inligting beskikbaar oor die kwesbaarheid van Suid-Afrikaanse onderstamme vir hierdie patogene. Daarom is in hierdie studie die vatbaarheid van snoeiwonde sowel as die beskerming van hierdie wonde van kommersiële onderstamme wat in Suid-Afrika gebruik word vir Petri-siekte, wat deur *Phaeomoniella chlamydospora* veroorsaak word, beoordeel.

Wondgevoeligheid is bepaal in gesertifiseerde onderstok moederblokke met twee van die onderstokvariëteite wat die meeste in Suid-Afrika geënt word, naamlik Ramsey en die US 8-7. Dit is bewerkstellig deur snoeiwonde met spoorsuspensies te inokuleer direk na snoei, sowel as 1, 7, 21 en 42 dae na snoei. Die proewe is na nege maande geëvalueer deur die voorkoms van *Pa. chlamydospora* in geïnokuleerde wonde te bepaal. 'n Algemene afname in die voorkoms van *Pa. chlamydospora* is tot 42 dae opgemerk met wonde wat 24 uur nadat dit gesnoei is die meeste vatbaar was.

In vitro-ondersoeke van mycelia- en ontkiemingsinhibisies is uitgevoer om te bepaal of geselekteerde fungisieds wat chemiese groepe soos; bensamidasool, triasool, piridien-karboksamied en strobilurien effektief is teen *Pa. chlamydospora*. Die inhibering van die swamgroei is bepaal vir ses swamdoders. Die resultate wat verkry is, is gebruik om die EC_{50}-waardes vir *Pa. chlamydospora* (LM310) te bepaal, en daar is waargeneem dat al die swamdoders effektief was teen *Pa. chlamydospora*. Verder is die lewensvatbaarheid van die selle beoordeel met behulp van drie van die swamdoders wat bekend is dat dit effektief is teen ontkieming deur middel van 'n mikrotiter-toets. EC_{50}-waardes vir *Pa. chlamydospora* is bepaal en effektief gevind. In 'n studie met afgesnyde lote is chemikalieë wat getoon het dat dit effektief is deur middel van mycelia- en ontkiemingsstudies, tesame met ander beheermiddels, beoordeel deur wonde te behandel en uit te daag met *Pa. chlamydospore*-spoorsuspensies (Ramsey, US 8-7 en 101-14 Mgt). Na 'n 4-week-inkubasietydperk is die voorkoms van *Pa. chlamydospora* deur middel van isolasies aangeteken, en daar is gevind dat verskeie van die chemikalieë baie effektief was om die voorkoms van *Pa. chlamydospora* te verlaag, en sodoende die wonde teen infeksie te beskerm. Gevolglik is hierdie beheermiddels verder geëvalueer in veldproewe wat uitgevoer is in 'n onderstok-moederkwekery. Snoeiwonde is onmiddellik na snoei met die geselekteerde beheermiddels behandel en met 'n *Pa.* chlamydospora-spoorsuspensie uitgedaag op 1 en 7 dae na snoei. Die proewe is na nege maande geëvalueer deur die voorkoms van *Pa. chlamydospora* van

geinokuleerde wonde te bepaal. Die studie kom tot die gevolgtrekking dat geïntegreerde beheerbehandelings waar die biologiese beheermiddel *Trichoderma atroviride* saam met bensamidasool swamdoders soos carbendazim of thiophante-methyl toegedien word, die doeltreffendste, getoon het om die voorkoms van *Pa. chlamydospora* in snoeiwonde van onderstok moederplante te verminder.

Resultate uit hierdie studie het nuwe inligting verskaf rakende die beskerming van snoeiwonde deur die integrasie van biologiese en chemiese beheertegnieke wat op onderstok snoeiwonde toegepas word op die mees vatbare tyd. Met inagneming van die resultate wat in hierdie studie verkry is, word voorgestel dat daar so vroeg as moontlik in die dormante seisoen, voor die einde van Mei, gesnoei word. Die beste chemiese groep wat beskerming bied vir die snoeiwonde van onderstokke, is bensamidasool in kombinasie met *Trichoderma atroviride* wat binne 24 uur na snoei toegedien moet word. Verdere navorsing sou egter nodig wees om 'n beter begrip van protokolle en kommersiële produkte te produseer. Die toediening van hierdie swamdoder saam met *Trichoderma* spp. op onderstok snoeiwonde beloof om beheer te verbeter. Dit sal verdere proewe benodig vir moontlike kommersialisering. Vir die suksesvolle voortplanting van wingerde en gevolglike lewensduur van nuwe gevestigde wingerde, speel die kwaliteit van die aangeplante materiaal 'n belangrike rol. Deur die bestaande inligting van wingerd stamsiekte-bestuur in die wingerdvoortplantingsproses te integreer met die kennis wat uit hierdie tesis verkry is, kan die kwaliteit van wingerdmateriaal dus verbeter word om die sukses van die Suid-Afrikaanse wingerdbedryf te verseker.

I dedicate this MSc thesis to my late grandmother **Ouma Nettie Groenewald**. Without her love, kindness, support, motivation and prayers over the years, I would not be where I am today.

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CONTENTS

CHAPTER 1

A review of Petri disease pathogens in South African nurseries and the management thereof

INTRODUCTION TO GRAPE PRODUCTION IN SOUTH AFRICA

Since the 17th- century grapevine (*Vitis vinifera* L.) production in South Africa was already initiated by Dutch explorers who imported propagation material from Europe and the Middle East. The Mediterranean climate with long summers and mild, wet winters formed the ideal habitat to produce grapes, a product that has given pleasure to man for many centuries. These explorers produced amongst the most popular wines and by the 18th century, these wines were already exported. Since then the production of grapes expanded exponentially and to date, approximately 119 181 ha of South African land is under grapevine, consisting of approximately 292 million vines, 106 018 ha for wine production, 18 212 ha to produce table grapes and 200 ha for rootstock mother fields (SAWIS, 2018).

The grape industry in South Africa is amongst the highest growing agricultural sectors, which generates a significant amount of foreign exchange. Grapevines are one of the most economically important perennial fruits grown in the world. The importance of the grape industry can be seen through its contribution to employment, an estimate of 353 000 people is employed either directly or indirectly in all sectors of the industry ranging from farm workers, retail to tourism (SAWIS, 2015). An annual harvest of 1 405 401 tons of wine grapes was recorded in the year 2017 of which 82% was used for winemaking. An estimate of R9.0 billion was generated in the year 2017 by wine exports alone (SAWIS 2018). South Africa contributes 3.9% of the world's wine, which places South Africa as the 7th largest wine producing country in the world (SAWIS 2018). According to The National Agricultural Marketing Council (NAMC) 225 thousand metric tons of table grapes are exported annually, mainly to European countries, placing South Africa in the top five table grape exporters in the world (NAMC, 2018).

Cultivation of grapevines requires extensive financial investment due to initial vineyard establishment and annual vineyard operations. A significant percentage of these costs are attributed to pest and disease management programs. The control programs of pest and disease include; cultural, chemical and biological control techniques. *Vitis vinifera* is known to be a host of a wide variety of pathogens including 29 fungal diseases (Bertsch *et al.,* 2013; Wilcox *et al.,* 2016). The most common and most destructive fungal disease complex known is grapevine trunk diseases (GTD's) (Mugnai *et al.,* 1999). Up to 133 fungal species have been associated with GTD although not all have been proven to be pathogenic using Koch's postulates (Gramaje *et al.,* 2018). Nonetheless, GTD is known to be the largest complex of

fungal pathogens to infect grapevines (Gramaje *et al.,* 2011; Cloete *et al.,* 2015). Grapevine trunk diseases are caused by fungi which primarily invades through annual pruning wounds, which provide infection sites each season during the life of the grapevine. Considering the impact of the grape industry, South Africa depends on the success and sustainability of this agricultural sector and therefore it is of utmost importance ensuring that our vineyards are free of disease, producing a high yield of excellent quality grapes.

Impact of grapevine trunk diseases

Grapevine trunk diseases have been affecting viticulture for many years and these fungal trunk disease pathogens represent one of the most important problems for grape production worldwide (Bertsch *et al.,* 2013; Mondello *et al.,* 2017). Although GTD's have been known for centuries their importance in grapevine health has only been recognized recently. A phenomenon that has been observed for the last 20 years in particular is the decline in the phytosanitary status of rootstock mother vines and propagation material (Fourie *et al.,* 2002). There are several problems linked to mother fields in South Africa including; high pathogen spore counts in the air and the presence of pathogens inside mother vines and their canes (Fourie *et al*., 2002; Fourie *et al.*, 2004a; Oliveira *et al*., 2004; Sieberhagen *et al*., 2017). The decline in the phytosanitary status of mother vines in South Africa contributes to a lower productivity of newly planted vines in vineyards. It is also evident at an earlier stage in the nursery as at this stage infected propagation material has a low take percentage due to several factors including the decline in the phytosanitary status. Consequently, these effects result in financial losses in South African nurseries along with costs associated with re-establishing infected vines (Scheck *et al.,* 1998; Sieberhagen *et al.,* 2017).

Three main diseases are responsible for vine decline during the propagation process and can act alone or as a complex in infected vines (Gramaje *et al.,* 2011; Bertsch *et al.,* 2013). These diseases include; Petri disease, black-foot disease and Botryosphaeria canker and dieback (Gramaje *et al.,* 2011). *Phaeomoniella chlamydospora* (W. Gams, Crous & M.J. Wingf. & L. Mugnai) Crous & W. Gams has been more often associated with dark-brown to black discoloration of xylem tissues in the wood, that is the typical Petri disease symptoms associated with rootstock mother plants (Fourie *et al.*, 2004a), with an important occurrence described on grapevine in South Africa (Fourie and Halleen, 2004a), and for these reasons *Pa. chlamydospora* was the primary focus in this study (Mugnai *et al.,* 1999; Gramaje *et al.,* 2011).

Petri disease

Petri disease has been increasingly reported in grapevine producing areas worldwide (Bertelli *et al.,* 1998; Halleen *et al.,* 2003; Fourie *et al.,* 2002). The decline of grapevines caused by Petri disease occurs frequently in young vines from 1 to 7 years of age. Petri disease, formerly also known as “black goo” was initially discovered in Italy over a 100 years ago (Mugnai *et al.,* 1999). This pathogen is also known as a major component of the esca disease complex in older vineyards (Surico *et al.,* 2008).

Casual pathogens

Petri disease is caused by mitosporic fungi *Phaeomoniella (Pa.) chlamydospora* and several *Phaeoacremonium (Pm.)* W. Gams, Crous & M.J. Wingf species, of which *P. minimum* is the most widely distributed *Phaeoacremonium* species (Spies *et al.,* 2018). Previously, Ferreira *et al.* (1994) identified the causal agent of ‘black goo” as *Phialophora parasitica* after which Crous *et al.* (1996) proposed a new genus; *Phaeoacremonium,* which included *Pm chlamydosporum.* Moreover, deepening the previous work with *Pm. chlamydosporum*, Crous and Gams (2000) proposed this pathogen as a new specie called as *Phaeomoniella chlamydospora*, based in cultural and morphological characteristics. Since then this disease was reported in a number of different countries including South Africa (Bertelli *et al.,* 1998; Surico *et al.,* 2008). Although several *Phaeoacremonium* species are associated with Petri disease, *Pa. chlamydospora* is generally regarded as the main causal agent of Petri disease in grapevine (Mugnai *et al.,* 1999; Crous *et al.,* 2000; Fourie *et al.,* 2002). However, *Pa. chlamydospora* also has been described as an important fungal trunk pathogen in adult grapevine associated with esca and esca-like symptoms (Mugnai et al., 1999; Díaz *et al.,* 2014). Where applicable, the remainder of this review focused on *Pa. chlamydospora* (Scheck *et al.,* 1998; Mugnai *et al.,* 1999; Spies *et al.,* 2018).

Symptoms

Petri disease is associated with a variety of external symptoms which all lead to the reduction of growth and the overall productivity of the infected plants. Symptoms such as dieback of shoots, slow decline and gradual death of vines are all common aerial symptoms which are related to Petri disease infection (Gramaje *et al.,* 2011).

The types of symptoms associated with this disease are quite general and therefore a thorough examination of the internal symptoms is required for accurate diagnosis. Some of these internal symptoms are easily identified as dark-coloured phenolic compounds in the xylem vessels of the cordons, trunks and rootstocks in cross-section. In severe cases, a black phenolic substance oozes from the discoloured tissue which is then referred to as “black goo”

(Mugnai *et al.,* 1999). These dark discolorations can be sparsely distributed or clustered in small groups around the growth ring and closer to the pith (Khan *et al.,* 2001). The pith itself can be darkened due to infection (Bertelli *et al.,* 1998; Mugnai *et al.,* 1999).

Epidemiology

Asymptomatic propagation material, infected soils, and aerial inoculum are potential sources of inoculum within vineyards (Khan *et al.,* 2001; Ridgway *et al.,* 2002; Halleen *et al.,* 2003; Oliveira *et al.,* 2004). Retief *et al.* (2006) reported that propagation material has the potential to get infected during the grafting process. Using molecular techniques, it has been confirmed that *Pa. chlamydospora* is present in hydration tanks, tools used for grafting as well as callusing media used in the propagation process (Retief *et al.,* 2006). Several species of *Phaeoacremonium,* as well as *Pa. chlamydospora,* have been frequently isolated from rootstocks before as well as after nursery establishment. This indicates that infections can derive from nursery operations and infected mother material (Halleen *et al.,* 2003; Rooney-Latham *et al.,* 2005b; Larignon *et al.,* 2006b). Both spores and hyphae of *Pa. chlamydospora* have been detected in tested rootstock canes and therefore it has been hypothesized that these spores are translocated in the sap flow of mother plants which then causes contamination of the harvested canes (Edwards *et al.,* 2004; Gubler *et al.,* 2004). Considering the above mentioned an overview of the potential inoculation points in the propagation process will be discussed later in this thesis.

The grapevine propagation process

Majority of South African grapevine nurseries are situated close the town of Wellington in the Western Cape Province due to the ideal soils and climatic conditions required for plant propagation. Farms in these regions produce millions of grafted vines of wine-, table- and raisin grapes annually (Van Niekerk, 2018).

Although the grapevine propagation process in principal is relatively easy, it requires a significant amount of skills and organisation to yield millions of viable vines which are needed each season for new plantings and replanting of unproductive vineyards, including diseased vines (Waite *et al.,* 2015). Some of the methods required for propagation includes; *in vitro* propagation, softwood cuttings and field grafting of the rooted rootstock cuttings. The most widely used method of propagation entails grafting dormant one bud *V. vinifera* cuttings onto rootstock (*Vitis* spp.) cuttings and propagating them as one plant (Gramaje *et al.,* 2011; Waite *et al.,* 2015). Despite the efforts for modernisation the challenge for obtaining a constant supply of healthy uniform vines remains a problem.

Steps in the propagation process

In grapevine propagation, the fundamental steps used within the process are parallel in major production areas across the world. These traditional techniques start with the planting and maintenance of rootstocks and scion mother blocks from which dormant cuttings are taken for grafting, rooting and budding (Gramaje *et al.,* 2011). Though, some common practices include hydration and cold storage they vary depending on the country and the individual nursery (Fourie *et al.,* 2006; Gramaje *et al.,* 2011).

In South Africa the first step in the grapevine production process is laboratory-based to produce nucleus plants. The aim of this step is to produce clean disease-free (mostly referring to viruses) plants before transferring them to foundation blocks which is the second step in the process. These foundation blocks are responsible for the multiplication and evaluation of vines according to the rules and regulation set by the South African Plant Improvement Act. Certified dormant rootings are then planted in mother blocks to produce cuttings which will form the rootstocks when grafted. Rootstock mother vines generally require 3 years to produce generous amounts of cuttings. Once these cuttings are collected and prepared (including de-budding, cutting in required lengths and tying in bundles of 100) during autumn and early winter they are subjected to a hydration period of between 1 and 12 hours following cold storage (1-6°C) at 90% humidity. Generally, fungicides such as Rovral [Iprodine: 3 – (3,5 – dichlorophenyl) – N - (1–methylethyl) – 2,4 – dioxo – 1 – imidazolidinecarboximade] and other biocides are added to the immersion water for the purpose of limiting superficial fungal growth during storage (Smith *et al.,* 2012; Gramaje *et al.,* 2015). Following cold storage in late winter or early spring, these cuttings are soaked for periods of 2 to 4 hours or up to 4 days before grafting. Immediately after grafting the graft unions are dipped in melted (70-85°C) wax formulations which in some cases may contain plant growth regulators or fungicides and packed in callus boxes containing pine sawdust soaked in broad spectrum fungicides. Sawdust is used to prevent dehydration of the grafted vines during storage. These callus boxes are held in humidified rooms (70% Relative Humidity) at 18°C-28°C for up to 3 to 5 weeks following a 1 to 2 week hardening off period under shade netting depending on the specific variety and grafting method used (Fourie *et al.,* 2006; Retief *et al.,* 2006). After successful callusing, around October, these graftlings are trimmed, dipped in a suitable grafting wax and transplanted in an open nursery field. They are covered with soil which is then later removed following bud burst (Fourie *et al.,* 2006; Retief *et al.,* 2006). This technique limits potential drying of the callus, but also causes an increase in the occurrence of soil borne pathogens in this plant zone, therefore it is rarely used in other countries (Gramaje *et al.,* 2011). At this point roots that develop from the graft union are removed as the removal of these roots promotes the formation of fibrous roots and also facilitates handling at the end of

the season (Waite *et al.,* 2015), but trimming also creates new wounds making the vine vulnerable to infection by common soil borne pathogens such as black foot pathogens (Waite *et al.,* 2015).

During the 8-month field nursery period, these graftlings are frequently irrigated and treated with fungicides for the control of powdery and downy mildew (Fourie *et al.,* 2006; Retief *et al.,* 2006). In autumn these dormant nursery plants are uprooted and cold stored until replanting in spring (Fourie *et al.,* 2006; Retief *et al.,* 2006; Gramaje *et al.,* 2011).

Potential inoculation points in the propagation process

Unitil recently, there was not much known about the disease cycle of *Pa. chlamydospore*, various aspects related to the source of inoculum, port of entry and the spread of *Pa. chlamydospora* during the propagation process of grapevines have come to the fore (Gramaje *et al.,* 2011).

1) *Rootstock and scion mother fields*

Although there are conflicting data both affirming and negating the contribution of scion mother vines to disease in young vines; the role of rootstock mother vines as a primary source of GTD's has been well documented by quite a few authors (Bertelli *et al.,* 1998; Halleen *et al.,* 2003; Giménez-Jaime *et al.,* 2006; Gramaje *et al.,* 2011). According to Edwards *et al.* (2004) and Petit *et al.* (2006) rootstocks and scion mother vines are both a source of infection. Some nursery practices in Australia and Spain allows mother vines to sprawl on the soil surface and with typical flood irrigation practices, it can result in water-soaked cuttings (Edwards *et al.,* 2004; Petit *et al.,* 2006; Gramaje *et al.,* 2011). In the past some nurseries in South Africa and California have cultivated rootstocks on a trellis system which although this method is much more labor intensive, avoids contact with any potential soil-surface pathogen infection, subsequently producing higher quality cuttings (Fourie *et al.,* 2002, 2004a; Retief *et al.,* 2006). Fourie *et al.* (2004a) have provided evidence that *Pa. chlamydospora* have been detected in rootstock mother plants leading them to conclude that the primary source of Petri disease is, in fact, *Pa. chlamydospora* hence providing evidence that rootstock mother plants are a primary source of inoculum within the grapevine propagation process (Gramaje *et al.,* 2011). Other species such as *Pm. minimum* and *Cylindrocarpon* have also been detected within these rootstocks mother plants, however, the incidence of Petri disease caused by these fungal trunk pathogens was extremely low (Ferreira *et al.,* 1999). Reports showing the presence of *Pa. chlamydospora* isolated from symptomless grapevines proves that this fungus has the ability to behave as a latent pathogen, only when the grapevines are stressed will the

disease symptoms become visible (Ferreira *et al.,* 1999). The last-mentioned highlights the possibility of mother vines as an inoculum source in the grapevine propagation process.

Studies have shown that infection can pass systemically within the xylem vessels as conidia and active mycelium of *Pa. chlamydospora* have been detected in these vessels within shoots, hence it has been hypothesized that pathogen spores are transported in the sap flow of mother plants consequently leading to the contamination of canes (Edwards *et al.,* 2004; Fourie *et al.,* 2006; Gramaje *et al.,* 2011). Therefore, the pathogen has the ability to move from the rootstock mother plant into the current season's growth. From these studies, it is evident that mother plants have the potential to perform as reservoirs of inoculum from which GTD pathogens infect cuttings.

The question is: how do these mother plants become infected? The first explanation could be the fact that soil could act as an inoculum source of the pathogen (Bertelli *et al.,* 1998; Surico, 2000; Gramaje *et al.,* 2011). It has been hypothesized that *Pa. chlamydospora* is a soil borne pathogen as it has the ability to produce chlamydospores in culture (Gramaje *et al.,* 2011). Rooney-Latham *et al.* (2001) showed that this pathogen species can withstand and survive a wide range water potential therefore aiding as a possible survival strategy in soil. Bertelli *et al.* (1998) suggests that these chlamydospores can potentially penetrate roots of grapevines in mother fields, nurseries and vineyards. Studies performed in South Africa supports the above-mentioned hypothesis, as *Pa. chlamydospora* conidia, mycelia and chlamydospores were detected originating from infected mother plants in infected soils (Retief *et al.,* 2006). Ridgway *et al*. (2002) also detected *Pa. chlamydospora* DNA from soils inoculated with spores; although not proven viable; it could indicate that these spores have the potential to persist in soils and that they might have the ability to build up over time.

Pathogenicity studies also revealed a second explanation to initial mother plant infection as this pathogen can infect dormant vines through pruning wounds (Adalat *et al.,* 2000; Rooney-Latham *et al.,* 2001; Fourie *et al.,* 2006; Eskalen *et al.,* 2007). Edwards *et al.* (2001, 2004) demonstrated that *Pa. chlamydospora* produces a Phoma-like synanamorph on debris which could potentially result in inoculum dispersal following rain and irrigation which would lead to infection of pruning wounds. Baloyi *et al.* (2016) showed that spores produced from pycnidia could infect pruning wounds and potentially cause disease. Until recently there was little evidence of insect transmission of GTD pathogens as observed in other systems (Gubler *et al.,* 2001). This type of dispersal has been confirmed by a study performed by Moyo *et al*. (2014) that demonstrated the transmission of *Pa. chlamydospora* by ants and Portuguese millipedes to grapevine pruning wounds.

2) Storage of propagation material, grafting and callusing

Besides rootstocks and scions being the earliest point of infection within the propagation process, another early stage where infection can take place is during the postharvest soaking period prior to the cold storage (Gramaje *et al.,* 2011). According to Gramaje *et al.* (2011) these soaking baths gets contaminated with field-acquired microorganisms on the bark of the cuttings dispersing into the soaking water. Whiteman *et al.* (2004) demonstrated that in New Zealand, the infection rate of *Pa. chlamydospora* detected in cuttings before the nursery process compared to after the processing was significantly higher. Whiteman *et al.* (2004) also demonstrated that pre-storage and pre-grafting hydration tanks as a potential source of inoculum. Another study performed by Retief *et al.* (2006) supported Whiteman *et al.* (2004) results showing a high rate of *Pa. chlamydospora* from water samples collected after the hydration period in South African nurseries. According to literature, conidia are present on the bark of cuttings which washes off into the water during hydrations (Edwards *et al.,* 2007). They also suggest that these conidia might also ooze from the xylem vessels during hydration (Retief *et al.,* 2006). The fungus *Pa. chlamydospora* has also been detected in hydration baths used for post-storage hydration purposes. In Italy, Pollastro *et al.* (2009) detected surface pathogens of pre-grafting and pre-callusing water baths. This study indicated that *Pa. chlamydospora* was detected in both instances. Commercial cool down baths is a common practice used at South African nurseries and it is suggested that even if these baths are treated with chlorine, the water is not sterile and therefore it is considered as a potential inoculation source of *Pa. chlamydospora* (Retief *et al.,* 2006; Edwards *et al.,* 2007; Gramaje *et al.,* 2011).

Throughout the cutting and grafting preparation stages, several wounds are created. These wounds leave the cuttings very susceptible to pathogens such as *Pa. chlamydospora* and as previously mentioned, GTD pathogens have the potential to colonize these wounds (Smith *et al.,* 1994; Graniti *et al.,* 2006; Larignon *et al.,* 2006b). Another study in Italy performed by Zanzotto *et al.* (2009) examined the occurrence of Petri disease-causing pathogens on rootstocks and scions during grafting and explained that *Pa. chlamydospora* was detected from grafted material and therefore indicated that grafting is a possible source of inoculum. Researchers in New Zealand stated that *Pa. chlamydospora* were detected during the grafting process and Retief *et al.* (2006) supported this statement proving that there is sufficient evidence of *Pa. chlamydospora* presence during grafting (Whiteman *et al.,* 2004). In Spain *Pa. chlamydospora* DNA was detected from grafting tools and in post grafting cuttings (Aroca *et al.,* 2010). Vigues *et al.* (2008) described that the incidence of *Pa. chlamydospora* in the propagating material increases throughout the nursery procedure.

The climatic conditions (26-28°C with high humidity) created by callusing boxes produce a thriving environment for pathogens such as *Pa. chlamydospora*. In South Africa,

Halleen *et al.* (2003) reported a high percentage of different *Phaeoacremonium* species and *Pa. chlamydospora* isolated from callused cuttings prior to planting. In correlation to this study Whiteman *et al.* (2004) found similar results from washings of callusing media. Another study performed in Australia reported a reduction in the quality of vines due to the inhibition caused by *Pa. chlamydospora* within the callus (Wallace *et al.,* 2001). Several studies noticed a potential of *Pa. chlamydospora* contamination during the callusing stage when bringing inoculated plants into contact with healthy ones (Larignon *et al.,* 2006a; Edwards *et al.,* 2007; Vigues *et al.,* 2008; Gramaje *et al.,* 2011).

Majority of these studies used molecular techniques for the detection of pathogen genomic DNA and therefore it is important to note that relying on the presence of DNA alone does not specify whether viable pathogen propagules are present (Gramaje *et al.,* 2011). Nevertheless, there are studies that detected viable propagules of *Pa. chlamydospora* from hydration baths, pruning equipment and machines used for grafting (Aroca *et al.,* 2010).

3) Planting of graftings and growth in the nursery and uprooted, dormant vines

Grapevine trunk diseases have been related to the failed establishment of vines in nurseries for years. Overall, infected cuttings have shown to be slow in establishment and in severe cases, these vines never make satisfactory growth (Gramaje *et al.,* 2011). Giménez-Jaime *et al.* (2006) performed several experiments and concluded that *Pa. chlamydospora* are present at each stage of the propagation process. Fourie *et al*. (2004a) has also suggested possible *Pa. chlamydospora* contamination during the propagation process using untreated water, soil or dust. In Spain, Giménez-Jaime *et al.* (2006) showed detection of *Pa. chlamydospora* from grafted plants two months after they were planted in the nursery field. A similar survey performed in Australia also isolated *Pa. chlamydospora* form 1-year old grafted plants which showed symptoms related to Petri disease (Edwards *et al.,* 2004). Studies testing for the incidence of *Pa. chlamydospora* from grafted vines in Spain, showing symptoms of Petri disease were also conducted by Gramaje *et al.* (2010a) and showed that *Pa. chlamydospora* caused vine decline in nursery fields. In Portugal it has also been shown that dormant grafted rooted cuttings which are ready to be commercialized were contaminated with *Pa. chlamydospora* and other GTD causing pathogens (Spagnolo *et al.,* 2011). Although Petri disease-causing agents are frequently isolated from failed gaftlings in South African nurseries (Fourie *et al.,* 2004a) and regardless of the amount of work proving the occurrence of GTD causing pathogens in the graftings at the end of the grapevine propagation process, there are authors still questioning the role of the isolated fungi in the poor performance of vines in the nursery fields and in the vineyards (Gramaje *et al.,* 2011). According to Rumbos *et al.* (2001) these GTD causing pathogens cannot by themselves be the cause of young grapevine

decline. Other factors including abiotic stresses such as poorly healed rootstocks, graft unions, and improper storage as well as transportation facilities essentially also plays a role contributing to the decline of these young vines (Gramaje *et al.,* 2011). According to Zanzotto *et al.* (2009) 1-year old grafted vines from the vineyard had significant lower rates of *Pa. chlamydospora* when compared to the original stock of grafted vines. Therefore, it has been hypothesized that in the first 12 months of cultivation within the vineyard the rate of *Pa. chlamydospora* infection could be masked due to competition with other microorganisms in the environment (Gramaje *et al.,* 2011).

Management of grapevine trunk diseases during the propagation process

The health of a grapevine essentially determines the success as well as the sustainability of vineyards. Grapevine farmers, therefore, trust nurseries to produce true to type and disease-free vine stock. This puts commercial nurseries under tremendous pressure as it is not possible for them to guarantee the production of fungal trunk pathogen-free stock. During the propagation process a great number of cuts and wounds are made consequently exposing the plant material to possible fungal trunk pathogens which could perhaps lead to infection. At present there are no curative measures for the control of Petri disease in grapevine nurseries and young vineyards. The management of these endogenous pathogens in woody plants such as vines is challenging. In South African vineyards, control tactics suggested for prevention and management of diseases focusses primarily on the prevention and the correction of stress situations (Fourie *et al.,* 2001). As previously mentioned, there are several opportunities for infection to take place during the grapevine propagation process as wounds are created at almost every stage of the process. Some other possible opportunities for infection are when graft unions do not heal properly, poor cold storage- and poor transport conditions.

There have been some improvements in the development of new measures, procedures and products which focusses on prevention and reduction of woody tissue infections caused specifically by fungal trunk pathogens during the grapevine propagation process. As a result, proper hygiene and wound protection are considered as the most important prevention mechanism. Other important strategies such as chemical-, physical- and biological control are also commonly used as well as hosts resistance. Therefore, Petri disease would be best managed using an integrated strategy which combines preventative measures, control techniques during the nursery mother blocks, the nursery process, propagation beds and newly established vineyards.

Nursery mother blocks

1) Pruning wound protection

When assessing current-year shoots, the reduction in *Botryosphaeriaceae* infection has been reported when treating trimmed wounds with fungicides during the mother block stage (Billones-Baaijens *et al.,* 2015). Unfortunately, protection strategies for the prevention of Petri disease in dormant pruning wounds remains scarce and it is unclear to what extent pruning wound protection is practiced. In South Africa it is not practiced in general and if used it is unclear which products are used. This issue is therefore to be addressed in this thesis.

2) Sanitation and cultural control

The role of mother vine management in the production of quality propagation material has been given little attention and there is also a lack of literature available on this topic. Gramaje *et al.* (2018) reported that several cultivation practices in mother plants can have a direct effect on trunk disease incidence and thus in the quality of graft material. According to Gramaje *et al.* (2015) nurseries that cultivates rootstock mother vines using a trellis system subsequently providing greater shoot mass as well as higher/longer quality shoots compared to those rootstock mother vines grown along the soil surface. Although using a trellis system might lower rates of infection with fungal pathogens it is reported that using this technique is very expensive and highly labour intensive and therefore not as commonly used (Hunter *et al.,* 1999). Whiteman *et al.* (2004) has shown that growing shoots along the soil surface exposes shoots to a variety of soil-borne pathogens and due to high temperatures and humidity levels these shoots are more susceptible compared to those shoots grown in a vertical position. Trellising also eliminate possible mechanical damage. Soil-borne pathogenic fungi favours high moisture levels and therefore it is of importance to expose these sprawling shoots to adequate aeration (Toussoun *et al.,* 1970). The above mentioned can be achieved by planting on elevated beds and insuring drip irrigation emitters does not overlap the vines (Gubler *et al.,* 2013). These irrigation systems are also commonly used in commercial production vineyards. Waite *et al.* (2015) suggested that overhead irrigation can also be used provided that these sprinklers uniformly distribute sprays at an adequate height to clear the foliage. But in California a study has shown that overhead irrigation also has the potential to initiate the release of *Botryosphaeriaceae* and *Pa. chlamydospora* conidia in vineyards (Rooney-Latham *et al.,* 2005a; Gubler *et al.,* 2013). Other cultural techniques such as removing dead wood and other plant debris from the surrounding soil surfaces is strongly recommended as these materials act as possible sources of fungal inoculum keeping in mind that many fungal fruiting bodies can persists in these dead plant materials (Boloyi *et al.,* 2016; Gramaje *et al.,* 2018).

Nursery propagation process

1) Cultural practices

A variety of studies has shown that viable propagules of fungal pathogens are present on washed pruning equipment and hydration tanks commonly used in the grapevine propagation process (Retief *et al.,* 2006; Gramaje *et al.,* 2011). Since the propagation process promotes GTD infection it threatens the phytosanitary statuses of these cuttings when they are exposed to contaminated water for long periods of time (Gramaje *et al.,* 2011). Gramaje *et al.* (2018) stated that contaminated wounds and poorly matched graft unions fails to heal properly, remain open to fungal infection, and create structural weakness in the finished vines. High temperatures are commonly used during the callusing stage of the propagation process and subsequently weakens callus unions also making them more vulnerable to infection (Waite *et al.,* 2015). The environmental conditions of these callusing rooms make it especially favourable for some fungal pathogens (Hartmann *et al.,* 2001). It is therefore suggested that these callusing rooms are disinfected on a regular basis. Waite *et al.* (2015) also suggested that any stress conditions including; dehydration, excessive wounding and extreme temperatures should be avoided during this time.

2) Chemical control

Hydration tanks, boxes, post-grafting and storage periods are some of the focal points important for chemical control in both mother blocks and newly established vineyards (Gramaje *et al.,* 2018). Unfortunately applying fungicides during the propagation process is much more difficult than anticipated as these chemical dips and sprays mainly eliminate external pathogens due to their inability of penetrating the cuttings (May *et al.,* 2005). Regardless of the reports stating the effectiveness of chemical applications and although fungicide application during the propagation process are commonly used in nurseries worldwide, there are still reports from countries such as Spain stating that fungicides for example Chinosol (Hydroxyquinolinesulfate) are ineffective for the control of *Pa. chlamydospora*, but to date, this fungicide remains the most important and commonly used fungicide used in grapevine nurseries worldwide (Gramaje *et al.,* 2009b, 2018).

3) Hot water treatment (HWT)

One of the most important disease control measures used in many grapevine nurseries globally is subjecting canes to hot water at 50°C for 30min (Crous *et al.,* 2001; Fourie *et al.,* 2004b). This treatment has been used successfully to disinfect numerous plant materials including seeds and other plant storage organs. In some cases, it remains the only effective

means of controlling n number of pests and pathogens in the grapevine propagation process. Phylloxera (Goussard, 1977), root-knot nematodes (Gokte *et al.,* 1995), mites (Szendrey *et al.*, 1995) and numerous other fungal pathogens, just to name a few, have been effectively controlled for many years by using HWT (Waite *et al.,* 2007).

Regardless of the success of this technique there are studies showing that exposing canes and rootings to long duration of HWT it could possibly lead to unacceptable losses (Ophel *et al.,* 1990; Bazzi *et al.,* 1991). A study performed in Italy by Habib *et al.* (2009) showed that after one season of growth, the shoot development as well as the growth of rootstocks and scion cuttings were severely affected when exposed to 50°C for 45min. Due to cooler environmental temperature the standard recommended HWT for nurseries in New Zealand was adjusted to 48°C for 30min (Bleach *et al.,* 2013). In warmer countries such as Spain the standard HWT recommend for nurseries is at 53°C for 30min (Gramaje *et al.,* 2008, 2010a). Therefore, it is hypothesized that cuttings harvested from vines grown in cooler temperatures are much more vulnerable to HWT which could lead to mortality. According to Crocker *et al.* (2002) cuttings from Australia collected from well-managed vineyards and rootstock plantings exposed to warm climates had a higher propagation success than those collected from cooler regions. Hot water treatment is applied at two stages during the propagation process; it is applied just before grafting rootstock and scion material and then again just prior to dispatch of dormant grafted vines (Fourie *et al.,* 2004b; Halleen *et al.,* 2016). The use of HWT remain controversial since exposing plant material to high temperatures makes it more susceptible to stresses such as prolonged storage periods (Gramaje *et al.,* 2010b). Some other negative effects linked to HWT includes; delayed callusing, rooting of cuttings, delayed development, bud death and failed graft unions (Laukart *et al.,* 2001; May *et al.,* 2005).

4) Biological control

Two of the most common fungal species investigated for the control of GTD's include; *Trichoderma atroviride* and *T. harzianum.* These biocontrol agents are available as commercial products in several formulations such as powders, granules as well as dowels. Powders are commonly used during the hydration stage as it is easily mixed with water. Fourie *et al.* (2004b) has reported that using *Trichoderma* formulation during the soaking of planting material reduced *Pa. chlamydospora* incidence on treated rootstock cuttings. In France, Mounier *et al.* (2014) reported a lower necrosis rate caused by *Pa. chlamydospora* when infected plants were subjected to *T. atroviride* strain I-1237. Other studies performed in Spain also showed that *T. atroviride* strain SC1 reduced infection by *Pa. chlamydospora* when applied at the hydration, callusing and pre-planting stages, hydration treatment being the most effective (Pertot *et al.,* 2016).

5) Resistant rootstocks

Intensive research has been implemented to evaluate the susceptibility of grapevine rootstocks to Petri disease pathogens and no cultivar have ever shown complete resistance to infection, but it has been hypothesized that there are differential levels of susceptibility that exists between rootstock cultivars (Khan *et al.,* 2001; Díaz *et al.,* 2009; Alaniz *et al.,* 2010; Sieberhagen *et al.,* 2017). Over-all it has been suggested that rootstock genotypes play a significant role in the incidence as well as the severity of this disease (Khan *et al.,* 2001; Gramaje *et al.,* 2010b).

According to Khan *et al.* (2001) did rootstocks originating from crosses of North America *Vitis* spp. not show resistance to infections caused by *Pa. chlamydospora*. In Spain, Gramaje *et al.* (2010c) showed 161-49 Couderc to be the least susceptible to *Pa. chlamydospora* among five grapevine rootstocks evaluated under field conditions. Studies performed in California suggests that grapevine crosses of *V. berlandieri* x *V. riparia* are least susceptible to Petri disease pathogens (Eskalen *et al.* 2001).

Recent studies performed in South Africa has shown that, Ramsey has the lowest level of disease severity against 11 important fungal trunk pathogens including *Pa. chlamydospora,* followed by US 8-7 and Paulsen 1103 (Sieberhagen *et al.,* 2017). Sieberhagen *et al.* (2017) has also stated that rootstock SO4 had the highest level of disease severity followed by 101-14 Mgt and Richter 110.

6) Integrated management strategies

Recently, Halleen *et al.* (2016), developed an integrated strategy for the proactive management of GTD pathogen infections in grapevine nurseries. This work compared several treatment regimes in nurseries with the purpose of eradicating trunk pathogens. Grapevine propagation material was subjected to treatments before cold storage, before and after grafting, before planting and after uprooting, following an integrated strategy, therefore reducing the incidence of the entire spectrum of fungal trunk disease pathogens. What they suggested was that *Trichoderma* performs best in an integrated approach, in combination with either benomyl or carbendazim, hot water treatment (HWT) at 50°C for 30min as well as applying sporekill, each treatment applied at different stages of the nursery process (Gramaje *et al.,* 2009a). This regime showed to effectively reduce several pathogens including *Pa. chlamydospora.* Halleen *et al.* (2016), recommended that benomyl be replaced with carbendazim when benomyl is no longer available. Studies done in Spain by Gramaje *et al.* (2009b), also showed the effectiveness of carbendazim when applied as a soak treatment during the hydration stage. In contrast, despite the increased availability of research proving the effectiveness of biocontrol products and HWT in combination with different fungicides, the

adoption and use of these techniques has been limited due to the entrenched belief that some of these techniques are less effective than conventional pesticides (Gramaje *et al.,* 2015). Consequently, there is a need for further research into the effects of these integrated strategies.

7) Other methods

Reducing disease progress and overall symptom expression has also been addressed using several ameliorative methods. One of the techniques is to apply electrolyzed acid water to fresh cuttings when subjected to the hydration period. Di Marco *et al.* (2009) proved that the application of this technique in Italy reduced the germination of *Pa. chlamydospora* conidia without any negative effect on the growth and development of plants in nursery fields. Therefore, further studies are required to evaluate the effectiveness of this methods against pathogens causing Petri disease in South African grapevines. A study investigated ozonated water to control conidia dispersal of the esca-associated fungus *P. minimum* (Pierron *et al.,* 2015), and what they have found is that not only did ozonated water totally suppress spore germination *in vitro* but they have also recorded a 50% reduction in fungal development. Therefore, making ozonated water a promising candidate for limiting grapevine infection in nurseries.

Nursery propagation beds

1) Crop rotation

Recently, grapevine nursery rotation studies in South Africa (Langenhoven *et al.,* 2017) has motivated the use of canola, white mustard, lupins and brassica plants as potential rotation crops for the protection of grapevines from soil borne pathogens in South African grapevine nurseries. Although not tested for the protection from *Pa. chlamydospora,* Langenhoven *et al*. (2017) has suggested that these crops could potentially lower the occurrence of pathogens such as black foot, crown and root rot pathogens in South African grapevine nurseries. It has been proposed that brassica plants release glycosinolates into the surrounding soil which is then broken down into isothiocyanates which in turn have the potential to suppress pathogenic fungi (Brown *et al.*, 1997). According to Brown *et al.* (1997) when incorporating alternative crops into nursery soils one must remember that organic matter in the surrounding soil may absorb released volatiles rendering them to be less effective against pathogens.

Jaspers *et al.* (2014) suggested that rotating field sites with mustard crops can possibly lower rates of black foot and Petri disease infection in nursery fields. Other biofumigation studies (Bleach *et al.,* 2010) also suggested mustard meal as a control measure effective against *Cylindrocarpon* spp. as it lowered disease incidence by 43% in artificially inoculated

grapevine rootstocks. *In vitro* studies also revealed that some mustard cultivars have the potential to reduce conidial and mycelial germination of *Dactylonectria* species as well as lowering counts of conidia and chlamydospores in soil (Agustí-Brisach *et al.,* 2011; Barbour *et al.*, 2014). More research is required to determine the role of other perennial crops and the duration of fallow periods in maintaining low fungal inoculum in grapevine nursery soils (Gramaje *et al.,* 2018). In countries such as Australia and New Zealand a lot of focus is given to the use of bio-fumigations (Indian mustard seed meal (*Brassica juncea*)) as an alternative for metham sodium and methyl bromide for the control of black foot pathogens. Bleach *et al.* (2010) proved that using bio-fumigation significantly improved growth and the over-all yield of treated diseased grapevines when planted in artificially inoculated soils.

Rootstocks used in the South African grapevine industry

Since the 1890's, there has been a wide range of rootstocks commercially used in South Africa. Originally Jacquez rootstocks were used to replace phylloxera devastated vineyards. Consequently, Jacquez dominated for almost 60 years before succumbing to phylloxera. Soon after, 101-14 Mgt and Richter 99 gained popularity and to date, these two varieties together with Richter 110, Ramsey and US 8-7 are still widely used as commercial rootstocks in South Africa. South African rootstocks have shown to be highly successful growing in a diverse range of soils, climates, and environments and therefore no significant changes are expected in the near future. Shifts between the use of rootstocks depending on the demand of the industry have been frequent over the last 100 years and a result of various factors, let in recent years mostly as a result of industry demands (SATI, 2018).

During the 2016/2017 season, South African nurseries produced 44.1 million grafted vines. Of these vines; 26.5 million were produced for the wine industry and 17.6 million vines for the table grape and the raisin industry (SATI, 2018). According to The Vine Improvement Association (VIA) the following rootstock cultivars are currently most commonly used in South African vineyards, namely Ramsey, US VIT 8-7, Paulsen 1103, Richter 110, Richter 99, 101-14 Mgt, Ruggeri 140, and SO4.

Ramsey (V. champinii)

In 1938 Ramsey was imported into South Africa under the name 'Salt Creek' (Carstens *et al.,* 1981; Loubser *et al.,* 1997). Ramsey has high vigour, good production abilities in a wide range environmental condition. Although Ramsey has a low affinity for important scion cultivars it remains specifically important to the commercial table grape industry in South Africa and is also the most frequently grafted rootstock in the wine grape industry in recent years (Carstens *et al.,* 1981; Loubser *et al.,* 1997). According to a recent study performed in South

Africa it has been concluded that Ramsey is the most tolerant to grapevine trunk diseases when compared to other commercially grown rootstocks currently used in South Africa (Sieberhagen *et al.,* 2017).

US 8-7 (Jacques x Richter 99) - [(Vitis aestivalis x Vitis cinerea x Vitis vinifera) x (V. berlandieri var. Las Sorres x V. rupestris var. du Lot)]

In 1949 US Vit 8-7 was successfully bred. This cultivar showed the most promising results compared to all the other crossings bred by Orffer (Carstens *et al.,* 1981; Loubser *et al.,* 1997). It has high vigour and is grown in a wide range of soils. It is resistant to calcareous soils which have salinity and drought problems. It also has high resistance to nematodes and pathogens such as *Phytophthora (Ph.) cinnamomi* (Carstens *et al.,* 1981; Loubser *et al.,* 1997). US VIT 8-7 rootstocks used in South Africa are also reported to be on the more tolerant end of the spectrum when comparing disease severity of common grapevine diseases amongst common rootstock cultivars (Sieberhagen *et al.,* 2017).

Paulsen 1103 (V. berlandieri var. Resseguier nr. 2 x V. rupestris var. du Lot)

Paulsen 1103 was imported from Sicily in 1962. This cultivar is comparable to Richter 99 as it has similar abilities and high vigour. However, it is less resistant to nematodes and is also more sensitive to waterlogged conditions (Carstens *et al.,* 1981; Loubser *et al.,* 1997).

Richter 110 (V. berlandieri var. Resseguier No 2 x V. rupestris var. Martin)

This cultivar was bred by Franz Richter, and only became available in South Africa in 1927 (Carstens *et al.,* 1981). Richter 110 can specifically be used to increase fertility due to its great vigour. It is reported that when comparing Richter 110 to Richter 99 that it has a higher tolerance to drought and it is also very resistant to phylloxera (Loubser *et al.,* 1997). However, this cultivar has shown some susceptibility to nematodes and therefore it is not recommended for planting in sandy soils, but rather in poorly drained soils.

Richter 99 (V. berlandieri var. Las Sorres x V. rupestris var. du Lot)

In 1927 this cultivar became an important rootstock in South Africa due to its good growing and production abilities (Carstens *et al.,* 1981). High performance in grafting and rooting along with excellent vigour, high affinity, and resistance to phylloxera and nematodes are just a few of the advantages accompanied by this cultivar (Loubser *et al.,* 1997). Unfortunately, there are some problems which are associated with this specific rootstock cultivar and that is its sensitivity to *Phytophthora cinnamomi.*

101-14 Mgt (V. riparia x V. rupestris)

101-14 Mgt was bred in France by Millardet in 1882 and was one of the first rootstock cultivars imported in South Africa after the phylloxera outbreak. It has abilities which enable it to adapt to a wide range of soil types especially soils with limited depth and that are poorly drained. As like other cultivars it also has some drawbacks as it has shown in the past to have poor grafting abilities compared to others and according to Sieberhagen *et al.* (2017) 101-14 Mgt is amongst the most susceptible rootstocks to GTDs (Loubser *et al.,* 1997).

Ruggeri 140 (V. berlandieri var. Resseguier nr. 2 x V. rupestris var. du Lot)

In 1964 Ruggeri 140 was imported into South Africa from Sicily. Ruggeri 140 shows excellent results when grown in dry calcareous soils. It performs well in soils which have problems with salinity and acidity (Loubser *et al.,* 1997).

SO4 (V. berlandieri x V. riparia)

SO4 stands for 'Selection Opperheim number 4' and was crossed by Teleki in France in 1941. This cultivar was included in South African studies in 1974 (Carstens *et al.,* 1981; Loubser *et al.,* 1997). It has high vigour and early ripening abilities and it also performs well with a variety of scion cultivars. Although this rootstock is resistant to nematodes, in recent studies it has been reported that SO4 is, however, on the most susceptible end of the scale to GTD (Sieberhagen *et al.,* 2017).

AIM AND OBJECTIVES OF THE CURRENT STUDY

The aim of the study is evaluating various chemical and biological control agents for effective pruning wound protection of rootstock mother vines. To our knowledge there has been no other study like this performed in South Africa and therefore this would aid in improving pruning wound protection and minimise the risks of spreading trunk disease pathogens through infected propagation material from mother blocks. The outcomes of this project will indicate which products can be further chosen for registration trials so that local grapevine nurseries, rootstock mother block owners and the Plant Improvement Scheme can more effectively protect pruning wounds.

The Objectives

1) To assess chemical and biological control products on detached rooted rootstock vines challenged with *Pa. chlamydospora* in controlled conditions;
2) To determine the duration of pruning wound susceptibility of rootstock mother plants when challenged with *Pa. chlamydospora* in the field;

3) To evaluate chemical and biological control products as wound protectants in rootstock mother vines when challenged with *Pa. chlamydospora* in the field.

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CHAPTER 2

Determining the susceptibility of grapevine rootstock cultivars to *Phaeomoniella chlamydospora* and the control thereof

ABSTRACT

Pruning wound protection of rootstock mother plants is important to prevent the infection of *Phaeomoniella (Pa.) chlamydospora*. Very little is known about rootstock mother plants' pruning wound susceptibility and pruning protection options that can be used for control of grapevine trunk diseases. Therefore, the temporal susceptibility of pruning wounds as well as the protection of these wounds of commercial rootstocks used in South Africa to Petri disease caused by *Pa. chlamydospora* were assessed. *In vitro* sensitivity towards selected benzimidazole, triazole, pyridine-carboxamide and strobilurin fungicides were done by determining mycelial inhibition (EC_{50}) on fungicide amended PDA plates and conidial germination by means of a microtiter assay. EC_{50} values were calculated and found to be well within the recommended field dosage of the fungicides. Chemical groups shown to be effective via mycelial and germination inhibition studies were evaluated in a 4-week detached shoot assay trial. Low *Pa. chlamydospora* incidence on treated shoots (Ramsey, US 8-7 and 101-14 Mgt) indicated possible pruning wound protection. Among the control agents tested, tebuconazole belonging to the chemical group triazole resulted in a 46.7% *Pa. chlamydospora* reduction compared to the untreated control therefore being the most effective pruning wound protectant; although not significantly different to T1 (*Trichoderma atroviride*), MT1 (*T. atroviride*) MT1 + carbendazim, MT1 + thiophanate-methyl, Eco77 (*T. atroviride***)**, carbendazim, thiophanate + epoxiconazole, pyraclostrobin, boscalid and boscalid + pyraclostrobin. These control agents were further evaluated in normal environmental conditions by means of field trials. Wound susceptibility was determined in certified mother vine blocks with Ramsey and US 8-7 rootstock varieties. This was achieved by inoculations of spore suspensions directly after pruning, as well as 1, 7, 21, and 42 days after pruning. The trials were evaluated after nine months by determining *Pa. chlamydospora* incidences from inoculated wounds. A general decline in *Pa. chlamydospora* incidence was recorded up to 42 days after pruning with wounds challenged 24 hours after pruning being the most susceptible. Protection field trials were conducted with two rootstock varieties (Ramsey and 101-14 Mgt) by treating wounds with selected control agents immediately after pruning and challenged with a *Pa. chlamydospora* spore suspension at 1 and 7 days after treatment application. The trials were evaluated after nine months by determining *Pa. chlamydospora* incidences from inoculated wounds in order to determine the potential of these chemicals to protect these

pruned wounds from *Pa. chlamydospora*. After completion of this study integrated control treatments with biological control *Trichoderma atroviride* and fungicides belonging to the benzimidazoles showed to be the most effective in reducing *Pa. chlamydospora* incidence in rootstock mother vine pruning wounds. The results obtained in this study have provided new and important information regarding the protection of pruning wounds via the integration of biological and chemical control techniques applied to protect rootstock pruning wounds at the most susceptible time period.

INTRODUCTION

Grapevine trunk diseases (GTD's) have been affecting vineyards for many years, not only in South Africa, but also internationally. GTD's responsible for the decline of young vines is a growing concern. Several diseases are responsible for causing young grapevine decline and they could either act individually or as a complex (Scheck *et al.,* 1998; Halleen *et al.,* 2003). One of the main causes of the decline is due to Petri disease caused by *Phaeomoniella (Pa.) chlamydospora* and *Phaeoacremonium* spp. (Mugnai *et al.,* 1999; Groenewald *et al.,* 2001; Rooney-Latham *et al.,* 2005; Halleen *et al.,* 2007; Rolshausen *et al.,* 2010).

The most common symptoms observed in the vineyards associated with young vine decline caused by Petri disease includes; poor establishment, stunted growth, shortened internodes, reduced vigour, wilting and dieback (Morton, 1995; Mugnai *et al.,* 1999). The major internal symptoms associated with Petri disease is black streaking in the wood when affected rootstocks, trunks and cordons are cut lengthwise. In cross-sections of the wood affected with *Pa. chlamydospora*, the symptoms can be observed as dark spots around the pith which is typically caused by the occurrence of tyloses, gums and phenolic substances which are produced in the xylem vessels by the plant in response to infection (Mugnai *et al.,* 1999; Edwards *et al.,* 2007). Subsequently, infections by *Pa. chlamydospora* on grapevines results in reduced yields, quality and the overall longevity of the infected vine and in some cases sudden death. Not only are nurseries experiencing a financial loss due to a lower take percentage, there are also costs associated with the re-establishment of infected vineyards. As *Pa. chlamydospora* is one of the major GTD causing agents, it is considered as an important factor detrimental to the success and sustainability of this agricultural sector worldwide (Groenewald *et al.,* 2001; Rooney-Latham *et al.,* 2005; Halleen *et al.,* 2007; Díaz *et al.,* 2014). A growing concern in the industry is the presence of infected vines in rootstock mother fields which results in systematic infection of grafting material resulting in infection in vineyards via propagation material (Halleen *et al.,* 2003; Edwards *et al.,* 2004; Aroca *et al.,* 2010; Gramaje *et al.,* 2011).

Phaeomoniella chlamydospora is known as an opportunistic pathogen and due to multiple wounds created during the propagation process it provides this pathogen with a competitive advantage, allowing the fungus to establish and manifest in the grafted material. It has been well defined by numerous authors that rootstock mother fields are the primary source of *Pa. chlamydospora* infection (Bertelli *et al.,* 1998; Fourie *et al.,* 2002; Retief *et al.,* 2006; Aroca *et al.,* 2010). Although some nurseries cultivated rootstocks on a trellis system in the past, all South African rootstock mother block owners currently make use of sprawling on the ground. Numerous authors suggested that the practice of sprawling vines freely on the soil surface contributes to the potential of pathogen contamination in general (Hunter *et al.,* 1999; Stamp, 2003; Waite *et al.,* 2015).

Two possible means of initial *Pa. chlamydospora* infection in mother vines have been proposed; the first is soil as a potential reservoir of chlamydospores of *Pa. chlamydospora* which allow it to survive for prolonged periods of time (Bertelli *et al.,* 1998; Mugnai *et al.,* 1999; Halleen *et al.,* 2003; Edwards *et al.,* 2004; Aroca *et al.,* 2010). In this sense, according to Feliciano *et al*. (2001) chlamydospores have the ability to penetrate uninjured roots of vines in nurseries and vineyards, but the extent to which root infections take place in the field remains unclear (Bertelli *et al.,* 1998). The second includes pruning wounds as potential infection point for aerial inoculum in mother vines (Larignon *et al.,* 2000; Edwards *et al.,* 2007). *Phaeomoniella chlamydospora* consequently establishes and grows through the wound inside the xylem vessels causing decay and slowly killing the vines (Carter, 1991; Pascoe *et al.,* 1998; Torres *et al.,* 2009*;* Pouzoulet *et al.,* 2014). Pycnidia, fruiting bodies formed on pruning debris as well as inside cracks and crevices of trunks and cordons of vines, is the inoculum source present in the vineyards (Mugnai *et al.,*1999; Edwards *et al.,* 2001; Baloyi *et al*., 2013).The spores are released from pycnidia following hydration during rainfall or irrigation (Mugnai *et al.,*1999; Eskalen *et al.,* 2001; Moyo *et al.,* 2013; Baloyi *et al*., 2013; Baloyi *et al.,* 2016). These spores are then easily spread via wind, splashing water, pruning shears as well as arthropods, leading to pruning wound infection (Eskalen *et al.,* 2001; Moyo *et al.,* 2013; Baloyi *et al.,* 2013). Both spores and hyphae of *Pa. chlamydospora* have been detected in rootstock canes (Baloyi, 2016). Therefore, it has been hypothesized that these spores are translocated in the sap flow from inside the head of the mother plant (Fourie *et al.,* 2004). Consequently, it can spread to the newly grown shoots which, in the case of rootstock mother vines, are cut and used as rootstocks in the propagation process (Fourie *et al.,* 2002; Edwards *et al.,* 2004; Gubler *et al.,* 2004).

Besides mother vine infection several potential inoculum sources have also been identified in the grapevine propagation process. These inoculum sources include; hydration practises, disbudding, grafting, improper healed graft unions, rooting and planting of finished

vines (Bertelli *et al.,* 1998; Halleen *et al.,* 2003; Zanzotto *et al.,* 2007). There has also been reports of the presence of *Pa. chlamydospora* on pruning shears, grafting machines and callusing media (Halleen *et al.,* 2003; Retief *et al.,* 2006; Aroca *et al.,* 2010; Gramaje *et al.,* 2011). Infections at such an early stage result in an increased risk that grapevine farmers are unknowingly establishing new vineyards with infected material also leading to the spread of *Pa. chlamydospora* to areas where the pathogen haven't been present before. According to Mugnai *et al.* (1999) there are no curative methods for the control of Petri disease and therefore it is essential to have preventative management practices in place. Pruning wound protection is a common practice in established vineyards but in South Africa it is not a standard practice in rootstock mother fields. To date, numerous studies have investigated the duration of grapevine pruning wound susceptibility and a variety of chemical and biological agents as potential pruning wound protectants have been studied (Halleen *et al.,* 2010; Rolshausen *et al.,* 2010). Currently, there are no available studies that have determined the susceptibility of pruning wound and protection of rootstock mother vines in South Africa. It is important to farmers to know when these wounds are most receptive to fungal infection. Therefore, this study addresses important issues regarding the duration of susceptibility of pruning wounds made on rootstock mother vines in South Africa along with identifying potential pruning wound protectants to prevent *Pa. chlamydospora* wound infection.

Unfortunately, previous studies have revealed that all commercial rootstock cultivars investigated thus far are susceptible to Petri disease, including all the rootstock cultivars currently used in the South Africa grapevine industry. No cultivar is completely resistant to *Pa. chlamydospora* infection, although varying levels of susceptibility or tolerance have been reported in different rootstock cultivars (Khan *et al.,* 2001; Aroca *et al.,* 2010; Shieberhagen *et al.*, 2017). Díaz *et al.* (2009) and Gramaje *et al.* (2015) found that various rootstock cultivars are more susceptible to GTD's in comparison to *Vitis vinifera* cultivars used as scion material and therefore more focus is given to rootstock cultivars as main sources of latent fungal trunk infections such as *Pa. chlamydospora.* There is a scarcity in literature available on the protection of wounds made on grapevine rootstock cultivars used in the South African industry and international studies typically included limited rootstock cultivars and are only tested against a limited range of fungal trunk pathogens (Gramaje *et al.,* 2011). Knowing the differences in rootstock cultivar susceptibility is important as it would assist farmers in choosing cultivars which are least susceptible to fungal trunk pathogens (Khan *et al.,* 2001; Gubler *et al.,* 2004; Alaniz *et al.,* 2010; Gramaje *et al.,* 2010).

Studies conducted in different vineyards all over the world suggests that although grapevine pruning wound susceptibility decreases after two months, these wounds remain susceptible to *Pa. chlamydospora* up to four months after pruning (Eskalen *et al.,* 2007; Serra

et al., 2008; Kotze *et al.,* 2011). Regardless of the decrease in susceptibility over time, any amount of infection is considered important as infected plant material is the primary source of inoculum subsequently leading to further spread of Petri disease in the following years. Regarding pruning times of vines in vineyards; there have been several studies both affirming and negating that pruning earlier in the dormant season lead to higher infection rates (Gramaje *et al.,* 2011; Van Niekerk *et al*., 2011) than compared to pruning wounds made later in the season, depending on the country where the study took place. These differences can be explained by the different environmental conditions including; climatic conditions, cultural practices as well as the plant age. According to Larignon *et al.* (2000) and Eskalen *et al.* (2001) wounds made early in the dormant season were more susceptible, and remained susceptible for longer, to infection by common GTD's including *Pa. chlamydospora*. In South Africa rootstock canes are harvested in April, May and June, although it is recommended to be done in April to July (Hunter *et al.,* 2004). The pruning periods of rootstock mother plants and commercial vineyards are therefore different, because vineyard pruning occurs between mid-June to August, depending on the cultivar. The only previous study conducted in South Africa investigating the duration of susceptibility of pruning wounds were conducted by Van Niekerk *et al.* (2011), during the month of June and August. No other information is available on rootstock pruning wound susceptibility in South Africa. Knowledge on the efficacy of pruning wound protectants made on rootstock mother plants, and specifically also the time period is unknown. As previously suggested inoculum sources such as conidia are available year-round (Eskalen *et al.,* 2007) and therefore the potential for infection remains a threat througout the growing season.

The aim of the study was to select and evaluate various chemical and biological control agents in order to formulate a protocol for local grapevine nurseries, rootstock mother block owners and the Plant Improvement Scheme on effective pruning wound protection of rootstock mother vines. In return to manage pruning wound infections and to minimise the risks of spreading trunk pathogens through infected propagation material. The first objective of the study was to conduct pruning wound protection assays with suitable chemical and biological control agents to determine if they have any potential to be evaluated under field conditions. The second objective was to determine the duration of pruning wound susceptibility in rootstock mother plants and the last was to evaluate selected chemical and biological control agents as wound protectants in rootstock mother vines.

MATERIALS AND METHODS

Fungal isolates and inoculum preparation

Highly virulent *Phaeomoniella chlamydospora* isolates were selected for the current study, namely; LM310 (STE-U 6384) and LM91 (STE-U 8276) (Table 1). These two specimens were previously isolated from grapevine pruning wounds located in Stellenbosch, Western Cape, South Africa and showed to be the most virulent *Pa. chlamydospora* strains on detcahed rootstock canes (Sieberhagen *et al.* 2017). These isolates are stored in the culture collection at Stellenbosch University, Department of Plant Pathology. Spore suspensions were prepared from 3-week-old *Pa. chlamydospora* cultures growing on potato dextrose agar (PDA) (Biolab, Wadeville, South Africa) and amended with chloromycetin (250 mg/L), by flooding the Petri dish with sterile water in order to dislodge spores. Conidia were counted using a haemocytometer and the concentration adjusted to 2000 conidia/mL. For each application spore suspensions were freshly prepared and after each trial application, 5 mL left over suspension were spread on PDA and incubated for 7 days to determine spore viability. Viability is defined as a spore that germinated and where the germ tube has exceeded one half the length of the spore.

Fungicides and fungicide preparation

Fourteen different fungicide treatments were selected for this study, based on their active ingredient and the chemical group they belong to. The fungicide treatments selected for this study (Table 2) were either registered for use on grapes in South Africa, known to be effective against *Pa. chlamydospora in vitro* or are known to be one of the main active ingredients in fungicides registered on grapevine in South Africa. The fungicides used were: carbendazim (Bendazid® 500SC, VILLA Crop Protection), thiophanate-methyl (Cercobin® M, BASF), epoxiconazole (Epoxicanozole 125 SC, VILLA Crop Protection), tebuconazole (Tebuzole® 250EW, VILLA Crop Protection), kresoxim-methyl (Stroby® 500WG, BASF), pyraclostrobin (Cabrio® EG, BASF) and boscalid/pyraclostrobin (Tessior®, 95% a.i., BASF). Other treatments included biological control agents based on *Trichoderma atroviride* strains, including; strain T1 (Department of Plant Pathology, Stellenbosch University), strain MT1 (Department of Plant Pathology, Stellenbosch University) and strain Eco77 (Plant Health Products, PTY Ltd., Nottingham Road, South Africa). Two biological control agent – fungicide combinations were also included; namely strain MT1 + carbendazim, and strain MT1 + thiophanate-methyl. Stock solutions were prepared by suspending these fungicides in 500 mL distilled water. Field rates recommended by the manufactures were followed (Table 2).

***In vitro* studies**

In vitro studies measuring inhibition of mycelial growth and germination by 50% (EC_{50} value) is a useful indication of pathogen sensitivity to fungicides and therefore *in vitro* studies

were performed prior to field trials to determine whether the selected treatments show potential to control *Pa. chlamydospora* LM310 and LM91 isolates.

Mycelial growth inhibition assays

The goal of the mycelial inhibition assay was to determine the EC_{50} values of selected chemicals known to be effective in *Pa. chlamydospora* mycelial inhibition. For each fungicide seven different concentrations were prepared, selected from a similar study described by Jaspers (2001) (Table 3). The final concentration of each of the chemicals were (µg/mL); carbendazim (0, 0.01, 0.05, 0.1, 0.5, 1, and 5), thiophanate-methyl (0, 0.01, 0.05, 0.1, 0.5, 1, and 1.5), epoxiconazole (0, 0.01, 0.05, 0.1, 0.5, 1, and 5), tebuconazole (0, 0.01, 0.05, 0.5, 1, 5, and 10), kresoxim-methyl (0, 0.5, 1, 10, 100, 500, and 1000), pyraclostrobin (0, 0.02, 0.5, 1.5, 2, 2.5, and 5). The fungicides were suspended in malten (50°C) potato dextrose agar and 20 mL aliquots suspended in Petri dishes. Within two hours after the plates solidified, they were inoculated with 5 mm diameter discs of actively growing mycelia of 2-week-old *Pa. chlamydospora* colonies LM310 and LM91. Three replicates per isolate were inoculated with each fungicide concentration and the trial was repeated. Plates were incubated for four weeks on a laboratory bench at 24°C +/- 1°C subject to natural light. Colonies were measured and the mean diameter were logged.

Percentage mycelial growth inhibition was calculated relative to the mean diameter of the control for each trial, fungicide and isolate. To mathematically describe inhibition patterns a power curve ($y=ax^b$) was fitted on percentage mycelial growth inhibition against chemical concentration over the three replicates per trial of each fungicide and isolate. Estimated EC_{50} values were calculated for each trial, fungicide and isolate using the regression parameters of fitted functions ($x=(50/a)^{1/b}$). Regression analyses were performed using the NLIN Procedure of SAS software (Version 9.4; SAS Institute Inc, Cary, USA).

Germination inhibition assays: Microtiter study

The EC_{50} values of selected chemicals known to be effective in inhibiting *Pa. chlamydospora* germination was tested with a microtiter assay. Resazurin-based microtitor assays were carried out using 96-well, rounded-bottom, polystyrene microtitor plates. In each well 80 µL potato dextrose broth (PDB, Biolab, South Africa) were added together with 80 µL (2000 conidia/mL) *Pa. chlamydospora* spores LM310 and LM91. In addition, 100 µL PDB amended with the respective chemicals at different concentration (µg/mL) were added to each of the wells followed by the addition of 20 µL of resazurin dye which acts as an indicator of cellular respiration. In the presence of actively growing cells, the resazurin indicator changes from an oxidized, non-fluorescent blue form to a reduced, fluorescent pink form. This indicates

the inhibition of germination therefore maintaining an oxidized environment, leaving the indicator blue. The three chemicals known to be effective against germination inhibition were included in this trial, namely kresoxim-methyl, pyraclostrobin and boscalid. The final concentration of each of the chemicals were (µg/mL); kresoxim-methyl (0, 0.5, 1.5, 5, 10, 100 and 1000), pyraclostrobin (0, 0.05, 0.5, 1, 10, 100 and 500) and boscalid (0, 0.05, 0.5, 1, 1.5, 5 and 10).

For the controls; internal standards of PDB and resazurin alone without *Pa. chlamydospora* spores at each of the chemical concentrations were used. These plates were then incubated using a shaker at 300 rpm for 80 hours (optimized) at 25°C. After incubation the respiration rate were determined using the absorbance of the dye at 570 nm (λ1 red reflectance, blue light absorbance) and 600 nm (λ2 blue reflectance, red light absorbance) on a tunable microplate reader (Versamax, Molecular Devices, Sunnyvale, CA, USA). The percentage resazurin reduction were calculated using the manufacturer's equation. The microtitor plate were prepared as described above with three absorbance measurements per isolate and the experiment was repeated. The percentage resazurin reduction were calculated using the manufacturer's equation.

Percentage resazurin reduction inhibition was calculated relative to the mean percentage resazurin reduction of the control for each trial, fungicide and isolate. To mathematically describe inhibition patterns a power curve ($y=ax^b$) was fitted on percentage resazurin reduction against chemical concentration over the two replicates per trial of each fungicide and isolate. Estimated EC_{50} values were calculated for each trial, fungicide and isolate using the regression parameters of fitted functions ($x=(50/a)^{1/b}$). Regression analyses were performed using the NLIN Procedure of SAS software (Version 9.4; SAS Institute Inc, Cary, USA).

Detached grapevine shoot assays

This experiment was performed to evaluate the protection of pruning wounds against infection from *Pa. chlamydospora* using different control agents at different time intervals (24 hours and 7 days) after pruning. This study was conducted with detached canes floating on foamalite boards in tubs with water. The experimental design was a completely random split plot with three tubs (random reps) for each of the three cultivars (main plot factor) and five canes each for the 15 fungicides x two challenge time combinations (subplot factors) randomly arranged per tub.

Dormant 1-year-old rootstock plant canes (4-6 mm-diameter) of Ramsey, 101-14 Mgt and US 8-7 were obtained from a certified nursery in Wellington, Western Cape, South Africa. These 4-node-length canes were hydrated by soaking them for 30 min in a hot water bath at

50°C after which they were soaked in cold water for 30 min and then dried at room temperature. The canes were pruned 10 mm above the third node and thereafter they were placed into 10 mm holes which were bored into 5 mm thick, 30 x 40 mm foamalite boards. The canes ware placed through the holes allowing 4 cm sections of the canes to be immersed into the water tubs. These boards were floated on water in 40 x 60 cm plastic containers and maintained at 22°C +/- 1°C and exposed to natural light. The water was changed every second day and the containers washed every 7 days. Each pruned wound was separately treated with the respective fungicides. All treatments were sprayed as a single application using a hand held 500 mL trigger spray bottle and control treatments received sterile water only. After treatment application treated wounds were challenged either 24 hours or 7 days after treatment application with *Pa. chlamydospora* LM310 by pipetting a 20 µL of 5 x 10^4 conidia/mL droplet per wound containing approximately 1000 spores using a micropipette. Four weeks after inoculation the treated canes were removed, triple sterilised by immersion in 70% ethanol for 30 seconds, then one minute in 3.5% sodium hypochlorite and in 70% ethanol for 30 seconds and aseptically split lengthwise after which fungal isolation was carried out. Twelve pieces of symptomatic wood were removed just below the wound surface of the healthy wood and placed onto PDA in 90 mm Petri dishes which were then incubated for four weeks at 12-hour day/night light cycles at 25°C. Sub-culturing took place to prevent overgrowth of emerging colonies. The plates were inspected for the presence of *Pa. chlamydospora* daily with the final assessment at four weeks (Moyo, 2013). The fungi were morphologically identified, and the incidences was recorded. The mean percent disease incidence was calculated compared to that of the control.

For each of the 15 x 2 treatment combinations per tub disease incidence of *Pa. chlamydospora* was calculated out of five canes. Analysis of variance (ANOVA) was performed per trial using the GLM (General Linear Models) Procedure of SAS software (Version 9.4; SAS Institute Inc, Cary, USA). Trial results were also combined after confirming trial homogeneity variance using Levene's test (Levene, 1960). The Shapiro-Wilk test (Shapiro *et al.,* 1965) was conducted to test for deviation from normality. Fisher's least significant difference was calculated at the 5% level to compare treatment means for significant effects (Ott, 1998). A probability level of 5% was considered significant for all significance tests.

Grapevine rootstock field trials

All the trials were conducted on rootstock mother vine vineyards at a nursery in Wellington, Western Cape Province, South Africa. The rootstock mother vines were 4-years-old when the trials commenced in 2017.

Wound susceptibility trials

Wound susceptibility was assessed in two rootstock blocks each of the cultivars Ramsey and US 8-7. Pruning occurred at the optimum time for collection of rootstock canes as recommended by Hunter *et al*. (2004) in May and July 2017 and 2018. Two-bud-spurs were cut with pruning shears on selected vines, instead of 1-bud-spurs as standard practice in order to observe any lesion formation. Pruned wounds were inoculated with *Pa. chlamydospora* LM310 (20 µL of 5 x 10^4 conidia/mL) spore suspension directly after pruning, as well as 1, 7, 21, and 42 days after pruning to assess the temporal susceptibility of pruning wounds. A non-inoculated control treatment was also included to record natural infection.

For each trial the experimental design was a split-plot with pruning time (May and July) as main plot factor, replicated at random on 10 mother vines each and inoculation time as subplot factor, applied on spurs within each mother vine. A total of 960 (10x2x6 per trial x 4 trials x 2 seasons) spurs were included over the four trials in two seasons in this study. After the 9-month incubation period the trials were evaluated by harvesting the inoculated spur and shoots from the mother vines. The number of shoots (1 or 2) originating from the inoculated spur was noted and shoot lengths were determined. The current year's growth was removed, and the original inoculated spurs were placed individually in plastic bags and immediately taken to the laboratory. The stubs were split lengthwise (Fig. 1) and fungal isolation was carried out after surface sterilisation as described above (detached grapevine shoot assay). Petri plates were incubated at 23 ± 2°C for four weeks on a laboratory bench and monitored daily for growth of *Pa. chlamydospora*. The cultures were identified based on morphological characteristics and the mean percentage incidence was recorded.

Disease incidence was calculated per trial as the percentage presence of *Pa. chlamydospora* out of the 10 mother vines per treatment combination. After verifying homogeneity variance using Levene's test (Levene, 1960), seasonal results were combined, considering trials as block replicates for treatments. The Shapiro-Wilk test (Shapiro *et al.,* 1965) was conducted to test for deviation from normality. Fisher's least significant difference was calculated at the 5% level to compare treatment means for significant effects (Ott, 1998). A probability level of 5% was considered significant for all significance tests.

Wound protection trials

The trials were conducted in two blocks each of the rootstock cultivars Ramsey and 101-14 Mgt. A total of 520 2-bud-spurs were cut at the end of June with pruning shears on selected vines, instead of 1-bud-spurs as standard practice. Wounds were treated with the selected products/formulations (Table 2) immediately after pruning by applying a single spray froma 500 mL spray bottle onto each wound. After, the pruning wounds were inoculated with

a spore suspension of *Pa. chlamydospora* LM310 by pipetting a 20 µL of 5 x 10^4 conidia/mL droplet containing approximately 1000 spores onto each wound at 1 and 7 days after pruning using a pipette. For each trial the experimental design was a split-plot with five block replicates of the 12 products/formulations (main plot factor) and challenge time (non-inoculated control, 1 and 7 days after pruning) as subplot factor, applied on spurs within each vine. A total of 1440 (5x12x3 per trial x 4 trials x 2 seasons) spurs were included over the four trials in two seasons in this study.

After the 9-month incubation period the trials were evaluated by harvesting the inoculated spur and shoots from the mother vine. Shoot lengths were determined where after the current year's growth were removed and placed individually in plastic bags and immediately taken to the laboratory. The remaining stubs were split lengthwise (Fig. 1) and fungal isolation was carried out after surface sterilisation as described above (Detached grapevine shoot assay). Petri plates were incubated at 23 ± 2°C for four weeks on a laboratory bench and monitored daily for growth of *Pa. chlamydospora*. The cultures were identified based on morphological characteristics and the mean percentage incidence was noted.

Disease incidence was calculated per trial as the percentage presence of *Pa. chlamydospora* out of the five vines per treatment combination. After verifying homogeneity variance using Levene's test (Levene, 1960), seasonal results were combined, considering trials as block replicates for treatments. The Shapiro-Wilk test (Shapiro *et al.,* 1965) was conducted to test for deviation from normality. Fisher's least significant difference was calculated at the 5% level to compare treatment means for significant effects (Ott, 1998). A probability level of 5% was considered significant for all significance tests.

RESULTS

In vitro trials

Mycelial growth inhibition assays

Treatment concentration ranges and EC_{50} values of the two *Pa. chlamydospora* isolates are summarised in Table 3. Of the two *Pa. chlamydospora* isolates that were used in the study, in general LM310 had higher EC_{50} values compared to LM91 except for tebuconazole where the EC_{50} value of LM310 was 0.082 µg/mL compared to LM91 at 0.129 µg/mL and kresoxim-methyl 22.9 µg/mL (LM91) versus 5.626 µg/mL (LM310). Of these fungicides, all resulted in EC_{50} values of less than 0.2 µg/mL which is much lower than the recommend field rate. On the other end of the spectrum, kresoxim-methyl, which had EC_{50} values of 5.626 µg/mL (LM310) and 22.9 µg/mL (LM91) and pyraclostrobin at 3.92 µg/mL (LM310) presented much higher EC_{50} values.

Germination inhibition assays: Microtiter study

Treatment concentration ranges and EC_{50} values of the two *Pa. chlamydospora* (LM310 and LM91) are summarised in Table 3. The pyridine-carboxamide fungicide, boscalid resulted in the highest EC_{50} values of 231.709 µg/mL (LM310) and 95.92 µg/mL (LM91). The strobilurin fungicide kresoxim-methyl resulted in EC_{50} values of 112.985 µg/mL (LM310) and 55.58 µg/mL (LM91) which is slightly lower than EC_{50} values of pyridine-carboxamide fungicide but considered relatively high when compared to pyraclostrobin at 0.034 µg/mL (LM310).

Detached grapevine shoot assays

The pruning wound treatments were able to reduce the incidence of *Pa. chlamydospora,* but with varying efficacy between the two trials (Table 4). Analysis of variance (Appendix, Table 1) revealed a significant (P=0.0001) trial x treatment interaction for the percentage *Pa. chlamydospora* incidence observed after the inoculated shoots were incubated for four weeks. This interaction was mainly caused by strain MT1 + carbendazim, epoxiconazole, tebuconazole and pyraclostrobin that produced different results between the two trials.

The mean incidence of *Pa. chlamydospora* on inoculated wounds ranged from 48.9 to 91.8% and from 36.2 to 76.9% for trial 1 and trial 2, respectively. Among all treatments, carbendazim had the lowest *Pa. chlamydospora* incidence of 36.2% in trial 2 which was significantly lower than the untreated control at 72.9%, reducing the pathogen incidence by 50.4% compared to the untreated control. Other treatments that had higher *Pa. chlamydospora* incidence, although not significantly different to carbendazim, includes; strain MT1 , strain MT1+ thiophanate-methyl, strain Eco77, thiophanate-methyl + epoxiconazole, epoxiconazole, boscalid and boscalid + pyraclostrobin. These treatments reduced *Pa. chlamydospora* incidence from 29.1 to 50.4%.

From trial 1 (Table 4), tebuconazole at an incidence of 48.9% had the lowest incidence amongst all treatments, although not significantly different to strain T1, strain MT1, strain MT1 + carbendazim, strain MT1 + thiophanate-methyl, strain Eco77, carbendazim, thiophanate + epoxiconazole, pyraclostrobin, boscalid and boscalid + pyraclostrobin. These treatments gave a reduction of 46.7% to 30.4% in *Pa. chlamydospora* compared to the untreated control (91.8%).

From trial 1, epoxiconazole had the highest *Pa. chlamydospora* incidence at 78.9%, although not significantly different to thiophanate-methyl (71.8%), kresoxim-methyl (65.1%) and boscalid (63.89%). In trial 2, treatments that showed no significant difference in incidence compared to the untreated control (Table 4) included; kresoxim-methyl at 76.9% which had

the highest *Pa. chlamydospora* incidence, followed by tebuconazole at 72.4%, pyraclostrobin (64.5%) and strain MT1 + carbendazim (59.1%).

Grapevine rootstock field trials

Wound susceptibility trials

Analysis of variance for mean shoot lengths and *Pa. chlamydospora* incidence (Appendix, Table 2) found a significant pruning time × challenge interaction (P=0.0415) in the incidence of *Pa. chlamydospora* also a significant (P=0.0439) incidence difference between the two seasons. A significant difference between the two seasons in shoot length was also found (P=0.0003 (basal shoot) and P=0.0095 (distal shoot)). These significant differences for the susceptibility trial are given in Table 5, 6 and 7.

The mean *Pa. chlamydospora* incidence was significantly higher in 2018 at 58.3% compared to 2017 at 51.9% (Table 5). Mean shoot lengths was significantly longer in 2018 at an average basal shoot length of 222.1 cm and distal shoot length of 205.5 cm, which is 51.5 cm (basal shoot) and 28.5 cm (distal shoot) longer than in 2017 (Table 6). The susceptibility of the wounds declined significantly as the length of time between pruning and inoculation increased but with varying incidence between the two pruning times (Table 7). Wounds pruned at the end of May and challenged at 42 days showed the lowest *Pa. chlamydospora* incidence at 41.3%, wounds that weren't significantly different to this includes wounds pruned at the end of May and challenged at 7 days (47.5%) and wounds pruned at the end of June and challenged at 42 days (47.5%). Wounds not challenged had a *Pa. chlamydospora* incidence of 11.3% when pruned at the end of May and 17.5% when pruned at the end of June which was significantly lower than all other pruned wounds. Wounds that resulted in the highest *Pa. chlamydospora* incidence was those pruned at the end of June and challenged at 7 days (78.8% incidence).

Wound protection trials

Analysis of variance (Appendix, Table 3) found significant (P<0.0001) treatment × challenge interactions in the incidence of *Pa. chlamydospore*, a significant (P<0.0001) year × challenge interaction, as well as a significant (P=0.0003 (basal shoot) and P<0.0001 (distal shoot)) difference between the two seasons in shoot length, similar to what was found in the susceptibility trial. Mean shoot lengths were significantly longer in 2018 at an average basal shoot length of 263.1 cm and distal shoot length of 291.9 cm, which is 44 cm (basal shoot) and 85.8 cm (distal shoot) longer than in 2017 (Table 8).

Wounds not challenged after treatment application had natural infection at an average incidence of 2.9% in 2017 and 1.7% in 2018 significantly lower than those challenged at 24

hours and 7 days after *Pa. chlamydospora* application (Table 9). Wounds challenged 24 hours after treatment application gave a mean incidence of 54.6% in 2017 and 70.4% in 2018. When wounds were challenged 7 days after treatment application, incidences of *Pa. chlamydospora* were recorded at a mean incidence of 55% in 2017 where as it was 78.3% in 2018. In 2017 lower incidences of *Pa. chlamydospora* were found in treated wounds.

Significant treatment × challenge interactions are summarised in Table 10. Wounds that received no treatment and challenged with *Pa. chlamydospora* after 24 hours had 80% *Pa. chlamydospora* incidence. The treatment that caused the lowest *Pa. chlamydospora* incidence when challenged with *Pa. chlamydospora* 24 hours after treatment application was MT1 + carbendazim (35%). The treatments that did not differ from this include MT1 + thiophanate-methyl (37.5%) and carbendazim (47.5%). Treatments T1 , Eco77 and thiophanate-methyl + epoxiconazole also had significantly lower *Pa. chlamydospora* incidence, than the control, but not similar to MT1 + carbendazim, MT1 + thiophanate-methyl and carbendazim. The treatments that differed from the control resulted in *Pa. chlamydospora* reduction of 21.9% to 56.3%. Wounds that received no treatment and challenged with *Pa. chlamydospora* after 7 days, had 80% *Pa. chlamydospora* incidence. The treatment that caused the lowest *Pa. chlamydospora* incidence when challenged with *Pa. chlamydospora* after 7 days was T1 (52.5%). Treatments that did not differ from T1 include; MT1, Eco77, MT1 + carbendazim, MT1 + thiophanate-methyl and tebuconazole. These treatments caused a reduction in *Pa. chlamydospora* incidence ranging from 25% to 34.3%.

DISCUSSION

The results of this study indicate that isolates of highly virulent *Phaeomoniella chlamydospora* are sensitive to certain fungicides, and protection of pruning wounds using a combination between biological control agents and benzimidazole fungicides are the best combination to reduce infections of *Pa. chlamydospora* on grapevine rootstock in South Africa. *In vitro* studies measuring inhibition of mycelial growth and germination by 50% are indicative of the pathogen's sensitivity to fungicides. *In vitro* studies were, therefore, performed prior to field trials to determine whether fungicides showed potential of pruning wound protection of rootstock mother vines from *Pa. chlamydospora* infection. The EC_{50} values determined for thiophanate-methyl and tebuconazole fell within the same sensitivity ranges reported by Groenewald *et al.* (2000) and Jaspers (2001). Moreover, Jaspers (2001) reported that PDA amended with 0.086 µg/mL kresoxim-methyl was sufficient to reduce mycelial growth of *Pa. chlamydospora* by 50%, however, in the current study EC_{50} values of 5.6 and 22.9 µg/mL were obtained. Although the EC_{50} value obtained for kresoxim-methyl was higher than that determined by Groenewald *et al.* (2000) and Jaspers (2001), it is still considered relatively low

and much lower than the recommended field rate of 500000 µg/mL. According to Jaspers (2001) PDA amended with 0.078 µg/mL carbendazim reduced mycelial growth of *Pa. chlamydospora* from New Zealand by 50%, a lower concentration was needed in this study at 0.0004 µg/mL. This is the first study to report fungicide sensitivity data for epoxiconazole and pyraclostrobin reducing *Pa. chlamydospora* mycelial growth *in vitro*.

The microtiter assays used to investigate the conidial germination inhibition was the first study performed in South Africa in which these chemicals were specifically tested against *Pa. chlamydospora* using this method. Higher EC_{50} values were observed for both kresoxim-methyl and boscalid compared to Jaspers (2001). The three chemicals tested are known to reduce not only mycelial growth, but also spore germination (Sauter *et al*., 1995; Leroux, 1996). Kresoxim-methyl and pyraclostrobin mode of action (MOA) is via a single specific site in the mitochondria, known as the quinol oxidation (Qo) site (or ubiquinol site) of cytochrome b. Thus, stopping the transfer of electrons from cytochrome b and cytochrome c, which in turn halts reduced nicotinamide adenine dinucleotide (NADH) oxidation and adenosine triphosphate (ATP) synthesis (Brandt *et al.,* 1993; Von Jagow *et al.,* 1982). Ultimately resulting in to complete energy production failure and cell death. A wider sample of isolates would be needed to confirm the EC_{50} ranges.

Pyraclostrobin showed a very low EC_{50} value indicating that this chemical might potentially be effective at reducing *Pa. chlamydospora*. Considering the relatively low EC_{50} values obtained for both the mycelial inhibition and the germination inhibition for pyraclostrobin, it is assumed that this chemical could be effective in controlling *Pa. chlamydospora* in pruning wounds. Considering the overall low EC_{50} values obtained in this study, these chemicals were chosen to be further evaluated in the following trials.

Although there was no treatment x challenge interaction from the detached shoot assay, in general we would expect the chemical treatments to show much lower incidence when challenged 24 hours after treatment application and the biological treatments agents to show lower incidence when challenged 7 days after treatment application. The reason for this being that chemicals break down after some time, thus, losing their effectiveness (Oostendorp *et al.,* 2000), whereas, biological control agents require some time to germinate and colonise the wound (Baker *et al.,* 1974; Cook *et al.,* 1983) which in turn would give them a competitive advantage when challenged 7 days after application. Consequently, incidences from the three cultivars challenged at different time intervals were combined and focus was shifted to the percentage incidence of *Pa. chlamydospora* in treated wounds from two separate trials. Trial 1 indicated a much higher *Pa. chlamydospora* incidence when isolated from the untreated wounds leading to believe that all other treated wounds from this trial will show a higher incidence compared to trial 2.

After four weeks of incubation no foliar symptoms were visible on any of the detached shoots inoculated with *Pa. chlamydospora,* possibly indicating that a longer incubation period is required to observe foliar symptoms and to find a probable correlation with internal infection. No studies have been done on the susceptibility and possible protection of pruning wounds of Ramsey, US 8-7 and 101-14 Mgt rootstock grapevines even though they are of the most commonly used rootstocks in South Africa (VIA, personal communication). Hence, studies focussing on the interaction of these rootstocks with *Pa. chlamydospora* by measuring incidence could deliver possible insight to potential control of this pathogen in the grapevine industry.

Different levels of incidence were recorded when isolations from wounds treated with different fungicides took place. From these results, it is suggested that the treatments chosen for the study have potential pruning wound protection ability against *Pa. chlamydospora.* In the detached grapevine assay, most of the treatments significantly reduced wound infection by *Pa. chlamydospora* at slightly higher levels than in the field trials. Although some of these treatments were tested further in the field trials, significantly reduced wound infection compared to the untreated control, the overall pathogen incidence reduction was slightly less in the field trial compared to the *in vitro* trial. This could easily be explained by environmental factors such as; rain, moisture, temperature, wind, sunlight and other abiotic factors which could potentially hinder the effectiveness of chemicals when applied in a natural environment (Somers, 1957; Staub *et al.,* 1984; Stehmann *et al.,* 1996;).

Results from the detached shoot assay showed that all *Trichoderma atroviride* strains were able to colonize all three the grapevine rootstock cultivars used and that all the *Trichoderma* treated wounds had lower pathogen incidence levels but with variation in percentage *Pa. chlamydospora* incidence. It is suggested that the success of *Trichoderma* as a biopesticide depends not only on its antagonistic activity alone, but a mixture of several factors which include; the method of application, the ability of the specific isolate to colonize the wound as well as the environmental conditions which include moisture availability and temperature (Mutawila *et al.,* 2011; Fourie *et al.,* 2001; Kotze *et al.,* 2009). Therefore, it's important to keep in mind that all these factors could have had a possible effect on the results and the overall outcome of the *Trichoderma* treated wounds. It was clear that there were a few chemical treatments showing promising protection capability with low *Pa. chlamydospora* incidences in both trial 1 and 2. These treatments include; carbendazim, , boscalid,boscalid + pyraclostrobin and thiophanate-methyl + epoxiconazole. Of these, carbendazim showed the highest reduction in *Pa. chlamydospora* incidence in trial 2.

In recent years a lot of focus was given to the development of protocols or products for the prevention or reduction of Petri disease infection of the woody tissues of grapevine

material, although none focussed on rootstock mother vines and to date no chemical product have been registered for this specific use despite promising results *in vitro* and *in vivo* (Gramaje *et al.,* 2011). According to Gramaje *et al.* (2009) carbendazim were effective against *Pa. chlamydospora* for both mycelial and conidial germination. Carbendazim belongs to the chemical group benzimidazoles which is known to be effective in reducing mycelial growth as it inhibits cell mitosis by targeting beta-tubulin (Lacey, 1990). The two *Pa. chlamydospora* isolates tested were sensitive to carbendazim and the high inhibition in the detached shoot trial confirms the efficacy of this fungicide. Results obtained from the detached shoot assay indicated that boscalid and the integrated treatment boscalid + pyraclostrobin could be used as potential chemical controls for *Pa. chlamydospora*. Although pyraclostrobin alone provided inconsistent results boscalid showed to be effective in both trial 1 and trial 2.

Boscalid belongs to the chemical group pyridine–carboxamide which is effective in reducing not only mycelial growth but also spore germination (Sauter *et al*., 1995; Leroux, 1996; Bartlett *et al.,* 2002) and therefore it is believed to have potential to reduce *Pa. chlamydospora* incidence in rootstock pruning wounds. Another treatment that showed potential as pruning wound protectant against *Pa. chlamydospora* was thiophanate-methyl + epoxiconazole. Epoxiconazole alone provided inconsistent results as the good reduction obtained in trial 2 could not be repeated in trial 1. Duet^{tm} Ultra contains thiophanate-methyl (benzimidazole) and epoxiconazole (triazole), which are effective against a wide spectrum of diseases as a preventative and curative control. Although Duet^{tm} Ultra is only registered for the use on wheat, maize and groundnuts in South Africa, it was decided to include it based on the benzimidazole combination with a triazole and the lack of registered thiophanate containing chemicals on grapevine.Considering the low EC_{50} values obtained for both these chemicals in the mycelial inhibition assays, the low *Pa. chlamydospora* incidence when wounds were treated with this combination during the detached shoot assays as well as supporting literature (Pitt, 2012; Díaz *et al.,* 2013; Olmo *et al.,* 2017), it was considered worthwhile to test them as potential pruning wound protectants against *Pa. chlamydospora* in rootstock mother vines. Unfortunately; in view of the fairly low success of this integrated control treatment during the field trials it is considered that this integrated treatment (Duet^{tm} Ultra [SC BASF South Africa (Pty) Ltd]) might potentially loose its effectiveness when applied in normal environmental conditions on rootstock mother vines. Overall, the treatments which most consistently resulted in relatively low *Pa. chlamydospora* incidence in detached shoot trials included; MT1, Eco77, MT1 + thiophanate–methyl, thiophanate-methyl + epoxiconazole, boscalid and boscalid + pyraclostrobin.

The field trials investigated the susceptibility and potential protection of three rootstocks used in South Africa against *Pa. chlamydospora.* Little is known about the

susceptibility of these rootstocks to *Pa. chlamydospora* especially in a South African perspective. Previous studies examining susceptibility and protection of these pruning wounds have either only focussed on mature vines or nursery vines, included other pathogens or were not conducted under field conditions (Eskalen *et al.,* 2001; Díaz *et al.,* 2009; Alaniz *et al.,* 2010). Therefore, this was the first study that has been conducted in South Africa focussing on *Pa. chlamydospora* infection on rootstock mother vines. It is also important to take this in mind when comparing the results of this trial to any other similar trials performed on either grafted or mature vines.

After the completion of the susceptibility and protection field trials, the two different seasons when comparing incidences were not evaluated separately although there was found to be higher *Pa. chlamydospora* incidences in 2018 compared to 2017 since a drought was experienced in 2017 which could potentially have influenced the results. A higher rainfall was experienced during the 2018 which could explain the longer shoot growth that was observed in both the susceptibility and the protection trials. The drought which was experienced during the 2017 season, as well as limited irrigation placed these rootstock mother vines under severe stress. Although the effect of *Pa. chlamydospora* infection on the new growth is unknown and could not be measured, the main objective for measuring shoot length was to determine whether the tested chemicals have any effect on shoot growth. Therefore, proper shoot growth showed that the chemicals tested in the protection field trial had no severe effect on the overall health and vegetative growth of the rootstock mother vine.

Wound susceptibility decreased as the period between pruning and inoculation of wound increased. Results of the present study were in accordance with findings of previous research in different countries and under different climate conditions, which confirmed that *Vitis vinifera* pruning wounds remained susceptible up to 42 days after pruning (Laringnon *et al.,* 2000; Eskalen *et al.,* 2007; Urbez-Torres *et al.,* 2011; Van Niekerk *et al.,* 2011). Although the gradual decline of susceptibility was seen for wounds challenged both at the end of May and the end of June, the decline in susceptibility was marginally more evident when pruning occurred at the end of May, possibly indicating that when wounds are pruned earlier in the dormant season, susceptibility of these wounds will be less and healing of the wounds might therefore be marginally faster. Although there is a lot of studies supporting this idea, slight differences in the susceptibility and duration have been found among pathogens, geographic regions, grape variety, age of the vineyard, inoculation time and pruning season (Elena *et al.,* 2016). The gradual decrease in the susceptibility of wounds are associated with the healing process of the wound which is in short, the drying of the tissue below the area where the pruning took place (Shigo, 1984; Doster *et al.,* 1988; Bostock *et al.,* 1989), which results in a dead wood area known as the drying- or the dead wood cone (Lafon, 1921; Galet, 2000; Dal

et al., 2008). In the case of improper pruning, the vascular system can be affected and may lead to weakening of the vine (Dal *et al.,* 2008) and therefore care should be taken for proper pruning ensuring good vine heath. Knowing the period of wound susceptibility helps the rootstock mother vine farmers understand when these potential protecting agents should be applied and for how long these agents should be applied for in order to optimize grapevine pruning wound protection.

Results from the protection field trial revealed a year x challenge interaction showing that there was a significant difference between challenging treated wounds 7 days after treatment application and challenging 24 hours after treatment application in 2018. A significant difference in incidence when wounds challenged at different time intervals in 2018 compared to 2017 was also revealed. These differences might again be explained due to the drought in 2017 and the higher rainfall experienced during 2018 as mentioned previously. The treatment having the least effect on *Pa. chlamydospora* challenged 24 hours and 7 days after treatment application when applied under normal environmental conditions was kresoxim-methyl. Kresoxim-methyl had similar incidence percentages to the untreated control. Not only did kresoxim-methyl have relatively higher EC_{50} values for both mycelial and germination inhibition, it has also shown to be inconsistent when applied in *in vitro* conditions on detached shoots.

Focussing on the treatments that showed to be effective as a potential pruning wound agent when applied in normal environmental conditions, includes; MT1 + carbendazim, MT1 + thiophanate-methyl and carbendazim. These treatments showed to be highly effective when challenged 24 hours after treatment application meaning that it has potential as a fast-acting protection agent. Although these treatments were within the group that showed the highest reduction in *Pa. chlamydospora* incidence (25% and 31.3% respectively), the reduction after 7 days was not as effective as during the 24 hour challenge. A study which also investigated the potential of these fungicides *in vitro* was done by Mutawila *et al.* (2015). Although Mutawila *et al.* (2015) found that carbendazim effects mycelial growth of wild type *Trichoderma,* it has little effect on *Trichoderma* germination. Mutawila *et al.* (2015) also suggested that wild type *Trichoderma* are naturally resistant to thiophanate-methyl. Therefore, both mycelial growth and germination of MT1 are not inhibited by thiophanate-methyl and germination of MT1 are not inhibited by carbendazim. Thereby integrating these two treatments could potentially provide short and long-term wound protection. From their study they have also shown the potential biological control of *Pa. chlamydospora* with MT1 as *in vitro* studies shown that MT1 could completely overgrow *Pa. chlamydospora* inoculated PDA plates leading to the assumption that MT1 is potentially an antagonist of *Pa. chlamydospora.* Not only did Mutawila *et al.* (2015) find a potential control for *Pa. chlamydospora* with the MT1 + carbendazim

combination *in vitro,* but they have also shown an overall decrease in *Pa. chlamydospora* incidence on *Vitis vinifera* pruning wounds treated with this combination. The two chemicals carbendazim and thiophanate-methyl have shown potential as *V. vinifera* and olive tree pruning wound protectants against different fungi in a variety of fungal trunk studies including; pruning wound evaluations done by Rolshausen *et al.* (2010), Pitt (2012), Díaz *et al.,* (2013) and Olmo *et al.* (2017). Considering results from Mutawila *et al.* (2015) and other studies and comparing their findings to results from this trial it is clear that these two integrated treatments have potential as pruning wound protectants against *Pa. chlamydospora* not only supported via *in vitro* studies but also with different field trials focussing on different fungal trunk pathogens (Billones-Baaijens *et al.,* 2015 and Halleen *et al.,* 2016). It is important to remember that these trials were artificially inoculated. Therefore, we would expect treated wounds to perform even better when they are only exposed to naturally occurring *Pa. chlamydospora* conidia. Results obtained in this study based on artificial inoculations showed that effectiveness of these different control treatments selected for the different trials varied from *in vitro* conditions to those applied in normal environmental conditions. The differences seen when comparing different treatments and their effectiveness in either inhibiting mycelial growth, germination inhibition or reducing *Pa. chlamydospora* incidence in the different trials evaluated in this study can be a result of different environmental condition, inoculation- and incubation protocols.

To date, there are limited number of registered chemical products available for grapevine pruning protection as it is difficult to control numerous taxonomically unrelated organisms and to protect wounds for the entire period of wound susceptibility. In view of the results of this study it is suggested that the most effective treatments; MT1 + carbendazim, MT1 + thiophanate-methyl, T1, Eco77, and tebuconazole which showed good reduction in *Pa. chlamydospora* incidence when challenged either at 24 hours or 7 days should be included in a repeated study. The same methods as used in this study should be used in the repeated study but including an additional *Pa. chlamydospora* challenge 42 days after pruning in order to determine whether these treatments are in fact effective against *Pa. chlamydospora.* Also, to determine whether combining these treatments as an integrated treatment could lead to even more efficient pruning wound protection of *Pa. chlamydospora* rootstock mother vines. It might also be worthwhile to investigate boscalid under field conditions as this chemical showed a potential against *Pa. chlamydospore* growth *in vitro* . To the best of our knowledge, this is the first time that pruning wound protection of rootstock mother vines were evaluated therefore, additional research would be needed to confirm these results in other grape growing regions of the world and under other climatic conditions. Focus should be given to the time and dosage of application of these different chemicals and it is also important to determine

whether these effective treatments are also effective against other common trunk pathogens as it is generally known that pruning wounds are susceptible to a broad range of fungal pathogens. Finally, a strategy for grapevine rootstock pruning wound protection could be optimized by applying effective treatments during the most susceptible period.

CONCLUSION

The success of grapevine pruning wound protection is a multifaceted process, and despite the bias towards the use of biological control agents alone, integrated disease and crop management should not be neglected. Chemicals are fast acting but break down after some time, thus, losing their effectiveness whereas, biological control agents require some time to germinate and colonise the wound therefore considered as a slow acting control agent. The ideal would be to combine these two control agents consequently protecting the wound directly after application and for an extended period. This goes without saying, applying these control agents at the right time is of fundamental importance. Results from this study showed the decrease of grapevine pruning wound susceptibility over a period of 42 days, and even at day 42 these wounds remained susceptible. It therefore is clear that grapevine pruning wounds should be protected for an extended period after pruning. Knowledge gained from previous research in accordance with the current results will assist in optimizing the application of the right control agents at the right time. Hence it can be concluded that the integration of *Trichoderma* with benzimidazole fungicide are effective when applied to wounds directly after pruning.

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TABLES AND FIGURES

Table 1. List of *Phaeomoniella chlamydospora* isolates chosen to use as inoculum in *in vitro*, detached shoot and field trials.

Organism	Culture number	STE-U number	Location	Isolation date	Collector	Collection
Phaeomoniella chlamydospora	LM91	8276	Paarl, South Africa	24/09/2001	L. Mostert	Stellenbosch University, Department of Plant Pathology
Phaeomoniella chlamydospora	LM310	6384	Paarl, South Africa	26/03/2002	L. Mostert	Stellenbosch University, Department of Plant Pathology

Table 2. List of products used *in vitro*, detached shoot and field trials against *Phaeomoniella chlamydospora.*

Trade name	Active ingredients	Chemical group	Mode of action	Formulation concentration	Formula
T1	*Trichoderma atroviride*	Biological fungicide	Antagonistic properties with enhanced plant growth action	25 g/l	Soluble powder
MT1	*Trichoderma atroviride* mutant-Benzimidazole resistant (US)	Biological fungicide	Antagonistic properties with enhanced plant growth action	25 g/l	Soluble powder
Eco77	*Trichoderma atroviride*	Biological fungicide	Antagonistic properties with enhanced plant growth action	25 g/l	Soluble powder
MT1+Bendazid 500SC	*Trichoderma atroviride*+ carbendazim				

Table 2. Continued

Trade name	Active ingredients	Chemical group	Mode of action	Formulation concentration	Formula
MT1+Cercobin M	*Trichoderma atroviride*+ thiophanate-methyl				
Bendazid 500SC	Carbendazim	Benzimidazole	Mycelia growth inhibition	500 g/l	Suspension concentrate systemic fungicide
Cercobin M	Thiophanate-methyl	Benzimidazole	Mycelia growth inhibition	700 g/kg	Wettable powder systemic application
Duet Ultra	Thiophanate-methyl+epoxiconazole	Benzimidazole+ Triazole			
Epoxiconazole 125SC	Epoxicanozole	Triazole	Mycelia growth inhibition	125 g/l	Suspension concentrate with systemic protective and curative action

Table 2. Continued

Trade name	Active ingredients	Chemical group	Mode of action	Formulation concentration	Formula
Tebuzole 250EW	Tebuconazole	Triazole	Mycelia growth inhibition	250 g/l	Emulsion oil in water fungicide with systemic action
Vista Flo	Kresoxim-methyl	Strobilurin	Mycelia/ Germination inhibition	500 g/l	Suspension concentrate translaminar fungicide
Cabrio	Pyraclostrobin	Strobilurin	Mycelia/ Germination inhibition	250 g/l	Emulsifiable concentrate contact and translaminar fungicide
Cantus	Boscalid	Pyridine-carboxamide	Mycelia/ Germination inhibition	500 g/l	Water dispersible granular systemic fungicide
Tessior	Boscalid+pyraclostrobin	Pyridine-carboxamide+ Strobilurin			

Table 3. Sensitivity of *Phaeomoniella chlamydospora* to selected fungicides based on *in vitro* inhibition of mycelial growth and germination viability.

Trade name	Active ingredient	Mycelial growth			Germination			Field rate (Recommended)
		Concentration range (µg/mL) and [number] [3]	EC_{50} (µg/mL)[1]		Concentration range (µg/mL) and [number]	EC_{50} (µg/mL)		
			LM310	LM91		LM310	LM91	
Bendazid 500SC	Carbendazim	0.01-5 [6][3]	0.0175	0.0004	-[2]	-	-	500 g/l
Cercobin M	Thiophanate-Methyl	0.01-1.5 [6]	0.0091	0.0017	-	-	-	700 g/kg
Epoxicanozole 125SC	Epoxiconazole	0.01-5 [6]	0.0161	0.0057	-	-	-	125 g/l
Tebuzole 250EW	Tebuconazole	0.01-10 [6]	0.082	0.129	-	-	-	250 g/l
Vista Flo	Kresoxim-methyl	0.5-1000 [6]	5.626	22.90	0.5-1000 [6]	112.985	55.575	500 g/l
Cabrio	Pyraclostrobin	0.02-5 [6]	3.92	0.197	0.05-500 [6]	0.034	-	250 g/l
Cantus	Boscalid	-	-	-	0.05-10 [6]	231.709	95.92	500 g/l

[1] The EC_{50}, is the effective concentration of the selected chemicals (in µg/mL) that inhibited radial mycelial growth and germination by 50%.
[2] - Indicates no EC_{50} value was recorded or the specific chemical was not evaluated in those trials.
[3] [ ] Number of fungicide concentrations used in the trial.

Table 4. Mean *Phaeomoniella chlamydospora* (LM310) incidence (%) from two detached shoot trials four weeks after treatment and challenge application from Ramsey, 101-14 Mgt and US 8-7 rootstock cuttings.

Fungicide	Mean *Phaeomoniella chlamydospora* incidence (%)[1]	
	Trial 1	Trial 2
T1	55.8 fghi	55.5 fghi
MT1	51.4 fghij	51.7 fghij
MT1+carbendazim	55.6 fghi	59.1 defghi
MT1+thiophanate-methyl	55.1 fghi	50.6 fghij
Eco77	54.8 fghij	50.5 fghij
Carbendazim	58.1 defghi	36.2 j
Thiophanate-methyl	71.8 bcde	56.1 efghi
Thiophanate-methyl+epoxiconazole	61.5 cdefgh	47.9 hij
Epoxiconazole	78.9 ab	47 hij
Tebuconazole	48.9 ghij	72.4 bcd
Kresoxim-methyl	65.1 bcdef	76.9 abc
Pyraclostrobin	61.2 cdefgh	64.5 bcdefg
Boscalid	63.9 bcdefg	43.5 ij
Boscalid+pyraclostrobin	54.5 fghij	48.9 ghij
Control	91.8 a	72.9s bcd

[1] Values followed by the same letter in the same column are not significantly different from each other (P = 0.05; LSD 15.882). A total of 2700 (15x5x2x3 per trial x 2 trials x 3 rootstocks) spurs were included over the two trials in this study and for each spur, symptomatic wood pieces were isolated onto approximately 8100 PDA plates.

Table 5. Mean *Phaeomoniella chlamydospora* (LM310) incidence (%) nine months after pruning and inoculation from Ramsey and US 8-7 rootstock mother vines from two separate seasons (year 2017 and 2018) in the susceptibility field trial.

Year	Mean *Phaeomoniella chlamydospora* incidence (%)[1]
2017	51.9 [b]
2018	58.3 [a]
LSD	6.3

[1] Values followed by the same letter are not significantly different (P = 0.05).

Table 6. Mean shoot length (cm) measured nine months after pruning and inoculation from Ramsey and US 8-7 rootstock mother vines from two separate seasons (year 2017 and 2018) in the susceptibility field trial.

Year	Mean length (cm)[1]	
	Basal shoot	Distal shoot
2017	170.5 [b]	177 [b]
2018	222.1 [a]	205.5 [a]

[1] Values followed by the same letter are not significantly different (P = 0.05; Basal LSD 26.86 Distal LSD 21.311).

Table 7. Mean *Phaeomoniella chlamydospora* incidence (%) nine months after pruning and inoculation at different time intervals (the end of May and the end of June) from Ramsey and US 8-7 rootstock mother vines in the susceptibility field trial.

Challenge	Mean *Phaeomoniella chlamydospora* incidence (%)[1]	
	End May	End June
No challenge	11.6 [e]	17.5 [e]
0 hours	73.8 [ab]	70 [ab]
24 hours	70 [ab]	71.3 [ab]
7 days	47.5 [cd]	78.8 [a]
21 days	62.5 [bc]	70 [ab]
42 days	41.3 [d]	47.5 [cd]

[1] Values followed by the same letter are not significantly different (P=0.05; LSD 15.373). A total of 960 (10x2x6 per trial x 4 trials x 2 seasons) spurs were included over the four trials in two seasons in this study and for each spur, symptomatic wood pieces were isolated onto approximately 2880 PDA plates.

Table 8. Mean shoot length (cm) measured nine months after treatment and challenge application from Ramsey and 101-14 Mgt rootstock mother vines from two separate seasons (year 2017 and 2018) in the protection field trial.

Year	Mean length (cm)[1]	
	Basal shoot	Distal shoot
2017	219.1 [b]	206.1 [b]
2018	263.1 [a]	291.9 [a]

[1] Values followed by the same letter are not significantly different (P = 0.05; Basal LSD 23.303 Distal LSD 26.685).

Table 9. Mean *Phaeomoniella chlamydospora* (LM310) incidence (%) nine months after treatment and challenge application from Ramsey and 101-14 Mgt rootstock mother vines from two separate seasons (year 2017 and 2018) in the protection field trial.

Challenge	Mean *Phaeomoniella chlamydospora* incidence (%)[1]	
	Year	
	2017	2018
No challenge	2.9 [d]	1.7 [d]
24 hours	54.6 [c]	70.4 [b]
7 days	55 [c]	78.3 [a]

[1] Values followed by the same letter are not significantly different (P = 0.05; LSD 6.908).

Table 10. Mean *Phaeomoniella chlamydospora* (LM310) incidence (%) nine months after treatment and challenge application from Ramsey and 101-14 Mgt rootstock mother vines from two separate seasons (year 2017 and 2018) in the protection field trial.

Treatment	Mean *Phaeomoniella chlamydospora* incidence (%)[1]		
	Challenge		
	No challenge	24 hours	7 days
T1	0 ^{k}	62.5 ^{cdefgh}	52.5 ^{ghi}
MT1	5 ^{k}	72.5 ^{bcde}	55 ^{fgh}
Eco77	2.5 ^{k}	62.5 ^{cdefgh}	57.5 ^{efgh}
MT1+carbendazim	0 ^{k}	35 ^{j}	60 ^{defgh}
MT1+thiophanate-methyl	7.5 ^{k}	37.5 ^{ij}	55 ^{fgh}
Carbendazim	0 ^{k}	47.5 ^{hij}	75 ^{abcd}
Thiophanate-methyl	0 ^{k}	67.5 ^{bdcefg}	72.5 ^{bcde}
Tebuconazole	2.5 ^{k}	75 ^{abcd}	55 ^{fgh}
Pyraclostrobin	5 ^{k}	70 ^{bcdef}	75 ^{abcd}
Kresoxim-methyl	2.5 ^{k}	77.5 ^{abc}	90 ^{a}
Thiophanate-methyl+epoxiconazole	0 ^{k}	62.5 ^{cdefgh}	72.5 ^{bcde}
Boscalid+pyraclostrobin	0 ^{k}	80 ^{ab}	75 ^{abcd}
Control	2.5 ^{k}	80 ^{ab}	80 ^{ab}

[1] Values followed by the same letter are not significantly different (P = 0.05; LSD 16.92). A total of 1440 (5x12x3 per trial x 4 trials x 2 seasons) spurs were included over the four trials in two seasons in this study and for each spur, symptomatic wood pieces were isolated onto approximately 4320 PDA plates.

Figure 1. Example of vascular discolouration caused by *Phaeomoniella chlamydospora* inoculated onto a pruning wound of grapevine rootstock cultivar, Ramsey, nine months after inoculation.

APPENDIX

Table 1. Analysis of variance for mean *Phaeomoniella chlamydospora* (LM310) incidence recorded during the detached shoot trial.

Source	Degreese of freedom	Incidence	
		***F*- value**	***P*-value**
Trial	1	8.61	0.0031
Trial (cultivar x block)	16	3.15	<0.0001
Treat[1]	14	4.82	<0.0001
Trial x Treat	14	3.07	**0.0001**
Challenge	1	1.72	0.1897
Treat x Challenge	14	0.66	0.8165
Trial x Challenge	1	0.23	0.6320
Trial x Treat x Challenge	14	1.10	0.3558

[1] Treat represents the different treatments tested in the trial.

Table 2. Analysis of variance for mean shoot lengths measured and mean *Phaeomoniella chlamydospora* (LM310) incidence recorded during the susceptibility field trial.

Source	Degreese of freedom	Basal shoot length		Distal shoot length		Incidence	
		***F*-value**	***P*-value**	***F*-value**	***P*-value**	***F*-value**	***P*-value**
Year	1	14.66	**0.0003**	7.13	**0.0095**	4.22	**0.0439**
Year (site)	6	10.93	<0.0001	4.48	0.0007	15.90	<0.0001
Pruning time	1	0.28	0.5973	0.69	0.4097	6.68	0.0120
Challenge	5	1.73	0.1410	0.77	0.5778	33.53	<0.0001
Pruning time x Challenge	5	1.65	0.1597	0.23	0.9472	2.46	**0.0415**
Year x Pruning time	1	0.87	0.3547	0.91	0.3448	0.22	0.6442
Year x Challenge	5	0.47	0.7992	0.79	0.5590	0.19	0.9637
Year x Pruning time x Challenge	5	0.67	0.6491	1.16	0.3383	0.15	0.9788

Table 3. Analysis of variance for mean shoot lengths measured and mean *Phaeomoniella chlamydospora* (LM310) incidence recorded during the protection field trial.

Source	Degreese of freedom	Basal shoot length		Distal shoot length		Incidence	
		***F*-value**	***P*-value**	***F*-value**	***P*-value**	***F*-value**	***P*-value**
Year	1	13.87	**0.0003**	40.19	**<0.0001**	39.03	<0.0001
Year (site)	6	10.34	<0.0001	6.04	<0.0001	5.10	<0.0001
Treat[1]	11	0.67	0.7676	1.38	0.1859	4.63	<0.0001
Challenge	2	1.36	0.2590	0.15	0.8626	422.80	<0.0001
Year x Challenge	2	0.21	0.8080	0.50	0.6098	12.93	**<0.0001**
Treat x Challenge	22	1.28	0.1899	0.45	0.9838	2.88	**<0.0001**
Year x Treat	11	1.58	0.1085	0.66	0.7751	0.55	0.8674
Year x Treat x Challenge	22	0.90	0.6016	0.70	0.8402	0.49	0.9760

[1] Treat represents the different treatments tested in the trial.