DETERMINING THE RESISTANCE OR SUSCEPTIBILITY OF GRAPEVINE ROOTSTOCKS USED IN SOUTH AFRICA TOWARDS FUNGAL TRUNK DISEASE PATHOGENS

by

MADELEINE SIEBERHAGEN

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Supervisor: Dr F. Halleen

Co-supervisor: Dr L. Mostert

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DECLARATION

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SUMMARY

Grapevine fungal trunk diseases are responsible for reduced grapevine production world-wide. Trunk diseases are caused by endophytic, xylem-inhabiting pathogens associated with a wide range of symptoms. Currently the largest concern is the presence of pathogens in rootstock mother vines and propagation material. Very little information is available on the susceptibility of grapevine rootstocks used in the South African industry.

The susceptibility of the most popular grapevine rootstocks in South Africa to Petri disease (*Phaeomoniella chlamydospora*, *Phaeoacremonium minimum, Pm. parasiticum, Cadophora luteo-olivacea* and *Pleurostoma richardsiae*), black-foot disease (*Ilyonectria liriodendri, Dactylonectria macrodidyma, Campylocarpon fasciculare* and *C. pseudofasciculare*) and Botryosphaeria dieback (*Neofusicoccum australe* and *N. parvum*) pathogens were evaluated.

Firstly, virulence screenings were conducted by plug inoculating dormant Paulsen 1103 cuttings with 10 isolates of each pathogen. The two most virulent isolates were identified according to lesion lengths that developed after six weeks of incubation at 22 °C. There was significant variability between the isolates of all the pathogens, except *Pleurostoma richardsiae.*

Dormant rootstock cuttings of Ramsey, Ruggeri 140, Paulsen 1103, Richter 99, Richter 110, US 8-7, 101-14 Mgt and SO_4 were vacuum inoculated with conidial suspensions (1 x 10^6 conidia mL) of the two most virulent isolates of each pathogen. After eight months in nursery fields, the dormant plants were uprooted and assessed for un-dried shoot and root weight. The percentage disease severity was determined by cutting the plant 10 cm from the base and assessing the internal vascular discolouration.

All of the rootstocks inoculated with Petri disease, black-foot disease and Botryosphaeria dieback pathogens showed a significant reduction in root mass, shoot mass and a significant increase in percentage disease severity. None of the rootstocks were completely resistant to fungal trunk disease pathogens, but differential levels of tolerance did exist. The reduction in shoot and root mass did not show consistent results between the rootstock cultivars, but rootstocks 101-14 Mgt and SO_4 were the most susceptible and Ramsey the most tolerant, in terms of percentage disease severity.

In an attempt to explain the variable tolerance of rootstocks to infection, suberin production and xylem morphology was investigated. Transversal cuts of Ramsey and 101-14 Mgt were made and stained using two suberin staining techniques. The important role suberin plays in the compartmentalization of a pathogen in grapevine wood was confirmed. Suberin was located around vessels filled with tyloses, tyloses itself was also suberized, ray parenchyma cells and cells located on the growth boundary ring. The suberized zones form

impermeable barriers that restrict pathogen spread to uninfected and newly developed vascular tissue.

The mean vessel diameters were determined for each cultivar using 40 µm thick transversal cuts stained with toluidine O. A strong correlation between mean vessel diameter and rootstock tolerance to fungal trunk pathogens was established. Ramsey had the smallest mean vessel diameter and 101-14 Mg the largest.

The quality of plant material is crucial in the success and longevity of newly established vineyards. By combining existing knowledge of disease management in the propagation process with knowledge obtained from this research, can the quality of plant material be optimized to ensure the sustainability of the South African grapevine industry.

OPSOMMING

Wingerd stamsiektes veroorsaak afname in wingerdproduksie wêreldwyd. Hierdie stamsiektes word veroorsaak deur endofitiese swamme en word geassosieer met ‘n wye reeks simptome. Tans skep die teenwoordigheid van patogene in onderstok moederplante en kwekery materiaal groot bekommernisse. Beperkte inligting is beskikbaar oor die vatbaarheid van onderstokke wat algemeen in Suid-Afrika gebruik word.

Die vatbaarheid van die gewildste onderstok kultivars in Suid-Afrika teenoor Petri-siekte (*Phaeomoniella chlamydospora*, *Phaeoacremonium minimum, Pm. parasiticum, Cadophora luteo-olivacea* en *Pleurostoma richardsiae*), swartvoet (*Ilyonectria liriodendri, Dactylonectria macrodidyma, Campylocarpon fasciculare* en *C. pseudofasciculare*) en Botryosphaeria terugsterwing (*Neofusicoccum australe* en *N. parvum*) patogene is geëvalueer.

Die virulensie van tien isolate per patogeen is bepaal deur dormante Paulsen 1103 stokkies te inokuleer met miselium skyfies. Die letsels wat die isolate veroorsaak het na ses weke teen 22 °C is gemeet en die twee virulentste isolate geïdentifiseer. Behalwe vir *Pleurostoma richardsiae,* was daar beduidende verskille tussen die isolate van elke patogeen.

Dormante stokkies van Ramsey, Ruggeri 140, Paulsen 1103, Richter 99, Richter 110, US 8-7, 101-14 Mgt en SO_4 is geïnokuleer deur spoor suspensies ($1x10^6$ spore mL) van elke isolaat met behulp van ‘n vakuumpomp deur die stokkies te suig. Nadat die stokkies vir agt maande in kwekery gronde gegroei het, is hulle gelig en ongedroogde loot- en wortelmassas bepaal. Die persentasie infeksie is ook vasgestel deur die stokkies 10 cm van die basis af deur te sny en die interne vaskulêre verkleuring te evalueer.

Al die onderstokke wat met die Petri-siekte, swartvoet en Botryosphaeria terugsterwing patogene infekteer was, het ‘n beduidende afname in wortel- en lootmassa en ‘n toename in persentasie infeksie getoon. Die onderstokke was nie een totaal weerstandbiedend teen swaminfeksie nie, maar verskillende vlakke van toleransie is waargeneem. Die afname in loot- en wortelmassas was nie konsekwent tussen die kultivars nie, maar in terme van persentasie infeksie was 101-14 Mgt en SO_4 die mees vatbaar en Ramsey die mees tolerant.

Om meer insig omtrent die verskillende vlakke van toleransie tussen die kultivars te bekom, is die suberien produksie en gemiddelde xileem vat deursnit bepaal. Deursnitte is gemaak van Ramsey en 101-14 Mgt en die snitte het twee suberien verkleuringstegnieke ondergaan. Die belangrike rol wat suberien speel in die kompartimentering van patogene in wingerdhout is bevestig. Suberien was geleë rondom xileem vate gevul met tilose (die tilose was ook gesuberiseer), in straalselle en selle geleë op die jaarring. Die zones met suberien vorm ondeurlaatbare versperrings wat die beweging van patogene na gesonde materiaal beperk.

Die gemiddelde vat deursnitte is bepaal vir elke kultivar deur 40 μm deursnitte te kleur met toluidine O en te evalueer onder ‘n ligmikroskoop. Daar is ‘n sterk korrelasie tussen ‘n kultivar se gemiddelde vaat deursnit en sy vatbaarheid vir stamsiektes gevind. Ramsey het die kleinste en 101-14 Mgt die grootste gemiddelde vat deursnit gehad

Die kwaliteit van plant materiaal is krities vir die sukses en langslewendheid van nuut gevestigde wingerde. Deur die bestaande kennis, met betrekking tot siekte-beheer in die kwekery prosesse, te kombineer met kennis wat verkry is in die huidige studie kan daar voortgegaan word om die kwaliteit van plantmateriaal te verhoog om die volhoubaarheid van die Suid-Afrikaanse wingerd industrie te verseker.

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CHAPTER 1

A review of young vine decline and trunk pathogen infections during grapevine propagation and the management thereof

INTRODUCTION TO GRAPE PRODUCTION IN SOUTH AFRICA

Grapevine (*Vitis vinifera* L.) production areas are located in temperate and cool climatic regions around the world. This was traditionally primarily located in Europe and the Middle-East. From here, propagation material was exported to North and South America, Australia, New Zealand and South Africa. In South Africa, viticulture and winemaking dates back to the 17th century when Dutch explorers arrived in the Cape and by the 18th century wines from South Africa, specifically from Constantia, were already being exported and was considered among the most popular wines. Today there are about 125 000 ha of land under grapevine in South Africa (excluding sultana production), of which 106 018 ha is used for wine production (SAWIS, 2015), 18 212 ha for the production of table grapes (SATI, 2015) and approximately 200 ha of rootstock mother fields (Raath, 2012). This growing industry employs approximately 353 000 people directly and indirectly (farm workers, packaging, retail and tourism) and the industry's contribution to South Africa's Gross Domestic Product (GDP) has had an annual growth of 10% per annum since 2003 (SAWIS, 2015).

Therefore, the success and sustainability of the grapevine industry is of the utmost importance to the South African economy. However, sustainable production is threatened by several factors of which fungal grapevine trunk diseases is one of the major threats. These infections result in the decline of quality and yield of infected vines and early death resulting in great financial losses.

IMPACT OF VINE DECLINE

Fungal trunk disease pathogens have been plaguing viticulture regions across the world for many years (Scheck *et al.,* 1998a; Waite and May, 2005; Gramaje and Armengol, 2011). An occurrence that has been increasing dramatically since the 1990's is the decline of young vines (Gramaje and Armengol, 2011). There are many propagation, cultural and environmental factors that contribute to the decline and failure of young grapevines, but the presence of fungal pathogens has been identified as one of the most prominent causes of this syndrome (Stamp, 2001, 2003).

There are management strategies in place to try and prevent grapevine trunk infection in vineyards, but no successful curative measures are available. A growing concern in the industry is, however, that young grafted grapevines are already infected in the nursery propagation process or from nursery mother vines (Fourie and Halleen, 2002, 2004b;

Gramaje and Armengol, 2011). Young vine decline results in great financial losses due to a significant reduction in productivity of infected plants and also the cost of re-establishing the infected vineyards (Hofstetter *et al.,* 2012).

The three main diseases responsible for young vine decline are Petri disease, black-foot disease and Botryosphaeria cankers and dieback (Giménez-Jaime *et al.*, 2006; Gramaje and Armengol, 2011). These diseases can act alone or as a complex in the infected grapevines (Cobos and Martin, 2008; Surico, 2009; Valtaud *et al*., 2009; Spagnolo *et al*., 2011; White *et al*., 2011a). General field symptoms associated with Petri and black-foot disease are often similar and include; reduced vigour, stunted growth, shortened internodes, leaf chlorosis with necrotic margins, wilting and dieback (Fourie *et al.,* 2001; Sheck *et al.,* 1998b). Even though these external symptoms overlap, there are more characteristic symptoms for each disease that is discussed in more detail below.

PETRI DISEASE

Petri disease, previously referred to as 'black goo', was discovered for the first time in Italy in 1912 (Mugnai *et al.,* 1999). The disease was later reported in South Africa (Ferreira *et al.,* 1994, 1998), the United States of America (Scheck *et al.,* 1998b) and in other countries (Surico, 2001). Petri disease affects vines aged up to 5-years-old and has been reported to cause major losses of young vines in newly established vineyards (Mugnai *et al.,* 1999; Eskalen *et al.,* 2001). The infection leads to great losses due to weak development and early dieback of the vines and has great financial implications in the agricultural industry.

Causal pathogens

Phaeomoniella chlamydospora (W. Gams, Crous, M.J. Wingf. And L. Mugnai) Crous and W. Gams and several *Phaeoacremonium* W. Gams, Crous and M.J. Winf. species, *Phaeoacremonium minimum* (Tul. And C. Tu.) D. Gramaje, L. Mostert and Crous being the most predominant, as well as some lesser known species like *Cadophora luteo-olivacea* (van Beyma) Harrington and McNew and *Pleurostoma richardsiae* (Nannf.) Réblová and Jaklitsch have been identified as the causal agents of this disease (Ferreira *et al*., 1994; Scheck *et al.,* 1998; Crous and Gams, 2000; Sidoti, *et al.,* 2000; Halleen *et al.,* 2007; Gramaje *et al.,* 2010a)

Symptoms

The external symptoms associated with Petri disease include a reduction in growth and productivity of the vine, general weakness; delayed budburst and short shoot internodes. Interveinal chlorosis and marginal necrosis as well as a reduction in leaf size and early senescence are common foliar symptoms associated with this disease (Ferreira *et al.,* 1994,

1998; Scheck *et al*., 1998b; Mugnai *et al*., 1999; Retief *et al*., 2005; Gramaje *et al*., 2008, 2013). Unfortunately, these symptoms are quite general and a thorough examination of the internal symptoms of an infected vine has to be made to establish an accurate diagnosis. The internal symptoms of infected vines are, discoloration as small black spots in the xylem tissue is visible and in some cases a dark phenolic substance is known to leach from the discoloured vessels also referred to as a 'black goo' (Mugnai *et al.,* 1999; Whiting *et al.,* 2001). The discolouration can be sparsely distributed or be clustered in groups around an annual growth ring or closer to the pith, which in itself can also be darkened (Mugnai *et al.,* 1999). Longitudinally, the xylem discolouration can appear as light brown to brown streaking (Mugnai *et al.*, 1999; Eskalen *et al*., 2001; Whiting *et al*., 2001).

Epidemiology

During the propagation of grapevines, there are various ways for plant material to be infected, usually involving infection through wounds. When such an infection occurs it is detrimental to the quality and success of the propagation material. Several studies have reported that *Phaeoacremonium* spp. and *P. chlamydospora* can partially or completely inhibit basal and grafting callus formation, which can later lead to very weak joints between the scion and rootstock as well as complete graft failure (Ferreira *et al*., 1994; Khan *et al*., 2000; Feliciano *et al*., 2004; Santos *et al*., 2005). Infections also result in a reduction in root emission and a reduction in graftling budburst. Internal streaking of the young grafted vines can already be visible early on in the production process (Ferreira *et al*., 1994; Khan *et al*., 2000; Eskalen *et al*., 2001; Feliciano *et al*., 2004).

The dissemination of the pathogens that are responsible for Petri disease has been investigated in great detail due to its devastating effect on grapevines. Studies have mostly focused on *Pm. minimum* and *P. chlamydospora,* as these species are most common and widely dispersed of all the Petri disease pathogens (Crous *et al*., 1996; Crous and Gams, 2000; Fourie and Halleen, 2002; Mugnai *et al*., 1999; Gramaje *et al.,* 2015). *Phaeoacremonium minimum* and *P. chlamydospora* are both endophytic vascular pathogens, which inhabit the grapevine xylem and pith. Here the pathogens have the ability to produce conidia that is then spread through the vascular system of the host by means of free flowing plant sap (Khan *et al*., 2000; Larignon and Dubos, 2000; Feliciano and Gubler, 2006).

Phaeoacremonium minimum and *P. chlamydospora* overwinters in pruning debris as well as deep cracks and crevices in cordons and spurs. These cracks provide the fungi with a humid environment that is ideal for growth, formation of pycnidia and perithecia as well as sporulation (Crous *et al*., 1996; Mugnai *et al*., 1999; Larignon and Dubos, 2000; Edwards and Pascoe, 2001; Edwards *et al*., 2001; Rooney-Latham *et al.,* 2005a, 2005b; Baloyi *et al.,*

2013). The presence of diseased pruning debris in and on top of soil makes it possible for Petri disease pathogens to become soil-borne pathogens (Bertelli *et al*., 1998; Mugnai *et al*., 1999; Taylor *et al*., 2005). The presence of *Pm. minimum* has been positively identified in soil and standing water samples taken from underneath grapevines (Rooney *et al*., 2001). *Phaeoacremonium minimum* along with *Pm. parasiticum* and *P. chlamydospora* has been isolated from the vascular tissue of young grapevine seedlings that were used as bait plants in testing nursery soils (Agustí-Brisach *et al*., 2013). The studies successfully established that grapevine roots can be infected by Petri disease pathogens present in infested soil.

When the air and soil temperatures rise in spring, the fungi become active and produce conidia and ascospores that can be transmitted through water, wind as well as arthropods (Larignon and Dubos 2000; Eskalen and Gubler 2001; Mostert *et al*., 2006; Moyo *et al*., 2014). After a period of adequate hydration, via rain or irrigation, ascospores are released from perithecia (Eskalen and Gubler, 2001). Whiting *et al.* (2001) found that *P. chlamydospora* and *Phaeoacremonium spp.* were able to adapt to a wide range of water potentials in order to survive. This survival mechanism is due to the fungi´s endophytic nature and makes it possible to be present in a wide range of climates (Whiting *et al.,* 2001). Aerial dispersal of spores has also been observed by analysing the spread of the disease in vineyards by means of spore traps placed in infected vineyards (Surico *et al.*, 2000a; Eskalen and Gubler, 2001; Baloyi *et al.*, 2013). Many studies have proved that it is possible for *Phaeoacremonium spp.* and *P. chlamydospora* to successfully infect grapevines through pruning wounds (Khan *et al.,* 2000; Larignon and Dubos, 2000; Gubler *et al.,* 2001, 2005; Eskalen *et al.,* 2007; Van Niekerk *et al.,* 2010). It was suggested by Edwards *et al.* (2001), that conidia can possibly be spread by insects like collembolans and mites. Recently Moyo *et al*. (2014) found the presence of Petri disease pathogens by washing arthropods gathered from vineyards in South Africa and plating on media and showed that *P. chlamydospora* could transmitted by Portuguese millipedes (*Ommattoiulus moreleti*) and cocktail ants (*Crematogaster peringueyi*) to infect fresh pruning wounds.

The main source of inoculum for Petri disease pathogens is still considered to be infected propagation material (Gramaje and Armengol, 2011). The presence of *Phaeoacremonium* spp., *P. chlamydospora*, *Co. luteo-olivacea* and *Pl. richardsiae* have been confirmed in grapevine mother plants as well as in various steps of the propagation process (Rego *et al*., 2000; Halleen *et al*., 2003; Fourie and Halleen, 2004b; Mostert *et al*., 2006; Retief *et al*., 2006; Díaz *et al.,* 2009).

BLACK-FOOT DISEASE

Causal pathogens

Black-foot disease of grapevines is a severe and common disease in several wine and grape-producing regions in the world and is a particularly big problem in nurseries and young vineyards (Alaniz *et al*., 2009; Agustí-Brisach *et al*., 2013; Cabral *et al*., 2012; Agustí-Brisach and Armengol, 2013). The common name, "black-foot" disease, was proposed by Sheck *et al.* (1998b) for the disease first known to be caused by *"Cylindrocarpon" destructans* (Zinnsm.) Scholten and "*C". obtusisporum* (Cooke and Harkn) Wollenw. Originally, these two agents were most commonly associated with basal or root necrosis on grapevines. Black-foot disease pathogens are common soil inhabitants that occur as saprobes or weak pathogens (Bayford, 1993). *Cylindrocarpon destructans* was first reported in France in 1961 (Maluta and Larignon, 1991) and has since been isolated from symptomatic vines in Tasmania (Sweetingham, 1983), Sicily (Grasso, 1984), Portugal (Rego, 1994; Rego *et al.,* 2001a) and the United States of America (Gugino and Travis, 2003). *Cylindrocarpon obtusisporum* has been reported in Sicily (Grasso and Magnano di San Lio, 1975) and the U.S.A. (Scheck *et al.*, 1998c). Another genus, *Campylocarpon* Halleen, Schroers and Crous was established as "*Cylindrocarpon*"-like in morphology and was associated with black-foot disease of grapevines in South Africa (Halleen *et al*., 2004). Of this genus, two species were specifically linked to black-foot disease namely *C. pseudofasciculare* and *C. fasciculare* and it has since been reported in Uruguay (Abrero *et al.*, 2010), Spain (Alaniz *et al.,* 2011a) and Turkey (Akgul *et al.,* 2014). Many more species have since been identified as pathogens causing black-foot disease symptoms. *"C". macrodidymum* (Halleen, Schroers and Crous) was one such species. It was, however, later confirmed by Halleen *et al.* (2006a) that "*C". macrodidymum* was identical to *"C". obtusisporum* and that the latter had been misidentified in the past and was in fact *"C". macrodidymum* that was isolated from symptomatic grapevines. After this case of miss-identification, "*C". destructans* as causal agent was brought into question. Halleen *et al.* (2006b) compared strains of *"C". destructans* isolated from grapevine with *"C". destructans*-like anamorphs that have been isolated from various woody and herbaceous hosts. It was found that *"C". destructans* isolated from grapevines was genetically identical to *"C". liriodendri*, originally associated with root rot of tulip poplar in California (Mac Donald and Butler, 1981). Thus isolates collected from asymptomatic and symptomatic grapevines were renamed to *"C". liriodendri* (Halleen *et al.,* 2006b). The sexual morphs of *"C". liriodendri* and "*C". macrodidymum* have been described as *Neonectria liriodendri* and *Neo. macrodidyma* (Halleen *et al*., 2004, 2006b). Several other *Cylindrocarpon* species have also been isolated from symptomatic grapevines in Australia (Edwards and Pascoe, 2004), Chile (Auger *et al.,* 1999), Greece (Rumbos and Rumbou, 2001), Spain (Armengol *et al.,* 2001) and South Africa (Fourie *et al.,* 2000; Fourie and

Halleen, 2001). A phylogenetic study of *Neonectria*, "*Cylindrocarpon*" and "*Cylindrocarpon*"-like anamorphs conducted by Chaverri *et al*. (2011) suggested that *Neonectria* and "*Cylindrocarpon*" represented more than one genus. Based on the results of the phylogenetic study and conidial septation and perithecial anatomy, *Neonectria* was divided into five genera. Consequently, *"C". liriodendri* and *"C". macrodidymum* was included into the *Ilyonectria* genus and re-identified as *Ilyonectria liriodendri* (Halleen, Rego and Crous) Chaverri and Salgado and *I. macrodidyma* (Halleen, Schroers and Crous) P Chaverri and Salgado (Chaverri *et al.*, 2011). More recently, a multi-gene study conducted by Lombard *et al*. (2014), indicated the genus *Ilyonectria* to be paraphylectic and as a result the genus *Dactylonectria* was introduced. This new genus contains ten new combinations which includes *Dactylonectria macrodidyma,* previously included in *Ilyonectria*. Therefore, in South Africa, the causal organisms of black-foot disease are *Campylocarpon fasciculare, Campylocarpon pseudofasciculare, Ilyonectria liriodendri* and *Dactylonectria macrodidyma.*

Symptoms

A large range of external and internal symptoms can be observed as a result of infection, unfortunately these symptoms greatly resembles that of Petri disease and makes it very difficult to distinguish between the two (Scheck *et al.*, 1998a; Rego *et al*., 2000; Halleen *et al*., 2006b; Abrero *et al*., 2010). Grapevines in established vineyards ranging from two- to ten-years-old can show symptoms. When younger vines are infected the plants die very quickly, but in older vines the decline of the vine is found to be more gradual (Gubler *et al*., 2004). Infected vines can show disease symptoms very early in the growing season. It is usually visible as poor new growth, failure to form new shoots and the infected vine dies by mid-summer (Sweetingham, 1983; Larignon, 1999). Internal symptoms of infection includes dark discolouration of wood at ground level with lesions extending upwards approximately 15 cm above the ground. The discolouration also stretches throughout the below ground portion of the trunk, rootstocks and into large roots (Sweetingham, 1983; Larignon, 1999). Roots at the basal crown can become necrotic and in some cases a second crown of roots can develop in an upper level as compensation to functional root loss below (Scheck *et al.,* 1998b; Larignon, 1999; Fourie *et al.,* 2000; Halleen *et al.,* 2006a). In older vines there are some examples where the diameter of the rootstock is thinner below the second tier (Fourie and Halleen, 2001). Removal of rootstock bark reveals black discoloration in cross section and necrosis of the wood tissue can be seen, which starts at the bark from the rootstock and continuous into the pith (Larignon*,* 1999; Fourie and Halleen, 2001; Bleach *et al.,* 2008; Alaniz *et al.,* 2009).

In the case of nursery vines or young vines that have been newly transplanted, vascular discolouration is generally evident. Black discolouration and gum inclusions of xylem tissue

as well as dark brown to black streaking of vascular tissue have been associated with propagation material infected with black-foot disease pathogens (Grasso, 1975; Scheck *et al*., 1998a). Other above ground symptoms have been reported as reduced vigour, small trunks, shortened internodes, uneven wood maturity, sparse foliage, small leaves with interveinal chlorosis and necrosis. Below-ground symptoms include a reduction in root mass and root hairs with sunken necrotic lesions (Scheck *et al*., 1998a; Rego *et al*., 2000; Halleen *et al*., 2006b; Alaniz *et al*., 2007, 2009; Abrero *et al*., 2010; Agustí-Brisach and Armengol, 2013). Observations of internal wood symptoms showed compaction of the pith and dark discolouration and streaks at the basal ends of the rootstocks (Scheck *et al.,* 1998a; Rego *et al.,* 2000).

Epidemiology

In a study done by Agustí-Brisach *et al.* (2013) the pathogens responsible for black-foot disease are commonly found in the soil of nurseries as well as established vineyards. The organisms can survive in the soil as saprobes, living on plant debris. Due to the fungi's ability to produce chlamydospores, it also makes it possible to survive in soil even after a crop has been removed (Agustí-Brisach and Armengol, 2012, 2013). The fungi also act as weak or opportunistic pathogens that infect hosts through wounds and natural openings (Fourie and Halleen, 2006; Halleen *et al*., 2006a, 2007a; Schroers *et al*., 2008; Probst *et al*., 2012). Black-foot disease pathogens mostly infect vines through soil, but also have the ability to live in seemingly healthy plant material as endophytes and the host will only show symptoms of infection once it is put under stress. This was confirmed by Halleen *et al.* (2003) and Fourie and Halleen (2004b) when these pathogens were isolated from asymptomatic rootstock mother vines. According to Agustí-Brisach and Armengol (2013), black-foot disease seems to prosper in temperate, sub-tropical, and tropical regions. The organisms responsible can grow at a wide range of temperatures from 5-30°C, where 20-25°C is found to be the optimal growing temperature.

BOTRYOSPHAERIA DIEBACK

Causal pathogens

Botryosphaeria dieback is caused by fungi of the family Botryosphaeriaceae which include species of *Botryosphaeria*, *Neofusicoccum*, *Lasiodiplodia*, *Diplodia*, *Dothiorella, Neoscytalidium, Phaeobotryosphaeria* and *Spencermartinsia* (Phillips, 2002, 2005; Van Niekerk *et al*., 2004; Crous *et al*., 2006; Úrbez-Torres *et al*., 2006; Larignon *et al*., 2009; Úrbez-Torres, 2011; Pitt *et al*., 2013; Rolshausen *et al.*, 2013). Botryosphaeriaceae spp. are described as cosmopolitan pathogens having the ability to infect an extended variety of hosts and has far-reaching geographical dissemination (Barr, 1972; Crous *et al*., 2006).

Infected hosts can develop a wide range of symptoms, but the pathogens involved have also been isolated from asymptomatic grapevines as well as non-woody hosts (Halleen *et al*., 2003; Van Niekerk *et al*., 2004; Wunderlich *et al*., 2011). Usually symptom expression will only develop once infected vines are put under stress (Van Niekerk *et al.,* 2004, 2006, 2010). An accurate identification between the various species is a complicated process, since sexual morphs are seldom found in nature and rarely form in culture (Boyer, 1995; Larignon and Dubos, 2001). Identification is primarily based on anamorph characterisation (Denman *et al*., 2000; Phillips, 2000) combined with molecular data (Jacobs and Rehner, 1998; Slippers *et al*., 2004a, b).

Symptoms

The diagnoses of Botryosphaeria dieback creates a range of problems since the symptoms associated with this disease closely resemble symptoms of other grapevine trunk diseases like Eutypa dieback (Van Niekerk *et al*., 2004). The persisting symptoms that result from infection with Botryosphaeriaceae species usually occur on vines older than 8 years and result in a gradual loss of vigour and yield (Phillips, 1998; Larignon and Dubos, 2001), but a study completed by Amponsah *et al.* (2008) revealed that symptoms can just as easily develop on younger vines. These symptoms include cankers, dieback of shoots and spurs, bud necrosis and mild leaf necrosis (Denman *et al*., 2000; Castillo-Pando *et al*., 2001; Larignon *et al*., 2001; Van Niekerk *et al*., 2004, 2006; Martos *et al.,* 2008). The bleaching of canes, which is usually associated with Phomopsis cane and leaf spot, can occasionally be observed (Phillips, 1998, 2000). Cankers can be chronic, resulting in a slow decline, or acute, causing severe and quick defoliation and wilt in the grapevine (Phillips, 1998; Gubler *et al.,* 2005). Cankers are mainly located on trunks, cordons and canes. They appear on the surface as dark sunken areas, often located close to a large wound or spur from where they extend (Phillips, 1998; Larignon and Dubos, 2001). When the bark from a canker is removed, it reveals a red-brown discoloration or wood necrosis (Larignon and Dubos, 2001; Gubler *et al.,* 2005). In a cross-section of an infected arm the streaking will be visible as wedge-shaped discolouration or dark spots (Van Niekerk *et al.,* 2006; Urbez-Torres *et al.,* 2008). These can be easily confused with Petri disease, but with Botryosphaeriaceae spp. the spots will be more diffuse (Larignon and Dubos, 2001; Gubler *et al*., 2005).

Epidemiology

Botryosphaeriaceae species are known to be endophytic fungi present in its host without any symptom expression until the host is under stress and symptoms become visible (Von Arx, 1987; Denman *et al*., 2000). The pathogens produce pycnidia and perithecia on diseased or dead wood and plant debris that can be present all year around (Hewitt and Pearson, 1988;

Amponsah *et al*., 2009). Once infection is established in a vineyard, it becomes more difficult to manage and infection is likely to spread in the vineyard through the release of conidia and ascospores (Michailides and Morgan, 1993; Amponsah *et al*., 2008). When spores are released, they have the ability to infect vines directly through lenticels, stomata, other natural openings as well as pruning and trimming wounds (Larignon and Dubos, 2000; Van Niekerk *et al*., 2005; Amponsah *et al*., 2008). The main modes of dispersal of the ascospores and conidia are through water splash and wind (Lehoczky, 1974; Hewitt and Pearson, 1988; Van Niekerk *et al*., 2010). During periods of rain and high relative humidity, the pycnidia and perithecia hydrates and starts to produce and release conidia and ascospores (Hewitt and Pearson, 1988; Michailides and Morgan, 1993). The longer the period of wetness and high humidity persists the more spores are produced (Hewitt and Pearson, 1988; Michailides and Morgan, 1993; Ahimera *et al*., 2004). A study conducted by Van Niekerk *et al*. (2010) in South African vineyards revealed the presence of conidia even in periods without rain, suggesting that the presence of pycnidia plays a major role in infection.

PRODUCTION OF VINES IN SOUTH AFRICAN NURSERIES

Traditionally, rootstock and scion mother vines are used to harvest dormant cuttings from use in the grafting process. Once the cuttings have been collected in autumn, they are usually cut according to specifications, buds are removed and the material is soaked in clean water for 8 hours before being put into cold storage (2 - 3°C with 90% relative humidity) until grafting takes place (Fourie and Halleen, 2006; Anonymous, 2011). Before grafting, after cold storage, the plant material is usually soaked again for periods of 2 – 4 hours (Van der Westhuizen, 1981; Le Roux, 1988; Fourie and Halleen, 2006). In South Africa, grapevines are usually hand grafted (tongue or long-wip hand grafting) and ‘omega-cut’ grafting machines are used less frequently (Van der Westhuizen, 1981; Le Roux, 1988; Anonymous, 2011). After the scion and rootstock combinations are grafted, the graft unions are dipped in melted (70 - 75°C) wax formulations that typically contain fungicides that inhibit fungal contamination during callusing (Van der Westhuizen, 1981; Le Roux, 1988; Anonymous, 2011). The grafted vines are kept under growth stimulating, warm and humid callusing conditions until callus tissue is formed around the base and graft union (Van der Westhuizen, 1981; Anonymous, 2011). The callusing-medium commonly used consists of pine sawdust soaked in broad-spectrum fungicides (Fourie and Halleen, 2006). In South African nurseries the practise is followed by cold-callusing conditions (18°C) for hand grafted vines for approximately 5 weeks and machine-grafted vines are hot-callused (26 - 28°C and 70% relative humidity) for 3 weeks (Van der Westhuizen, 1981; Fourie and Halleen, 2006). After callusing is finished, around October, the callused vines are planted in open field nursery fields. The planting distances of cuttings usually vary according to the equipment

used by the nursery. In South Africa the common planting distances are 50 mm between vines with an 850 mm row spacing (Van der Westhuizen, 1981; Anonymous, 2011). A furrow is ripped along each row and the cuttings are placed in the furrow in. In South Africa it is common practice to cover the graft unions with soil for the first couple of weeks after planting to protect the callus tissue around the graft union from drying out (Van der Westhuizen, 1981; Fourie and Halleen, 2006; Anonymous, 2011).

Early in the season, the vines are watered frequently (twice a week) until the young graftlings' root systems have established. Over-head sprinkling is common practise in South African nurseries, providing a uniform distribution (Van der Westhuizen, 1981). Weed control is also of utmost importance, since weeds compete with the graftlings for nutrients and water and can also be potential hosts for fungal pathogens (Van der Westhuizen, 1981; Agustí-Brisach *et al.,* 2011; Anonymous, 2011). Herbicides (napropamide, oryzalin and trifluralin) are applied until budburst takes place (Anonymous, 2011). As soon as the vine canopy had grown, the warmth, humidity and close proximity of the vines is favourable for the development of downy and powdery mildew infection. Copper-sulphur-based sprays are applied from budburst to control these infections (Van der Westhuizen, 1981). After the growing season the dormant vines are uprooted in winter (between the end of May and start of June). This is conducted using a U-shaped digger fitted to a tractor that cut the roots of the vines and lift them out of the soil (Van der Westhuizen, 1981; Anonymous, 2011). The uprooted vines are graded according to the standards set by the Vine Improvement Association (VIA, Paarl, South Africa) and then cold stored or heeled-in until they are replanted in spring (Fourie and Halleen, 2006).

GRAPEVINE TRUNK DISEASES IN THE PROPAGATION PROCESS

The decline of young vines can be a result of numerous causes. The severity of symptoms caused by decline can be enhanced by two groups of factors. The first involves nursery induced stress, including extended cold storage, structural vine defects and limited vine carbohydrate storage. Finally, biological stresses like root colonizing fungal pathogens, nematodes, viruses, fungal trunk pathogens and scion/rootstock incompatibilities have a major influence on decline (Scheck *et al*., 1998a; Morton, 2000; Fourie *et al*., 2001; Stamp, 2003; Waite and May, 2005; Gramaje and Armengol, 2011).

Even though infection can occur in established vineyards, many studies confirm that plant material can already be infected in rootstock mother fields (Fourie and Halleen, 2002, 2006) and nurseries (Halleen *et al.,* 2003). There are several potential inoculum sources of fungal trunk disease pathogens present in the propagation process that can result in the infection of plant material. In most cases the infections are latent and disease establishment and symptom expression only occurs after new vineyards have been established. Infected

vines in newly established vineyards cause a reduction in vine vigour and yields which in turn result financial losses (Fourie and Halleen, 2001; Waite and May, 2005; Abbatecola *et al*., 2006; Aroca *et al*., 2010).

Rootstock mother fields

In South Africa there are approximately 200 ha of rootstock mother fields, providing grafting material for 44.1 million grafted vines per year (PlantSA, 2016). It has been confirmed by various authors, that the primary source of inoculum of trunk disease pathogens are found in rootstock mother fields (Fourie and Halleen, 2002, 2004; Edwards and Pascoe, 2004; Gramaje and Armengol, 2011). As previously discussed, most of the fungal trunk disease pathogens have the ability to live as endophytes in seemingly healthy plant material. *Phaeomoniella chlamydospora, Phaeoacremonium spp.* and Botryosphaeriaceae species have strong endophytic qualities and are commonly found in un-grafted plant material, demonstrating that mother vines are a high risk inoculum source (Fourie and Halleen, 2002, 2004b; Edwards and Pascoe, 2004; Halleen *et al*., 2007a; Aroca *et al*., 2010).

There are two different methods that can be followed when cultivating rootstock mother vines. With the first method the vines are allowed to sprawl freely on the ground. Even though this method is not labour intensive or expensive, the vines have a very high risk of getting infected by soil pathogens, especially during and after irrigation, when wet and humid conditions persist. Previously vines were cultivated on a trellis system (Stamp, 2003; Hunter *et al*., 2004). This method is more expensive and costly, but the risk of infection by soil borne pathogens is reduced and the microclimate in the foliage is improved dramatically.

Research has shown that *Pm. minimum* and *P. chlamydospora* can move via sap flow through xylem vessels to new summer growth (Feliciano and Gubler, 2001; Edwards *et al*., 2003; Edwards *et al.*, 2004; Whiteman *et al.,* 2005). If a latent infection is present in a vine, there is a very high probability that the pathogen will move through the xylem vessels and infect the new growth that will be used as grafting material (Whiteman *et al*., 2007). This can result in the spread of infected plant material all over the country and could later also infect healthy vineyards after establishment.

Soil is one of the pathogen sources present in mother blocks (Bertelli *et al*., 1998; Surico *et al*., 2000b). Black-foot disease pathogens can be present as saprobes in soil, dead plant material or it has the ability to act as a weak pathogen of plants infecting wounds in roots and stems (Halleen *et al*., 2006a; Schroers *et al*., 2008). The production of chlamydospores gives *Ilyonectria, Dactylonectria* and *Campylocarpon* spp. the ability to survive in soil for extended periods of time (Halleen *et al*., 2004). *Phaeomoniella chlamydospora* also has the ability to produce chlamydospores thus suggesting the potential for it to be a soil borne pathogen (Bertelli *et al*., 1998; Mugnai *et al*., 1999; Taylor *et al*., 2005). These

chlamydospores have the ability to form conidia that penetrate uninjured roots of vines in nursery vineyards (Bertelli *et al*., 1998; Feliciano and Gubler, 2001). *Phaeoacremonium minimum* has also been found to be present in soil (Eskalen *et al*., 2001) as well as standing water found under grapevines (Rooney *et al*., 2001).

Some *Phaeoacremonium* species produce perithecia in infected tissue, pruning wounds and cracks in the cordon, trunks and spurs in vineyards as well as rootstock mother blocks (Eskalen *et al*., 2005a, 2005b; Rooney *et al*., 2001; Baloyi *et al*., 2013). Under the ideal conditions, ascospores are released from the structures and can infect new trimming and pruning wounds via wind, splashing water and arthropods (Eskalen and Gubler, 2001; Eskalen *et al*., 2005a, 2005b, 2007; Moyo *et al*., 2014). There are also many studies that have shown that species of Botryosphaeriaceae can infect grapevines through pruning wounds (Lehoczky, 1974; Smith *et al*., 1994; Van Niekerk *et al*., 2004, 2010). Apart from effecting wounds, these fungi can successfully infect the host directly through lenticels, stomata or other natural openings on healthy plants (Brown and Hendrix, 1981; Michailides, 1991). Species of Botryosphaeriaceae survive in rootstock mother fields by producing pycnidia and pseudothecia on wood and pruning debris (Lehoczky, 1974; Van Niekerk *et al*., 2010).

Hydration

After the plant material is cut from the mother vines, there are many steps in the propagation process where infected shoots can cross contaminate other healthy cuttings. The first step where infection can take place is during the first soaking period of the cuttings coming from the mother blocks (Gramaje and Armengol, 2011).

During hydration, the water itself can be a source of micro-organisms. Even if the water is clean initially, it can become contaminated by micro-organisms from the hydration bath, field, soil attached to cuttings or plant sap containing spores leaching from cuttings that then disperse into the water (Waite and Morton, 2007). Several studies have confirmed that pre-storage, pre-grafting and post-storage hydration processes prove to be potential inoculum sources of vascular fungi (Whiteman *et al*., 2004; Retief *et al*., 2006; Aroca *et al*., 2010). If there are even only a couple of infected cuttings in a batch, the pathogens can spread to the healthy cuttings when mycelium or spores are washed from the surface of the cutting or plant sap that has leached into the water (Retief *et al*., 2006; Waite and Morton, 2007). Various studies have detected Botryosphaeriaceae species and pathogens associated with Petri disease in pre- and post-harvest hydration baths (Fourie and Halleen, 2004a; Ridgway *et al*., 2002; Whiteman *et al*., 2004, 2007; Aroca *et al*., 2010; Gramaje and Armengol, 2011). Aroca *et al*. (2010) found viable propagules of *Phaeoacremonium* spp., P. *chlamydospora* and *Cadophora luteo-olivacea* in hydration tanks, pruning and grafting equipment. The

occurrence of viable propagules of *Cadophora luteo-olivacea* in these stages of the propagation process was described by Gramaje *et al.* (2011).

According to the study done by Retief *et al*. (2006), the management of all the initial propagation steps are crucial. They sampled from the cuttings in mother blocks, and found that 25% of these cuttings were infected by *P. chlamydospora*. By the time these cuttings were ready for grafting, 42% of the cuttings were infected with this fungus. To reduce these infections, Retief *et al*. (2006) suggested some management strategies that included the sterilisation of hydration tanks after every period of hydration and also to add effective chemical and biological control agents to the water during soaking.

Grafting and callusing

The whole propagation process is stressful on the vine cuttings, and result in many wounds (Gramaje and Armengol, 2011). These wounds are made during disbudding, grafting, poorly healed graft unions and also during the rooting process (Bertelli *et al*., 1998; Halleen *et al*., 2003; Gramaje and Armengol, 2011). All these wounds leave the cuttings vulnerable and are highly susceptible for fungal infections. *Phaeoacremonium* spp., *P. chlamydospora* and Botryosphaeriaceae spp. have all been proven to be strong pruning wound colonizers, suggesting that they can infect wounds made during the propagation process (Smith *et al*., 1994; Bertelli *et al*., 1998; Larignon and Dubos, 2000).

The grafting process as well as the tools involved can be responsible for further contamination of propagation material. Botryosphaeriaceae species and *P. chlamydospora* have been detected in plant sap and grafting tools (Aroca *et al*., 2010). Since these fungi grow in the vascular system of vines they could be present in the plant sap. The plant sap remains behind on the tools and transfers to the next graft that is made. The cross-contamination can be managed by the frequent sterilisation of the grafting tools, preferably after every cut that is made. Research has shown that the amount of infected vines increases as the propagation process progresses (Vigues *et al*., 2008).

The condition of high temperature and humidity that persists in callusing boxes and rooms during the warm callusing method provides the ideal climate to favour pathogen growth. High levels of *Phaeoacremonium* spp. and *P. chlamydospora* have been isolated from callused shoots prior to planting in nursery fields, confirming the callusing period provides subsequent conditions for fungal growth (Halleen *et al*., 2003). A reduction of certifiable vines due to callus inhibition caused by infection with *P. chlamydospora* has been reported in the past (Vigues *et al*., 2008).

Nursery fields

In South African nurseries, after the grafted cuttings have finished the callus period, they are planted out into open nursery fields from September to October. Here they will be left to grow until they are uprooted in the following winter (June), subjected to certification by the Vine Improvement Association of South Africa and then sold in the industry. Infected vines can be identified during this period as being slow to establish or never make satisfactory growth. Graft unions can even fail in severe cases.

Studies in South Africa have established that black-foot pathogens (*Ilyonectra* spp., *Dactylonectria* spp. and *Campylocarpon* spp.) from nursery soils infect young grafted vines once they are planted in the nursery fields (Halleen *et al*., 2003). In a study done by Halleen *et al*., (2003), they found that less than 1% of the grafted vines were infected by black-foot pathogens before they were planted in the nursery soil. When the vines were uprooted in the following winter, they found that more than 50% of the vines were infected by these soil borne fungi. A study conducted by Cardoso *et al.* (2013) to investigate inoculum sources of black foot disease pathogens in the grapevine propagation process, found viable propagules of black-foot pathogens in fallow soils after the regulatory rotation cycle before planting newly grafted vines in open field nurseries. Therefore, during the propagation process, the primary source of black-foot pathogen inoculum is considered to be nursery soil. Black foot pathogens had also been isolated from graft unions, which suggest that the practise of covering this part of the plant with soil for approximately 5 weeks is responsible for these infections. This practise prevents the drying out of the callus material (Halleen *et al*., 2003). Many other studies have confirmed that black-foot pathogens are usually only present in the plant material after planting out in nursery fields rather than before, suggesting nursery soils being the primary inoculum source (Rego *et al*., 2000; Fourie and Halleen, 2002; Gubler *et al*., 2004).

When the material is planted out in the nursery field, the basal ends of the shoots are usually partly or sometimes fully exposed. Callus roots can also break during the planting process leaving small wounds. The vines are therefore pre-disposed to possible infection through these access points (Rego *et al*., 2000; Halleen *et al*., 2003)

MANAGEMENT OF TRUNK PATHOGENS IN NURSERIES

There are many opportunities for infection in the propagation process. Wounds are made on the plant material at nearly every step, from collection from mother blocks, disbudding before grafting, grafting, lifting the vines from the nursery field and trimming of the vines. Graft unions that are not properly healed can also be an access point for infection. Certain stress factors like poor cold storage and improper transport conditions can also make vines

sensitive for cross-contamination. Therefore good hygiene and wound protection are crucial to manage possible infections.

Chemical control

Pruning wounds have been identified as a focus point of preventative management in mother blocks; unfortunately this practice is not common in South African rootstock mother blocks (Fourie and Halleen, 2004b). In a study by Halleen *et al*. (2010) looking at fungicides for wound protection in vineyards, it was found that benomyl and flusilazole proved to be effective in reducing natural infections of *P. chlamydospora* in pruning wounds. Rolshausen *et al*. (2010) conducted a study of certain fungicides against Botryosphaeriaceae and Petri disease pathogens. The study revealed that thiophanate-methyl was most effective against infection by these pathogens. In a recent study conducted by Halleen and Fourie (2016) in South African grapevine nurseries, they found that both benomyl and carbendazim used during pre-cold storage hydration reduced the incidence of *P. chlamydospora, Phaeoacremonium* spp*.* and Botryosphaeriaceae species isolated form propagation material. There are still many challenges regarding chemical control methods. The range of registered products to use for protection is limited and do not always provide broad spectrum and long-lasting protection, as was found in a study conducted by Kotze *et al.* (2011), which showed that *Trichoderma* products was more successful in preventing infection by trunk disease pathogens than benomyl. Application of these fungicides can also prove to be labour intensive and very costly (Rolshausen *et al*., 2010).

Chemical applications to control fungi during the propagation process can also be very problematic. There are methods available to control surface pathogens of plant material. The chemical sprays and dips don't, however, have the ability to penetrate deep enough into the material to effectively control fungi present in vascular tissue (Caudwell *et al.,* 1997). During the periods of hydration, prior to cold-storage and grafting, shoots can be soaked in benomyl, carbendazim, dedicyldimethylammonium chloride or captan. This has proven to have an effect in reducing *P. chlamydospora* and *Phaeoacremonium* spp. infection in the graft unions and basal ends of cuttings (Fourie and Halleen, 2004a, 2006a; Gramaje *et al*., 2008). Rego *et al*. (2009) found that soaking naturally infested cuttings in a mixture of pyraclostrobin and metiran or cyprodinil and fludioxonil before grafting, reduced *Ilyonectria* spp., *P. chlamydospora* and Botryosphaeriaceae spp. The effects of fungicides during the callus and rooting phase have also been investigated. Alaniz *et al*. (2011b) conducted *in vitro* pot assay studies, testing several fungicides for their potential to prevent infection by *Ilyonectria liriodendri* and *Dactylonectria macrodidyma*. Results showed that captan, carbendazim and didecyldimethylammonium chloride showed to effectively reduce disease

severity and levels of inoculum of *I. liriodendri* and prochloraz was effective in the case of *D. macrodidyma*.

Biological control

Growth stimulation and wound protection using biological agents have been reported having good potential as part of an integrated management strategy (Fourie and Halleen, 2001, 2004a, 2006). In a trial conducted by Fourie *et al.* (2001) it was demonstrated that commercial *Trichoderma* products have beneficial growth stimulating qualities as well as the ability to notably reduce the incidence of *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora* in the roots of nursery vines. The *Trichoderma* treatments also significantly improved root development, making the plants more tolerant to infection by black-foot disease pathogens.

The ability of *Trichoderma* spp. to colonize and protect pruning wounds has been proved by multiple studies (Fourie and Halleen, 2006; Kotze *et al*., 2009, 2011; Mutawila *et al*., 2011). *Trichoderma harzianum* Rifai, *T. longibrachiatum* Rifai and *T. atroviride* P. Karst have all been reported to effectively protect wounds against *P. chlamydospora* and *T. atroviride* also proved to be effective against some Botryosphaeriaceae species (Kotze *et al.*, 2009). Apart from pruning wound protection, the soaking of cuttings in *Trichoderma* formulations before cold storage, have the ability to successfully reduce the incidence of *P. chlamydospora* and *Phaeoacremonium* spp. in graft unions and basal ends of young grafted vines (Fourie and Halleen, 2004a). In a study conducted by Halleen *et al*. (2007a) different chemical products were tested alone and amended with *Trichoderma* treatments or hot water treatments. It was found that none of the treatments were able to significantly and consistently reduce the incidence of both black-foot and Petri disease pathogens except for the graftlings that underwent hot water treatment.

Hot water treatment (HWT)

There has been a lot of focus on HWT as a potential control method against Petri disease and black-foot pathogens in planting material. Using HWT in rootstock cuttings before grafting (Edwards *et al*., 2004b; Halleen and Fourie, 2004a) or on dormant plants after lifting from nursery fields (Fourie and Halleen, 2002, 2004a, 2006a; Gramaje *et al*., 2009) has been recommended to effectively reduce infection in nursery vines. HWT is known to also eradicate *Phytophthora cinnamomi* (Von Broembsen and Marais, 1978) and *Meloidogyne javanica* (Barbercheck, 1986). Some nurseries prefer to use HWT as a pre-callus treatment to avoid problems with unpredictable failure of vines in vineyards (Waite and May, 2005; Waite, 2010).

The standard HWT protocol in South Africa was 50°C for 30 min but was recently changed to 50°C for 45 min to eliminate Aster yellows. It has, however, been suggested by Halleen and Fourie (2016) that HWT (50 °C) forms a vital part of the integrated management of fungal trunk diseases in South African nurseries. In their study HWT effectively reduced the incidence of black-foot disease pathogens as well as *P. chlamydospora, Phaeoacremonium* spp. and Botryosphaeriaceae.

Contradicting evidence has revealed that this protocol (50°C for 30 min) may not always effectively eradicate target pathogens (Rooney and Gubler, 2001; Whiting *et al.*, 2001). These conditions can be detrimental to the cuttings and grafted plants. It has been suggested that the tolerance of plants and the pathogens present are influenced by the climatic conditions in which the material was grown (Waite and Morton, 2007). This has been confirmed in studies done in New Zealand (Graham, 2007; Bleach *et al*., 2009) and Spain (Gramaje *et al*., 2008, 2009). Cuttings grown in cool climates in New Zealand proved to be more susceptible to HWT as well as the pathogens present in the material. Temperatures below 50°C were able to reduce the presence of *P. chlamydospora* and *Ilyonectria* spp. (Graham, 2007; Bleach *et al*., 2009). Vines growing in warmer regions like Spain were able to withstand HWT at 53°C for 30 min without detrimental effects to the cuttings and successfully control Petri disease pathogens (Gramaje *et al*., 2008, 2009).

ROOTSTOCK RESISTANCE TO TRUNK PATHOGENS

Since most of the preventative management strategies are not completely effective, natural host resistance is the next step in obtaining effective management strategies. Unfortunately it has been revealed that all major commercial rootstock and scion cultivars tested thus far are susceptible to infection by fungal trunk pathogens (Armengol *et al*., 2001; Eskalen *et al*., 2001; Aroca *et al*., 2006; Díaz *et al.,* 2009; Úrbez-Torres *et al*., 2009).

Studies have revealed that rootstocks possess different levels of susceptibility. In these studies mostly Petri disease pathogens had been evaluated. Gramaje *et al*. (2010b) found that 161-49 Couderc was the least susceptible against *Cadophora luteo-olivecea*, five species of *Phaeoacremonium* and *P. chlamydospora* under field conditions. Ruggeri 140 and Richter 110 had been reported to be very sensitive to Petri disease pathogens. Theoretically, crosses of *V. riparia x V. berlandieri* could potentially be tolerant to infection by Petri disease pathogens (Gramaje *et al*., 2010b). A study comparing susceptibility of rootstock cultivars and *Vitis vinifera* cultivars to Petri disease pathogens revealed that the rootstock cultivars were significantly more sensitive to infection, confirming the theory that infections of fungal trunk disease pathogens originate from rootstock propagation material (Wallace *et al*., 2004).

Gubler *et al*. (2004) investigated rootstock susceptibility to black-foot disease and found *Vitis riparia* 039-16 and Freedom possessed a good level of tolerance toward this disease. In contrast, other studies conducted in New Zealand (Jaspers *et al*., 2007) and Spain (Alaniz *et al*., 2010) reported that all rootstock cultivars included in the trials were affected by black-foot disease.

ROOTSTOCKS IN SOUTH AFRICA

History of rootstocks

Phylloxera (*Phylloxera vitifoliae*) is a root louse that was first identified in 1868 in France by JE Planchon. The pest is located in soil and attacks the roots of *Vitis vinifera* vines leading to a decrease in vine performance and death. By the second part of the 19th century phylloxera had decimated European vineyards that were all growing on their own roots at that point (Heyns, 2014). Phylloxera was believed to have been introduced into Europe through the import of non-*vinifera* species from the United States of America (especially from the Mississippi region where it was present). Phylloxera was later introduced into South Africa through the import of contaminated planting material. The presence of the pest in the Cape wine region was first confirmed by Mr Louis Peringuy on the 1st of January 1886 and by the end of the 19th century the South African grapevine industry had been almost completely wiped out (De Klerk, 1974; Heyns, 2014). This lead to the practise of grafting scion cultivars on phylloxera resistant rootstocks (De Klerk, 1976; Carstens *et al*., 1981). Many attempts were made to import phylloxera resistant *Vitis vinifera* from France where grafting trials were already in progress since the late 1870's, but none were forthcoming (Pienaar, 1966). Finally seeds of American *Vitis* species were imported from France. The reproduction and multiplication of the seeds lead to the production of rootstocks that were specific to South Africa (Pienaar, 1966; Burger and Deist, 1981). The seeds that were reproduced were all *Vitis rupestris* and there were a large range of genetic diversity that occurred when propagating through seeds, resulting in seedlings that display different characteristics. The most important rootstock cultivars unique to South Africa was Constantia Metallica, Donkie-Rupestris, Mooikelder and Schabort 1 and 2 (Pienaar, 1966). Other European bred rootstocks were later imported in the 1900's to be evaluated in the Cape area (Pienaar, 1966; Burger and Deist, 1981; Heyns, 2014).

Choosing a rootstock

The goal of a rootstock is to be able to produce grapes of high quality in conditions that are not suitable otherwise. There are two main factors to consider when choosing a rootstock scion combination. The first factor to take into consideration is how the rootstock cultivar might influence the quality and growth of the scion. Lastly is to determine how the rootstock

will perform in the specific growing site. The first factor is mainly influenced by the affinity of the rootstock/scion combination and the second factor is influenced by a combination of biological, physical and chemical soil qualities.

Affinity and vigour

The term 'affinity' is sometimes used differently between researchers and producers. Good affinity is the connective growing of a rootstock and scion to form an anatomic and physiological unit that ensures normal function of the plant. If a graft union is successful, the movement of nutrients to and from the roots can proceed as normal. When a rootstock/scion combination did not form a successful graft, the negative results can usually be seen at an early stage. Some of the problems that occur can be dieback or complete death of the vines. If a successful graft union seemed to have formed but without good affinity, the implications might only become apparent after a couple of years. This can have a negative impact on vigour, bud fertility and grape quality.

The vigour of a rootstock is always important to take into consideration. It should be compared to the type of soil involved to determine how the scion cultivar will react. As an example; a rootstock with high vigour should not be considered to use on a high quality soil. This can result in a decrease in fertility and quality of grapes.

Soil quality (biological, chemical and physical)

Secondly the soil type on which the vines will be established also plays an important role. After the soil profile of the chosen site has been determined, a rootstock should be chosen that will be able to grow and adapt to the specific site's physical and chemical properties. Some of the limiting factors that may occur in certain soils are shallow top soils, draught, weak drainage and salinity (Carstens *et al*., 1981). The water holding capacity, which is determined by the amount of clay in the soil, should also be part of the decision since some cultivars can be sensitive to drought or waterlogged conditions. Apart from soil properties, the location of the site as well as the climate should also be taken into consideration.

Popular rootstock cultivars in South Africa

In the 2014/2015 nursery production season, 44.1 million grafted vines were produced in South Africa of which 26.5 million was for the wine industry and 17.6 million for the table grape and raisin industries. According to the official statistics of the Vine Improvement Association, the following cultivars were the most popular cultivars used in the South African industry, namely Richter 99, Richter 110, Ramsey, 101-14 Mgt, Ruggeri 140, Paulsen 1103, SO_4 and US VIT 8-7.

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

The cultivar was crossed in 1889 by Franz Richter and imported to South Africa in 1927 (Carstens *et al*., 1981). This cultivar is very popular in South Africa due to good growing and production abilities. The advantages associated with this cultivar include: good vigour, high production ability, good affinity, exceptional resistance to phylloxera and nematodes and performs excellent with grafting and rooting (Loubser and Uys, 1997). The only problem associated with this cultivar is its sensitivity to *Phytophthora cinnamomi*. Poor growth is sometimes associated with soils that are wet and have poor drainage. Richter 99 requires soil that is deep, fertile and well drained and has exceptional resistance to soil compaction. This cultivar is not recommended in sandy soil types (Loubser and Uys, 1997).

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

Richter 110 was also crossed in1889 by Franz Richter and imported to South Africa in 1927 (Carstens *et al*., 1981). Richter 110 can be used to control excessive vigour to increase fertility. Richter 110 has better drought resistance than Richter 99 (Loubser and Uys, 1997). The cultivar is highly resistant to phylloxera but only moderately resistant to nematodes and will therefore not do as well on sandy soils that have a high nematode count (Loubser and Uys, 1997). The cultivar is only moderately susceptible to *Phytophthora cinnamomi* and will do better in poorly drained soils than Richter 99.

Ramsey (*V. champinii)*

Ramsey was imported into South Africa in 1938 under the name of ‘Salt Creek’ (Carstens *et al*., 1981; Loubser and Uys, 1997). This cultivar has proven to have great vigour and production abilities in a wide range of soil types and climates. The cultivar utilises poor soil types extremely well. Under dry land conditions the vigour and production seem to be more moderate. It has excellent resistance against nematodes and makes the cultivar ideal for use in sandy soil with irrigation and is popular in the table grape growing industry. The one great negative when it comes to Ramsey, is its bad affinity to many important scion cultivars (Carstens *et al*., 1981; Loubser and Uys, 1997).

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

This rootstock was crossed in 1882 by Millardet and de Grasset in France (Carstens *et al*., 1981) and was one of the first cultivars to be imported to South Africa after the phylloxera devastation in 1886. The cultivar is very important due to its ability to adapt in a wide range of soil types especially poorly drained soils. The rootstock is ideal in soils with limited depth and a changing water table. The cultivar has some problems with grafting. The cultivar is

highly resistant against phylloxera and moderately resistant to nematodes and *Phytophthora cinnamomi* (Loubser and Uys, 1997).

Ruggeri 140 (*V. berlandieri* var. Resseguier nr. 2 *x V. rupestris* var. du Lot)
This cultivar was crossed in Sicily by Ruggeri and imported to South Africa in 1964. This rootstock is described as a strong growing; tempered rootstock that shows good results on dry calcareous soils (Carstens *et al*., 1981). The cultivar does well in soils with problems like salinity, high acidity and drought, but should not be considered or soils with poor internal drainage (Loubser and Uys, 1997).
Paulsen 1103 (*V. berlandieri* var. Resseguier nr. 2 x *V. rupestris* var. du Lot)
This rootstock was also crossed in Sicily, but by Paulsen, in 1892. Paulsen 1103 was imported to South Africa in 1962. In many ways it is comparable to Richter 99 in terms of vigour and does well on heavy clay-loam soils with a wet underlying soil. It does well in saline soils and generally recommended for use under dry land conditions (Carstens *et al*., 1981; Loubser and Uys, 1997). The rootstock shows a moderate sensitivity to nematodes and waterlogged conditions (Loubser and Uys, 1997).

SO4 (*V. berlandieri* x *V. riparia*)
SO4 is short for 'Selection Oppenheim nr. 4'. The cultivar was selected from seedlings of crossings done by Teleki. It has been cultivated in France since 1941 and was included in South African studies since 1974 (Carstens *et al*., 1981; Loubser and Uys, 1997). It is described as a rootstock with great vigour and is known to cause early ripening. The rootstock has good affinity with most scion cultivars. It is well adjusted to wet clay soils but can have good results in sandy soils with proper irrigation. The cultivar has good resistance against nematodes (Loubser and Uys, 1997).

US VIT 8-7 (Jacquez x Richter 99)
Since 1949 a wide range of rootstock crossings were made by Orffer with Jacquez and Richter 99. Of all the crossings made US VIT 8-7 showed the most promising results and was further cultivated (Carstens *et al*., 1981). The cultivar has strong vigour and can be cultivated in a wide variety of soil types. The rootstock is able to withstand problems like: calcareous soils, salinity, drought and wetness. It is also highly resistant against nematodes and *Phytophthora cinnamomi* (Loubser and Uys, 1997).

AIMS OF STUDY

The aim of this study is to determine the possible resistance of commonly used commercial rootstock varieties used in South African nurseries to all the economically important fungal

trunk diseases pathogens. The specific objectives of this study were to 1) select the most virulent grapevine trunk disease isolates and 2) assess eight rootstock cultivars for their susceptibility/resistance towards grapevine trunk disease pathogens in the field. The knowledge obtained from this study can then form part of integrated management strategies to further prevent grapevine losses due to fungal trunk pathogens.

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CHAPTER 2

Determining the susceptibility or resistance of grapevine rootstock cultivars to fungal trunk disease pathogens

ABSTRACT

The susceptibility of the most commonly used grapevine rootstocks in South Africa to Petri disease (*Phaeomoniella chlamydospora*, *Phaeoacremonium minimum, Pm. parasiticum, Cadophora luteo-olivacea* and *Pleurostoma richardsiae*); black-foot disease (*Ilyonectria liriodendri, Dactylonectria macrodidyma, Campylocarpon fasciculare* and *Campylocarpon pseudofasciculare*) and Botryosphaeria dieback (*Neofusicoccum australe* and *Neofusicoccum parvum*) pathogens were evaluated. Dormant rootstock cuttings of Ramsey, Ruggeri 140, Paulsen 1103, Richter 99, Richter 110, US 8-7, 101-14 Mgt and SO_4 were vacuum inoculated with conidial suspensions (1 x 10^6 conidia mL) of the two most virulent isolates of each pathogen, determined by virulence screenings, and planted in nursery field sites. The virulence screenings were conducted by plug inoculating dormant Paulsen 1103 cuttings with 10 isolates of each pathogen. The most virulent isolates were identified according to the lesion lengths that developed after six weeks of incubation at 22°C. There was significant variance seen between the isolates of all the pathogens, except *Pleurostoma richardsiae.* After eight months in nursery fields, the dormant plants were uprooted, washed and assessed for un-dried shoot and root weight. A disease rating was given by cutting the plant 10 cm from the base and assessing the internal vascular discolouration to determine percentage disease severity. All of the rootstocks inoculated with Petri disease, black-foot disease and Botryosphaeriaceae pathogens showed a significant reduction in root mass (8.00 – 64.91%), shoot mass (17.69 – 83.11%) and a significant increase in percentage disease severity (28.92 – 77.04%). It is clear that none of the rootstocks are resistant to fungal trunk disease pathogens, but differential levels of tolerance do exist. The reduction in shoot and root mass was not significantly different between the rootstock cultivars, but rootstocks 101-14 Mgt and SO_4 were the most susceptible and Ramsey the most tolerant in terms of percentage disease severity. In an attempt to explain the variable tolerance of rootstocks to infection, suberin production and xylem morphology was investigated. Transversal cuts were made of the most tolerable and most susceptible cultivars and stained using two suberin staining techniques. The results showed that suberin production is not cultivar specific but the important role suberin plays in the compartmentalization of a pathogen in grapevine wood was confirmed. Suberin was located around vessels filled with tyloses, tyloses itself was also suberized, ray parenchyma cells and cells located on the growth boundary ring of both rootstocks. The suberized zones form impermeable barriers

that restrict pathogen spread to uninfected and newly developed vascular tissue. The mean vessel diameters were determined for each of the eight cultivar using 40 µm thick transversal cuts stained with toluidine O. A strong correlation between mean xylem vessel diameter and rootstock tolerance to fungal trunk pathogens was established.

INTRODUCTION

The decline and failure of older, and especially younger vines, have become a growing concern in grape producing regions around the world. Poor establishment of vines, reduction in vigour and early decline of vineyards infected with fungal trunk pathogens, cause reductions in yield, quality and the longevity of vineyards. The global economic cost for replacing dead vineyards runs in excess of 1.5 billion dollars per annum (Hofstetter *et al*., 2012). Therefore, vine trunk diseases are detrimental to the sustainability of all grape growing regions worldwide. Botryosphaeria dieback have been reported to be caused by species of the genera *Diplodia, Dothiorella, Fusicoccum, Lasiodiplodia* and *Neofusicoccum* (Van Niekerk *et al*., 2004, 2006; Taylor *et al*., 2005; Úrbez-Torres *et al*., 2006, 2007a, 2007b; Aroca *et al*., 2008; Úrbez-Torres *et al.,* 2009a), Petri disease which is caused by *Phaeoacremonium* spp*., Phaeomoniella chlamydospora* and recently also *Cadophora luteo-olivacea* and *Pleurostoma richardsiae (*Mugnai *et al*., 1999; Crous and Gams, 2000; Groenewald *et al*., 2001; Rooney-Latham, 2005; Halleen *et al*., 2007a; Rolshausen *et al*., 2010) and black-foot disease caused by *Ilyonectria liriodendri, Dactylonectria macrodidyma* and *Campylocarpon* spp. (Halleen *et al*., 2004, 2006a, 2006b; Schroers *et al*., 2008) are some of the main role players in this global dilemma (Giménez-Jaime *et al*., 2006; Gramaje and Armengol, 2011). These grapevine trunk disease pathogens may act in synergy or succession to produce an array of symptoms in grapevines (Cobos and Martin, 2008; Surico, 2009; Úrbez-Torres *et al*., 2009b; Valtaud *et al*., 2009; Spagnolo *et al*., 2011; White *et al*., 2011).

In young vines the general external symptoms of fungal trunk infection include stunted growth, reduced vigour, shortened internodes, chlorotic and sparse foliage with necrotic margins, wilting, dieback and sudden death. These symptoms overlap for Petri and black-foot disease and are often impossible to differentiate (Sheck *et al*., 1998a, 1998b; Mugnai *et al.,* 1999; Fourie and Halleen, 2001; Retief *et al*., 2005; Gramaje and Armengol, 2011). In addition to the above-mentioned symptoms, characteristic symptoms of black-foot disease are sunken and necrotic root lesions, with an overall reduction in root biomass and root hairs (Halleen *et al*., 2006a; Alaniz *et al*., 2007). The removal of infected rootstock bark usually reveals black discolouration that develops from the base of the rootstock. When this lesion is cut transversally, it reveals necrosis inside the wood tissue that stretches from the bark to the pith (Larignon, 1999; Fourie and Halleen, 2001; Halleen *et al.,* 2004). In comparison,

dissection of vines infected with Petri disease shows a typical black discolouration of the xylem vessels, usually caused by the presence of tyloses, gums and phenolic substances present in the vessels (Mugnai *et al.,* 1999; Edwards *et al.,* 2007a; Mutawila *et al.,* 2011). The occlusions are formed in reaction to the presence of fungi in and around the vessels (Bruno *et al.,* 2007; Mutawila *et al.,* 2011). The typical disease symptoms associated with Botryosphaeriaceae species include cankers and other dieback symptoms like leaf chlorosis, bud mortality, shoot dieback, wedge-shaped wood necrosis and graft union failure (Kummuang *et al*., 1996; Phillips, 2000; Larignon and Dubos, 2001; Larignon *et al*., 2009).

Currently the largest concern is the presence of infected vines in rootstock mother fields and infected nursery vines resulting in infections in vineyards via propagation materials (Fourie and Halleen, 2002, 2004a; Halleen *et al*., 2003; Edwards and Pascoe, 2004; Gramaje and Armengol, 2011). The propagation material and young rooted vines can be infected either systemically, from infected mother vines (Fourie and Halleen, 2002, 2004a; Halleen *et al*., 2003; Aroca *et al*., 2010), or by contamination during the propagation process (Bertelli *et al*., 1998; Giménez-Jaime *et al*., 2006; Aroca *et al*., 2010; Billones-Baaijens *et al*., 2010; Gramaje *et al*., 2011). The pathogens associated with Botryosphaeria dieback, Petri disease and black-foot disease are known to be endophytic or opportunistic fungi (Ferreira *et al*., 1999; Stamp, 2001; Halleen *et al.,* 2003) and since the entire propagation process induces stress on the young grafted vines, the pathogens can establish and manifest in the vines at an early stage. Some of the factors that induce stress on nursery propagation material include extended cold storage, failed or weak graft unions, pathogen infection and harvesting cuttings too early leading to limited carbohydrate storage (Stamp, 2001, 2003).

Rootstock mother fields as a primary source of grapevine trunk pathogens has been well defined by many authors. Many nurseries allow mother vines to sprawl freely on the ground, yet in some nurseries in South Africa rootstocks are cultivated on trellis systems to try and eliminate potential pathogen contamination that originate from the soil resulting in rootstock cuttings of a higher quality (Stamp, 2003; Hunter *et al*., 2004). Rootstock mother vines have been identified as a source of Petri disease pathogens although it have been mainly focused on the isolation of *P. chlamydospora* and *Pm. minimum* from rootstock mother vines and cuttings (Fourie and Halleen, 2002, 2004a; Retief *et al*., 2005, 2006; Aroca *et al*., 2010). Some black foot disease pathogens have been detected in rootstock mother vines, but the occurrences were very low in all the studies. (Rego *et al*., 2001; Halleen *et al*., 2003; Fourie and Halleen, 2004a; Oliveira *et al*., 2004). Infections caused by Botryosphaeriaceae species have been observed regularly in rootstock mother vines (Halleen *et al*., 2003; Fourie and Halleen, 2004a; Oliveira *et al*., 2004; Aroca *et al*., 2010; Billones-Baaijens *et al*., 2010). In most cases the presence of the above mentioned grapevine trunk diseases did not correspond with observed external symptoms (Halleen *et*

al., 2003; Edwards and Pascoe, 2004; Fourie and Halleen, 2004a). These pathogens were usually found in symptomless mother vines with only signs of internal black streaking of the vascular system, demonstrating its high potential risk as a source of inoculum in the propagation process (Ferreira *et al*., 1999).

There are two ways in which rootstock mother vines can become infected. Soil can act as a reservoir for infection (Morton, 1997; Bertelli *et al*., 1998; Surico *et al*., 2000). Black-foot disease pathogens are known to be saprobes in soil; they can occur on dead plant substrata and can also act as weak pathogens by infecting wounds on roots and stems (Halleen *et al*., 2006b; Schroers *et al*., 2008). The ability of these pathogens to produce chlamydospores allows them to survive in soil for extended periods of time (Halleen *et al*., 2004). *Phaeomoniella chlamydospora* also has the ability to produce chlamydospores, suggesting the potential for it to be a soil borne pathogen (Bertelli *et al*., 1998; Mugnai *et al*., 1999; Taylor *et al*., 2005). These chlamydospores form conidia that have the ability to penetrate uninjured roots of vines in nurseries and vineyards (Bertelli *et al*., 1998; Feliciano and Gubler, 2001). *Phaeoacremonium minimum* has also been found to be present in soil (Eskalen *et al*., 2001a) as well as standing water found under grapevines (Rooney *et al*., 2001). Pruning wounds are the second potential infection point of mother vines (Christen *et al*., 2007; Edwards *et al*., 2007a; Gramaje and Armengol, 2011). The fungal pathogens consequently establish and grow inside the wounds, causing decay and slowly killing the vines (Carter, 1991; Gubler *et al*., 2005; Christen *et al*., 2007). Inoculum sources in vineyards can be present as fruiting bodies growing on pruning debris and also in the cracks and crevices of the trunk and cordon (Crous *et al*., 1996; Mugnai *et al*., 1999; Edwards and Pascoe, 2001; Edwards *et al*., 2001; Rooney *et al*., 2001; Baloyi *et al*., 2013). From here the spores are released following hydration during periods of rainfall or sprinkler irrigation (Eskalen and Gubler, 2001; Eskalen *et al.,* 2005a, 2005b). The spores are then dispersed through wind, splashing water, pruning shears (Larignon and Dubos, 2000; Eskalen and Gubler, 2001; Mostert *et al*., 2006) and also arthropods, as recently established by Moyo *et al*. (2014). Many studies have confirmed that *Phaeomoniella chlamydospora, Phaeoacremonium* spp. and Botryosphaeriaceae species have the ability to colonize and infect pruning wounds (Lehoczky, 1974; Khan *et al*., 2000; Larignon and Dubos, 2000; Eskalen *et al*., 2007a; Van Niekerk *et al*., 2010).

Several potential inoculum sources of vine trunk disease pathogens have been identified in the grapevine propagation process. Post harvest hydration is considered as one of the earliest stages where infection can take place. In this hydration phase, as well as during pre-grafting hydration, the water itself used for hydrating can be a source of micro-organisms and even if the water is clean initially, it can become contaminated from micro-organisms on the bark of bud sections or plant sap containing spores can leach into the

water (Mugnai *et al*., 1999; Larignon and Dubos, 2000; Feliciano and Gubler, 2006; Retief *et al*., 2006; Waite and Morton, 2007; Aroca *et al*., 2010). Fungicide tanks used in the nursery process have also been identified as potential risks for contamination with *Phaeomoniella chlamydospora* (Retief *et al*., 2006).

There are many opportunities for infection by fungal trunk pathogens during the nursery production process. Disbudding, grafting, improperly healed graft unions, rooting and planting of finished vines (that can result in the breaking off of callus roots) are all steps in the propagations process that result in multiple wounds, causing the young graftlings to be vulnerable and exposed to infection (Bertelli *et al*., 1998; Zanzotto *et al*., 2001; Halleen *et al*., 2003). This is problematic since the entire range of pathogens connected to the decline of young vines, are known to colonize wounds (Khan *et al*., 2000; Eskalen *et al*., 2007a; Van Niekerk *et al*., 2010). The presence of trunk pathogens has been found on pruning shears, grafting machines and even callusing medium (Halleen *et al*., 2003; Retief *et al*., 2006; Pollastro *et al*., 2009; Aroca *et al*., 2010; Gramaje *et al*., 2011). In a study conducted in South Africa by Halleen *et al*. (2003), isolation studies revealed that soils infected with black-foot pathogens have the ability to infect grafted vines once they are planted in open nursery fields. The occurrence of these pathogens in graft unions can be explained by the nursery practice of covering the graft union with soil for approximately five weeks as a measure to prevent callus tissue from drying out. At the time of planting, the susceptible basal ends of most graftlings are still partially exposed and callus roots can also break off in the planting process, resulting in the formation of small wounds that are vulnerable to infection (Rego *et al*., 2000; Halleen *et al*., 2003, 2007b). Infections at such an early stage result in a heightened risks that grapevine producers could be establishing new vineyards with infected vines and also the spread of these vine trunk diseases to areas where the diseases haven't been present before.

There are no curative treatments for fungal trunk infections and therefore, preventative management is of the utmost importance (Mugnai *et al*., 1999; Di Marco *et al*., 2004; Hunt, 2004). In scion mother fields, pruning wound protection forms an essential part of managing and preventing infection with fungal trunk disease pathogens, but in South Africa this is not standard practice in rootstock mother fields. Studies have been conducted investigating various chemical fungicides that can be applied to pruning wounds to assist in reducing natural infection of pathogens (Halleen *et al*., 2010; Rolshausen *et al*., 2010). There are however, still many challenges regarding these chemical control methods since the range of registered products are very limited, products do not always provide broad-spectrum and long-lasting protection and the application of these fungicides can prove to be costly and labour intensive (Rolshausen *et al*., 2010; Kotze *et al*., 2011). As an alternative, the use of *Trichoderma* spp. as a biological control agent on pruning wounds have been proven to be

effective (Fourie and Halleen, 2001; Hunt *et al*., 2001; Kotze *et al*., 2011; Mutawila *et al.,* 2016). Good sanitation practices, to remove and reduce potential inoculum sources, are also an essential part of integrated disease management (Edwards and Pascoe, 2001; Eskalen and Gubler, 2001; Rooney *et al*., 2001). These measures include remedial pruning, uprooting of dying or dead vines and the removal of pruning debris from the vineyard floor (Mugnai *et al*., 1999).

Chemical control can also be utilized in the grapevine propagation process. These strategies involve drenching and dipping of propagation material in fungicides during the various propagation stages (Fourie and Halleen, 2004b, 2006; Gramaje *et al*., 2009). The propagation material can be soaked in fungicides prior to cold-storage and grafting, to protect the vines from fungal infection (Fourie and Halleen, 2004b, 2006; Eskalen *et al*., 2007b). The vascular pathogens involved are, however, known to reside in the vine xylem vessels and the efficacy of fungicide treatments are limited due to the inability to penetrate wood tissue (Waite and May, 2005). The treatment of propagation material with hot water for 30 minutes at 50°C followed by 30 minutes in cold water has been found to be an effective method for disinfecting shoots (Crous *et al*., 2001; Fourie and Halleen, 2004b; Waite and May, 2005). There are contradicting reports questioning the ideal water temperature and duration to ensure the treatment is effective, as well as reports of negative effects and losses of planting material due to hot water treatment (Rooney and Gubler, 2001; Whiting *et al.*, 2001; Gramaje *et al*., 2008, 2009; Habib *et al*., 2009). In South Africa, propagation material currently undergoes hot water treatment at 50°C for 45 minutes for the eradication of Aster Yellows, but the effect of this treatment on grapevine fungal trunk disease pathogens is unknown.

Since most of the preventative management strategies are not completely effective, investigating natural host resistance and host responses to fungal infection is vital. Unfortunately previous studies have revealed that all the major commercial scion and rootstock cultivars tested thus far are not completely resistant to fungal trunk infections, but varying levels of susceptibility or tolerance was found (Eskalen *et al*., 2001b; Aroca *et al*., 2006; Úrbez-Torres *et al*., 2009a). It has been established that grapevine rootstock cultivars are generally more susceptible to fungal trunk disease pathogens compared to *Vitis vinifera* cultivars, moving the focus to rootstock cultivars as the main source of latent fungal trunk infections (Wallace *et al*., 2004; Zanzotto *et al.,* 2008; Diaz *et al*., 2009).

Very little information is available on the susceptibility of grapevine rootstock cultivars commonly used in the South African industry and studies conducted in other countries usually only included a couple of the popular rootstock cultivars used in South Africa, or were only tested against a limited number of fungal trunk pathogens. If differences in tolerance can be established between the cultivars, this information can assist growers in

choosing the ideal rootstock cultivar when establishing new vineyards. Previous studies conducted abroad have established that none of the rootstock cultivars used in those trials was completely resistant to fungal trunk infections, but differences in susceptibility did exist between rootstock cultivars (Eskalen *et al*., 2001b; Gubler *et al*., 2004; Alaniz *et al*., 2010; Gramaje *et al*., 2010a). Gramaje *et al*. (2010a) found 161-49 Couderc was the least susceptible of five rootstock cultivars (161-49 Couderc, 41 B Millardet Grasset, Ruggeri 140, Paulsen 1103 and Richter 110), to infections of *Ca. luteo-olivacea*, five species of *Phaeoacremonium* spp. and *P. chlamydospora*. The two cultivars, Richter 110 and Ruggeri 140 were found to be the most susceptible in the study. Santos *et al*. (2006) also reported that 3309 Couderc was more tolerant to infection by *P. chlamydospora* and *Pm. augustius* than the cultivars Baga and Maria Gomes. A study conducted by Billiones-Baaijens *et al*. (2014) evaluated the effect Botryosphaeriaceae species have on six different rootstock cultivars (101-14 Mgt, 5C Teleki, 3309 Couderc, Riparia Gloire, Schwarzmann and SO_4). It was concluded that 5C Teleki and SO_4 was the most susceptible in this study. The effect of *I. liriodendri* and *D. macrodidyma* on seven different rootstock cultivars (Richter 110, Paulsen 1103, Ruggeri 140, 161-49 Couderc, 196-17 Couderc, Fercal and SO_4) was investigated by Alaniz *et al*. (2010) and they found that all the rootstocks were affected by the pathogens to some degree, but Richter 110 was the most susceptible cultivar.

The objective of this study is to investigate the susceptibility or resistance of the most commonly used rootstock cultivars in South Africa against the most economically important fungal trunk disease pathogens. The mean xylem vessel diameters of each cultivar and the presence of suberin produced as a host response was also investigated in this study to better understand the differences in susceptibility found between cultivars.

MATERIALS AND METHODS

Virulence screenings were conducted with fungal trunk disease pathogens in order to select virulent isolates for inoculation of nursery plant shoots in large-scale nursery trials to determine whether differences in susceptibility of rootstocks exist.

Virulence screenings

Collection of fungal material

Eleven economically important fungal trunk disease pathogens were identified, namely *I. liriodendri*, *D. macrodidyma*, *C. fasciculare*, *C. pseudofasciculare*, *Pl. richardsiae*, *Co. luteo-olivacea*, *P. chlamydospora*, *Pm. minimum, Pm. parasiticum, N. parvum* and *N. australe.* Ten isolates of each of the pathogens were retrieved from the culture collection of the Department of Plant Pathology (STEU, Stellenbosch University) and Nietvoorbij fungal culture collections (Table 1).

The isolates were plated onto potato dextrose agar (PDA, Biolab, South Africa) amended with chloromycetin (250 mg/L). Cultures were left to grow for two to three weeks at 24-25 °C after which fungal plugs (4 mm in diameter) were prepared to use in the inoculation process.

Collection and sterilization of plant material

The virulence screenings were conducted on dormant Paulsen 1103 (*Vitis berlandieri x Vitis rupestris*) rootstock cuttings. This was the cultivars of which we had the most material available at the time of the screenings. The one-year-old grapevine cuttings were harvested in 2013 from a rootstock mother block in Wellington. The rootstock cuttings were stored at 3 °C at high humidity to prevent the shoots from drying out. The shoots, in total 2200 dormant cuttings, were cut into uniform lengths with four to five buds (approximately 30cm long).

The plant material underwent hot water treatment (HWT) at 50 °C for 30 min. After HWT the material was hydrated in cool sterile distilled water for another 30 min. Before inoculation, the grapevine cuttings were surface sterilized with Sporekill and left to air dry in a laminar flow hood.

Inoculation and incubation

Each rootstock cutting was inoculated with one fungal isolate. A wound (4mm in diameter) was made in the middle of the central internode of the cutting, using a cork borer. The wound was made deep enough to reach the xylem tissue, but not so deep as to reach the pith. A fungal disk was then placed inside the wound and the wound was then closed with parafilm. The 4mm fungal discs were obtained from the margin of two to three week old cultures growing on PDA. Control treatments only received un-colonised PDA discs. The inoculated shoots were placed inside closed containers (29 x 23.5 x 5 cm) on top of moist sterilised tissue paper and incubated for 6 weeks at 22°C on a lab bench. During the six week period, the tissue paper was kept moist with sterile distilled water to obtain a high relative humidity inside the incubation containers. Each box contained 1 inoculated shoot of each of the 10 isolates of a specific species as well as one control shoot. Ten replications were completed for each pathogen and the entire experiment was repeated once.

Determining lesion length

After the six week incubation period the trials were evaluated by splitting the shoots lengthwise through the inoculated holes and then measuring the lesions lengths with the use of a calliper. The 4mm diameter of the wound created by the cork-borer was deducted from the measured lesions before statistical analysis was conducted. Mean lesion lengths were determined for each isolate and the control and then used for further statistical analysis.

Re-isolation and identification

After measurements were completed, wood samples were surface-sterilised in 70% ethanol for 30 seconds, then 2 minutes in 0.35% sodium hypochlorite and again in 70% ethanol for 30 seconds. Fungal isolations were conducted in a laminar flow cabinet by aseptically removing small wood fragments (approximately 1 x 1 mm) from the symptomatic (brown discolouration or streaking) vascular tissue on the wound inter-phase as illustrated in Fig. 1. From each wood sample, eight wood fragments were obtained and placed onto 90 mm Petri dishes (four fragments per plate) with PDA amended with chloromycetin (250mg/L). The isolated samples were then incubated at 25°C and monitored for four weeks. Inoculated fungi were identified using cultural and morphological characteristics (Crous *et al*., 1996, 2006; Crous and Gams, 2000; Halleen *et al*., 2004, 2007; Chaverri *et al.,* 2011; Lombard *et al.,* 2014). Representative cultures were sub-cultured, DNA was extracted from the cultures and PCR products were sequenced (Damm *et al.,* 2008). Different genes were amplified for the fungal groups: the translation elongation factor 1-α for the Botryosphaeriaceae (Carbone and Kohn, 1999), partial β-tubulin for *Phaeoacremonium*, *Ilyonectria*, *Dactylonectria* and *Campylocarpon* spp. (Glass and Donaldson, 1995; Mostert *et al.,* 2006, 2010). *P. chlamydospora* and *Pl. richardsiae* were identified based on cultural and morphological characters (Crous *et al.,* 1996; Halleen *et al.,* 2007).

Data analysis

Analysis of variance (Anova) was performed on the data obtained from all the experiments according to their experimental designs, using GLM (General Linear Models) Procedure of SAS statistical software version 9.4 (SAS Institute Inc., Cary, NC, USA)). Experimental results were combined in one analysis of variance after testing for experiment homogeneity of variance using Levene's test (Levene, 1960). If experiments were not of comparable precision a weighted Anova was performed (John & Quenouille, 1977). Shapiro-Wilk test was performed to test data for deviation from normality of (Shapiro, 1965). 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.

In the isolate screenings, level of isolate virulence (or level of infection) was determined by the mean lesion length formed by the pathogen from the point of inoculation.

The data obtained from root and shoot mass were expressed as grams (g) and disease severity scores as percentages (%). It was performed with rootstock cultivars and treatments as independent variables and the following dependant variables; shoot mass (g), root mass (g) and disease severity (%). The corrected efficiency percentages were determined as follows: percentage reduction in shoot mass = 100 – (Trootmass – Crootmass) x 100

(Abbot's formula); percentage reduction in shoot mass = 100 – (Tshootmass – Cshootmass) x 100 (Abbots's formula); Corrected % disease severity = ((Tseverity – Cseverity) / (100 – Cseverity)) x 100 (Schneider-Orelli formula) in which T is treatment values and C is control values.

Nursery field trials

Fungal isolates

The fungal isolates used in the nursery field trials were selected according to the results of the virulence screenings described above. The two most virulent isolates of each pathogen species (Table 2) were identified and plated out onto PDA amended with chloromycetin (250 mg/L). The isolates were left to grow at 25 °C for two weeks after which sporulation were induced. For *N. parvum* and *N. australe* the two isolates that showed the highest virulence as well as ability to produce pycnidia were selected.

Each pathogen required its own unique conditions and methods to ensure sporulation. *Phaeoacremonium minimum*, *Pm. parasiticum*, *P. chlamydospora*, *Co. luteo-olivecea* and *Pl. richardsiae* were grown on PDA as described above, and incubated for approximately 3 to 4 weeks at 25 °C until sporulation. *Ilyonectria liriodendri, D. macrodidyma, C. fasciculare* and *C. pseudofasciculare* isolates were plated out onto V-8 agar and incubated for three to four weeks at 28 °C with 12 hours of normal light and 12 hours of black light until sporulation. *Neofusicoccum australe* and *N. parvum* sporulation was achieved by inoculating rootstock cuttings. Dormant Paulsen 1103 rootstock cuttings were cut into 20 cm pieces. The cuttings first underwent HWT at 50 °C for 30 min and were then placed in cold water for another 30 min. Mycelium discs (4mm) growing from the edge of three-day-old PDA cultures were placed inside wounds (4mm) made in the middle of an internode at the centre of the shoot using a cork borer. The entire shoot was then covered with parafilm and placed inside a moisture chamber and incubated at 23 °C for six to eight weeks until pycnidia had formed on the shoot surface.

Sourcing and preparation of rootstock material

The rootstock material used in the field trials was sourced from rootstock mother fields of approximately the same age and from the same geographical region. The rootstock cultivars included in this trial were chosen at the onset of this trial according to the official statistics of the Vine Improvement Association of South Africa. The cultivars were Richter 110, Richter 99, Ramsey, Ruggeri 140, SO_4, Paulsen 1103, US 8-7 and 101-14 Mgt. The rootstock material, cut in 30 cm lengths, was stored at 3°C at high humidity to prevent the shoots from drying out until inoculation (approximately two months). Prior to inoculation, the material was hydrated for 24 hours to ensure optimal budburst.

Preparation of conidial suspensions

A conidial suspension was prepared for each isolate. For the Petri and black-foot disease pathogens it was done by flooding the agar with sterile distilled water (SDW) and scraping the mycelium with a sterile spatula. In the case of the Botryosphaeriaceae species, the fruiting bodies formed on the detached shoots were removed and crushed to release the conidia inside. The initial conidial suspensions were then filtered through a double layer of cheesecloth. The concentration of conidia in each filtrate was determined using a haemocytometer and adjusted to 1 x 10^6 conidia per mL.

Validation of the vacuum inoculation protocol against different fungal groups

A vacuum inoculation method was followed in this study to inoculate rootstock cuttings. This entailed, fitting rubber tubing to the top of each cutting while the base of the cutting was immersed in a conidial suspension. Preliminary studies with dye showed that seven seconds is efficient to suck the dye to the top of the 30 cm cutting. Previous studies also established that this is enough time to ensure that uniform inoculation takes place through the cuttings' vascular system (Rooney and Gubler, 2001; Gramaje *et al*., 2010a).

Prior to large-scale inoculations, laboratory trials were conducted using the vacuum inoculation method to inoculate Paulsen 1103 rootstocks cuttings with one pathogen of each of the disease groups, to ensure the efficiency of the method with different conidial types. For this trial we used isolates of *P. minimum* to represent Petri disease, *C. fasciculare* to represent black-foot disease and *N. australe* to represent Botryosphaeria dieback and cankers. A conidial suspension was made of each of the pathogens as described above, using SDW and adjusting the concentration to 1 x 10^6 conidia per mL. Following the vacuum inoculation protocol described above, three shoots were inoculated per pathogen and three control shoots were inoculated with SDW. The shoots were left to incubate in moist chambers for five days at 25 °C. After the incubation period, the shoots were surface-sterilised in 70% ethanol for 30 seconds, then two minutes in 3.5% sodium hypochlorite and again in 70% ethanol for 30 seconds. The shoots were then split lengthwise and three points of isolation were determined, the first 2 cm from base of the cutting, the second in the middle and the third 2 cm from the top. Fungal isolations were conducted by aseptically removing small wood fragments (approximately 1 x 1 mm) from the three isolation points and placing it on to PDA in Petri dishes. The isolated wood samples were incubated at 25 °C for four weeks and the emerging fungal growth was identified and confirmed as previously described.

Vacuum inoculation of rootstock cuttings used in field trials

Using the conidial suspensions of each isolate, the cuttings (30 cm) of each of the eight rootstock cultivars were vacuum inoculated using a lab bench vacuum apparatus (40 mm Hg) as indicated above and described by Rooney and Gubler (2001) as well as Gramaje *et al*. (2010a). Only the two isolates of one pathogen were inoculated per day, to limit the risk of cross contamination between the different fungal groups. Control cuttings were vacuum infiltrated with SDW in the same manner. The cuttings were planted in nursery fields on the same day of inoculation.

Experimental design

In total there were eight different rootstock cultivars evaluated in this trial. Each cultivar was treated with 23 different treatments, two isolates of each of the 11 pathogens and one was a control treatment. Sixty shoots were randomly picked out and inoculated for each treatment x rootstock combination per site. The inoculated shoots were immediately taken to the nurseries and planted out in two nursery sites (2014 and 2015 season). The cuttings were planted 5 cm apart from each other and with an inter-row spacing of 80 cm. Each site had three rows each with 23 groups (11 000 cuttings per site). The experimental design was a randomised block split plot with pathogen as main plot factor, replicated at random within each of three row replicates, and cultivar as split plot factor, randomly allocated within each main plot. Standard nursery cultural practices were followed in both sites during the eight month nursery season. Each season, the two sites were less than one kilometre apart having very similar climates. In total, 44 000 rootstock cuttings were used the field trials conducted over two seasons.

Assessment of field trials

At the end of the growing season (June/July of 2015 and 2016), the dormant plants were uprooted following normal nursery practices. The uprooted plants were immediately taken to the laboratory where they were washed to remove any excess soil. The un-dried root and shoot mass was determined for each rootstock x treatment combination. An internal disease assessment was then conducted. The internal assessment was conducted by making a transversal cut 10 cm from the base of the plant and giving an estimated rating according to the percentage of vascular discolouration observed. The rating system was described by Gramaje *et al*. (2010a) using a scale from 0 to 4, in which 0 = no discolouration, 1 = 1 – 25% discolouration, 2 = 26 – 50% discolouration, 3 = 51 – 75% discolouration and 4 = 76 – 100% discolouration. The severity was then calculated using McKinney's index and rating given according to area of vascular discolouration (Gramaje *et al*., 2010a). McKinney's index

expresses the percentage of the maximum severity of disease according to the formula below;

$$MI = \frac{[\sum(R\ x\ N)]\ x\ 100}{H\ x\ T}$$

where R = disease rating, N = number of plants with this rating, H = the highest rating, and T = total number of plants counted (KcKinney, 1923).

Re-isolation and identification of fungal pathogens

Re-isolations were conducted on 20% of the cultivar x treatment combinations. Sterilisation and isolation was completed as described above. The emerging colonies were then sub-cultured onto PDA to allow identification of fungal cultures.

Histological studies

Determining mean vessel diameter

The mean vessel diameters of each of the eight rootstock cultivars were determined according to the method described by Pouzoulet *et al*. (2014). Six shoots with internode lengths ranging between 8 to 10 mm were selected for each cultivar. Cross-sections of 40 µm thick were made of the fresh wood material using a slide stage freeze-microtome (Leitz Wetzlar, Germany). The cross sections were stained with toluidine O (Ruzin, 1999) and observed under a bright field microscope (Nikon Eclipse E600) under 100x magnification. Micrographs were assembled to create pictures covering a quarter of each stem section (NIS Elements). These micrographs were then used to realise morphological measurements (Fig. 2).

The large vessel areas were determined for three fascicular portions, 45° from each other. One fascicular portion had to be positioned on the dorso-ventral symmetry axis and another on the lateral symmetry axis. The data collected were then pooled by stems and the vessel areas were converted to their arithmetic diameters (Scholz *et al*., 2013). The mean vessel diameters and standard deviations were determined for each cultivar. Statistical analysis was carried out using the Student's test.

Investigating suberin

Two different staining techniques were followed to localize suberin in the rootstocks, namely Sudan black B and Sudan IV staining. Transversal cuts were made (40 µm) from the inoculated material from the nursery field trials described above. The fresh cuts were then stained using Sudan black B (Jensen, 1962). The cuts were immersed in 0.1% Sudan black B solution made with 70% ethanol for 15 minutes. The excess Sudan dye was then removed by washing the cuts three times for five minutes in phosphate buffered saline with 0.02%

Tween 20. The stained cuts were fixed onto microscope slides with glycerol and observed under a fluorescence microscope (340 – 380 nm excitations; Olympus live cell imaging epifluorescence microscope) under 400x magnification. Sudan black B stained suberin blue under bright field and quenched suberin autofluorescence (Biggs, 1984, 1985). For the Sudan IV staining, transversal cuts (40 µm) were made from the same material as described above. Suberin was then examined using Sudan IV as previously described (Jensen, 1962; Biggs, 1986). Sections were treated with Sudan IV (Sigma) saturated in 70% ethanol for 15 min. The sections were then rinsed rapidly for three minutes with 50% ethanol and fixed onto slides (glycerine) to be observed under a light microscope (Nikon Eclipse E600) under 400x magnification.

RESULTS

Virulence screenings

Lesion length measurements

The goal of the virulence screenings was to identify the two most virulent isolates to use in large-scale field trials. All the isolates of the 11 pathogens included in the virulence screenings produced lesions (Fig. 3) that differed significantly from their respective controls (Appendix A, Table 1; $P < 0.05$). The mean lesion lengths (mm) of the isolates of each pathogen measured six weeks after inoculation are shown in Figs. 4-14. There was significant variation seen between the isolates of all the pathogens ($P < 0.05$) except for *Pleurostoma richardsiae* where there were no significant differences in lesion lengths between the isolates (Fig. 8). For *P. chlamydospora* (Fig. 4), STEU 8276 (17.68 mm) and STEU 6384 (16.65 mm) were selected as the two isolates to use in the field trials, although they were not significantly different from LM 310 (15.97 mm). For *Pm. minimum* (Fig. 5), STEU 8272 (36.13 mm) and STEU 8273 (30.94 mm) were selected, although the mean lesion measurement of STEU 8273 was not significantly different from all the other isolates except FH-P 118 (13.37 mm). For *Pm. parasiticum* (Fig. 6), STEU 8275 (46.94 mm) and STEU 8274 (43.65 mm) were selected, although all the other isolates, except STEU 6990 (25.81 mm) did not differ significantly from them. For *Co. luteo-olivacea* (Fig. 7), STEU 8277 (14.64 mm) and STEU 8278 (13.05 mm) were selected, although STEU 8278 were not significantly different from FH-P 542 (8.72 mm), FH-P 543 (11.85 mm) and FH-P 551 (12.37 mm). For *Pl. richardsiae,* STEU 8271 (67.78 mm) and STEU 8270 (66.84 mm) were the isolates selected to be used in the nursery field trials even though there was no significant difference in lesion lengths between the isolates. For *I. liriodendri* (Fig. 9), STEU 8266 (28.42 mm) and STEU 8267 (25.88 mm) were selected, although STEU 8267 were only significantly different from STEU 7539 (14.08 mm). For *D. macrodidyma* (Fig. 10), STEU 8264 (28.51 mm) and STEU 8265 (23.72 mm) were selected, although STEU 8265 was not

significantly different from STEU 7543 (20.00 mm), FH-C 98 (20.79 mm), STEU 7618 (16.28 mm), FH-C 217 (18.44 mm) and FH-C 279.2 (17.01 mm). For *C. fasciculare* (Fig. 11), STEU 8281 (59.30 mm) and STEU 8282 (60.30 mm) were selected and was only significantly different from FH-C 251 (39.79 mm) and FH-C 393 (33.10 mm). For *C. pseudofasciculare* (Fig. 12), although the lesion lengths caused by STEU 8280 (28.13 mm) and STEU 8279 (31.13 mm) were selected, although STEU 8280 did not significantly differ from any of the other cultures. For *N. australe* (Fig. 13), STEU 7029 (49.43 mm) and STEU 8269 (39.12 mm) were selected based on their ability to sporulate successfully, although STEU 8269 was not significantly different from STEU 7030 (46.00 mm, but no sporulation was observed), PMM 92 (27.31 mm), PMM 6 (33.84 mm), PMM 9 (34.48 mm) and PMM 95 (24.24 mm). For *N. parvum* (Fig. 14), STEU 7021 (26.85 mm) and STEU 8268 (17.18 mm) were selected due to their ability to sporulate successfully, although the lesion length produced by STEU 8268 was not significantly different from that produced by any of the other isolates.

Re-isolation percentages

All the pathogens had re-isolation percentages of above 76% (Table 3), except for *Pm. parasiticum* (64%) and *C. fasciculare* (64%). The re-isolation percentages confirm the presence of the pathogens initially inoculated causing the observed lesions (Fig. 2).

Results from nursery field trials

The two cultivars, Ramsey and US 8-7, were not correctly prepared and failed to grow in the 2015 season. These two cultivars are therefore not present in the 2015 data sets.

Percentage reduction in root mass

Analysis of variance revealed a significant year x site x pathogen interaction (P = 0.0482; Appendix A, Table 2) for the reduction in root mass seen after the inoculated shoots were grown in nursery fields for eight months. The mean percentage reduction for each site, year and pathogen are shown in Table 4. The control treatments were already taken into consideration in the mean values as described above. Due to the interaction between the different sites and years the results will be further discussed per individual site.

Dactylonectria macrodidyma in site two of 2016 caused the largest reduction in root mass (64.91%) and *C. fasciculare* in site one of 2016 caused the smallest reduction in root mass (7.99%). There was no significant differences in reduction induced by the different fungal trunk pathogens in site one and site two of 2015, respectively (Table 4). In site one of the 2016 season, *P. chlamydospora* (37.15%) caused the highest reduction in root mass, and was significantly higher than all other pathogens. *Campylocarpon fasciculare* (7.99%) caused the lowest percentage reduction of root mass, but this was not significantly different

from *I. liriodendri* (13.89%), *Pm. minimum* (10.36%), *C. pseudofasciculare* (24.57%), *Co. luteo-olivacea* (22.29%), *N. parvum* (21.56%) and *Pm. parasiticum* (18.84%). In site two of the 2016 season, *D. macrodidyma* (64.91%) caused the highest reduction in root mass, the reduction was however not significantly different from *C. fasciculare* (58.90%), *C. pseudofasciculare* (62.90%), *I. liriodendri* (49.38%), *N. australe* (59.65%), *N. parvum* (51.56%) and *Pm. minimum* (57.77%). At this site *P. chlamydospora* (20.53%) caused the lowest percentage of root reduction, but this was, however, not significantly different from *Pm. parasiticum* (46.78%), *Pl. richardsiae* (29.39%) and *Co. luteo-olivacea* (47.64%).

Analysis of variance revealed a significant year x site x cultivar interaction ($P < 0.001$; Appendix A, Table 2) for the reduction in root mass. The mean percentage reduction for each site, year and pathogen are shown in Table 5. The control treatments were already taken into consideration in the mean values as described above. Due to the interaction between the different sites and years the results will be further discussed per individual site.

The cultivar SO_4 in site two of 2016 had the largest reduction in root mass (68.11%) and Ramsey in site one of 2016 had the smallest reduction in root mass (17.32%). There were no significant differences in root mass reduction induced by the different fungal trunk pathogens in site one of 2015. In site two of 2015, 101-14 Mgt (44.72%) had the highest reduction in root mass, but was not significantly different from Paulsen 1103 (43.89%), Ruggeri 140 (42.18%) and SO_4 (42.54%). Richter 110 (22.60%) had the lowest reduction in root mass, but this was, however, not significantly different from Richter 99 (26.21%). In site one of 2016, Ruggeri 140 (47.66%) had the highest reduction in root mass and was significantly higher than all the other rootstock cultivars. Ramsey (17.32%) had the lowest reduction in root mass, but was not significantly lower than 101-14 Mgt (30.70%), Paulsen 1103 (18.93%), Richter 110 (26.08%), Richter 99 (23.48%) and US 8-7 (32.80%). In site two of 2016, SO_4 (68.11%) had the highest reduction in root mass, but was, however, not significantly different from Richter 110 (52.22%), Ruggeri 140 (62.69%) and US 8-7 (55.66%). Ramsey (24.04%) had the lowest reduction in root mass, but was not significantly different from 101-14 Mgt (30.01%), Paulsen 1103 (38.16%) and Richter 99 (36.72%).

Analysis of variance revealed no significant ($P = 0.9893$; Appendix A, Table 2) pathogen x cultivar interaction for the reduction in root mass.

Percentage reduction in shoot mass

Analysis of variance revealed a significant year x site x pathogen interaction ($P < 0.0317$; Appendix A, Table 3) for the reduction in shoot mass. The mean percentage reduction for each site, year and pathogen are shown in Table 6. The control treatments were already taken into consideration in the mean values as described above. Due to the interaction between the different sites and years the results will be further discussed per individual site.

Dactylonectria macrodidyma in site two of 2016 caused the largest reduction in shoot mass (83.11%) and *Pm. minimum* in site one of 2016 caused the smallest reduction in shoot mass (13.69%). There were no significant differences in reduction induced by the different fungal trunk pathogens in site one of 2015. In site two of 2015, *N. australe* (61.58%) caused the highest reduction in shoot mass, but was not significantly higher than *C. fasciculare* (50.11%), *C. pseudofasciculare* (49.13%), *Co. luteo-olivacea* (50.61%), *I. liriodendri* (50.41%), *D. macrodidyma* (55.60%), *N. parvum* (56.94%) and *Pm. minimum* (43.05%). *Pleurostoma richardsiae* (30.80%) caused the lowest reduction in shoot mass, but this reduction was not significantly different from *Pm. parasiticum* (36.99%), *P. chlamydospora* (35.00%), *C. fasciculare*, *C. pseudofasciculare*, *Co. luteo-olivacea* and *I. liriodendri.* In site one of 2016, *D. macrodidyma* (55.16%) caused the highest reduction in shoot mass, but the reduction was not significantly different from *C. pseudofasciculare* (42.92%), *Co. luteo-olivacea* (45.81%), *I. liriodendri* (37.24%), *N. australe* (39.27%), *P. chlamydospora* (47.48%) and *Pl. richardsiae* (36.76%). *Phaeoacremonium minimum* (13.69%) caused in the smallest percentage reduction of shoot mass, but this was not significantly different from *C. fasciculare* (23.27%). In site two of 2016, *D. macrodidyma* (83.11%), caused the highest reduction in shoot mass, the reduction was, however, only significantly different from *P. chlamydospora* (35.06%), *Pm. parasiticum* (62.41%) and *Pl. richardsiae* (42.12%).

Analysis of variance revealed a significant year x site x cultivar interaction ($P < 0.0001$; Appendix A, Table 3) for the reduction in shoot mass. The mean percentage reduction for each site, year and pathogen are shown in Table 7. The control treatments were already taken into consideration in the mean values as described above. Due to the interaction between the different sites and years the results will be further discussed per individual site.

The cultivar SO_4 in site two of 2016 had the largest reduction in shoot mass (77.57%) and SO_4 in site one of 2016 had the smallest reduction in shoot mass (7.83%). Due to the interaction between the different sites and years the results will be further discussed per individual site. In site one of 2015, Richter 110 (60.81%) had the highest reduction in shoot mass, but was however not significantly different from Ruggeri 140 (51.69%). Rootstock 101-14 Mgt (29.64%) had the lowest reduction in shoot mass, but was not significantly different from Paulsen 1103 (39.20%), Richter 99 (33.94%) and SO_4 (42.94%). In site two of 2015, Ruggeri 140 (54.87%) had the highest reduction in shoot mass, but was not significantly different from Paulsen 1103 (47.36%) and SO_4 (46.60%). Richter 99 (35.66%) had the lowest reduction in shoot mass, but this was however not significantly different from Richter 110 (43.83%) and 101-14 Mgt (43.83%). In site one of 2016, Ruggeri 140 (53.84%) had the highest reduction in shoot mass but was not significantly different from Ramsey (40.34%), 101-14 Mgt (40.44%), Richter 110 (39.13%) and US 8-7 (42.78%). SO_4 had the lowest reduction in shoot mass and was significantly lower than all the other cultivars. In site

two of 2016, SO_4 (77.57%) had the highest reduction in shoot mass, but was however not significantly different from Richter 110 (66.11%), Ruggeri 140 (75.51%) and US 8-7 (71.44%). 101-14 Mgt (38.53%) had the lowest reduction in shoot mass, but was not significantly different from Ramsey (42.64%) and Paulsen 1103 (49.42%).

Analysis of variance revealed no significant (P = 0.9991; Appendix A, Table 3) pathogen x cultivar interaction for the reduction in shoot mass.

Percentage disease severity in the eight different rootstock cultivars

Analysis of variance revealed a significant year x site x pathogen interaction (P < 0.0001; Appendix A, Table 4) for the percentage disease severity seen after the inoculated shoots were grown in nursery fields for eight months. The mean percentage for each site, year and pathogen are shown in Table 8. The control treatments were already taken into consideration in the mean values as described above. Due to the interaction between the different sites and years the results will be further discussed per individual site.

Phaeomoniella chlamydospora in site one of 2015 had the largest percentage disease severity (73.27%), but was however not significantly different from *P. chlamydospora* in site one (72.87%) and two (69.82%) of 2016. *Ilyonectria liriodendri* in site two of 2016 had the lowest percentage disease severity (41.56%). In site one of 2015, *P chlamydospora* (73.27%) had the highest level of disease severity and was significantly higher than all the other fungal trunk pathogens. In site two of 2015, *C. pseudofasciculare* (62.50%) had the highest percentage disease severity, but was not significantly different from any of the other fungal trunk pathogens. In site one of 2016, *P. chlamydospora* (72.87%) had the highest percentage disease severity and was significantly higher than all the other fungal trunk pathogens. In site two of 2016, *P. chlamydospora* (69.82%), had the highest percentage disease severity and was significantly higher than all the other fungal trunk pathogens. *Ilyonectria liriodendri* (41.56%) had the lowest disease severity, but was however not significantly different from *Co. luteo-olivacea* (41.67%), *Pm. parasiticum* (44.21%) and *N. parvum* (43.12%).

Analysis of variance revealed a significant year x site x cultivar interaction (P < 0.0001; Appendix A, Table 4) for the percentage disease severity seen. The mean percentage for each site, year and pathogen are shown in Table 9. The control treatments were already taken into consideration in the mean values as described above. Due to the interaction between the different sites and years the results will be further discussed per individual site.

The cultivar SO_4 in site one of 2015 had the highest percentage disease severity (65.91%) and Ramsey in site two of 2016 had the lowest percentage disease severity (29.23%). In site one of 2015, SO_4 (65.91%) had the highest percentage disease severity, but was however not significantly different from Richter 110 (62.44%). Paulsen 1103

(46.60%) had the lowest percentage disease severity and was significantly different from all the other rootstock cultivars. In site two of 2015, SO_4 (62.15%) had the highest percentage disease severity, but was not significantly different from 101-14 Mgt (58.06%). Richter 99 (50.46%) had the lowest percentage disease severity, but this was however not significantly different from Richter 110 (51.30%) and Ruggeri 140 (52.81%). In site one of 2016, 101-14 Mgt (59.84%) had the highest percentage disease severity but was not significantly different from SO_4 (55.76%). Ramsey (35.62%) had the lowest percentage disease severity and was significantly lower than all the other cultivars. In site two of 2016, 101-14 Mgt (58.62%) had the highest percentage disease severity, but was however not significantly different from SO_4 (55.00%). Ramsey (29.23%) had the lowest percentage disease severity and was significantly lower than all the other cultivars.

Analysis of variance revealed no significant (P = 0.4071; Appendix A, Table 4) pathogen x cultivar interaction for the percentage disease severity seen after the inoculated shoots were grown in nursery fields for eight months.

Histological studies

Mean vessel diameter

There were significant differences found between the cultivars in terms of mean vessel diameter (Table 10). Ramsey (51.92 µm) had the smallest vessel diameter followed by US 8-7 (58.14 µm) and both were significantly different from each other and remaining rootstock cultivars. The mean vessel diameter of 101-14 Mgt (99.23 µm) was significantly larger than all the other cultivars, followed by SO_4 (85.53 µm) which was significantly larger than Richter 110, Richter 99, Ruggeri 140, Paulsen 1103, US 8-7 and Ramsey.

Presence of suberin in infected vascular tissue

After staining control and infected transversal cuts of Ramsey (Fig. 15) and 101-14 Mgt (Fig. 16) it was clear that the suberization of cells form an intricate part of the grapevine host reaction. No suberization was observed in the control treatments. In Figs. 15 and 16 the cuts stained with Sudan black B indicated the presence of suberin around xylem vessels and parenchyma cells located around xylem vessels filled with tyloses. The tyloses itself was also suberized. Suberization was also seen in the cells located on growth ring boundary as well as ray parenchyma cells. The presence of suberin was confirmed by following a second suberin staining protocol using Sudan IV. There was no differences observed between Ramsey and 101-14 Mgt. Both cultivars showed suberization in the same reaction zones.

DISCUSSION

This study evaluated the susceptibility of the eight most popular rootstocks used in the South African grapevine industry, against 11 economically important fungal trunk disease pathogens namely, *Phaeomoniella chlamydospora, Phaeoacremonium minimum, Pm. parasiticum, Cadophora luteo-olivacea, Pleurostoma richardsiae, Ilyonectria liriodendri, Dactylonectria macrodidyma, Campylocarpon fasciculare, C. pseudofasciculare, Neofusicoccum australe, N. parvum.* There is very little information available on the susceptibility of grapevine rootstock cultivars to trunk pathogens, especially in the South African context. Past studies investigating susceptibility have been either limited to only a few cultivars or only included a small range of pathogens and most were not conducted under field conditions (Eskalen *et al.,* 2001b; Diaz *et al.,* 2009; Alaniz *et al.,* 2010). This is the first study that has been conducted in South Africa under field conditions that included such a high number of rootstock cultivars and large range of fungal trunk pathogens. All of the pathogens induced symptoms in the grapevine rootstocks studied; however, the reaction to infection differed among the cultivars. This is in agreement with past studies conducted on grapevine rootstocks where it was also found that none of the cultivars were completely resistant to infection, but differential levels of susceptibility existed between rootstock cultivars (Eskalen *et al.,* 2001b; Jaspers *et al.,* 2007; Diaz *et al.,* 2009; Alaniz *et al.,* 2010; Gramaje *et al.,* 2010a; Billones-Baaijens *et al.,* 2014).

After the completion of the experiments, the years and sites were evaluated separately, due to the fact that two of the rootstock cultivars failed in 2015 and that a drought was experienced in 2016 which influenced the results. In site two of 2016, much higher reductions in root and shoot mass were observed compared to the other site. Unfortunately due to the drought that was experienced during that time, limited irrigation was provided to the site putting the young vines under further stress, allowing the pathogens to flourish (Von Arx, 1987; Scheck *et al.,* 1998a; Fourie *et al.,* 2001, Halleen *et al.,* 2003; Fourie and Halleen, 2004b; Van Niekerk *et al.,* 2004). With the results of this study, which include the percentage reduction in root and shoot mass and percentage disease severity (level of vascular discoloration), most of the grapevine rootstock cultivars that were evaluated were significantly affected by all 11 fungal trunk pathogens. This corroborates previous studies that found no grapevine rootstock cultivar to be completely resistant to pathogen infection (Jaspers *et al.,* 2007; Diaz *et al.,* 2009*;* Alaniz *et al.,* 2010; Gramaje *et al.,* 2010a*;* Billones-Baaijens *et al.,* 2014). A study conducted by Amponsah *et al.* (2011) had shown that the infection rate of fungal pathogens are slow when inoculating with conidial suspensions, due to a lag phase involved in conidial attachment, germination and penetration in the host tissue. Therefore, it was expected that the pathogens would not have such a detrimental effect on bud burst at such an early stage of infection. The percentage of cuttings emerging

from dormancy was not included in the results, as the bud burst of vines are determined by climate; sufficient cold in winter and enough heat in late spring (Samish, 1954; Antcliff and May, 1961; Pouget, 1972).

All of the infected rootstock cuttings showed a reduction in root and shoot mass. Even though differences were seen between the cultivars, the results were not consistent. The vegetative growth of a grapevine is partially determined by the starch reserves that were stored in canes of the mother vines before the canes were harvested for propagation (Yang and Hori, 1979; Keller and Koblet, 1994; Murisier and Aerny, 1994). The starch reserves stored in the canes not only takes part in vegetative and reproductive development and respiration (energy requirements), but is also utilized in grapevine defence reactions against abiotic and biotic stresses (Murisier, 1996; Jermini *et al.,* 2010a, 2010b). Therefore, a pathogen's affect on the reduction in root and shoot mass would be smaller in the first growth season, but could affect the vines ability to store reserves for the next season and therefore influencing the root and shoot growth in the season following infection.

The overall root and shoot reduction that was seen in all the cultivars inoculated with fungal pathogens can be explained by the host utilising its nutrients to establish a host response in the plant. A grapevine consists of a set of 'sink' organs (leaves, roots, fruits, fine roots and a perennial structure consisting of the stem, canes and woody shoots) that obtains carbon from photosynthesis and the mobilization of reserves (Vivin *et al.,* 2002). The vine then grows as a collection of interacting organs that constantly compete for resources (Grossman and De Jong, 1994). During the first growing season, majority of the starch reserves are utilized between bud burst and flowering (Zufferey *et al.,* 2015). During this period, a young grafted vine has very few leaves and a very small developing root system and is therefore highly dependent on stored reserves to develop since photosynthesis is minimal. To induce a host reaction, the vine needs to utilize resources and nutrients to successfully compartmentalize the fungi present in the plant resulting in a partitioning of resources to the detriment of growth (Grossman and De Jong, 1994), possibly explaining the overall reduction in root and shoot mass observed in all of the cultivars.

Gramaje *et al.* (2010a) conducted a study in Spain following the same inoculation method as the current study and after the analysis of the inoculated vines, great variability between the rootstock cultivars was observed when measuring the percentage of cuttings emerging from dormancy and shoot weight. Similar observations were made in the current study in terms root and shoot mass measurements. The analysis of disease severity produced more consistent results. Various parameters to determine disease severity in grapevines have been investigated in the past including root initiation, callus production, presence of leaf chlorosis and necrosis and bud break (Adalat *et al.,* 2000; Wallace *et al.,* 2004; Zanzotto *et al.,* 2008; Gramaje *et al.,* 2010a). The effectiveness of fungal trunk

pathogens to induce xylem discoloration and cause lesions suggest that the evaluation of internal vascular discolouration (Mugnai *et al.,* 1999; Adalat *et al.,* 2000; Eskalen *et al.,* 2001b; Halleen *et al.,* 2007a), according to the method of inoculation either through a wound inoculation or vacuum inoculation, would provide the most appropriate and 'true' insight into the infection present in a vine.

Between the different rootstock cultivars, the percentage disease severity revealed the most insight in terms of susceptibility towards fungal trunk disease pathogens. Ramsey had the lowest level of disease severity against all the pathogens followed by US 8-7 and Paulsen 1103. Rootstock SO_4 had the highest level of disease severity followed by 101-14 Mgt and Richter 110. In a rootstock tolerance study conducted by Gramaje *et al.* (2010a) in Spain, the effect of Petri disease pathogens were evaluated following the same vacuum inoculation method used in the current study. A different set of rootstock cultivars were evaluated, but it was determined that all the cultivars were influenced by the inoculated pathogens and Richter 110 was found to be the most susceptible which is in agreement with our results having Richter 110 at the bottom spectrum of the cultivars we evaluated.

In other studies conducted using a mycelium plug inoculation method under greenhouse conditions, Richter 110 and SO4 were the most susceptible cultivars of the rootstocks evaluated (Martos, 2008; Diaz *et al.,* 2009; Billones-Baaijens *et al.,* 2014). In contrast with the results obtained from our study, Diaz *et al.* (2009) and Billones-Baaijens *et al.* (2014) found 101-14 Mgt to show smaller lesion lengths when inoculated with *Phaeoacremonium minimum* and Botryosphaeriaceae species, compared to other rootstock cultivars in the respective studies. The difference in the results found, compared to our study, could be due to the use of different inoculation and incubation protocols.

Very little investigation has been done into the susceptibility of Ramsey, also known as Salt Creek even though it is one of the most commonly used rootstock cultivars used in South Africa, especially in the table grape industry, due to its strong vigour, high drought tolerance and ability to perform well in poor quality soils (Loubser and Uys, 1997). Most rootstock cultivars used today are crosses of *V. berlandieri, V. rupestris* and *V. riparia* and since Ramsey is a clone of *V. champinii,* it is possible that this specie could have a higher tolerance to infection and further studies into its interaction with fungal pathogens could deliver promising insights.

No significant interaction was found in our study between pathogen and cultivar and this supports the theory that grapevine reaction to infection is not specific to the pathogen, but dependant on wood anatomy (Deflorio *et al.,* 2009; Travadon *et al.,* 2013).

The results of this study revealed that *P. chlamydospora* caused the greatest symptom expression (percentage disease severity) which is in agreement with previous studies that found that *P. chlamydospora* induced the largest areas of vascular discolouration compared

to other Petri disease pathogens (Mugnai *et al.,* 1999; Halleen *et al.,* 2007a; Aroca and Raposo, 2009; Laveau *et al.,* 2009). In terms of shoot mass, both *P. chlamydospora* and *Co. luteo-olivacea* caused high reduction in shoot mass. This emphasises the importance of *Co. luteo-olivacea* to grapevine disease (Halleen *et al.,* 2007a). In terms of root mass, *P. chlamydospora* again caused the highest reduction in site one of 2015 and 2016, compared to the other Petri disease pathogens. In site two of 2015, *Co. luteo-olivacea* caused the largest reduction and in site two of 2016, *Pm. minimum* caused the highest reduction in root mass. Of the two Botryosphaeriaceae species tested, no significant differences were observed between the levels of vascular discolouration that were observed. These results were consistent with Úrbez-Torres and Gubler (2009) that reported *N. australe* and *N. parvum* were equally pathogenic when inoculated onto trunks of raisin, table and wine grapes. The shoot and root mass revealed that *N. australe* caused greater reduction in both shoot and root mass compared to *N. parvum*. However, many pathogenicity studies have reported that *N. parvum* is one of the most pathogenic Botryosphaeriaceae species on grapevine (Phillips, 2002; Van Niekerk *et al.,* 2004; Úrbez-Torres and Gubler, 2009; Billones-Baaijens *et al.,* 2013, 2014). Differential levels of virulence in the *Neofusicoccum* spp. has been reported in past studies (Van Niekerk *et al.,* 2004; Úrbez-Torres and Gubler, 2009; Billones-Baaijens *et al.,* 2013). The four black-foot disease pathogens induced vascular discolouration of around the same level, which is in agreement to Alaniz *et al.* (2010) who did not observe any differences in the level of symptom expression induced in rootstocks by *I. liriodendri* and *D. macrodidyma.* In contrast to this, *D. macrodidyma* caused the greatest reduction in shoot mass and *D. macrodidyma* and *C. pseudofasciculare* caused the greatest reduction in root mass over the other black-foot pathogens. Generally black foot pathogens are associated with below ground symptoms and are usually isolated from roots and from the base of the rootstock. In South Africa these pathogens have also been isolated from graft unions. This occurrence is a result of the common South African Nursery practise of this part of the plant covered with soil for approximately 5 weeks to prevent the drying out of the callus material (Halleen *et al*., 2003). More recently, in a study conducted by Cardoso *et al.* (2013) that investigated the fungal inoculum sources in the nursery process, they had a high incidence of black foot disease pathogens from scion canes. They suggested that aerial inoculum of black foot disease pathogens should be considered as a possible source of infection. Our study confirmed that black foot pathogens have the ability to infect and produce symptoms in grapevine vascular tissue.

No foliar symptoms were observed on any of the cultivars during the nursery growing season. This is in agreement to what was reported by Gramaje *et al.* (2010a) and it was suggested that a longer growing period would be necessary to observe typical foliar symptoms to find a possible correlation with internal infection.

Using the vacuum inoculation method as described by Gramaje *et al*. (2010a) and Rooney and Gubler (2001), it was possible to ensure the most accurate and natural scenario of infection. Grapevine vascular structures are asymmetrical, with larger xylem vessels in the dorsal/ventral sections than the vessels found in the lateral sections (Brodersen *et al.,* 2011). These vessels are then clustered in fascicular portions radiating from the central pith. Travadon *et al.* (2013) suggested that both the depth and location (lateral or dorsal/ventral) of a wound made (with a cork borer or power drill) to inoculate a pathogen, could influence the subsequent colonization of vessels with the pathogen and influence results. Thus, a wound located in the proximity of larger vessels could promote faster colonization of the pathogen and therefore larger lesions. Billones-Baaijens *et al.* (2014) also suggested that to ensure the most realistic scenario of what natural infection might appear like, it is most ideal to inoculate grapevines with conidial suspensions under field conditions.

The virulence screenings that were conducted before the nursery field trials proved to be an effective method in determining deferential levels of pathogenicity between isolates of the same pathogen. All of the isolates produced significant lesions with deferential lesion lengths between isolates of the same pathogen and was possible to identify the most virulent isolates of each of the pathogens to be further investigated in the field trials. The different virulence levels observed between isolates indicates the importance of conducting preliminary virulence screenings prior to field trials to ensure reliable results. The differences in virulence also reflect the genetic diversity of all the included pathogens and the possibility that they might also react differently to control measures and an integrated management strategy might be necessary to combat problems with grapevine fungal trunk diseases. All the included fungal pathogen isolates were consistently isolated from the brown streaking that was produced upward and downward from the point of inoculation. The high re-isolation incidences are a reflection to the ease with which the pathogens can colonize and degrade the grapevine vascular tissue.

Numerous studies investigating wounds as an infection point have been conducted in the past, focusing mainly on pathogens associated with esca and Petri disease as well as Botryosphaeriaceae species, and have confirmed pruning wounds, suckering wounds and mechanical wounding to be one of the main infection points in established vineyards (Lehoczky, 1974; Larignon and Dubos, 1997; Eskalen and Gubler, 2001; Van Niekerk *et al.,* 2010; Makatini, 2014). Most of these studies mainly focused on the susceptibility of wounds made on scion cultivars. The results of the virulence screenings indicate that fungal trunk pathogens can also colonize and infect rootstocks through wounds, emphasizing the need to incorporate wound protection protocols in rootstock mother field cultural practices to protect the mother vines and prevent them from becoming pathogen reservoirs. It would be useful to conduct trials in rootstock mother fields to determine how susceptible pruning wounds are

under field conditions and also to determine how long they are susceptible and what wound protections would be most effective in protecting the wounds.

In our study the analysis of the mean xylem vessel diameters of the eight rootstock cultivars revealed that there were significant differences in the vessel size of the eight rootstock cultivars. Rootstocks with smaller vessel diameters also had a smaller percentage disease severity, confirming that vessel diameter has a direct influence on rootstock susceptibility to fungal trunk disease pathogens. Pouzoulet *et al.* (2014) proved that xylem vessel diameters differed among *V. vinifera* cultivars and found a correlation between susceptibility to drought and susceptibility to vascular disease due to xylem vessel size. No rootstock cultivars were, however, included in their study. The principles of the CODIT model (Compartmentalization Of Decay In Trees) was used to better understand the host reaction of grapevines in the presence of pathogen infection. The model consists of four 'Walls' that aim in restricting pathogen spread. Wall 1 restricts longitudinal movement of pathogens and is primarily associated with vessel occlusions through gels and tyloses. Wall 2 includes the growth ring boundary and prevents centripetal pathogen movement between seasonal growth. Wall 3 prevents tangential pathogen movement and is mainly associated with ray parenchyma cells. These three walls form the reaction zone and occur mainly in lignified tissue before infection and/or wounding takes place (Shigo and Marx, 1977). Finally, Wall 4, is called the barrier zone and is the newly modified cells which form after an injury in the tissue to provide a more impermeable barrier to prevent pathogen spread (Pearce, 1996).

Vessel occlusion (Wall 1) as a host response in grapevine has been studied extensively. The occlusion of vessels in grapevine occurs through the development of tyloses and gels that originate from paratracheal parenchyma cells (Sun *et al.,* 2008) as a response to hormonal signals like ethylene that are associated with wounding and infection (Sun *et al.,* 2007). Pouzoulet *et al*. (2014) confirmed that vessel dimensions play a role in the ability of a vine to compartmentalize a pathogen. This correlates with the results of our study. In their study three scion cultivars were evaluated namely, Merlot, Cabernet Sauvignon and Thompson Seedless (no rootstock cultivars were included). Merlot was the most tolerant to fungal trunk pathogens and showed to have the lowest mean vessel diameter, whereas Thompson Seedless had the largest and was also the most susceptible scion cultivar. A grapevines success in developing tyloses and gels in infected conduits increases its tolerance toward trunk pathogens (Yadeta and Thomma, 2013). This is due to the volume of material needed to fully occlude a vessel increases with the square of the infected vessel diameter. The slightest increase in vessel diameter would result in a substantial increase in material needed to fully occlude the vessel (Pouzoulet *et al.,* 2014). Grapevine cultivars that have smaller vessel diameters would have to utilize fewer resources to establish a host

reaction and therefore be able to restrict the spread of pathogens quicker and more efficiently.

When evaluating suberin production in the two cultivars, Ramsey and 101-14Mgt, no differences in production was observed and we concluded that suberin production is part of the general host response of grapevines. In our study suberization was observed in cells located on the growth ring boundary and ray parenchyma cells, form part of Wall 2, Wall 3 and Wall 4 of the CODIT model (Biggs, 1987; Shigo and Marx, 1977). Suberin deposits can also occur in tyloses as was observed in our studies (Rioux *et al.,* 1995). Defence mechanisms in vines can be divided into response to wounding and response due to pathogen perception (Shigo and Marx, 1977). The plant response on a cellular level involves barrier formation and results in a reaction zone that develops near injuries and areas of infection. These reaction zones are usually enriched with lignin and suberin (Hawkins and Boudet, 1996). In parenchyma cells, rays and vessels, intracellular suberin is produced near cell walls (Biggs, 1987; Pearce, 2000; Pouzoulet *et al.,* 2014). Suberin is known to form an impermeable barrier restricting water movement as well as pathogen spread (Pearce, 1996). Therefore the suberized ray and growth ring boundaries prevent the movement of fungi to another fascicular portion or to newly developed vascular tissue, successfully compartmentalizing the pathogen. The direct interaction between suberin and fungal tissue is not well documented and further investigation is needed to fully understand this component of the grapevine host reaction.

To make it possible for grapevine producers to make the most informed decision when choosing a rootstock to establishing a new vineyard, it would be essential to conduct further trials investigating the effect of the pathogens on grafted vines, focusing especially on the effect fungal trunk pathogens would have on callus formation that would influence the success of graft union formation.

The results show that pathogens associated with the three major fungal trunk diseases (Petri disease, black-foot disease and Botryosphaeria dieback and cankers) all have the ability to reduce percentage root mass, shoot mass and increase percentage disease severity and that none of the rootstock cultivars were completely resistant. The quality of plant material is crucial in the success and longevity of newly established vineyards. Combining existing knowledge of disease management in the propagation process with the knowledge obtained from this research in terms of disease susceptibility will assist in optimizing plant material quality and the sustainability of the South African grapevine industry as well as others worldwide. This study has shown that Ramsey could form a useful part of an integrated management strategy for fungal trunk disease pathogens.

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TABLES AND FIGURES

Table 1. List of fungal isolates from *Vitis vinifera* used in preliminary virulence screenings

	Accession Number			Isolation date	
Organism	**STE-U[1]**	**Other**	**Location**		**Collector**
Phaeomoniella chlamydospora	6384	LM 91	Farm 1, Paarl, South Africa	24/09/2001	L. Mostert
	8276	LM 310	Farm 2, Paarl, South Africa	26/03/2002	L. Mostert
		LM 312	Farm 2, Paarl, South Africa	26/03/2002	L. Mostert
		C.blanc	Farm 3, Stellenbosch, South Africa		C. Mutawila
		C64C	Farm 4, Constantia, South Africa	14/15/2013	P. Moyo
		C4F	Farm 4, Constantia, South Africa	06/15/2013	P. Moyo
		C5(A)F	Farm 4, Constantia, South Africa	06/15/2013	P. Moyo
		FH-Pc 30	Farm 5, Ashton, South Africa	19/03/2014	F. Halleen
		FH-Pc 31	Farm 5, Ashton, South Africa	19/03/2014	F. Halleen
		FH-Pc 32	Farm 6, Worcester, South Africa	13/03/2014	F. Halleen
Phaeoacremonium minimum		FH-P 32	Stellenbosch, South Africa	11/06/2012	A. Baloyi
		FH-P 44	Farm 14, Durbanville, South Africa	29/05/2012	A. Baloyi
		FH-P 118	Farm 8, Slanghoek, South Africa	10/07/2012	A. Baloyi
		FH-P 108	Farm 8, Slanghoek, South Africa	24/07/2012	A. Baloyi
	8273	FH-P 116	Farm 15, Rawsonville, South Africa	10/07/2012	A. Baloyi
		FH-P 76	Farm 16, Wellington, South Africa	25/06/2012	A. Baloyi
		FH-P 112	Stellenbosch, South Africa	10/07/2012	A. Baloyi
	8272	FH-P 104	Farm 16, Wellington, South Africa	10/07/2012	A. Baloyi
		FH-P 46	Farm 14, Durbanville, South Africa	25/06/2012	A. Baloyi
		FH-P 34	Stellenbosch, South Africa	29/05/2012	A. Baloyi

Continued

Table 1. Continued

Organism	Accession Number STE-U[1]	Other	Area	Isolation date	Collector
Phaeoacremonium parasiticum		LM 36	Farm 7, Porterville, South Africa	12/03/2001	L. Mostert
	6990		Klawer, South Africa	31/01/2008	F. Halleen
	6993		De Rust, South Africa	31/01/2008	F. Halleen
		FH-P 606	Stellenbosch, South Africa	18/03/2013	A. Baloyi
	8275	FH-P 625	Durbanville, South Africa	26/03/2013	A. Baloyi
		FH-P 631	Farm 1, Paarl, South Africa	18/03/2013	A. Baloyi
	8274	FH-P 632	Farm 8, Slanghoek, South Africa	18/03/2013	A. Baloyi
		FH-P 64	Stellenbosch, South Africa	29/05/2012	A. Baloyi
		FH-P 64	Stellenbosch, South Africa	29/05/2012	A. Baloyi
		FH-P 99	Stellenbosch, South Africa	07/02/2012	A. Baloyi
		LM 209	Farm 7, Porterville, South Africa	12/03/2001	L. Mostert
Cadophora luteo-olivacea		FH-P 502	Stellenbosch, South Africa	24/07/2012	F. Halleen
		FH-P 350	Stellenbosch, South Africa	10/07/2012	F. Halleen
		FH-P 529	Slanghoek, South Africa	25/07/2005	F. Halleen
		FH-P 517	Constantia, South Africa	31/01/2008	F. Halleen
		FH-P 551	Slanghoek, South Africa	31/01/2008	F. Halleen
	8278	FH-P 511	Wellington, South Africa	05/06/2007	F. Halleen
		FH-P 510	Paarl, South Africa	06/07/2012	F. Halleen
		FH-P 542	Paarl, South Africa	18/03/2013	F. Halleen
	8277	FH-P 533	Wellington, South Africa	29/05/2012	F. Halleen
		FH-P 543	Wellington, South Africa	6/15/2013	F. Halleen

Continued

Table 1. Continued

Organism	Accession Number STE-U[1]	Other	Location	Isolation date	Collector
Pleurostoma richardsiae		LM 355	Paarl, South Africa	24/04/2002	F. Halleen
		LM 32	Farm 1, Paarl, South Africa	24/07/2001	L. Mostert
	8271	LM 27	Farm 1, Paarl, South Africa	24/07/2001	L. Mostert
		FH-P 500	Wellington, South Africa	05/06/2012	F. Halleen
	8270	FH-P 21	Paarl, South Africa	06/03/2000	F. Halleen
		FH-P 52	Stellenbosch, South Africa	06/03/2000	F. Halleen
		FH-P 36	Paarl, South Africa	06/03/2000	F. Halleen
		FH-Pr 12	Stellenbosch, South Africa	20/01/2014	F. Halleen
		FH-Pr 10	Stellenbosch, South Africa	20/01/2014	F. Halleen
		FH-Pr 11	Stellenbosch, South Africa	13/03/2014	F. Halleen
Ilyonectria liriodendri		FH-C 25	Paarl, South Africa	25/07/2005	F. Halleen
	8267	FH-C 226	Nursery 1, Wellington, South Africa	21/06/2012	S. Langenhoven
	8266	FH-C 204	Farm 9, South Africa	21/06/2012	S. Langenhoven
		FH-C 14	Paarl, South Africa	13/05/2003	F. Halleen
		FH-C 17	Stellenbosch, South Africa	13/05/2003	F. Halleen
		FH-C 247	Farm 9, South Africa	21/06/2012	S. Langenhoven
		FH-C 404	Nursery 2, Wellinton, South Africa	20/06/2013	F. Halleen
		FH-C 342	Nursery 3, Wellington, South Africa	21/06/2013	F. Halleen
		FH-C 363	Nursery 2, Wellinton, South Africa	21/06/2013	P. Lesuthu
	7539		Western Cape, South Africa	05/06/2007	F. Halleen

Continued

Table 1. Continued

Organism	Accession Number STE-U[1]	Other	Location	Isolation date	Collector
Dactylonectria macrodidyma	8264	FH-C 106	Paarl, South Africa	20/07/2011	F. Halleen
		FH-C 98	Paarl, South Africa	15/06/2011	F. Halleen
		FH-C 217	Nursery 4, South Africa	09/07/2012	P. Lesuthu
		FH-C 279.2	Nursery 3, Wellington, South Africa	03/07/2012	P. Lesuthu
		FH-C 292	Farm 10, Stellenbosch, South Africa	27/11/2012	P. Lesuthu
	8265	FH-C 241	Nursery 2, Wellington, South Africa	05/07/2012	P. Lesuthu
		FH-C 215	Nursery 4, South Africa	06/07/2012	P. Lesuthu
	7543		Wellington, South Africa	06/05/2000	F. Halleen
	7618	C 12	Wellington, South Africa	27/08/2000	F. Halleen
		FH-C 270	Nursery 2, Wellinton, South Africa	05/06/2012	F. Halleen
Campylocarpon pseudofasciculare		FH-C 566	Nursery 3, Wellington, South Africa	03/07/2013	F. Halleen
		FH-C 206	Nursery 3, Wellington, South Africa	21/06/2012	F. Halleen
		FH-C 372	Nursery 3, Wellington, South Africa	21/06/2013	F. Halleen
	8280	FH-C 397	Nursery 3, Wellington, South Africa	21/06/2013	F. Halleen
		FH-C 508	Nursery 3, Wellington, South Africa	27/06/2013	F. Halleen
	8279	FH-C 558	Nursery 5, South Africa	27/06/2013	F. Halleen
		FH-C 323	Nursery 2, Wellinton, South Africa	24/05/2013	F. Halleen

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Table 1. Continued

Organism	Accession Number STE-U[1]	Other	Area	Isolation date	Collector
Campylocarpon fasciculare		FH-C 251	Farm 9, South Africa	21/06/2012	F. Halleen
	8281	FH-C 403	Nursery 2, Wellinton, South Africa	21/06/2013	F. Halleen
		FH-C 331	Nursery 1, Wellington, South Africa	20/06/2013	F. Halleen
		FH-C 387	Nursery 2, Wellinton, South Africa	20/06/2013	F. Halleen
		FH-C 390	Nursery 2, Wellinton, South Africa	21/06/2013	F. Halleen
	8282	FH-C 575	Nursery 3, Wellington, South Africa	03/07/2013	F. Halleen
		FH-C 360	Nursery 2, Wellinton, South Africa	13/06/2013	F. Halleen
		FH-C 583	Nursery 2, Wellinton, South Africa	04/07/2013	F. Halleen
		FH-C 393	Nursery 2, Wellinton, South Africa	13/06/2013	F. Halleen
		FH-C 343	Nursery 2, Wellinton, South Africa	20/06/2013	F. Halleen
Neofusicoccum parvum	7021	Bot 48	Paarl, South Africa	14/02/2005	F. Halleen
	7022	Bot 50	Paarl, South Africa	14/02/2005	F. Halleen
	7023	Bot 52	Paarl, South Africa	14/02/2005	F. Halleen
	7036	Bot 126	Darling, South Africa	22/10/2007	F. Halleen
	7037	Bot 128	Constantia, South Africa	16/10/2007	F. Halleen
	4584	Bot 71.6	Saint Julien, Bordeaux, France	01/08/1999	P. Larignon
		PMM 319	Constantia, South Africa	10/06/2013	P. Moyo
	8268	PMM 330	Constantia, South Africa	10/06/2013	P. Moyo
		PMM 336	Constantia, South Africa	10/06/2013	P. Moyo
		PMM 337	Constantia, South Africa	10/06/2013	P. Moyo

Continued

Table 1. Continued

Organism	Accession Number STE-U[1]	Other	Area	Isolation date	Collector
Neofusicoccum australe	7024		Stellenbosch, South Africa	15/02/2005	F. Halleen
	7027	Bot 60	Porterville, South Africa	02/03/2005	F. Halleen
	7030		Porterville, South Africa	14/02/2005	F. Halleen
		PMM 4	Farm 11, Grabouw, South Africa	06/03/2006	P. Moyo
		PMM 6	Farm 11, Grabouw, South Africa	06/03/2006	P. Moyo
	8269	PMM 8	Farm 12, Grabouw, South Africa	28/05/2005	P. Moyo
		PMM 9	Farm 12, Grabouw, South Africa	28/05/2005	P. Moyo
		PMM 79	Farm 13, Grabouw, South Africa	15/06/2007	P. Moyo
	7025	Bot 59	Porterville, South Africa	07/03/2005	F. Halleen
	7029	Bot 91	Stellenbosch, South Africa	24/05/2005	F. Halleen

[1]STE-U: Stellenbosch University, South Africa (Culture collection of the Department of Plant Pathology).

Table 2. List of isolates chosen to use as inoculum in nursery field trials according to preliminary virulence screenings and their ability to produce conidial inoculum.

Organism	Isolates used in nursery field trials (treatment nr)	
P. chlamydospora	STEU 8276	STEU 6384
Pa. minimum	STEU 8272	STEU 8273
Pa. parasiticum	STEU 8275	STEU 8274
Pl. richardsiae	STEU 8270	STEU 8271
Ca. luteo-olivacea	STEU 8278	STEU 8277
I. liriodendri	STEU 8266	STEU 8267
D. macrodidyma	STEU 8264	STEU 8265
C. fasciculare	STEU 8281	STEU 8282
C. pseudofasciculare	STEU 8279	STEU 8280
N. australe	STEU 7029	STEU 8269
N. parvum	STEU 7021	STEU 8268

Table 3. Re-isolation percentage for grapevine trunk disease pathogens evaluated in virulence screenings.

	Re-isolation percentages
Organism	**(%)**
P. chlamydospora	76
Pa. minimum	84
Pa. parasiticum	64
Pl. richardsiae	82
Ca. luteo-olivacea	86
I. liriodendri	86
D. macrodidyma	86
C. fasciculare	64
C. pseudofasciculare	84
N. australe	84
N. parvum	86

Table 4. Mean percentages for reduction in vine root mass caused by 11 fungal pathogens for two different experimental sites in 2015 and 2016.

Pathogen	Reduction in root mass (%)[xy]			
	2015		2016	
	Site 1	Site 2	Site 1	Site 2
C. fasciculare	37.43^{c-k}	41.43^{c-i}	7.99^{n}	58.90^{a-d}
C. pseudofasciculare	36.46^{c-k}	48.71^{a-g}	24.57^{i-n}	62.90^{ab}
Co. luteo-olivacea	38.68^{c-k}	44.33^{b-i}	22.29^{h-n}	47.64^{b-h}
I. liriodendri	36.71^{c-k}	38.01^{c-k}	13.89^{l-n}	49.38^{a-e}
D. macrodidyma	35.62^{d-k}	48.55^{a-g}	29.73^{e-m}	64.91^{a}
N. australe	48.22^{a-g}	45.13^{b-h}	26.16^{g-m}	59.65^{ab}
N. parvum	30.08^{e-l}	49.78^{a-f}	21.56^{j-n}	51.56^{c-e}
P. chlamydospora	44.07^{b-i}	33.97^{e-l}	37.15^{c-k}	20.53^{f-m}
Pm. minimum	43.35^{b-i}	39.58^{c-j}	10.36^{l-n}	57.77^{abc}
Pm. parasiticum	41.74^{c-i}	28.46^{e-m}	18.84^{k-n}	46.78^{a-g}
Pl. richardsiae	43.52^{b-i}	26.34^{f-m}	29.15^{f-m}	29.39^{e-l}

[x] Values followed by the same letter are not significantly different from each other (P <0.05; LSD 23.65).
[y] The root mass of each plant was determined after the rootstocks were uprooted in June/July 2015 and 2016.

Table 5. Mean percentages for root mass in eight different cultivars inoculated with fungal trunk pathogens for two different experimental sites in 2015 and 2016.

Rootstock[z]	**Reduction in percentage root mass (%)[xy]**			
	2015		2016	
	Site 1	Site 2	Site 1	Site 2
101-14 Mgt	32.76^{e-j}	44.72^{cde}	30.70^{e-j}	30.01^{e-i}
Paulsen 1103	35.17^{e-h}	43.89^{cde}	18.93^{j}	38.16^{c-h}
Ramsey	-	-	17.32^{j}	24.04^{ghi}
Richter 110	42.83^{cde}	22.60^{h-j}	26.08^{f-j}	52.22^{abc}
Richter 99	32.83^{e-i}	26.21^{f-j}	23.48^{g-j}	36.72^{c-h}
Ruggeri 140	39.10^{c-g}	42.18^{c-f}	47.66^{bcd}	62.69^{ab}
SO_4	35.13^{e-h}	42.54^{cde}	25.81^{ghi}	68.11^{a}
US 8-7	-	-	32.80^{e-j}	55.66^{ab}

[x] Values followed by the same letter are not significantly different from each other ($P < 0.05$; LSD 16.18).

[y] The root mass of each plant was determined after the rootstocks were uprooted in June/July 2015 and 2016.

[z] Dormant un-grafted rootstock cuttings were inoculated in October 2014 and 2015 by sucking up a spore solution of each pathogen through the plants vascular system.

Table 6. Mean percentages for reduction in vine shoot mass caused by 11 fungal pathogens for two different experimental sites in 2015 and 2016.

Pathogen	**Reduction in shoot mass (%)[xy]**			
	2015		2016	
	Site 1	Site 2	Site 1	Site 2
C. fasciculare	50.81^{d-m}	50.11^{d-m}	23.27^{op}	70.22^{a-d}
C. pseudofasciculare	45.49^{f-n}	49.13^{d-m}	42.92^{j-o}	76.70^{abc}
Co. luteo-olivacea	41.49^{h-n}	50.61^{d-m}	45.81^{f-n}	69.75^{a-d}
I. liriodendri	35.67^{j-o}	50.41^{d-m}	37.24^{k-o}	65.89^{a-f}
D. macrodidyma	51.55^{d-m}	55.60^{c-k}	55.16^{d-l}	83.11^{a}
N. australe	57.24^{c-j}	61.58^{b-h}	39.27^{k-o}	76.15^{ab}
N. parvum	38.50^{i-o}	56.94^{c-j}	30.89^{no}	68.19^{a-e}
P. chlamydospora	51.79^{d-m}	35.00^{k-o}	47.48^{e-n}	35.06^{i-o}
Pm. minimum	48.47^{d-n}	43.05^{h-n}	13.69^{p}	65.26^{a-g}
Pm. parasiticum	50.92^{d-m}	36.99^{j-o}	32.54^{mno}	62.41^{b-i}
Pl. richardsiae	44.31^{g-n}	30.80^{mno}	36.76^{l-o}	42.12^{g-n}

[x] Values followed by the same letter are not significantly different from each other ($P < 0.05$; LSD 21.64).

[y] The shoot mass of each plant was determined after the rootstocks were uprooted in June/July 2015 and 2016.

Table 7. Mean percentages for reduction in shoot mass in eight different cultivars inoculated with fungal trunk pathogens for two different experimental sites in 2015 and 2016.

Rootstock[z]	**Reduction in shoot mass (%)**[xy]			
	2015		2016	
	Site 1	Site 2	Site 1	Site 2
101-14 Mgt	29.64^{l}	43.83^{e-l}	40.44^{e-l}	38.52^{g-l}
Paulsen 1103	39.20^{e-l}	47.36^{d-j}	32.99^{jkl}	49.42^{d-i}
Ramsey	-	-	40.34^{e-l}	42.64^{e-l}
Richter 110	60.81^{bcd}	31.84^{jkl}	39.13^{f-l}	66.11^{abc}
Richter 99	33.94^{i-l}	35.66^{h-l}	31.01^{kl}	55.37^{c-e}
Ruggeri 140	51.69^{c-h}	54.87^{c-f}	53.84^{c-g}	75.51^{ab}
SO_4	42.94^{e-l}	46.60^{d-k}	7.83^{m}	77.57^{a}
US 8-7	-	-	42.78^{e-l}	71.44^{ab}

[x] Values followed by the same letter are not significantly different from each other ($P < 0.05$; LSD 16.23).

[y] The shoot mass of each plant was determined after the rootstocks were uprooted in June/July 2015 and 2016.

[z] Dormant un-grafted rootstock cuttings were inoculated in October 2014 and 2015 by sucking up a spore solution of each pathogen through the plants vascular system.

Table 8. Mean percentages of corrected percentage disease severity of rootstock cultivars caused by 11 different fungal trunk pathogens for two different experimental sites in 2015 and 2016.

Pathogen	Corrected disease severity (%)[xy]			
	2015		2016	
	Site 1	Site 2	Site 1	Site 2
C. fasciculare	64.92^{b}	59.71^{b-g}	51.12^{h-l}	49.37^{i-m}
C. pseudofasciculare	58.97^{b-h}	62.50^{bcd}	50.48^{f-k}	48.22^{i-l}
Co. luteo-olivacea	62.38^{bcd}	59.04^{b-h}	49.86^{i-m}	41.67^{n}
I. liriodendri	64.43^{bc}	60.41^{b-f}	52.33^{f-k}	41.56^{n}
D. macrodidyma	62.53^{bcd}	59.74^{b-g}	50.53^{g-k}	51.47^{e-k}
N. australe	62.29^{bcd}	59.60^{b-g}	48.24^{j-m}	45.23^{klm}
N. parvum	61.15^{b-e}	58.61^{c-i}	53.27^{d-j}	43.12^{lmn}
P. chlamydospora	73.27^{a}	65.03^{b}	72.87^{a}	69.82^{a}
Pm. minimum	64.42^{bc}	59.95^{b-g}	48.45^{i-m}	48.28^{klm}
Pm. parasiticum	62.58^{bcd}	57.37^{d-j}	51.99^{i-l}	44.21^{mn}
Pl. richardsiae	61.97^{b-e}	59.82^{b-g}	50.18^{g-k}	47.62^{klm}

[x] Values followed by the same letter are not significantly different from each other ($P < 0.05$; LSD 6.36).
[y] The percentage disease severity of each plant was determined after the rootstocks were uprooted in June/July 2015 and 2016.

Table 9. Mean percentages of corrected percentage disease severity of eight rootstock cultivars inoculated with fungal trunk pathogens prior to planting in a nursery field and grown for a period of eight months.

Rootstock[z]	**Corrected disease severity (%)[xy]**			
	2015		2016	
	Site 1	Site 2	Site 1	Site 2
101-14 Mgt	58.68^{b-e}	58.06^{b-e}	59.84^{bcd}	58.62^{b-e}
Paulsen 1103	46.40^{ijk}	55.91^{c-f}	46.07^{ijk}	38.19^{l}
Ramsey	-	-	35.62^{l}	29.23^{m}
Richter 110	62.44^{ab}	51.30^{fgh}	48.32^{h-k}	44.22^{k}
Richter 99	60.53^{bc}	50.46^{ghi}	49.29^{hij}	44.57^{jk}
Ruggeri 140	55.24^{d-g}	52.81^{fgh}	45.22^{jk}	38.58^{l}
SO_4	65.91^{a}	62.15^{ab}	55.76^{c-f}	55.00^{e-g}
US 8-7	-	-	46.11^{ijk}	45.31^{jk}

[x] Values followed by the same letter are not significantly different from each other ($P < 0.05$; LSD 4.79).

[y] The percentage disease severity of each plant was determined after the rootstocks were uprooted in June/July 2015 and 2016.

[z] Dormant un-grafted rootstock cuttings were inoculated in October 2014 and 2015 by sucking up a spore solution of each pathogen through the plants vascular system.

Table 10. Mean vessel diameters (µm) of eight grapevine rootstock cultivars.

Rootstock cultivar	Mean vessel diameter (µm)[x]
Ramsey	51.92[g]
US 8-7	58.14[f]
Paulsen 1103	65.14[e]
Ruggeri 140	69.01[de]
Richter 99	73.57[cd]
Richter 110	75.54[c]
SO_4	85.53[b]
101-14 Mgt	99.23[a]
LSD (p = 0.05)	5.44

[x] Values within the respective columns followed by the same letter do not differ significantly (P = 0.05).

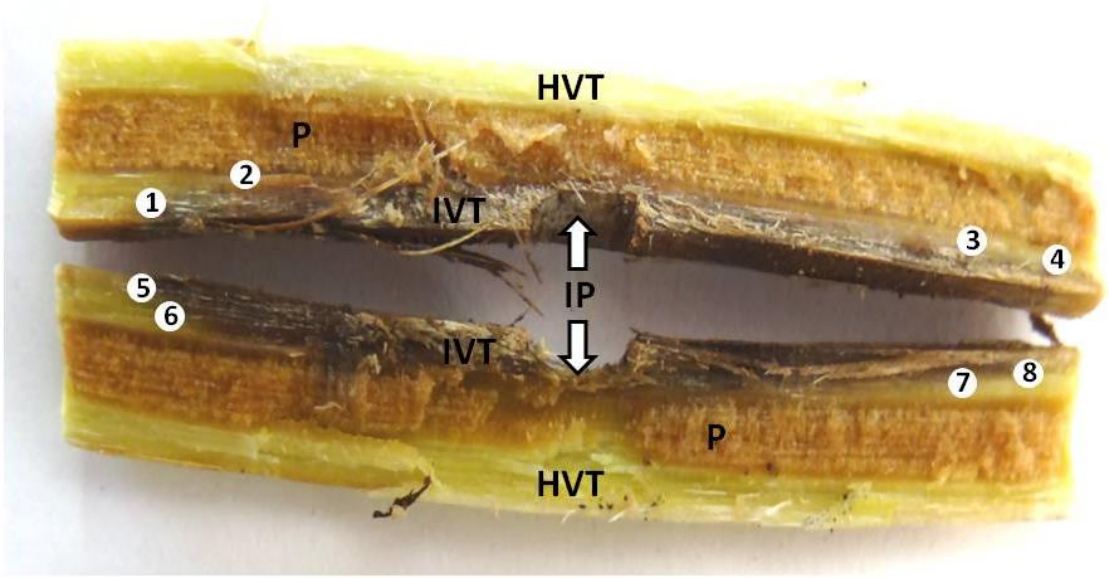

Figure 1. Example of vascular discolouration caused by *Neofusicoccum parvum* inoculated on grapevine rootstock cultivar, Paulsen 1103, six weeks after inoculation. ○ - Numbered white circles indicate points of isolation; pith (**P**); Infected and discoloured vascular tissue (**IVT**); Healthy vascular tissue (**HVT**); Infection point (**IP**).

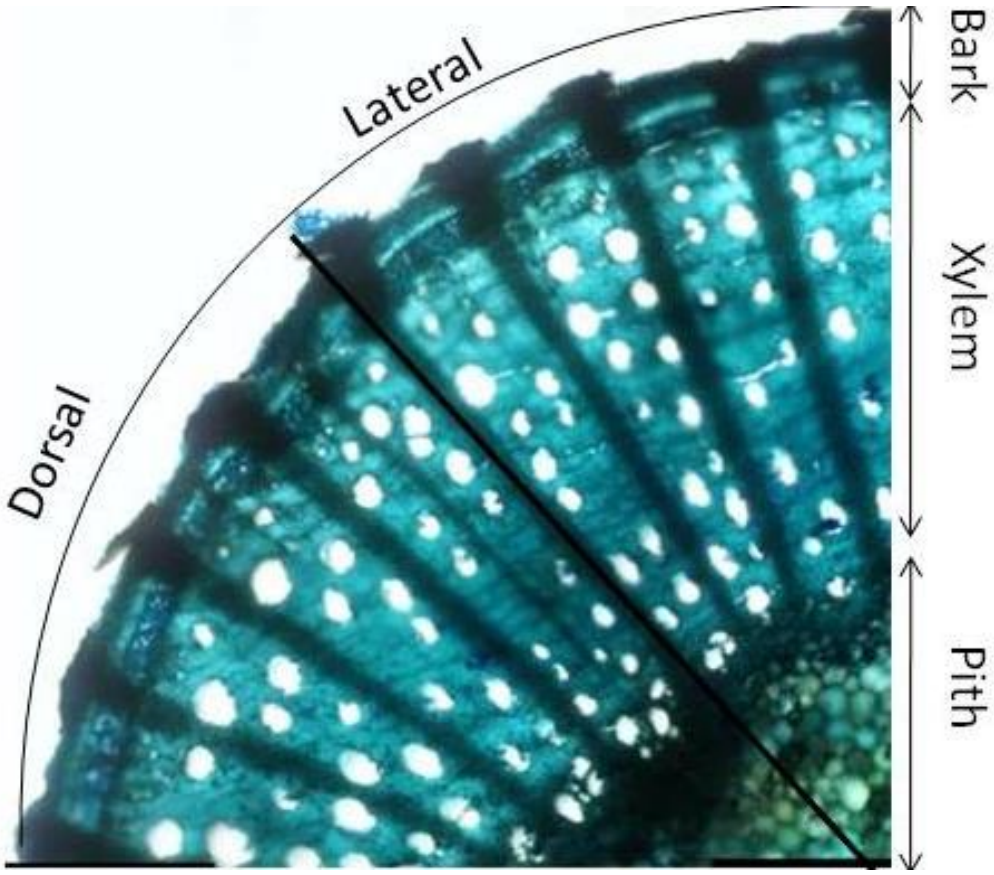

Figure 2. Micrograph of the cultivar Ramsey showing the stem tissue in cross-section (stained with toluidine O). Notice the change seen in vessel diameter between the dorsal and lateral side of the shoot.

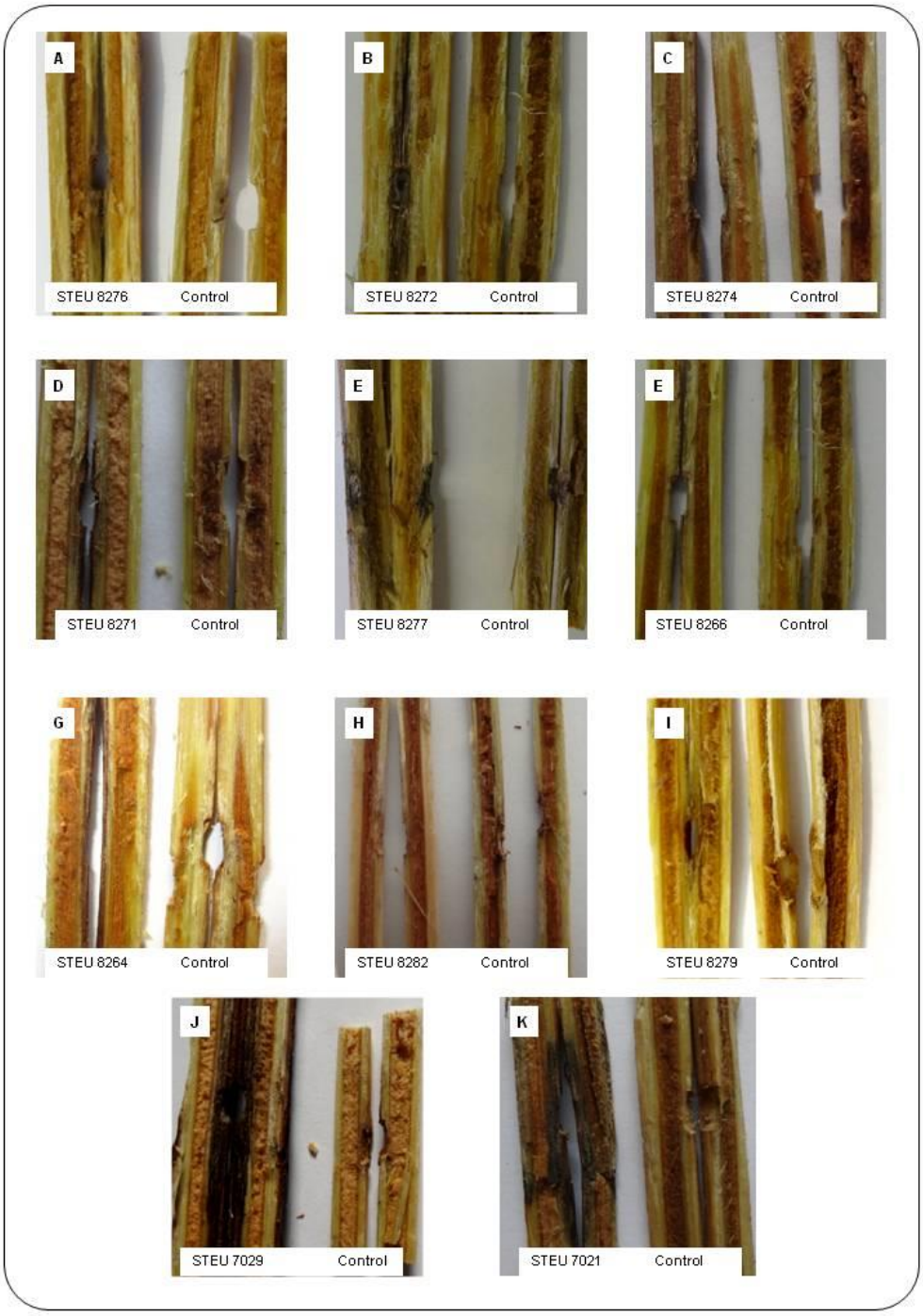

Figure 3. Lesion development and streaking caused by the various pathogens six weeks after inoculation of Paulsen 1103 rootstock cuttings. A variation in the severity and colour of lesions were observed between the different pathogens. *P. chlamydospora* (STEU 8276) (A); *Pm. minimum* (STEU 8272) (B); *Pm. parasiticum* (STEU 8274) (C); *Pl. richardsiae* (STEU 8271) (D); *Ca. luteo-olivacea* (STEU 8277) (E); *I. liriodendri* (STEU 8266) (F); *D. macrodidyma* (STEU 8264) (G); *C. fasciculare* (STEU 8282) (H); *C. pseudofasciculare* (STEU 8279) (I); *N. australe* (STEU 7029) (J); *N. parvum* (STEU 7021) (K).

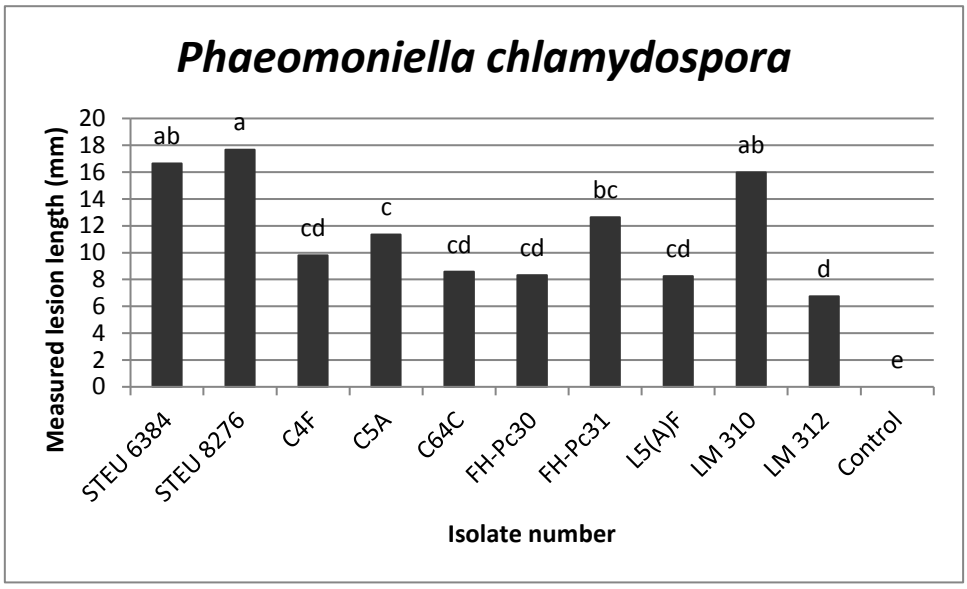

Figure 4. Mean lesion lengths of ten *P. chlamydospora* isolates six weeks after inoculation of Paulsen 1103 rootstock cuttings. Each bar shows the mean of 20 independent biological replicates. Bars with the same letter on top indicate means that are not significantly different. LSD = 4.54 ($P < 0.05$)

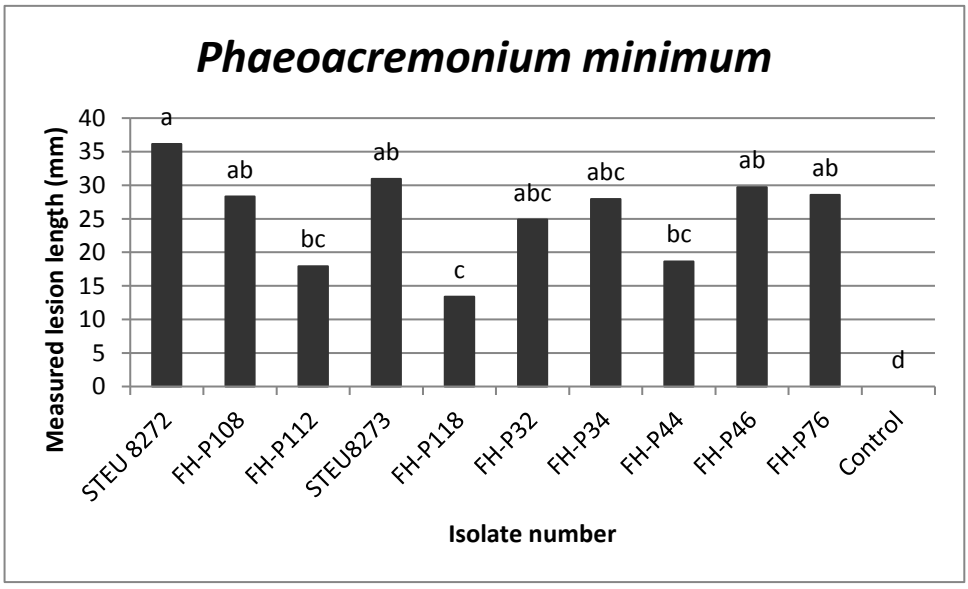

Figure 5. Mean lesion lengths of ten *Pm. minimum* isolates six weeks after inoculation of Paulsen 1103 rootstock cuttings. Each bar shows the mean of 20 independent biological replicates. Bars with the same letter on top indicate means that are not significantly different. LSD = 14.61 ($P < 0.05$)

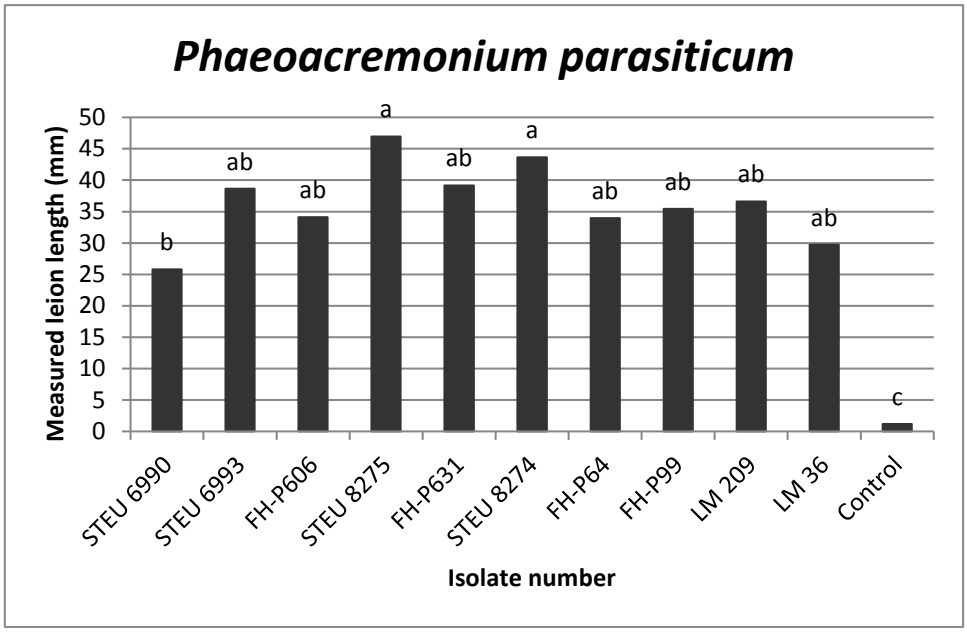

Figure 6. Mean lesion lengths of ten *Pm. parasiticum* isolates six weeks after inoculation of Paulsen 1103 rootstock cuttings. Each bar shows the mean of 20 independent biological replicates. Bars with the same letter on top indicate means that are not significantly different. LSD = 17.27 ($P < 0.05$)

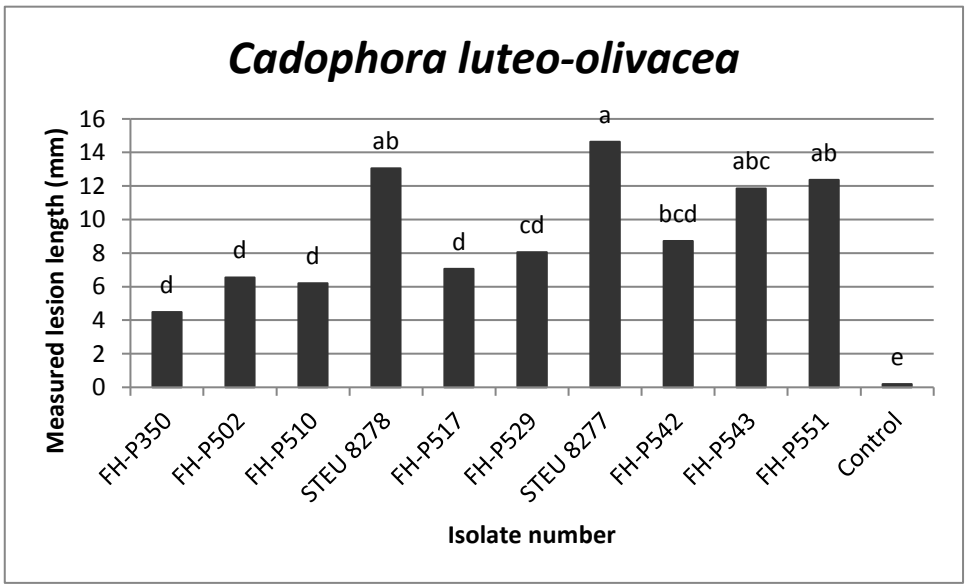

Figure 7. Mean lesion lengths of ten *Ca. luteo-olivacea* isolates six weeks after inoculation of Paulsen 1103 rootstock cuttings. Each bar shows the mean of 20 independent biological replicates. Bars with the same letter on top indicate means that are not significantly different. LSD = 4.57. ($P < 0.05$)

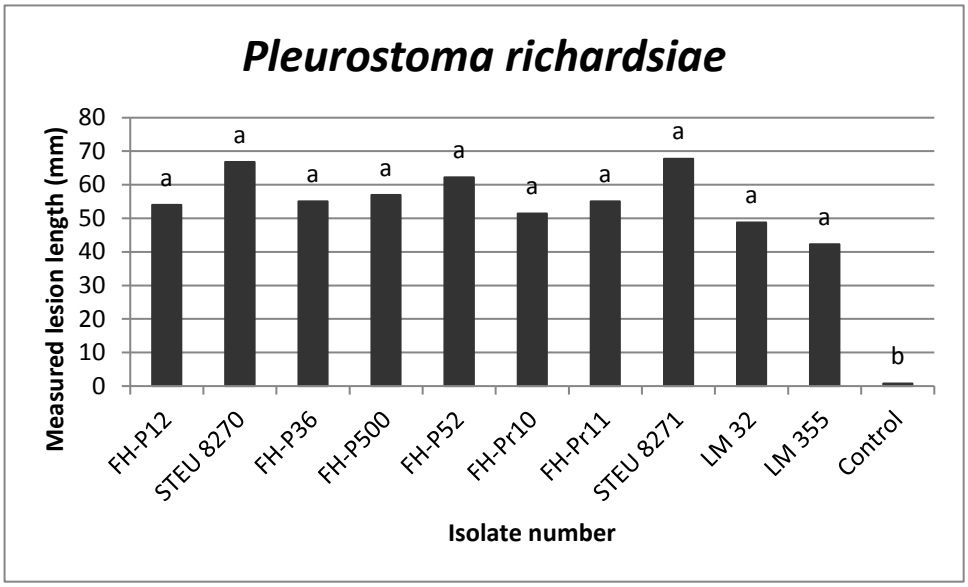

Figure 8. Mean lesion lengths of ten *Pl. richardsiae* isolates six weeks after inoculation of Paulsen 1103 rootstock cuttings. Each bar shows the mean of 20 independent biological replicates. Bars with the same letter on top indicate means that are not significantly different. LSD = 27.50 ($P < 0.05$)

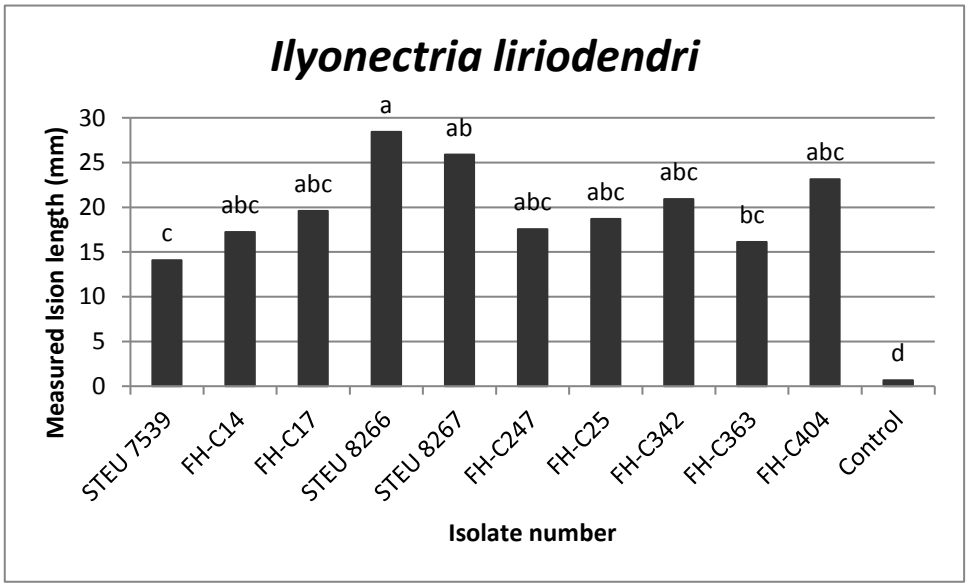

Figure 9. Mean lesion lengths of ten *I. liriodendri* isolates six weeks after inoculation of Paulsen 1103 rootstock cuttings. Each bar shows the mean of 20 independent biological replicates. Bars with the same letter on top indicate means that are not significantly different. LSD = 11.44 ($P < 0.05$)

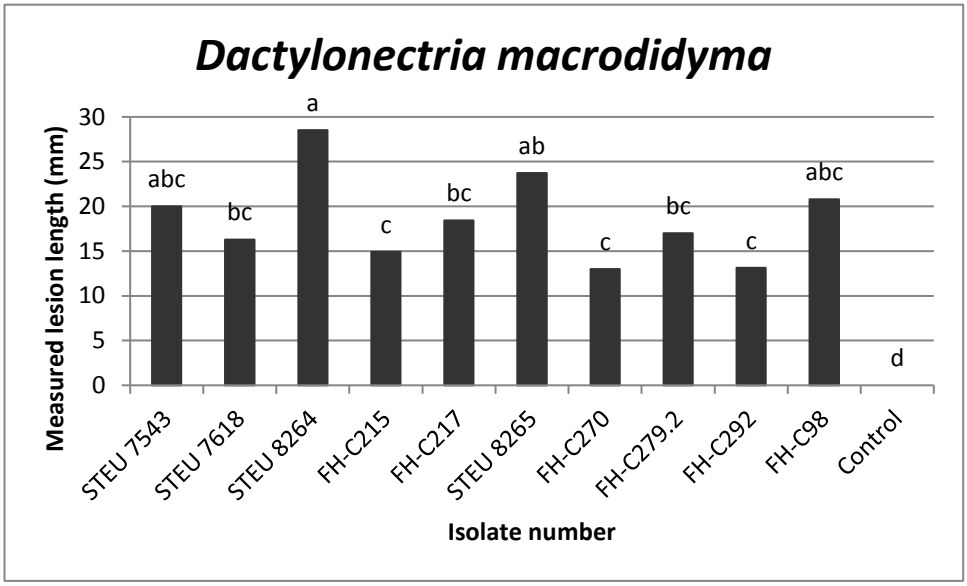

Figure 10. Mean lesion lengths of ten *D. macrodidyma* isolates six weeks after inoculation of Paulsen 1103 rootstock cuttings. Each bar shows the mean of 20 independent biological replicates. Bars with the same letter on top indicate means that are not significantly different. LSD = 8.81 ($P < 0.05$)

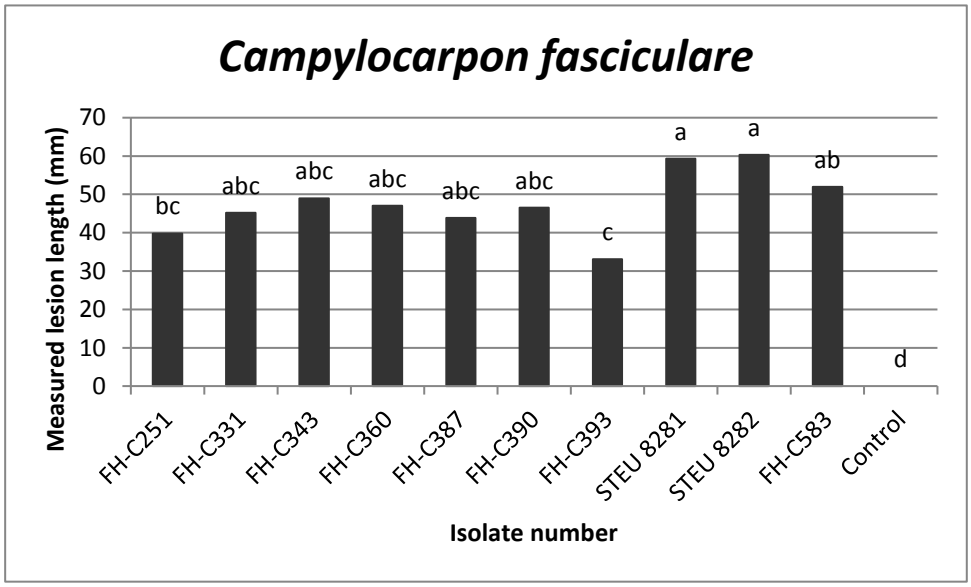

Figure 11. Mean lesion lengths of ten *C. fasciculare* isolates six weeks after inoculation of Paulsen 1103 rootstock cuttings. Each bar shows the mean of 20 independent biological replicates. Bars with the same letter on top indicate means that are not significantly different. LSD = 18.55 ($P < 0.05$)

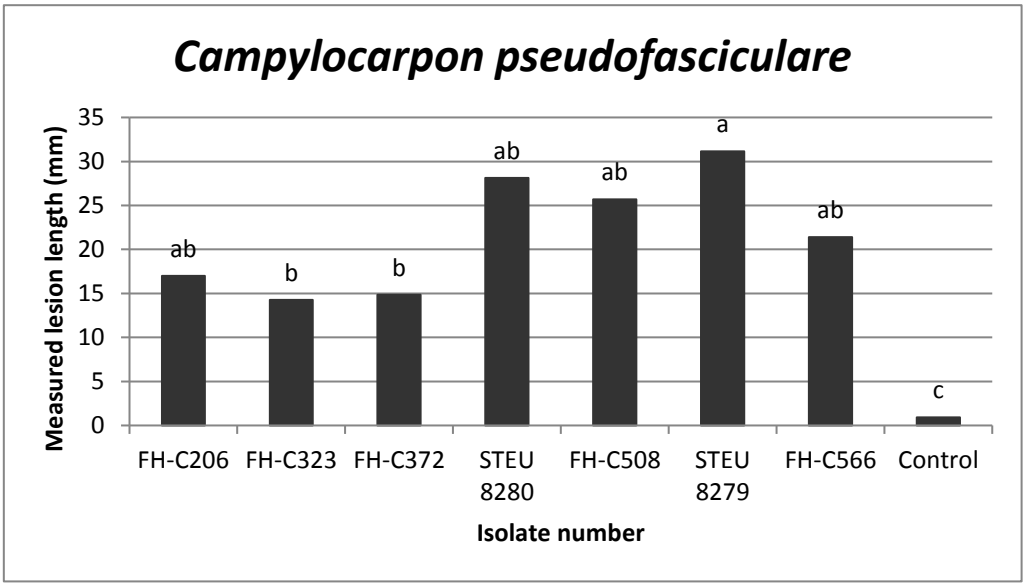

Figure 12. Mean lesion lengths of seven *C. pseudofasciculare* isolates six weeks after inoculation of Paulsen 1103 rootstock cuttings. Each bar shows the mean of 20 independent biological replicates. Bars with the same letter on top indicate means that are not significantly different. LSD = 15.17 ($P < 0.05$)

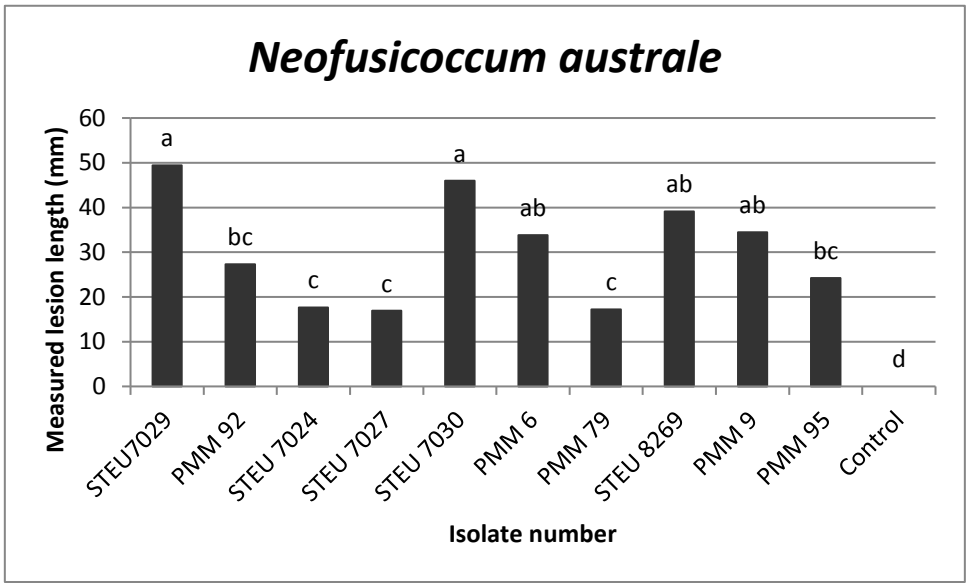

Figure 13. Mean lesion lengths of ten *N. australe* isolates six weeks after inoculation of Paulsen 1103 rootstock cuttings. Each bar shows the mean of 20 independent biological replicates. Bars with the same letter on top indicate means that are not significantly different. LSD = 15.96 ($P < 0.05$)

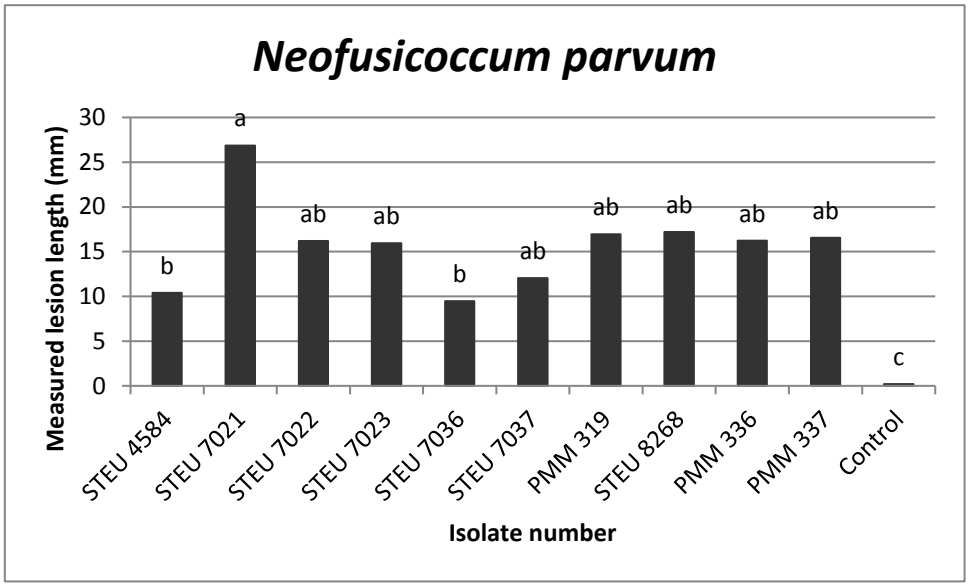

Figure 14. Mean lesion lengths of ten *N. parvum* isolates six weeks after inoculation of Paulsen 1103 rootstock cuttings. Each bar shows the mean of 20 independent biological replicates. Bars with the same letter on top indicate means that are not significantly different. LSD = 16.21 ($P < 0.05$)

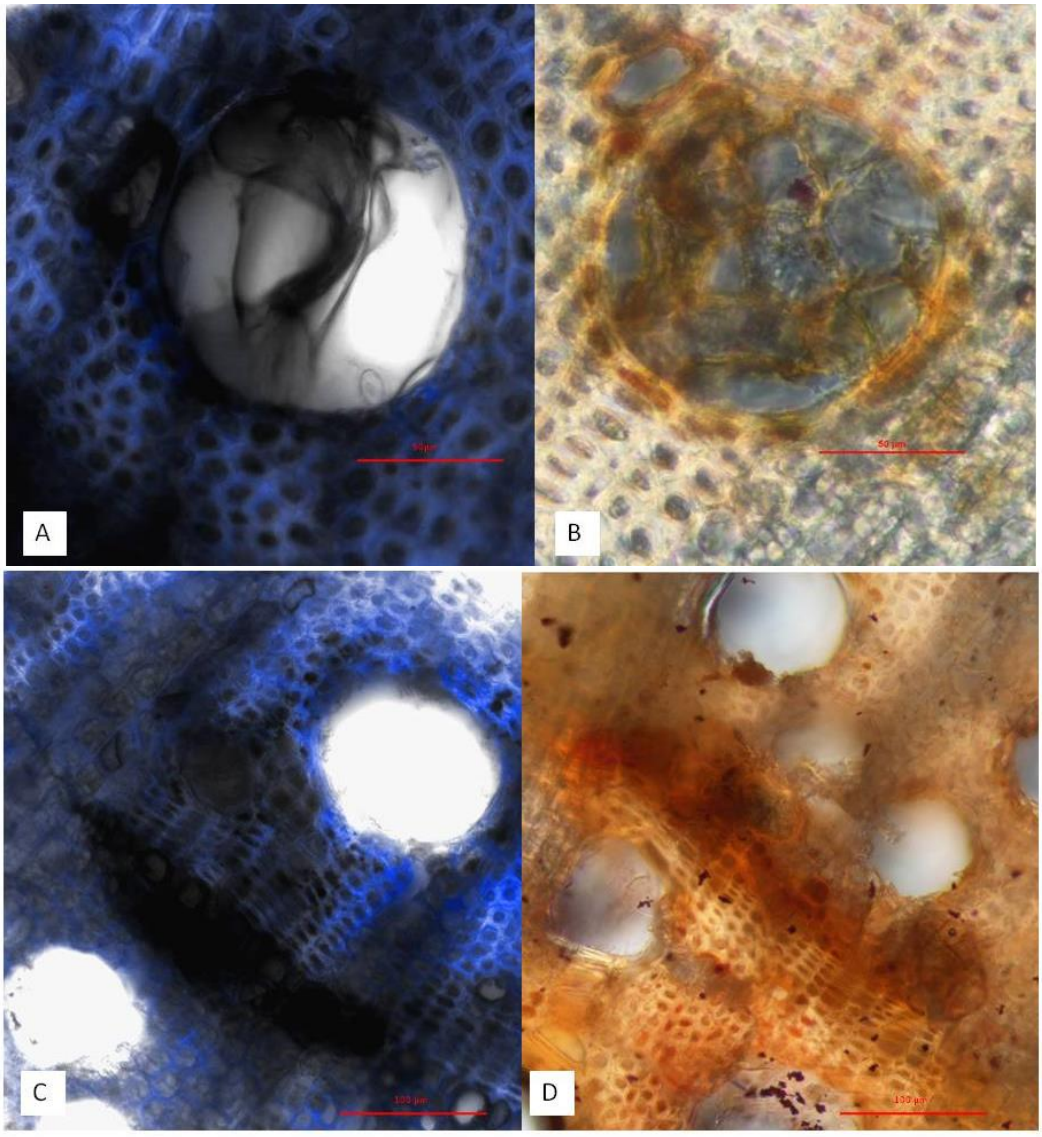

Figure 15. Transversal sections of Ramsey inoculated with *P. chlamydospora.* Sections have been treated with Sudan black B to quench suberin autofluorescence and photographed under ultraviolet excitation. The dark black areas indicate the presence of suberin (A and C). Sections have been stained with Sudan IV to stain suberin orange brown (B and D). In A and B suberin is located in parenchyma cells around vessel filled with tyloses. In C and D suberin is present in cells located on the growth ring boundary between the initial inoculated vascular tissue and new growth.

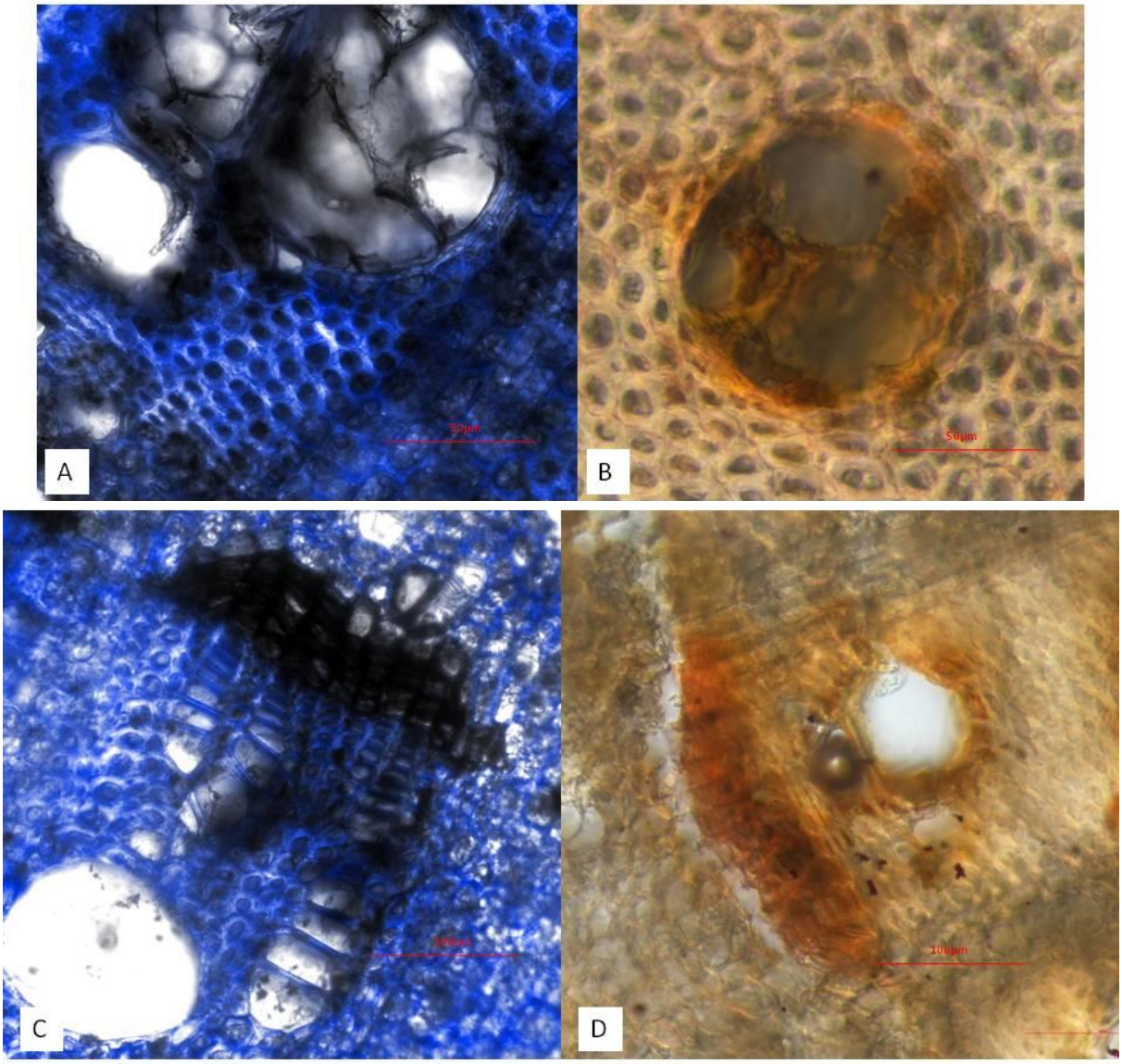

Figure 16. Transversal sections of 101-14 Mgt inoculated with *P. chlamydospora.* Sections have been treated with Sudan black B to quench suberin autofluorescence and photographed under ultraviolet excitation. The dark black areas indicate the presence of suberin (A and C). Sections have been stained with Sudan IV to stain suberin orange brown (B and D). In A and B suberin is located in parenchyma cells around vessel filled with tyloses. In C and D suberin is present in cells located on the growth ring boundary between the initial inoculated vascular tissue and new growth.

APPENDIX A

Table 1. Analysis of variance for mean lesion lengths produced by the different fungal isolates during preliminary detached shoot virulence screenings.

Factor	Degrees of Freedom	*F*-value	*P*-value
P. chlamydospora	9	6.19	<0.01
Pm. minimum	9	1.85	0.089
Pm. parasiticum	9	1.05	0.420
Ca. luteo-olivacea	9	4.60	<0.01
Pl. richardsiae	9	0.68	0.725
I. liriodendri	9	1.26	0.289
D. macrodidyma	9	2.50	0.023
C. fasciculare	9	1.54	0.168
C. pseudofasciculare	7	1.64	0.173
N. australe	9	4.52	<0.01
N. parvum	9	0.74	0.668

Table 2. Analysis of variance for the reduction in rootstock cultivar root mass caused by fungal trunk pathogen infection during nursery field trials.

Factor	Degrees of Freedom	*F*-value	*P*-value
YearxSite	3	14.49	<0.0001
YearxSite(Row)	6	14.12	<0.0001
Pathogen[x]	11	16.65	<0.0001
YearxSitexPathogen[x]	33	1.62	0.0482
Cult	5	5.36	<0.0001
Pathogen[x] xCult	55	0.60	0.9893
YearxSitexCult	15	7.79	<0.0001
YearxSitexPathogen[x] xC	165	0.57	1.0000

[x] The factor Isolate is nested in Pathogen (two isolates for each pathogen)

Table 3. Analysis of variance for the reduction in rootstock cultivar shoot mass caused by fungal trunk pathogen infection during nursery field trials.

Factor	Degrees of Freedom	*F*-value	*P*-value
YearxSite	3	19.28	<0.0001
YearxSite(Row)	6	15.28	<0.0001
Pathogen[x]	11	25.64	<0.0001
YearxSitexPathogen[x]	33	1.71	0.0317
Cult	5	8.59	<0.0001
Pathogen[x] xCult	55	0.49	0.9991
YearxSitexCult	15	5.95	<0.0001
YearxSitexPathogen[x] xC	165	0.49	1.0000

[x] The factor Isolate is nested in Pathogen (two isolates for each pathogen)

Table 4. Analysis of variance of the corrected percentage disease severity in rootstock cultivars shoot caused by fungal trunk pathogen infection during nursery field trials.

Factor	Degrees of Freedom	*F*-value	*P*-value
YearxSite	3	56.88	<0.0001
YearxSite(Row)	6	6.48	<0.0001
Pathogen[x]	11	300.90	<0.0001
YearxSitexPathogen[x]	33	3.09	<0.0001
Cult	5	40.32	<0.0001
Pathogen[x] xCult	55	1.04	0.4071
YearxSitexCult	15	7.42	<0.0001
YearxSitexPathogen[x] xC	165	0.85	0.8788

[x] The factor Isolate is nested in Pathogen (two isolates for each pathogen)

Table 5. Analysis of variance for differences in mean vessel diameter and mean vessel count for eight different grapevine rootstock cultivars

Factor	Degrees of Freedom	*F*-value	*P*-value
Vessel diameter	7	79.79	<0.05
Vessel count	7	41.04	<0.05