Botryosphaeria dieback in vineyards: conidial dispersal, infection, disease development and control

_____

A thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy (Plant Pathology)

at
Lincoln University

by
Amna Shafi

_____

Lincoln University

2016

Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy (Plant Pathology)

Botryosphaeria dieback in vineyards: conidial dispersal, infection, disease development and control

By

Amna Shafi

Botryosphaeria dieback is a major trunk disease of grapevines worldwide that has been recognised for its importance during the last 15-20 years. This disease causes direct losses by killing canes, arms and vines, which ultimately reduce yields in the vineyards. There are five Botryosphaeriaceae species that commonly infect grapevines in New Zealand vineyards, although many more species have been reported worldwide. Reports on pathogenicity and epidemiology overseas have shown large variation between the predominant species in different countries and so this research aimed to improve understanding of spore production, dispersal and infection dynamics for the most common species within a Marlborough vineyard setting.

Pathogenicity testing with multiple isolates of *Neofusicoccum luteum* and *N. parvum* showed that all isolates caused disease of different grapevine stem tissues, with great differences in symptoms and conidial release between isolates of each species. Overall trends showed that *N. luteum* caused longer lesions and produced more conidia than *N. parvum* and isolates of both species released conidia at all temperatures (10-25ºC) and relative humidities (80-100%), although maximum conidial release occurred at 25ºC and 100% RH. Cirrhi were also observed in vineyards in moist conditions.

Rainwater run-off and Burkard spore traps collected Botryosphaeriaceae conidia in three Marlborough vineyards in 2013 and 2015. The conidia were found only during rainfall and up to 2 h after rainfall ceased, release occurring after as little as 0.2 mm rain and conidial numbers were generally greater for greater rainfall. Identification of species trapped by single stranded conformational polymorphism (SSCP) indicated presence of *N. luteum, N. parvum/ribis, N. australe, D. mutila* and *D. seriata.* Within vineyards, conidia of a marker isolate of *N. parvum* were shown to disperse during 2 days rain, up to 10 m in the wind direction and up to 5 m in other directions. Further, these conidia caused many infections of newly trimmed shoots up to 2 cm from the conidial source.

Conidia of *N. luteum* and *N. parvum* were able to infect 100% of wounds on green shoots and trunks of potted vines for 7 days after wounding. Incidence decreased with increasing age of wounds, with hard shoots being susceptible until 56 days old and trunk wood until 28 days old. Incidences and colonisation distances were also affected by the seasons, being highest in autumn and spring, and lowest in winter and summer. Also different conidial numbers (2-100) affected incidences and colonisation distances on wounded stems of vines with as little as 2 conidia causing 100% infection incidence of wounded green shoots and 67% incidence of trunks. The pathogen infected the underlying tissues after spray application of conidia onto non-wounded cane and trunk bark, and progressed rapidly towards nearby wounds made later. Also infection through lenticels was observed by fluorescence microscopy. Wounded leaf buds, and berries on potted vines and in the vineyards were able to be infected at all stages of growth tested, but non-wounded buds and berries also become infected at late stages of development. Infection progressed from the buds into the developing green shoots and supporting canes, and from berries into the bunch stems and supporting canes.

Experiments using a range of treatments to prevent infection of wounds in stem and trunk tissues of vines grown in pots and a in a vineyard showed that Cheif®, Megastar® and Folicur® were most effective at reducing infection by *N. luteum* and *N. parvum,* when inoculation was carried out 2 h, 7 and 14 days after treatment and with 2-600 conidia. The biological control product, TRI D25, was as effective as Megastar® when inoculation occurred 7-14 days after treatment, but only on potted vines. These treatments also reduced colonisation distances greatly within inoculated tissues which indicated potential for control by pruning.

This research has provided new information on the disease cycle of Botryosphaeriaceae diseases in vineyards. It has elucidated effects of environmental conditions on release and dispersal of conidia. These studies also demonstrated the effects of some host, environmental and pathogen factors on infection of pruning and trimming wounds and disease development in the various types of stem tissues, and that infection may take place in non-wounded shoots of living vines. The potential infection of leaf buds and berries and sporulation from berries were also demonstrated. Some control products were shown to be effective in preventing infection but limitations on their use indicates the need for integrated control methods which also aim to reduce inoculum sources in vineyards.

Keywords: *Diplodia, Neofusicoccum*, spore traps, environmental factors, wounding, fungicides and biocontrol agents.

Acknowledgements

First and foremost, I would like to express my utmost gratitude and appreciation to my supervisors. I would like to thank Assoc. Prof. Marlene Jaspers, for her constant encouragement, support, guidance, practical help and thesis editing which she provided during my Ph.D study. I could not have imagined having a better supervisor. To Professor Eirian Jones, who has been always there to listen and advise me, I am deeply grateful for the discussions that helped me clarify the technical details of my work and for carefully reading and commenting on this manuscript. To Assoc. Professor Hayley Ridgway, I am grateful for assisting me in all the molecular work throughout this study and for reading my drafts and helping me to understand the technology which has enriched my ideas.

To my advisor, Andrew Naylor, whose expertise, understanding, and patience, have added considerably to my postgraduate experience. I truly appreciate his vast knowledge and skills in viticulture. A big thank you to Roland Jaspers, who made an irrigation system for my many grapevine plants, and to Seona Casonata who has provided me with statistical advice at times of critical need. Thank you to Candice Hume for all the technical support and her prompt actions in sending me the materials I needed in Blenheim.

During the course of my studies, I have been fortunate to have had help and care from a number of talented individuals at Plant & Food Research, Marlborough who also let me use their laboratory for my entire study. In particular, Rob Agnew, who has provided me with weather data; Marc Greven and Mike Trought who provided office spaces; Dion Mundy, Sharlene Haycock, Abby Albright, Claire Grose, Lily Staurt and Rafida Horner, have helped me in the laboratory and provided materials when needed; Cherryl Fitzgerald who has organised many things for me throughout my research at Marlborough.

I would also like to thank the Ministry of Science & Innovation for my scholarship, and Pernod-Ricard who have sponsored me to pursue my study in New Zealand. Also, thanks to ICCP for travel funds and New Zealand Plant Protection for their scholarship grant.

Many other people have also contributed in some way to my work, including the friends who have supported me and helped me in overcoming setbacks and staying focused on my study. I greatly value their friendship.

Last but not least, I would also like to thank my family for their support throughout my entire life and in particular, I must acknowledge my brother, Tehseen Khan who is also my best friend. Without his love and encouragement, I would not have finished this thesis. My father Shafi Khan and mother Majreha Khan to whom this dissertation is dedicated have been a constant source of love, support and strength throughout my life.

Table of Contents

List of Tables

List of Figures

Chapter 1

General introduction

1.1 Viticulture industry in New Zealand

Grapes for wine production are one of the most economically important crops in New Zealand, with about $1.42 billion worth of wine being exported in 2015 (New Zealand Winegrowers Annual Report, 2015). Further, the 2014 report indicated that the New Zealand wine industry had provided $700 million annual contribution to GDP and employment for more than 7,700 people on a full time basis (New Zealand Winegrowers Annual Report, 2014). In 2015 there were 35,859 hectares in vineyards which provided the grapes for 673 wineries (New Zealand Winegrowers Annual Report, 2015). The varieties grown in New Zealand are predominantly of white wine grapes, with Sauvignon blanc and Chardonnay covering 66% and 8.3%, of the total producing hectares, respectively, while Pinot noir is the dominant red wine variety (8%) followed by Merlot (2.7%) (Figure 1.1) (New Zealand Winegrowers Annual Report, 2015).

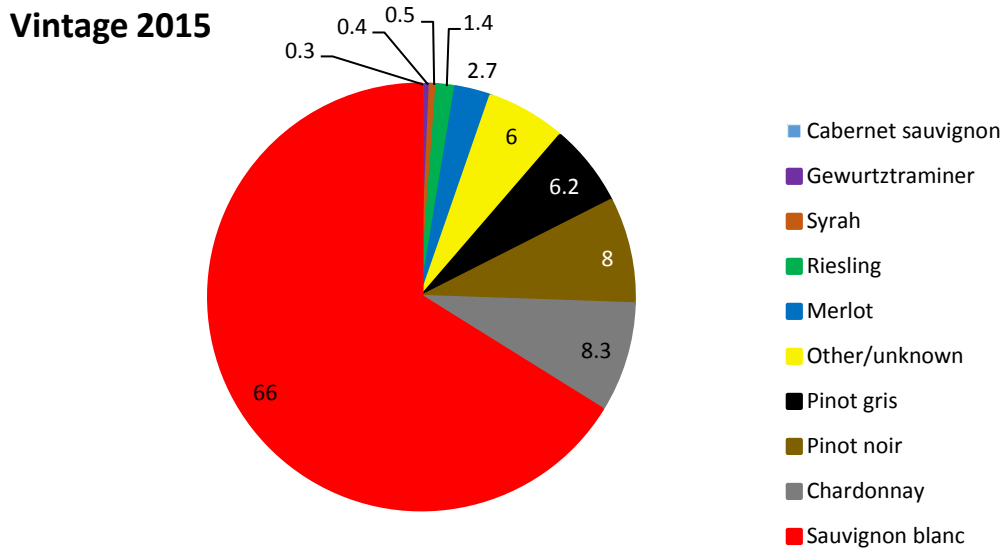


Figure 1.1 Percentage production of different grapevine variety during the 2015 vintage (New Zealand Winegrower Annual Report 2015).

The very quick expansion of about 60% in the overall industry during the last 15 years is reflected by a relatively young national vineyard (Figure 1.2); about 70% of New Zealand vines are now estimated to be aged 8–25 years (New Zealand Winegrowers Statistical Annual, 2015). Overseas experience has shown that as vines enter this age profile they

become more susceptible to woody trunk diseases, such as those caused by the Botryosphaeriaceae and *Eutypa* spp. Sosnowski *et al*., 2009; Úrbez-Torres and Gubler, 2011).

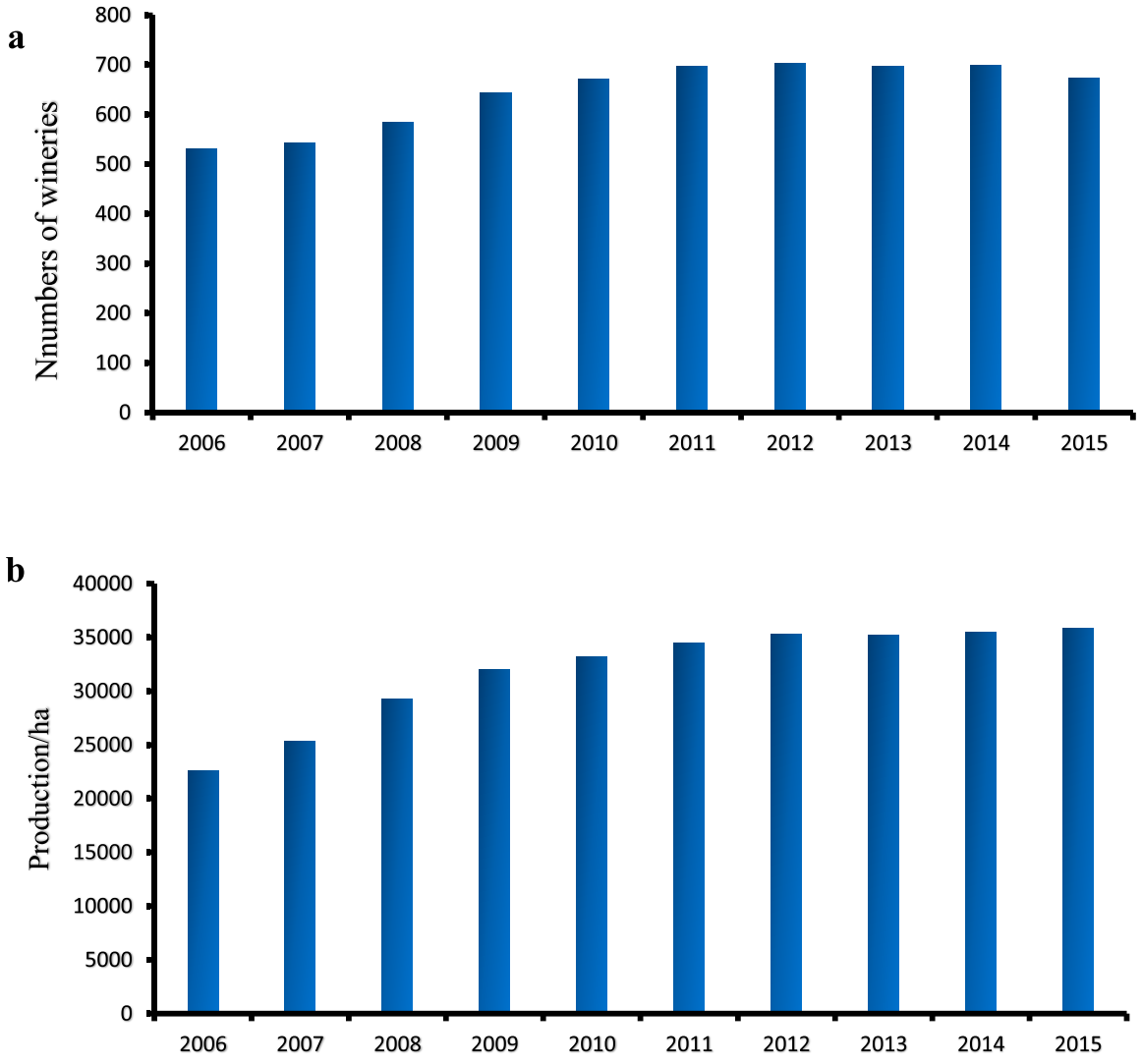


Figure 1.2 (a) Increase in the number of wineries and (b) producing vineyard area from 2006 to 2015 (New Zealand Winegrowers Statistical Annual, 2015).

In New Zealand's cool climate, it is necessary to maintain grapevines with narrow open canopies that provide adequate light penetration to the fruit and air flow to reduce humidity, thereby lowering incidence of diseases, especially Botrytis rot which is considered to be the most damaging disease of grapevines in New Zealand (Jackson and Schuster, 2001). The two training systems commonly used, vertical shoot positioning (VSP) and Scott Henry (Jackson and Schuster, 1987) provide for narrow canopies which are maintained by frequent summer trimming. Further, about 90% of the canopy growth is removed each winter by pruning which provides space among the shoots.

1.2 Grapevine trunk diseases

Worldwide, significant economic losses to the wine industry result from grapevine trunk diseases (Eutypa, Botryosphaeria dieback, Esca and Phomopsis blight), which are caused by different wood-infecting fungi. Symptoms of these diseases include dead spurs, arms and cordons and eventual vine death due to canker formation which occludes the vascular tissue. Until recently, *Eutypa lata* was thought to be responsible for most canker development in vineyards. However, recent findings showed that the Botryosphaeriaceae were also recovered from many cankers, and they were determined to be the main cause of canker diseases in vineyards overseas (Phillips, 2002; van Niekerk *et al*., 2004; Savocchia *et al*., 2007) and in New Zealand (Baskarathevan *et al*., 2012).

1.2.1 Eutypa dieback

Eutypa dieback of grapevines, formerly referred to as "dead arm", was for many years thought to be caused by *Phomopsis viticola*. However, Carter and Price (1974) reported that the pathogen which commonly caused cankers and dieback in Australian grapevines was *Eutypa armeniacae.* More recently other diatrypaceaous fungal species such as *Eutypa lata*, *Cryptosphaeria pullmanensis, Cryptovalsa ampelina* and *Diatrype oregonensis* have been shown to cause similar cankers and dieback symptoms (Trouillas *et al.,* 2010). The symptoms of this disease usually appear 4-10 years after infection (New Zealand Winegrowers Fact Sheet, 2013). The obvious symptoms on infected vines are the clusters of stunted shoots with small deformed chlorotic leaves which often develop as new growth in spring. The pathogen however is present in the trunks and arms where necrosis, often visible as cankers, often develops surrounding old pruning wounds. Cross-sections through the cankers on infected trunks reveal typical wedge shaped wood necrosis (Figure 1.3a) (Carter, 1988).

The pathogen produces perithecia on the surface of infected dead wood. When moistened, they release air-borne ascospores which spread the disease (Carter *et al.,* 1983). When these ascospores land on pruning wounds they germinate and grow into the xylem vessels and surrounding wood tissues, which results in the formation of cankers and death of vines (Figure 1.3b) (New Zealand Winegrowers Fact Sheet, 2013).

Figure 1.3 (a) A typical wedge shaped necrosis in wood that has extended annually and (b) death of the whole vine caused by *Eutypa lata* (Photographs: Marlene Japers).

1.2.2 Esca

Esca disease of grapevine is believed to be caused initially by *Phaeomoniella chlamydospora* and *Phaeoacremonium* species, leading to Petri disease which is associated with declining symptoms in young vines. The characteristic symptom of this disease is seen in cross-sections of infected wood as black spots which ooze a tar like substance soon after cutting. Esca develops when secondary infections occur by basidiomycete fungi, such as *Fomitiporia mediterranea* (Mugnai *et al.,* 1999; Tabacchi *et al*., 2000).

Esca is a complex disease whose leaf symptoms are associated with structural and biochemical changes, leading to the leaves becoming yellow brown or red brown in colour, leaving only a narrow strip of green tissue along the main veins. Commonly referred to as 'tiger-striped' leaf symptomsFigure 1.4a). The most common symptoms inside the trunks are a spongy white rot. In spring, the shoots and branches of infected vines show delayed or weak growth. Tiny dark brown or purple coloured spots which develop on the berries, referred to as "black measles", have been reported in California (Mugnai *et al.,* 1999). During the summer, rapid wilting of the entire vine may be seen, a condition called "vine apoplexy", which is often followed by the death of the entire vine (Figure 1.4b) (Mugnai *et al.,* 1999).

In infected vineyards the conidia of the primary fungi (*P. chlamydospora* and *Phaeoacremonium* spp.) have been trapped using spore trapping methods, with large numbers of spores trapped during rainfall, while spores of *F. mediterranea* are wind-borne. However, factors influencing disease spread are not fully understood (Eskalen *et al.,* 2007). Esca disease has not been reported in New Zealand.

Figure 1.4 (a) Esca symptoms on leaves and (b) esca "vine apoplexy" resulting in death of the whole vine (Photographs: Marlene Jaspers).

1.2.3 Phomopsis

Phomopsis cane and leaf spot disease is widely distributed in grape growing areas throughout the world (Phillips, 1998). This disease has been recently proposed to be referred as Phomopsis dieback due to its association with die back of grapvines (Urbez-Torres *et al.,* 2013). The disease symptoms include small yellow or light green spots with dark centres usually more common on the lower leaves of shoots (Figure 1.5a). Severely infected leaves are smaller in size than the normal leaves and may drop early in the season. This pathogen also attacks shoots, pedicles and rachides, on which symptoms appear initially as small brownish black spots which develop into dark oval shaped lesions. When numerous lesions occur at the base of the shoots, they are subject to wind breakage and stunted growth (Phillips, 2000). Systemically infected mature shoots which appear bleached in winter, with black raised spots which are the pycnidia. When berries become infected they develop a light brown rot, on which black pycnidia are later produced. The berries eventually shrivel and develop into mummies (Figure 1.5b) (Erincik and Madden, 2001).

The pathogen produces pycnidia and perithecia on infected canes, berries and rachides in which it may overwinter. Spores oozed during wet weather are spread to healthy tissues by rain splash (Emmett *et al.,* 1998).

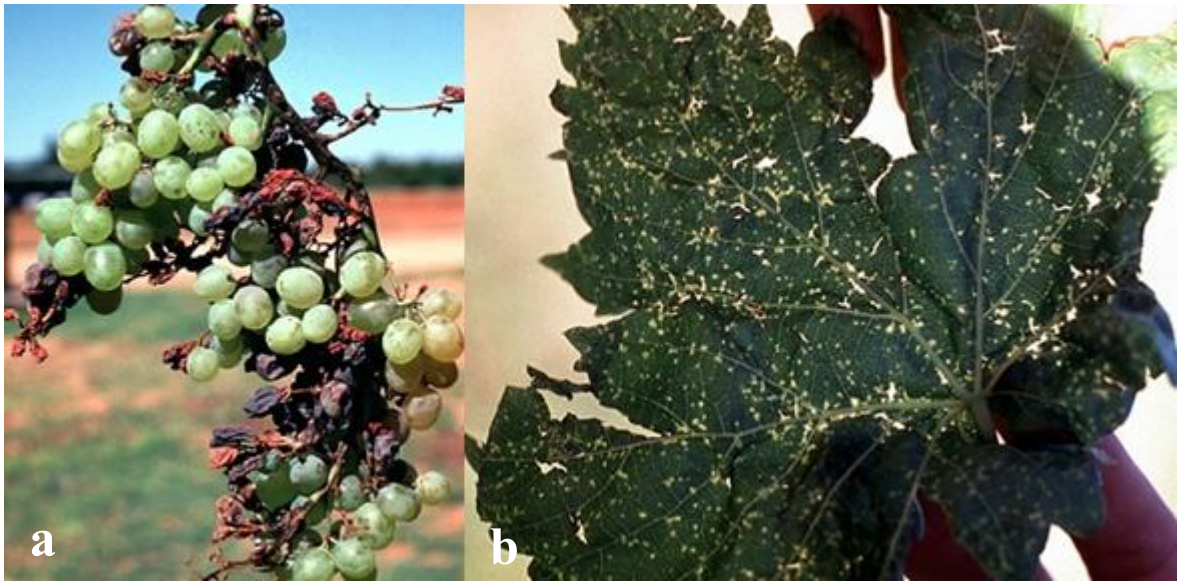


Figure 1.5 (a) Fruit rot and (b) leaf spots caused by *Phomopsis viticola* [Photographs from the Crop Protection Compendium reproduced with kind permission of: Bob Emmett (a) Trevor Wicks (b)].

1.3 Botryosphaeriaceae species as trunk pathogens

Species of the Botryosphaeriaceae are important pathogens on a wide range of agricultural, horticultural and forestry plant hosts worldwide. They cause a range of symptoms, including leaf spots, fruit rots, dieback, perennial cankers, and eventual death in economically important woody perennial crops and ornamental plants (Farr and Rossman, 2011), although symptoms vary according to the host species, cultivar and pathogen species. However, in grapevines, Botryosphaeriaceae species were originally considered to be saprophytes, secondary or weak pathogens in the wood. Despite this, evidence has slowly been gathered worldwide which indicated that they might be serious pathogens (Phillips, 2002).

The names of the pathogens have been revised during the last 10-20 years and so the names of the Botryosphaeriaceae species reported in published literature as able to infect grapevines are shown in Table 1.1 to provide some clarity.

Table 1.1 Species of Botryosphaeriaceae known to infect grapevines [adapted from Urbez-Torres *et al.,* 2011].

Botryosphaeriaceae species	Anamorph - Teleomorph connection	Reference
Botryosphaeria dothidea (Moug. ex Fr.) Ces. & De Not.	*Fusicoccum aesculi*	Cesati and De Notaris, 1863; Slippers *et al*., 2004b;
Diplodia corticola A.J.L. Phillips, A. Alves & J. Luque	*Botryosphaeria corticola*	Alves *et al*., 2004
Diplodia mutila (Fr.) Mont.	*Botryosphaeria stevensii*	Montagne, 1834; Shoemaker, 1964;Alves *et al*., 2004
Diplodia seriata De Not.	*Botryosphaeria obtusa*	De Notaris, 1845; Phillips *et al*., 2007
Dothiorella iberica A.J.L. Phillips, J. Luque & A. Alves	*Botryosphaeria iberica*	Phillips *et al*., 2005
Dothiorella americana J.R. Úrbez-Torres, F. Peduto & W.D. Gubler	Unknown	Úrbez-Torres *et al*., 2012
Guignardia bidwellii (Ellis) Viala & Ravaz	Unknown	Viala and Ravaz, 1892
Lasiodiplodia crassispora T.I. Burgess & Barber	Unknown	Burguess *et al*., 2006
Lasiodiplodia missouriana J.R. Úrbez-Torres, F. Peduto & W.D. Gubler	Unknown	Úrbez-Torres *et al*., 2011
Lasiodiplodia theobromae (Pat.) Griff. & Maubl.	*Botryosphaeria rhodina*	Griffon and Maublanc, 1909; Punithalingam, 1976
Lasiodiplodia viticola J.R. Úrbez-Torres, F. Peduto & W.D. Gubler	Unknown	Úrbez-Torres *et al*., 2011
Neofusicoccum australe (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips	*Botryosphaeria australis*	Slippers *et al*., 2004b; Crous *et al*., 2006
Neofusicoccum luteum (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips	*Botryosphaeria lutea*	Pennycook and Samuels, 1985; Phillips *et al*., 2002; Crous *et al*., 2006;
Neofusicoccum macroclavatum (T.I. Burgess, Barber & Hardy) T.I. Burgess, Barber & Hardy	Unknown	Billones *et al*., 2010 Burgess *et al*., 2005; Crous *et al*., 2006
Neofusicoccum mediterraneum Crous, M.J. Wingf. & A.J.L. Phillips	Unknown	Crous *et al*., 2007
Neofusicoccum parvum (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips	*Botryosphaeria parva*	Pennycook and Samuels, 1985;Crous *et al*., 2006
Neofusicoccum ribis (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips	Unknown	Slippers *et al*., 2004; Crous *et al*., 2006
Neofusicoccum viticlavatum (van Niekerk & Crous) Crous, Sl ippers & A.J.L. Phillips	Unknown	van Niekerk *et al*., 2004; Crous *et al*., 2006
Neofusicoccum vitifusiforme (van Niekerk & Crous) Crous, Slippers & A.J.L. Phillips	Unknown	van Niekerk *et al*., 2004; Crous *et al*., 2006
Phaeobotryosphaeria porosa (van Niekerk & Crous) Crous & A.J.L. Phillips	Unknown	van Niekerk *et al*., 2004; Phillips *et al*., 2008
Spencermartinsia viticola (A.J.L. Phillips & J. Luque) A.J.L. Phillips, A. Alves & Crous	*Dothiorella viticola*	Luque *et al*., 2005;Phillips *et al*., 2008

In 1964 Chamberlain *et al.* (1964) isolated *Diplodia mutila* from lesions on the trunks and pruning stubs of grapevines. In 1970, El-Goorani and El-Meleigi (1972) isolated *Botryodiplodia theobromae* (renamed *Lasiodiplodia theobromae*) from failing grapevines, and a few years later Lehockzy (1974) reported that *D. mutila* was pathogenic in mature canes on field vines and those used for nursery grafts. Then in Italy Cristinzio (1978) showed that *Diplodia seriata* was associated with grapevine dieback. In Chile, Latorre *et al.* (1986) reported that *Botryosphaeria dothidea, D. mutila* and *L. theobromae* caused grapevine canker and dieback and in Brazil Paradela Filho *et al.* (1995) isolated *B. dothidea* from trunk cankers of grapevines. In Portugal, Phillips (1998) reported that *B. dothidea, D. mutila* and *D. seriata* were associated with symptoms of excoriose and dieback in grapevines. During the same year Pascoe (1998) isolated *L. theobromae, D. seriata* and *D. mutila* from wedge shaped trunk symptoms in Australia. Since then the Botryosphaeriaceae have gained importance as grapevine trunk disease pathogens (Larignon and Dubos, 2001; Phillips, 2002; van Niekerk *et al*., 2004; Taylor *et al*., 2005; van Niekerk *et al*., 2006; Urbez-Torres *et al.,* 2008; Urbez-Torres and Gubler, 2009a; Pitt *et al.,* 2010; Urbez-Torres and Gubler, 2011; Morales *et al.,* 2012; Yan *et al.,* 2013). On grapevines, 21 Botryosphaeriaceae species have so far been reported to cause disease symptoms in grapevines across the different production regions of the world (Urbez-Torres *et al.,* 2012). Losses due to grapevine disease caused by the Botryosphaeriaceae were estimated to be 4-20% in Bordeaux vineyards in France (Larignon *et al.,* 2001) and 25-30% in South Africa (Pearson & Goheen, 1998). Together with Eutypa dieback, the annual losses they caused were reported to be in excess of $USA 260 million in California, USA (Siebert, 2001).

1.3.1 Symptoms of Botryosphaeria diseases

The dieback symptoms that cause most damage on grapevines develop slowly and lead to a gradual decline in vigour and yield (Phillips, 1998), which is probably why the most severe losses occur in grapevines that are eight years and older (Larignon and Dubos, 2001). The most characteristic symptoms are arm and trunk dieback, in which a dark brown wood necrosis frequently starts at the pruning wounds and spreads down the trunks or arms of the vines. The cankers which develop on trunks where limbs were pruned off characteristically appear as flattened areas with loosening bark. Removal of the bark around a canker reveals the wood beneath to be discoloured brown to reddish-brown instead of white (Figure 1.6) (Phillips, 2002). When the canker is cut across, the necrotic wood shows as a wedge shaped lesion (Figure 1.7g) which is also characteristic of Eutypa dieback. Other shapes of wood

necrosis have also been reported which reflect the shape of the wound site and wood architecture (van Neikerk *et al.,* 2004; Amponsah *et al.,* 2011). Cankers can encircle the vine arms or trunks, resulting in the death of the arms or whole vines. Some losses attributed to Eutypa dieback in vineyards in the past are now believed to have been caused by Botryosphaeriaceae species (Phillips, 2002; van Niekerk *et al.,* 2004; Savocchia *et al*., 2007).

Infection of buds causes their mortality and leads directly to a reduction in yield but can also lead to shoot dieback (Amponsah *et al.,* 2012a). When mature, systemically infected shoots may appear bleached with black fruiting structures immersed in the host tissue (Phillips, 1998), which are similar to the symptoms of Phomopsis cane blight (Phillips, 2002). Vines infected by Botryosphaeriaceae species may also show mild chlorosis of the leaves, depending on the extent of wood colonisation (van Niekerk *et al.,* 2004). In young grapevines, symptoms of Botryosphaeriaceae spp. infection may also be seen as stunted and chlorotic growth, necrosis of leaf and flower buds (Figure 1.7a and b) and delayed bud burst (1.7c), a condition often called "young vine decline" that has also been associated with other diseases (Aroca *et al*., 2006).

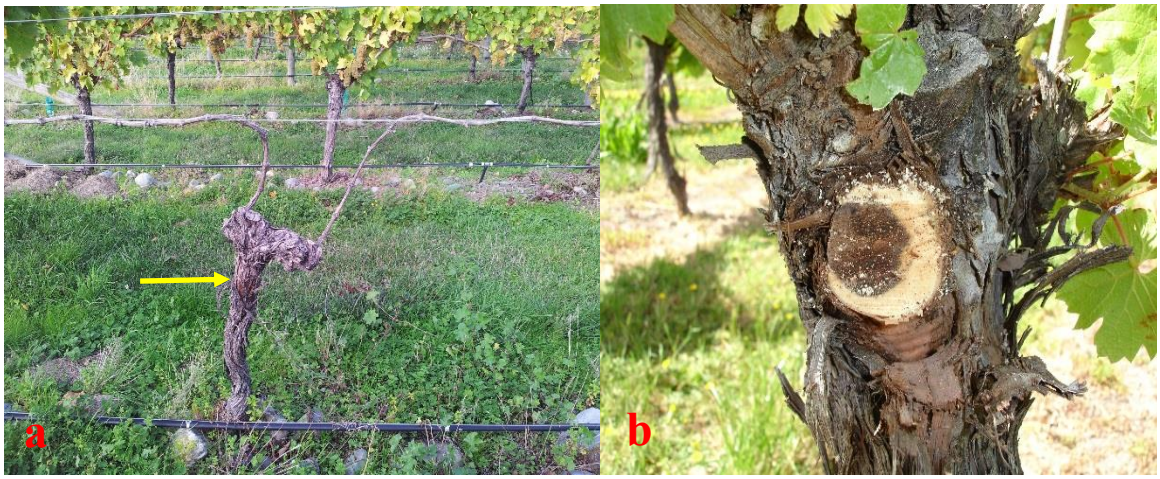


Figure 1.6 (a) Trunk canker shown by arrow, and (b) discolouration of trunk wood from cordon dieback above the canker caused by Botryosphaeriaceae species (Photographs: Marlene Jaspers).

Berry rots are an important Botryosphaeriaceae spp. symptom that has been reported from France (Larignon and Dubos, 2001) and the USA (Hewitt, 1988) as well as being experimentally induced in New Zealand (Amponsah *et al.,* 2012a). When berries become infected, they develop a light brown rot and eventually dry out and become mummified with black pycnidia emerging on the surface (Figure 1.7i, j). Berries of Sauvignon Blanc and Pinot Noir were able to be infected by conidia of *N. luteum* by Amponsah *et al.* (2012a) from pea-size to completely ripe.

Figure 1.7 Symptoms of Botryosphaeriaceae spp. infection (a) leaf necrosis, (b) necrosis of flower buds, (c) delayed bud burst, (d) shoot die back, (e) dead arm, (f) bleached cane, (g) wedge shaped necrosis, , (h)internal wood necrosis , (i) infected bunch, (j) mummified berries with pycnidia, and (k) pycnidial ooze [Photographs: Nicholas Amponsah (a, b, f, h, i), Marlene Jaspers (c, d, e, k) and Seelan Baskarathevan (g)].

After berries had rotted, the pathogen was also found to have progressed into the pedicels and rachides, and the shoot supporting the bunch.

In vineyards, berries were reported to be usually infected near the ripening stage followed by rot, development of black pycnidia, and shrivelling (Hewitt, 1994). However, these berries usually became infected with secondary fungi and yeasts, so often pycnidia and typical symptoms of Botryosphaeria rot were difficult to see as the bunches turned into a rotten mass known as a "summer bunch rot" (Hewitt, 1988).

Green shoots have also been reported to be infected with Botryosphaeriaceae species, which has been observed in vineyards as dieback (1.7d) after summer trimming (Amponsah *et al.,* 2011). Inoculation of soft and hard green shoots of potted vines has been shown to cause light to dark brown lesions which were clearly visible within 1-2 weeks. On soft green shoots, the lesions were clearly visible (Figure 1.8a), but on hard green shoots and more mature canes, the lesions were frequently visible only when the bark had been peeled off, or the stems split open (Figure 1.8b) (Amponsah *et al.,* 2011; Billones-Baaijens *et al.,* 2014).

Figure 1.8 (a) Internal lesions that developed 60 days after inoculating attached green shoots on 1-year-old potted Pinot noir grapevines with conidia of *Neofusicoccum luteum*, (b) Lesions 10 days after inoculating wounded green shoots of grapevine (Pinot noir) with mycelium colonised agar plugs of *N. luteum , N. parvum N. australe,* and *Diplodia mutila* (left to right) (Photographs: Nicholas Amponsah).

The many research reports on species pathogenicity (lengths of lesions), with respect to the inoculated plants and tissues, have differed greatly between species and countries (van Niekerk *et al.,* 2004; Savocchia *et al.,* 2007; Urbez-Torres and Gubler, 2009; Amponsah *et al.*, 2009a). This has made it difficult to provide a valid comparison between species. Table 1.2 shows a summary of the symptoms reported for each species.

Table 1.2 Symptoms associated with different Botryosphaeriaceae species as reported in published literature [adapted from van Niekerk *et al.* (2006)].

Symptoms	*B. d*[a]	*N. p*	*D. s*	*D. m*	*N. l*	*N.rib*	*L. theo*	*N. a*	*N. vitic*	*N. vitif*	*D. p*	Refs[b]
Bud mortality	x	x	x									4; 10; 11; 15
Shoot dieback	x					x	x					
Elongated black lesions	x					x	x					
Bleached canes	x	x	x	x	x		x					
Trunk dieback	x	x	x	x	x		x					1; 9; 11; 12; 13; 14; 15
Wedge-shaped necrotic lesion	x	x	x	x	x	x	x	x				
Arch-shaped lesions								x				
Dark brown wood discolouration	x	x	x	x	x		x			x		
Brown streaking, black spots	x	x	x	x	x		x					
Infected pruning wounds		x	x						x			
Leaf chlorosis		x	x	x		x						4; 5; 6
Fruit rot	x					x	x					2; 3; 7; 8
Graft union failure	x	x	x	x	x		x					1; 9; 11; 12
Pruning debris											x	14
Asymptomatic								x				

[a]*B. d – Botryosphaeria dothidea; N. p – Neofusicoccum parvum; D. m – Diplodia mutila; D. s – Diplodia seriata; N. l –Neofusicoccum luteum; N. rib – Neofusicoccum ribis; L. theo –\Lasiodiplodia theobromea; N. a – Neofusicoccum australis; F. vitic –Neofusicoccum viticlavatum; N. vitif – Neofusicoccum vitifusiforme; D. p – Diplodia porosum.* [b] 1. Castillo-Pando *et al*., 2001; 2. Kummuang *et al*., 1996a; 3. Kummuang *et al*., 1996b; 4. Larignon and Dubos, 2001; 5. Lehoczky1974; 6. Lehoczky, 1988; 7. Milholland, 1988; 8. Milholland, 1991; 9. Pascoe, 1998; 10. Phillips, 2002; 13. Taylor *et al*., 2005; 14. van Niekerk *et al*., 2004; 15. Wood and Wood, 2005.

In New Zealand, Amponsah *et al.* (2011) showed that inoculation of Pinot noir green shoots with *N. luteum, N. australe, N. parvum* and *D. mutila* caused significant differences in lesion

lengths, being 73, 58, 57 and 40 mm, respectively. Further, the lesion colours differed between species, being light brown for *N. luteum* and dark brown for *N. parvum, N. australe* and *D. mutila* (1.8b). In contrast, Billones-Baaijens *et al.* (2014) who investigated the susceptibility of rootstock and scion cultivars commonly grown in New Zealand, using three isolates each of *N. luteum, N. parvum* and*, N. australe* reported that *N. parvum* produced the longest lesions on trunks of potted plants of all varieties, but that there was also significant variation between isolates of *N. parvum*. However, studies in California using multiple isolates of each species to inoculate rooted canes showed that *L. theobromae* was the most virulent species followed by *N. luteum*, *N. parvum*, and *N. australe* (Urbez-Torres and Gubler, 2009). They considered these species to be highly virulent while *B. dothidea* was of intermediate virulence, and *D. mutila*, *D. seriata*, *Dothiorella iberica*, and *Do. viticola* were weakly virulent. In Australia, a study by Pitt *et al.* (2013) reported that *N. parvum* and *L. theobromae* were the most pathogenic based on lesion length developed in 15-year-old vines, followed by *N. australe*, *B. dothidea* and *D. mutila*, with *Do. viticola, Do. iberica* and *D. seriata* being the least pathogenic.

1.3.2 Prevalence of Botryosphaeriaceae species in New Zealand

In New Zealand, Baskarathevan *et al.* (2012) surveyed the six main grape-growing regions and showed that the distribution of species was different in the different regions surveyed (Table 1.3). The predominant species was *N. parvum* (34%) which was found in all six regions. This was followed by *D. mutila* (18%) recovered in Gisborne, Nelson, Blenheim and Canterbury, *D. seriata* (16%) in the north and central areas of the South Island, *N. luteum* (14%) from Auckland, Gisborne, Nelson and Otago and *N. australe* (11%) in Auckland, Gisborne, Blenheim and Canterbury. The less frequently isolated species were *N. ribis* (3%) in Auckland and Nelson, *Do. iberica* (2%) in Otago, *Do. sarmentorum* (1%) in the central and southern areas of the South Island, and *B. dothidea* (1%) in Nelson.

This nation-wide sampling of the New Zealand vine regions showed that Botryosphaeriaceae spp. were:

- in 68% of necrotic wood samples,
- in 23% of the declining vines,
- in 38 of the 43 vineyards (88%) that contributed samples,
- in all regions (Auckland, Gisborne, Nelson, Marlborough, Canterbury and Otago),
- in vines of all ages, but mostly from the 6-10 and 11-15 years age groups (34 and 23% of positive samples, respectively),
- in all varieties, tested, but mostly from Sauvignon blanc and Pinot noir (58% of samples).

Table 1.3 Incidence of Botryosphaeriaceae species isolated from diseased grapevines from six major wine growing regions in New Zealand [Adapted from Baskarathevan *et al.,* 2012].

	Number of samples yielding botryosphaeriaceous species								
	Neofusicoccum				*Diplodia*		*Dothiorella*		*Botryosphaeria*
Region	*luteum*	*parvum*	*australe*	*ribis*	*mutila*	*seriata*	*sarmentorum*	*iberica*	*dothidea*
Auckland*	15	24	1	4					
Gisborne*	11	18	1		3				
Blenheim#		9	16		20	11			
Nelson#	2	14		2	10	21			3
Canterbury#		4	6		4	2	1		
Otago#	2	3					1	4	
Totals	30	72	24	6	37	34	2	4	3

*North Island and #South Island

1.3.3 Identification of Botryosphaeriaceae species

Although morphological characteristics have been used for the identification of the Botryosphaeriaceae spp. some species have similar morphological characteristics (Crous *et al.*, 2006; Qiu *et al.*, 2008). Some species such as *N. luteum, N. parvum* and *N. australe* showed similar mycelial growth characteristics and colour as well as similar conidial morphology (Slipper *et al.,* 2004b). In addition, some species of the Botryosphaeriaceae are unable to produce conidia in artificial media (Denman *et al.,* 2000; Amponsah *et al.,* 2008) meaning that identification based on conidial morphology is not possible. Therefore, for accurate identification of Botryosphaeriaceae species it is necessary to use a combination of morphological and molecular methods (Denman *et al.,* 2000, Phillips *et al*., 2002; Slippers *et*

al., 2004). Molecular based species identification which involves the sequencing of the ribosomal internal transcribed spacer (rRNA ITS) region, β-tubulin and elongation factor 1-α (EF1-α) genes have proved to be very useful for accurately identifying Botryosphaeriaceae species (Phillips *et al.,* 2002; van Niekerk *et al*., 2004; Slippers *etal.,* 2004). Ribosomal DNA sequences at different taxonomic levels are very useful for discrimination of fungi (Guarro *et al.,* 1999). However, the non-coding regions such as internal transcribed spacer (ITS) are more reliable and generally used for species identification (Guarro *et al.,* 1999). Some domains like the D1 and D2 of the 28S rRNA gene are also variable within the coding regions (Alves *et al.,* 2005) and so universal fungal primers and polymerase chain reaction (PCR) can be used to easily distinguish species (Alves *et al.,* 2005).

When the region amplified by PCR is digested with a restriction enzyme, it cuts the DNA at specific sites which can differ between species (Guarro *et al*., 1999) and gel electrophoresis is used to visualise the resulting fragments. This technique called amplified ribosomal RNA restriction analysis (ARDRA) was used by Alves *et al.* (2005) to differentiate between species of Botryosphaeriaceae, whereby the amplified fragment of the 28S rRNA gene was digested using restriction endonucleases *Alu*I, *Asu*I, *Hae*III, *Mbo*I, *Nci*I and *Taq*I. The techniques of Alves *et al.* (2005) were further expanded to allow the differentiation between *N. luteum* and *N. australe* with the *SacII* enzyme (Baskarathevan *et al*., 2012).

Nowadays multi species specific primers have been developed, which are more commonly used for detection and differentiation of Botryosphaeriaceae species. Spagnolo *et al*. (2011) developed multi species specific primers that could detect 17 Botryosphaeriaceae species in grapevine wood. Ridgway *et al.* (2011) developed multi species primers BOT100F and BOT472R which amplified a 371-372 bp portion of the ribosomal RNA gene region and were able to detect six Botryosphaeriaceae species (*N. luteum, N. parvum/N.ribis, N. australe, Diplodia mutila* and *Diplodia seriata*) in wood, rain water and soil samples. Further, use of single stranded confirmation polymorphism (SSCP) allowed researchers to identify individual Botryosphaeriaceae spp. in complex samples (Ridgway *et al.,* 2011).

1.3.4 Disease cycle

The disease cycle of Botryosphaeriaceae pathogens has generally more fully demonstrated on other crops than on grapevines. Michailides (1991) described the asexual disease cycle of *B. dothidea* on pistachio trees (Figure 1.9). The disease cycle begins with survival of the pathogen over winter within blighted shoots, petioles, buds, blighted and mummified fruit

and perennial shoot cankers. In spring during favourable conditions (rainfall), conidia are released from pycnidia and infect female panicles, male blossoms and young shoots. Buds infected the previous summer also resulted in blighted flowers and infection extending into the supporting shoots. In summer the pathogen conidia which are released during moist conditions and spread by wind or rain could infect mature panicles and leaves through stomata, developed fruit through lenticels and developing buds through the scales. Pycnidia which form on blighted shoots, petioles, leaf lesions and mummified fruits could provide further conidia for autumn infections under favourable conditions (Epstein *et al.,* 2008).

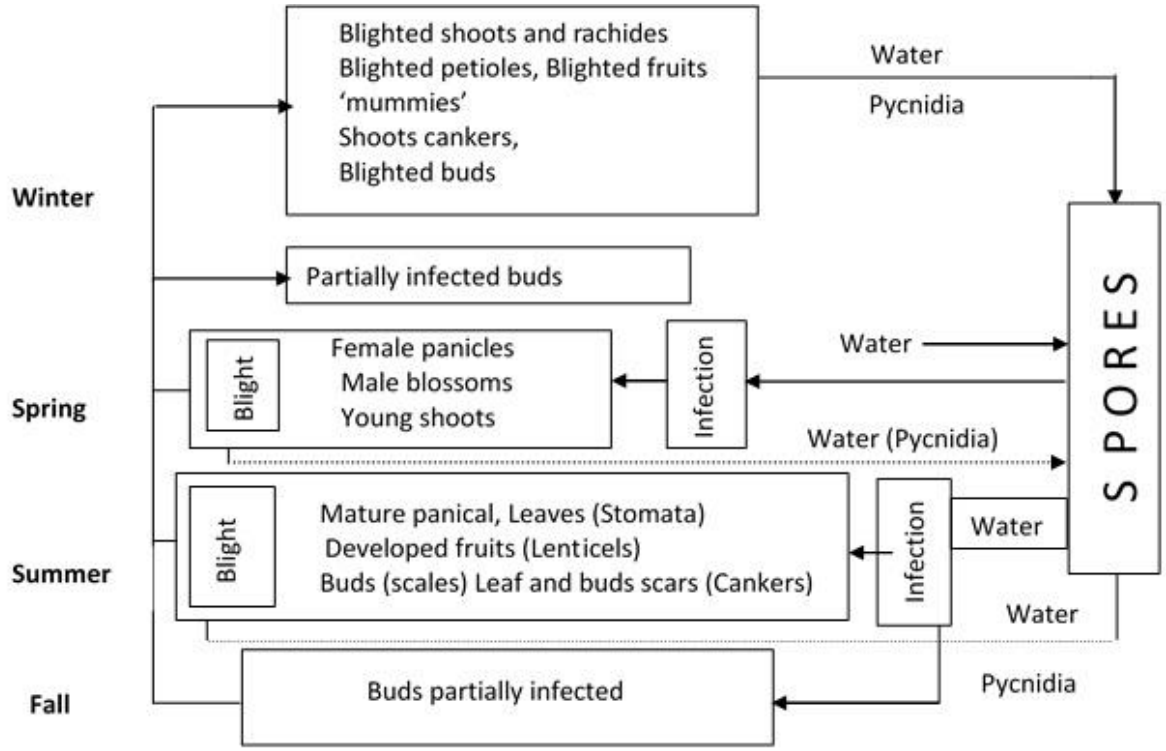


Figure 1.9 The disease cycle of Botryosphaeria panicle and shoot blight of pistachio caused by an asexual stage (a *Dothiorella* spp.) of *B. dothidea* (Slightly modified Michailides, 1991). Boxes represent tissues infected by the pathogen.

On peach trees, the pathogen has been found to survive as perithecia on cankers. *Botryosphaeria dothidea* (anamorph *Fusicoccum aesculi* ascospores were reported to infect new shoots, blossoms and fruit under favourable temperatures of 12-39°C and at 12-32°C, when moisture was available, (Pusey, 1993: Copes and Hendrix, 2004). Brown-Rytlewski and McManus (1999) also reported that *B. obtusa* (anamorph *D. seriata*) ascospores and conidia, which are formed in perithecia and pycnidia, respectively, on infected fruit and twigs of apple trees, are disseminated in late spring or summer rainfall. These can infect other apple

trees through natural openings and wounds. In avocado orchards, Hartill and Everett (2002) reported that *N. luteum* pycnidia were present throughout summer, with potential for ongoing conidial production and release.

In grapevines, the disease cycle has not been fully described. The pathogen almost certainly survives overwinter in diseased wood. The sexual stages of Botryosphaeriaceae spp. are rarely found in vineyards (Taylor *et al.*, 2005) however pycnidia are frequently observed in diseased wood. The pycnidia are known to release conidia when moistened by mist, rain or irrigation, since they have been found by spore-trapping during these conditions. They are dispersed by wind and rain to cause new infections, which are generally believed to be through fresh pruning wounds (Urbez-Torres, 2011). In a vineyard in Canterbury, New Zealand, Botryosphaeriaceae species conidia were trapped in rain water run-off traps but not on Vaseline™ coated slides suitable for collecting air-borne spores, which had been placed on vineyard posts (Amponsah *et al*., 2009b). The conidia were trapped during an entire year, and were most abundant during December to February, probably due to the rise in temperature as well as the availability of a larger canopy of mature canes during that period (Amponsah *et al.*, 2009b). The role of rainwater in the dispersal of conidia of Botryosphaeriaceae species has been reported by Pusey (1989) and Sutton (1981) who showed that waterborne conidia of *B. dothidea* and *D. seriata* were abundant in the air of peach and apple orchards, respectively, during rainy periods, and that the amount and duration of rainfall were the most important factors in waterborne dispersal of ascospores and conidia. However, Urbez-Torres *et al.* (2010b) reported trapping air-borne conidia on Vaseline™ coated slides placed within a grapevine canopy in California. They concluded that most spores were trapped following rain events during winter and very few or no spores were trapped in late spring and summer when conditions were much drier. Further, use of a volumetric spore trap showed that Botryosphaeriaceae spp. conidia were trapped from 1 h after onset of rain or irrigation and "not usually" after 2 h from the end of rain or irrigation. In South Africa, van Niekerk *et al.* (2010a) also reported trapping conidia of Botryosphaeriaceae spp. with a volumetric spore trap during winter and early spring, during and just after rainfall events.

The conditions under which Botryosphaeriaceae species produce and disperse conidia from infected grapevine tissues has not been fully investigated. Copes and Hendrix (2004), who extracted conidia from stem cultures, reported that conidia were produced over a wide temperature range of 6–30°C with maximum sporulation and spore maturation at 18-24°C. In

New Zealand, production of pycnidia and conidial ooze has been induced on infected green shoots and canes when placed in high RH at room temperature, with repeated periods of conidial ooze possible from lesions over a few weeks (Amponsah *et al*., 2008). However, the specific effects of different environmental conditions on this have not been determined. Further studies are needed to fully investigate the effects of environmental conditions on sporulation as it affects Botryosphaeria dieback in vineyards. This has important implications for potential infection of grapevines during pruning or trimming operations in vineyards during the year. Such information will assist with the development of control strategies.

1.3.4.1 The effect of tissue wetness duration on disease development

Relative humidity and leaf wetness significantly influence the production and transport of inoculum as well as the infection success of many fungi. However, for Botryosphaeriaceae conidia which are dispersed with moisture, there is some evidence to show that they do not require prolonged moisture for infection. Amponsah *et al*. (2010) demonstrated that conidia of *N. luteum* within the ooze exuded from infected shoots were able to germinate when spread on glass slides without the presence of free moisture. The moisture present in relatively high RH was sufficient at 25°C; germination was optimum at 100% RH and much reduced at 93% RH, with no germination at 84% RH. Further infection studies using wounded green shoots on potted Pinot noir grapevines showed that continuing surface wetness was not an important factor in infection because the conidial suspension of *N. luteum* infected the grapevine shoots at 95% RH and 24°C without the need of a further wetness period (Amponsah *et al*., 2014). However, Parker and Sutton (1993) reported that in orchards the incidence of apple fruit infection caused by *B. dothidea* increased with increasing wetness periods, and Arauz and Sutton (1989) also reported that in orchard conditions apple fruit and leaf infection caused by *D. seriata* increased with increasing wetness periods.

1.3.4.2 The effect of wounds and wound age on susceptibility

Botryosphaeriaceae species are generally considered as wound pathogens (Smith *et al.*, 1994; Taylor *et al*., 2005; Amponsah *et al*., 2011; Urbez-Torres, 2011). However, in pistachio Michailides (1991) reported that penetration by *B. dothidea* conidia occurred through lenticels or stomatal openings on healthy leaves, petioles, fruit and rachides. Further, Kim *et al.* (1999) reported that *B. dothidea* penetrated through apple lenticels. They observed that the germ tubes also penetrated the fruit directly; in some cases, globose appressoria were formed at the tip of germ tubes on the fruit surface, where no lenticels or surface cracks occurred.

Pusey (1993) also showed that lenticels of peach trees became infected with *B. dothidea* and thus caused canker symptoms in the wood. *Botryosphaeria dothidea* was also shown to infect apple trees through lenticels and reside there; when the trees were exposed to water stress symptoms developed (Connor, 1968). Sutton (1991) also reported that *B. dothidea* infected apple fruit in middle and late parts of the season through cracks in the cuticle, wounds or lenticels. The conidia of *B. dothidea* have also been reported to enter into non-wounded elm and blueberry stems through stomata (Luttrell, 1950; Milholland, 1972).

In grapevines, Phillips (1998) demonstrated that when *B. dothidea* conidia were inserted under the scales of dormant non-wounded buds on excised canes bud mortality resulted. Further, Amponsah *et al*. (2012a) reported that buds did not require wounds for infection since wounded and non-wounded buds had similar incidence and symptoms when inoculated in spring with conidia of *N. luteum*. However, attempts to infect non-wounded green shoots or trunks of potted vines with *N. australe, N. luteum, N. parvum* and *D. mutila*, were unsuccessful, although all fungi were pathogenic on the wounded tissues (Amponsah *et al*., 2011). Despite these findings, Billones-Baaijens *et al.* (2015a) demonstrated that grapevine shoots grown in mothervine blocks became infected during the season through another route other than wounds, since fungicide protection of trimming wounds did not significantly reduce incidence of infection. Further, they were able to show that multiple species and genotypes were sited within the bark of canes more often than in the wood, which indicated latency on surface tissues. However, presence of the same genotypes in some adjacent wood and bark infections indicated that some wood infection may have originated from the bark (Billones-Baaijens *et al.,* 2015b).

The age of wounds also has a significant effect on susceptibility to Botryosphaeriaceae species infections. On wounds made in trunks of 18-month-old potted Pinot noir grapevines in New Zealand in spring, susceptibility to infection by *N. luteum* conidia applied at 10^4 conidia/mL decreased with wound age. Infection was 100% on fresh to 2-day-old pruning wounds, 40% when wounds were 7-day-old and 0% on 14-day-old wounds. In the same study, wounds remained susceptible to mycelial infection for up to 30 days (Amponsah *et al.*, 2014). In California, Úrbez-Torres and Gubler (2011) demonstrated prolonged susceptibility of wounds in Cabernet Sauvignon and Chardonnay canes to conidia of *L. theobromae* and *N. parvum*; susceptibility remained higher when pruning was done in early winter (up to 84 days) than in early spring (up to 12 days). In Italy, a vineyard study by Serra *et al.* (2008) demonstrated that wounds made on grapevine spurs remained susceptible to infection by *D.*

seriata, even 8 weeks after pruning in late winter. Clearly, knowledge of the timing of pruning with respect to season and rainfall events which disperse fungal spores could provide growers with a strategy for preventing infection.

1.3.4.3 The effect of tissue age, weather conditions and inoculum concentration on susceptibility to infection

Initial studies with soft green tissues showed that lesion development was faster in detached than in attached tissues inoculated with *N. luteum, N. australe, N. parvum* and *D. mutila* (Amponsah *et al.*, 2011). Further studies on effects of physiological age of attached grapevine stems on development of lesions caused by *N. luteum* showed that lesions were not always visible on 2-year-old trunks and 1-year-old canes, however pathogen progression, as determined by isolation, was greatest in older tissues, with distances of 39.1, 31.6 and 23.2 mm for 2-year-old trunks, 1-year- old canes and current green shoots, respectively, (Amponsah *et al.*, 2014). In California, Úrbez-Torres and Gubler (2011) showed no differences between susceptibility of wounds on 1-year-old and 2-year-old wood to infection by *L. theobromae* and *N. parvum*. Van Niekerk *et al*. (2004) also demonstrated pathogenicity of different Botryosphaeriaceae species on green shoots, mature canes and mature wood but they did not compare susceptibility among the different aged tissues. Urbez-Torres and Gubler (2011) found that infection of pruning wounds by *L. theobromae* and *N. parvum* varied throughout the dormant season (November versus March). However in 2007 dormant season, wounds remained susceptible up to 7 weeks in November, December and January, whereas in the 2008 dormant season, wounds remained susceptible up to 12 weeks in November and December.

Effect of tissue age on susceptibility of grapevines to infection by Botryosphaeriaceae spp. has not been reported, although other studies have reported effects of wood age on infection by other pathogens. Balci *et al.* (2008) showed that lesions formed by *Phytophthora* species on 20-year-old oak tree stems were considerably larger than the stem lesions on stems of 1 and 2-year-old seedlings of the same hosts. Increased susceptibility with tree trunk age was also reported for *Phytophthora cinnamomi* infected *Quercus rubra* plants (Robin *et al.*, 1992). Moller and Kasimatis (1980) and Trese *et al.* (1980) also showed that pruning wounds made on 1-year-old grapevine wood were more resistant to infection by *Eutypa armeniaceae* compared to wounds made in older wood. However, Munkvold and Marois (1995) reported that age of grapevine wood at the time of wounding did not significantly affect the susceptibility of the wood to infection from *E. lata,* although wound susceptibility was

highest directly after pruning and decreased over time. Also, wounds made in early winter remained susceptible for a longer time than wounds made in January or February (late winter).

Conidium concentration has also been reported to affect infection incidence, with *B. dothidea* incidence in apple fruit increasing as the inoculating concentration increased from 10^4 to 10^7 conidia/mL (Biggs, 2004). However, Amponsah *et al*. (2014) showed that inoculation of fresh pruning wounds with 20 µL drops of *N. luteum* conidial suspension at a range of concentrations (10^2-10^6/mL) resulted in 100% infection incidence for all concentrations, although lesion development was slower for the lower concentrations than the higher concentrations.

1.3.4.4 Infection of other grapevine tissues

Amponsah *et al.* (2012a) demonstrated that wounded and non-wounded buds on potted vines became infected when inoculated with conidia of *N. luteum*; most were killed, with downward progression of the pathogen into the supporting shoots. Berries wounded and inoculated at the pre-bunch closure stage and later in the season were also shown to be susceptible to *N. luteum* infection, with isolation incidence increasing over the season and peaking at harvest, when infected berries became mummified and produced pycnidia with many conidia. The pathogen was also able to progress from berries into bunch stems and supporting canes. Castillo-Pando *et al.* (2001) who isolated Botryosphaeriaceae spp. from dead buds concluded that budburst failure was due to Botryosphaeriaceae spp. infections. In pistachio, Michailides (1991) found that conidia of *B. dothidea* were able to enter through stomata of leaves and shoots and through lenticels on fruits thus aggressively infecting uninjured fruit.

1.3.5 Control

To prevent infection of stem tissues it is essential that the pruning wounds are protected from infection by Botryosphaeriaceae species (Bester *et al.,* 2007). However, the studies that investigated use of fungicides for wound protection have been limited; they were mostly conducted under protected growing conditions and only the important species of the region were used for inoculation. Researchers from different countries had reported the efficacy of tebuconazole, flusilazole, spiroxamine and fluazinam, carbendazim, fludioxonil, fluazinam, penconazole, procymidone, thiophanate methyl, fenarimol and iprodione *in vitro* (Denman *et al*., 2004; Savocchia *et al.*, 2005; Luque *et al.,* 2008; Pitt *et al.*, 2010b; Amponsah *et al.,*

2012b). Leavitt (2003) demonstrated the efficacy of iprodione, benomyl, captan and penconazole when applied to pruning wounds prior to inoculation with *L. theobromae* in a glasshouse experiment with potted grapevine plants. Pitt *et al.* (2010b) also showed that pruning wounds were protected from infection by *D. seriata*, *B. parva*, *L. theobromae* and *B. dothidea* by the fungicides tebuconazole, cyproconazole, flusilazole, carbendazim and benzimidazole. Bester *et al.* (2007) in South Africa reported that when shoots treated with prochloraz, tebuconazole and benomyl, were inoculated with species of Botryosphaeriaceae the incidence of disease was reduced from the relatively high levels in the water treated shoots. In addition, Rolshausen *et al.* (2005) also demonstrated that Topsin (thiophanate-methyl) applied as a paint prior to inoculation was able to provide 77-82% protection against the inoculated pathogens, *B. dothidea, D. seriata* and *L. theobromae* in a vineyard. Luque *et al*. (2008) investigated the efficacy of different fungicides in field trials in Spain and found that carbendazim was the most effective, followed by thiophanate-methyl at reducing numbers of lesions (83.4 and 70.1%, respectively) caused by *D. corticola* on cork oak trees after cork removal. Diaz and Latorre (2013) studied the efficacy of paste and spray formulations of fungicides in Chile and reported that both spray and paste applications of benomyl, pyraclostrobin, tebuconazole and thiophanate-methyl reduced the infection rate of *D. seriata*, *Inocutis* sp. and *Pa. chlamydospora* in pruning wounds of grapevine (Cabernet Sauvignon) but paste formulations of these fungicides provided better control than spray applications. They also reported that application of the fungicides 1 day pre-inoculation provided better control than the post inoculation applications. The efficacy of fungicides has also been shown by Pitt *et al.* (2012) in Australia. They tested 20 fungicides *in vitro* and *in vivo* and found that the fungicides, flusilazole, tebuconazole, fluazanim, fludioxonil and iprodione and carbendazim not only inhibited mycelial growth of Botryosphaeriaceae spp. *in vitro* but were also effective in the field where they reduced the incidence of *D. seriata* or *D. mutila* by 32, 54, 55, 18-32, 41 and 27-41%, respectively. In addition to fungicides they also evaluated wound protectant paints in which Garrison and Bacseal Super, provided 42-65% and 38% control, respectively. In some trials they used such as tree wound dressings and acrylic paints which reduced Botryosphaeriaceae infection of pruning wounds by about 46%.

The apparent lack of field efficacy found by many researchers was possibly due to the continuous levels of natural inoculum, which a single fungicide application could not protect against during the long period of wound susceptibility. This was demonstrated by the infection incidence of non-inoculated control vines, for which 63% were reported to be

infected by Pitt *et al.* (2012). However, Amponsah *et al.* (2012b) demonstrated the efficacy of flusilazole, carbendazim, tebuconazole, thiophanate-methyl and mancozeb in a New Zealand vineyard where natural inoculum had not been found. When sprayed onto cane pruning wounds on potted and field grapevines, which were subsequently inoculated with *N. luteum* conidia, these fungicides prevented the inoculated pathogen from infecting healthy wood in 100, 93, 87, 83 and 80% of field vines, respectively. Clearly, the longevity of fungicides and the re-application frequency need to be investigated in an environment where there are continuously high levels of conidia.

Biological control agents have been used as wound protectants to provide control of some grapevine trunk diseases (Di Marco *et al.,* 2004; Fourie and Halleen, 2004b; Sosnowski *et al.*, 2008). Pitt *et al.* (2012) showed that the mean percent recovery of *D. seriata* was significantly reduced by 13% by the application of Vinevax® in a trial conducted in the Hunter valley. *Trichoderma* spp. were shown to be mycoparasites of several *Botryosphaeria* species, with isolates of *T. harzanium* and *T. atroviride* reported to control infection of apple stem and fruit by *Botryosphaeria berengeriana* f. sp. *piricola* (Kexiang *et al*., 2002). They found that reisolation of *B. berengeriana* after co-inoculation and pre-inoculation was reduced by 27.0-42.3% and 22.2-47.1%, respectively due to *Trichoderma* spp. application. Chen *et al*. (2003) showed that when *Paenibacillus lentimorbus* was applied to pistachio pruning wounds before inoculation with *B. dothidea*, it greatly reduced wound infection. Biological control agents such as *Fusarium lateritium* (Carter and Price, 1974; John *et al.,* 2005), *Trichoderma* spp. (John *et al.,* 2005; Kotze *et al.,* 2011)*, Bacillus subtilis* (Ferreira *et al.,* 1991; Schmidt *et al.,* 2001; Kotze *et al.,* 2011) and *Erwinia herbicola* (Schmidt *et al.,* 2001) have also shown antagonistic activity against these pathogens. Recently a study in France used bacterial strains isolated from grapevines as biocontrol agents against *B. cinerea* and *N. parvum*. The *in vivo* assays showed the effectiveness of *Pantoe agglomerans* and an *Enterobacter* sp. as potential biocontrol agents against *N. parvum* (Haider *et al*., 2016). However, in New Zealand, no studies have been carried out to test the effectiveness of biological protectants to prevent infection of grapevine pruning wounds by Botryosphaeriaceae species.

Another control measure that is widely recommended is the use of sanitary practices. Babadoost (2005) stated that pruning debris should either be buried or removed from the vineyard and preferably burnt, as this material can be a source of inoculum that can cause infection. However, they did not provide any data to support this statement. Hygiene methods

may also be effective. In avocado, frequent sterilization of the pruning instruments used to harvest the fruit reduced stem end rot infections caused by *N. parvum* and *N. luteum* (Hartill and Everett, 2002). Pitt *et al.* (2010a) reported that the incidence of trunk diseases can be reduced by avoiding pruning during and immediately after rainfall, pruning later in the season and removing infected wood as well as the trimmed branches because the pathogens can survive on dead tissues for a long period of time.

1.4 Aims and objectives of this research

The aim of this research programme is to improve the understanding of how pruning time, environmental conditions and the stages of the disease cycle affect rates of infection, information from which to develop control strategies. The objectives of this research are as follows:

Objective 1: Investigate factors associated with optimum conidium production. These experiments will provide understanding of which grapevine tissues produce conidia and under which environmental conditions.

Objective 2: Investigate conidium dispersal and infection in vineyards. These experiments will monitor dispersal and pathogenicity of conidia dispersed naturally from infected tissues.

Objective 3: Investigate the host factors that affect infection of grapevine tissues. These experiments will determine the lengths of time that the normal pruning and trimming wounds, made on a range of tissues, can remain susceptible to infection by the dispersed conidia and whether conidial numbers and season affect infection incidence of wounded stem tissues.

Objective 4: Investigate potential for infection of non-wounded tissues. These experiments will determine whether infection can occur in buds and fruits at different developmental stages, and whether infection of stem tissues can occur through lenticels.

Objective 5: Determine the efficacy of chemical and biological wound protectants during the period of susceptibility.

Chapter 2

Factors associated with conidium production

2.1 Introduction

Environmental factors are very important in the epidemiology of fungal pathogens. Of these, relatve humidity (RH) and wetness of surfaces are known to be especially important for fungal infection as well as production and transport of inoculum. For the Botryosphaeriaceae species which infect grapevines, the conidia which have been trapped during moist conditions, are believed to be dispersed mainly by rain splash (Amponsah *et al.,* 2009b; Úrbez-Torres *et al.,* 2010b; Baskarathevan *et al.,* 2013). The effect of relative humidity (RH) and sunlight on survival of Botryosphaeriaceae spp. conidia were also investigated by Amponsah *et al.* (2010). They showed that conidial germination of *Neofusicoccum luteum*, *N. australe* and *Diplodia mutila* was highest in 100% RH (91.8% within 3 h), moderate at 97% (67.2% within 6 h), and 93% (43.9% after 24 h), with no germination at 84% RH. They also showed that increasing exposure times to UV radiation caused decreases in the viability of conidia of *N. luteum*, *N. australe* and *D. mutila*. Further studies showed that prolonged surface wetness was not an important factor in *N. luteum* infection of wounded Pinot noir grapevine canes. However, under *in vitro* conditions, Parker & Sutton (1993) reported that incidence of apple fruit infections caused by *Botryosphaeria dothidea* increased with increasing wetness period and Arauz & Sutton (1989) also reported greater incidence of apple fruit and leaf infections caused by *D. seriata* with increasing wetness period (92-100% RH).

Temperature may also affect development of pathogenic fungi. Urbez-Torres *et al*. (2010a) reported that Botryosphaeriaceae conidia could germinate over a range from 5 to 35°C. However, only conidia of *B. dothidea*, *D. seriata*, and *L. theobromae* could germinate at 40°C. An understanding of how these factors contribute to disease progression in vineyards is important for the management of disease. Further, previous studies have indicated that disease progression differs between isolates of some *Neofusicoccum* spp. (Billones-Baaijens *et al*., 2014), so conidial production may also differ between isolates. The aims of this study were to (a) investigate the pathogenicity and sporulation of different isolates of *N. luteum* and *N. parvum* on different grapevine tissues and cultivars, and (b) investigate the effects of environmental factors on sporulation by these isolates on different grapevine stem tissues.

2.2 Materials and Methods

2.2.1 Production of grapevine plants and their management

For some of the experiments in this chapter and for most of the experiments in this thesis, about 1,500 Sauvignon blanc and 500 Pinot noir grapevine plants were grown in pots in a Marlborough tunnel house and used as needed. The dormant canes from which they were grown were removed by winter pruning from the Lincoln University vineyard and soaked for 30 min with occasional agitation in a solution of the systemic fungicide carbendazim (MBC 500 Flo; 0.25 g a.i./L) (Agronica). The bases of the canes were then placed in containers with 15 cm of vermiculite for rooting on a heat pad (27°C for 6 weeks) at ambient temperature in the Lincoln University Nursery. Two weeks later, the canes were washed in sterile water and the disease status checked by removing the top 5-7 mm from each shoot, which was plated on PDA and incubated at 25°C for 5 days before being examined for presence of colonies that resembled Botryosphaeriaceae spp. Since none of the canes were infected all rooted cuttings were transferred to the Marlborough Fairhall Vineyard, Blenheim where they were transferred to 5 L pots with a commercial potting mix (Morgans, Marlborough; (Appendix A.1). The plants were grown in two tunnel houses (Figure 2.1a) which were not heated and were cooled by opening the doors at each end during summer. In mid-summer, they were also covered in a shade-cloth screen to reduce the temperature. Plants were drip-irrigated every second day as needed. In spring when the buds had broken, the plants were sprayed with sulphur (Kumulus DF) (ORION Crop Protection) (40 g/8 L) and Impulse [(Spiroxamine/benzyalcohol) (Bayer CropScience) (9.6 mL/8 L)] alternatively every second week to prevent powdery mildew. After 10 months growth and every 12 months after that, each plant was side-dressed with approximately 10 g of the fertilizer Agroblen®Mg (Everris). Plants were also sprayed with Nitrosol (Yates) (8: 3: 6; N: P: K, respectively, plus minerals and trace elements) twice during each growing season. Pots were also weeded by hand every third week. Plants were pruned and trimmed several times during each growing season and tied to stakes for support. After pruning, wounds were sprayed with VineVax (Agrimm®Technologies Limited) at 10 g/L. Potted plants were transferred to an open gravelled area 1 week prior to setting up each experiment (Figure 2.1b).

Figure 2.1 a) Potted vines growing in tunnel house b) Plants transferred to outside area for experiments.

2.2.2 Lesion development and production of conidia from different isolates of *N. luteum* and *N. parvum* on different stem tissues

2.2.2.1 Pathogenicity and sporulation of multiple isolates on detached green shoots

In late summer 2013, green shoots (15-20 cm long from the tip) and semi-hard green shoots (30-60 cm from the shoot tip) were cut from Sauvignon blanc vines in the Lincoln University vineyard and the bases were immediately placed in water where they were held until inoculated (1-3 days). Each shoot was inserted into a Universal bottle containing sterilized water when required for the experiment. The Universal bottles were sealed with Parafilm® (American National Can™, Sigma-Aldrich) around the stems of the shoots. The six isolates of *N. luteum* (CC445, G51b1, G31d2, ICMP 16678, A526 and MM558) and eight isolates of *N. parvum* (G69a1, A75a1, A122, G61a1, B2141, A842, MM562 and G652), were used to inoculate the shoots. They had been stored at -80°C and were revived by subculture on potato dextrose agar (PDA; Difco^{TM} New Jersey, United States) and plates incubated at 25°C in a 12 h dark and light 12 h cycle, with illumination by fluorescent and near ultra violet light (366 nm) for 3 days, were used to inoculate the shoots (Figure 2.2a). A central area of each shoot was sterilised by swabbing with 70% ethanol and allowed to air dry and was then wounded (~3 mm deep and long) between the nodes using a sterilised scalpel blade. The wounded areas were inoculated with colonised agar plugs (3 mm diam.) cut from the margins of 3-day-old cultures. Control plants were wounded and inoculated with sterile PDA using the same method. These inoculated soft green shoots were incubated for 7 days and semi-hard green shoots for 14 days at room temperature (23-24°C) under natural light, with frequent misting for the first 4 days. For each treatment, two replicates (blocks) were set up per day on three consecutive days. The six replicates per isolate and tissue type were arranged in a randomized block design (RBD). The lesions which developed (Figure 2.2b) were measured with a digital calliper (Mitutoyo).

The stem pieces containing the lesions and pycnidia were removed and surface sterilized in 70% alcohol for 30 s and washed for 30 s in sterile water, before being air dried in a laminar flow unit overnight. The dried stem lesions were placed on sterile paper in a sterile plastic container and incubated on the laboratory bench for 5-7 days. A 1 cm section was cut from the centre of each lesion and placed in a sterilized Universal bottle also containing a moist filter paper strip. After 24 h incubation at 23-24°C, the filter paper strip was removed and 2 mL sterile tap water added to each bottle with the 1 cm stem sample. It was shaken for 3 min by hand and the suspended conidia were counted using a haemocytometer, with three

replicate counts for each treatment. Assessment of conidial concentrations was conducted for two replicates per day.

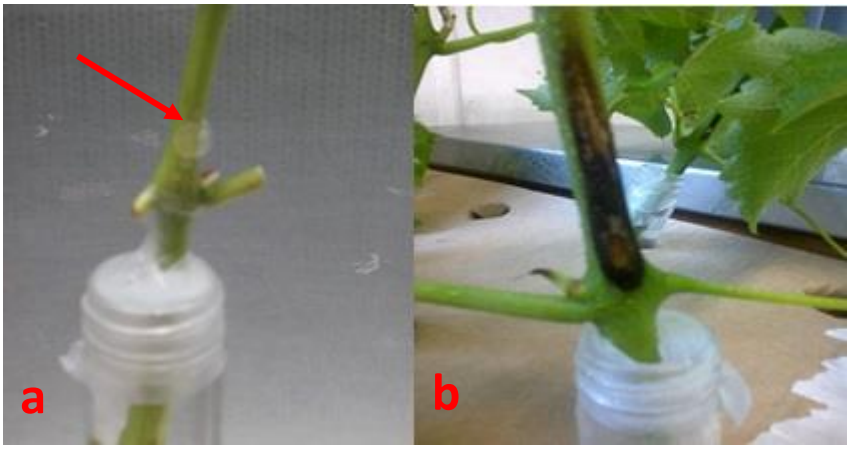


Figure 2.2 Inoculation of grapevine stem tissue (a) mycelium agar plug inoculated onto wounded shoot denoted by an arrow, (b) lesion that developed by 1 week after inoculation.

2.2.2.2 Pathogenicity and sporulation of selected isolates of *N. luteum* and *N. parvum* on attached soft green shoots

The isolates for this experiment were selected according to the length of the lesions which developed in Section 2.2.2.1, which indicated levels of virulence. Three highly virulent isolates of *N. luteum* (CC445, ICMP 16678 and MM558) and *N. parvum* (G69a1, MM562 and G652) and one moderately virulent isolate for *N. luteum* and *N. parvum,* G51b1 and A122 respectively, were grown as described in Section 2.2.2.1. The potted Sauvignon blanc plants which had been growing for 4 months were wounded between the first and second nodes of the green shoots emerging from the trunks and inoculated as described in Section 2.2.2.1, using mycelium plugs of 3-day-old cultures. Control plants were inoculated with sterile PDA. The six replicates per isolate were arranged in a (RBD). The plants were incubated for 2 weeks, on a gravel area outside the tunnel house at Fairhall Vineyard, Blenheim and were assessed for lesion development and sporulation as described in Section 2.2.2.1.

2.2.2.3 Pathogenicity of mixed isolate mycelium of *N. luteum* and *N. parvum* on green soft and semi-hard shoots of two different grapevine varieties

One-year-old potted plants were selected from the tunnel house for inoculation. For each species, the 3-day-old mycelium of the same three most pathogenic isolates as in Section 2.2.2.1 were mixed by chopping together and used to inoculate wounded areas of the green shoots and semi-hard shoots of Sauvignon blanc and Pinot noir in late October, 2014 at the

Fairhall Vineyard**.** Control plants were inoculated with sterile PDA. To prevent drying of the mycelium plugs, each inoculated wound was wrapped with Parafilm. The 10 replicates of each species and grapevine variety were laid out in a RBD and allowed to grow for 2 and 4 weeks (green shoots and semi-hard shoots, respectively) outside the tunnel house at the Fairhall Vineyard. They were then assessed for lesion development and conidial numbers as described in Section 2.2.2.1.

2.2.2.4 Pathogenicity of mixed isolate mycelium of *N. luteum* and *N. parvum* on woody trunks of potted vines of two different grapevine varieties

In November 2014 the 2-year-old trunks of potted grapevines (Sauvignon blanc and Pinot noir) were wounded and inoculated with mycelium as described in Section 2.2.2.3 (Figure 2.3). Each wounded trunk was inoculated with a mixed mycelium of either species. Control plants were inoculated with sterile PDA. The 10 replicates per species and variety were laid out in a CRBD and plants were allowed to grow for 6 weeks outside the tunnel house at the Fairhall Vineyard, and then assessed for lesion development as described in Section 2.2.2.1. After 6 weeks the bark was removed from each trunk to allow the lesion length to be assessed.

Figure 2.3 Inoculated area (arrow) of main trunk of potted grapevine with *Neofusicoccum parvum* and *N. luteum*.

Pieces (0.5 cm) cut from lesion edges were surface sterilized in 70% alcohol for 30 s and rinsed in sterile water for 30 s. The pieces were placed onto PDA plates and incubated at 25°C for 3-7 days, then observed for characteristic growth of *N. parvum* and *N. luteum* (Amponsah *et al*., 2011).

2.2.2.5 Pathogenicity of *N. luteum* and *N. parvum* mycelium on Pinot noir and Sauvignon blanc in the vineyard

In November 2014, hard green shoots and canes (woody, 1-year-old stems) of Pinot noir and Sauvignon blanc vines growing at Kaituna Vineyard, Blenheim were wounded and inoculated with a mixed mycelium of the same isolates of *N. luteum* or *N. parvum*, eight replicate plants each, as described in Section 2.2.2.3. On each vine two semi-hard green shoots and one cane were selected for each species. Control vines were inoculated with sterile PDA. After 3 weeks the inoculated hard green shoots were removed from vines and assessed for lesion development and conidium production as described in Section 2.2.2.1. The inoculated canes were assessed for lesion development after 8 weeks as described in Section 2.2.2.4.

2.2.3: Effects of environmental conditions on sporulation

2.2.3.1 Detached hard green shoots

Detached hard green shoots collected from the Lincoln University vineyard in late summer 2013 were inoculated with the isolates which produced the most conidia of *N. parvum,* (G69A1, MM562, and G652) and *N. luteum,* (CC445, ICMP 16678, and MM558) (Section 2.2.2.1). After 2 weeks, when lesions and pycnidia had developed, the 1 cm sections cut from the centres of the lesions on these infected shoots were incubated under relative humidities (RH) of 80-81, 92.5-96 and 100%, using saturated solutions of salts, $(NH_4)_2SO_4$, KNO_3 and water, respectively, based on the methods of Dhingra and Sinclair (1985). The lesion sections which had been tied to a thread were surface sterilised and air dried as described in Section 2.2.2.1, then suspended in the air above the salt solutions in the tubes for 24 h after the air had been allowed to equilibrate to the appropriate RH (24 h) (Figure 2.4). These sealed tubes were placed in a RBD in different incubators set at 10, 15, 20 and 25°C. The six replicates were set up on alternate days, i.e. on days 1, 3, 5, 7, 9, and 11. The incubators retained the same temperatures throughout, and because there were limited numbers of incubators available the tubes were not randomly allocated to temperatures with respect to blocks. After 2 days incubation, the numbers of conidia that oozed from the pycnidia were determined as described in Section 2.2.2.1.

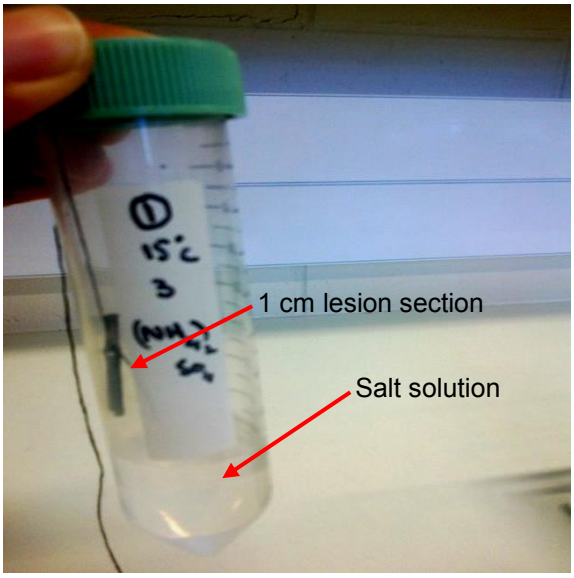


Figure 2.4 Grapevine shoot section (1 cm length) containing a *Neofusicoccum parvum* lesion suspended in the air above the salt solution.

2.2.3.2 Attached soft stem tissues on potted vines

To determine the effects of environmental conditions on release of conidia from living plants, soft green shoots on 1-year-old potted vines were inoculated with mycelium of selected isolates of *N. parvum* (G69a1, MM562, and G652) and *N. luteum* (CC445, ICMP 16678, and MM558) as described in Section 2.2.2.1 and allowed to develop lesions and pycnidia by incubating for 1 week in a shade house. The lesion lengths were measured using a digital calliper, and the plants were then transferred to growth chambers (Conviron PGV36 with H319UV-B lighting system, floor area 1.37 x 2.45 m, lighting max. 960 u mols/m^2/s; Controlled Environments Limited), for sporulation. The conditions in the growth chambers were 93 and 100% RH at both 20 and 25°C (selected based on the results of Section 2.2.3.1). The chamber was set to 93% RH for the first set of potted grapevines and then to 100% RH with the second set of potted grapevines. However, the chamber could not achieve this so 100% RH was achieved by hanging wet cotton fabric around the inner walls of the growth chamber and placing water buckets, which were refilled frequently, inside the chamber. The floor was also wetted with water every 4 h during the day. Tinytag® relative humidity (0-100%) and temperature (-40- +75°C) data loggers (Gemini Data Loggers, UK) were also placed in the growth chambers and they confirmed RH and temperature. After 2 days, stem lesion segments were assessed for numbers of conidia as described in Section 2.2.2.1. It was not possible to randomly allocate plants to temperatures with respect to blocks because there were only two chambers available for a short time.

2.2.3.3 Release of conidia from naturally infected vines in vineyards

Vines within two vineyards (Fairhall and Brancott Estate Vineyards) which were known to have infected vines (Sections 3.2.1.1 and 3.2.1.2) were assessed during the year to observe the effects of moist conditions in Marlborough. In each of the vineyards, symptomatic tissues were identified on the basis of morphological characteristics and these tissues were marked on 24 vines for ongoing observation. The lesions on these tissues were observed with a hand lens for presence of characteristic pycnidia. After each period of rainfall or cloudy, humid weather, the same tissues were observed with a hand lens for conidial ooze (Figure 2.5). To identify the spores, the ooze was removed, placed on a microscope slide and observed at ×100 with a microscope. When conidia characteristic of Botryosphaeriaceae species were observed, the slides were washed with a few drops of sterile water, using a sterile 'hockey stick' and the liquid spread onto PDA containing Triton-X 100 (1 mL/L) for growth. Colonies characteristic of Botryosphaeriaceae species were then sub-cultured on PDA and identities of sub-cultured isolates characteristic of Botryosphaeriaceae species were confirmed by molecular methods as described in Section 3.2.2.4. Those tissues that produced conidia characteristic of Botryosphaeriaceae species were observed continuously during spring to winter.

At the end of the season, these tissues were removed, washed under tap water and incubated under high relative humidity for 3 days at room temperature. However, assessment of conidial numbers could not be conducted because the overgrowth by mycelium obscured any conidial ooze. The HortPlus database (http://hortplus.metwatch.co.nz/) provided the data of temperature and rainfall during the periods of observation.

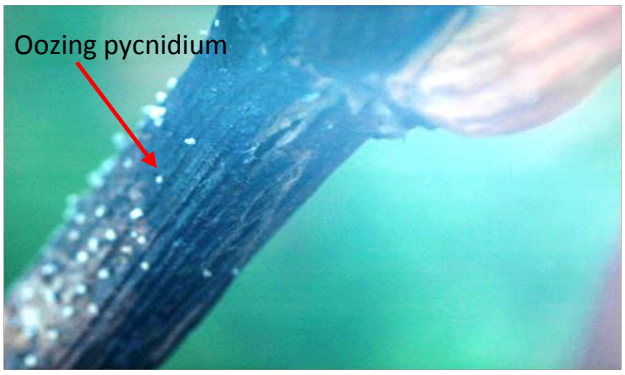


Figure 2.5 Infected Sauvignon blanc cane with oozing pycnidia from Brancott Vineyard.

2.2.3.4 Identification of strains isolated from conidial ooze on shoots in vineyards

2.2.3.4a DNA extraction

The colonies characteristic of Botryosphaeriaceae species grown from the collected ooze were sub-cultured on PDA and 1 mm mycelium plugs cut from the edges of the pure colonies were used to inoculate potato dextrose broth (PDB; Difco™ New Jersey, United States) in deep (25 mm) Petri dishes. After 3 days, superficial tufts of mycelium were removed from each PDB culture with a sterile micropipette tip and added to PowerPlant® Bead tubes (MO BIO laboratories, California, USA) which contained small beads. Then 450 µL of solution PD1, 50 µL of solution PD2 and 3 µL of RNase solution were added to each PowerPlant® Bead tube. After briefly vortexing to mix, the tubes were vortexed at maximum speed for 15 min in the vortex adaptor tube holder (MO BIO laboratories, California, USA). The tubes were then centrifuged at 13,000 × *g* for 2 min and 550 µL of each supernatant was transferred to a clean 2 mL tube. To these tubes 175 µL of solution PD3 was added, they were vortexed for 5 s and the tubes were incubated at 4°C for 5 min. After centrifugation at 13,000 × *g* for 2 min, 600 µL of each resulting supernatant was transferred to a clean 2 mL tube, to which were added 600 µL of solution PD4 and 600 µL of solution PD6 which caused the proteins to precipitate. The tubes were briefly vortexed for 5 s and the resulting supernatant from each tube was then filtered through a spin filter by centrifugation at 10,000 × *g* for 1 min. The flow through from each solution was discarded, leaving the DNA bound to the silica membrane in the filter. To each spin filter, 500 µL of solution C5 was added and centrifuged at 10,000 × *g* for 30 s. The wash solution that flowed through was discarded. This step was repeated three times until all of the lysate had been passed through the spin filter. To each spin filter tube, 500 µL of solution PD5 was added and it was centrifuged at 10,000 × *g* for 30 s. The flow through was discarded and the spin filter was placed back into the same collection tube. To each spin filter tube, 500 µL of solution PD6 was added and the tubes were centrifuged at 10,000 × *g* for 30 s. The flow through was discarded and each spin filter was returned to the same collection tube. To remove the residual PD6, the spin filter tubes were again centrifuged at 13,000 × *g* for 4 min. Each spin filter was placed in a clean 2 mL collection tube, 100 µL of solution PD7 was added to the centre of the white filter membrane and it was incubated for 2 min at room temperature. These tubes were centrifuged at 10,000 × *g* for 30 s and the flow through was re-loaded again to the centre of the white filter membrane followed by centrifugation at 10,000 × *g* for 30 s. The spin filter was discarded and the concentration of DNA in the flow through liquid was determined using a Nanodrop 3.0.0 spectrophotometer

(Nanodrop Techologies Inc., Delaware, USA). DNA samples were then diluted to 20 ng/µL by diluting the original extract with UltraPure™ Distilled water (Invitrogen). The resulting DNA from this process was stored at -80°C for PCR amplification.

2.2.3.4b PCR amplification with ITS primers

The DNA extracted from each culture was amplified using primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3'). Each tube of PCR mixture contained 200 µM of each dNTP, 1 U Taq DNA polymerase (FastStart® Roche), 1 x PCR buffer sufficient for each reaction tube (with 1.5 mM $MgCl_2$), 0.4 µM of each primer and 1µL of DNA template. Each reaction volume was made up to 25 µL with UltraPure™ distilled water. Negative control tubes which contained sterile water instead of the template DNA were included in every PCR. The amplification conditions were as follows: initial denaturation of 5 min at 95°C, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C, and a final extension period of 10 min at 72°C. After amplification, 1 µL of each PCR product was separated by electrophoresis on a 1% agarose gel for 50 min at 10 V/cm in 1 x TAE buffer with a 1 Kb plus DNA ladder (Invitrogen Life Technologies, Carlsbad, California, USA). The gel was stained with ethidium bromide (0.05µg/mL, AMRESCO) for 15 min, destained in water for 10 min, and then photographed using a UV transilluminator (Versodoc™, BioRad Laboratory).

2.2.3.4c Restriction Analysis

The amplified ribosomal DNA restriction analysis (ARDRA) technique was used for all the amplicons as described by Baskarathevan *et al.* (2012). The PCR products of 1200 bp were obtained following amplification using ITS1 and NL4 primers, were digested with restriction enzymes in an iterative and sequential process (Figure 2.6). To distinguish the *N. parvum*/*N.ribis* group from other *Neofusicoccum* species, each reaction contained 10 µL of PCR product that was digested with 2 U of *Hae*III (BioLabs) enzyme for 12 h at 37°C and the resulting fragments were separated by electrophoresis on a 1.5 % agarose gel for 1 h at 10 V/cm in 1 x TAE. The gels were visualized as described in Section 2.2.2.4b. Digestion with *Hae*III distinguished *N. parvum* from all other Botryosphaeriaceae species, with five visible bands (258, 254, 203, 157 and 58-83 bp) (Group A) (Baskarathevan *et al.,* 2012).

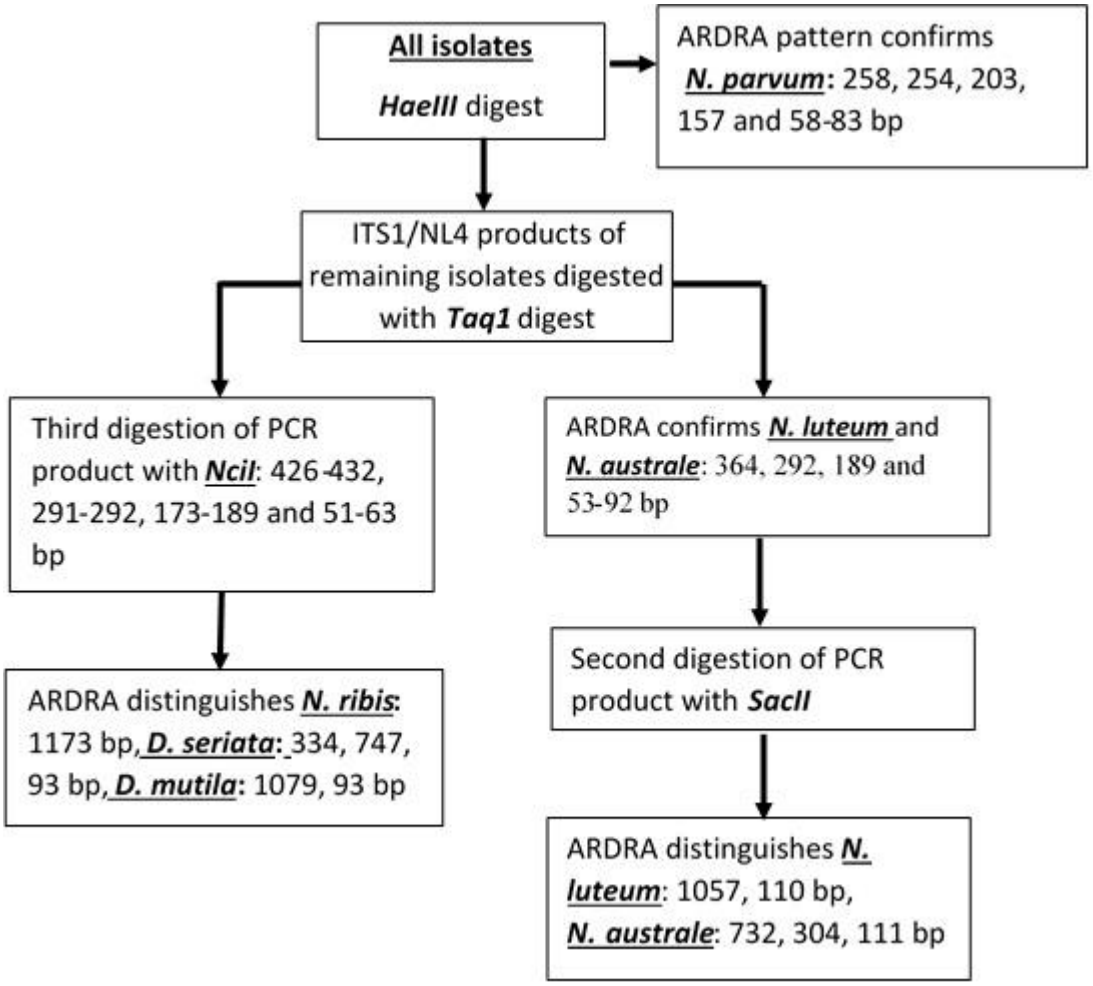


Figure 2.6 Diagram of the iterative restriction enzymes digestion to identify the Botryosphaeriaceae species isolates from oozing pycnidia.

For the remaining *Neofusicoccum* isolates, a second 10 µL aliquot of the PCR product was digested with 2 U *Taq*I (BioLabs) restriction endonuclease at 65°C for 2 h followed by heat inactivation at 80°C for 20 min which produced four bands of 364, 292, 189 and 53-92 bp which indicated that isolates were either *N. australe* or *N. luteum* (Group C). The PCR products of these isolates were differentiated with 2 U of *Sac*II (Fermentas) at 37°C for 12 h, which resulted in two banding patterns, with two bands of 1071 and 102 bp indicating *N. luteum* and three bands of 766, 304 and 102 bp indicating *N. australe*. After *Taq*I digestion, the isolates with four bands of 426-432, 291-292, 173-189 and 51-63 bp were placed in Group B (*N. ribis, D. mutila* or *D. seriata*). For Group B isolates the PCR product was further digested using the *Nci*I (BioLabs) enzyme at 37°C for 14 h, which differentiated *D. mutila* (with two bands of 1079 and 93 bp), *D. seriata* (with three bands of 334, 747 and 93 bp) and *N. ribis* which was not cut by the enzyme (with one band of 1173 bp).

2.2.4 Statistical analysis

Data was analysed by General Linear Model (Minitab 17th edition). For the analysis, data of the lesion length and conidia counts were transformed to $\log_{10}$ to improve the homogeneity in variance. The comparison responses were examined with Sidak Pairwise Comparisons at $P \leq 0.05$. Only the differences between means that met this criterion are considered as being significantly different in the following results section.

2.3 Results

2.3.1 Lesion development and production of conidia from different isolates of *N. luteum* and *N. parvum* on different stem tissues

2.3.1.1 Pathogenicity and sporulation of multiple isolates on detached green shoots

There was a block effect for lesion lengths on soft and semi-hard shoots ($P<0.001$ for both $P<0.001$, respectively) (Appendix B.1.1, and B.1.2, respectively), due to shoots inoculated on the day after cutting producing longer lesions than those held in water for 1 and 2 days. However, the lesion lengths produced by the isolates followed the same relative trends on all days (blocks). For all *N. parvum* isolates, mean lesion lengths for soft green shoots differed significantly between those inoculated on the day of cutting and after 1 and 2 days, being 2.6, 1.5 and 1.3 cm, respectively and for semi-hard shoots being 3.5, 1.9 and 1.4 cm, respectively. For all *N. luteum* isolates, mean lesion lengths for semi-hard shoots also differed significantly for days 0, 1 and 2, being 4.9, 1.7 and 1.5 cm, respectively, and for soft green shoots being 3.4, 1.5 and 1.4 cm, respectively. Numbers of conidia produced on lesion sections were also affected by block for soft but nor for semi-hard shoots ($P<0.001$ and $P=0.370$, respectively) (Appendix B.1.4 and B.1.6, respectively). For all *N. parvum* isolates, mean numbers of oozed conidia for soft green shoots differed significantly between days 0, 1 and 2, being 3.4×10^4, 1.7×10^4 and 1.3×10^4 conidia/cm lesion, respectively, and for semi-hard shoots being 3.5×10^4, 2.9×10^4, and 3.0×10^4 conidia/cm lesion, respectively. For all *N. luteum* isolates, mean numbers of oozed conidia also differed significantly for semi-hard shoots being 5.1×10^4, 5.4×10^4 and 2.5×10^4 conidia/cm lesion, respectively, and for soft green shoots were 5.2×10^4, 2.9×10^4 and 2.0×10^4 conidia/cm lesion, for days 0, 1 and 2, respectively. Control plants did not show any symptoms typical of Botryosphaeriaceae species

On soft green shoots, lesion lengths were not affected by species ($P=0.192$; Appendix B.1.1) but were significantly affected by isolate ($P<0.001$; Appendix B.1.3) with longest mean

lesions being caused by *N. luteum* CC445 (3.9 cm) and *N. parvum* G69a1 (2.9 cm), and shortest lesions caused by *N. luteum* isolate G31d2 (1.1 cm), and *N. parvum* isolate A842 (1.2 cm), both being approximately 30% of the mean length for isolate CC445 (Figure 2.7). There was a significant effect of isolate on numbers of oozed conidia/cm for soft tissues, however the effect of species was not significant (P<0.001 and P= 0.051, respectively; Appendix B.1.4). The highest mean numbers of conidia were oozed for isolates CC445 (5.8 x 10^4 conidia/cm lesion) and G69a1 (5.6 x 10^4 conidia/cm lesion), and the lowest mean number was 5.4 x 10^3 conidia/cm lesion for *N. parvum* isolate A842 (Figure 2.8).

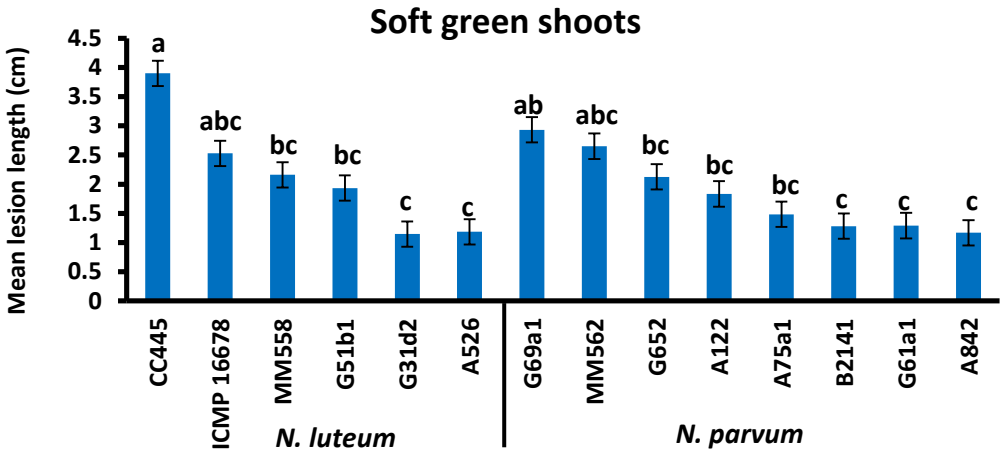


Figure 2.7 Mean lesion lengths after inoculation of detached Sauvignon blanc grapevine soft green shoots with isolates of *Neofusicoccum luteum* and *N. parvum*. Error bars represent standard errors of the means and bars with different letters are significantly different at P≤0.05.

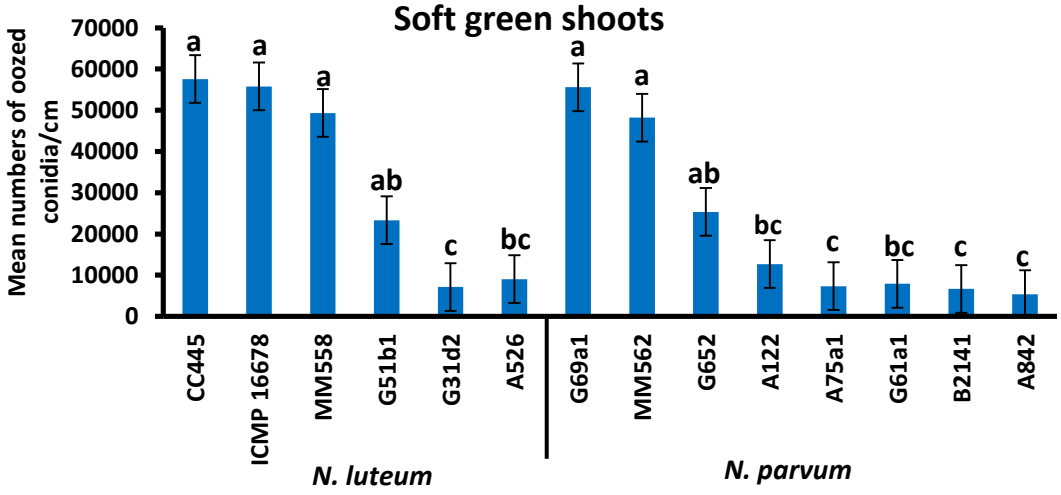


Figure 2.8 Mean numbers of oozed conidia from 1cm lesion lengths which developed on detached Sauvignon blanc grapevine soft green shoots after inoculation with different isolates of *Neofusicoccum luteum* and *N. parvum*. Error bars represent standard errors of the means, and bars with different letters are significantly different at P≤0.05.

On semi-hard shoots, lesion lengths were not affected by species (P=0.139; Appendix B.1.2 but were significantly affected by isolate (P<0.001; Appendix B.1.5), with longest mean lesions caused by *N. luteum* CC445 (4.25 cm) and *N. parvum* G69a1 (3.8 cm), and shortest mean lesions caused by *N. luteum* G31d2 (1.5 cm), and *N. parvum* A842 (1.6 cm), being approximately 37% of the mean length for isolate CC445 (Figure 2.9). There was no significant effect of species (P= 0.285; Appendix B.1.6) on the number of oozed conidia/cm lesion on semi-hard tissue. However, there was a significant effect of isolate (P<0.001; Appendix B.1.6), with highest numbers of conidia being oozed by *N. parvum* G69a1 (1.0 x 10^5 conidia/cm lesion) and *N. luteum* CC445 (8.6 x 10^4 conidia/cm lesion). The lowest numbers of conidia oozed were for *N. parvum* isolate A842 (6.4 x 10^3 conidia/cm lesion), both being approximately 5% of the numbers for isolate G69a1 (Figure 2.10).

For *N. parvum,* the three most pathogenic isolates with highest sporulation were G69a1, MM562 and G652 and for *N. luteum* they were CC445, ICMP 16678 and MM558. Isolates of moderate pathogenicity and sporulation were G51b1 (*N. luteum*) and A122 (*N. parvum*) Overall, the remaining four isolates of *N. parvum* and two isolates of *N. luteum* consistently produced short lesions and very low numbers of conidia. (Figures 2.7, 2.8, 2.9 and 2.10).

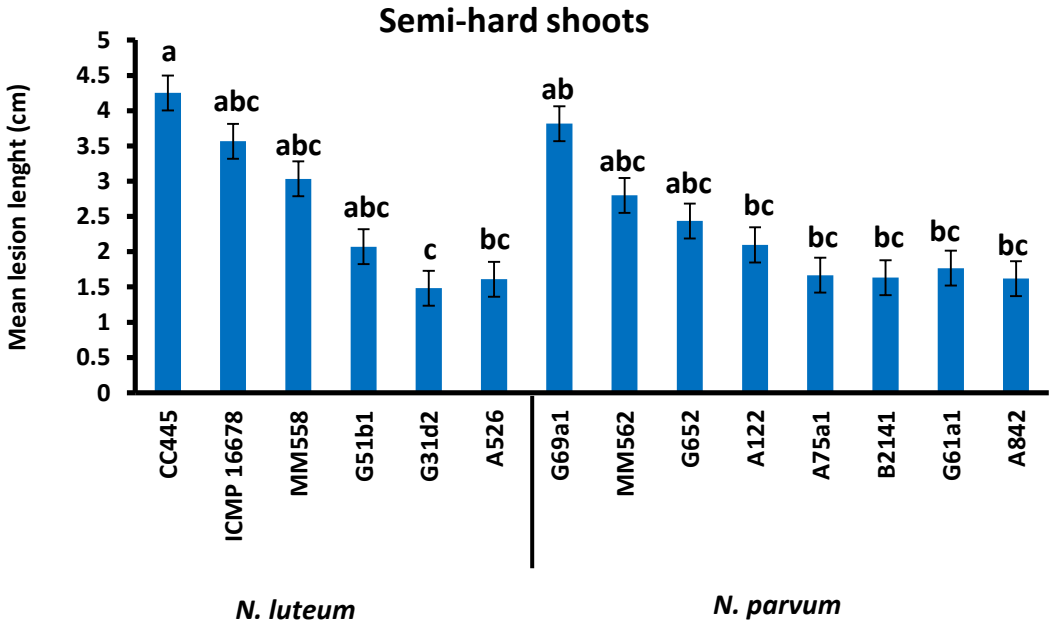


Figure 2.9 Mean lesion lengths after inoculation of detached Sauvignon blanc grapevine semi-hard shoots with isolates of *Neofusicoccum luteum* and *N. parvum.* Error bars represent standard errors of the means and bars with different letters are significantly different at $P \leq 0.05$.

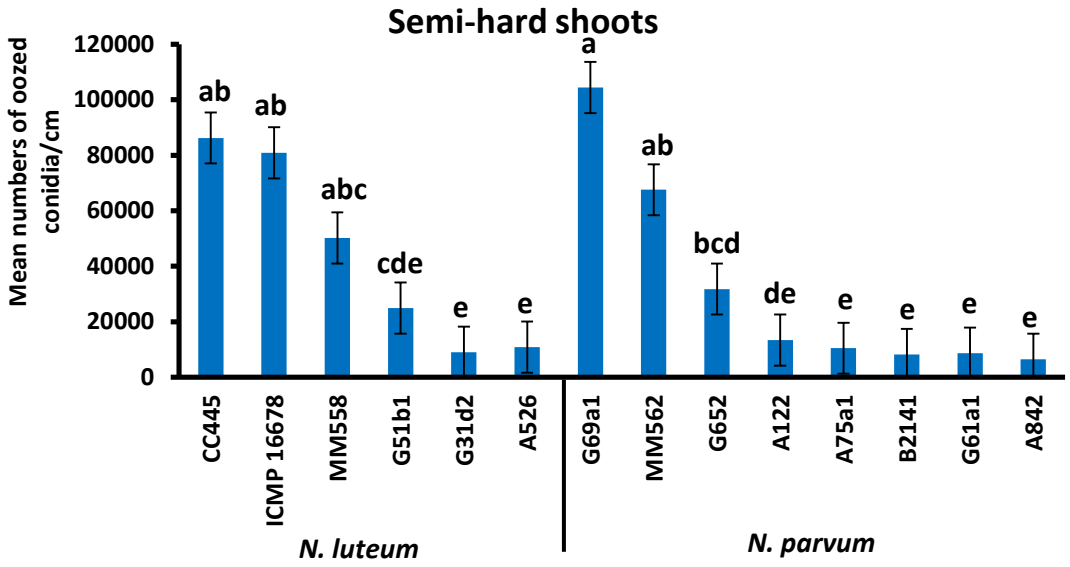


Figure 2.10 Mean numbers of oozed conidia from 1cm lesion lengths which developed on detached Sauvignon blanc grapevine semi-hard shoots after inoculation with different isolates of *Neofusicoccum luteum* and *N. parvum*. Error bars represent standard errors of the means, and bars with different letters are significantly different at $P \leq 0.05$.

2.3.1.2 Pathogenicity and sporulation of selected isolates of *N. luteum* and *N. parvum* on attached soft green shoots

On soft green shoots, there was a significant isolate effect on lesion lengths and numbers of oozed conidia ($P<0.001$ for both; Appendix B.2.1, A.2.2). The greatest mean lesion lengths were caused by the same isolates as for the detached tissue (Section 2.3.1.1) being *N. luteum* CC445, ICMP 16678 and MM558 and *N. parvum* G69a1, MM562 and G652, which were not significantly different with lesions of 2.5-2.8 cm (Figure 2.11a). The greatest sporulation was from *N. luteum* CC445 (1.6×10^5 conidia/cm lesion) and ICMP 16678 (1.4×10^5 conidia/cm lesion), followed by *N. parvum* G69a1 (9.9×10^4 conidia/cm and MM562 (9.5×10^4 conidia/cm lesion) (Figure 2.11b). Control plants did not show any symptoms typical of Botryosphaeriaceae species

2.3.1.3 Pathogenicity of mixed isolate mycelium of *N. luteum* and *N. parvum* on soft green and semi-hard shoots of two different grapevine varieties

On attached soft green shoots of Sauvignon blanc and Pinot noir, mean lesion lengths differed significantly between the species ($P=0.032$; Appendix B.2.3), with *N. luteum* causing significantly longer lesions (1.92 cm) compared with *N. parvum* (1.67 cm). However, mean lesion lengths were not significantly affected by grapevine varieties ($P=0.859$; Appendix B.2.3) nor a variety and species interaction ($P=0.361$; Appendix B.2.3).

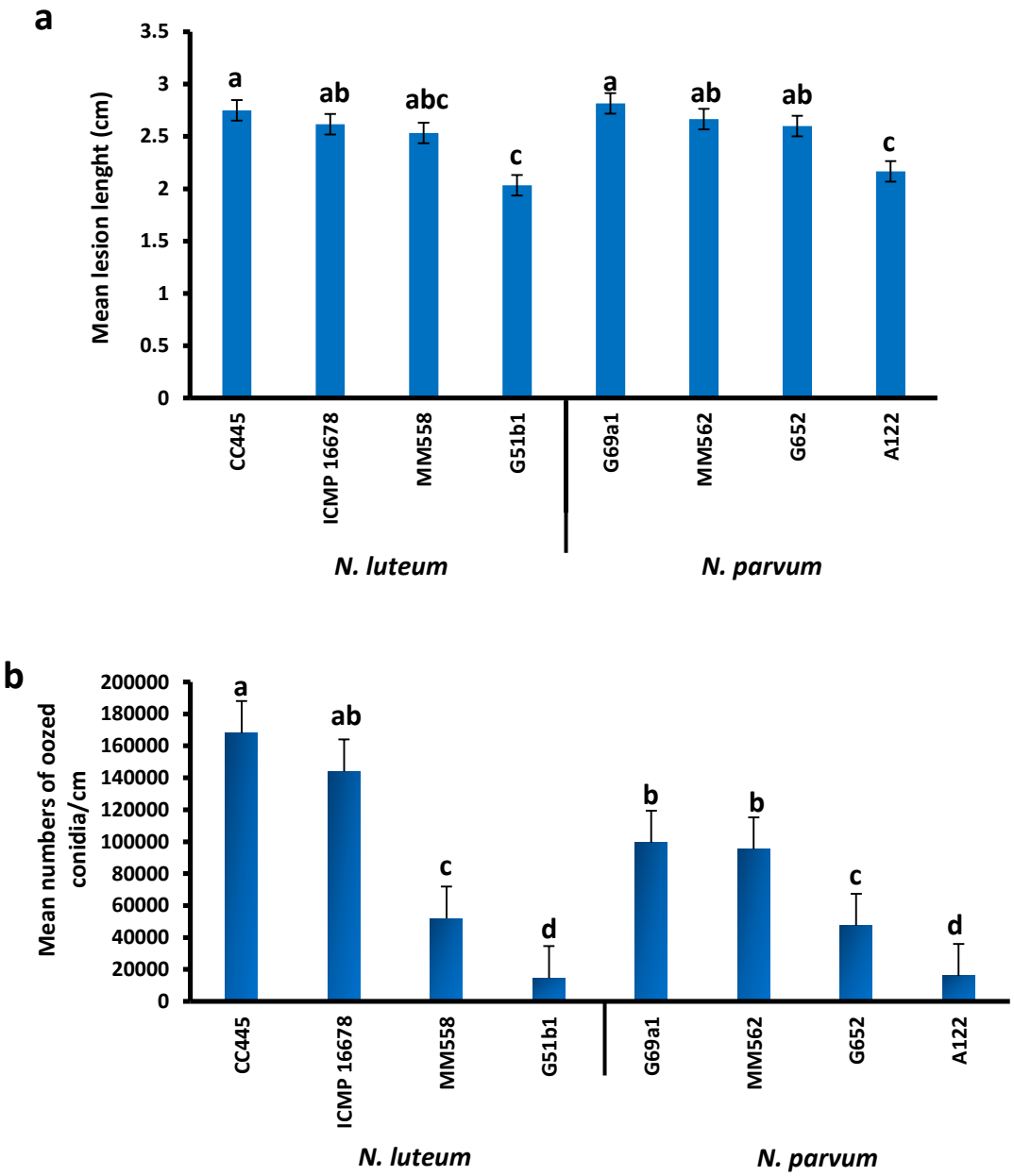


Figure 2.11 (a) Mean lesion lengths and (b) mean numbers of conidia oozed from 1cm lesions on attached soft green stem shoots of Sauvignon blanc grapevine plants infected with isolates of *Neofusicoccum luteum* and *N. parvum.* Error bars present standard errors of the means, and bars with different letters are significantly different at $P \leq 0.05$.

The mean lesion lengths on soft tissues of Sauvignon blanc were 1.98 and 1.64 cm for *N. luteum* and *N. parvum,* respectively and the mean lesion lengths on soft tissues of Pinot noir were 1.87 and 1.71 cm for *N. luteum* and *N. parvum,* respectively. The numbers of oozed conidia differed significantly between the species and grapevine varieties on soft tissues of Sauvignon blanc and Pinot noir ($P<0.001$, $P=0.008$, respectively; Appendix B.2.4). Inoculation with *N. luteum* resulted in significantly higher numbers of oozing conidia (2.5 x

10^4 conidia/cm lesion) compared with *N. parvum* (1.0 x 10^4 conidia/cm lesion), and inoculation of Sauvignon blanc resulting in significantly higher numbers of oozing conidia (2.3 x 10^4 conidia/cm lesion) compared with Pinot noir (1.9 x 10^4 conidia/cm lesion). However, there was no interaction between grapevine varieties and species (P=0.418; Appendix B.2.4). The mean numbers of oozed conidia on soft tissues of Sauvignon blanc for *N. luteum* and *N. parvum* were 2.8 x 10^4 and 1.8 x 10^4 conidia/cm lesion, respectively and the mean numbers of oozed conidia on soft tissues of Pinot noir for *N. luteum* and *N. parvum* were 2.2 x 10^4 and 1.6 x 10^4 conidia/cm lesion, respectively.

On attached semi-hard tissues, lesion lengths on Sauvignon blanc and Pinot noir were not significantly affected by species (P=0.114; Appendix B.2.5), but were significantly affected by grapevine varieties (P=0.016; Appendix B.2.5). Lesions which developed after inoculation of Sauvignon blanc (2.6 cm) were significantly longer than those which developed on Pinot noir (2.4 cm). However, there was no interaction between the grapevine varieties and species (P=0.377; Appendix B.2.5). The number of oozed conidia was also significantly affected by species (P<0.001; Appendix B.2.6), with significantly higher numbers of oozing conidia for *N. luteum* (3.1 x 10^4 conidia/cm lesion) compared with *N. parvum* (2.1 x 10^4 conidia/cm lesion). However, mean numbers of oozed conidia were not affected by grapevine varieties (P=0.600; Appendix B.2.6), and there was no interaction between grapevine varieties and species (P=0.548; Appendix B.2.6). The mean numbers of oozed conidia on semi-hard tissues of Sauvignon blanc were 3.2 x 10^4 and 2.3 x 10^4 conidia/cm lesion for *N. luteum* and *N. parvum,* respectively and the mean number of oozed conidia on soft tissues of Pinot noir were 3.1 x 10^4 and 2.1 x 10^4 conidia/cm lesion for *N. luteum* and *N. parvum,* respectively. Control plants did not show any symptoms typical of Botryosphaeriaceae species.

2.3.1.4 Pathogenicity of mixed isolate mycelium of *N. luteum* and *N. parvum* on woody trunks of two different grapevine varieties.

The lesions were hard to see so the bark was removed from each trunk prior to measuring the lesion length (Figure 2.12). Lesion lengths differed significantly between the species (P<0.001; Appendix B.2.7) and grapevine varieties (P=0.046; Appendix B.2.7) but there was no variety and species interaction (P=0.963; Appendix B.2.7). Significantly longer lesions developed on Sauvignon blanc (1.7 cm) compared with Pinot noir (1.5 cm), and *N. luteum* caused significantly longer lesions (1.9 cm) compared with *N. parvum* (1.3 cm). The mean lesion lengths on Sauvignon blanc for *N. luteum* and *N. parvum* were 2.0 and 1.4 cm, respectively and on Pinot noir were 1.8 and 1.2 cm for *N. luteum* and *N. parvum,*

respectively. Characteristic Botryosphaeriaceae colonies were recovered when small pieces from infected trunk lesions were plated onto PDA. No lesions characteristics of Botryosphaeriaceae species were observed and no characteristic colonies were isolated from control plants.

Figure 2.12 De-barked trunks of 2-year-old vines grown in pots showing lesions 6 weeks after inoculation with *Neofusicoccum luteum* and *N. parvum* onto wounded trunks of two grapevine cultivars (Sauvignon blanc (SB), Pinot noir (PN).

2.3.1.5 Pathogenicity of *N. luteum* and *N. parvum* mycelium on Pinot noir and Sauvignon blanc in the vineyard

On attached hard green shoots of Sauvignon blanc and Pinot noir, lesion lengths differed significantly between the species (P<0.001; Appendix B.2.8) and grapevine varieties (P<0.001; Appendix B.2.8). Significantly longer lesions developed after inoculation of Sauvignon blanc (3.1 cm) compared with Pinot noir (2.1 cm), with *N. luteum* (3.0 cm) causing significantly longer lesions compared with *N. parvum* (2.7 cm). However, there was no significant interaction between varieties and species (P=0.136; Appendix B.2.8). The mean lesion lengths on hard green shoots of Sauvignon blanc were 3.6 and 2.8 cm for *N. luteum* and *N. parvum,* respectively, on Pinot noir were 2.3 and 2.0 cm for *N. luteum* and *N. parvum,* respectively (Figure 2.13a). The number of oozed conidia was not significantly affected by the species (P=0.428; Appendix B.2.9), but were affected by the grapevine variety (P<0.001; Appendix B.2.9). Inoculation of Sauvignon blanc resulted in 6.9 x 10^4

conidia/cm lesion which was significantly greater than for Pinot noir (2.6 x 10^4 conidia/cm lesion). There was no interaction between grapevine varieties and species (P=0.863; Appendix B.2.9). The mean numbers of conidia oozed from Sauvignon blanc were 7.1 x 10^4 and 6.7 x 10^4 conidia/cm lesion for *N. luteum* and *N. parvum,* respectively, which were significantly different from Pinot noir with 2.4 x 10^4 and 2.0 x 10^4 conidia/cm lesion for *N. luteum* and *N. parvum,* respectively.

On attached canes, lesion lengths on Sauvignon blanc and Pinot noir were significantly affected by species (P<0.001; Appendix B.2.10) and grapevine varieties (P=0.021; Appendix B.2.10). Significantly longer lesions developed after inoculation of Sauvignon blanc (1.7 cm) compared with on Pinot noir (1.5 cm), with significantly longer lesions caused by *N luteum* (1.8 cm) compared with *N. parvum* (1.4 cm). However, there was no interaction between the grapevine varieties and species (P=0.253; Appendix B.2.10). The mean lesion lengths on Sauvignon blanc for *N. luteum* and *N. parvum* were 1.9 and 1.5 cm, respectively, and on Pinot noir were 1.7 and 1.4 cm, respectively.

2.3.2 Effects of environmental conditions on sporulation

2.3.2.1 Detached semi-hard green shoots

There were significant effects of isolate, temperature and relative humidity on numbers of conidia oozed by the isolates of *N. luteum* and *N. parvum* (P<0.001 for all; Appendix B.3.1). Overall, the species effect was not significant (P=0.895; Appendix B.3.1). The isolate effect followed a similar trend to that shown in Figure 2.11b, with *N. parvum* G69a1 producing the greatest number of conidia *(*P<0.05) (Figure 2.13). The greatest numbers of conidia were oozed at 25°C (3.1 x 10^4 conidia/cm lesion) and least at 10°C (5.6 x 10^3 conidia/cm lesion). The greatest number of conidia were oozed at 100% RH (2.9 x 10^4 conidia/cm lesion) and the least at 80-81% RH (1.0 x 10^4 conidia/cm lesion). There were significant interactions between isolates and temperature (P=0.010; Appendix B.3.1), isolates and RH (P=0.002; Appendix B.3.1), and temperature and RH (P<0.001), as well as an interaction between isolates, RH and temperature (P=0.046; Appendix B.3.1), which seemed to be associated with the apparent preferences of some isolates for specific conditions (Figure 2.14). There were significant interactions between species and temperature (P=0.004; Appendix B.3.1), species and RH (P=0.003; Appendix B.3.1), and temperature and RH (P<0.001; Appendix B.3.1), but not between species, RH and temperature (P=0.179; Appendix B.3.1). Overall, higher conidial numbers were oozed at 25°C by both species (Table 2.1). However, *N. parvum*

isolate G69a1 oozed most conidia at 20°C, while all other *N. parvum* isolates oozed most conidia at 25°C (Figure 2.14).

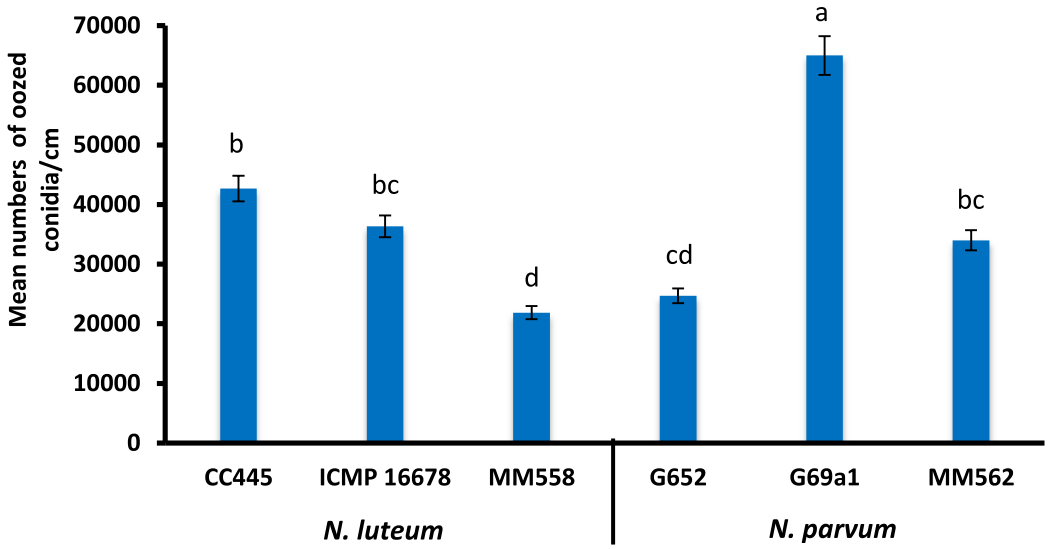


Figure 2.13 Mean numbers of conidia oozed from 1 cm lesion lengths which developed on detached semi-hard shoots of Sauvignon blanc infected by different isolates of *Neofusicoccum luteum* and *N. parvum*. Error bars represent standard errors of the means, and bars with different letters are significantly different at $P \leq 0.05$.

Table 2.1 Mean numbers of conidia (x10^3) oozed from 1 cm sections of Sauvignon blanc grapevine stem tissue infected with *Neofusicoccum luteum* and *N. parvum,* after incubation at 10, 15, 20 and 25°C and 80-81, 93-96 and 100% relative humidities (RH) for 2 days.

Species	**Mean numbers of oozed conidia oozed from 1 cm infected shoots (10^3)**					**RH Means both species**
	RH	10°C	15°C	20°C	25°C	
N. luteum	81-81 %	3.2	10.0	9.8	21.2	**10.2c[b]**
	93-96 %	3.6	1.03	11.6	30.6	**17.0b**
	100 %	9.2	18.0	25.9	43.8	**29.0a**
N. parvum	81-81 %	3.2	9.6	9.4	14.6	
	93-96 %	4.4	19.4	20.8	31.0	
	100 %	9.6	27.2	50.0	46.4	
Temperature		**5.6d[a]**	**16.4c**	**21.4b**	**31.6a**	

Means followed by different letters, across [a]temperatures in row or [b]RH in column, are significantly different at $P \leq 0.05$.

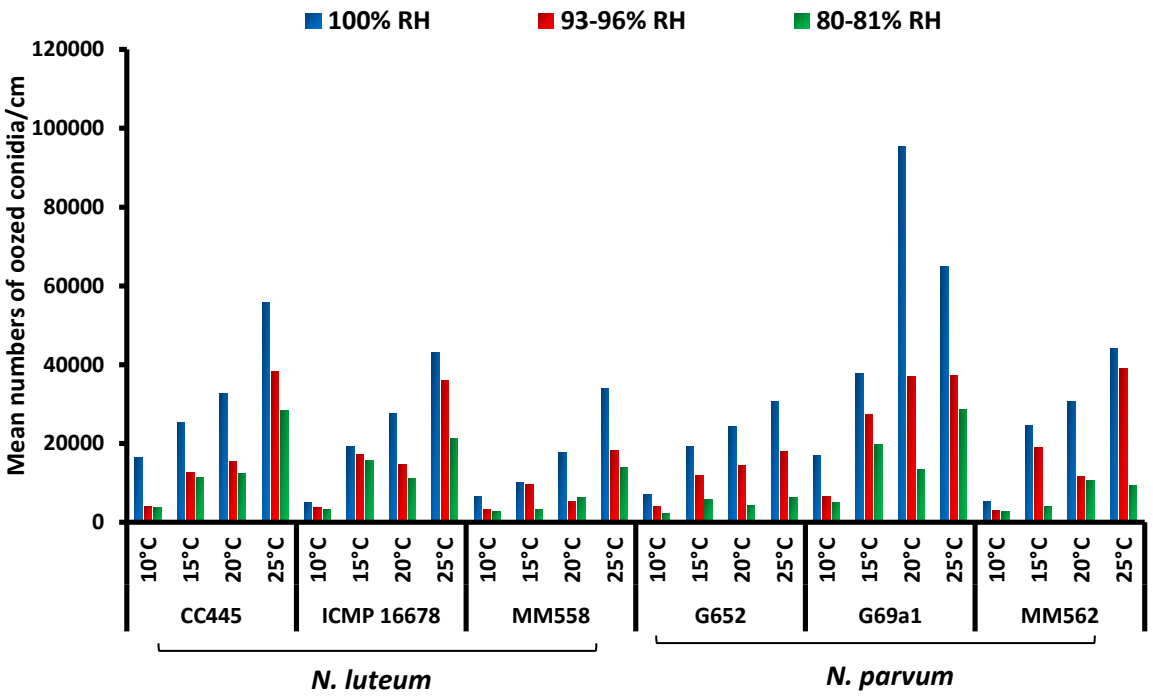


Figure 2.14 Mean numbers of conidia oozed from detached 1 cm semi-hard Sauvignon blanc grapevine stem tissues infected with three isolates each of *Neofusicoccum luteum* and *N. parvum*, and incubated at four different temperatures and three different relative humidities (RH).

2.3.2.2 Attached soft green shoots on potted vines

There was a significant effect of isolates on numbers of conidia oozed but not of species (P<0.001, P=0.362, respectively; Appendix B.3.2), with *N. luteum* CC445 producing significantly (P<0.05) greater numbers of conidia compared with all other isolates, followed by *N. parvum* G69a1, which was significantly higher than the remaining isolates (Figure 2.15). There were significant effects of temperature and relative humidity (both P<0.001; Appendix B.3.2) on conidial numbers oozed by the isolates of *N. luteum* and *N. parvum* (Appendix B.3.2), with highest numbers of conidia oozed at 25°C (3.6 x 10^4 conidia/cm lesion) and 100% RH (3.8 x 10^4 conidia/cm lesion) (Table 2.2). There was a significant interaction between temperatures and isolates (P=0.020; Appendix B.3.2) which appeared to be associated with *N. parvum* isolate G69a1 oozing higher numbers of conidia at 20°C than at 25°C (Figure 2.16), in contrast to the rest of the isolates. There was also a significant interaction between temperature and relative humidity (P=0.031; Appendix B.3.2), which was associated with a greater increase in numbers of conidia at 100% than 93% RH being evident at 20°C than at 25°C. There was no significant interaction between isolates and RH (P=0.137; Appendix B.3.2). There was also a significant interaction between isolates, temperature and RH (P=0.039; Appendix B.3.2).

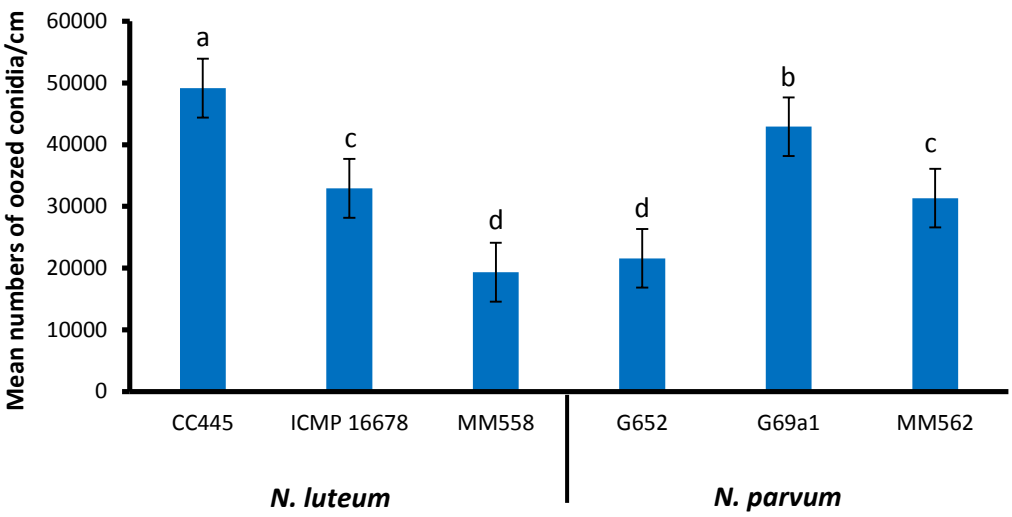


Figure 2.15 Mean numbers of conidia oozed/cm from attached Sauvignon blanc soft green shoots containing lesions caused by *Neofusicoccum luteum* and *N. parvum*. Error bars represent standard errors of the means, and bars with different letters are significantly different at $P \leq 0.05$.

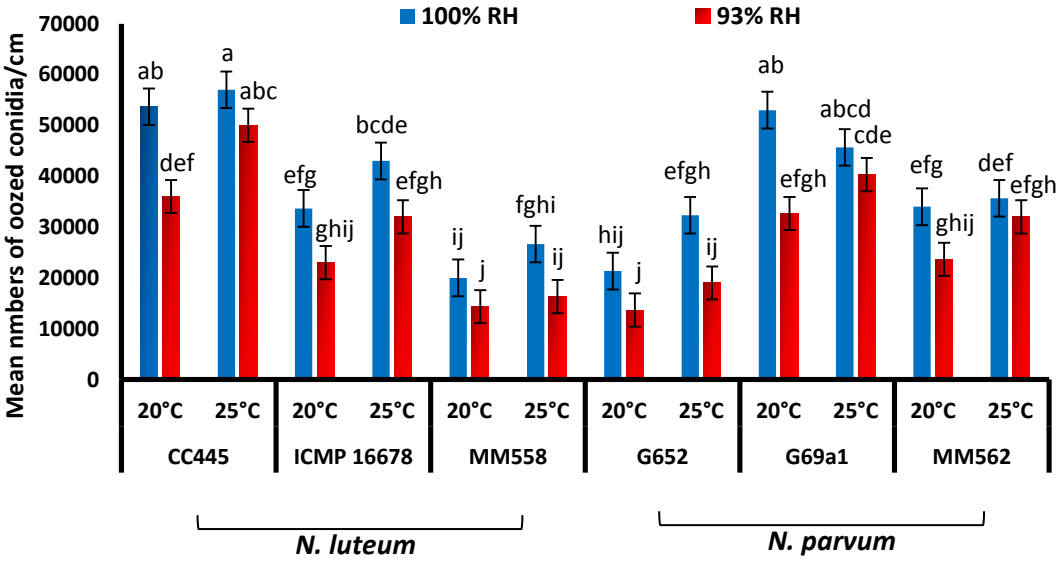


Figure 2.16 Mean numbers of conidia oozed from attached Sauvignon blanc soft green shoots of grapevine infected with isolates of *Neofusicoccum luteum* and *N. parvum*, when incubated at two different temperatures and two different relative humidities (RH).

Table 2.2 Mean numbers of conidia (x10^3) oozed from 1 cm sections of infected Sauvignon blanc soft green shoots of grapevine, which were incubated at two different temperatures (20 and 25°C) and two relative humidities (RH) (93 and 100%) for 2 days.

	Mean numbers of oozed conidia (x 10^3) from 1cm infected shoots			**RH Mean Both Species**
Species	RH	20°C	25°C	
N. luteum	93%	24.4b[a]	32.8ab[a]	**28.1b[b]**
N. luteum	100%	35.8ab	42.2a	**38.0a**
N. parvum	93%	23.3b	30.4ab	
N. parvum	100%	3 6.1ab	37.8a	
Temperature Mean		**29.9b**	**35.8a**	

Means followed by different letters within columns and for the [a]temperature and [b]RH means, are significantly different at $P \leq 0.05$.

2.3.3 Release of conidia from naturally infected vines in vineyards

During the 6 months when vineyards were observed, pycnidia were found oozing five times (Table 2.3). The effects of environmental variables [average dew point, leaf wetness, RH, mean air temperature and total rainfall/day] at the time and previous night are shown in Table 2.3. Ooze from infected canes was collected during or just after rain fall and also on dry mornings when there had been rain the previous night. When the ooze was observed under the microscope, conidia characteristic of *Neofusicoccum* and *Diplodia* species were observed (Figure 2.17). At Brancott and Fairhall Vineyards, 66.7% and 50% of ooze samples, respectively, were positive for Botryosphaericeae species conidia.

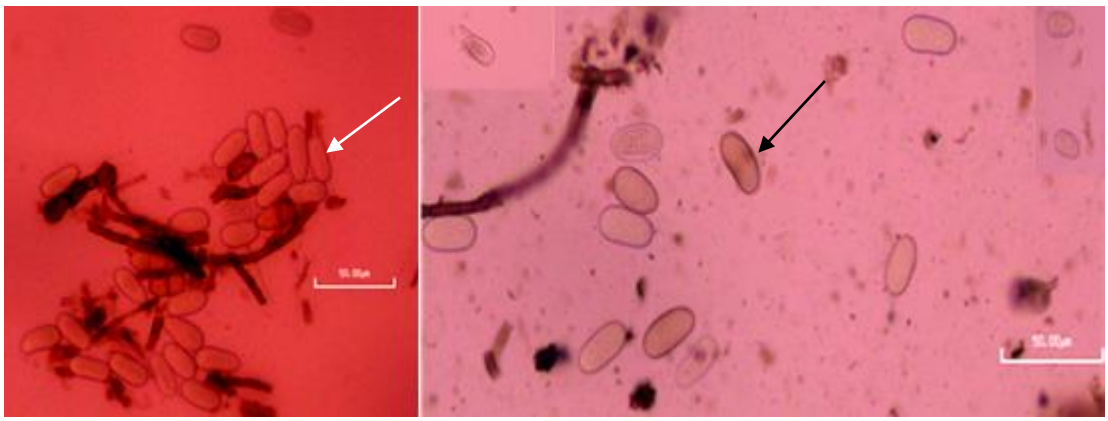


Figure 2.17 Conidia characteristic of *Neofusicoccum* (white arrow) and *Diplodia* species (black arrow). Scale bars represent 50 µm.

Table 2.3 Average environmental variables for the previous night and in the morning when conidia were seen to ooze from pycnidia on infected canes in the Brancott and Fairhall Vineyards, Blenheim on specific dates.

Envir. variables	Dates in 2014 and 2015									
	28th Sep		10th Dec		31th Dec		6th Mar		27th April	
	Prev. Night	Morn.	Prev. Night.	Morn.	Prev. Night.	Morn.	Prev. Night.	Morn.	Prev. Night.	Morn.
Max. dew point (°C)	9.1	4.4	14.2	14.2	10.7	18.5	14.6	16.2	14.4	14.1
Total rain (mm/day)	7		24.8		10.2		40.2		18.1	
Max. leaf wetness (%)	90.5	80.5	87.4	100.9	0.6	14.1	78.4	104.9	98.3	93.8
Max RH (%)	93.2	86.7	82.1	90.9	79.1	81.2	77	91.5	94.7	90.8
Max. air temp. (°C)	10.2	8.4	18.2	16.9	15.6	18.4	19.9	19.4	16.8	18.5

2.3.3.1. Identification of strains isolated from conidial ooze on shoots in vineyards

Of the 69 samples, the frequency of recovered species was *D. mutila* (39.1%), *N. australe* (30.4%), *N. parvum* (14.5%), *N. luteum* (5.8%), *D. seriata* (7.2%) and *N. ribis* (3.0%) (Table 2.4). At Brancott Vineyard, the predominant species was *D. mutila* (47.5%), followed by *N. australe* (25.0%), *N. parvum* (12.5%), *N. luteum* (7.5%), *D. seriata* (5.0%) and *N. ribis* (2.5%). At Fairhall, the predominant species was *N. australe* (38%) followed by *D. mutila* (27.7%), *N. parvum* (17.2%), *D. seriata* (10.3%), *N. luteum* (3.4%) and *N. ribis* (3.4%).

Table 2.4 Incidence of botryosphaericeae spp. being isolated from conidial ooze recovered from infected canes in the Brancott (Br) and Fairhall (FH) vineyards, Blenheim on specific dates.

	Detection of different Botryosphaeriaceae species									
	28th Sept 2014		10th Dec 2014		31st Dec 2014		6th March 2015		27th April 2015	
	Br	FH	Br	FH	Br	FH	Br	FH	Br	FH
N. luteum	1	-	-	-	-	1	2	-	-	-
N. parvum	-	1	2	2	1	-	1	2	1	-
N. australe	2	3	2	-	-	3	3	3	3	2
D. seriata	-	-	1	2	-	-	-	-	1	1
D. mutila	5	2	4	2	2	1	5	2	3	1
N. ribis	-	-	-	-	1	1	-	-	-	-
Total	**8**	**6**	**9**	**6**	**4**	**6**	**11**	**7**	**8**	**4**

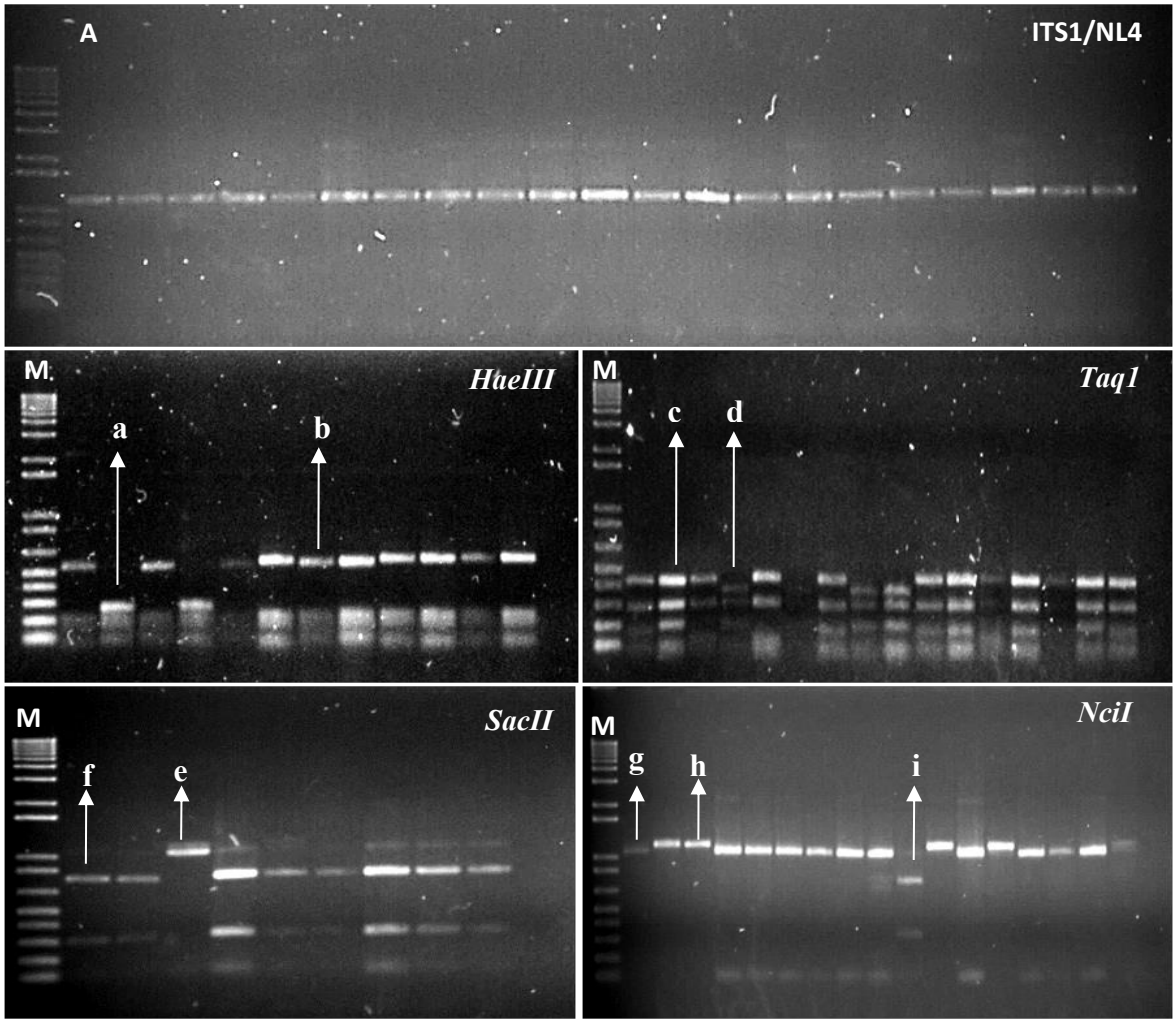


Figure 2.18 Representative gels of the amplified 1200 bp fragment of the ribosomal DNA using ITS1/NL4 primers (A). Gels below show PCR products with ITS1/NL4 primers which were digested with restriction enzymes (*HaeIII, Taq1, SacII* and *NciI*). Characteristic patterns for a) *N. parvum*, b) all non-*N. parvum*, c) *N. luteum*/*N. australe*, d) all non-*N. luteum*/*N. australe*, e) *N. luteum*, f) *N. australe*, g) *D. mutila*, h) *N. ribis*, i) *D. seriata*, M= 1 kb^+ ladder.

2.4 Discussion

In this study detached grapevine shoots of Sauvignon blanc were infected by different isolates of *N. luteum* and *N. parvum* to determine the variation in lesion development and conidial production by different isolates of the two species, with the results also allowing selection of the best isolates for further experiments. The results indicated that *N. luteum* lesions were able to progress faster than those of *N. parvum* on both soft and semi-hard shoots. The same pattern was shown by Amponsah *et al*. (2011) who reported that the mean lesion lengths produced on green shoots by isolates of *N. luteum* (73.2 mm) were longer than for isolates of *N. parvum* (57.9 mm) using Pinot noir plants. Similar results were shown by Urbez-Torres and Gubler (2009); when they examined the pathogenicity of nine Botryosphaeriaceae species, on 1-year-old dormant grapevines of variety Crimson in California they ranked *L. theobromae* as being the most pathogenic, *N. luteum* as the second and *N. parvum* as the third most pathogenic species overall. In contrast, Billones-Baajiens *et al.* (2014) showed that *N. parvum* was the most pathogenic species among the three isolates each of *N. parvum, N. luteum* and *N. australe*, with lesion means of 79.9, 48.2 and 47.8 mm, respectively. Clearly, several of the Botryosphaeriaceae species should be considered major pathogens of grapevines. The differences in species pathogenicity reported in the different countries may have been associated with a number of experimental factors, including the differences between selected isolates, the conditions of the experiments and the grapevine varieties chosen, which have generally varied between countries. Further, use of mycelium inoculum which was used in all the above studies as well as the current study may also have improved efficiency of infection, as shown by Amponsah *et al.* (2014). With *N. luteum* inoculation of detached soft green shoots, they showed that mycelial inoculum caused faster pathogen progression than conidial inoculum and that greater concentrations of conidia in the inoculum led togreater pathogen progression than the lower concentrations tested. Overall, this study has illustrated the importance of pathogenicity testing with a range of isolates, and with different scion varieties, and indicates that the outcome of lesion development is dependent on isolate capability as well as host factors.

In this study, different isolates within a species had different pathogenicity levels. Isolates CC445 for *N. luteum* and G69a1 for *N. parvum* produced lesion lengths at least twice as long as for other isolates within the same species. Further, five of the eight *N. parvum* isolates and two of the six *N. luteum* isolates produced only moderate lesions on detached soft shoots and produced minimal lesions on detached semi-hard shoots. Van Niekerk *et al.* (2004) also

showed differences between isolates since *D. seriata* isolate STE-U 5139 produced lesions twice as long as for other isolates of the same species. They also reported that for *N. australe* one of the isolates produced lesion lengths twice as long as the other isolates in their study. Isolate differences were also reported by Billones-Baajiens *et al.* (2014) for *N. parvum,* with isolate P069 causing the longest overall lesions (mean of 43.3 mm) followed by P178 (23.1 mm) while the other isolates were generally similar in their effects (8.6-17.5 mm) on six scion varieties of potted plants. In a study by Amponsah *et al.* (2011), with three isolates each of *N. australe, N. luteum, N. parvum, D. mutila* and *D. seriata*, longest mean lesions were produced by *N. luteum* and *N. australe,* but there was significant variation between isolates of *N. luteum* and *D. mutila.* Further, similar observations were reported by Urbez-Torres and Gubler (2009); on rooted cuttings of the table grape variety Crimson there was significant variation in lesion lengths between the eight isolates each of all nine species tested. The canes inoculated with *N. luteum* showed highest variability among isolates, ranging from 7.3 cm to 31.3 cm of vascular discolouration. The variation in disease responses seen in this study and other studies is likely to be due to inherent variation between isolates of factors involved in pathogenicity such as production of enzymes and toxins involved in infection of host tissue and growth rates (Saldanha *et al.,* 2007). When Srivastava *et al.* (2013) added naturally occurring plant phenolic compounds to growth media, their *in vitro* results showed that these compounds inhibited growth of mycelium for *Botryosphaeria* isolates. Further they decreased laccase production and pectinase activity, which are associated with pathogenicity.

The production of conidia from 1 cm lesion sections caused by all isolates on detached stem tissues, when placed in high relative humidity for 1 day, showed that more conidia were produced from the more pathogenic isolates (longer lesions produced) of any species, except for one *N. parvum* isolate (G69a1) which produced more conidia than the most pathogenic isolate of *N. luteum*. The release of more conidia from more pathogenic isolates might be due to production of more pycnidia on lesions caused by these isolates which ultimately resulted in the release of more conidia. However, in the current study, the 1 cm pieces used for sporulation comprised most of the lesion tissue for the least pathogenic species (which had lesions of 1.1 and 1.6 cm, respectively, for the soft and semi-hard shoots). Since the generally observed pattern during the disease process in this study, was for extension of a lesion followed by later sporulation from the necrotic areas of the lesion, these small lesions may not have been mature enough for optimum sporulation to occur (Sammonds *et al*., 2016). These researchers also reported in their study that pycnidial production for *B. dothidea* was

close to the inoculation point (the oldest part of the lesion). However, the relationship between lesion pathogenicity and conidial production was not consistently reported by Amponsah (2011) who found that *N. parvum* isolates caused relatively long lesions on detached green shoots of Pinot noir, but no pycnidia or conidia.

The attached soft green, semi-hard green shoots and trunks on potted plants of two grapevine varieties in this study were susceptible to infection by two species of *Neofusicoccum* (mixed isolates each of *N. luteum* and *N. parvum)*, with the mean lesion lengths being significantly longer for *N. luteum* than for *N. parvum.* However, on attached semi-hard green shoots and on woody trunks mean lesion lengths differed significantly between varieties, showing Sauvignon blanc to be more susceptible than Pinot noir with both species tested. A similar result occurred when mature canes in a vineyard were inoculated with the same type of inoculum. Billones-Baajiens *et al.* (2014) also reported differences between varieties when 1-year-old trunks of six grapevine scion varieties were inoculated with three isolates each of *N. luteum*, *N. parvum* and *N. australe.* Lesion lengths were significantly different among varieties, with Merlot and Pinot noir having significantly longer lesions of 2.1 and 2.0 cm, respectively, than the 1.6 cm for Sauvignon blanc. However, when Amponsah *et al.* (2011) investigated varietal susceptibility of Cabernet Sauvignon, Chardonnay, Sauvignon blanc, Pinot noir and Riesling they found no varietal differences on excised green shoots inoculated with one isolate each of *N. luteum*, *N. australe*, *N. parvum* and *D. mutila*. The 2003-2004 survey in California, conducted by Gubler *et al*. (2005) found that Botryosphaeriaceae species were isolated most often from Sauvignon blanc (63.6%), followed by Chardonnay (54.5%) and few Botryosphaeriaceae infections were found with Thompson seedless (12.2%).

In the current study, inoculation with *N. luteum* and *N. parvum* onto the woody trunks of 15 month old potted grapevines caused no external symptoms except at the inoculated wounded point. However, when the bark was removed from these woody trunks, brown necrosis was observed. Potted plants in this study did not develop bark symptoms during the 1 month period of incubation, however external symptoms were visible in field vines when lesions of unknown age were much larger than in the potted vines used here. Amponsah *et al.* (2009a) also showed that inoculation of 2-year-old trunks of grapevines with *N. australe*, *N. luteum* and *D. mutilia* caused no external symptoms except at the inoculation point; however, inoculation with only *N. parvum* caused external wood necrosis 7 months after inoculation. In the study by Billones-Baajiens *et al.* (2014), it was also found necessary to peel the bark from

the canes so that lesions caused by *N. luteum, N. parvum* and *N. australe* could be measured 18 days after inoculation. Amponsah *et al.* (2014) also reported that dieback due to inoculation by *N. luteum* did not develop in potted plants until after plants were water stressed for 2 months (15% field capacity). In another experiment, they also showed that lesions developed only after 8 months when plants had entered winter dormancy. These results suggest that *N. luteum* and *N. parvum* may have a tendency to penetrate and colonize inner wood layers, and that external symptoms occur only when the lesions are well-developed and the plants not able to grow vigorously.

These pathogenicity experiments showed that *N. luteum* consistently produced longer mean lesions than *N. parvum* when these species were used to infect wounded attached and detached tissues of grapevines and at different phenological stages (soft green shoots, semi-hard green shoots and woody trunks). The pathogenicity assay on detached tissues provided a useful method to determine the pathogenicity of different isolates, despite the differences in lesion lengths between detached and attached tissues, as also reported by previous researchers (Savocchia *et al.,* 2007; Amponsah *et al.,* 2008; Urbez-Torres and Gubler, 2009). Van Niekerk *et al*. (2004) also reported that Botryosphaeriaceae species could infect different stem tissues of wounded grapevines (green shoots, mature canes and mature wood) but they did not compare the pathogenicity levels on these tissues. However, future experiments should inoculate the detached cuttings within 1 day of removal from the plants as this study has shown that inoculating cuttings held over several days led to differences in pathogen development.

This study investigated some environmental factors that may affect release of Botryosphaeriaceae conidia. Isolates of *N. luteum* and *N. parvum* released relatively high numbers of conidia (>10^4 /cm stem tissue) at all temperatures and relative humidities tested in this study. Maximum conidial ooze occurred at 25°C except for one isolate of *N. parvum* (G69a1) which produced more conidia at 20°C. Fewest conidia were released at 10°C for all isolates of *N. parvum* and *N. luteum,* and similar numbers of conidia were oozed at 15 and 20°C for all isolates except G69a1. Copes and Hendrix (2004) determined the release of conidia of *D. seriata, B. dothidea* and *L. theobromea* at temperatures of 6, 12, 18, 24 and 30°C with constant moisture and found that maximum numbers of conidia oozed at 24°C for *B. dothidea,* 18-24°C for *D. seriata* and at 12, 18 and 24°C for *L. theobromea.* However, the set-up of their experiment was very different to that described here. The autoclaved water shoots of apple and peach were cut into 1 cm segments, inoculated and placed into glass

tubes plugged with cotton wool. Assessment of conidia oozed by these 'stem cultures' involved the removal of pycnidia which were crushed in water and then the conidial solution used for counting. The current experiment showed that moisture was essential for release of conidia and that more conidia were released in warm conditions than cool conditions. Since northerly (warm) winds commonly occur during rainfall in Marlborough this indicates the greater potential for conidial release in Marlborough than in other regions such as Canterbury where southerly winds often accompany rainfall.

Although no other studies have been done on release of Botryosphaeriaceae conidia in relation to effect of RH and temperature, there are some studies on trapping of conidia in the field, which concur with results of the current study. Knutazman *et al.* (2009) reported conidial dispersal of *Diplodia* species at field temperatures from 9°C upwards in France and Urbez-Torres *et al*. (2010b) reported the release of conidia in California at temperatures of 3-7°C during the winter months. However, Amponsah *et al.* (2009b) reported that greatest numbers of Botryosphaeriaceae species conidia were trapped in summer in rain water run-off, which they concluded was due in part to the greater amount of grapevine canopy which could produce more conidia from the infected wood. Xu *et al.* (1998) investigated the effects of several factors on the incidence of Nectria canker on potted apple trees of varieties Bramley's and McIntosh grown from seedlings. They found that temperature was not a limiting factor for inducing conidial release as many conidia were trapped in weeks when the weekly median (of average daily temperatures) was as low as 10°C. Further, temperature had little effect on disease incidence but moisture did. Incidence decreased with increasing duration of wet periods, being 60, 51, 48 and 45% for 6, 12, 24 and 48 h of wet periods following inoculation, which apparently conflicted with other studies. However, these conflicting results are consistent with the concept that all mature conidia are released as soon as conditions are sufficiently moist, with little further release until new conidia have matured. This was clearly demonstrated by Ramos *et al*. (1975) who showed that ascospores of *Eutypa armeniacae* were less in spring after a large release in January.

In this study, high RH was necessary for oozing of conidia. Maximum numbers of conidia oozed at 100%, moderate numbers at 93-96% and low numbers at 83% RH. Also, Amponsah *et al.* (2008) showed that when stem lesions containing pycnidia were maintained at ambient RH, they did not ooze conidia, but when placed in high relative humidity for 36 h at 23-24°C they oozed conidia. Similar results were reported by Gough and Lee (1985) who showed that numbers of conidia oozing from pycnidia of *Septoria tritici* were also affected by RH. On

leaf lesions, pycnidia discharged 4.0 x 10^3 conidia at 100%, while much lower numbers were oozed at 86% RH (1.7 x 10^2 conidia). Since conidia are not usually considered to be survival spores, factors that promote release are likely to be similar to factors optimum for germination. Amponsah *et al*. (2010) reported that maximum germination of *N. luteum*, *N. australe* and *D. mutila* conidia occurred at 100% RH with no germination at 84% RH. Aruaz and Sutton (1991), who studied the germination of *D. seriata* conidia in different RH levels, reported different moisture effects on conidial germination for *D. seriata* isolates. Although conidia of one isolate did not germinate at all, most conidia from two of the three isolates were able to germinate in free water, while conidia of only one isolate were able to germinate under high RH, with declining rates as RH reduced from 100 to 92% and no germination at 88.5% RH. Arauz and Sutton (1990) also observed that 4 h continuous wetness at 24°C was required for conidial germination of *D. seriata* conidia on apples. However, as little as 1 h of subsequent dryness was sufficient to prevent infection of apple leaves. They also reported that 4.5 to 13 h wetness was required at the optimum temperature of 26.6°C for leaf infection on Golden Delicious seedlings and 9 h of wetting at 20-24°C for infection of Golden Delicious apple fruit. Moisture availability is therefore important for conidial release, germination and infection by Botryosphaeriaceae species.

In the current study, pycnidia on infected grapevines in the vineyards were also observed to ooze cirrhi of conidia at maximum air temperatures of 8.2-19.9°C and RH 77-94.7%, respectively. The conidial ooze collected when the lowest temperature of 8.2°C might indicate that conidia are released from pycnidia at lower temperature than was tested in the *in vitro* study, or alternatively, conidial release might have occurred the previous night when the air temperature was 10.2°C. Further, the grapevine tissue could have retained some heat and so affected the rate of exudation. No other researchers have reported conidial ooze in the vineyards under natural environmental conditions, probably because the ooze, which is exuded in moist conditions, is often removed by rain splash. Studies on effects of environmental conditions on release of conidia have reported that high relative humidity could initiate sporulation by Botryosphaeriaceae spp. (Michailides and Morgan, 1993; Aruaz and Sutton, 1989). Van Niekerk *et al*. (2010) studied the spore dispersal pattern of Botryosphaeriaceae species in South Africa and found that air-borne spores of *Diplodia* and *Dothiorella* spp. were present after periods of high relative humidity at or above 70%. Pusey (1989) conducted an experiment in peach orchards and found that air-borne ascospores of Botryosphaeriaceae species were able to be trapped after periods of dew or mist but not

measureable rainfall. Studies by Kuntzamann *et al.* (2009) in France reported that weather conditions such as fog and snow could also induce the release of Botryosphaeriaceae spp. spores. Spore-trapping studies in France and New Zealand reported an abundance of Botryosphaeriaceae species spores throughout the year, maximum release being associated with rainfall during summer months (Amponsah *et al.,* 2009b; Kuntzamann *et al.,* 2009). Further, Valencia *et al.* (2015) demonstrated in Chile, that large numbers of air-borne conidia were trapped mainly after or during precipitation events of at least 0.2 mm during autumn and winter. However, in the current study, 7 mm of rain was the minimum which led to visible oozing of cirrhi in the field, but it is possible that less rainfall had caused release of fewer conidia in minute cirrhi which were not obvious to the naked eye.

Conclusion

This study showed that isolates of the two most commonly found species of the Botryosphaeriaceae on grapevine in New Zealand, *N. parvum* and *N. luteum,* were able to infect different kinds of grapevine excised and attached tissue (green and semi-hard shoots, canes and trunks), although there were differences in lesion lengths between isolates. This study therefore provided evidence for the selection of isolates for further experimentation in this research programme, and using mainly *N. luteum* which was more pathogenic than *N. parvum.* The *in vitro* study of effects of temperature and relative humidity on release of conidia has also indicated the best conditions for release of conidia (100% RH and 25°C) to be used in these studies. Since Sauvignon blanc was more susceptible than Pinot noir, this variety was selected for most of the planned studies. Both *N. luteum* and *N. parvum* produced lesions on stems of potted grapevines within practical timeframes. Although lesions were produced more rapidly on detached shoots, the potted vines were considered to better reflect natural conditions so it was decided that they would be used in future experiments. Conidial ooze was found to be produced in natural conditions, and further investigations were planned to examine conidial dispersal in vineyards. Chapter 3 of this thesis is therefore focused on rain splash dispersal and the effects of wind direction on the dispersal distances of Botryosphaeriaceae conidia in the vineyards.

Chapter 3
Conidium dispersal in Marlborough vineyards

3.1 Introduction

Conidia and ascospores of Botryosphaeriaceae spp. are released during and following rainfall and then splash-borne and air-borne (Urbes-Torres and Gubler, 2008; Urbes-Torres *et al*., 2010a; Eskalen *et al.,* 2013). In New Zealand, many observations made in vineyards have not found perithecia but have shown that dispersal of these pathogens is mainly by conidia which are exuded from pycnidia in sticky tendrils called 'cirrhi', during moist conditions. These tendrils are able to mix with rain water for subsequent splash dispersal, with potential for aerial dispersal of droplets containing conidia. In a Canterbury vineyard, conidia from species of the Botryosphaeriaceae were trapped in rain water run-off during an entire year, but were not trapped in late summer on Vaseline™-coated slides, indicating that they were not air-borne in dry conditions (Amponsah *et al.*, 2009b).

The role of rainwater in the dispersal of conidia of the Botryosphaeriaceae has been reported for other crops by Pusey (1989) and Sutton (1981) who showed that conidia of *Botryosphaeria dothidea* and *D. seriata* were abundant in the air of peach and apple orchards, respectively, during rainy periods, and that the amount and duration of rainfall were the most important factors in dispersal of ascospores and conidia. In California, spores of Botryosphaeriaceae species were trapped with a Burkard spore trap during rainfall and during overhead sprinkler irrigation (Urbez-Torres *et al.,* 2010a). In that study conidia were trapped from mid fall to early spring and maximum numbers were trapped in winter. Kuntzmann *et al.* (2009) studied dispersal of Botryosphaeriaceae spp. spores (*D. mutila, D. seriata, B. quercuum* and *N. parvum*) in France and reported that the spores were trapped from spring to winter during periods of rain. In their study 90% of the spores of *D. mutila* and *D. seriata* were trapped during the grapevines' vegetative growth. In another study in South Africa, spores of Botryosphaeriaceae spp. were trapped with a Quest volumetric spore trap (Interlock Systems, Pretoria, South Africa) during or after rain, being trapped after as little as 0.25 mm rainfall, with more spores being trapped in 2005 when 339.2 mm rain fell in 48 rain days than in 2004 when 207.3 mm of rain fell in 44 rain days (van Niekerk *et al.,* 2010a).

This study was carried out to investigate the dynamics and efficacy of conidium dispersal and colonisation of grapevines by Botryosphaericeae spp. as pathogens in Marlborough vineyards.

3.2 Materials and Methods

3.2.1 Spore trap set-up in commercial vineyards (2013-2015)

3.2.1.1 Rain water spore traps set-up

In 2013, rainwater run-off traps were set up to catch Botryosphaeriaceae spp. conidia in the Waipara, Brancott, Renwick and Fairhall Vineyards of Pernod-Ricard Ltd to identify vineyards with natural infection. These were chosen as potential sites for experimentation so that the later planned release and recapture experiments with Botryosphaeriaceae pathogens would not deliberately introduce the pathogen to trunk-disease free vineyards. In each vineyard, two blocks were selected (18-22 years old symptomatic vines) (one Sauvignon blanc and one Pinot noir) and in each block four vines at least 20 m apart were randomly selected. Each rainwater spore trap consisted of a 210 × 297 mm transparent acetate sheet which funnelled the trapped water into a container below. The top straight edge of the acetate sheet was positioned by the head of the vine, the centre being stapled onto the trunk, and the two opposing corners stapled onto the wrapped-down canes. The lower corners of the sheet were folded inwards and stapled together to create a funnel. Each funnel base was placed into a 200 mL container tied onto the vine 20-50 cm above the ground (Figure 3.1a). After any full day of rain (6 hof rain), the collected water was removed for assessment as described by Amponsah *et al*. (2009b).

The trapped water was held at 4ºC for about 1 h to allow spores to settle, the supernatant (up to 60 mL) was discarded and the remaining ~40 mL was centrifuged at 10,000 × *g* for 15 min at 10°C. The supernatant was discarded and the pellet divided into two approximately equal portions. One portion was stored frozen at -20°C to be used in molecular identification with single-stranded conformation polymorphism (SSCP). The remaining portion of the pellet was added to 5 mL sterile water, vortexed briefly to mix and the suspension observed with a light microscope (Leitz LABORUX S, Germany) at ×100 magnification for presence of characteristic Botryosphaeriaceae spp. conidia. Aliquots (100 µL) of the suspension and the 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions were spread onto PDA amended with streptomycin, penicillin and chloramphenicol (0.05 g/L of each) (Sigma-Aldrich™ Co. St. Lous, MO USA) to suppress the bacterial growth, as well as Triton-X (1 mL/L) (Labchem, Ajax Finechem Pty Ltd) to restrict colony growth. Plates were incubated at 25°C in a 12:12 h light:dark cycle, with illumination by fluorescent and near ultra violet light (366 nm). After 3 days the plates were observed for typical colonies of Botryosphaeriaceae species.

In 2015, the same process was repeated in Brancott, Fairhall and Renwick Vineyards. The characteristic spores of *Diplodia* and *Neofusicoccum* spp. were counted using a haemocytometer as described in Chapter 2 Section 2.2.2.1 and identified by molecular methods as described below.

Figure 3.1 (a) Rain water spore trap, (b) Burkard spore trap placed in the vineyards to trap air-borne spores of Botryosphaericeae species.

3.2.1.2 Botryosphaeriaceae spp. conidia trapping 2014 (Burkard spore sampler)

Naturally released spores of Botryosphaeriaceae spp. were collected with a Burkard spore trap (Burkard Scientific Ltd, Uxbridge, Middlesex, UK). The Burkard spore trap (Figure 3.1b) has a built in vacuum pump which draws in air that may have air-borne fungal spores and pollen. The air containing the spores is passed over a sticky transparent plastic tape (Melinex tape) which is supported on a drum. The spores are trapped onto the tape that unwinds and is wound onto a second clockwork-driven drum, which is removed for assessment of the tape. The Burkard trap was run during and after rainfall events in the 3 months of autumn, 2014, at Brancott, Fairhall and Renwick Vineyards, two randomly selected blocks in each (one Sauvignon blanc and one Pinot noir), being rotated amongst the vineyards. The Melinex tape preparation and mounting was done according to instructions provided with the Burkard spore trap sampler. The Melinex tapes were cut into segments (48 mm), each representing one 24 h period, which were put onto glass slides that were examined with a microscope (Leitz LABORUX S) at 400× magnification. All conidia characteristic of Botryosphaeriaceae spp. were counted on each segment. The segments of Melinex tape positive for Botryosphaeriaceae spp. conidia were stored at -20°C for DNA extraction and

PCR analysis (Sections 3.2.2.1, 3.2.2.2 and 3.2.2.3). The numbers of Botryosphaeriaceae spp. conidia trapped at each weekly interval were compared with the temperature, relative humidity, dew point and rainfall data obtained from the weather station (http://hortplus.metwatch.co.nz/) nearest to the vineyard, to enable conidial release to be correlated with weather events.

3.2.2 Molecular confirmation of Botryosphaeriaceae spp. identity for conidia collected by rain water and Burkard spore traps

3.2.2.1 Extraction of DNA from rain water samples

DNA was extracted from all the rain water pellets using a PowerSoil™ isolation kit (MO BIO laboratories, California, USA) according to the manufacturer's instructions. Each pellet held at -20°C was thawed at room temperature for an hour, centrifuged at 30,000 × *g* for 10 min and the supernatant discarded. Aliquots of approximately 250 µL from all samples were added to PowerBead™ tubes which contained small beads and 2 mL of an aqueous solution of acetate and salts (to protect nucleic acid from degradation). These tubes were gently mixed by vortexing and 60 µL of solution C1 was added to each. After briefly vortexing to mix, the tubes were vortexed at maximum speed for 10 min in the vortex adaptor tube holder (MO BIO). The tubes were centrifuged at 10,000 × *g* for 30 s and then 500 µL of each supernatant was transferred to a clean 2 mL tube. To these tubes 250 µL of solution C2 was added, they were vortexed for 5 s and the tubes were incubated at 4°C for 5 min. After centrifugation at 10,000 × *g* for 1 min, the resulting supernatants (about 600 µL) were transferred to clean 2 mL tubes. To precipitate non DNA material, 200 µL of C3 solution was added to each tube. After briefly vortexing, the tubes were incubated at 4°C for 5 min and centrifuged at 10,000 × *g* for 1 min. The supernatants from each tube (approximately 750 µL) were then transferred to clean 2 mL tubes. Then 1200 µL of solution C4 was added to each tube and they were vortexed for 5 s. The resulting solution from each tube was then filtered through a spin filter by centrifugation at 10,000 × *g* for 1 min. The flow through from each solution was discarded, leaving the DNA bound to the silica membrane in the filter. To each spin filter 500 µL of solution C5 was added and centrifuged at 10,000 × *g* for 30 s. The wash solution that flowed through was discarded and the spin filter was then again centrifuged at room temperature for 1 min. The wash solution that flowed through from each spin filter was again discarded and these spin filter were then transferred to clean 2 mL tubes. To these tubes, 100 µL of solution C6 was added and the tubes were centrifuged at 10,000 × *g* for 30 s to release the DNA into solution.

A Nanodrop 3.0.0 spectrophotometer (Nanodrop Techologies Inc., Delaware, USA) was used to determine the concentration of extracted DNA by measuring absorbance at 280 nm. DNA samples were diluted to 20 ng/µL with UltraPure™ distilled water (Invitrogen). To check DNA quality, 5 µL of this DNA was mixed with 3 µL of loading dye (Appendix A.2.1) and separated by electrophoresis on a 1% agarose gel in 1 × TAE (Appendix A.2.2) at 10 V/cm for 50 min. The gel was processed as described in Section 3.2.2.3. The extracted DNA was stored at -80°C until used.

3.2.2.2 Extraction of DNA from melinex tape (Burkard spore trap)

DNA was extracted from the stored melinex tape for all Burkard spore samples obtained in Section 3.2.1.2 following the method of Kaczmarek *et al.* (2009). Each melinex tape sample was placed in a sterile 2 mL tube with glass beads (0.15 g; particle size 425–600 µm; Sigma, UK), 1000 µL CTAB buffer (Appendix A.2.3) and 0.2 volumes (200 µL) of 5% sarcosyl. The tubes were placed in a FRITSCH Pulverisette 23 (John Morris Scientific Ltd.) cell disrupter twice (1 min maximum each time) and were then incubated for 30 min at 70°C. The tubes were centrifuged for 5 min at 11,400 × g and the supernatant (approximately 700 µL) from each tube was transferred to a clean 1.6 mL tube. To each tube 800 µL chloroform:isoamyl alcohol (24:1) was added, mixed by inversion 50 times, and centrifuged for 10 min at 11,400 × *g*. The top aqueous layers (approximately 450 µL) from these tubes were then transferred to clean 1.6 mL tubes, 500 uL of 100% isopropanol was added to each and they were mixed by inversion 20 times. The tubes were then placed on ice for 10 min and centrifuged for 5 min at 11,400 × *g* to obtain a DNA pellet. The supernatants were discarded and the DNA pellets were washed with 200 µL ice-cold 70% (v/v) ethanol. After centrifugation for 1 min at 11,400 × *g* the tubes were inverted on paper towels to dry. Each DNA pellet was re-hydrated by adding 50 µL of 1 mM TE buffer (tris-ethylene diamine tetra acetic acid, pH 7.5; Sigma, UK) and the tubes were incubated at room temperature overnight. The concentrations of DNA were determined as described in Section 3.2.2.1 and the extracted DNA stored at -80°C until used.

3.2.2.3 PCR amplification of DNA from rain water and Burkard spore trap

The extracted DNA from each rainwater and Burkard spore trap sample was amplified in a PCR mixture containing 200 µM of each dNTP, 1 U Taq DNA polymerase (FastStart® Roche), 1 × PCR buffer (with 1.5 mM $MgCl_2$), 0.4 µM of each primer and 1 µL of DNA template. Each reaction volume was made up to 25 µL with UltraPure™ distilled water. The

negative control consisted of the same reagents but used UltraPure™ distilled water instead of template DNA. The positive control contained DNA template from a Botryosphaeriaceae spp. sample identified by Baskarathevan (2011) as *N. parvum* (G62b1). The primers used were the universal primers ITS1-F (5'-CTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') described by White *et al.* (1991). The amplification conditions were: initial denaturation of 5 min at 95°C, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C and 1 min at 72°C, and a final extension period of 10 min at 72°C. The resulting PCR product was diluted 1:200 in UltraPure™ distilled water and 1 µL was used as template for secondary amplification with multi specific primers BOT100F (5'-AAACTCCAGTCAGTRAAC-3') and BOT472R (5'-TCCGAGGTCAMCCTTGAG-3') using the same reagents and thermal cycle as listed above. Then 5 µL of the resulting PCR product was separated by electrophoresis on a 1% agarose gel for 50 min at 10 V/cm in 1 × TAE buffer and stained with ethidium bromide (0.05µg/mL, AMRESCO) for 10 min, destained in water for 5 min, and then photographed using a UV transilluminator (Versodoc™, BioRad Laboratory). The rest of the PCR product was stored at -20°C for single stranded conformational polymorphism (SSCP).

3.2.2.4 Identification of Botryosphaeriaceae species from rain water and Burkard spore traps by single stranded conformational polymorphism (SSCP)

SSCP was used to distinguish the species in the amplimers of all the positive bands (Ridgway *et al.,* 2011). The SSCP also included amplicons of *N. parvum* (P179), *N. luteum* (L125), *D. seriata* (N230), *D. mutila* (M232) and *N. australe* (B319) produced from pure cultures that were present in the Lincoln University Culture Collection whose identity had been confirmed by Baskarathevan *et al.* (2012). For each PCR product 0.4 µL was mixed with 20 µL of SSCP loading dye (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, 10 mM NaOH and 0.8 mM EDTA; pH 8). These samples were mixed with brief vortexing and were then heat denatured at 99°C for 7 min in a thermal cycler to separate the double stranded DNA into single strands. These tubes were immediately put into wet ice and when cool were loaded into wells of a MDE™ acrylamide gel (Appendix A.2.4). The gel was run at 22°C, 250V for 17 h in a BioRad Protean II xi vertical electrophoresis unit using 1 x TBE buffer (Appendix A.2.5). The gel was immersed in fixer (900 mL distilled H_2O, 100 mL 99% ethanol and 5 mL acetic acid) for 3 min and then silver stained (250 mL of fixer and 0.5 g of $AgNO_3$) for 5 min. The gel was briefly rinsed with distilled H_2O (dH_2O) for 2 min and then put into a developer solution (500 mL of dH_2O, 15 g NaOH and 0.5 mL of formaldehyde) for 10-40 min until the

bands became visible. This gel was again fixed for 5 min as decribed before and rinsedwith dH_2O. The gel was then put onto a filter paper and wrapped with cling film before being dried on a gel dryer (SGD210D Speed Gel™ System) at 68°C for 2-3 h. The dried gel was scanned with a photocopier with maximum dpi and colour and the file saved as TIFF/PDF. The band positions were compared with bands of the standard isolates, *N. parvum* (P179), *N. luteum* (L125) *D. seriata* (N230), *D. mutil*a (M232) and *N. austale* (B319).

3.2.3 Dispersal distances of *Neofusicoccum parvum* conidia during rainfall

3.2.3.1 Production of marker isolate conidia on green shoots

Apparently healthy semi-hard green shoots obtained from Sauvignon blanc vines growing in the Fairhall Vineyard were inoculated with a marker strain of *N. parvum* (B2141) described by Baskarathevan *et al.* (2013), using the methods described in Section 2.2.2.1. After 14 days, the lesion sections of the shoots (10-15 cm) were excised, surface sterilised by soaking for 30 s in 70% ethanol, washed with sterile water and dried overnight in a laminar flow unit. These shoots were then stored in a sterile sealed container at 4°C until used for the dispersal experiment. Two days prior to forecasted rainfall these shoots were placed in an enclosed sterile container with a moist sterile paper towel to stimulate oozing of conidia.

3.2.3.2 Rainwater dispersal experiments

In 2014, a bundle of ten shoot sections with lesions that oozed conidia of *N. parvum* isolate B2142 was fixed to the top of a post within the Brancott Vineyard at the beginning of the first forecast rainfall period after summer trimming and again after winter pruning. Containers (2 L) which collected rainwater (spore traps) were set up at distances of 0.5, 1, 5, 10 and 20 m in the direction of the prevailing wind, which was forecast (http://hortplus.metwatch.co.nz/) as easterly (9.6 km/h wind, autumn) (4.6 km/h, spring), and at 0.5, 1 and 5 m in three other equidistant directions around the post (Figure 3.2). If other grapvines posts were in the way, traps were placed to one side of the post. There were three 20 m apart experimental replications set up at the same times. The same process was repeated in autumn 2015 in the Fairhall Vineyard with easterly forcasted wind with an average speed of 4.2 km/h.

Figure 3.2 Ice cream containers (2L) for rain water collection placed on the ground around the *Neofusiccocum parvum* isolate B2141 (marker isolate) inoculum source.

In 2014, rain water samples were collected after 2 days, and the three replications for each distance and direction were combined prior to DNA extraction. In 2015, rain water samples were collected after 2 days and replicate samples were treated separately. Each rainwater sample was passed through a sieve with a 50 µm pore size and the resultant solution centrifuged at 10,000 × *g* for 15 min. The supernatant was discarded and the pellet divided into two portions, half for spore identification by light microscope (×100 and ×400) and the other half was stored frozen at -80°C for molecular identification.

3.2.4 Molecular confirmation of *N. parvum* (B2141) conidia in collected rain water

3.2.4.1 Genomic DNA extraction (positive control)

To provide DNA of the marker isolate (B2141), for control purposes, a mycelium plug of a single spore culture, which had been stored at -80°C was thawed and plated onto PDA, and incubated at 25°C in a 12:12 h light and dark cycle for 3 days. Mycelium plugs (3 mm) were cut from the edge of the culture and sub-cultured onto potato dextrose broth (PDB; Difco™ New Jersey, United States) in deep Petri dishes. After 3 days, the mycelium from each PDB

culture was removed with a sterile micropipette tip and transferred on to sterile Miracloth™ (Calbiochem) on a stack of 10-15 absorbent paper towels. The paper towel stack was folded over the mycelium and pressed for a few seconds to absorb excess PDB. The dried mycelium was removed, wrapped in aluminum foil and snap frozen in liquid nitrogen. It was then ground to a fine powder with a sterile pestle and mortar and processed using the Puregene® system (Gentra systems, Minneapolis, USA) to extract genomic DNA. About 200 mg of this powder was added to a 1.5 mL tube with sterile tweezers. To this tube, 400 µL of cell lysis solution was added and mixed by pippeting the liquid up and down several times. Then 1.5 µL of RNAase (10 mg/mL, Invitrogen) was added to it, swirled to mix and it was incubated at 37°C for 15 min. The tube was cooled to room temperature and 167 µL of the 'protein precipitation' solution was added to it. The tube was vortexed for 20 seconds and centrifuged at 13,000 × *g* for 3 min. The supernatant containing the DNA was poured into a clean 1.5 mL tube and 500 µL of 100% isopropanol was added and mixed by inverting several times. This tube was then centrifuged at 13,000 × *g* for 1 min. The supernatant was decanted and the remaining liquid was drained by inverting the tube onto absorbent paper. The pellet was then washed by adding 300 µL of 70% ethanol and centrifuging at 13,000 × *g* for 1 min. The liquid was drained as above and the tube containing the DNA was allowed to air-dry for 25-30 min. After drying, 30 µL of sterile distilled water was added to this tube and it was allowed to rehydrate overnight at 4°C.

3.2.4.2 DNA extraction from conidia in traps and PCR amplification with the nested PCR-RFLP process

DNA was extracted from all rain water samples as in Section 3.2.2.2. The primary PCR used primers forward (BP-1-42F; 5′-AGAGCGGATACAACGTCAGT-3′) and reverse (BP-1-42R; 5′-GGAGAGGAGAAAGTAGTGTG-3′), which were designed to amplify a product of 1550 bp (Baskarathevan *et al.,* 2012). Each 25 µL PCR tube contained 200 µM of each dNTP, 1 U Taq DNA polymerase (FastStart® Roche), 1 × PCR buffer (with 1.5 mM $MgCl_2$), 0.4 µM of each primer and 1 µL of DNA template. and UltraPure™ distilled water was added to make the final volume to 25 µL. The negative control contained UltraPure™ distilled water instead of template DNA. The positive control contained DNA template extracted from marker isolate B2141 as described in Section 3.2.4.1. Amplification of *N. parvum* was conducted as follows: denaturation at 94°C for 5 min, 40 cycles of 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 90 s, with a final extension at 72°C for 10 min. The resulting PCR product was separated by electrophoresis on a 1% agarose gel as described in Section 3.2.2.3.

The secondary PCR was conducted with the internal (nested) forward primer BP-1-42intF (5′-CCATGTGAAGTGGACCCAGA-3′) and reverse primer BP-1-42intR (5′-CTTCCTGAAATGACACCCGA-3′) which would amplify a 403 bp internal segment from the *N. parvum*. For this the primary PCR products were diluted 50-fold in UltraPure™ distilled water. A 1 µL aliquot of the diluted primary product was used as the template for the secondary PCR using the same amplification conditions and reagents as in the primary PCR (Baskarathevan *et al.,* 2012). Genomic DNA from the marker strain of *N. parvum* (B2141) was used as a positive control and distilled water as the negative control.

Restriction digestion with the *Taq1* restriction enzyme was carried out for all PCR products to detect the presence of a fragment length specific to marker isolate *N. parvum* B2141 which differentiated it from other *N. parvum* isolates (DAR78999, STE-U-5856, G62b1, A242 and UCD64S0) from Lincoln University Culture collection, which had been identified by Baskarathevan *et al.* (2012).

For the *Taq*I digestion, the reaction contained 10 µL of PCR product, digested with 2U *Taq1* restriction endonuclease at 65°C for 2 h followed by heat inactivation at 80°C for 20 min. The resulting fragments were separated by electrophoresis on a 1.5% agarose gel run for 1 h and 15 min at 10 V/cm in 1 x TAE buffer and stained as described in Section 3.2.2.3.

3.2.5 Necrotic cane collection after dispersal experiment with marker isolate (2015)

At the Fairhall Vineyard, one lignified hard shoot (50 mm) was collected from the vine nearest to the spore source after summer trimming, which showed characteristic dieback and for each replicate, at 0.5, 1, 2, and 5 cm in each of the four directions from the conidial source (48 in total), 4 weeks after the dispersal experiments with marker isolate of *N. parvum* (B2141). These stem tissues were each placed in a container with 50 mL sterile distilled water and rubbed vigorously with fingers for one minute. They were observed under the stereo microscope for characteristic symptoms and presence of pycnidia, then cut into 10 mm pieces (5 pieces), surface sterilized in 70% alcohol for 30 s and rinsed in sterile water for 30 s, and these pieces used for isolation onto PDA amended with chloramphenicol (0.05 g/L). Plates were observed after 3-7 days incubation at 25°C for characteristic growth of *N. parvum*. The 50 mL wash water for each shoot was centrifuged at 10, 000 × *g* for 15 min and the pellet resuspended in sterile water prior to examination with a light microscope (×100 and ×400).

3.2.5.1 Identification of *N. parvum* isolates based on morphological characteristics

All colonies characteristic of Botryosphaeriaceae species were sub-cultured onto PDA, and after 4 and 15 days incubation at room temperature the colour of the colonies which grew on the plates were observed to morphologically confirm the presence of *N. parvum* isolates.

3.2.5.2 Molecular confirmation of *N. parvum* (B2141) from collected necrotic shoots with nested PCR and PCR-RFLP process

3.2.5.2.1 Genomic DNA extraction

All the colonies which were morphologically characteristic of *N. parvum* were used for extraction of DNA as previously described in Chapter 2, Section 2.2.3.4. Colonies were identified to species level using the ARDRA system as described in Section 2.2.3.4 and the presence or absence of the marker strain (B2141) was determined using the nested PCR-RFLP process as described in Sections 3.2.4.2.

3.2.6 Infection of pruned grapevine canes

To determine whether apparently healthy pruned canes left on the vineyard floor could become infected by *Neofusicoccum* spp. and so become active within the disease cycle of Botryosphaeria dieback, canes (48) were collected from the floor of the Fairhall Vineyard 1 day after winter pruning. The canes were cut into 150 mm sections, washed under tap water to remove dirt then air-dried on paper towels laid out on a laboratory bench overnight. To prevent overgrowth by natural contaminants, surface sterilization was needed. Because surface sterilisation methods had not been tested on dormant canes, several methods were assessed. The samples were divided into four portions (12 each) which were (1) autoclaved; (2) surface sterilised by dipping in 70% ethanol; (3) washed with sterile water; (4) untreated and not inoculated. After air-drying as before, samples from Treatments 1-3 were inoculated onto the freshly cut upper end with a mixed conidial droplet comprising 40 µL (10^4/mL) of the same isolates of *N. luteum* or *N. parvum* as used in Section 2.2.3.1. The control non-inoculated pieces were only washed in tap water. The six replications (two 150 mm sections each) were incubated in sterile containers with moist sterile paper towels and placed outside in a shaded area in a complete block design. After 1 month, the upper 2 mm portion from each shoot was removed and a 10 mm piece cut from the same end was surface sterilized and isolated onto PDA as described in Section 3.2.5.

3.2.7 Statistical analysis

Data were analysed by General Linear Model (Minitab 17th edition). Data of conidial numbers from traps were transformed to $\log_{10}$ to improve the homogeneity of variance. For significant differences between treatments, means were examined with Sidak Pairwise Comparisons at $P \leq 0.05$.

3.3 Results

3.3.1 Spore trap set-up (2013-2015)

3.3.1.1 Rain water spore trap set-up

In 2013, no conidia characteristic of Botryosphaeriaceae species were observed in any of the rain water samples collected from the Waipara Vineyard. However, conidia characteristic of both *Neofusicoccum* and *Diplodia* spp. (Figure 3.3) were found in rain water collected on 28th March in the Brancott and Fairhall Vineyards, but not the Renwick Vineyard. On 3rd May there were no conidia typical of Botryosphaeriaceae spp.in the samples collected from any of the vineyards but rainwater collected on 5th May in Brancott, Fairhall and Renwick Vineyards had conidia characteristic of *Neofusicoccum* and *Diplodia* spp. conidia. Rainwater collected on 17th May had no conidia characteristic of the Botryosphaeriaceae species. In all cases, the PDA plates were covered in bacteria and yeasts and no colonies characteristics of Botryosphaeriaceae spp. were observed. In the repeated experiment in 2015, collected rain water contained conidia characteristic of Botryosphaeriaceae spp. for all traps and trapping months. Total rainfall, average relative humidity and average temperature are shown in Figure 3.4a. Overall conidial proportions showed more *Diplodia* spp. (53.6%) than *Neofusicoccum* spp. (46.4%) (Figure 3.4b). Suspensions plated onto PDA developed colonies characteristic of Botryosphaeriaceae spp.

Numbers of *Neofusicoccum* conidia trapped in 2015 were significantly affected by months and varieties, but not vineyards (P=0.004, P=0.035, P=0.076, respectively; Appendix C.2.1). Overall, highest mean numbers of conidia were trapped in April (4.9) and least in March (4.7) (Figure 3.4b). Overall highest mean numbers of conidia were trapped in the Pinot noir variety (1.0×10^5) and lowest in the Sauvignon blanc (6.3×10^4). The interactions between months and vineyards, and months and variety were not significant (P=0.318, P=410, respectively). However, the interaction between vineyards and variety was significant (P<0.001), which seemed to be associated with the high numbers of conidia trapped at Fairhall Vineyard in the

Pinot noir block and the low numbers trapped at Renwick Vineyard in the Sauvignon blanc block (Figure 3.4b).

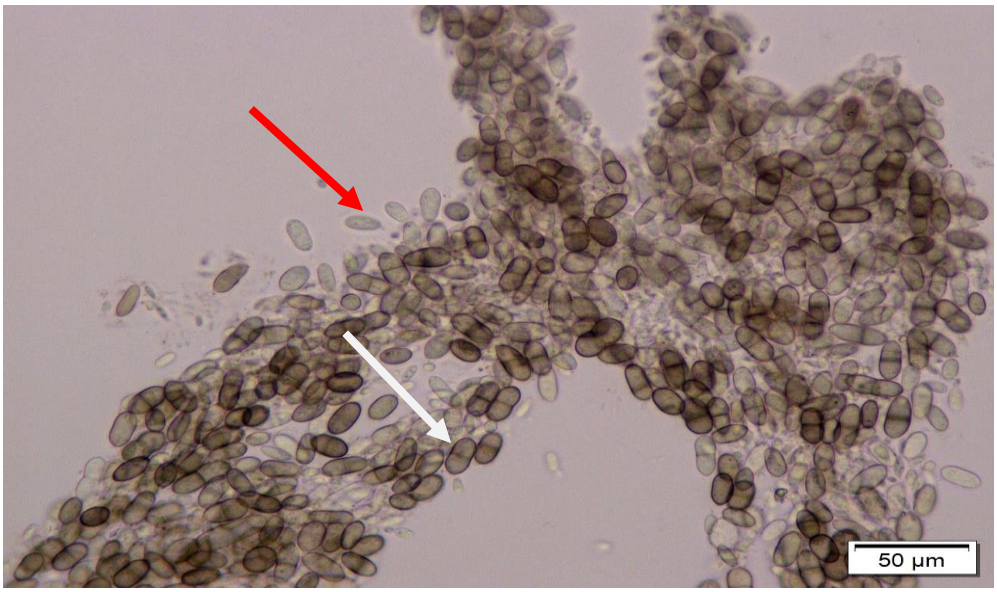


Figure 3.3 Botryosphaeriaceae spp. conidia caught in a rain water spore trap set up in a vineyard in Marlborough. Conidium indicated by red arrow is typical of a *Neofusicoccum* spp. and by white arrow a *Diplodia* spp.

Numbers of *Diplodia* conidia trapped in 2015 were significantly affected by months and vineyards, but not varieties (P=0.048, P=0.002, P=0.822, respectively; Appendix C.2.2). Highest (P<0.05) mean numbers of conidia were trapped in April (1.2 x 10^5) and least (P<0.05) in March (6.3 x 10^4) and most (P<0.05) at Fairhall (1.0 x 10^5) and least (P<0.05) at Renwick (5.0 x 10^4). The interactions between months and variety, and vineyards and variety were not significant (P=0.684, P=0.070, respectively). However, the interaction between months and vineyards was significant (P=0.041), which seemed to be associated with high numbers of conidia trapped at Fairhall Vineyard in April and low numbers trapped at Renwick Vineyard in May (Figure 3.4b).

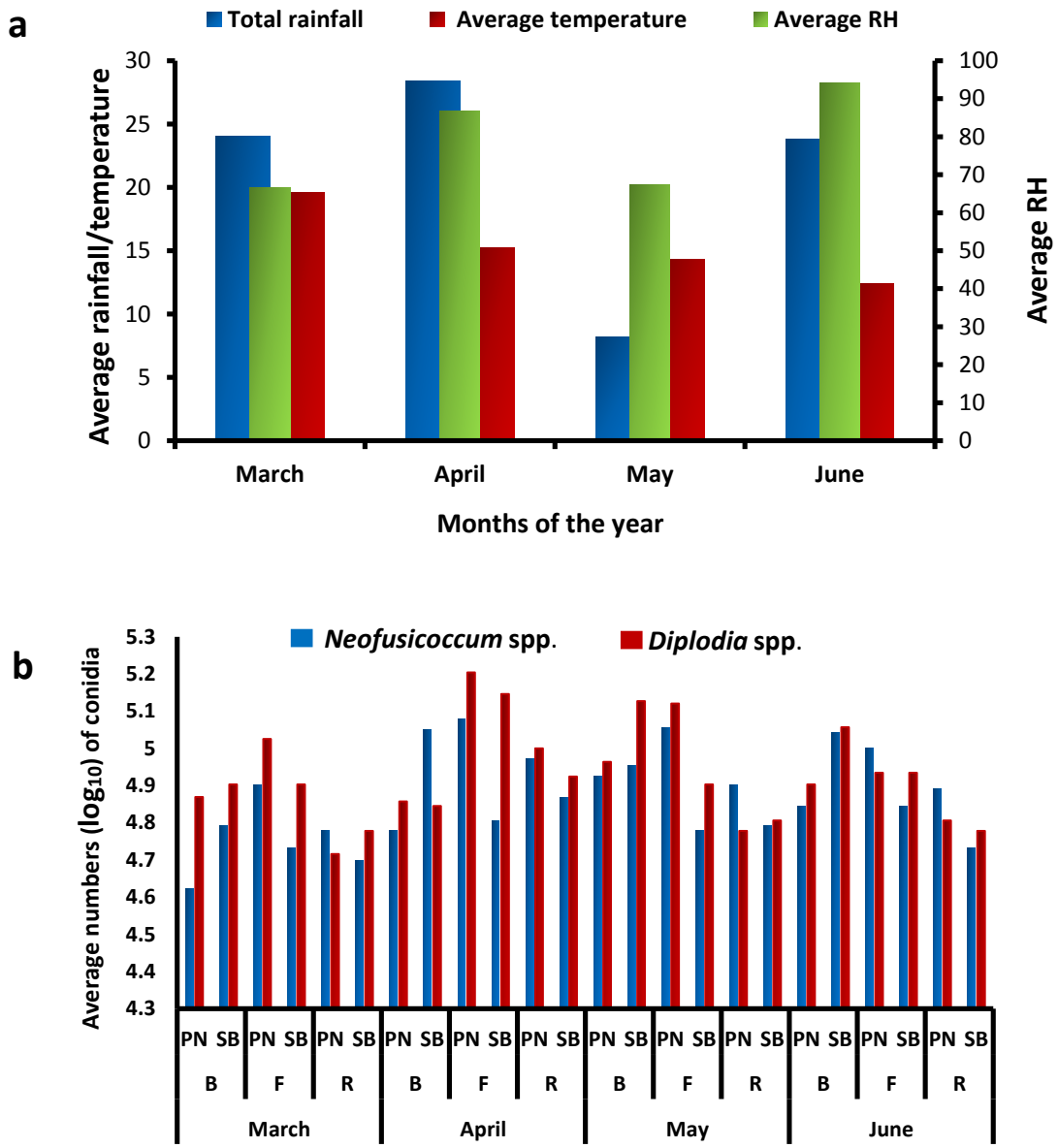


Figure 3.4 (a) Total rainfall, average relative humidity and average temperature. (b) Mean numbers of conidia (log_{10}) characteristic of *Neofusicoccum* spp. and *Diplodia* spp. collected in rain water spore traps during March-June, 2015 for varieties Pinot noir (PN) and Sauvignon blanc (SB), and in vineyards Brancott (B), Fairhall (F) and Renwick (R).

3.3.1.2 Botryosphaeriaceae spp. conidia trapping in 2014 (Burkard spore sampler)

The numbers of Botryosphaeriaceae conidia trapped were low (Figure 3.5) for all days except for 9th and 10th June when average conidial numbers reached 62 and 67 per Melinex segment (per day) for Pinot noir and Sauvignon blanc blocks, respectively (Figure 3.5). The two peaks coincided with the high rain fall events (19.4 and 32.6 mm/day, respectively). The maximum average daily temperature was 15.4°C and the minimum average daily temperature was 9.0°C during the trapping period. There was no linear relationship between temperature and conidial numbers trapped with R^2 of 0.36. Conidia of Botryosphaeriaceae spp. were trapped (1 per Melinex segment) when as little as 0.6 mm rain had fallen and maximum numbers of conidia (67 per Melinex segment) were trapped when higher rainfall (32.6 mm) occurred during the trapping period, and only up to 2 h after rainfall ceased. A linear relationship was observed between number of conidia trapped and rainfall with R^2 of 0.95.

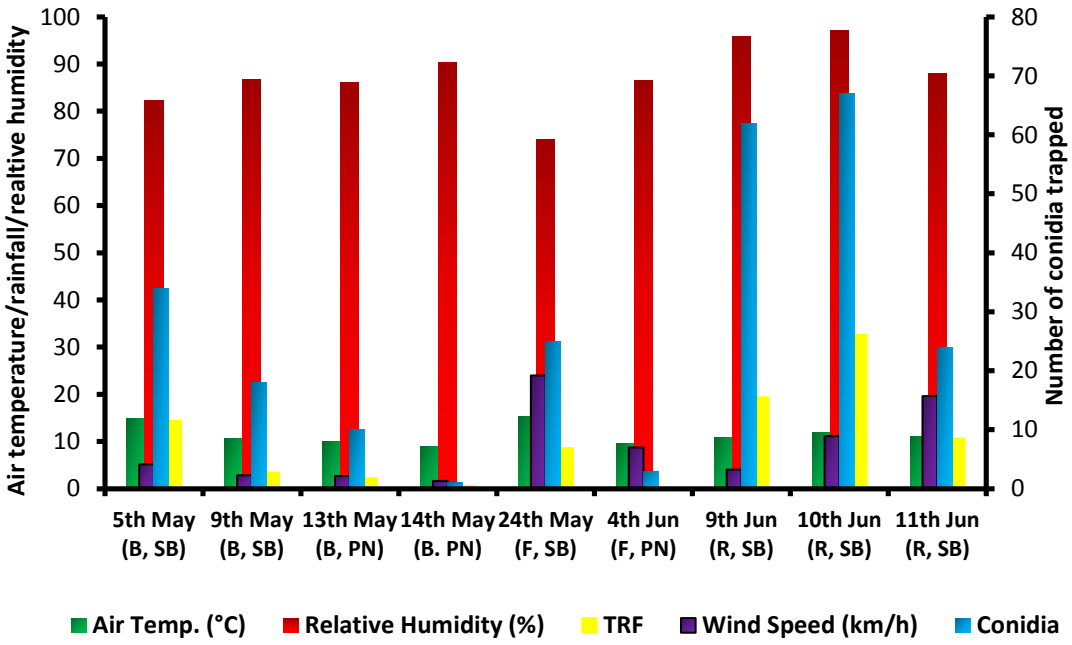


Figure 3.5 Daily total number of Botryosphaeriaceae spp. conidia trapped in the vineyards using a 7 day Burkard spore sampler over 6 weeks trapping period, from 5th May until 16th June 2014. The air temperature (°C), relative humidity (%), wind speed (Km/h) and total rainfall (TRF; mm) were those recorded from the nearest weather station (Hortplus). B-Brancott, F-Fairhall, R-Renwick, SB-Sauvignon blanc and PN-Pinot noir.

3.3.2 Molecular confirmation of Botryosphaeriaceae spp. identity for conidia in collected rain water and Burkard spore traps

3.3.2.1 Confirmation of *Diplodia* and *Neofusicoccum* species in rainwater and Burkard spore traps by PCR using Botryosphaeriaceae multi-species primers

As illustrated in Figure 3.6 the PCR for all samples from the Fairhall, Brancott and Renwick Vineyards produceda single band of molecular weight, approximately 372 bp, which indicated the presence of Botryosphaeriaceae spp.

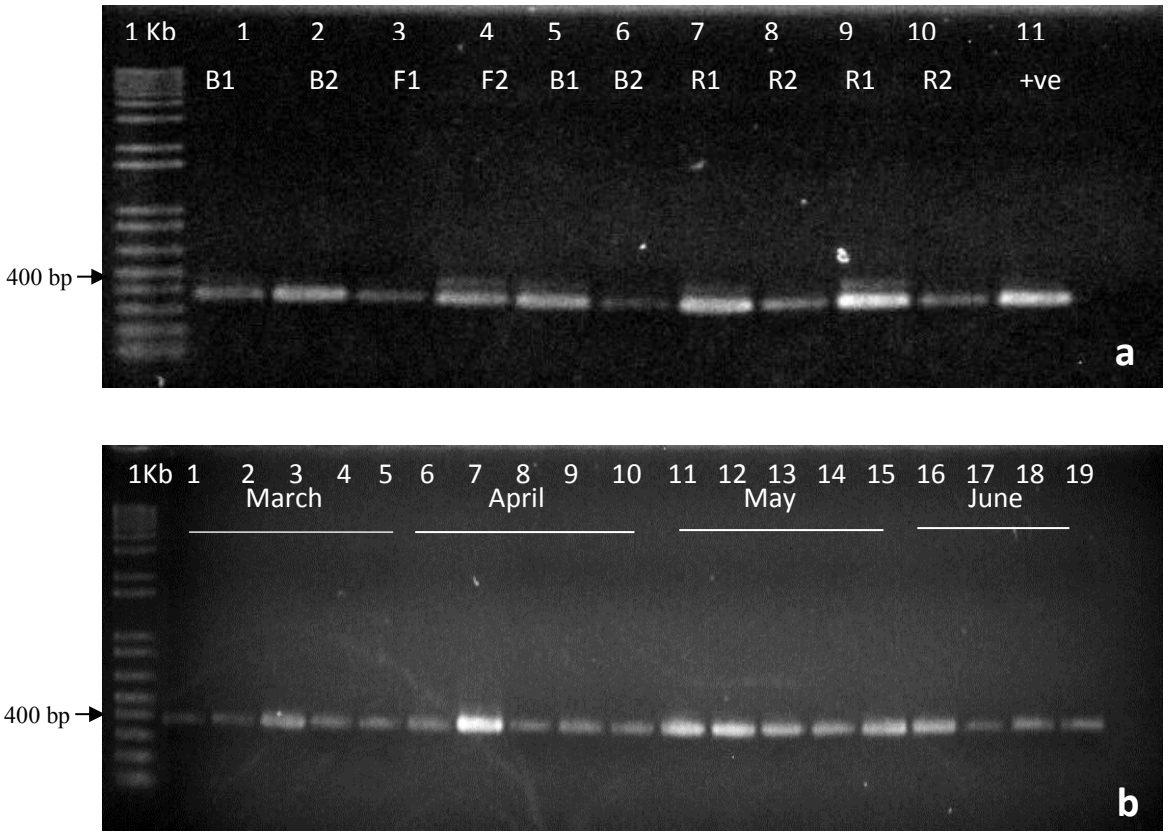


Figure 3.6 Agarose gels showing the presence of amplimers specific for Botryosphaeriaceae spp. produced using the multispecies primers with rain water samples from Brancott, Fairhall and Renwick Vineyards (labelled as B, F and R, respectively) on (a) (28th March and 5th May, 2014 and (b) March to June 2015). The far left lane contains the 1 kb plus DNA ladder.

3.3.2.2 Identification of Botryosphaeriaceae species from rain water and Burkard spore trap samples by single stranded conformational polymorphism (SSCP)

This method detected *N. luteum*, *N. australe*, *D. mutila* and *D. seriata* which had ≥ 3 bp differences in the 372 bp amplicon; the unique conformations were easily differentiated on acrylamide gels. However, *N. parvum* and *N. ribis* were identical within the 372 bp amplicon and therefore could not be distinguished by SSCP. These species were identified from the 2013 rainwater traps across all the sampling times and vineyards (Figure 3.7).

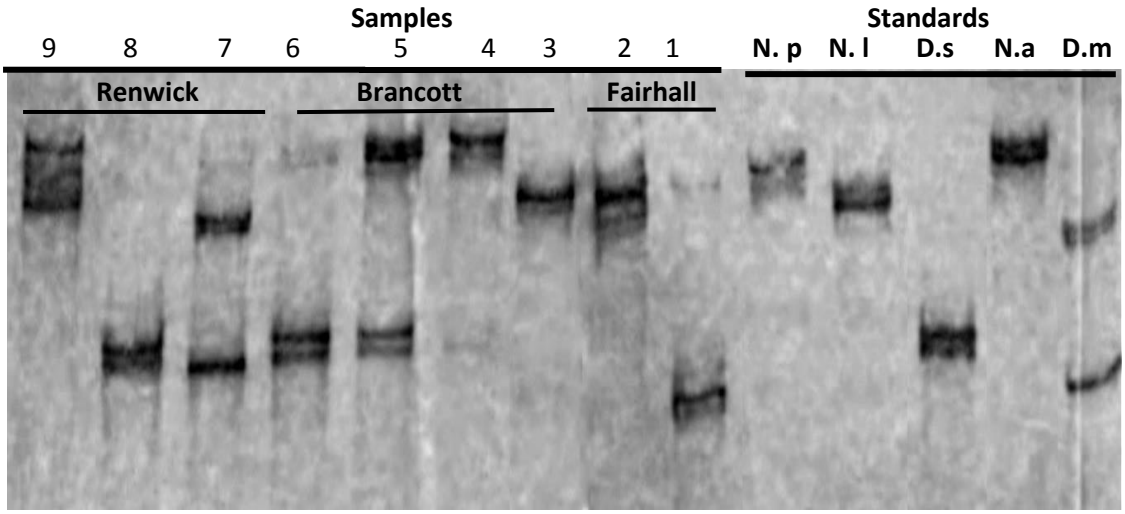


Figure 3.7 SSCP analysis using primers BOT 100F/BOT 427R showing different amplicons. Lanes 1-9 represent Burkard spore trap samples from different vineyards and the standard isolates are as follows: N.p=*Neofusicoccum parvum* (P179), N.l=*N. luteum* (L125), D.s=*Diplodia seriata* (N331), N. a=*N. australe* (B3192) and D.m=*D. mutila* (M212).

Table 3.1 Botryosphaeriaceae spp. conidia trapped (✓) in the vineyards using a 7 days Burkard spore sampler over 6 weeks trapping period, from 5th May until 16th June 2014.

			[a]**Botryosphaeriaceae spp.**				
Date of rain	**Vineyard**	**Variety**	**N. rib/N. p**	**N.a**	**N. l**	**D. s**	**D. m**
5th May	Brancott	SB		✓		✓	
9th May	Brancott	SB		✓		✓	
13th May	Brancott	PN	✓				✓
14th May	Brancott	PN			✓		
24th May	Fairhall	SB	✓		✓		
4th June	Fairhall	PN		✓		✓	
9th June	Renwick	SB	✓		✓		
10th June	Renwick	SB				✓	
11th June	Renwick	SB		✓			✓

[a]N. rib= *Neofusicoccum ribis*, N.p= *Neofusicoccum parvum*, N.a= *Neofusicoccum australe*, N. l= *Neofusicoccum luteum*, D.s= *Diplodia seriata*, D. m= *Diplodia mutila.*

The species most commonly found with the Burkard spore trap, from all vineyards, were *N. australe* and *D. seriata* (26.7%) (Tables 3.1 and 3.2). The next most common species were *N. parvum/N. ribis, N. luteum* (20%) and *D. mutila* (13.3%).

Table 3.2 Botryosphaeriaceae spp. conidia trapped (✓) in the vineyards in rain water sample trapping period, from March until June 2015.

			[a]Botryosphaeriaceae spp.				
Date of rain event (2015)	**vineyard**	**Variety**	**N. rib/N. p**	**N.a**	**N. l**	**D. s**	**D. m**
6th March	Brancott	SB		✓			✓
10th April	Brancott	SB		✓	✓		✓
6th March	Brancott	PN					
10th April	Brancott	PN		✓			
12th May	Brancott	SB					✓
2nd June	Brancott	SB				✓	
12th May	Brancott	PN					
2nd June	Brancott	PN					✓
6th March	Fairhall	SB					
10th April	Fairhall	SB		✓		✓	✓
6th March	Fairhall	PN					
10th April	Fairhall	PN				✓	
12th May	Fairhall	SB		✓			✓
2nd June	Fairhall	SB					
12th May	Fairhall	PN		✓			
2nd June	Fairhall	PN				✓	✓
6th March	Renwick	SB					
10th April	Renwick	SB					
6th March	Renwick	PN		✓			✓
10th April	Renwick	PN					
12th May	Renwick	SB		✓	✓		✓
2nd June	Renwick	SB					
12th May	Renwick	PN					✓
2nd June	Renwick	PN					

[a]N. rib =*Neofusicoccum ribis*, N.p= *Neofusicoccum parvum*, N.a= *Neofusicoccum australe*, N. l= *Neofusicoccum luteum*, D.s= *Diplodia seriata*, D. m= *Diplodia mutila.*

In the rain water spore traps in 2013, the species most commonly found were *D. mutila* (70%) and *N. australe* (55%) followed by *D. seriata* (25%). In 2015, the most commonly found species in rain water spore traps were *D. mutila* (41.7%) and *N. australe* (33.3%), followed by *D. seriata* (16.7%) and *N. luteum* (8.3%). All six species were found with the Burkard spore trap at the Renwick and Brancott Vineyards while at the Fairhall Vineyard, *N. parvum/N.ribis, N. luteum* and *N. australe* were found.

3.3.3 Dispersal distances of *Neofusicoccum parvum* conidia during rainfall

3.3.3.1a Microscopic identification of *Neofusicoccum* spp. in rain water (2014)

Microscopic examination confirmed presence of conidia characteristic of *Neofusicoccum* and *Diplodia* spp. in samples set at all distances, up to 20 m from the source in the direction of the prevailing wind and up to 5 m from the source in the three other directions.

3.3.3.2a Molecular confirmation of *N. parvum* isolate B2141 in 2014 rain water samples with nested PCR and PCR-RFLP

From samples collected in 2014 a single band of approximately 1500 bp was amplified in six rainwater samples. These were collected at up to 1 m after both pruning and trimming in the three directions away from the prevailing wind (Figure 3.8) and in the wind direction at up to 10 m from the source after pruning and up to 5 m after trimming. When a nested PCR with internal primers (BP-1-42int) was done, this produced amplimers from samples (after pruning and trimming) up to 10 m from the source in the wind direction and up to 5 m in the other three directions (Figure 3.9). When the primary PCR products of the trap samples were digested with *Taq1* restriction enzyme, and run on a 1.5% agarose gel, four visible bands (51-64, 97-157, 405 and 776 bp) were produced for some samples which were clearly different from other *N. parvum* isolates, which had four visible bands of 51-97, 221, 405 and 776 bp (Figure 3.10). When the nested PCR products of the trap samples were digested with *Taq1* restriction enzyme it produced two visible bands (95 bp and 159-210 bp). This confirmed recovery of *N. parvum* marker isolate (B2141) from rain water samples up to 10 m in wind direction and 5 m away from prevailing wind (Figures 3.10 and Table 3.3, 3.4).

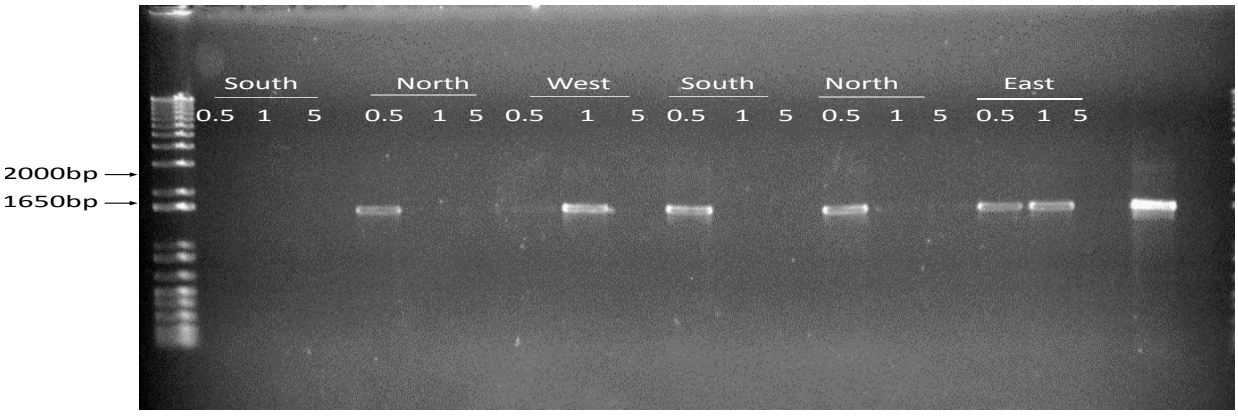


Figure 3.8 Initial PCR amplification using external primers BP-1-42F and BP-1-42R and extracted DNA from rain water samples trapped at different distances from the source in direction of the downwind (west) and other directions (south, north and east). All lanes contain rain water samples, except that the far right lane contains the marker strain B2141 and the far left lane contains the 1 kb plus DNA ladder.

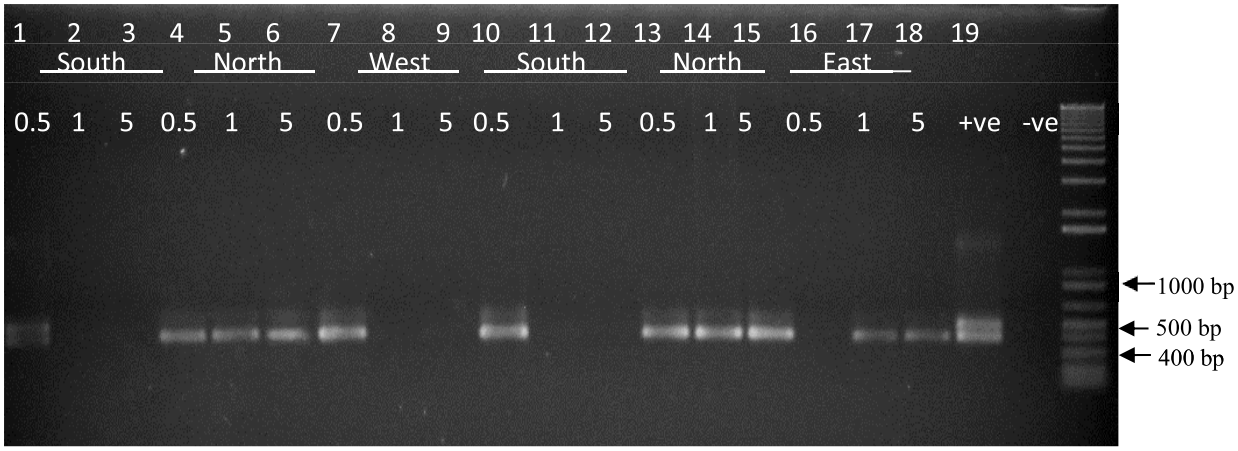


Figure 3.9 Agarose gel of the nested PCR of rain water samples using primers BP-1-42intF and BP-1-42intR. The first 18 lanes (from left) contain rain water samples and lane 19 the marker strain B2141. The numbers on the far right denote the molecular weights of the 1 kb plus DNA ladder.

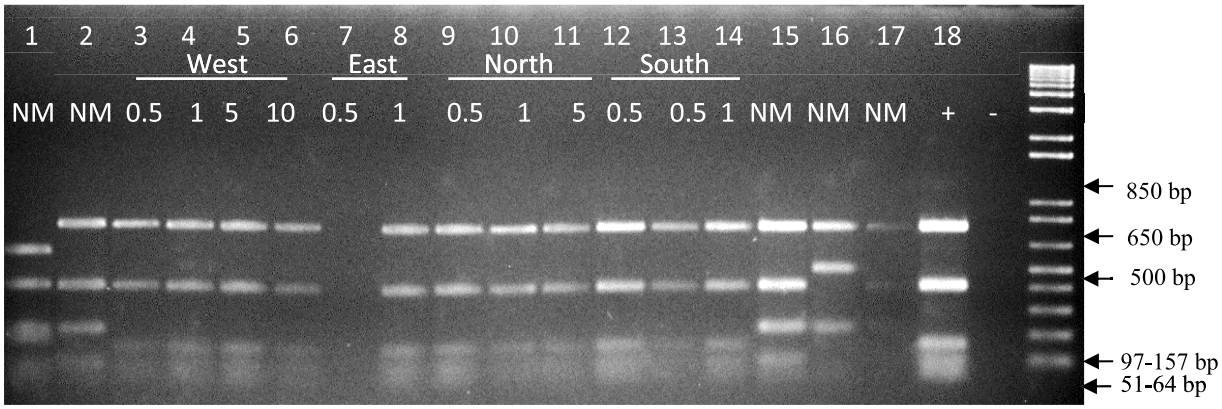


Figure 3.10 Agarose gel of *Taq1* digested PCR products produced by primers BP-1-42F and BP-1-42R from DNA extracted from rain water samples. The five non-marker *Neofusicoccum parvum* isolates are in the following lanes: 1 NM is non- marker *N. parvum* isolates (UCO642S), 2 (G62b1), 15 (DAR 7899) 16 (STE-U 5856) and lane 17 (A242). Lanes 3-14 contain rain water samples and lane 18 contains *N. parvum* marker isolate B2141. The numbers on the far right denote the molecular weights of the 1 kb plus DNA ladder.

Table 3.3 Dispersal distances of marker isolate B2141 after rainfall in autum and spring (2014) and autumn of 2015 as confirmed by PCR-RFLP.

	Maximum distance spores trapped		
	2014		2015
	Autumn	Spring	Autumn
Direction	Water trap	Water trap	Water trap
S	0.5	0.5	5
E	1	0.5	1
N	5	0.5	1
Downwind	10	10	10

Table 3.4 Dispersal distances of marker isolate B2141 after pruning (indicated by red colour) after trimming (indicated by black colour) in 2014 as confirmed by PCR-RFLP.

(✓) Presence of marker strain at different distances (m)													
180°			**270°**			**360°**			**Downwind**				
0.5 m	**1 m**	**5 m**	**0.5 m**	**1 m**	**5 m**	**0.5 m**	**1 m**	**5 m**	**0.5 m**	**1 m**	**5 m**	**10 m**	**20 m**
✓			✓	✓		✓	✓	✓	✓	✓	✓	✓	
✓			✓			✓	✓	✓	✓	✓	✓	✓	

3.3.3.1b Molecular confirmation of *N. parvum* isolates in 2015 rain water samples with nested PCR and PCR-RFLP process

In 2015, external PCR using BP-1-42 primers for *N. parvum* (B2141) detected *N. parvum* in traps at up to 5 m from the source in the wind direction, and up to 1 m in the other three directions (Figure 3.11). Subsequent nested PCR with internal primers (BP-1-42int) detected *N. parvum* at up to 10 m from the source in the wind direction for one replicate and up to 5 m for most of the other traps. In the three other directions, nested PCR with internal primers detected *N. parvum* at 1 m in all replicate samples except for one sample, in which it was found at up to 5 m (Figure 3.12) (Table 3.5). The *Taq1* restriction digestion of primary PCR products produced four visible bands (51-64, 97-157, 405 and 776 bp) for the marker isolate

on a 1.5% agarose gel which clearly differentiated it from other *N. parvum* isolates, which had four visible bands (51-97, 221, 405 and 776 bp) (Figure 3.15). The *Taq1* restriction digestion of secondary PCR products produced two visible bands (95 bp and 159-210 bp) (Figure 3.16). This confirmed the recovery of *N. parvum* marker isolate (B2141) from rain water samples up to 5 m in wind direction and 1 m away from prevailing wind (Figure 3.15).

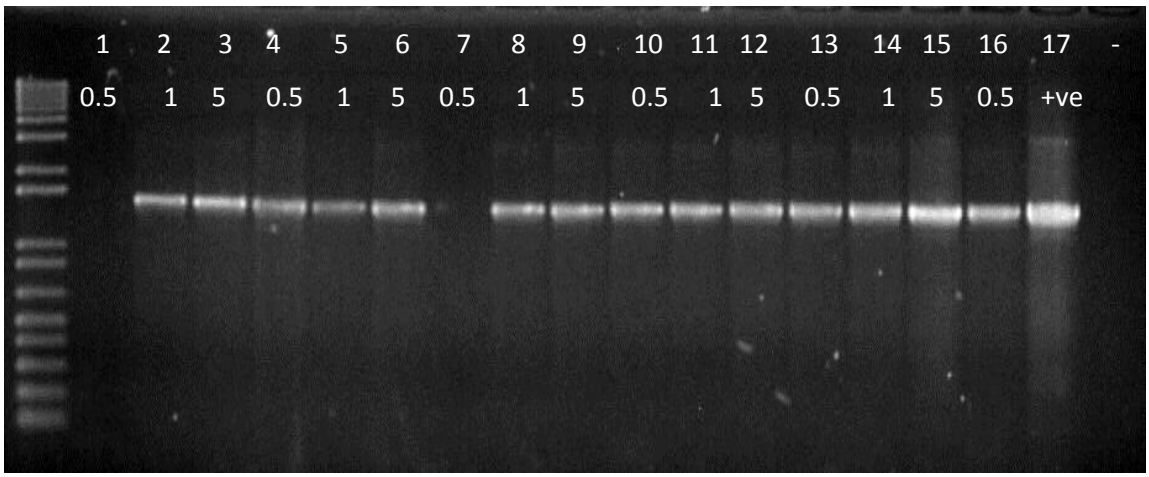


Figure 3.11 Initial PCR amplification, using external primers BP-1-42F and BP-1-42R, of extracted DNA from rain water samples set at different distances from the source in the direction of the prevailing wind after pruning and trimming. Lanes 1-16 represent rain water samples and lane 17 represents the marker strain B2141. The numbers on the far left denote the molecular weights of the 1 kb plus DNA ladder.

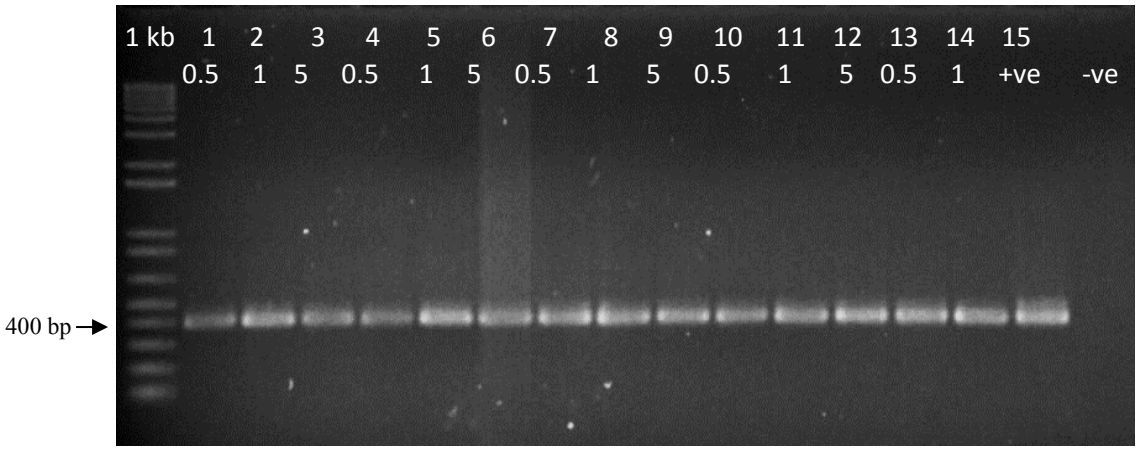


Figure 3.12 Secondary PCR amplification, using internal primers BP-1-42intF and BP-1-42intR, of extracted DNA from rain water samples set at different distances from the source in the direction of the prevailing wind, and in other directions, after pruning and trimming. Lanes 1-14 represent rain water samples and lane 15 the marker strain B2141. The numbers on the far left denote the molecular weights of the 1 kb plus DNA ladder.

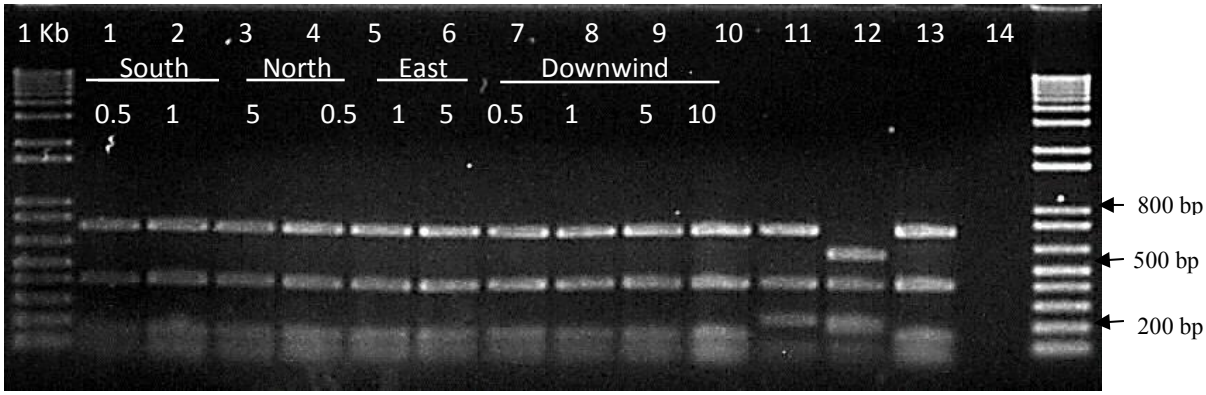


Figure 3.13 Agarose gel of *Taq1* digested PCR products, generated by primers BP-1-42F and BP-1-42R, from DNA extracted from rain water samples set at different distances from the source in the direction of the prevailing wind after pruning. Lanes 1-10 represent rain water samples, lane 11 is non- marker *N. parvum* isolate A842, lane 12 is non- marker *N. parvum* isolate G69a1, lane 13 represents the +ve control and lane 14 the –ve control. The numbers on the far right denote the molecular weights of the 1 kb plus DNA ladder.

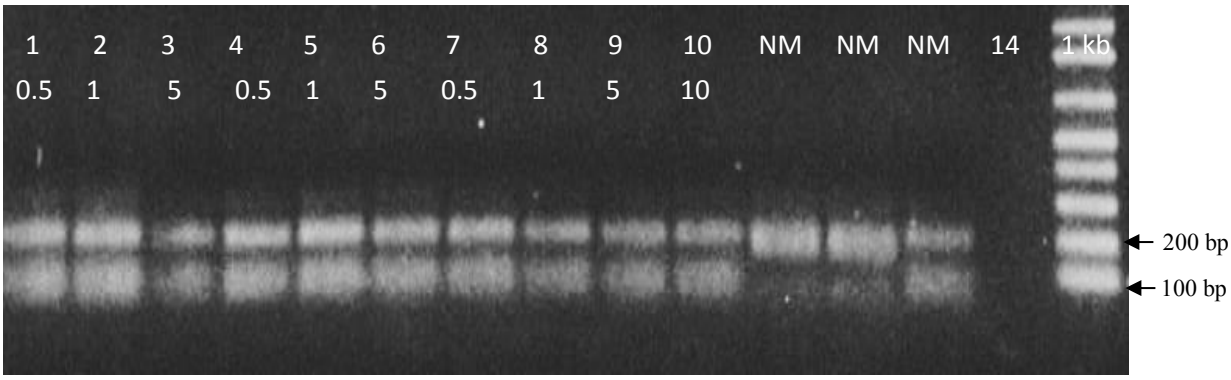


Figure 3.14 Agarose gel of *Taq1* digested PCR products, generated by internal primers BP-1-42intF and BP-1-42intR, from extracted DNA from rain water samples set at different distances from the source in the direction of the prevailing wind after pruning and trimming. Lanes 1-9 represents rain water samples and lane 10 the marker strain B2141. The *N. parvum* isolates are: Lane 11 isolate STE-U 5856, lane 12 isolate G69a1, lane 13 isolate A842. Lane 14 represents the –ve control and the numbers on the far right denote the molecular weights of the 1 kb plus DNA ladder. NM= non-marker.

Table 3.5 Dispersal distances of marker strain (B2141) in different directions in 2015, as confirmed by PCR-RFLP.

Rep	(✓) Presence of marker strain at different distances (m)													
	180°			270°			360°			Downwind				
	0.5 m	1 m	5 m	0.5 m	1 m	5 m	0.5 m	1 m	5 m	0.5 m	1 m	5 m	10 m	20 m
1	✓				✓			✓		✓	✓			
2	✓	✓	✓	✓						✓	✓	✓		
3		✓		✓	✓		✓	✓			✓		✓	

3.3.4 Recovery of *Neofusicoccum parvum* isolate B2141 from necrotic canes collected from the vineyard after the dispersal experiment

All centrifuged water samples from washing the necrotic canes (Figure 3.15) showed conidia characteristic of Botryosphaeriaceae spp. (*Neofusicoccum* and *Diplodia*). All the 1 cm shoot sections plated onto PDA also showed colonies characteristic of Botryosphaeriaceae species (Figure 3.16).

Figure 3.15 Necrotic canes collected after the dispersal experiment.

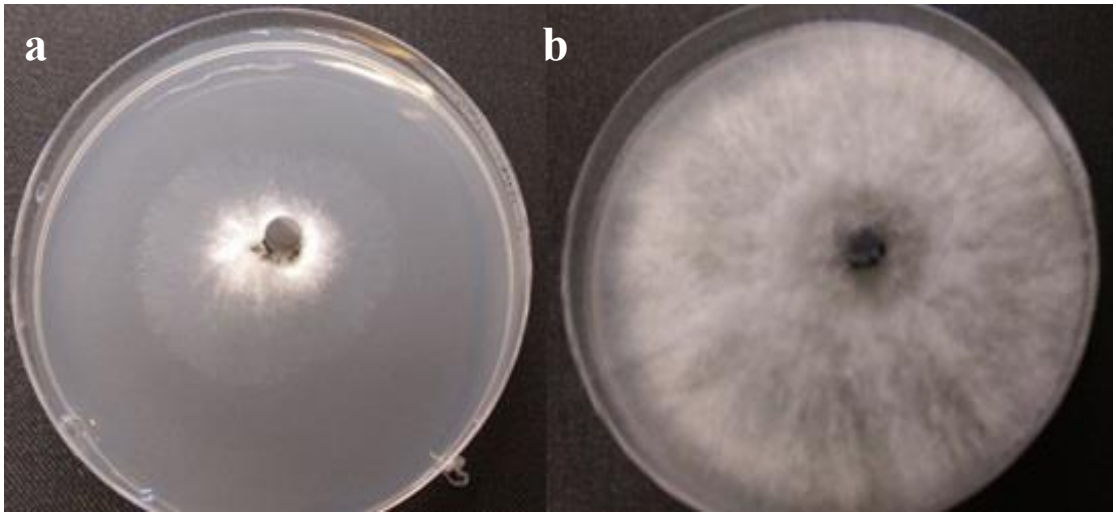


Figure 3.16 Characteristic *Neofusicoccum parvum* colonies isolated from necrotic shoots onto PDA plates a) 3-day-old, b) 7- day-old.

3.3.4.1 Molecular confirmation of *N. parvum* (B2141) from collected necrotic shoots with nested PCR-RFLP process

PCR products of 1200 bp were obtained using the ITS1 and NL4 primers; when digested with the *Hae*III restriction endonuclease, the products visible on a gel were five bands of 258, 254, 203, 157 and 58-83 bp (Figure 3.17), which clearly distinguished *N. parvum* from all other Botryosphaeriaceae species.

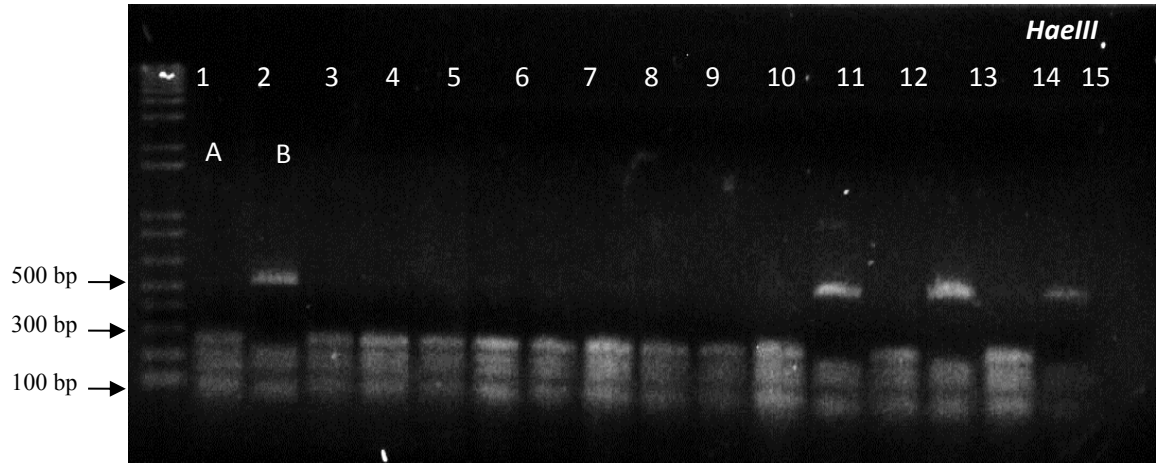


Figure 3.17 Agarose gel of *HaeIII* digested PCR products generated by amplification with primers ITS1 and NL4 from colonies recovered from necrotic shoots samples. Band pattern A represents *Neofusicoccum parvum*, and pattern B represents all non- *N. parvum*. The far left lane contains the 1 kb plus DNA ladder.

The external primers BP-1-42 detected *N. parvum* in infected shoots up to 1 cm from the source in the wind direction, and at up to 0.5 cm in the three other directions. Subsequent nested PCR with internal primers (BP-1-42int) detected *N. parvum* up to 1 cm from the source in the wind direction except for one sample, which also produced a band at 2 cm. In the three other directions the nested PCR detected *N. parvum* up to 1 cm.

The *Taq1* restriction digestion of the primary PCR products produced four visible bands (51-64, 97-157, 405 and 776 bp) for the marker isolate (B2141) on a 1.5% agarose gel which clearly differentiated it from the other *N. parvum* isolates with four visible bands (51-97, 221, 405 and 776) (Figure 3.18). Digestion of the secondary PCR products with *Taq1* produced two visible bands (95 bp and 159-210 bp) (Figure 3.19). The samples were positive for marker isolate (B2141) for all isolates obtained from infected canes within 1 cm of the source in the wind direction and for one isolate within 2 cm. This clearly confirmed that the shoots were infected by conidia from the source. Out of 45 samples 13 were positive for the marker isolate (Table 3.6).

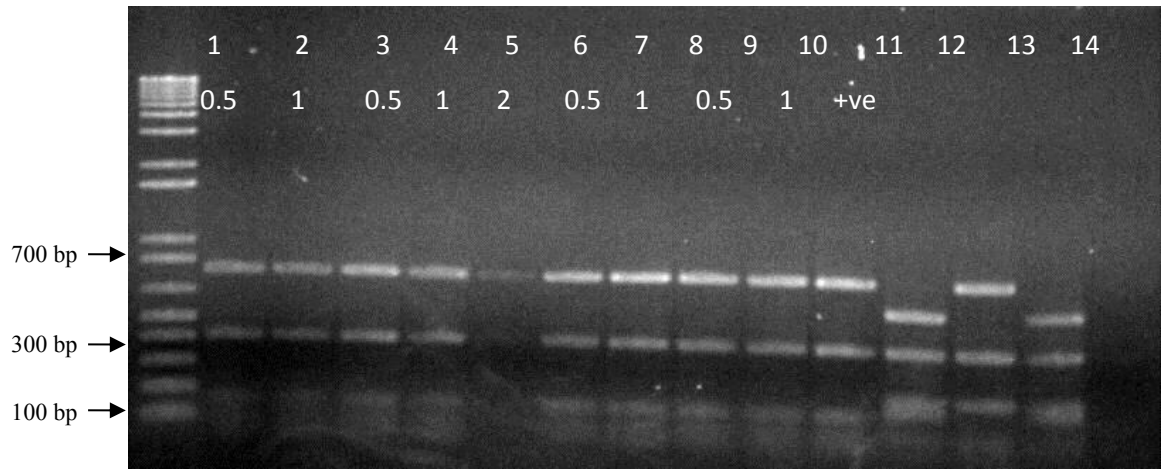


Figure 3.18 Agarose gel of *Taq1* digested PCR products generated by external primers BP-1-42F and BP-1-42R from DNA extracted DNA from necrotic shoots samples. Lanes 1-9 represent rain water samples, lane 10 represents marker strain B2141 (+ve control), lane 11 G69a1, lane 12 A842, lane 13 STE-5886 and lane 14 represents the –ve control. The numbers on the far left denote the molecular weights of the 1 kb plus DNA.

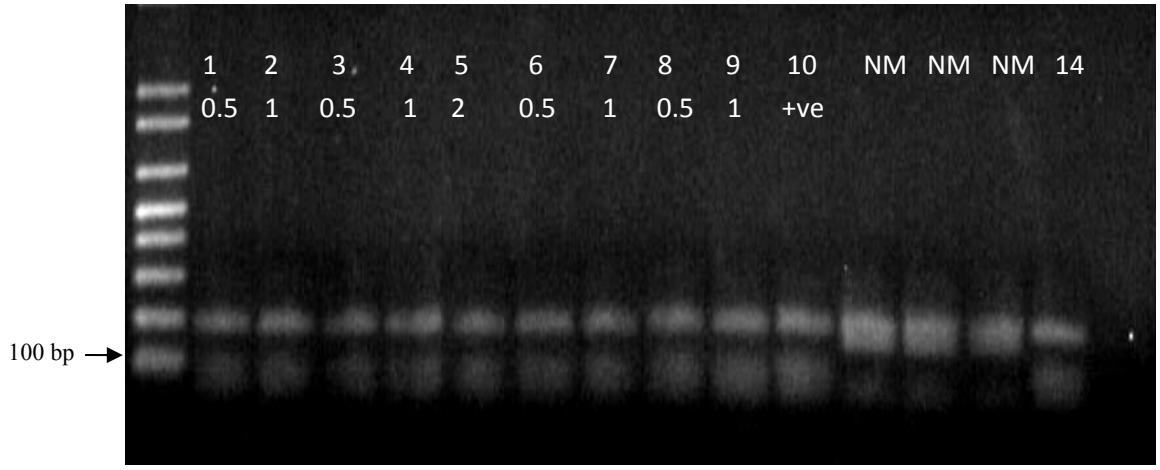


Figure 3.19 Agarose gel of *Taq1* digested PCR products generated by internal primers BP-1-42intF and BP-1-42intR of extracted DNA from necrotic shoots samples set at different distances from the source in the direction of the prevailing wind after pruning and trimming. Lanes 1-9 represent rain water samples and lane 10 represents the marker strain B2141. Lane 11 represents G69a1, lane 12 A842, lane 13 and lane 14 represent the +ve control (B2141). The numbers on the far left denote the molecular weights of the 1 kb plus DNA ladder.

Table 3.6 Infection of necrotic shoots by the *Neofusicoccum parvum* marker strain (B2141) at distances (cm) from the inoculum source after dispersal in different directions, 2015.

	(✓) Presence of marker strain at different distances (cm)															
Rep	**180°**				**270°**				**360°**				**Downwind**			
	0.5	**1**	**2**	**5**	**0.5**	**1**	**2**	**5**	**0.5**	**1**	**2**	**5**	**0.5**	**1**	**2**	**5**
1	✓					✓				✓			✓			
2		✓			✓									✓	✓	
3		✓			✓	✓			✓	✓						

3.3.5. Infection of pruned grapevine canes

Pruned shoots inoculated after being autoclaved showed 41.6% infection by *N. parvum* and 50% infection by *N. luteum* as shown by characteristic colonies when reisolated on PDA. Shoots inoculated after surface sterilisation with 70% ethanol showed 91.6% infection by *N. parvum* and 100% by *N. luteum*. Shoots just washed in water and inoculated showed highest level of infection, 100% for both *N. parvum* and *N. luteum*. The few contaminants found on these plates were mostly *Alternaria, Fusarium* and *Ulocladium* spp.

3.4 Discussion

The aim of this study was to improve understanding of the processes of Botryosphaeriaceae conidium dispersal and survival and thereby the dynamics of disease epidemics.

Microscopic examination of rainwater run-off collected from the Fairhall, Brancott and Renwick Vineyards in 2013 and 2015 revealed conidia characteristic of *Neofusicoccum* and *Diplodia* species. Amponsah *et al.* (2009b) also reported *Neofusicoccum* and *Diplodia* type species in the rainwater run-off of a Canterbury vineyard throughout the year. Pusey (1989) and Sutton (1981) identified *B. dothidea* and *D. seriata* ascospores in rain water run-off from peach and apple trees, respectively, and largely during spring and summer. In the study of Urbez-Torres *et al*. (2010b) Botryosphaeriaceae spores were trapped during rainfall and overhead sprinkler irrigation periods in California. Thus, this study confirms previous work demonstrating the presence of these species in rainwater both in New Zealand and overseas for grapevines and other woody hosts. In this study Botryosphaeriaceae spores were not found in the sampling of Waipara, which might be because it represents a relatively young vineyard and viticultural region.

In the present study, conidia of *Diplodia* spp. were more numerous than for *Neofusicoccum* spp. This contrasts with results of Amponsah *et al.* (2009b) who reported 59.8% *Neofusicoccum* spp. and 40.2% *Diplodia* spp. in a Canterbury vineyard. However, those results were from a single vineyard in a different region. Further, the species incidence within a single vineyard may reflect the initial infection status of the young plants used for establishing that vineyard. The variation in data from two regions may also indicate that the distribution of Botryosphaericeae species is influenced by climatic conditions as reported from a New Zealand survey of species in necrotic vineyard tissues by Baskarathevan *et al.* (2012). In that study *Neofusicoccum* spp. predominated in the North Island (95%) while in the South Island they comprised only 45% of the pathogens isolated. Further, they also showed that the proportions of *Neofusicoccum* spp. and *Diplodia* spp., respectively, were 45 and 55% in Blenheim and 60 and 32% in Canterbury. Differences in species distribution pattern have been suggested to be the result of different temperature preferences of different species (Taylor *et al.,* 2005; Urbez-Torres *et al.,* 2006a, 2008; Pitt *et al*., 2010). Baskarathevan *et al.* (2012) also reported that different species of Botryosphaeriaceae had different optimum temperatures for mycelial growth. In their study growth of *N. parvum, N. luteum* and *D. seriata* was optimum at temperatures of 26.1°C, 26.3°C and 25.9°C, while for

D. mutila and *N. australe* the optimum temperatures were 24.6°C and 23.4°C, respectively. In another study, Billones *et al*. (2013) reported that all the above species were present in nursery materials, so nurseries could be responsible for providing the primary sources of vineyard Botryosphaeria disease. Therefore, regional nurseries which provide the plants for nearby vineyards, might also account for the predominating species in those regions.

Microscopic examination of the Melinex tape which trapped air-borne spores showed very few Botryosphaeriaceae spp. conidia in comparison with the rainwater traps. However, the Burkard spore trap was able to show the timing of conidial release. The conidia were trapped during or immediately after rainfall, being found after as little as 0.2 mm rain had fallen, but higher numbers of conidia were trapped with higher rainfall. Similar results were shown by a spore trapping study in South Africa (van Niekerk *et al.*, 2010). In their study, conidia of Botryosphaeriaceae spp. were captured by a Quest volumetric spore trap after as little as 0.25 mm rainfall, with higher number of conidia in higher rainfall. However, they also reported that in some cases no conidia were trapped even when rainfall did occur. In another volumetric spore trapping study in California by Urbez-Torres *et al.* (2010b) they showed that there was a positive relationship between conidial numbers released and amount of precipitation. In their study highest numbers of conidia were trapped in February (50.4% in Monterey and 65% in Sonoma) which coincided with the maximum amount of rainfall during that period. In another study Sutton (1981) observed similar results, with numbers of conidia and ascospores of *Physalospora obtusa* (syn. *D. seriata*; Phillips *et al.,* 2007) and *Botryosphaeria dothidea* in an apple orchard having a positive relationship with amount and duration of rainfall. However, these results conflict with a study by Holmes and Rich (1970) in apple orchard, who observed no positive relationship between number of conidia of *D. seriata* and amount of rainfall. They showed that maximum numbers of *D. seriata* 'spores' were trapped when the relative humidity was 100%. Moisture effects on conidial release differ between regions. The current study has demonstrated that conidial release was associated with rainfall in Marlborough for which spore trapping had not previously been investigated.

In this study, conidia of Botryosphaeriaceae spp. were not trapped beyond 2 h after rainfall ceased. The same result was shown by Urbez-Torres *et al*. (2010b); they also could not trap any Botryosphaeriaceae spp. conidia beyond 2 h after termination of rainfall or overhead irrigation. This is in contrast with a study of Sutton (1981) who reported that air-borne ascospores of *D. seriata* and *B. dothidea* were often trapped in apple orchards for 33hours

after the rainfall ended. In his study, ascospores of *D. seriata w*ere often the most commonly found spores at the end of rain period or after rain fall ceased. Since the ascospores are not produced in ooze but forcibly ejected into the air as air-borne spores, this probably accounts for the difference reported. Since apple trees are largely composed of more permanent and/older wood than grapevines in New Zealand, this may also account for the presence of ascospores in apple orchards but not in vineyards. However, there are no reports of the season and time during which perithecia and ascospores develop.

Ascospores of Botryosphaeriaceae spp. were not found in spore traps in the current study, which was consistent with the study of Michailides and Morgan (1992), who reported that perithecia of Botryosphaeriaceae spp. are rarely observed in perennial crops, including grapevines. Smith *et al.* (2001) also reported that the sexual stages of Botryosphaeriaceae spp. were rarely found in nature. However, Pusey (1989) reported that ascospores of *B. dothidea, D. seriata* and *L. theobromae* were abundant in peach orchards in April 1984. They also reported that in 1985 the maximum numbers of *B. dothidea* ascospores were trapped in mid-March to mid-May, while ascospores of *D. seriata* and *L. theobromae* were trapped in high numbers in all months except in January (winter). Further, Sutton (1981) found similar numbers of conidia and ascospores of both *D. seriata* and *B. dothidea* in a trapping study in apple orchards, although the ascospores were predominant in spring and the conidia in mid- to late-summer. The lack of ascospores in New Zealand might be due to the lack of suitable mating types as found for grape powdery mildew. In New Zealand, only asexual spores of powdery mildew were found until summer 2014 when Johnston *et al.* (2014) reported finding chasmopara (formerly called cleistothecia), which are the sexual form of this fungus, in all the major grape-growing regions, due to the recent introduction of the Group B population. However, no research has reported the mating types of Botryosphaericeae spp. so this indicates a useful area for future research. Presence of two mating types in a country might indicate likely presence of ascospores which could mean that growers need to protect pruning wounds for longer periods after rainfall.

In the present study, conidia of Botryosphaeriaceae spp. were trapped during or immediately after rainfall, when the average daily temperature was 9.0 -15.3°C and the daily average relative humidity was 74-97%. Spore-trapping studies in New Zealand and France reported an abundance of Botryosphaeriaceae species conidia throughout the year, maximum release being associated with rainfall during summer months (Amponsah *et al.,* 2009b; Kuntzmann *et al.,* 2009). The greater release of conidia during mild or warm conditions indicates that the

reported optimum growth temperatures of Botryosphaericeae might also apply to sporulation processes. However, conidial release has also been reported at low temperatures. Valencia *et al.* (2015) demonstrated in Chile that maximum conidial release (*Diplodia seriata*, *Spencermartinsia viticola* and *Neofusicoccum sp*.) occurred in weeks when the weekly median (of average daily temperatures) was 10°C. Kuntzmann *et al.* (2009) reported conidial dispersal of *Diplodia* species from 9°C in France and Urbez-Torres *et al*. (2010) reported that release of conidia (*D. seriata* and *B. dothidia*) in California occured at temperatures of 3-7°C during the winter months. In another study in North America by Pusey (1989), maximum numbers of *B. dothidea* conidia were detected during periods of high temperature, but not in winter when the temperature was below -10°C. However, Weaver (1979) found no correlation between air temperature and number of *B. dothidea* conidia collected in peach orchards. The effect of temperature on sporulation of three Botryosphaeriaceae spp. grown on detached apple and peach stems *in vitro* was studied by Copes and Hendrix, (2004). They reported that conidia were produced over the range of 6 to 30°C, with peak sporulation of *B. dothidea* at 24°C, *D. seriata* at 18-24°C and *D. seriata* at 12, 24, and 30°C. Numbers of conidia released were lowest at 6°C for all species. They also reported that in apple and peach orchards conidia of *B. dothidea* and *D. seriata* had been recovered during late fall, winter, spring and early summer months. In contrast, Urbez-Torres *et al.* (2010b) showed that in vineyards Botryosphaeriaceae spp. spores were released in almost all rainfall events, although highest numbers of released spores usually followed a rain-free period. However, in the current study numbers of air-borne conidia of Botryosphaeriaceae spp. were relatively low in autumn 2014 (May) and higher in early winter (June), even though the amounts of rainfall were similar. It is possible that there were insufficient conidia available for dispersal in autumn (26 mm total rainfall in May) because they had been released by earlier rain fall events, (156 mm total rainfall in April) and that by June they were more abundant and ready for release. Similar patterns were reported by van Niekerk *et al.* (2010a) who showed that after a high conidial release event, few or no spores were released during the following 1-3 weeks, even when rainfall and relative humidity were adequate. Further, the rain events following such 'spikes' usually resulted in much lower numbers of spores. From these studies, it seems likely that conidia could be released during most day-time temperatures found in New Zealand, requiring moisture for exudation of conidial cirrhi and rain for splash dispersal which might also be wind-assisted.

The presence of Botryosphaeriaceae spp. isolates were confirmed by molecular studies. The amplification of a 372 bp band from collected rain water and the Burkard spore trap confirmed the presence of Botryosphaeriaceae species. Further confirmation of species by SSCP indicated the presence of five Botryosphaeriaceae spp. (*N. luteum, N. parvum/ribis, N. australe, D. mutila* and *D. seriata*). The ability of SSCP to distinguish fungal species present in collected rain water was demonstrated by Ridgway *et al.* (2011) who developed the SSCP system for identification of Botryosphaeriaceae spp. in environmental samples. They showed that up to three different Botryosphaeriaceae spp. were present in each sample and that the predominant species were *N. luteum* and *N. parvum/ribis* throughout the sampling period. The use of SSCP to differentiate species present in samples of mixed amplimers has been reported for many fungi. Rasmus and Rosendahl (2000) used *Glomus* specific primers and the SSCP technique to differentiate species of *Glomus* in root tissues. The SSCP technique was also used by Kumeda and Asao (1996) to differentiate *Aspergillus* species within the *flavi group,* including *A. flavus, A. oryzae, A. tamarii, A. parasiticus, A. sojae,* and *A. nomius*. However, in the current study this technique could not differentiate between *N. parvum* and *N. ribis*, because DNA of these two species was identical within the 372 bp amplicon produced by BOT100F and BOT472R primers. *Neofusicoccum parvum* and *N. ribis* have been considered closely related species because they could not be confidently separated by ITS sequence data alone, which is the method most commonly used in molecular identification of the Botryosphaeriaceae (Crous *et al.,* 2006). In the current study, SSCP technology was used to differentiate between species of Botryosphaeriaceae because morphological techniques cannot be used to identify different species of Botryosphaeriaceae which often exhibited overlap between the species (Alves *et al.,* 2006; Crous *et al.,* 2006) and cloning and sequencing of the PCR product from each sample is cumbersome and costly process. However, when Pavlic *et al.* (2009) conducted sequence comparisons of gene segments in RNA polymerase II subunit (RPB2), using isolates of the *N. luteum/ N. parvum* complex, this enabled them to resolve the identity, and to introduce three new species from within the complex as *N. cordaticola* sp. nov., *N. umdonicola* sp. nov. and *N. kwambon ambiense* sp. nov. Similar studies could be conducted with *N. parvum* and *N. ribis*.

Naturally occurring marker isolates are useful for studying population genetics and epidemiology of phytopathogenic fungi. Since the topic was reviewed by Michelmore and Hulbert (1987), who discussed the potential of isozymes and RFLPs to identify polymorphisms in specific isolates, many different genetic techniques for isolate

identification have been developed which have allowed for environmental and population studies. In this study, a marker isolate of *N. parvum* (B2141) was used to investigate the dispersal success of Botryosphaeriaceae spp. The investigation was made possible through use of specific primers followed by restriction enzyme analysis which produced a unique RFLP pattern with isolate B2141 and thus was able to differentiate between the marker isolate and other isolates naturally present in the vineyards. Marker isolates were detected in both standard and nested PCR but the detection capacity of nested PCR was greater. Rasmus and Rosendahl (2000) also used *Glomus* specific primers in nested PCR to amplify known fungi in their study. A similar approach was used by Helgason *et al.* (1998), who used Glomales specific primers and RFLP. In two other studies a species specific molecular marker had been developed and was used to detect *P. chlamydospora* (Ridgway *et al.,* 2002; Ridgway *et al*., 2005).

In the 2014 study, the dispersal success of *N. parvum* and the detection capability of the marker isolate were unknown, so the samples from the three replications for each distance were combined prior to molecular identification. The results of this showed that conidia of the marker isolate of *N. parvum* had dispersed up to 10 m in the wind direction and 5 m in other directions. In the repeated study in 2015, in which the replicate samples were analysed separately, conidia were shown to have dispersed up to 5 m in the wind direction in two replications and up to 10 m in one replication, with dispersal up to 1 m in the other directions. This result is in contrast to the report by Baskarathevan *et al*. (2013), who could only track the marker isolate up to 2 m from the source of conidia. When Ahimera *et al.* (2004) conducted a single-drop experiment, in which 200 water drops were dispensed onto infected pistachio nuts under laboratory conditions; they found that *B. dothidea* conidia were dispersed over quite short distances. The median travel distance (distance travelled by 50% of droplets) was 20 mm. Only 6% of droplets were splashed further than100 mm, and less than 0.2% of droplets reached 300 mm. Overall, more than 56% of conidia were carried in droplets that landed within 15 mm of the inoculum source and more than 95% of the conidia counted were splashed within 60 mm from the source. However, in this study one entire cirrhus, or a part of it, was found at 55 mm away from the source, indicating the potential for resplash in natural environments, which was likely to be occurring in the current experiment. Rainwater traps were also set up by Ahimera *et al.* (2004) at distances of 25, 50 and 100 cm from infected pistachio trees and after single rainfall events conidia were trapped in all cases at 100 cm. However, the numbers trapped were much lower than for traps set under the tree

canopy, maximum numbers being 6 x 10^3 and 4 x 10^4, respectively. However, in these two studies there is a lack of information regarding the role that wind plays in dissemination of Botryosphaeriaceae spp. conidia, as high wind speed is likely to affect the dispersal distance of conidia in water droplets, as observed by van Neikerk *et al.* (2010a). This was also demonstrated in the current study, in which dispersal of marker strain conidia occurred over longer distances in the direction of prevailing wind than in the other directions.

When the infection success of the dispersed conidia was investigated by isolating necrotic shoots onto PDA after the dispersal experiment, the marker isolate was found colonising plants at up to 1 cm for most directions, although one sample was found at 2 cm from the source. The shorter distances of isolation than for spore traps might be because the number of conidia that landed on the vines was not enough to infect them. Biggs (2004) showed that infection incidence of apple was increased as the inoculum concentration of *B. dothidea* increased. The other reason for this might be that conidia were not viable by the time they reached those shoots. Amponsah *et al.* (2010) reported that solar radiation has a negative effect on germination of *N. luteum*, *N australe* and *D. mutila*, with 35% germination after 7 h exposure to sunlight and, increasing exposure caused decreasing germination. Further they showed that relative humidity (RH) had a great effect on germinability of conidia, since 92% of conidia germinated within 3 h when RH was 100%, whereas in 97% RH it took 6 h for germination to reach 67.2%, and at 93% RH no conidia had germinated after 3 and 6 h. Air-borne conidia of Botryosphaeriaceae spp., potentially within rain droplets, have been trapped by Burkard spore traps, which could be used to detect air-borne conidia of the marker isolate at greater distances. However, in the current research programme, only one Burkard spore trap was available so this type of experiment was not attempted. Further distances of dispersal, which would have required resplashing from water films containing conidia, were not detected in these experiments. However, the resplash model may not be effective with conidia of Botryosphaeriaceae spp. since Sammond *et al.* (2016) demonstrated that most of the conidia of *N. luteum, N. parvum* and *B. dothidea* adhered onto a range of experimental surfaces, including cellulose, and were unable to be washed off within 5 minutes of deposition.

In the current study, freshly pruned canes from a vineyard were able to be infected by conidia of *N. luteum,* and *N. parvum* when held in an outdoor environment, thereby indicating that the disease cycle includes a saprophytic phase involving the canes commonly left on vineyard floors. Elena *et al.* (2016) also showed that canes naturally infected with *D. seriata* and

pruned out 2 years previously, and then held under natural vineyard conditions, were able to produce viable conidia from the pycnidia for another 18 months. Although numbers of conidia reduced over time, these results confirmed the role of pruning debris in inoculum production. The current study showed that *N. luteum,* and *N. parvum* are also likely to colonise pruning debris, which may then act as an important long-lasting inoculum source. Further experiments need to be conducted to determine whether removal of this debris from vineyard floors causes reduction in the numbers of splashed and air-borne conidia within Marlborough vineyards.

Conclusion

This study has shown that many more conidia were trapped in rainwater than in air by the Burkard spore trap and that the splash-dispersed conidia were able to infect wounds in the vineyard. Botryosphaeriaceae spp. are generally acknowledged as pathogens of wounds which are caused on grapevine tissues by the common practices of trimming (conducted in summer) and pruning (conducted from late May to late August), which coincide with some major rainfall periods. Further, this study and other studies of spore trapping in New Zealand have shown that Botryosphaeriaceae spp. conidia are present throughout the year. Although spore trapping has provided valuable information, further information is needed about the effects of biological and environmental factors on the potential of dispersed conidia to infect the grapevine tissues. The next chapter in this thesis will describe investigations into how susceptibility of wounds in different tissues is affected by the time period after pruning, tissue age, variety, conidial concentration and environmental conditions.

Chapter 4

Factors affecting infection of grapevine stems, buds and fruit

4.1 Introduction

Botryosphaeriaceae species are known to infect wounds of soft green and woody stems of grapevines, which occur on grapevine plants several times throughout the year during pruning and trimming. Studies by Munkvold and Marois (1995) and Chapuis *et al.* (1998) showed that susceptibility of grapevine stem wounds to *Eutypa lata* infection varied with wood age; wound susceptibility was highest directly after pruning and decreased over time. Also, wounds made in early winter remained susceptible for a longer time than wounds made in January or February (late winter). Biggs **(**2004) also showed that inoculum concentration was an important factor since incidence of *B. dothidea* infections on apple fruit increased as conidium concentration increased from 10^4 to 10^7 spores/mL. Petzoldt *et al.* (1981) also reported higher infection incidence in grapevine pruning wounds inoculated with 10^3 ascospores/mL of *E. lata* as compared to 10^2 ascospores/mL. However, Amponsah *et al.* (2014) showed that when 20 µL drops of *Neofusicoccum luteum* conidia (10^2-10^6/mL) were applied onto fresh trunk pruning wounds, all concentrations were able to cause 100% incidence of infection, but that lesion development was slower for the lower concentrations than the higher concentrations.

Different tissues may also vary in their susceptibility to infection by the Botryosphaeriaceae. Flowers *et al*. (2001) reported that *Diplodia sapinea* was rarely isolated from xylem and pith tissues of pine trees as compared to bark and phloem tissues. Azouaoui *et al.* (2012) also reported being able to isolate *Botryosphaeria iberica* from the necrotic bark surrounding the inoculation sites in cypress. Botryosphaericeae spp. are generally regarded as wound pathogens (Smith *et al.*, 1996; Taylor *et al.*, 2005; van Niekerk *et al.*, 2006), however Amponsah *et al.* (2012a) reported that a conidial suspension of *N. luteum* could infect even non-wounded buds on detached canes, killing most of the buds and progressing down into the supporting shoots. Phillips (1998) also reported that when a conidium suspension was 'inserted' between grapevine bud scales and stipules without being directly wounded, the buds became infected by *B. dothidea*. Amponsah *et al.* (2012a) also showed that wounded, attached berries could be infected by *N. luteum* conidia, with greater incidence of berries inoculated at harvest time than at pre-bunch closure time. Wunderlich *et al.* (2011) also

showed that detached berries were susceptible to infection when inoculated at harvest time with *D. seriata*, *N. parvum, N. luteum, Dothiorella viticola*, *L. theobromae, D. mutila* and *Fusicoccum aesculi*. In mango fruits, disease symptoms caused by *Botryosphaeria* spp. appeared only when sugar levels increased during ripening of fruit (Johnson *et al.,* 1992).

The aim of this study was to find how susceptibility of wounds in different tissues is affected by the time period after pruning, tissue age and type, Botryosphaeriaceae species, conidial concentration and environmental conditions. Unlike many other reported experiments with Botryosphaeriaceae species, the inoculated sites were not protected by Parafilm™ nor the plants covered with plastic bags to provide high relative humidity. Instead, inoculation was conducted late afternoon or early morning to reduce dehydration but still ensure that the results were relevant to natural infection conditions.

4.2 Materials and Methods

4.2.1 Effect of wound age on susceptibility

In February 2014, the potted plants of Sauvignon blanc were acclimatised for 2 weeks outside of the tunnel house at Fairhall Vineyard. Tissues of various ages (semi-hard shoots and 2-year-old lignified trunk wood) were wounded and inoculated with 20 and 40 µL drops, respectively, of conidial suspensions (10^4/mL*)* using a mixture of the three most pathogenic isolates of *N. luteum* (CC445, MM558, ICMP 16678) or *N. parvum* (MM562, G69a1, G652)*,* which were prepared as described in Section 2.2.2.1. Semi-hard shoots were wounded by removing the shoot tops with sterilized secateurs and the main stems of woody trunks were wounded by drilling a 2.5 mm diam. and 3 mm deep hole into the centre of each trunk. Inoculation was performed at 0, 7, 14, 28, 42, 56 and 70 days after wounding. Sterile water was applied to non-inoculated control plants for all treatment days. The same plants were used to provide semi-hard shoot and trunks tissues for each inoculation day with six replicate plants per treatment arranged in a RBD. After 14 days the top ~6 cm of each semi-hard shoot was harvested. The upper 2 mm of the shoot was discarded and the shoot cut into segments at 1 cm intervals up to 5 cm below the wounded area. These segments were surface sterilized, by dipping in 70% ethanol for 30 s followed by 30 s in sterile water and air drying under the laminar flow unit for 1 h, and then used for isolation onto PDA amended with chloramphenicol (0.05 g/L). After 28 days, trunk sections, up to 5 cm below the wounded inoculated areas, were harvested and cut into 1 cm segments, which excluded the ~0.5 cm area of the wound. These were surface sterilized, by dipping in 70% ethanol for 30 s,

followed by 30 s in sterile water and air drying under the laminar flow unit for 1 h. Bark and wood pieces were plated separately onto PDA amended with chloramphenicol (0.05 g/L). All plates were incubated at 25°C for 3-7 days and then typical colony appearance allowed identification of *N. parvum* and *N. luteum*. Assessment was based on incidences and lengths of colonised tissues for *N. parvum* and *N. luteum* in treated tissues.

4.2.2 Effects of season and wound age on wound susceptibility

To check the infection efficacy of pathogens at different plant growth stages and under different environmental conditions, the same process as described in Section 4.2.1 was repeated with Sauvignon blanc semi-hard green shoots and trunks (both on the same plant). Wounding, inoculation and incubation were conducted outside the tunnel house in different weather and growth conditions as occurred in the different seasons (winter, spring, summer and autumn). Dates and average environmental conditions are shown in Appendix F.1. The wounds were inoculated at 0, 7, 14, 28 and 42 days after wounding with only *N. luteum* conidia and water controls, since both species (*N. luteum* and *N. parvum*) showed similar levels of infection in Section 4.2.1. Assessment was the same as described in Section 4.2.1 except that wood and bark were not separated prior to isolation.

4.2.3 Effects of conidial number and wound age on wound susceptibility

To further check the effect of wound age and the infection efficacy of *N. luteum* with different conidial concentrations, in December, 2014 the experiment in Section 4.2.1 was repeated with Sauvignon blanc semi-hard green shoots and trunks (both on the same plant), with different conidial numbers per wound (2, 10, 20, 40 and 100). Serial dilution of a mixed isolate conidial suspension of *N. luteum* provided concentrations from 1 x 10^2 conidia/mL to 5 x 10^3 conidia/mL, for the 20 µL conidial droplets used for inoculation onto wounded hard shoots. A further dilution series of 5 x 10^1 conidia/mL to 2.5 x 10^3 conidia/mL, provided the same conidial numbers in the 40 µL conidial droplets used for inoculation onto wounded trunks. Inoculation was conducted at 0, 7, 14 and 28 days after wounding the plants as described in Section 4.2.1. Sterile water was used for the control plants, with the same tissues and wound ages. Experimental layout and assessment were the same as described in Section 4.2.1 except that wood and bark were not separated prior to isolation.

4.2.4 Bark as a saprophytic link to wound infection

In June, 2015 bark of the trunks and canes of Sauvignon blanc and Pinot noir were inoculated with isolates of *N. parvum* (isolate B2141) and *N. luteum* (isolate G51a2). Inoculation was conducted using a spray bottle to apply conidial suspensions (10^4/mL) of *N. parvum* and *N. luteum* to an area of 3 cm length (requiring about 0.5 or 1 mL of suspension, respectively) onto the bark of grapevine canes and trunks. Control vines were inoculated with sterile water only. Cuts were made into the bark and through to the wood about 1 cm above the inoculation area using a sterile scalpel, after 1 h (T1), 2 days (T2) or 1 week (T3) (Figure 4.1). They were then left for 24 h and isolation was conducted using five 1 cm segments cut from beyond the horizontal cuts (one segment), at the inoculated areas (three segments) and 1 cm below the inoculated areas (one segment), using the same isolation methods as described in Section 4.2.1. Typical colony appearance allowed identification of *N. parvum* and *N. luteum.* Assessment was based on incidences and positions of colonised tissues for *N. parvum* and *N. luteum*. In addition, Pinot noir potted plants with pruning cuts on main trunks of different ages [0 days (T1), 7 days (T2) and 14 days (T3)] were inoculated on the bark as above, with a pruning cut 1 cm below the inoculated area, and the pathogens allowed to colonise for 2 days before removing the relevant 5 cm section for cutting into 1 cm segments from which sequential isolations of bark and wood were made as in Section 4.2.1.

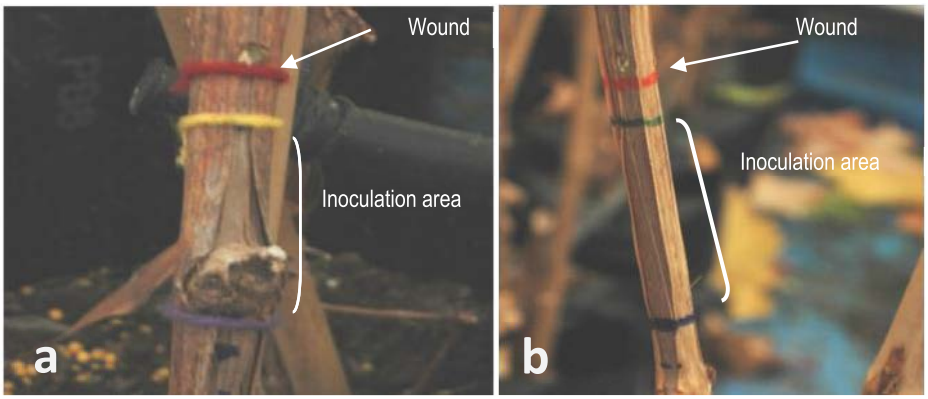


Figure 4.1 Experimental tissues showing inoculation area on bark and position of wound site made after allowing bark to be colonised; a) trunk, b) cane.

4.2.5 Lenticels infection

Since the previous experiment in Section 4.2.4 had shown bark infection seemed to have occurred through lenticels, semi-hard green shoots, canes and trunks of 2-year-old Sauvignon blanc and Pinot noir grapevines grown in the Lincoln University shade house were selected in October, 2015 to investigate infection development through lenticels. Three replicate plants were inoculated on the bark of hard green shoots, canes and trunks, selecting areas with obvious lenticels. The conidial suspensions (10^4/mL) of *N. parvum* (isolate B2141) or *N. luteum* (isolate G51a2) were applied using a spray bottle to cover an area of 3 cm length (about 0.5 to 1 mL of suspension). Control plants were inoculated with sterile water only. After 2 weeks these tissues were removed from the stems, so that infecting mycelium could be observed by fluorescence microscopy following the method of Williamson *et al.* (1998). Three thin (~0.3 mm thick) longitudinal sections of the bark and underlying wood as well as transverse sections in the lenticel areas were cut on the inoculated semi-hard green shoots, canes and trunks using a new sterile scalpel blade. The sections were fixed in Carnoy solution (ethanol: chloform: glacial acetic acid 6: 3: 1 v/v/v) for 1 h for semi-hard green shoots and for 24 h for cane and trunk tissues. Sections were removed, drained and placed in clean Universal bottles containing NaOH (1 Normal), and softened and cleared in a water bath at 60°C for 1 h (soft shoots) or 1 h 40 min (hard tissues). The NaOH was discarded and the sections were transferred to clean Petri dishes, where they were rinsed in 10 mL sterile water to remove the residual NaOH. A paint brush was used to pick up the individual sections and transfer them to a microscopic slide for mounting. Excess water was absorbed by blotting the sections gently with a new sheet of Kimwipe™ (Kimberly-Clark, Science+Brand). A drop of freshly prepared 0.1% aniline blue in 0.1 N K_2HPO_4 was used to stain each specimen section and then a cover slip was gently pressed onto the tissue section. The specimens were observed by fluorescence microscopy with an Olympus SZX12 microscope with UV light source model U-LH100HG, with a supplementary barrier filter (excitation wave length of 460-495 nm, emission of 510-550 nm) and without UV light. Image analysis was performed using the Cell/F (Olympus soft imaging solutions, Athens, Greece) image software. Data for presence of mycelium in the tissues adjacent to lenticels (incidence) were analysed by ANOVA.

4.2.6 Leaf bud infection

In this experiment, conidial suspensions (10^4/mL) were prepared for both *N. parvum* (isolate B2141) and *N. luteum* (isolate G51a2) and 20 µL drops of each applied to seven leaf buds on a single cane of each 2-year-old Sauvignon blanc and Pinot noir vines at various developmental times [tight bud (August, 2014), woolly bud (September, 2014) and green tip visible stage (October, 2014)], with and without superficial wounding. For wounding, each bud was pricked thrice with a needle on the tip to a depth of 2-3 mm. Inoculation was done immediately with 20 µL drops of conidia which were lightly spread over the surface of the wounded area with the pipette tip. Sterile water applied to controls following wounding. For each treatment there were six replicate vines arranged in a RBD outside the tunnel house at Fairhall Vineyard Blenheim. After 2 weeks, five of the treated buds were cut from each vine and used for isolation onto PDA after surface sterilization as described in Section 4.2.1. Two buds were left for observation. After 3 months (tight buds) 2 months (woolly buds) and 1 month (green tip visible buds), leaves developing from all bud stages were examined for necrosis, and the n eight, 5 mm segments cut from the lesion edges of each necrotic leaf were surface sterilised in 70% ethanol for 30 s, followed by 30 s in sterile water, dried and plated onto PDA. The green shoots which developed from each bud, were also removed, with five 1 cm segments being cut for surface sterilisation and isolation onto PDA, starting from the leaf petiole to the junction with the supporting shoot. A further 5 cm section was cut from each cane containing the buds, at 0.5 cm above the top inoculated bud on the cane, and this was cut into five 1 cm segments for isolation. These segments were surface sterilised and used for sequential isolation of 1 cm segments as described in Section 4.2.1.

This experiment was repeated in September 2015, with wounded woolly buds of 2-year-old, potted Sauvignon blanc and Pinot noir vines in the Lincoln University shade house. For each treatment (species, wounding and non-wounding) there were four replicate plants with three buds used on each plant. After 1 week these inoculated buds were removed and prepared by fixing, clearing and staining for observation under a fluorescent microscope as described in Section 4.2.4.

4.2.7 Susceptibility of berries on potted vines at different stages of development

In this experiment, potted vines of Sauvignon blanc and Pinot noir were selected in 2014 at various bunch developmental stages [pre-bunch closure (January, 2015; 4-6 mm in diameter), veraison (February, 2015) and 1 week before harvest (March, 2015)]. Grape sugar content

was measured to determine the harvest stage. Each week from 4 weeks after veraison one berry was selected from each bunch and its juice squeezed out within a new polythene bag. One drop of this juice (repeated three times) was put onto the glass face of a digital Atago Pocket PAL-1 refractometer (Atago Co., Ltd, Japan) and the reading was noted. When the mean sugar content was about 22-23%, the berries for harvest stage were inoculated. For each potted vine 12 berries were inoculated on one bunch for non-wounded berries and on another vine for wounded berries (4 superficial needle pricks per berry). The conidial suspensions (10^4/mL) prepared for both *N. parvum* (isolate B2141) and *N. luteum* (isolate G51a2) were applied with a spray bottle to the selected berries. The 12 replicate vines per treatment were arranged in a RBD outside the tunnel house at Fairhall Vineyard, Blenheim. One week after inoculation, 10 treated berries were removed and detached from their rachii. Berries and their rachii were surface sterilized as in Section 4.2.1 by dipping into 70% ethanol for 30 s, then washed with sterile water, dried and used for isolation onto PDA.

The remaining two berries were left for 1 month to observe formation of pycnidia and infection progression into shoots. They were removed from the bunch, washed with sterile water for one minute and then incubated separately in Universal bottles with moist filter paper at room temperature for 2 days to allow oozing of pycnidia. The material that appeared to ooze from the pycnidia of berries was mounted on a glass slide and examined for conidia similar to those of *N. luteum* and *N. parvum*. For the berries inoculated 1 week before harvest, the supporting shoot of each bunch was immediately excised at 5 cm below and above the bunch stem position, surface sterilized by washing with sterile water for one minute and cut into 1 cm sections, which were sequentially placed onto PDA, followed by incubation at 25°C for 3-7 days. Presence of fungal colonies characteristic of *N. luteum* and *N. parvum* indicated the distances moved by the pathogen.

4.2.8 Susceptibility of berries on field vines at different stages of development

In the Brancott Vineyard, Blenheim, 11 replicate Sauvignon blanc vines 19 years old were allocated for this experiment by the vineyard manager, of which one arm per vine was inoculated with *N. parvum* (isolate B2141) and the other with *N. luteum* (isolate G51a2). Ten bunches per vine were randomly selected for treatment at each development time (pre-bunch closure (February, 2015), veraison (March, 2015) and 1 week before harvest (April, 2015), of which four inoculated bunches were wounded (4 needle pricks/berry), four non-wounded, and two bunches (one wounded and one non-wounded) were inoculated with sterile water (control). On each bunch 12 berries were used for treatment. Conidial suspensions (10^4/mL)

were prepared for both species and applied with a spray bottle to the selected berries. After 1 week, six berries were taken from each bunch for surface sterilization with ethanol (as in Section 4.2.7) and isolation onto PDA. The rest of the berries were left for 2 weeks to observe for development of pycnidia. For the berries inoculated 1 week before harvest, the supporting shoot of each bunch was excised at 5 cm below and above the bunch stem position, surface sterilized by washing with sterile water for one minute and cut into 1 cm sections, which were sequentially placed onto PDA, followed by incubation at 25°C for 3-7 days. Presence of fungal colonies characteristic of *N. luteum* and *N. parvum* indicated the distances moved by the pathogen.

4.2.9 Statistical analysis

The lengths of colonised tissues were analysed by General Linear Model (Minitab 17th edition) to determine significant main effects, and the significance of differences between related treatments was determined using Sidak pairwise comparisons at $P \leq 0.05$. The proportion of infected berries and buds per plant were arcsine transformed to improve the homogeneity in variance. Data of infection incidence in inoculated grapevine shoots were analysed by General Linear Model (GLM) using GenStat (16th edition), which is considered appropriate for binomial data (McCue *et al*., 2008), using the logit link function. Bernoulli distribution defined as a binary variable was assumed. Standard errors of differences (SEDs) derived from GLM analysis were used to show differences between treatment means.

4.3 Results

4.3.1 Effect of wound age on susceptibility

For semi-hard green tissues, wound age at the time of inoculation significantly affected infection incidence ($P<0.001$; Appendix D.1.1). Pathogen mean infection incidence was 100% for wounds inoculated at 0 and 7 days and 83.3% at 14 days, which were all significantly greater ($P<0.05$) than the 50.0% at 28 days. The lowest ($P<0.05$) mean incidences were 16.6% at 56 days and 0% at 70 days. (Table 4.1). Pathogen mean infection incidence was not affected significantly by species ($P=0.531$) and the interaction between wound age and species was not significant ($P=0.912$).

For wood of trunks, wound age at the time of inoculation also significantly affected infection incidence ($P<0.001$; Appendix D.1.2). Mean incidence was 100% at 0 and 7 days, which was significantly greater ($P<0.05$) than the 50% at 14 days, which was significantly greater ($P<0.05$) than the 16.6% at 28 days and 0% for the remaining inoculation times (Table 4.1).

Pathogen mean infection incidence was not affected significantly by species (P=1.000) and the interaction between wound age and species was not significant (P=1.000).

For bark, wound age at the time of inoculation significantly affected infection incidence (P<0.001; Appendix D.1.3). Mean incidence was 100% for wounds inoculated at 0 and 7 days, which was significantly greater (P<0.05) than the incidence of 58.3% at 14 days and 41.6% at 28 days. The lowest mean incidences were 8.3% at 42 days, and 0% for the remaining inoculation times (Table 4.1). Pathogen infection incidence was not affected significantly by species (P=0.425) and the interaction between wound age and species was not significant (P=0.998). Control plants showed minimal internal necrosis (Figure 4.2) but no colonies characteristic of Botryosphaeriaceae spp. were isolated from any of the control plants.

Table 4.1 Mean infection incidences (%) of *Neofusicoccum luteum* and *N. parvum* after inoculation with conidial suspensions (10^4) onto different wound ages made in semi-hard green shoots and trunks of potted Sauvignon blanc grapevines. Values in parentheses are logit transformed data.

Wound age	[a]***Neofusicoccum* spp. incidence (%)**		
	Semi-hard shoots	**Trunks**	
		Wood	**Bark**
0	100 (0.00) d	100 (0.00) c	100 (0.00) c
7	100 (0.00) d	100 (0.00) c	100 (0.00) c
14	83.3 (0.34) d	50.0 (1.10) b	58.3 (0.90) b
28	50.0 (1.15) c	16.6 (8.76) a	41.6 (1.36) b
42	33.3 (1.61) bc	0 (15.90) a	8.3 (9.65) a
56	16.6 (8.26) ab	0 (15.90) a	0 (16.90) a
70	0 (14.90) a	0 (15.90) a	0 (16.90) a
SED	**(0.351)**	**(0.346)**	**(0.440)**

[a]Values within columns followed by the same letter are not significantly different according to the SEDs derived from GLM analysis of logit transformed data.

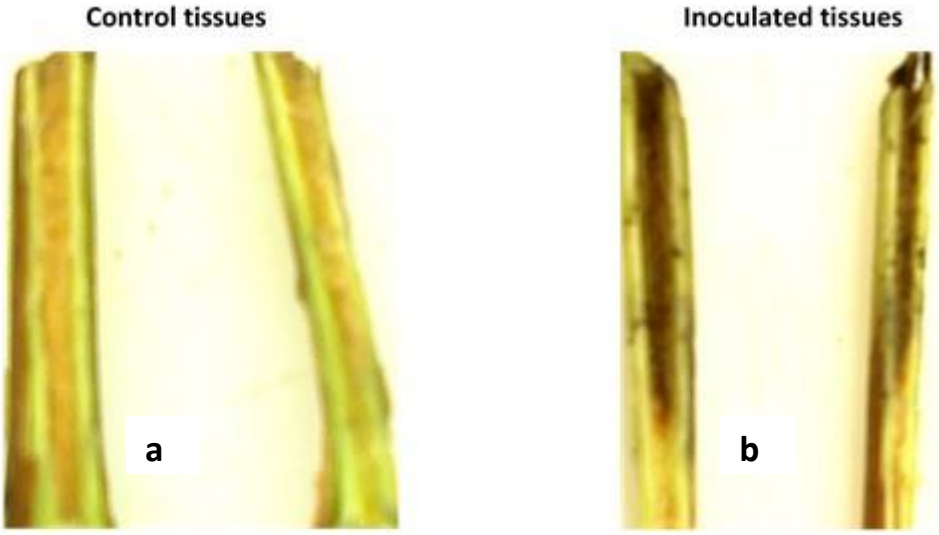


Figure 4.2 Effect of inoculation with *Neofusicoccum parvum* on semi-hard green shoots with internal necrosis (b) as compared to non-inoculated control (a).

For infection of semi-hard green tissues, the lengths of colonised tissues were significantly affected by wound age ($P<0.001$; Appendix D.2.1). The greatest ($P<0.05$) mean length of colonisation was for the 0 day inoculation (3.0 cm) and the least was for 56 and 70 days, mean similar ($P>0.05$) lengths being 0.2 cm and 0 cm, respectively (Table 4.2). Colonisation length was not affected by species ($P=0.153$) and there was no interaction between wound age and species ($P=0.486$).

For infection of the wood of trunks, the lengths of colonised tissues were affected significantly by wound age ($P<0.001$; Appendix D.2.2). The greatest ($P<0.05$) mean lengths of colonisation were for 0 and 7 days inoculation with similar ($P>0.05$) lengths of 1.4 and 1.3 cm. The least ($P<0.05$) mean lengths were for 56 and 70 days, being 0 cm for both (Table 4.2). The length of colonised tissues was not affected by species ($P=1.00$) and there was no interaction between wound age and species ($P=0.459$).

Table 4.2 Mean lengths of colonised tissues after inoculation with conidial suspensions (10^4) of *Neofusicoccum luteum* and *N. parvum* onto different wound ages made in semi-hard green shoots and trunks of potted Sauvignon blanc grapevines.

Wound age	[a]*Neofusicoccum* spp. colonised tissues (cm)		
	Semi-hard shoots	Trunks	
		Wood	Bark
0	3.0a	1.4a	1.8a
7	1.9b	1.3a	1.6ab
14	1.1bc	0.9ab	1.0abc
28	0.4cd	0.3bc	0.7bc
42	0.3cd	0c	0.2c
56	0.2d	0c	0c
70	0d	0c	0c
Means	**0.5**	**0.4**	**0.7**

[a]Values within columns followed by the same letter are not significantly different according to the Sidak test at 95% CIs.

For infection of the bark of trunks, the lengths of colonised tissues were significantly affected by wound age ($P<0.001$; Appendix D.2.3). The greatest ($P<0.05$) mean colonised length was for 0 day inoculation (1.8 cm) and the least ($P<0.05$) were for 42, 56 and 70 days, all being 0 cm (Table 4.2). The length of colonised tissues was not affected by species ($P=0.207$). There was no interaction between wound age and species ($P=0.965$).

4.3.2 Effects of season and wound age on wound susceptibility

On semi-hard green shoots, *N. luteum* infection incidence was significantly affected by seasons ($P=0.018$; Appendix D.3.1), with greatest ($P<0.05$) mean infection incidence in autumn (73.3%) and lowest ($P<0.05$) in summer (40.0%) (Table 4.3). Infection incidence was also affected by wound age ($P<0.001$), with greatest ($P<0.05$) mean infection incidences for 0 and 7 days (100 and 87.5%, respectively) and least ($P<0.05$) for 42 days (4.1%) (Table 4.4). The interaction between seasons and wound age was not significant ($P=0.877$), although in each season there were different trends for decreasing infection as wound aged (Table 4.5).

Table 4.3 Mean infection incidences of *Neofusicoccum luteum* after inoculation with conidial suspensions (10^4) onto wounds made in hard green shoots and trunks of potted Sauvignon blanc grapevines in different seasons. Values in parentheses are logit transformed data.

Seasons	[a]*Neofusicoccum* spp. incidence (%)	
	Green shoots	**Trunks**
Autumn	73.3 (0.55) c	66.6 (0.62) b
Spring	66.4 (0.70) bc	57 (0.85) ab
Winter	53.3 (1.02) ab	46.6 (1.10) a
Summer	46.2 (1.20) a	40 (1.34) a
SED	**(0.093)**	**(0.515)**

[a]Values within columns followed by the same letter are not significantly different according to the SEDs derived from GLM analysis of logit transformed data.

Table 4.4 Mean infection incidences of *Neofusicoccum luteum* after inoculation with conidial suspensions (10^4) onto different wound ages made in semi-hard green shoots and trunks of potted Sauvignon blanc grapevines. Values in parentheses are logit transformed data.

Wound age	[a]*Neofusicoccum* spp. incidence (%)	
	Semi-hard shoots	**Trunks**
0	100 (0.00) d	100 (0.00) d
7	87.5 (0.26) d	83.3 (0.36) d
14	62.5 (0.85) c	50 (1.13) c
28	37.5 (1.57) b	25 (4.75) b
42	12.5 (7.97) a	4.1 (11.03) a
SED	**(0.392)**	**(0.523)**

[a]Values within columns followed by the same letter are not significantly different according to the SEDs derived from GLM analysis of logit transformed data.

Table 4.5 Mean infection incidences of *Neofusicoccum luteum* after inoculation with conidial suspensions (10^4) onto wounds made on different days on Semi-hard green shoots and trunks of potted Sauvignon blanc grapevines in different seasons. Values in parentheses are logit transformed data.

		[a]*Neofusicoccum* spp. incidence (%)				
Tissue type	**Seasons**	**Wound age**				
		0	**7**	**14**	**28**	**42**
Green	**Autumn**	100 (0.00)	100 (0.00)	83.3 (0.34)	50 (1.12)	33.3 (1.63)
	Spring	100 (0.00)	100 (0.00)	66.7 (0.70)	50 (1.12)	16.7 (2.43)
	Summer	100 (0.00)	66.7 (0.70)	33.3 (1.63)	33.3 (1.63)	0 (13.90)
	Winter	100 (0.00)	83.3 (0.34)	66.7 (0.70)	16.7 (2.40)	0 (13.90)
Trunk	**Autumn**	100 (0.00)	100 (0.00)	66.7 (0.69)	50 (1.099)	16.7 (2.40)
	Spring	100 (0.00)	100 (0.00)	50 (1.10)	33.3 (1.61)	0 (13.90)
	Summer	100 (0.00)	50 (1.10)	33.3 (1.61)	16.7 (2.40)	0 (13.90)
	Winter	100 (0.00)	83.3 (0.34)	50 (1.10)	0 (13.90)	0 (13.90)

[a]Values within columns are not significantly different according to the SEDs derived from GLM analysis of logit transformed data.

On woody trunks, *N. luteum* infection incidence was significantly affected by seasons (P=0.024; Appendix D.3.2), with greatest (P<0.05) mean infection incidence in autumn (66.6%) and lowest (P<0.05) in summer (46.6%) (Table 4.3). Infection incidence was also affected by wound age (P<0.001), with mean infection incidence being greatest (P<0.05) for 0 days (100%) and least (P<0.05) for 42 days (16.7%) (Table 4.4). The interaction between seasons and wound age was not significant (P=0.734).

For semi-hard green shoots, length of colonised tissues was significantly affected by season (P<0.001; Appendix D.4.1), with greatest (P<0.05) mean length in autumn (2.0 cm) and lowest (P<0.05) in summer (1.0 cm) (Table 4.6). Length of colonised tissues was also affected by inoculation day after pruning (P<0.001) with greatest (P<0.05) length for 0 day inoculation (2.9 cm) and lowest (P<0.05) for 42 day inoculation (0.2 cm) (Table 4.7). The interaction between the seasons and time of wound age was not significant (P=0.173).

For trunks, the length of colonised tissues was also significantly affected by season (P<0.001; Appendix D.4.2), with greatest (P<0.05) mean length in autumn (1.3 cm) and lowest in summer (0.6 cm) (Table 4.6). Length of colonised tissues was also affected by inoculation

day after pruning (P<0.001), with greatest (P<0.05) length for 0 day inoculation (2.1 cm) and lowest (P<0.05) for 42 day inoculation (0.1 cm) (Table 4.7). The interaction between the seasons and time of wound age was not significant (P=0.173).

Table 4.6 Mean lengths of colonised tissues after inoculation with conidial suspensions (10^4) of *Neofusicoccum luteum* onto wounds made in semi-hard green shoots and trunks of potted Sauvignon blanc grapevines in different seasons.

Seasons	[a]***Neofusicoccum* spp. colonised tissues (cm)**	
	Semi-hard shoots	**Trunks**
Autumn	2.0a	1.3a
Spring	2.0a	1.0ab
Winter	1.1b	0.7bc
Summer	1.0b	0.6c
Means	**0.5**	**0.3**

[a]Values within columns followed by the same letter are not significantly different according to the Sidak test at 95% CIs.

Table 4.7 Mean lengths of colonised tissues after inoculation with conidial suspensions (10^4) of *Neofusicoccum luteum* onto different wound ages made in semi-hard green shoots and trunks of potted Sauvignon blanc grapevines.

Wound age	[a]***Neofusicoccum* spp. colonised tissues (cm)**	
	Semi hard shoots	**Trunks**
0	2.9a	2.1a
7	2.6a	1.3b
14	1.4b	0.8c
28	0.7c	0.3cd
42	0.6c	0.1d
Means	**10.9**	**20.2**

[a]Values within columns followed by the same letter are not significantly different according to the Sidak test at 95% CIs.

4.3.3 Effects of conidial number and wound age on wound susceptibility

For semi-hard green shoots, mean infection incidence was significantly affected by wound age (P<0.001; Appendix D.5.1), with greatest (P<0.05) mean infection incidence for 0 day inoculation (100%) and lowest (P<0.05) for 28 days (36.6%) (Table 4.8). Mean infection incidence was not affected by different inoculum levels (P=0.772) and the interaction between wound age and inoculum level was not significant (P=0.885).

For woody trunks, mean infection incidence was also significantly affected by wound age (P<0.001; Appendix D.5.2), with greatest (P<0.05) mean infection incidence for 0 day inoculation (93.3%) and lowest (P<0.05) for 28 days (23.3%) (Table 4.8). Mean infection incidence was not affected by different inoculum levels (P=0.093) and the interaction between wound age and inoculum levels was not significant (P=0.930).

The trends for greater incidence with higher inoculum levels, which seemed more evident with lower range of conidial numbers and for 0 and 7 day wounds are shown in Table 4.11

Table 4.8 Mean infection incidences of *Neofusicoccum luteum* after inoculation with conidial suspensions (10^4) onto different wound ages made in semi-hard green shoots and trunks of potted Sauvignon blanc grapeviness. Values in parentheses are logit transformed data.

Wound age	[a]*Neofusicoccum luteum* infection incidence (%)	
	Green shoots	Trunks
0	100 (0.00) c	93.3 (0.14) c
7	86.6 (0.41) bc	80.0 (0.43) c
14	70.0 (0.92) b	50.0 (1.14) b
28	36.6 (1.23) a	23.3 (2.08) a
SED	**(0.499)**	**(0.338)**

[a]Values within columns followed by the same letter are not significantly different according to the SEDs derived from GLM analysis of logit transformed data.

For semi-hard green shoots, length of *N. luteum* colonised tissues was significantly affected by inoculation time (P<0.001; Appendix D.6.1), with greatest (P<0.05) mean length for 0 day inoculation (2.8 cm) and shortest (P<0.05) mean length for 14 and 28 days (0.9 and 0.5 cm, respectively; Table 4.9). There was a significant effect of inoculum level (P=0.020), with longest (P<0.05) length for 100 conidia/wound site (1.8 cm) and shortest (P<0.05) length for 2 conidia per wound site (1.1 cm) (Table 4.10). There was no significant interaction between

wound age and inoculum level (P=0.105). Figure 4.6 shows the greater lesion length for 100 conidia than for 2 conidia per wound site.

Table 4.9 Mean lengths of colonised tissues after inoculation with conidial suspensions of *Neofusicoccum luteum* onto different wound ages made in hard green tissues and trunks of potted Sauvignon blanc grapevines.

Wound age	[a]*Neofusicoccum luteum* colonised tissues (cm)	
	Green shoots	**Trunks**
0	2.8a	2.4a
7	1.5b	1.3b
14	0.9c	0.6c
28	0.5c	0.3c
Means	**0.3**	**0.4**

[a]Values within columns followed by the same letter are not significantly different according to the Sidak test at 95% CIs.

Table 4.10 Mean lengths of colonised tissues after inoculation with different numbers of conidia of *Neofusicoccum luteum* onto wounded semi-hard green shoots and trunks of potted Sauvignon blanc grapevines.

Inoculum conc. conidia/wound	[a]*Neofusicoccum luteum* colonised tissues (cm)	
	Green shoots	**Trunks**
100	1.8a	1.4a
20	1.6ab	1.3ab
40	1.5ab	1.3ab
10	1.2ab	0.8bc
2	1.1b	0.7c
Means	**0.4**	**0.3**

[a]Values within columns followed by the same letter are not significantly different according to the Sidak test at 95% CIs.

For woody trunks, length of *N. luteum* colonised tissues was significantly affected by wound age (P<0.001; Appendix D.6.2), with greatest (P<0.05) mean length for 0 day inoculation (2.4 cm) and the shortest (P<0.05) mean lengths for 14 and 28 days (0.6 and 0.3 cm,

respectively) (Table 4.9). There was a significant effect of inoculum level (P<0.001; Appendix D.6.2), with longest (P<0.05) mean length for 100 conidia/wound site (1.4 cm) and shortest (P<0.05) mean length for 2 conidia per wound site (0.8 cm) (Table 4.10). The significant (P=0.027; Appendix D.6.2) interaction between wound age and inoculum level seemed to be associated with the higher conidial numbers causing similar colonisation distances for 0 and 7 day wound ages (Table 4.11).

Table 4.11 Mean lengths of colonised tissues and infection incidence in trunks of potted Sauvignon blanc grapevines inoculated with different doses of conidia of *Neofusicoccum luteum* onto wounds of different ages. Values in parentheses are logit transformed data.

		[a]Colonised tissues (cm)	[b]Infection incidence (%)
Inoculum dose	**Wound age**	**Mean**	**Mean**
2	0	1.2cdef	66.7 (0.70)
2	7	0.6def	50.0 (1.10)
2	14	0.4def	33.3 (1.61)
2	28	0.4def	33.3 (1.61)
10	0	2.1abc	100 (0.00)
10	7	0.7def	66.7 (0.70)
10	14	0.4def	33.3 (1.61)
10	28	0.2f	16.7 (2.40)
20	0	2.8ab	100 (0.00)
20	7	1.5bcdef	100 (0.00)
20	14	0.8cdef	50.0 (1.10)
20	28	0.2f	16.7 (2.40)
40	0	2.7ab	100 (0.00)
40	7	1.8abcd	83.3 (0.34)
40	14	0.5def	66.7 (0.70)
40	28	0.3ef	16.7 (2.40)
100	0	2.9a	100 (0.00)
100	7	1.7abcde	100 (0.00)
100	14	0.8cdef	66.7 (0.70)
100	28	0.2f	33.3 (1.61)

[a]Values for colonised tissues within columns followed by the same letter are not significantly different according to the Sidak test at 95% CIs. [b]Values for infection incidence are not significantly different according to the SEDs derived from GLM analysis of logit transformed data, shown in brackets.

4.3.4 Bark as saprophytic link to wound infection

For canes, infection incidence of the first piece beyond the inoculated area (where the wounds were made) was significantly affected by treatments (*P*=0.050; Appendix D.7.1), with incidences for T1 (1 h), T2 (2 days) and T3 (1 week) being 33.3%, 41.6% and 70.8%, respectively (Table 4.12), which indicated movement of the pathogen from the inoculated point, through the bark upwards to where the wounds were made, with greater penetration over time. The central pieces which were inoculated had 100% infection incidence, which indicated that the pathogen had remained latent in the bark. The pieces from below and 1 cm beyond the inoculated areas had 0% infection incidence, indicating no movement into the new tissues. The infection incidence was not significantly affected by species or grapevine variety (*P*=0.529, *P*=0.826, respectively). All the interactions were non-significant. Non-inoculated controls did not show any symptoms of Botryosphaericeae.

For trunk wood, infection incidence of the first piece beyond the inoculated area was significantly affected by treatments (*P*=0.009; Appendix D.7.2), with incidences for T1, T2 and T3 being 25%, 33.3% and 70.5%, respectively (Table 4.12). The central pieces which were underneath the inoculated bark area had a mean of 83.3% infection incidence, with 76.3%, 83.3% and 90.2% for T1, T2 and T3 respectively. Infection in the wood below indicated that bark infection had progressed rapidly into adjacent wood. The pieces from below and 1 cm beyond the inoculated areas had 0% infection incidence. Infection incidence was not significantly affected by species or variety (*P*=0.871, *P*=0.791, respectively). All the interactions were non-significant. Non-inoculated controls did not show any symptoms of Botryosphaericeae

For bark, infection incidence of the first piece beyond the inoculated area was significantly affected by treatments (*P*=0.044; Appendix D.7.3), with incidences for T1, T2 and T3 being 24.9%, 49.5% and 66.6%, respectively (Table 4.12). The central pieces which were inoculated had 100% infection incidence and the pieces from below and 1 cm beyond the inoculated areas had 0% infection incidence Infection incidence was not significantly affected by species or variety (*P*=0.946, *P*=0.365, respectively). All the interactions were non-significant. Non-inoculated controls did not show any symptoms of Botryosphaericeae

Table 4.12 Mean infection incidences for *Neofusicoccum luteum* and *N. parvum* 1 cm above the inoculation areas of bark after wounding at different times [(T1 (1 h), T2 (2 days) and T3 (7 days)] on canes and wood tissues of potted Sauvignon blanc and Pinot noir grapevines. Values in parentheses are logit transformed data.

Treatments	[a]Mean infection incidence (%) 1 cm segment above the inoculation point		
	canes	wood	bark
T1	33.3 (1.61) a	25 (2.10) a	24.9 (2.03) a
T2	41.6 (1.36) a	33.3 (1.82) b	49.5 (1.14) b
T3	70.8 (0.60) b	70.5 (0.64) c	66.6 (0.72) c
SED	**(0.195)**	**(0.231)**	**(0.220)**

[a]Values within columns followed by the same letter are not significantly different according to the SEDs derived from GLM analysis of logit transformed data.

In the repeated experiment with trunks of Pinot noir potted plants, infection incidence in wood of the first piece beyond the inoculated area was significantly affected by treatments (P=0.041; Appendix D.8.1), with incidences for T1 (0 day), T2 (7 days) and T3 (14 days) being 25%, 50% and 83.3%, respectively (Table 4.13). The central pieces which had been inoculated had 84.2% mean infection incidence, with 72.2%, 88.8% and 91.6% for T1, T2 and T3, respectively, and the pieces from below the inoculated areas had 0% infection incidence. Infection incidence for trunk wood was not significantly affected by species (P=0.422). All the interactions were non-significant. Non-inoculated controls did not show any symptoms of Botryosphaericeae

Table 4.13 Mean infection incidences for *Neofusicoccum luteum* and *N. parvum* 1 cm above the inoculation area after wounding at different times (T1 (1 h), T2 (2 days) and T3 (1 week) on trunk tissues of potted Pinot noir grapevines. Values in parentheses are logit transformed data.

Treatments	[a]Mean infection incidence (%) 1 cm above the inoculation point	
	Wood	Bark
T1	25.0 (2.01) a	16.6 (2.40) a
T2	50.0 (1.10) b	41.6 (1.35) b
T3	83.3 (0.35) c	83.3 (0.35) c
SED	**(0.363)**	**(0.496)**

[a]Values within columns followed by the same letter are not significantly different according to the SEDs derived from GLM analysis of logit transformed data.

Infection incidence for bark of the first piece beyond the inoculated area was significantly affected by treatments (P=0.023; Appendix D.8.2), with incidences for T1, T2 and T3 being16.6%, 41.6% and 83.3%, respectively. The three central pieces which were inoculated had 100% infection incidence and the pieces from below the inoculated areas had 0% infection incidence. The infection incidence was not significantly affected by species (P=0.318). All the interactions were non-significant. Non-inoculated controls did not show any symptoms of Botryosphaericeae

4.3.5 Lenticel infection

The fixation and clearing of specimens made it possible to visualize hyphae and germinating conidia in longitudinal and transverse sections of cane bark, wood and green shoots under the light component of the fluorescent microscope. Although the plant tissues also fluoresced it did not interfere with the differentiation of mycelium (Figure 4.3). However, it was not possible to discern mycelium in the bark of the tissues of trunks.

Overall infection incidence was not significantly affected by species or variety (P=0.586, P=0.111; Appendix D.9.1, respectively), but it was significantly effect by tissue types (P=0.008), with overall mean infection incidences of semi-hard green shoots, canes and trunks being 97.9%, 93.0% and 67.5%, respectively (Table 4.14). Infection incidence was also significantly affected by segment types (P<0.001; Appendix D.9.1) being 77.9%, 48.1% and 72.1% of cross-sections and longitudinal sections (bark and underlying wood), respectively.

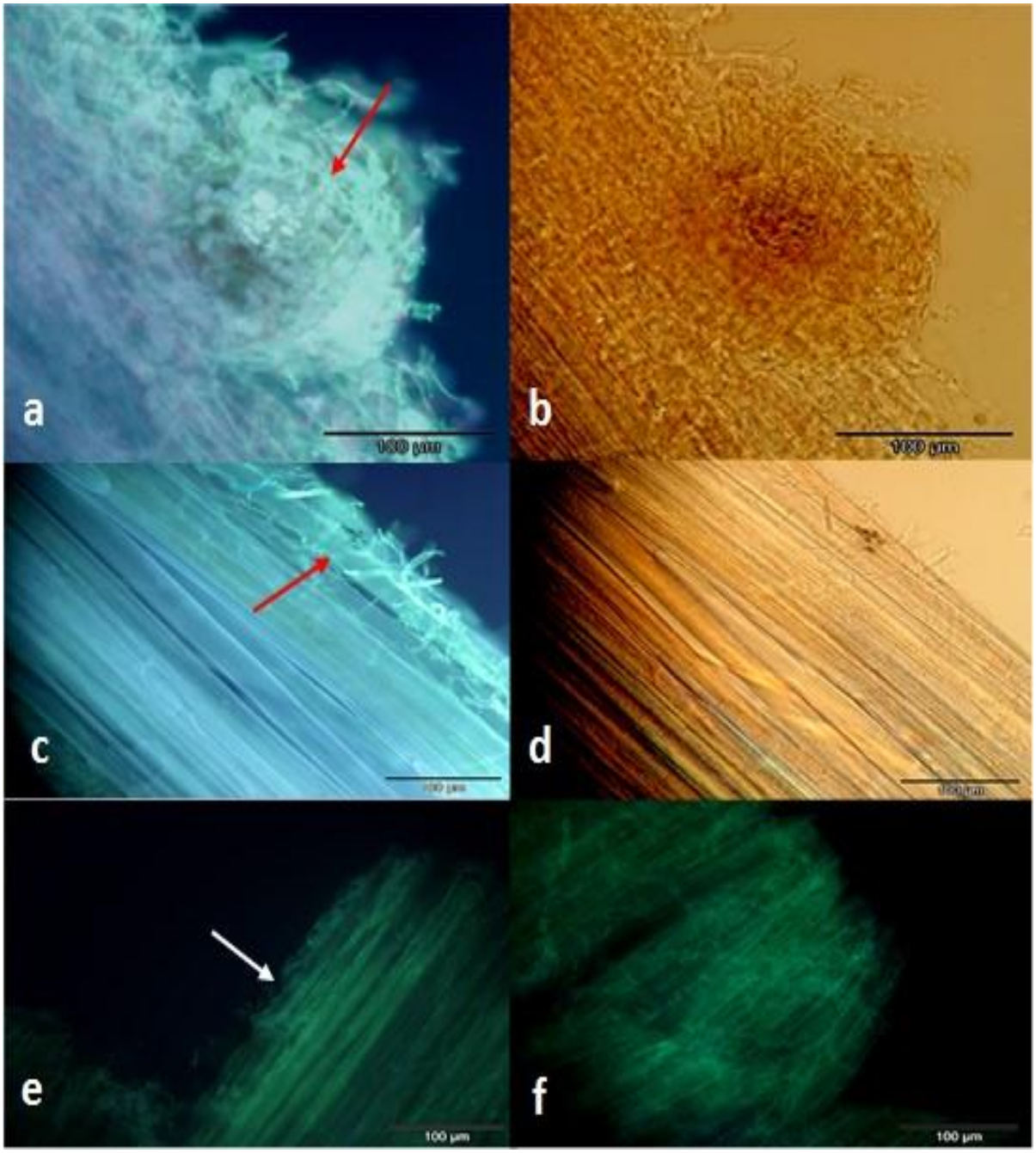


Figure 4.3 Representative fluorescent microscope observations of ~ 0.3 mm sections cut from grapevine tissues inoculated with *Neofusicoccum luteum* and *N. parvum* a) longitudinal section of bark of cane showing lenticel (arrow) with hyphae of *N. luteum* under UV light, b) same image without UV light, c) longitudinal section cut of green shoot showing mycelium of *N. parvum* under UV light, d) same image without UV light, e) longitudinal section of cane with germinating conidia of *N. parvum* under UV light, f) cross section of green shoot with mycelium of *N. luteum* under UV light. Scale bars represent 100 µm.

Table 4.14 Mean percent of segments showing mycelium in thin sections cut at lenticels of different stem tissues when examined by fluorescent microscopy, 7 days after inoculation with conidia of *Neofusicoccum luteum* and *N. parvum* onto 2-year-old plants of Sauvignon blanc and Pinot noir.

	[a]Stem tissue types			
	Longitudinal Sections		Crosssections	**Mean**
Tissues types	Bark	Wood		
Green shoots	74.9	69.4	97.9	**97.9a**
Canes	69.4	77.7	93.0	**93.0a**
Trunk	0	69.4	67.5	**67.5b**
Means	**48.1b**	**72.1a**	**77.9a**	

[a]Values for infection incidence within columns and rows followed by the same letter are not significantly different according to the Sidak test at 95% CIs.

4.3.6 Leaf Bud infection

Non-wounded buds yielded colonies of *N. luteum* and *N. parvum* only from the buds inoculated at the green tip visible stage, therefore non-wounded buds were not included in the overall data analysis and the data for non-wounded green tip visible stage buds were analysed separately. For the wounded buds over all bud development stages, infection incidence was significantly affected by bud stage (P=0.003; Appendix D.10.1), with overall mean incidences at dormant, woolly and green tip visible stages of 43.3%, 46.7% and 55%, respectively. There was significant effect of species (P=0.037), with overall mean infection incidences of *N. luteum* and *N. parvum* being 50.6% and 46.1%, respectively. The effect of variety on mean infection incidence was also significant (P=0.006), with overall mean incidences for Sauvignon blanc (52.0%) being greater than for Pinot noir (45.0%). These incidence data were also analysed according to the bud stage inoculated to further investigate the effects.

Table 4.15 Mean infection incidence on *Neofusicoccum luteum* and *N. parvum* potted grapevines (Sauvignon blanc and Pinot noir) at different bud developmental stages.

Species	Developmental bud stages at inoculation	[a]Mean infection incidence (%)
N. luteum	Dormant	46.7a
N. parvum	Dormant	40.0a
N. luteum	Woolly	48.3a
N. parvum	Woolly	45.0a
N. luteum	Green tip visible	56.6a
N. parvum	Green tip visible	53.3a

[a]Values within columns followed by the same letter are not significantly different according to the Sidak test at 95% CIs.

At the dormant stage, incidences of *N. luteum* and *N. parvum* in wounded buds were not significantly affected by species (P=0.238; Appendix D.10.2) or grapevine variety (P=0.238). Mean incidences of *N. luteum* and *N. parvum* were 46.7 and 40.0%, respectively (Table 4.15) and for Sauvignon blanc and Pinot noir were 46.7 and 40.0%, respectively. The interaction between species and variety was not significant (P=0.261; Appendix D.10.2).

At the woolly bud stage, incidences of *N. luteum* and *N. parvum* were not significantly affected by species (P=0.506; Appendix D.10.3) or grapevine variety (P=0.182). Mean incidences of *N. luteum* and *N. parvum* were 48.3 and 45.0%, respectively (Table 4.15), and for Sauvignon blanc and Pinot noir were 50 and 43.3%, respectively. The interaction between species and variety was not significant (P=0.506) ().

At the green tip visible stage, incidences of *N. luteum* and *N. parvum* were not significantly affected by species (P=0.491; Appendix D.10.4) or grapevine variety (P=0.339). Mean incidences of *N. luteum* and *N. parvum* were 56.6% and 53.3%, respectively (Table 4.15), and for Sauvignon blanc and Pinot noir were 58.8% and 51.7%, respectively. The interaction between species and variety was not significant (P=0.659) ().

For non-wounded buds at the green tip visible stage, incidences of *N. luteum* and *N. parvum* were not significantly affected by species or grapevine variety (P=0.552, P=0.976; Appendix D.10.5, respectively). Mean incidences of *N. luteum* and *N. parvum* were 33 and 30%,

respectively, and was 32.5% for both Sauvignon blanc and Pinot noir. The interaction between species and variety was not significant (P=0.976).

The pathogens colonised all the green shoots growing out of the wounded buds and all canes supporting the buds (100% incidence for both tissues). Over all buds, mean lengths of colonised tissues in green shoots that developed from the buds were significantly affected by different bud stages of inoculation (P<0.001; Appendix D.11.1), with similar (P<0.05) mean lengths of colonised shoots inoculated at dormant and woolly stages (1.7 cm and 1.9 cm, respectively) which were less (P<0.05) than for the green tip visible (2.3 cm). There was a significant effect of species (P<0.001), with mean lengths of colonised tissues for *N. luteum* and *N. parvum* being 2.2 cm and 1.7 cm, respectively. The effect of grapevine variety on mean length of colonised shoots was also significant (P<0.001), with mean lengths of colonised shoots for Sauvignon blanc and Pinot noir being 2.1 and 1.8 cm, respectively.

Over all wounded buds, mean lengths of colonised tissues in supporting canes showed a significant effect of different bud stages used for inoculation (P<0.001; Appendix D.11.2). Over all buds, mean lengths of colonised canes at dormant, woolly and green tip visible stage were 1.7 cm, 2.0 cm and 2.4 cm, respectively, which were all significantly different (P>0.05). There was a significant effect of species (P<0.001), with mean lengths of colonised canes for *N. luteum* and *N. parvum* being 2.3 cm and 1.7 cm, respectively. The effect of variety on mean length of colonised canes was also significant (P<0.001), with mean lengths for Sauvignon blanc and Pinot noir being 2.2 cm and 1.9 cm, respectively. These data for green shoots and canes were also analysed according to the inoculated bud stage to further investigate the effects.

For dormant wounded buds, the lengths of colonised tissues in the green shoots were significantly affected by species (P<0.001; Appendix D.11.3), with mean lengths for *N. luteum* and *N. parvum* being 1.9 cm and 1.4 cm, respectively (Table 4.16). The mean lengths of colonised shoots were also affected by variety (P=0.006), with mean lengths for Sauvignon blanc and Pinot noir being 1.8 cm and 1.5 cm, respectively (Table 4.17). The interaction between species and variety was not significant (P=0.678). For supporting canes, the lengths colonised were significantly affected by species (P<0.001; Appendix D.11.4), with mean lengths for *N. luteum* and *N. parvum* being 2.0 cm and 1.5 cm, respectively (Table 4.16). The lengths of colonised canes were also affected by variety (P=0.002), with mean lengths for Sauvignon blanc and Pinot noir being 1.9 cm and 1.6 cm, respectively (Table 4.17). The interaction between species and variety was not significant (P=0.410).

Table 4.16 Mean lengths of colonised tissues (Green shoots and canes) on *Neofusicoccum luteum* and *N. parvum* potted grapevines (Sauvignon blanc and Pinot noir) at different bud developmental stages.

Species	Developmental bud stages at inoculation	[a]Mean distance colonised (cm)	
		Green shoots	Cane tissues
N. luteum	Dormant	1.9a	2.0a
N. parvum	Dormant	1.4b	1.47b
N. luteum	Woolly	2.2a	2.2a
N. parvum	Woolly	1.6b	1.68b
N. luteum	Green tip visible	2.6a	2.7a
N. parvum	Green tip visible	1.6b	2.0b

[a]Values within columns followed by the same letter are not significantly different according to the Sidak test at 95% CIs.

Table 4.17 Mean lengths of colonised tissues on Sauvignon blanc and Pinot noir potted grapevines at different bud developmental stages.

Variety	Developmental bud stages at inoculation	[a]Mean distance colonised (cm)	
		Green shoots	Cane tissues
Sauvignon blanc	Dormant	1.8a	1.9a
Pinot noir	Dormant	1.5b	1.58b
Sauvignon blanc	Woolly	2.0a	2.0a
Pinot noir	Woolly	1.78b	1.8b
Sauvignon blanc	Green tip visible	2.5a	2.57a
Pinot noir	Green tip visible	2.16b	2.2b

[a]Values within columns followed by the same letter are not significantly different according to the Sidak test at 95% CIs.

For wounded woolly buds, the lengths of colonised green shoots were significantly affected by species (P<0.001; Appendix D.11.5), with mean lengths for *N. luteum* and *N. parvum* being 2.2 cm and 1.6 cm, respectively (Table 4.16). The lengths of colonised shoots were also affected by variety (P=0.005), with mean lengths for Sauvignon blanc and Pinot noir being 2.0 cm and 1.8 cm, respectively (Table 4.17). The interaction between species and variety was not significant (P=0.608). For the supporting canes, the lengths colonised were

significantly affected by species (P<0.001; Appendix D.11.6), with mean lengths for *N. luteum* and *N. parvum* being 2.2 cm and 1.7 cm, respectively (Table 4.16). The lengths of colonised canes were also affected by variety (P=0.006), with mean lengths of colonised canes for Sauvignon blanc and Pinot noir being 2.0 cm and 1.8 cm, respectively (Table 4.17). The interaction between species and variety was not significant (P=0.540).

For wounded green tip visible buds, the lengths of colonised green shoots were significantly affected by species (P<0.001; Appendix D.11.7), with mean lengths for *N. luteum* and *N. parvum* being 2.6 cm and 1.6 cm, respectively (Table 4.16). The lengths of colonised shoots were also affected by variety (P=0.005), with mean lengths of colonised tissues for Sauvignon blanc and Pinot noir being 2.5 cm and 2.1 cm, respectively (Table 4.17). The interaction between species and variety was not significant (P=0.503). For supporting canes, the lengths colonised were significantly affected by species (P<0.001; Appendix D.11.8), with mean lengths for *N. luteum* and *N. parvum* being 2.7 cm and 2.0 cm, respectively (Table 4.16). The lengths of colonised canes were also affected by variety (P=0.004), with mean lengths for Sauvignon blanc and Pinot noir being 2.6 cm and 2.2 cm, respectively (Table 4.17). The interaction between species and variety was not significant (P=0.472).

Leaf and petiole necrosis was observed in leaves which emerged from inoculated wounded buds after 1 month (Figure 4.4).

Figure 4.4 Necrosis on leaves (red arrows) and petioles (yellow arrows) on shoots developed 1 month after inoculation of wounded green tip visible bud stage.

When woolly buds on Sauvignon blanc vines were inoculated at Lincoln University, the sections prepared after 1 week and observed by fluorescent microscope showed presence of

germinating conidia and development of mycelia within the buds (Figure 4.5). Overall infection incidence was 88.8%.

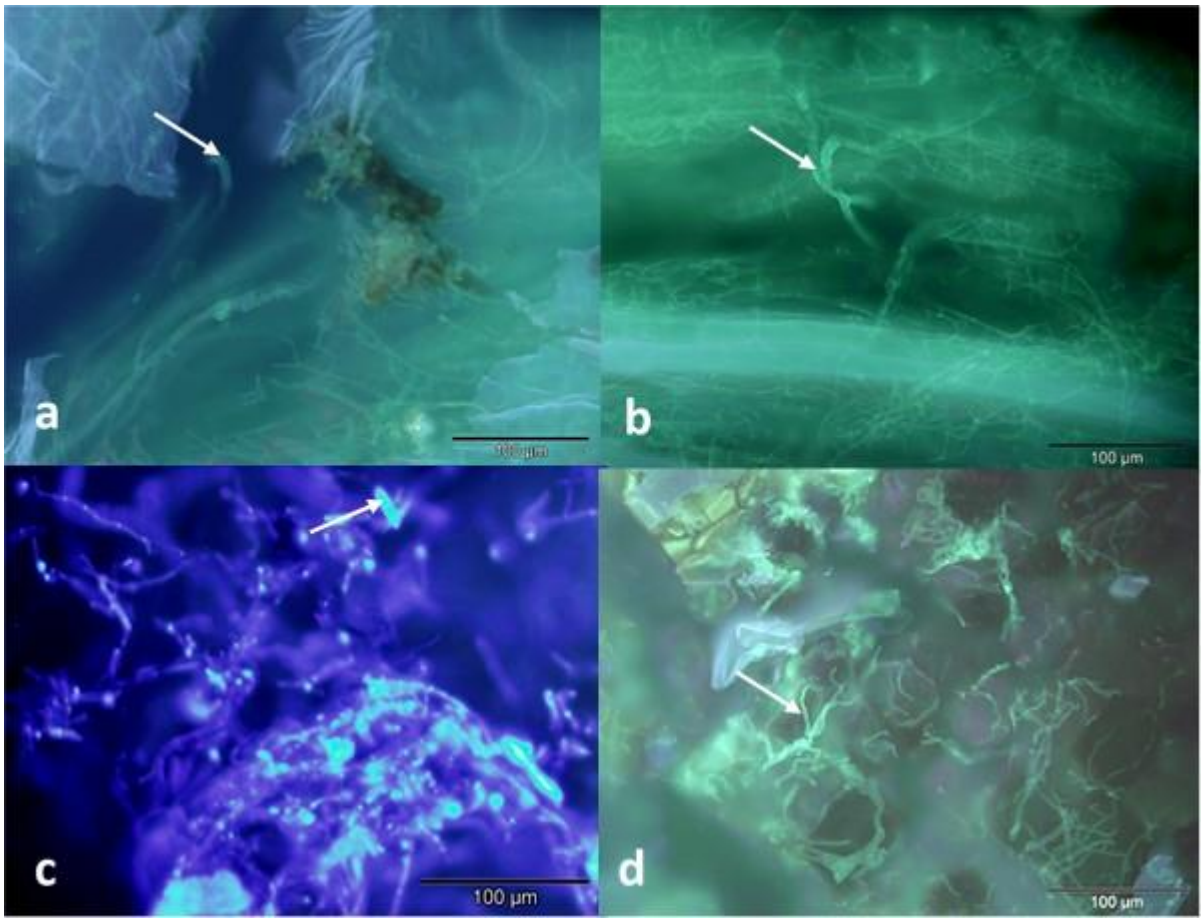


Figure 4.5 Representative fluorescent microscope observations of woolly buds inoculated with *Neofusicoccum luteum* and *N. parvum* a) and c) Cross sections of buds with germinating conidia of *N. parvum* b) and d) cross sections with growing mycelium of *N. luteum*, Scale bars represent 100 µm.

4.3.7 Susceptibility of berries on potted vines at different stages of development

No colonies characteristic of *N. luteum* and *N. parvum* were recovered from any of the non-wounded berries or their rachii at pre-bunch closure or veraison therefore non-wounded berries were not included in the overall data analysis. For the wounded berries at all berry development stages, infection incidence was not significantly affected by species. There was a significant effect of grapevine variety (P=0.003; Appendix D.12.1), with incidence for Sauvignon blanc (65.3%) being greater than for Pinot noir (56.1%). There was a significant effect on infection incidence of the different berry stages (P<0.001), with incidences of berries infected at pre-bunch closure (38.0% and 31.0%), verasion (53.5% and 51.1%), and 1 week before harvest time (97.5% and 93%) for *N. luteum* and *N. parvum*respectively, which were significantly different (P>0.05) (Figure 4.6a).

Infection incidences of wounded berries inoculated at pre-bunch closure stage were significantly affected by species (P=0.018; Appendix D.12.2), with mean incidences for *N. luteum* and *N. parvum* being 38% and 31%, respectively (Figure 4.6a). The effect of grapevine variety was also significant (P=0.017), with mean incidences for Sauvignon blanc and Pinot noir being 38% and 31%, respectively (Figure 4.6b). The interaction between species and variety was not significant (P=0.678). For rachii of these berries, infection incidences were significantly affected by species (P=0.012; Appendix D.12.3), with mean incidences for *N. luteum* and *N. parvum* being 42.5% and 34.1%, respectively (Figure 4.7a). Infection incidence was also significantly affected by variety (P=0.022), with mean incidences for Sauvignon blanc and Pinot noir being 42.0% and 34.5%, respectively (Figure 4.7b). The interaction between species and variety was not significant (P=0.364).

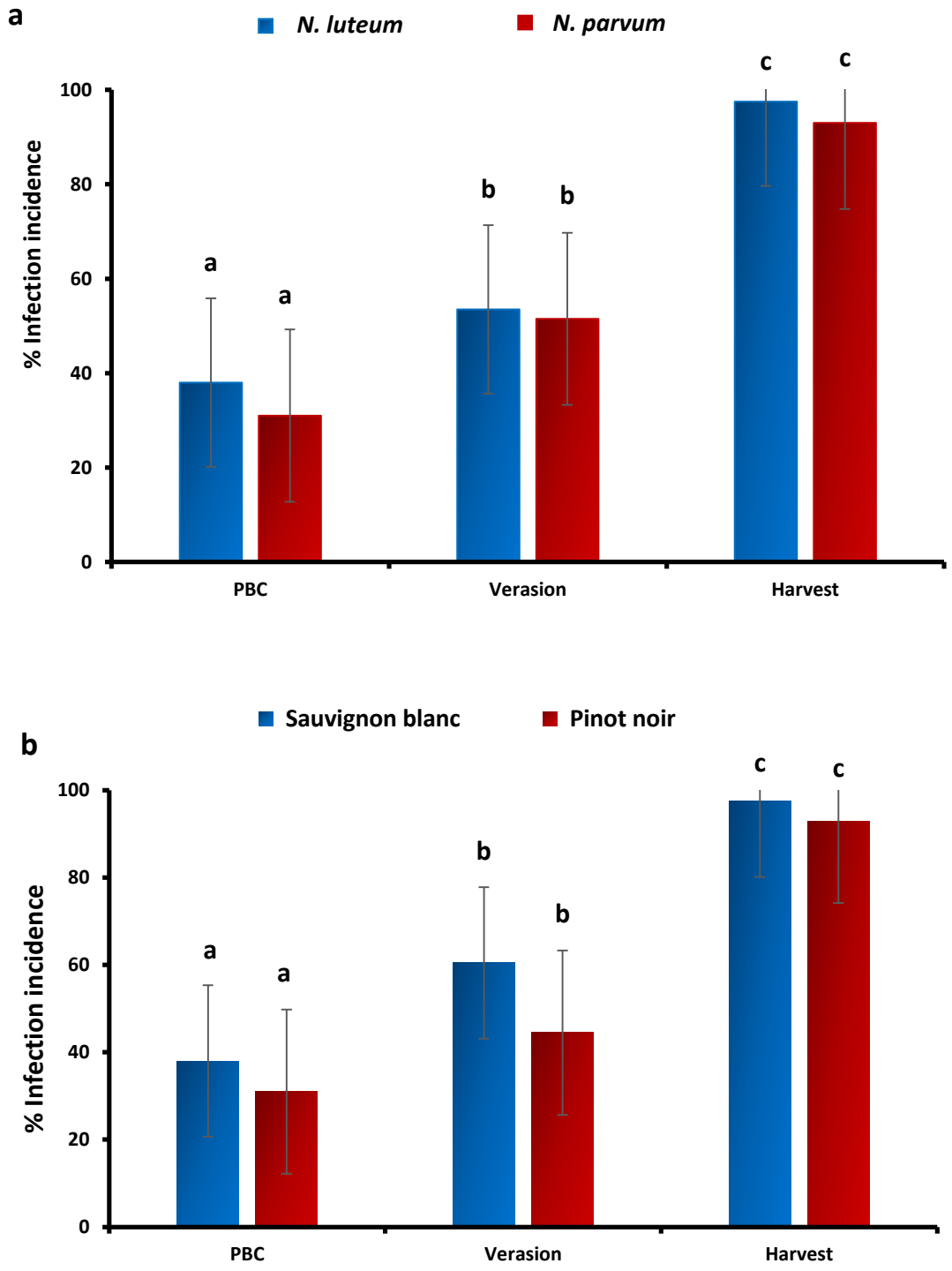


Figure 4.6 Mean infection incidences of a) *Neofusicoccum luteum* and *N. parvum* b) Sauvignon Blanc and Pinot noir in potted grapevines on wounded berries inoculated at pre-bunch closure (PBC), veraison and 1 week before harvest. Error bars represents standard error of the mean and barswith different leters are significantly different at $P \leq 0.05$.

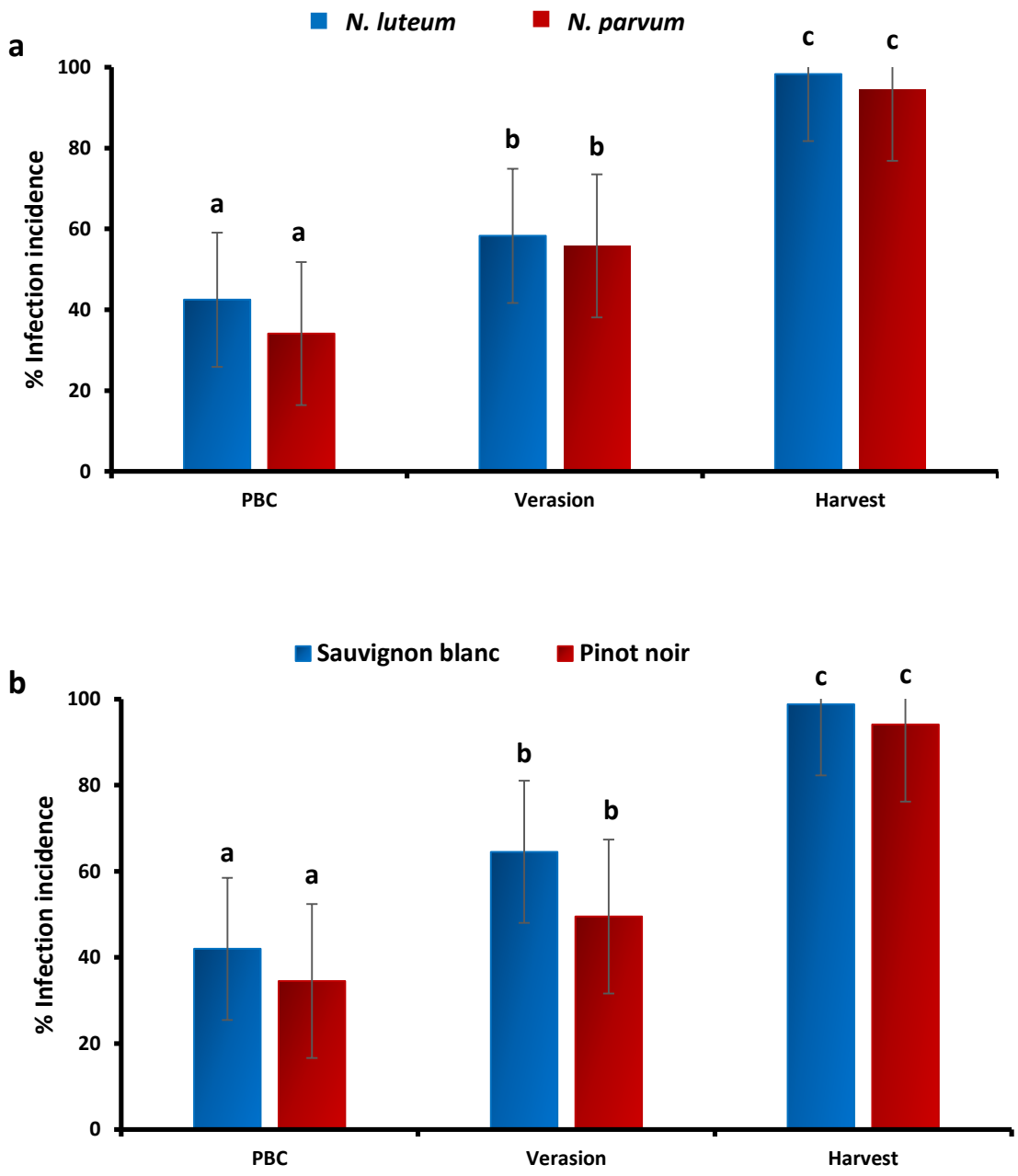


Figure 4.7 Mean infection incidence of a) *Neofusicoccum luteum* and *N. parvum*, b) Sauvignon Blanc and Pinot noir on rachii of potted grapevines when berries were inoculated at pre-bunch closure (PBC), veraison and 1 week before harvest. Error bars represents standard error of the mean and barswith different leters are significantly different at $P \leq 0.05$.

Infection incidences of wounded berries inoculated at veraison stage were not affected significantly by species, (P=0.662; Appendix D.12.4) but were affected by variety (P<0.001), with mean incidences for Sauvignon blanc and Pinot noir being 60.5% and 44.5%, respectively (Figure 4.6b). The interaction between species and variety was not significant (P=0.964). For rachii of these berries, infection incidences were not significantly affected by species (P=0.609; Appendix D.12.5) but were significantly affected by variety (P<0.001), with mean incidences for Sauvignon blanc and Pinot noir being 64.5% and 49.5%,

respectively (Figure 4.7b). The interaction between species and variety was not significant (P=0.976).

At 1 week before harvest stage infection incidences were significantly affected by species (P=0.016; Appendix D.12.6), with mean incidences for *N. luteum* and *N. parvum* being 97.5% and 93%, respectively (Figure 4.6a). Infection incidences were significantly affected by variety (P=0.019; Appendix D.12.6), with mean incidences for Sauvignon blanc and Pinot noir being 97.5% and 93%, respectively (Figure 4.6b). The interaction between species and variety was not significant (P=0.743). For rachii of these berries, infection incidences were significantly affected by species (P=0.015; Appendix D.12.7), with mean incidences for *N. luteum* and *N. parvum* being 98.3% and 94.5%, respectively (Figure 4.7a). Infection incidences were significantly affected by variety (P=0.003), with mean incidences for Sauvignon blanc and Pinot noir being 98.7% and 94.1%, respectively (Figure 4.7b). The interaction between species and variety was not significant (P=0.655). For the non-wounded berries, colonies characteristic of *N. luteum* and *N. parvum* were isolated from 4.3% of the inoculated berries but not the rachii for both Sauvignon blanc and Pinot noir.

Infected berries were mummified, brown to black in colour with raised black pycnidia which oozed when placed in high relative humidity (Figure 4.8). For the infected berries incidence of pycnidia, which oozed over all berry development stages, was not significantly affected by species or variety (P=0.090 and P=0.670, respectively; Appendix D.12.8). There was a significant effect of the different berry stages (P<0.001), with incidences of berries with oozing pycnidia at pre-bunch closure, verasion, and 1 week before harvest time being 71.8%, 89.5% and 98.9%, respectively.

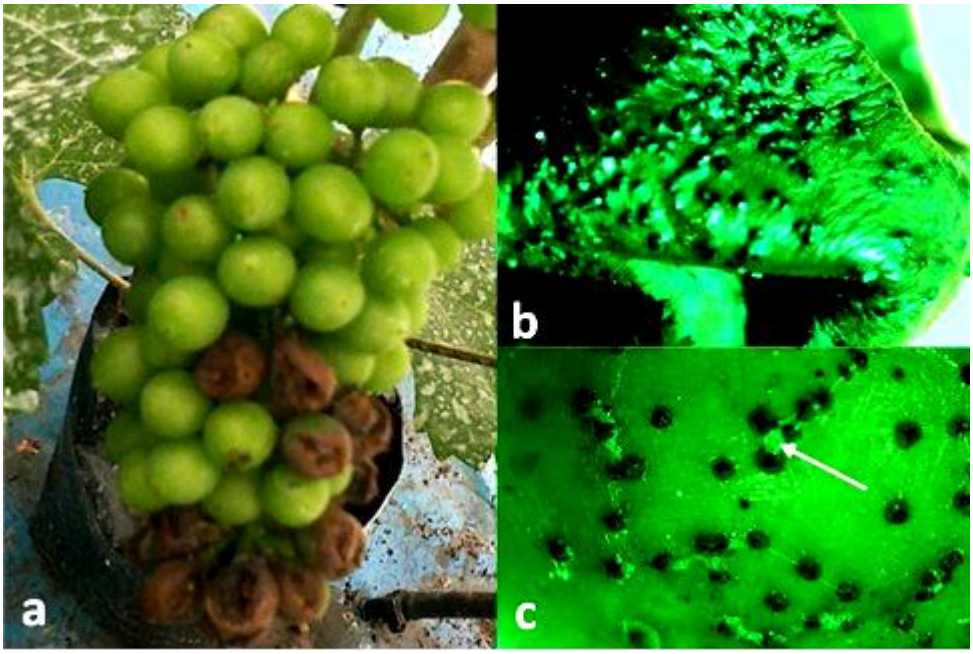


Figure 4.8 a) Symptoms developed on Sauvignon blanc grapevine berries after wounding and spray inoculation with *Neofusicoccum luteum,* b) infected berry, showing black raised pycnidia of *N. luteum*, c) pycnidial ooze showing by white arrow.

For wounded berries inoculated 1 week before harvest, the pathogens progressed from the infected berries and rachii into supporting shoots. The mean upward lengths of colonised stems were not significantly affected by species (P=0.094; Appendix D.12.9) (Figure 4.9a) but were significantly affected by variety (P=0.021; Appendix D.12.9), with mean shoot lengths colonised for Sauvignon blanc and Pinot noir being 3.1 cm and 1.9 cm, respectively (Figure 4.9b). The interaction between variety and species was not significant (P=0.237). For downward colonisation, lengths of stems were also not affected by species (P=0.118; Appendix D.12.10) but were affected by variety (P=0.013), with mean lengths of colonised stems for Sauvignon blanc and Pinot noir being 2.4 cm and 1.4 cm, respectively (Figure 4.9b). The interaction between variety and species was not significant (P=0.170).

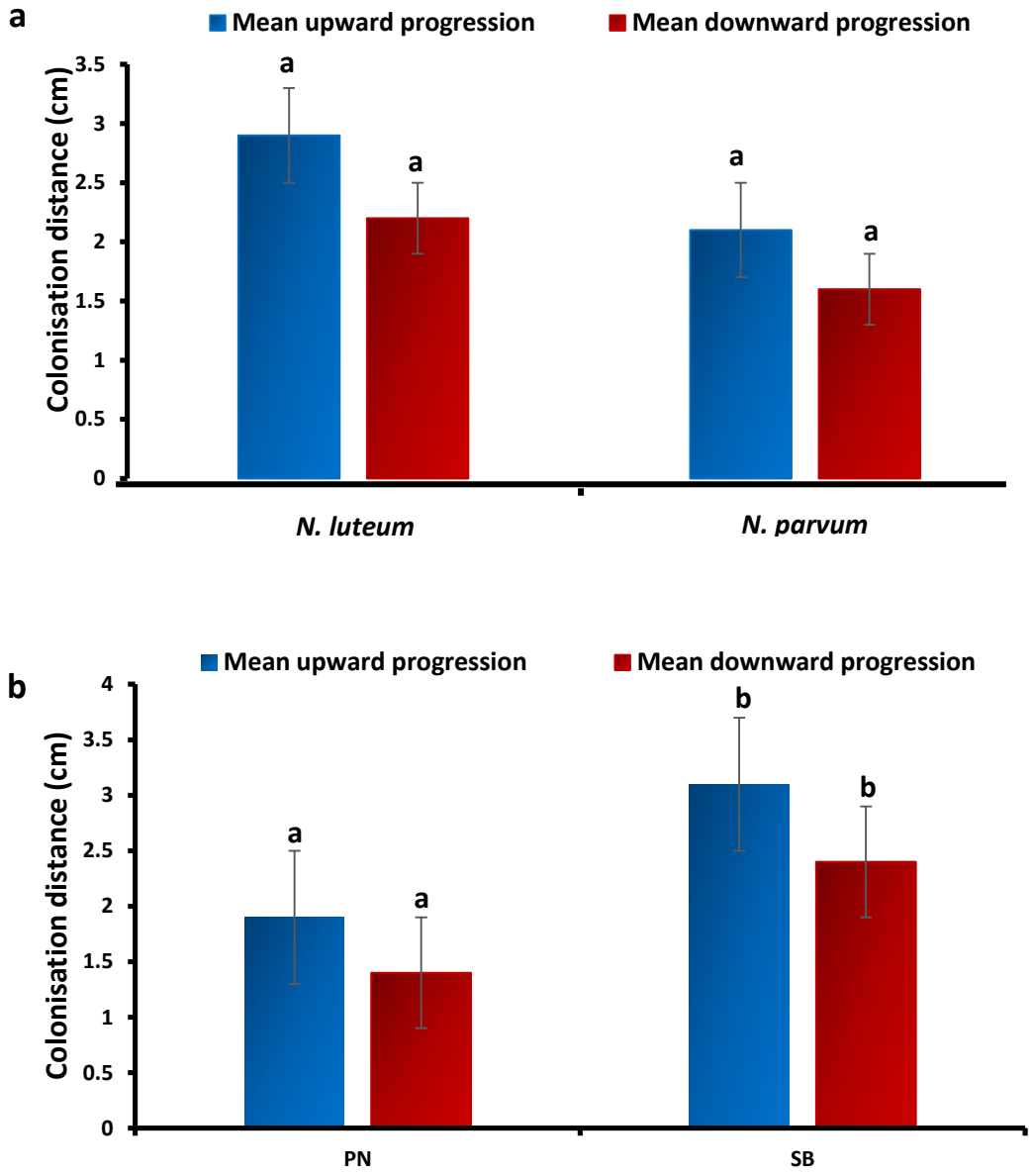


Figure 4.9 Mean upward and downward progression a) on *Neofusicoccum luteum* and *N. parvum* b) on Sauvignon blanc (SB) and Pinot noir (PN) potted grapevines. Error bars represents standard error of the mean and barswith different leters are significantly different at $P \leq 0.05$.

4.3.8 Susceptibility of berries on field vines at different stages of development

No colonies characteristic of *N. luteum* and *N. parvum* species were recovered from the non-wounded berries or their rachii at pre-bunch closure and veraison, therefore non-wounded berries were not included in the overall data analysis. All infected, wounded berries became rotten, and then after 1 month were mummified with raised black pycnidia (Figure 4.10). For the infected berries, incidence of pycnidial ooze over all berry development stages was

significantly affected by species (P<0.001; Appendix D.13.1), with incidence of ooze being 78.2% for *N. luteum* and 69.1% for *N. parvum*. There was a significant effect on ooze incidence of the different berry stages (P=0.027; Appendix D.13.1), with incidences for berries infected at pre-bunch closure, verasion, and 1 week before harvest time being significantly different (P<0.05) from each other (46.9%, 75.7% and 93.9%, respectively).

Figure 4.10 Infected mummified berries of Sauvignon blanc with *Neofusicoccum luteum* with raised black pycnidia 1 month after inoculation in the vineyard.

For the wounded berries at all berry development stages, infection incidence was significantly affected by species (P<0.001; Appendix D.13.2), with incidence for *N. luteum* (46.9%) being greater than for *N. parvum* (36.1%). There was a significant effect on infection incidence of the different berry stages (P<0.001), with incidences of berries infected at pre-bunch closure, verasion, and 1 week before harvest time being significantly different (P<0.05) from each other (27.8%, 41.6% and 55.1%, respectively).

At pre-bunch closure, incidences of infection in wounded berries were not significantly affected by species (P=0.110; Appendix D.13.3) but incidences in rachii were significantly affected by species (P<0.001; Appendix D.13.4), with mean incidences for *N. luteum* and *N. parvum* being 39.0% and 23.0%, respectively.

At veraison, incidences were significantly by affected by species (P<0.026; Appendix D.13.5), with 64.3% incidence for *N. luteum* and 46.0% for *N. parvum*. Incidences in rachii were significantly affected by species (P<0.001; Appendix D.13.6), with mean incidences for *N. luteum* and *N. parvum* being 81.0% and 54.0% respectively.

One week before harvest, incidences in wounded berries were significantly affected by species (P<0.001; Appendix D.13.7), with mean incidences for *N. luteum* and *N. parvum*

being 80.3% and 60.2%, respectively. For rachii, incidences were significantly affected by species (P<0.001; Appendix D.13.8), with means for *N. luteum* and *N. parvum* being 100% and 69.0%, respectively. For wounded berries inoculated 1 week before harvest, the pathogens progressed from the infected berries and rachii into supporting shoots. The mean downward lengths of colonised tissues were significantly affected by species (P=0.026; Appendix D.13.9), with mean lengths for *N. luteum* and *N. parvum* being 3.4 cm and 1.6 cm respectively. The upward lengths of colonised tissues were also affected by species (P=0.029; Appendix D.13.10), with mean lengths for *N. luteum* and *N. parvum* being 2.9 cm and 1.4 cm respectively.

In non-wounded berries at 1 week before harvest, infection incidences were significantly affected by species (P=0.036; Appendix D.13.11), with 11.7% incidence for *N. luteum* and 10.9% for *N. parvum*, but no infection was found in the rachii.

4.4 Discussion

In this study, the effect of different factors on infection processes of *N. luteum* and *N. parvum* conidia on attached grapevine tissues was investigated. Wound age in late summer was shown to affect infection, since incidence decreased with increasing age from 100% immediately after wounding; for hard green shoots, incidence for 42 day and 70 day wounds decreased to 33% and 0%, while for wood of trunks, incidence decreased to 0% by 42 days. Colonisation distances also reflected the same trends. Studies by Urbez-Torres and Gubler (2011) and van Niekerk *et al.* (2011) also reported decreasing incidence of Botryosphaeriaceae species and pathogen colonisation distances with increasing wound age in grapevine woody tissues. Decline in infection with increasing wound age is related to wound healing. Biggs (1986) reported that infection incidence decreased with wound age by *Cytospora leucostoma* on peach, with infection incidence being 100% for wounds up to 10 days old when inoculated, which reduced to 10% for 14 day wounds and 0% for older wounds. The microscopy studies conducted by Biggs (1986) on these tissues showed that reduced incidence was related to wound healing processes, which involved lignification and formation of lignosuberized tissues. He observed that a lignosuberized layer was evident by day 3 after wounding which was well developed by day 7. After day 10, a phellem cell layer began to develop which was six cells thick by day 21. He concluded that these wound healing processes had decreased the rate of colonization rather than preventing colonization of peach by *C. leucostoma*. Sun *et al.* (2006) also reported that 1 or 2 days after pruning of grapevines, tyloses started to develop in exposed xylem vessels of the pruned canes,

continuing until almost all xylem vessels were totally or partially (85%) occluded with tyloses after about 6 days. The same phenomenon was reported by Biggs (1987), who reported that tyloses were formed in response to wounding in peach, forming impenetrable layers in the xylem vessels by developing lignified and /or suberized secondary walls. This process has also been reported to be dependent on temperature. Biggs (1990) also showed that the rate of suberised tissue formation in wounded bark was greater at higher average temperatures than at lower temperatures.

A further experiment which examined the effect of wound age and season on infection by *N. luteum*, found that incidence in hard green shoots and woody trunks declined from 100% immediately after wounding to 12.5 and 4.1%, respectively, for inoculation of 42-day-old wounds. Incidences and colonisation distances were also affected by the seasons, being highest in autumn and spring, and lowest in winter and summer. Van Niekerk *et al.* (2011) reported from their study in South Africa that Chenin blanc wounds inoculated with *N. australe* directly after pruning in July (mid-winter) had lower infection incidence than wounds made in August (late winter). However, in California, USA, Urbez-Torres and Gubler (2011) showed that wounds inoculated with *N. parvum* directly after pruning in winter had higher incidences (~80%) than those inoculated in late winter (~50%) and in early spring (~30%). This conflicted with the current study which showed a trend for higher incidence and pathogen progression in spring than in winter. The differences in results may be associated with the differences in temperature, average daytime temperatures in spring, summer, autumn, and winter in the current study were 18.5ºC, 23.7ºC, 19.1ºC, and 14.7ºC, respectively. The vine growth conditions may also have affected the results as the temperatures within the tunnel houses was likely to be higher than outdoors and these vines were observed to develop shoots earlier in spring than the outdoor vines of the Fairhall Vineyard. Further, tissues wounded and varieties used were also different to the study by Urbez-Torres and Gubler (2011); the field vines (1 and 2-year-old) used in the Napa Valley, California were spur pruned Chardonnay and Cabernet Sauvignon, in contrast to the hard shoot and trunk wounds made on Sauvignon blanc and Pinot noir in the current study.

In the current study, the lower overall pathogen progression and incidence in summer might be due to higher concentrations of lignin compounds in the wood as the weather became warmer. At this time there were also likely to be greater numbers of epiphytes colonising the wounds, which Munkvold and Marois (1995) concluded were partly responsible for inhibiting infection of grapevine wounds by *Eutypa lata.* Munkvold and Marios (1995) also

reported a high positive correlation between higher mean temperature after pruning and rate of suberin accumulation in the pruning wounds. However, a number of other studies (Moller and Kasimatis, 1980; Munkvold and Marois, 1995; Chapuis *et al*., 1998; Largnon and Dubos, 2000), have reported that grapevine wounds made in late winter were less susceptible to infection than wounds made in early winter. In contrast, Serra *et al.* (2008) showed that in Italy the susceptibility of pruning wounds to infection by *D. seriata* in January, February and March (winter) was similar over the three years of the study. Further, the effects on incidences in months when pruning/inoculation was conducted varied between years, which they concluded was associated with temperature directly after wounding, with higher incidences when temperatures were colder. Van Neikerk *et al*. (2011) also concluded that the climatic conditions after pruning affected wound susceptibility and that period of wound susceptibility may not be determined necessarily by the time of year that pruning was done. These climatic factors may account for the contrasting report by Trese *et al*. (1980), who observed that pruning wounds made in late winter in Michigan USA were more susceptible to infection by *E. lata* compared to early winter pruning wounds.

Another possible explanation for the higher susceptibility of the wounds in some months can be found in differences during these months in the nutritional status of grapevine tissues. Ferreira (1999) noted that carbohydrate and nitrogen concentrations in the vine were greatest in the dormant season, making the environment more favourable for the establishment of infections. Increased carbohydrate in the vine rootstock and higher nitrogen in canes, shoots and wood during the dormancy period were also reported by Uys (1981). In the current study, the higher infection incidence of grapevines in which wounds were inoculated after leaf fall in autumn, could be due to higher nitrogen and carbohydrate content.

In this study, an experiment was also conducted on the effects of different conidial concentrations of *N. luteum* and pruning wound age on susceptibility of wounds made on potted vines of Sauvignon blanc. All concentrations caused 100% infection incidence on fresh wounds in green tissues and 93% incidence in trunks, but lesion lengths increased with increasing concentrations. Also lesion lengths were longer in green tissues than in the woody tissues. Amponsah *et al.* (2014) also reported similar results on detached soft green shoots of grapevines after *N. luteum* inoculation. In their study, all the conidial doses caused 100% infection incidence, with 2 conidia causing shortest lesions of 8 mm and longest lesions caused by 200 conidia (42 mm) after 10 days. Similar effects were reported by Pusey and Bertrand (1993), who showed that increasing concentrations (10^4-10^6 conidial/mL) on 1-year-

old peach bark caused increasing lengths of necrosis by *B. dothidea*. Elena *et al.* (2015) also reported the effect of the inoculum dose of *D. seriata* (10-4000 conidia per wound) on pruning wounds of dormant 5-year-old Tempranillo vines in two potted grapevines experiments. Isolations showed increasing incidence of 10-100% and 44-100% for the increasing conidial concentrations for the two experiments, which was different to the current experiment and that reported by Amponsah *et al.* (2014). The differences may be due to differences in tissue type, as Elena *et al*. (2015) used dormant canes and the current study, used hard green shoots and trunks of 2-year-old Sauvignon blanc potted vines in summer. Further, the current study has shown multiple times that the Botryosphaeriaceae species progress more quickly in soft than harder tissues. Also, species, variety, host growth stage, season and methods of isolation have differed between the reported studies. Several researchers have shown that wound infection in grapevines depends on several factors, including, variety, age of the plant, virulence of the isolates, experimental and environmental conditions (van Niekerk *et al.,* 2004; Sosnowski *et al.,* 2007 and Serra *et al.,* 2008), which indicates that further studies should be conducted with a range of Botryosphaeriaceae species and tissue type through all seasons of the year to provide a better understanding of this topic.

The initial study into effects of wound age by *N. luteum* and *N. parvum* also showed that incidence and lengths of colonisation were greater in bark than wood of the trunks, which indicated the potential for saprophytic colonisation of the bark, as reported by Billones-Baaijens *et al.* (2012)**.** A further experiment in the current study showed that conidial infection and saprophytic presence in the bark, could allow for infection of the wood from 1 h to 7 days later when a wound was made. In this study, the pathogen remained latent in the bark and when the wound was made it progressed towards the wound. Also in this study pathogen incidence was highest when wounds were made after 7 days. Billones-Baaijens *et al.* (2015) isolated from bark and wood segments separately from an entire shoot (3-4 m long) taken from a grapevine mothervine in a nursery and found few isolates of the Botryosphaeriaceae within the wood and most occurred in the bark, which suggested that they were latent on surface tissues. In addition, Billones-Baaijens (2011) showed that *N. luteum* conidial suspensions applied to the surfaces of canes adhered rapidly and could not be totally washed from the surfaces after a few minutes, with minimal recovery of conidia after 1, 2 and 4 h incubation at room temperature. Dissection and isolation of the bark and outer wood layers showed that during this time they had adhered and then germinated, progressively colonising the periderm, phloem and xylem but not the pith of the cuttings

(Billones-Baaijens., 2011). In the current study, mycelium and germinating conidia were observed in samples inoculated onto the bark near lenticels, but without subsequent wounding. The mycelia were present in the bark, in underlying wood and cross-sectioned tissues, which indicated that pathogen had entered through lenticels. Pusey and Bertrand (1993) also reported that non-wounded bark of 1 to 2-year-old peach trees were susceptible to infection by *B. dothidea,* as did Sutton (1990) who observed lenticels as a common entry point for *B. dothidea* infection on apple and pear fruit. Prusky *et al*. (1983) also reported that mango fruit became infected with conidia of *Alternaria* through lenticels and that the pathogen then invaded the intercellular spaces. It therefore seemed likely that infection of grapevines was also able to occur through lenticels.

This study showed that wounded leaf buds, but not non-wounded ones, could be infected at the dormant and woolly bud stages. However, buds with emerging green tips had high incidences if wounded and low incidences when non-wounded. Wounded buds of Sauvignon blanc were slightly more susceptible than buds of Pinot noir. A similar study was conducted by Amponsah *et al.* (2012a) who inoculated wounded woolly buds on Pinot noir vines with 2000 conidia of *N. luteum* per bud. Their results showed infection incidence was 88% and 73% for wounded and non-wounded buds, respectively, in contrast to the current study for which incidence after inoculation with 200 conidia was 46.7% and 0%, respectively. Also in their study, most of the inoculated buds failed to burst and some buds developed into asymptomatic shoots with internal discolouration from the inoculated point. In the current study, buds were not killed by the inoculated pathogen, but the pathogen progressed into 100% of the supporting canes and emerging shoots, on which necrosis was seen to develop. As well as the difference in inoculum concentration, another likely reason for the differences in results between this study and that of Amponsah *et al.* (2012a) is that they covered the treated plants with clear polythene bags that were misted inside with sterile water for 24 h to provide high relative humidity which would have improved germination and infection. In contrast, the buds in the current study were left exposed after inoculation. Wunderlich *et al.* (2011) were also unable to cause disease on non-wounded dormant flower buds of Chardonnay and Shiraz when inoculated onto the bud surfaces with 10^5 conidia of *N. parvum* or *D. seriata* in the greenhouse and vineyard. They covered buds with plastic bags for 48 h to maintain high relative humidity. Smith and Hendrix (1984) showed that when 10^6 conidia/mL of *B. dothidea* were applied to the entire trees, dormant non-wounded apple leaf buds were not susceptible to infection by *B. dothidea,* but that buds became susceptible to infection at

the beginning of silver tip visible stage, and most buds were able to be infected at the green tip visible stage. Phillips *et al.* (1998) also showed the infection of grapevine buds occurred when they inoculated a conidial suspension of *B. dothidea* under bud scales without directly wounding them, which may however have provided a more conducive environment that the open air of the current study. A study by Ntahimpera *et al.* (2002) also showed that buds collected from pistachio orchards were naturally infected with *B. dothidea* immediately after their formation. In their study they collected 100 symptomless buds from commercial pistachio orchards, which were surface sterilised, cut and isolated onto PDA. The results in this study showed that non-wounded buds could become infected at the green tip visible stage and also that bud infection could progress into green shoots and canes.

Berries with pinprick wounds were susceptible at all stages of development tested, being most susceptible when ripe (1 week before harvest) when some of the non-wounded berries also became infected. Overall Sauvignon blanc berries were slightly more susceptible than those of Pinot noir. Amponsah *et al.* (2012a) also reported that attached wounded berries of Sauvignon blanc and Pinot noir became infected by *N. luteum* conidia, with moderate incidences (16-28%) at pre-bunch closure and verasion, and high incidences (65-70%) at harvest. They also reported rotting of the berries, formation of pycnidia and pathogen progression into the supporting shoots of a very few specimens, which was similar to the current study, in which berries also oozed conidia when placed under high relative humidity. In Australia, Wunderlich *et al.* (2011) demonstrated that six species of Botrysophaeriaceae were able to cause infection of non-wounded, detached ripe berries of Chardonnay and Shiraz although infection varied between isolates and species. They also reported that symptomatic berries were soft and developed black raised pycnidia and mycelial growth. Steel *et al.* (2007) also reported isolation of Botryosphaeriaceae spp. from grape flowers and berries in low frequency throughout the growing season in Australian vineyards. Lorenzini *et al*. (2015) reported infection capacity of two strains of *N. parvum* on Garganega grapes when the individual, detached berries were incubated in plastic bins. They used unwounded and wounded berries and found that virulence of strains was higher in wounded than non-wounded berries. In another study, Wunderlich *et al.* (2011) reported that these Botryosphaeriaceae species (*L. theobromae, N. parvum, N. luteum, D. seraita, B. dothidia* and *D. viticola*) are common on reproductive structures. They found that dormant flower buds, flowers and pea-sized berries which appeared asymptomatic still yielded low levels of Botryosphaeriaceae species. However, many bunches with symptoms of Botryosphaeria

bunch rot prior to harvest also showed mycelial growth and formation of black pycnidia on berry surfaces. Of their 330 Botryosphaeriaceae isolates, the most common species were *D. seriata*, *N. parvum, N. luteum* and *B. dothidea*, which were also found to be common causes of canker and dieback. This study has also shown that the same isolates which were pathogenic on wounded shoots and canes were also able to infect berries and leaf buds. However, the importance of berry and bud infection in New Zealand vineyards as sources of infection is unclear and needs further work.

Conclusion

This study has provided information about the susceptibility of most grapevine tissues to Botryosphaeriaceae species. It has shown that wound susceptibility declined with increased wound age, and differed between seasons, with wounds tending to remain susceptible for longer in autumn and spring. On fresh wounds, all conidial inoculum doses were found to cause 100% incidence of green shoots, and trunk wounds inoculated with two conidia had 67% incidence while higher doses of conidia caused 100% incidence, and resulted in longer lesions. Wounded leaf buds of Sauvignon blanc and Pinot noir were susceptible to infection at the dormant woolly stage, and at the green tip visible stage, when non-wounded buds also became infected. Wounded berries were also susceptible from pre-bunch closure to harvest, being more susceptible at the 1 week before harvest stage when some of the non-wounded berries also became infected. Overall, Sauvignon blanc was more susceptible than Pinot noir. This study has provided information on wound susceptibility to Botryosphaeriace infection which was useful in the next chapter when developing investigations into Botryosphaericeae management strategies by fungicides and biocontrol agents.

Chapter 5

Control methods for protecting wounds

5.1 Introduction

For the pathogens associated with grapevine trunk diseases, wounds, especially pruning wounds, are regarded as the main point of infection and so require protection (van Niekerk *et al*., 2005*a*; Urbez-Torres and Gubler, 2011). Bester *et al.* (2007) investigated the *in vitro* efficacy of ten fungicides against 16 isolates of *D. seriata, N. australe, N. parvum* and *L. theobromae*, followed by greenhouse studies using young grapevine plants. Their studies indicated that benomyl, tebuconazole, flusilazole and prochloraz as liquid formulation reduced incidence and the length of colonised tissues in treated shoots, but their efficacy varied between isolates and species. Amponsah *et al.* (2012b) demonstrated the *in vitro* efficacy of 14 fungicides, out of which carbendazim, tebuconazole, procymidone, iprodione and flusilazole were effective against *N. luteum, N. australe* and *D. mutila.* In a study on potted vines they showed that spray application of carbendazim, iprodione, mancozeb and flusilazole were most effective at preventing infection by *N. luteum* conidia. From their subsequent field experiment, they reported that spray applications of flusilazole, carbendazim, tebuconazole and thio-phanate methyl were effective at preventing infection by conidia of *N. luteum*. Similarly, Pitt *et al.* (2010b) reported a field experiment with carbendazim, flusilazole, cyprodinil+fludioxonil, fluazinam, iprodione and tebuconazole applied to wounds by paint brush to prevent infection by conidia of *D. seriata.* Isolations after 10 months showed that use of fungicides at rates 10 x the recommended rates provided 20-55% reduction in incidence of *D. seriata.*

Biological control agents have also been used as wound protectants to provide some control of some grapevine trunk diseases, although the efficacy has been reported to vary significantly (Fourie and Halleen, 2004b; Sosnowski *et al.,* 2008). Latorre *et al.* (2013) reported *Bacillus subtilis* and *Trichoderma* spp. to be ineffective at controlling *N. parvum* infection of blueberry wounds. In contrast, John *et al*. (2008) reported *T. harzianum* to variable reduction in infection of grapevines by *E. lata* and Kotze *et al*. (2011) reported that a *B. subtilis* isolate to reduce infection by a range of grapevines trunk pathogens including Botryosphaeriaceae species.

All fungicide studies have investigated the efficacy of one application prior to inoculation, however when spores were continuously produced in vineyards the fungicides did not give prolonged protection (Pitt *et al.*, 2010b). Further, efficacy has varied between reports, largely due to the different isolates and Botryosphaeriaceae spp., grapevine varieties and experimental conditions used. For effective and economical use of wound protectants in vineyards where infection has been reported, knowledge about how long pruning wounds remain susceptible to infection will indicate for how long protection is required. The products already reported to be effective against some Botryosphaeriaceae spp. should be tested for their efficacy and longevity, by spraying with multiple inoculations over time, against the most prevalent species. The potential of biocontrol products to effectively protect pruning wounds should also be investigated.

In this study, products already reported to be effective against some Botryosphaeriaceae spp. will be tested for their efficacy by spraying, and longevity with multiple inoculations over time, against the most prevalent species. Investigations into how long pruning wounds remain susceptible to infection will also be conducted to show for how long protection is required. These will be supported by repeated inoculations of the treated wound to demonstrate the period of protection provided by the treatments.

5.2 Materials and Methods

5.2.1 Efficacy of fungicides and biocontrol agents on wounds of potted grapevines placed outdoors

In September 2014, 1-year-old potted vines (Sauvignon blanc) were acclimatised to an outdoor environment at the Fairhall Vineyard, Blenheim as described in Section 2.2.1. The semi-hard green shoots of the vines were pruned to approximately 15 cm using ethanol sterilised secateurs and the trunks were wounded by drilling holes of 2.5 mm diameter and 3 mm deep. The wounds were immediately sprayed with the fungicides Folicur®, Chief®, Gem®, Dithane® or Megasta®r, or commercial biocontrol agents (TRI D25 and Serenade®) using rates recommended by the manufacturers (Appendix E.1). After 1-2 h (0 days), 7 and 14 days, the wounds were drop inoculated with conidial suspensions (10^4/mL) of *N. parvum* (isolate B2141) or *N. luteum* (isolate G51a2) which were produced from inoculated detached green shoots as described in Chapter 2, Section 2.2.2.1. The amounts of spore suspension varied with the size of pruned tissue, being 20 µL (200 conidia) and 60 µL (600 conidia) for hard green shoots and 2-year-old trunks, respectively. Control plants were sprayed with sterile water and inoculated with the fungi (I) or sprayed with sterile water and not inoculated

(NI). The six replicate plants of all treatments were arranged in randomised block design (RBD).

The efficacy of treatments was determined after 14 days for hard green shoots and 28 days for trunks by isolation. After removal of the end tissue (2 mm), 12 discs were cut at 5 mm intervals from the pruned and wounded end down to 60 mm and surface sterilized, which involved dipping in 70% ethanol for 30 s, followed by 30 s in sterile water and air drying in a laminar flow unit for 1 h. Bark and wood pieces were plated separately on PDA amended with chloramphenicol (0.05 g/L) and incubated at 25°C for 3-7 days. Typical colony appearance allowed identification of *N. parvum* and *N. luteum.* Efficacy assessments of the fungicides and biocontrol agents were based on incidences and length of tissues colonised by *N. parvum* and *N. luteum*.

5.2.2 Efficacy of fungicides and biocontrol agents on wounds of potted grapevines in a tunnel house

In April 2015, another experiment was conducted inside the tunnel house at the Fairhall Vineyard, Blenheim, to reduce levels of natural infection. The hard green shoots of potted vines (Sauvignon blanc) were pruned to approximately 15 cm using ethanol sterilised secateurs, and immediately sprayed with Folicur®, Chief®, Gem®, Dithane® or Megastar®, fungicides, or commercial biocontrol agents (TRI D25 and Serenade®). Two-year-old trunks were wounded by drilling a 2.5 mm diam. and 3 mm deep hole into each trunk and the same treatments applied. The wounds were drop inoculated with conidial suspensions [(10^4/mL) of *N. parvum* (B2141) or *N. luteum* (G51a2)] which were produced from inoculated detached green shoots as described in Chapter 2, Section 2.2.2.1. These spore suspensions were applied 1 and 24 h after the application of fungicides and biocontrol agents. The amounts of spore suspension varied with the size of pruned tissue, being 20 and 60 µL (hard green shoots and 2-year-old trunks, respectively). Control plants were sprayed with sterile water and inoculated with either fungus (I) or not inoculated (NI). The six replicate plants were arranged in a CRBD.

The infection incidence of *N. luteum* and *N. parvum* was determined after 7 and 14 days (hard green shoots and trunks, respectively). From each inoculated tissue, a 1 cm section between the dead and live wood was cut and surface sterilized in 70% ethanol for 30 s, followed by 30 s in sterile water and air drying in the laminar flow unit for 1 h. Bark and wood were plated separately on PDA amended with chloramphenicol (0.05 g/L) and incubated at 25°C for 3-7

days. Typical colonies of the inoculating fungi indicated incidence and so efficacy of the treatment.

5.2.3 Longevity of fungicides on wounds of potted grapevines using low inoculum of a marker isolate in a tunnel house

The previous experiment (Section 5.2.1) was repeated with some modifications. Green shoots and 2-year-old trunks were wounded as in Section 5.2.1, sprayed with the fungicides and drop inoculated with the least and intermediate conidial numbers (2 and 40 conidia per wound) that were found to cause infection, as determined in a previous experiment (Section 4.3.3), using *N. luteum* (G51a2). For all treatments, fungicides (Folicur®, Chief®, Gem®, Dithane® or Megastar®) were applied immediately after wounding and wounds were inoculated after ~2 h (Treatment 1) or after 2 weeks (Treatment 2), 4 weeks (Treatment 3), 8 weeks (Treatment 4) or at 10 weeks (Treatment 5) after wounding. Control plants were sprayed with sterile water and inoculated with *N. luteum* (I) for the first two treatments (Treatments 1 and 2) and not inoculated (NI) for all treatments. The infection incidence and the length of tissue colonised by *N. luteum* were determined in the green shoots and trunks (both wood and bark) at 14 and 28 days, respectively, after treatment by making isolations. After removal of the end tissue (2 mm), 12 discs were cut at 5 mm intervals from the pruned and wounded ends of each treated stem, down to 60 mm. These discs were surface sterilized by dipping in 70% ethanol for 30 s, followed by 30 s in sterile water and air drying in a laminar flow unit for 1 h. Bark and wood pieces were separated with steile scalpel and plated on PDA amended with chloramphenicol (0.05 g/L) and incubated at 25°C for 3-7 days. Typical colony appearance allowed identification of *N. luteum.* Efficacy assessments of the fungicides were based on incidences and the length of tissue colonised by *N. luteum* from treated tissues. The six replicate plants per treatment were arranged in a CRBD inside the tunnel house.

5.2.4 Vineyard experiment

In September 2014, a vineyard experiment was conducted at the Kaituna Vineyard, Marlborough, to evaluate the efficacy of five fungicides (Folicur®, Cheif®, Gem®, Dithane® and Megastar®) and two biocontrol agents commercially available as pruning wound protectants (TRI D25 and Serenade®). The field experiment was established with Sauvignon blanc vines planted in 1996. Lignified canes in the vineyard were tagged with different colour ribbons to represent the different treatments. There were ten replicate vines

(due to logistical issues the experiment was not replicated across blocks) on which four canes per grapevine were pruned to two buds using ethanol sterilized secateurs and immediately sprayed with the appropriate treatment using a 2 L pressurized home garden sprayer. Wounds were drop inoculated with 60 µL conidial suspensions (10^4/mL) of *N. luteum* (G51a2) or *N. parvum* (B2141) after 0 h, 7 and 14 days. Control plants were sprayed with sterile water and inoculated (I) or sprayed with sterile water and not inoculated (NI).

Inoculated canes and control canes were harvested from the vines 7 months after inoculation, and for each cane a 1 cm section cut from between dead and live wood used for isolation. These sections were surface sterilized in 70% ethanol for 30 s, followed by 30 s in sterile water and air drying in a laminar flow unit for 1 h and then bark and wood were placed separately on PDA amended with chloramphenicol (0.05 g/L) and incubated at 25°C for 3-7 days.

5.2.5 Statistical analysis

Statistical analysis was conducted without the data from non-inoculated control plants which were used simply to indicate absence of natural infection. The length of colonised tissues were analysed by General Linear Model (Minitab 17th edition) to determine significant main effects, and the significance of differences between related treatments was determined using Sidak pairwise comparison at $P \leq 0.05$. Data of infection incidence in inoculated grapevine shoots were analysed by General Linear Model (GLM) using GenStat (16th edition), which is considered appropriate for binomial data (McCue *et al*., 2008), using the logit link function. A Bernoulli distribution defined as a binary variable was assumed. Standard errors of differences (SEDs) derived from GLM analysis were used to show differences between treatment means.

5.3 Results

5.3.1 Efficacy of fungicides and biocontrol agents on wounds of potted grapevines placed outdoors

For semi-hard green shoots on potted plants inoculated at 0, 7 and 14 days after application of the fungicides and biocontrol agents, incidence of infection was not affected by species (P=0.456; Appendix E.2.1) or inoculation time (P=0.769). However, it was affected by treatment (P<0.001; Appendix E.2.1). All treatments significantly reduced mean incidence as compared to the control (Table 5.1). The least mean incidence was for Cheif® (21.2%) which was significantly less (P<>0.05) than for all other treatments, followed by Folicur® (41.0%)

which was significantly less (P<0.05) than all other treatments apart from Cheif®. The greatest mean incidences were for Gem® (72.0%), TRI D25 (72.9%) and Serenade® (76.9%) which did not differ significantly from each other. The interactions were not significant (P>0.05).

Table 5.1: Efficacy of fungicides and biocontrol agents on mean infection incidences of *N. luteum* and *N. parvum* which were inoculated onto green shoots and trunks of potted Sauvignon blanc vines placed outside. Values in parentheses are logit transformed data.

Treatments	[a]Botryosphaeriaceae spp. incidence (%)		
	Green shoots	Trunks	
		Wood	Bark
Cheif®	21.2 (2.14) e	38.1 (1.45) g	40.9 (1.36) e
Gem®	72.0 (0.57) b	71.5 (0.59) cd	68.4 (0.65) c
Megastar®	55.2 (0.96) c	57.7 (0.90) e	56.8 (0.92) d
Dithane®	59.3 (0.86) c	69.1 (0.64) d	68.3 (0.64) c
Serenade®	76.9 (0.47) b	80.0 (0.41) b	82.9 (0.34) b
Folicur®	41.0 (1.35) d	51.6 (1.05) f	58.3 (0.89) d
TRI D25	72.9 (0.56) b	73.9 (0.53) c	69.2 (0.66) c
Control	97.1 (0.06) a	91.5 (0.17) a	91.5 (0.17) a
SED	**(0.091)**	**(0.072**	**(0.071)**

[a]Values within columns followed by the different letter are significantly different according the SEDs derived from GLM analysis of logit transformed data.

For trunks, incidence in wood was not significantly affected by species (P=0.690) or inoculation time after treatment application (P=0.462; Appendix E.2.2). However, there was a treatment effect on incidence (P=0.003). All treatments significantly reduced mean incidence as compared to the control, with lowest mean infection incidence for Cheif® (38.1%) which was significantly lower (P<0.05) than all other treatments (Table 5.1). This was followed by Folicur® (51.6%) and Megastar® (57.7%), with incidence for Folicur® being significantly less (P<0.05) than Megastar®. The greatest mean incidence was for Serenade® (80%) which was significantly greater (P<0.05) than for all other treatments apart from the control (Table 5.1). The interactions were not significant (P>0.05).

For the bark, incidence was not significantly affected by species (P=0.606) or inoculation time after treatment application (P=0.617; Appendix E.2.3). However, there was a treatment

effect on incidence (P<0.001). All treatments significantly reduced mean incidence as compared to the control, with lowest (P<0.05) mean infection incidence for Cheif® (40.9%) (Table 5.1). The greatest, (P<0.05) mean incidence was for Serenade® (82.9%). The interactions were not significant (P>0.05).

For the lengths of colonised tissues on green shoots, there was no significant effect of species (P=0.561; Appendix E.3.1), however there was a significant effect of inoculation time after treatment application (P<0.001; Appendix E.3.1). Mean distances were 2.4 cm, which was greater (P<0.05) than 1.6 cm and 1.1 cm which were similar (P>0.05), for the 0, 7 and 14 day inoculation time, respectively. There was a significant treatment effect (P<0.001), with shorter mean lengths of colonised tissues for all treatments compared to the control (P<0.05). The shortest, mean lengths of colonised tissues were for Cheif® (0.4 cm), Folicur® (0.7 cm) and Megastar® (1.0 cm) which did not differ significantly (P>0.05) from each other (Table 5.2). The longest mean lengths of colonised tissues were for TRI D25 and Serenade® (2.0 cm and 2.4 cm, respectively) which did not differ significantly (P>0.05) from each other (Table 5.2). All the interactions were not significant (P>0.05) except the interaction between treatment and inoculation time after application (P<0.001). This interaction was associated with a trend for greater efficacy at 14 days for the two biocontrol products (TRI D25 and Serenade®) whereas efficacy was similar for the different inoculation times for the other treatments (Table 5.3).

Table 5.2: Effect of fungicides and biocontrol products on the lengths of tissue colonised by *N. luteum* and *N. parvum* which were inoculated onto green shoots and trunks of potted Sauvignon blanc vines at different times after treatment.

Treatments	[a]Mean length of colonised tissues (cm)		
	Green shoots	Trunks	
		Wood	Bark
Cheif®	0.4e	0.5d	0.7d
Gem®	1.5cd	1.7b	1.4bcd
Megastar®	1.0de	0.8cd	1.3cd
Dithane®	1.6cd	1.2bcd	1.5bc
Serenade®	2.4b	1.8b	2.9a
Folicur®	0.7e	0.7cd	1.3cd
TRI D25	2.0bc	1.6bc	2.0b
Control (I)	4.0a	3.1a	3.4a
Means	**1.7**	**1.4**	**1.8**

[a]Values within the columns followed by the different letter are significantly different according to Sidak 95% CIs at $P \leq 0.05$.

For length of colonized tissues in wood of the trunks, there was no significant effect of species (P=0.259; Appendix E.3.2). There was a significant effect of inoculation time (P=0.043), with different (P<0.05) means of 1.8 cm, 1.3 cm and 1.1 cm, respectively for the 0, 7 and 14-day inoculation times. There was a significant treatment effect on colonization distance (P<0.001; Appendix E.3.2), with shorter mean lengths of colonised tissues for all treatments compared to the control (P<0.05) with shortest mean distances colonised for Cheif® (0.5 cm), Folicur® (0.7 cm), Megastar® (0.8 cm) and Dithane® (1.2 cm) which were not significantly different from each other (Table 5.2). The longest colonisation distances were for TRI D25 (1.6 cm), Gem® (1.7 cm) and Serenade® (1.8 cm) which did not differ significantly from each other. There was a significant interaction (P<0.011; Appendix E.3.2) between treatment and inoculation time after treatment application, which was associated with a trend for greater efficacy at 14 days for the biocontrol products (Serenade® and TRI D25) whereas for Gem® efficacy decreased at 14 days and efficacy was similar for the different inoculation times for the other treatments (Table 5.3).

Table 5.3: Efficacy of fungicides and biocontrol products when pruning wounds on Sauvignon blanc were inocuated with *N. luteum* and *N. parvum* 0, 7 or 14 days after treatment on the mean length of colonised tissues.

Treatments	Days	[a]Mean length of colonised tissues (cm)		
		Green shoots	Woody	Bark
Cheif®	0	0.4c	0.4d	0.7cd
Gem®	0	2.0b	1.3abcd	1.4bcd
Megastar®	0	1.3bc	0.9abcd	1.5bcd
Dithane®	0	1.5bc	1.3abcd	1.8bcd
Serenade®	0	4.5a	2.8abc	4.1a
Folicur®	0	0.8bc	0.8bcd	1.7bcd
TRI D25	0	4.0a	2.7ab	3.0ab
Control (I)	0	4.8a	4.1a	4.3a
Cheif®	7	0.3c	0.6cd	0.8cd
Gem®	7	1.8bc	1.4abcd	1.7bcd
Megastar®	7	1.3bc	0.8bcd	1.7bcd
Dithane®	7	1.7bc	1.4abcd	1.7bcd
Serenade®	7	1.5bc	1.3abcd	3.0ab
Folicur®	7	0.7bc	0.7cd	1.4bcd
TRI D25	7	1.3bc	1.3abcd	2.1bc
Control (1)	7	4.0a	3.2ab	3.5ab
Cheif®	14	0.4c	0.7cd	0.7cd
Gem®	14	1.3bc	2.5abc	1.1cd
Megastar®	14	0.7bc	0.9bcd	1.1cd
Dithane®	14	1.1bc	0.9bcd	1.0cd
Serenade®	14	1.0bc	1.1abcd	1.5bcd
Folicur®	14	0.6bc	0.7cd	0.8cd
TRI D25	14	0.7bc	0.4d	0.4d
Control (I)	14	3.2ab	2.1abcd	2.3bcd
Means		**1.7**	**1.4**	**1.8**

[a]Values within the columns followed by the different letter are significantly different according to Sidak 95% CIs at $P \leq 0.05$.

In addition, colonies characteristic of *Trichoderma* and *Bacillus* were observed growing out from the tissue from the plants treated with TRI D25 and Serenade®, respectively. All the other interactions were not significant ($P > 0.05$).

For lengths of colonized tissues in bark of the trunks, there was a significant effect of species (P=0.029; Appendix E.3.3), with mean lesion length of tissue colonized being greater (P<0.05) for *N. luteum* (2.0 cm) than for *N. parvum* (1.6 cm). There was a significant effect of inoculation time after treatment application (P<0.001; Appendix E.3.3), with different (P<0.05) means of 2.7 cm, 1.8 cm and 1.6 cm, respectively for the 0, 7 and 14 day inoculation times. There was a significant effect of treatment (P<0.001), with shorter (P<0.05) mean lengths of colonised tissues for all treatments compared to the control except Serenade®. The shortest, mean lengths of colonised tissues were for Cheif® (0.7 cm), Megastar®e (1.3 cm), Folicur® (1.3 cm) and Gem® (1.4 cm; Table 5.2). Longest mean lengths of colonised tissues were for TRI D25 (2.0 cm) and Serenade® (2.9 cm). All the interactions were not significant (P>0.05) except between treatments and inoculation times after application (P<0.038; Table 5.3), which was again associated with a trend for greater efficacy at 14 days for the biocontrol products compared with similar efficacy for the different inoculation times for the other treatments.

5.3.2 Efficacy of fungicides and biocontrol agents on wounds of potted grapevines in a tunnel house

Wounds inoculated on hard green shoots of potted plants with *N. luteum* and *N. parvum,* 1 h and 24 h after application of the fungicides and biocontrol products, showed similar infection incidences for the two species (P=0.295; Appendix E.4.1) but inoculation time was significant (P=0.047), with overall mean infection incidences of 59% for 1 h and 73% for 24 h. There was a significant effect (P<0.001) of treatments on infection incidence. All treatments significantly (P<0.05) reduced mean incidences as compared to the control, with least mean incidence being for Cheif® (23.7%), which was significantly less (P<0.05) than for all other treatments (Table 5.4). This was followed by Folicur® (41.0%) while the greatest means were for TRI D25 and Serenade® (both 83.0%). The interactions were not significant (P>0.05).

For wood of the trunks of potted plants, there was no significant effect on infection incidence of species (P=0.757; Appendix E.4.2) or inoculation time (P=0.332). There was a significant effect (P<0.001; Appendix E.4.1) of treatment on infection incidence. All treatments significantly reduced mean incidence as compared to the control, with least mean incidence being for Cheif® (40.7%), which was significantly less (P<0.05) than for all other treatments (Table 5.4). This was followed by Folicur® and Megastar® (both 53.5%), while the greatest

mean incidences were for TRI D25 and Serenade® (both 91.5%). The interactions were not significant (P>0.05).

On bark there was no significant effect on infection incidence of species (P=0.785; Appendix E.4.3) or inoculation time (P=0.390). The treatments significantly affected infection incidence (P<0.001; Appendix E.4.3). All treatments significantly reduced mean incidence as compared to the control, with least, (P>0.05) mean incidences being for Cheif® (41.0%), followed by Folicur® (48.7%) and Megastar® (60.6%), while TRI D25 (87.4%) and Serenade® (91.6%) had highest mean incidences which were not significantly different (P>0.05) (Table 5.4). The interactions were not significant (P>0.05).

Table 5.4: Effect of fungicides and biocontrol products on mean infection incidences of *N. luteum* and *N. parvum* which were inoculated onto hard green shoots and trunks of potted vines of Sauvignon blanc in a tunnel house. Values in parentheses are logit transformed data.

Treatments	[a]Botryosphaeriaceae spp. isolated (%)		
	Green shoots	Wood	Bark
Cheif®	23.7 (2.00) e	40.0 (1.36) e	41.0 (1.35) f
Gem®	64.4 (0.74) c	74.5 (0.52) c	70.1 (0.62) c
Megastar®	57.9 (0.90) c	53.5 (1.01) d	60.6 (1.13) e
Dithane®	64.4 (0.74) c	73.7 (0.54) c	70.6 (0.60) c
Serenade®	83.0 (0.34) b	91.5 (0.17) b	91.6 (0.17) b
Folicur®	41.0 (1.35) d	53.5 (1.01) d	48.7 (0.83) d
TRI D25	83.0 (0.34) b	91.5 (0.17) b	87.4 (0.25) b
Control	100 (0.00) a	99.9 (0.00) a	100 (0.00) a
SED	**(0.012)**	**(0.085)**	**(0.095)**

[a]Values within the columns followed by the different letter are significantly different according to SED derived from GLM analysis of logit transformed data.

5.3.3 Fungicide longevity

The effect of inoculum concentration on infection incidence of green shoots was significant (P=0.044 Appendix E.5.1), with mean infection incidences being 46.2% with 2 conidia/wound and 57.2% with 40 conidia/wound. There was a significant effect of treatment time on infection incidence (P=0.031); the most effective treatment was Treatment 5, which was inoculated after 10 weeks and the least was Treatment 3, which was inoculated after 4

weeks with mean infection incidences of 41.0% and 62.4%, respectively (Table 5.5). There was a significant effect of fungicide on infection incidence (P<0.001), with the least mean incidence being for Cheif® (16.2%), which was significantly less (P<0.05) than for Folicur® (32.6%) which was less (P<0.05) than for Megastar® (41.0%; Table 5.6). The least effective fungicide was Gem® (72.8%). The interactions were not significant at P=0.05.

Table 5.5: Effect of different treatments on mean infection incidences of *N. luteum* and *N. parvum* which were inoculated onto green shoots. Fungicides were applied and wounds inoculated after ~2 h (Treatment 1) or after 2 weeks (Treatment 2), 4 weeks (Treatment 3), 8 weeks (Treatment 4) or at 10 weeks (Treatment 5) after wounding. Values in parentheses are logit transformed data.

Treatments	[a]**Infection incidence %**
T1	46.2 (1.20) a
T2	61.7 (0.81) b
T3	62.4 (0.80) b
T4	47.6 (1.16) a
T5	41.0 (1.40) c
SED	**(0.050)**

[a]Values within the columns followed by the different letter are significantly different according to SED derived from GLM analysis of logit transformed data.

In the wood of trunks, the effect of inoculum concentration on infection incidence was not significant (P=0.498) nor was the effect of treatment time (P=0.443). However, there was a significant effect of fungicide treatment (P<0.001; Appendix E.5.2) with all fungicides significantly reducing (P<0.05) incidence compared with the control. The least mean incidence being for Cheif® (32.6%), which was significantly less (P<0.05) than for Megastar® (49.2%), which was less (P<0.05) than for Folicur® (52.6%; Table 5.6). The least effective fungicide was Gem® (76.1%). The interactions were not significant at P=0.05.

In the bark of trunks, the effect of inoculum concentration on infection incidence was not significant (P=0.586) nor was the effect of treatment time (P=0.350). There was a significant effect of fungicide treatment on infection incidence (P<0.001; Appendix E.5.3), with all fungicides significantly reducing (P<0.05) incidence compared with the control. The least mean incidence being for Cheif® (32.0%), which was significantly less (P<0.05) than for Megastar® (49.0%) which was less (P<0.05) than for Folicur® (52.5%; Table 5.6). The least

effective fungicide was Gem® with mean infection incidence of 76.1%. The interactions were not significant at P=0.05.

Table 5.6: Effect of fungicides on mean infection incidences of *N. luteum* and *N. parvum* which were inoculated onto green shoots and trunks. Values in parentheses are logit transformed data.

Fungicides	[a]Botryosphaeriaceae spp. isolated (%)		
	Green shoots	Wood	Bark
Cheif®	16.2 (2.43) f	32.6 (1.66) f	32.0 (1.66) f
Gem®	72.8 (0.56) b	76.1 (0.49) b	76.1 (0.49) b
Megastar®	41.0 (1.36) d	49.2 (1.12) e	49.0 (1.12) e
Dithane®	66.1 (0.70) c	69.4 (0.64) c	69.1 (0.64) c
Folicur®	32.6 (1.63) e	52.6 (1.03) d	52.5 (1.03) d
Control	83.6 (0.33) a	82.8 (0.40) a	80.1 (0.40) a
SED	**(0.069)**	**(0.047)**	**(0.055)**

[a]Values within the columns followed by the different letter are significantly different according to SED derived from GLM analysis of logit transformed data.

For the length of colonised tissues, the effect of inoculum concentration on green shoots was significant (P<0.001: Appendix E.6.1), with longer mean lengths for 40 conidia/wound than for 2 conidia/wound (0.9 cm and 0.6 cm, respectively) (Table 5.7). There was a significant effect of treatment time on the length of colonised tissues (P=0.012). Overall the most effective was Treatment 1, which was inoculated after 2 h with a mean length of 0.5 cm and the least effective was Treatment 2, which was inoculated after 2 weeks with a mean length of 1.0 cm (Figure 5.7). There was a significant effect of fungicide (P<0.001), with all fungicides significantly reducing (P<0.05) incidence compared with the control. The least (P<0.05), mean lengths were for Cheif® (0.3 cm), Folicur® (0.5 cm) and Megastar® (0.7 cm) which were not significantly different (P>0.05) to each other (Table 5.8). The longest similar (P>0.05) mean lengths of colonised tissues were for Gem® and Dithane® (1.3 cm and 1.2 cm, respectively, which were not significantly different (P>0.05) to each other. The interactions were not significant at P=0.05.

Table 5.7: Effect of different treatments on mean length of *N. luteum* and *N. parvum* which were inoculated onto different tissues. Fungicides were applied and wounds inoculated after ~2 h (Treatment 1) or after 2 weeks (Treatment 2), 4 weeks (Treatment 3), 8 weeks (Treatment 4) or at 10 weeks (Treatment 5) after wounding.

Treatments	[a]Green shoots	[a]Wood	[a]Bark
T1	0.5b	0.6b	0.8c
T2	1.1a	1.0a	1.5a
T3	1.0ab	1.0a	1.2ab
T4	0.8ab	0.9a	0.7bc
T5	0.6b	0.9a	0.7bc
Means	**0.8**	**0.9**	**1.0**

[a]Values within the columns followed by the different letter are significantly different according to Sidak. 95% CIs at $P \leq 0.05$

For wood of trunks, there was a significant effect of inoculum concentration (P=<0.035; Appendix E.6.2) on the length of colonised tissues, with longer mean distances for 40 conidia/wound than for 2 conidia/wound (0.8 cm and 0.7 cm, respectively). The effect of treatment time was also significant (P=0.025). Overall the most effective treatment was Treatment 1 with a mean length of colonised tissues of 0.6 cm and the least effective was Treatment 2 with a mean length of 1.0 cm (Table 5.7). There was a significant effect of fungicide (P<0.001), with shortest (P<0.05) mean lengths being for Cheif® (0.4 cm), Folicur® (0.6 cm) and Megastar® (0.7 cm) which were not significantly different (P>0.05) to each other (Table 5.8). Lengths for the remaining treatments were similar (P>0.05) to each other with a trend showing longest, mean lengths of colonised tissues for Dithane® and Gem® (1.1 cm and 1.2 cm, respectively). The interactions were not significant at P=0.05.

For bark of trunks, there was a significant effect of inoculum concentration on the length of colonised tissues (P<0.043; Appendix E.6.3), with longest mean length of colonised tissues for 40 conidia/wound and least for 2 conidia/wound (1.1 cm and 0.9 cm, respectively). There was a significant effect of treatment time (P<0.001) on the length of colonised tissues, with the most effective treatment being Treatment 1 (0.8 cm) and the least effective being Treatment 2 (1.5 cm) (Figure 5.7). There was a significant effect of fungicide on lengths of colonised tissues (P<0.001). The shortest (P<0.05) mean lengths of colonised tissues were for Cheif® (0.5 cm; Table 5.8). The longest (P<0.05) mean lengths of colonised tissues were for Gem® and Dithane® (1.4 cm and 1.3 cm, respectively) which were not significantly different

(P>0.05). The interactions were not significant at P=0.05. Overall trends for all fungicides, treatment times and inoculum doses are shown in Appendix F.2.

Table 5.8: Effect of fungicides on the mean length of colonisation by *N. luteum* on different wound tissues of potted Sauvignon blanc grapevines.

Fungicides	[a]Mean length of colonised tissues (cm)		
	Green shoots	Wood	Bark
Cheif®	0.3c	0.4b	0.5b
Gem®	1.3a	1.2a	1.4a
Megastar®	0.7b	0.7b	1.0a
Dithane®	1.2a	1.1a	1.3a
Folicur®	0.5bc	0.6b	1.0a
Means	**0.083**	**0.093**	**0.1**

[a]Values within the columns followed by the different letter are significantly different according to Sidak 95% CIs at P≤0.05.

Table 5.9: Effect of fungicides on the mean length of colonisation by *N. luteum* when applied at 2 or 40 conidia per wound to different tissues of potted Sauvignon blanc grapevines.

Fungicides	[a]Mean length of colonised tissues (cm)			
	Inoculum conc.	Green shoots	Wood	Bark
Cheif®	2	0.2d	0.3d	0.4c
Gem®	2	1.1ab	1.1ab	1.2abc
Megastar®	2	0.5bcd	0.6bcd	1.0abc
Dithane®	2	1.1ab	0.9abc	1.1ab
Folicur®	2	0.4cd	0.6bcd	1.0abc
Cheif®	40	0.3yz	0.4yz	0.7xy
Gem®	40	1.5w	1.3w	1.5w
Megastar®	40	1.0wxy	0.7wxyz	1.0wxy
Dithane®	40	1.4w	1.2w	1.4wx
Folicur®	40	0.6xyz	0.7wxyz	1.0wxy
Means		**0.835**	**0.935**	**1.0**

[a]For each inoculum concentration, values within the columns followed by the same letter (a-d for inoculum concentration 2 conidia/wound, and x-y for inoculum concentration 40 conidia/wound) are not significantly different according to Sidak 95% CIs at P≤0.05.

5.3.5 Vineyard experiment

When wounded canes in the vineyard were inoculated with *N. luteum* and *N. parvum* at 0, 7 and 14 days after application of the fungicides and biocontrol products there was no significant effect of species or inoculation time (P=0.776, P=0.194, respectively; Appendix E.7.1) on infection incidence of the cane wood. However, there was a significant effect (P<0.001) of treatment, with all treatments significantly reducing (P<0.05) mean incidence compared with the control. The lowest mean incidence was for Cheif® (23.3%), which was significantly less (P<0.05) than for Folicur® (25.0%), which was less (P<0.05) than for Megastar® (26.2%) (Table 5.10). The least effective treatments (P<0.05) were Serenade® and TRI D25, with mean infection incidences of 76.6 and 72.9%, respectively. The interactions were not significant at P=0.05, except the interaction between the fungicides and time (P<0.001), which was associated with greater efficacy at 7 and 14 days of Serenade® and TRI D25 than for the 0 day treatment, whereas for most other treatments efficacy remained similar or decreased with application after 7 and 14 days compared with 0 days (Table 5.11).

Table 5.10: Mean infection incidences of *N. luteum* and *N. parvum* applied after fungicides and biocontrol products on wounded canes. Values in parentheses are logit transformed data.

Treatments	**[a]Botryosphaeriaceae spp. isolated (%)**	
	Wood	**Bark**
Cheif®	23.3 (2.75) h	27.9 (2.47) g
Gem®	43.7 (1.84) d	51.2 (1.55) d
Megastar®	26.2 (2.60) f	30.0 (2.37) f
Dithane®	32.9 (2.28) e	38.3 (2.01) e
Serenade®	76.6 (0.76) b	78.3 (0.71) b
Folicur®	25.0 (2.66) g	30.0 (2.37) f
TRI D25	72.9 (0.88) c	74.1 (0.83) c
Control	86.9 (0.54) a	89.0 (0.64) a
SED	**(0.015)**	**(0.014)**

[a]Values within the rows and columns followed by the different letter are significantly different according to SED derived from GLM analysis of logit transformed data.

On cane bark, infection incidence was not significantly affected by species (P=940; Appendix E.7.2) or time of fungicides application (P=0.608) but was affected (P<0.001) by fungicide treatment, with all treatments significantly reducing incidence compared with the

control. The least mean incidence was for Cheif® (27.9%), which was significantly less (P<0.05) than for Folicur® and Megastar® (both 30.0%). The least effective treatments (P<0.05) were Serenade® and TRI D25 (78.3% and 74.1%, respectively; Table 5.10).

Table 5.11: Mean infection incidences of *N. luteum* and *N. parvum* applied 0, 7 and 14 days after treatments on wounded canes of Sauvignon blanc in the vineyard. Values in parentheses are logit transformed data.

Treaments	Time	[a]Botryosphaeriaceae spp. isolated (%)	
		Wood	Bark
Cheif®	0	20.0 (2.95) e	23.7 (2.67) f
Gem®	0	36.2 (2.13) c	43.7 (1.42) d
Megastar®	0	21.2 (2.87) e	23.7 (2.20) e
Dithane®	0	26.2 (2.59) d	32.5 (2.25) e
Serenade®	0	92.5 (0.29) b	93.7 (0.26) b
Folicur®	0	20.0 (2.95) e	23.7 (2.67) f
TRI D25	0	92.5 (0.29) b	92.5 (0.29) c
Control	0	96.1 (0.09) a	99.9 (0.00) a
Cheif®	7	25.0 (2.66) e	30.0 (2.36) e
Gem®	7	47.5 (1.69) d	55.0 (1.42) c
Megastar®	7	28.7 (2.46) e	32.5 (2.25) e
Dithane®	7	36.2 (1.13) c	41.2 (1.90) d
Serenade®	7	71.2 (0.92) b	72.5 (0.90) b
Folicur®	7	27.5 (2.53) bc	32.5 (2.25) e
TRI D25	7	65.0 (1.11) bc	66.2 (1.07) bc
Control	7	86.2 (0.29) a	85.0 (0.52) a
Cheif®	14	25.0 (2.66) d	30.0 (2.36) e
Gem®	14	47.5 (1.69) c	55.0 (1.42) c
Megastar®	14	28.7 (2.46) e	33.7 (2.20) e
Dithane®	14	36.2 (2.13) c	41.2 (1.90) d
Serenade®	14	66.2 (1.08) b	68.7 (1.07) b
Folicur®	14	27.5 (2.52) bc	33.7 (2.20) e
TRI D25	14	61.2 (1.23) b	63.7 (1.14) bc
Control	14	78.5 (0.35) a	82.1 (0.44) a
SED		**(0.043)**	**(0.040)**

[a]Values within the columns followed by the different letter are significantly different according to SED derived from GLM analysis of logit transformed data.

All the interactions were not significant at P=0.05 except the interaction between the fungicides and time (P<0.001), which was associated with greater efficacy at 7 and 14 days of Serenade® and TRI D25 than for the 0 day treatment, whereas for most other treatments

efficacy remained similar or decreased with application after 7 and 14 days compared with 0 days (Table 5.11).

5.4 Discussion

In this study the efficacy of fungicides and biocontrol agents, which were reported to have some potential for control of the Botryosphaeriaceae, was investigated in potted vines and in a vineyard on different tissues and at various times with respect to inoculation by *N. parvum* and *N. luteum*. Overall, the effects of the fungicides were similar for all tissues, semi-hard shoots and wood and bark of trunks. In the vineyard experiment, pathogen incidence was high in the inoculated control (86.9%) and was reduced by all tested products, with lowest incidence in tissues treated with Cheif® (23.3%), Folicur® (25%) and Megastar® (26.2%). The length of colonised tissues after 1 month in potted vines and same varietyshowed the same overall trends (0.53, 0.90 and 1.03 cm, respectively). Amponsah *et al.* (2012b) also reported that the most effective fungicides for protecting wounds of 2-year-old potted grapevines (variety Pinot noir), against infection by conidia of *N. luteum* applied after 24 h, were carbendazim, iprodione, mancozeb and flusilazole (all 0% incidence) although tebuconazole was less effective (20% incidence). In another vineyard experiment assessed after 6 months they reported incidences to be 0, 2.7 and 3.7%, for flusilazole, carbendazim and tebuconazole, respectively. The differences in incidence between this study and that of Amponsah *et al.* (2012b) are hard to explain, as both studies used manufacturers' recommended field rates applied as sprays. However, Amponsah *et al.* (2012b) set up their field studies in vineyards where Botryosphaeriaceae spp. had not been found. They also used Pinot noir for their studies, although this variety was shown to have similar susceptibility to Sauvignon blanc by Amponsah *et al.* (2011). The inoculum concentrations in the current study (200-600 conidia per wound) were about 10% of those used by Amponsah *et al.* (2012b), however the results presented in Chapter 4 and by Amponsah *et al.* (2014) showed that incidence was not affected by conidial numbers used for inoculation. The same lower conidial inoculum concentration of 10^4 was used throughout the experiment in this Chapter to allow comparison between the

different experiments.

In this study, the colonisation lengths by *N. luteum* and *N. parvum* across all tissues showed similar trends with regards to incidences for the fungicides, with mean distances in the outdoor experiment being 0.53, 0.90 and 1.03 cm, respectively, for Cheif®, Folicur® and

Megastar®. Amponsah *et al.* (2012b) reported lengths of dieback lesion for these fungicides after 3 months to be 3.32, 5.02 and 4.14 cm, respectively. The very low colonisation distances in the current study were probably due to the short incubation period of 1 month during which pathogen did not have adequate time to establish inside the grapevine tissues. Luque *et al.* (2008) also reported that carbendazim was the most effective fungicide in their study, with 83.4% reduction in infection of oak trees by *B. corticola*. Pitt *et al.* (2012) also reported carbendazim as the most effective fungicides for prevention of grapevine pruning wounds by *D. seriata* and *D. mutila*. Sosnowski *et al.* (2008) reported that benomyl (a benzimidazole; same chemical group as carbendazim) applied onto pruning wounds of field vines reduced the infection incidence of naturally occurring Botryosphaericeae spp. by 27-41% when applied at 1 mL/L, which was a similar rate as used in the current experiment. They also reported that, of 15 fungicides tested in the field, benomyl was the most effective at reducing colonisation of pruning wounds by *E. lata.* Further, applications of 2 mL/L were shown to provide twice this level of control against *E. lata*. Other fungicides, such as fluazinam, pyrimethanil and pyraclostrobin also reduced colonisation of wounds by *E. lata* but only when applied at much higher concentrations than the recommended field rates. Ayres and Sosnowski (2013) reported that when wounds on 1-year-old canes of Cabernet Sauvignon were treated with 2-10 times the recommended field rates of tebuconazole with a paint brush and inoculated 1 day later with *E. lata* spores (500/wound) the treatment reduced the infection incidence by 83-87%. However, Pitt *et al.* (2012) showed that 10 mL/L of carbendazim did not significantly reduce incidence of Botryosphaericeae spp. in comparison to the lower dose of 1 mL/L. This was probably because assessment was carried out one year later, when natural inoculum had fallen on susceptible wounds, long after the likely efficacy period of the fungicides. If assessments had been conducted after a few weeks or months, they might have been able to demonstrate greater effects. Application of fungicides at higher doses may increases efficacy as shown by Sosnowski *et al*. (2008) and Ayres and Sosnowski (2013), however if applied at higher dosages there is a possible risk of phytotoxicity that will influence the long-termvine health. Fludioxonil at higher application concentrations was reported to increase the phytotoxicity effects on grapevine leaves (Petit *et al*., 2009). Similarly, the phytotoxicity of captan on pepper, and carbendazim on tobacco was reported to be more pronounced with higher application concentrations (Garcia *et al*., 2002; Tort and Tu'rkyilmaz, 2003).

In the current study treating wounds with TRI D25 and Serenade® failed to reduce the infection incidence and pathogen colonisation when inoculation was done just after their application. However, in the experiment which inoculated wounds at 0, 7 and 14 days after treatment, there were reduced colonisation distances for TRI D25 applied 7 and 14 days before pathogen inoculation, which were the same as those for flusilazole (13 and 7 mm, respectively), although at 0 days distances were similar to the control (40 and 48 mm, respectively). Serenade® also reduced colonisation distances for 7 and 14 days compared to the 0 day treatment, being 15, 10 mm and 45 mm, respectively. Biocontrol agents need time to establish and to colonize the wound before they are effective at preventing infection (Lo *et al.,* 1998). Similar results were reported by Latorre *et al.* (2013), who used same biocontrol agents as in this thesis, *Bacillus subtilis* (Serenade®) and *Trichoderma* spp. (Trichonativa®) provided no or weak protection (incidences of 100 and 50%, respectively) when applied 24 h before inoculation of *N. parvum* on 1-year-old blueberry stems. However, benomyl and iprodione applied at 0.1% and 0.06%, respectively, were both highly effective (0% incidence). John *et al*. (2005) reported the efficacy of biocontrol agents against *E. lata* ascospores. They reported that that when spores were applied to grapevine pruning wounds in the field, 14 days after wounding and application of *T. harzianum*, *F. lateritium* or Vinevax (*T. harzianum)*, they reduced the recovery of the pathogen as compared to pathogen inoculation on the day after wounding and biocontrol application. Kexiang *et al.* (2002) also observed greater control of apple ring rot caused by *Botryosphaeria berengeriana* f. sp *piricola* by *Trichoderma* spp. when applied 3 days before the pathogen than when inoculated at a same time. Further, Kotze *et al*. (2011) demonstrated that some biocontrol agents were as effective as benomyl when applied 7 days before pathogen inoculation in field vines assessed 8 months later. *Bacillus subtilis* isolate EE 1/10 reduced the overall mean incidences for four Botryosphaeriaceae spp. compared with the water control (15.2 and 34.2%, respectively). However, the *Trichoderma* spp. isolates USPP-T1 and USPP-T2 reduced mean incidences of the four Botryosphaeriaceae spp. even further, to 5.6 and 12.5%, respectively when the mean incidence for the benomyl treatment was 16.5%. The results of the current study however showed lower efficacy for the biocontrol agents than the Cheif® and Folicur® treatments and Pitt *et al.* (2012) also reported that Vinevax provided limited protection against Botryosphaeriaceae species. In contrast to the results of this study in which Serenade® (*B. subtilis*) was not effective, Ferreira *et al.* (1991) reported that a *B. subtilis* strain isolated from grapevine applied to wounds on 2-year-old canes, provided 100% suppression of *E. lata* infection. The greater success of this treatment might have been because they covered the

wounds with aluminium foil after inoculation, whilst in the current study the wounds were left open. Also using different bacterial isolates may have been a factor, as different isolates are known to have different efficacy against pathogens. Alternatively, there may have been differences in the susceptibility of the pathogens used, *E. lata* compared with Botryosphaeriaceae spp., to antibiotics produced by *B. subtilis.*

In the current study, TRI D25 was not as effective in the vineyard experiment as when used with potted plants placed in an outdoor but sheltered position. Incidences of 65.0 and 61.2% for the 7 and 14 day inoculations, respectively, indicated much lower efficacy than for the potted plant assays. Kredics *et al*. (2003) reported that the *Trichoderma* strains used in biocontrol are often sensitive to environmental conditions, especially low temperatures and dry conditions which are likely to inhibit their activity when used in aerial environments. Therefore, these products may need to be applied under particular environmental conditions which allow for germination and mycelia growth as well as production of anti-fungal compounds which prevent establishment of pathogens.

The factors which may affect efficacy are the formulation and concentration of the products. In the current study fungicides and biocontrol agents were applied as a spray, mixed to manufacturers' recommended field rates. When spray and paste formulations were compared by Diaz and Latorre (2013) in Chile, they found that paste applications containing (w/v) 1% benomyl, 0.1% pyraclostrobin, 0.5% tebuconazole and 1% thiophanate-methyl reduced the infection rate of *D. seriata*, *Inocutis* sp. and *Pa. chlamydospora* in pruning wounds of grapevine (Cabernet Sauvignon) better than spray applications. Future work is warranted to investigate the efficacy of different formulations of the most effective treatments identified in the current study.

In the first experiment on potted vines outdoors, there was no effect of inoculation time (0, 7 and 14 days) on incidence (67.7%, 68.4% and 62.1% respectively). In the second experiment when vines were retained in the tunnel house incidence was greater for 24 h (73%) than 1 h (59%). These effects were probably due to the sapwood dripping from wounds (bleeding). Serra *et al.* (2008) reported that susceptibility of grapevine pruning wounds to *D. seriata* on 15-year-old Sauvignon blanc plants in Alghero, northern Sardinia, Italy pruned in January, February and March was very high during most of the trial. However, in March, infection percentages decreased when inoculated soon after pruning, which they considered was due to the bleeding of grapevine spurs reducing infection. Although the inoculation time after fungicide application did not affect incidence it did affect the length of colonised tissues,

which were longest when freshly treated wounds were inoculated than when wounds were inoculated 7 and 14 days after treatment. However, this time effect was not evident for the systemic fungicides Cheif® and Folicur®.

In this study the efficacy of fungicides was tested to see whether they could provide protection over a longer period to repeated inoculations with *N. luteum* conidia. The most effective fungicide was Cheif® in green shoots and wood/bark and all fungicides were most effective at preventing infection by the inoculum applied 2 h later. The present study showed that when fungicide treated wounds were challenged by the pathogens 2 weeks after treatment, its efficacy was poorer than with the 2 h inoculation (incidences of 61.7% and 46.2%, respectively). The reduced efficacy of fungicides observed in the present study might be attributed to breakdown of the fungicide in the wound sites, as was observed by other researchers. Price and Carter (1975) reported that the extractable amount of methyl benzimidazole-2-yl carbamate in the sapwood had reduced by 45% 2 weeks after application to pruning wounds. Another study by Kotze *et al.* (2011) showed that when benomyl-treated wounds were infected with the *N. australe*, *N. parvum*, *Diplodia seriata*, *Lasiodiplodia theobromae*, *Eutypa lata*, *Phaeomoniella chlamydospora* or *Phomopsis viticola* 1 week after treatment, its efficacy was poor. In the current study, the reduced infection incidences when inoculations were done at week 8 and 10 after treatment were probably due to the wounds having healed sufficiently to become impervious to conidial infection. This corresponds to the results in Chapter 4 where infection incidence was reduced when 28 day wounds on green canes and trunks were inoculated with *N. luteum* conidia compared with inoculation of wounds immediately. This reduction in infection incidence of pruning wounds due to the healing process in the wounds was also shown by other researchers in different host plants and with different filamentous fungi (Eskalen *et al*., 2007; Urbez-Torress and Gubler, 2011). Since the less effective control was observed with 40 conidia/wound than with two conidia/wound, this may indicate survival of some conidia long enough to outlast the fungicides.

In the vineyard experiment Cheif®, Folicur® and Megastar® were the most effective fungicides. They provided reductions in infection incidences (25.0, 27.5 and 28.7%, respectively) compared to the control (78.5%) even when inoculated 14 days after treatment with two *Neofusicoccum* spp. which indicated that they are effective for at least that length of time.

Conclusion

In summary, these results showed that all treatments were able to reduce infection of pruning wounds but with differences in efficacy between treatments. Although the biological agents provided some control in potted vines, they were not as effective as Cheif®, Folicur® or Megastar® in the field. Although total protection was not achieved with any of the applications, Cheif® allowed greatest reduction in infection of grapevine pruning wounds. From these results, as well as other reports in the literature, Cheif®, Folicur® and Megastar® can be suggested to grape growers as fungicides to be evaluated as pruning wound protectants. Folicur is now registered in New Zealand as a wound protectant as Gelseal Ultra Spray-on (New Zelanad Winegrower Magazine, 2016). Further, since they greatly reduced the lengths of colonised tissue, this might result in removal of infected areas on shoots in Marlborough vineyards when they trim the upper shoots and side shoots of vine several times during summer.

Chapter 6

Concluding discussion

The overall aim of this study was to improve understanding of the spore production, dispersal infection dynamics, wound susceptibility and evaluation os treatments to protect wounds from nfection of the most common *Neofusicoccum* species in a New Zealand vineyard setting. In this chapter the key findings of this research are discussed with respect to future research needs and development of sustainable control programmes that are practical enough to meet the needs of grape growers.

Pathogenicity and conidium production on grapevine tissues

This study has demonstrated great variation in pathogenicity of different isolates of *N. luteum* and *N. parvum* used to infect different stem tissues of grapevine, and is the most extensive comparison, worldwide, of multiple isolates of *N. luteum* and *N. parvum* in terms of pathogenicity and conidial production. Pathogenicity was generally similar for the two species tested although there was a trend for production of more conidia from *N. luteum* than *N. parvum.* Variability in virulence between isolates and species has also been observed by other researchers, although only Urbez-Torres and Gubler (2009) used large numbers of isolates as used in this study to investigate pathogenicity. Greater pathogenicity of some species has also been reported, for example *Lasiodiplodia theobromae* by Urbez-Torres and Gubler (2009), *N. parvum* by Billones-Baajiens *et al.* (2014) and *N. luteum* by Amponsah *et al.* (2014). Further, other researchers have reported different outcomes in species occurrence, biology, and epidemiology from country to country and even between different grape-growing areas within the same country (van Niekerk *et al*., 2004; Urbez-Torres and Gubler, 2009; Amponsah *et al*., 2011; Billones-Baajiens *et al*., 2014), probably due in part to differences in climatic conditions and grape varieties. Billones-Baajiens *et al*. (2013) demonstrated that the assay parameters affected the lesion lengths caused when 114 Botryosphaeriaceae isolates were used to inoculate excised green shoots and 1-year-old rooted canes of Sauvignon blanc in different batches over time. Their experiments also showed that all isolates and species were pathogenic although the *Diplodia* species isolates were pathogenic on canes but not on green shoots. Since lengths of lesions were significantly affected by experimental batch, which reflected inherent host and environmental factors over time, this demonstrates that pathogen characteristics are only one type of factor to affect the

final disease outcome. Most of the subsequent experiments in this thesis used mixed isolate inoculum to infect grapevine tissues in case the individual isolates were affected differently by the outdoor environments of the assays. Further, natural inoculum in the field is likely to involve multiple isolates from different necrotic tissues.

In the current study, the numbers of conidia released from lesions on detached stems infected by the isolates of the two species also varied greatly between isolates and was directly proportional to their demonstrated pathogenicity. This is a novel finding as there are no similar published reports of differing sporulation capacity of Botryosphaeriaceae species and isolates. Amponsah *et al.* (2008) used three isolates of each species for their sporulation study but did not show differences between isolates, only between species. Knowledge of the sporulation capacity of isolates was important in this study because selected isolates were to be used for production of inoculum in further assays. Further, the evidence of overall sporulation capacity from necrotic tissues provided here gives some understanding of potential inoculum levels which may occur in the field.

Temperature and relative humidity were shown in this study to affect the release of *N. luteum* and *N. parvum* conidia from pycnidia on detached shoots under laboratory conditions and on attached shoots in growth chambers. All isolates of *N. luteum* and *N. parvum* were able to release conidia at all temperatures (10-25°C) and relative humidities (80-100%) tested but numbers were higher in high RH (~100%) and temperature (25°C). In the vineyard, oozing by pycnidia was also observed from naturally infected canes and shoots soon after rainfall events. This novel research provides further understanding of factors which may affect inoculum levels in the field. However, further investigations should be conducted to validate these results under natural conditions in vineyards. These should include studies that monitor individual pycnidia on marked stem tissues, which are fixed onto platforms for ongoing observation, to observe the effects of moisture, temperature, light and host tissues on numbers of conidia released, periods of repeated release and productive period of the pycnidia.

Conidium dispersal of Botryosphaeriaceae species in Marlborough vineyards

Rainwater traps collected Botryosphaericeae conidia many times while trapping in Blenheim vineyards, with *D. mutila* and *N. australe* being the most common species, as was also reported by Baskarathevan *et al*. (2012) who sampled necrotic tissues. A Burkard spore trap was also able to confirm presence of these conidia, during rainfall and up to 2 h after rainfall ceased, although it trapped comparatively few conidia. Since this type of trap can accurately

show the timing of spore release it should be used to trap spores over an entire year. The effects of temperature and relative humidity in the canopies could be recorded with Tinytag® data loggers and with reference to the nearest weather stations, which could also provide information on the minimum level of rainfall needed for spore release and dispersal.

This study also showed the dispersal distances of conidia released in vineyard settings. However, the experiments were conducted over limited periods. Further experiments which used marker strains and a Burkard trap might be able to demonstrate longer distance dispersal as well as timing of the secondary spread cycle. If the experiment was set up in vineyards with different climatic conditions, it could also provide useful information about how other factors affect dispersal distance, particularly wind speed and direction. Development of control strategies might also be improved by information derived from spore trapping over a number of years, especially during trimming and pruning. Further, the role of insects in the spread of conidia should be investigated since they have been shown to affect dispersal of fungal spores. Moyo (2013) caught arthropods in traps placed next to fresh cane pruning wounds in South African vineyards during winter. When the washings from millipedes, ants, spiders and beetles were used for molecular identification, most samples from all these insects contained DNA of Petri disease pathogens but 10% also contained DNA of *D. seriata*.

This study has provided information on the pathogenicity of Botryosphaeriaceae species, and potential sources of inoculum as well as the dispersal distances and how the environmental factors such as temperature and relative humidity affect release of conidia. The data from this study do not directly contribute to improving management practices of the Botryosphaeria disease, but they do provide some important information to be used in combination with spore trapping data, temperature, humidity, wind speed, rainfall and seasonal trapping of pathogens from infected vineyards to better understand the seasonal behaviour of conidia. These factors are vital to the spread and development of disease.

In this study, ascospores were not collected during sampling nor found when inspecting necrotic tissues in vineyards. However, future research should sample the air of vineyards in different regions and seasons using a Burkard trap to determine whether ascospores are released in New Zealand vineyards, and the species identity by molecular methods. Since perithecia and ascospores identified as belonging to the Botryosphaeriaceae have rarely been found under natural vineyard conditions, further studies should investigate whether Botryosphaeriaceae species are homothallic or heterothallic. A study by Bihon *et al.* (2011) reported that intensive scrutiny of the natural symptomatic tissues in pine had failed to find

the sexual state of *D. pinea* and thus the fungus had been considered exclusively asexual. However, their more recent population genetics study Bihon *et al*. (2014) showed high genotypic diversity, suggesting that the pathogen had a cryptic sexual stage. When they interrogated the structure of the *MAT* locus within sequenced genomic DNA of *D. pinea* isolates they found complete and apparently functional copies of the *MAT* genes (*MAT1-2-1* and *MAT1-1-1*), with both idiomorphs having a 1:1 ratio in the populations examined, as would be expected in heterothallic sexually reproducing populations. However, they did not attempt to induce sexual reproduction in the laboratory. Similar investigations into presence of different mating types among the Botryosphaeriaceae species, with a view to inducing sexual reproduction and ascospore production, could form the basis of an interesting study. The Plant Pathology Culture Collection at Lincoln University has several hundred Botryosphaeriaceae species which could be used to search for mating types and perhaps induce ascospore production under laboratory conditions. However, Urbez-Torres (2011) commented that induction of sexual reproduction was difficult under laboratory conditions, and recent studies by Elena *et al.* (2015) failed to produce sexual fruiting bodies under experimental conditions even though they had observed vegetative compatibility reactions among *D. seriata* isolates from grapevines. Phillips *et al*. (2005) reported that some of the *Botryosphaeria iberica* isolates used in their study were from perithecia and ascospores of these pathogens found infecting *Quercus* species in Spain, so sexual reproduction studies should be widened to include pathogenicity studies on other hosts.

Factors that affect infection of grapevine stems, buds and fruit

Wound age in late summer was shown to affect rate of infection and differed between tissues, with hard shoots being susceptible to infection by *N. luteum* conidia until 56 days old and trunk wood until 28 days old, which also demonstrated decreasing incidence and colonisation distances during the ageing process. However, the season when wounds were made also affected incidence, which was higher in autumn and spring than summer, when trunk wounds were minimally susceptible at 42 days old. Another study showed that inoculum density was also an important factor; inoculation of freshly wounded grapevine tissues with as little as 2 conidia/wound caused 100% incidence in hard shoots and 66.7% incidence in trunks of young potted vines. However, as the wounds aged, more conidia were required to cause high infection incidence and pathogen colonisation distance. These studies have provided new information on the factors affecting infection of canes in a Marlborough vineyard in much more detail than the single-factor experiments conducted by Amponsah *et al*. (2014), who

inoculated trunk wounds of Pinot noir potted vines with 250 conidia of *N. luteum* in Canterbury and in summer only. Further, they covered inoculated wounds with Parafilm™ which was not used in current experiments which were considered to be representative of natural infection in vineyards. Urbez-Torres and Gubler (2011) also reported that pruning wounds of Chardonnay and Cabernet Sauvignon in California, USA were susceptible to *N. parvum* and *L. theobromae* for up to 84 days when inoculated monthly over 5 months in autumn and winter with 5 x 10^4 conidia per wound. However, their studies used much higher numbers of conidia and focussed on the timing of pruning with different varieties to those used in this study. This study has also shown that infection by low numbers of *N. luteum* conidia is likely and represents a great risk to grape growers, especially as this species is known to progress rapidly through grapevine stems, often without development of obvious symptoms (Amponsah *et al*., 2011). However, these experiments were conducted with just one species and on potted vines, so further studies are needed to determine the effects of these factors on infection rates of different species in the field.

The effects of season and pruning wound age, which has not been investigated previously, showed that pruning wounds remained susceptible to infection by *N. luteum* for up to 28 days after pruning in summer and winter and 42 days after pruning in spring and autumn, with decreasing susceptibility over time. Grapevine pruning in Marlborough typically occurs in the dormant season from mid-May through to the end of August. When large cuts are required, such as for reshaping the heads of the vines, they are usually made in September (pers. comm. Andrew Naylor 2014). Since many reports have concluded that Botryosphaericeae spp. spores were present whenever rainfall had occurred to trigger release and to disperse them, the susceptibility of pruning wounds is of great concern. Urbez-Torres *et al.* (2010) showed in their study that spores were trapped from the mid autumn to early spring in Californian vineyards and Eskalen *et al.* (2013) reported that Botryosphaeriaceae spp. conidia were trapped in all seasons in Californian avocado orchards. In New Zealand, Amponsah *et al*. (2009b) also showed that Botryosphaericeae spp. conidia were present in rainfall traps throughout the year. Since colonisation distances in hard shoots were shown by this study to be 2.9 cm after 2 weeks and by Amponsah *et al.* (2011) to be 7.3 cm after 9 weeks, it seems likely that infection of trimming wounds could lead to infection of the vine structure. These studies should be repeated in vineyards where infection of trimmed shoots should be tracked and dieback measured, so that growers can be made aware of the timeframe for pruning out the infected shoots and the symptoms to watch out for. If experiments were set up in

vineyards destined for removal, these may also provide demonstration sites for grower workshops.

This study showed that inoculation of bark with conidia could result in infection of the wood below and that the germinating conidia grew rapidly in the direction of cuts made into the wood of the canes. When sections of inoculated lenticels were observed by microscope, hyphal invasion through lenticels was indicated by the large amount of mycelium covering the surface and internal tissues about the lenticels. This novel result indicates that wounds are not necessary for infection, as it occurred in the unprotected tissues used in these experiments. However, these results appear to contradict the findings of Amponsah *et al.* (2011) who was not able to isolate the inoculated pathogen from non-wounded green shoots or trunks. Further, it is the general understanding reported by other researchers that the Botryosphaeriaceae are wound pathogens. Clearly, these results have implications for the potential control of this disease, for which most studies have focussed on protection of pruning wounds. Further studies are required to validate these results, and they should be conducted in vineyards with marker strains of pathogens to exclude other types of natural infection.

In the current study, leaf buds were infected with *N. luteum* and *N. parvum* conidia throughout all stages of development for superficially wounded buds, with a meanof 48.3%. Non wounded buds were only able to be infected (~30%) at the green tip visible stage. These results contrast with the findings reported by Amponsah *et al*. (2012a), who reported higher incidences in non-wounded "swelling" buds (73%) after wounding and inoculation with *N. luteum* conidia. However, the inoculated area was protected by covering with a moist plastic bag in that study which may have allowed for higher incidence by providing high relative humidity and temperature. The studies by Phillips (1998) in Portugal, who 'inserted' 10^5 conidia of *P. viticola* and *B. dothidea* under the scales of each bud, were conducted on detached shoots of variety Espadeiro and held in a protected environment and so were quite dissimilar from the current work. Further, the work by Wunderlich *et al*. (2011) in New South Wales, Australia, which inoculated non-wounded buds at the dormant stage with 1000 conidia of *N. parvum* and *D. seriata*, and covered them with plastic bags after inoculation, was relatively unsuccessful as they recovered the pathogens from only 6% and 12%, respectively, of the buds, and observed no necrotic symptoms. In the current study, the supporting canes were shown to be colonised rapidly by the pathogen, leading to diseased shoots and potential inoculum production early in the growing season. If these shoots were

not detected and removed, the infection is likely to spread into the canes and even vine trunks, in which these pathogens have been shown to move downwards and upwards in the wood of living vines. Clearly, bud infection could occur in the field and lead to dieback of developing shoots and canes. Therefore, future research should investigate ways to protect buds from infection. If this is not possible, growers might need to monitor the health of new shoots in vineyards so that they can remove any symptomatic shoots to prevent further disease progression into the structure of the vine.

Berries also became infected after inoculation with *N. luteum* conidia. The berries at the intermediate development stages required wounding but berries at the pre-harvest stage were able to be infected without wounding. Further, the subsequent colonisation of rachii and supporting shoots means that the pathogen can spread rapidly through a bunch. Since infected berries also produced pycnidia, which can ooze conidia in all temperatures and relative humidities likely to be found during berry development, these tissues can act as another secondary source of inoculum. Amponsah *et al*. (2012a) also demonstrated similar effects when they wounded the berries which were protected with plastic bags and held in a greenhouse. Clearly, these results have shown that field berries may become infected with Botryosphaericeae species with the potential for secondary bunch rots, such as those caused by *Botrytis cinerea.* Further investigations should determine whether Botryosphaericeae species pre-dispose berries to infection by other extremely damaging pathogens. Also, if the canes colonised by the Botryosphaericeae species which infected bunches were not detected and removed during winter pruning, the infection is likely to spread into the vine structure. Amponsah *et al.* (2011) reported that after winter dormancy began, dieback developed rapidly in asymptomatic but infected tissues. In addition, the colonisation distances always extended further than the visible lesions; this evidence indicates the potential for spread from peripheral tissues into vine arms and trunks, ultimately resulting in vine death. Further research is required to validate these pathways of Botryosphaeriaceae infection in various grapevine tissues within vineyards and to develop pruning strategies which may eliminate the infected tissues.

Efficacy of fungicides and biocontrol agents for protecting wounds

The fungicide sensitivity study conducted on potted and field grown vines showed that Cheif®, Folicur® and Megastar® were effective at reducing infection by *N. luteum* and *N. parvum,* even when inoculation was carried out 14 days after treatment. The biological control product, TRI D25, was as effective as Megastar® but less effective than Cheif®,

which was the most effective overall. These treatments are also likely to control many other Botryosphaeriaceae species, since *in vitro* and field studies by Bester *et al.* (2007), Amponsah *et al.* (2012b) and Pitt *et al.* (2012) showed that these fungicides were effective against a wide range of these species. However, none of the fungicides were able to completely prevent infection in this study, even when inoculum doses were reduced to 2 conidia per wound. However, with low inoculum levels the fungicides reduced the colonisation distances to very low levels (3-8 mm) after a few weeks. Therefore, the slower pathogen progression might allow for removal of the infections by trimming and pruning. These studies have shown that only a few conidia are needed for successful infection and conidia are likely to be available whenever rainfall has occurred. Therefore, effective management of these pathogens with chemicals is likely to be extremely difficult. Further research should be done to investigate ways to make these products more effective at preventing bud, fruit, bark and wound infection. It is possible that repeated applications of the systemic fungicides found effective in this study might prevent colonisation of the bark by these pathogens. Further, higher concentrations might be more effective at preventing bark and wound infection than the normal field rates used in the current study. However, before such fungicide strategies can be adopted by grape growers, residue testing of the fruit will be necessary to establish the withholding periods.

Since TRI D25 was shown to prevent infection if allowed to colonise the tissues prior to challenge by the pathogen, further studies should investigate whether this biocontrol agent can colonise bark for prolonged periods when applied to vines Such a strategy might reduce infection via bark and even prevent infection of wounds made through that bark. Further studies should be conducted to investigate ways to provide good coverage and to ensure establishment of these agents. Investigations should also aim to determine the longevity of the biocontrol agents within bark of different stem tissues to determine the potential need for reapplication. Further, the efficacy of such treatments should be investigated with different varieties and in different regions since Mutawila *et al*. (2011) showed that in South Africa, reduction of pathogen incidence by *Trichoderma* spp. varied between varieties, being 10.3% in Chardonnay and 66.7% in Chenin blanc.

Summary

This study has provided new information on the disease cycle of Botryosphaeria diseases in vineyards. It has also shown how environmental conditions affect sporulation which occurs on grapevine stems of different ages as well as on fruit. This study also showed the effective dispersal distances of conidia and their infection efficacy on wounds in Marlborough vineyards. Further, it has demonstrated that infection can progress rapidly into the vine structure which can ultimately lead to vine death. The information about the length of time for which pruning and trimming wounds remained susceptible, and the capacity for other tissues to become infected without the need for wounds has demonstrated that management of this disease requires a multi-pronged strategy. Further monitoring of how weather conditions affect sporulation in the field using a Burkard spore trap might allow for timing strategies that avoid pruning during such conditions. This could be used for deciding trimming times and timing of large cuts but is unlikely to be used for normal pruning which needs to be continuous in winter due to shortage of labour.

Vineyard sanitation could reduce inoculum sources; this might mean removal of all cane pruning, rather than just mulching them as is currently practised in Marlborough vineyards. Elena and Luque (2015) reported that grapevine debris already infected with *D. seriata* were able to produce conidia for up to 3.5 years after pruning when placed in natural vineyard conditions, and the viability of these conidia decreased significantly between 2 and 3.5 years. Further, the current study showed that fresh cane prunings were able to be infected. Therefore, removal of these tissues should be investigated along with Burkard spore trapping, to determine whether this practice reduces dispersed inoculum. Frequent inspection of vines and removal of necrotic canes and shoots could prevent disease progression into the permanent wood of the vines. However, strategies for protection of tissues from infection with chemical and biological products must also be considered as necessary for more than just wounds; overall protection is clearly necessary for prolonged periods throughout the year.

References

Ahimera, N., Gisler, S., Morgan, D. P., & Michailides, T. J. (2004). Effects of single-drop impactions and natural and simulated rains on the dispersal of Botryosphaeria dothidea conidia. *Phytopathology,* 94: 1189-1197.

Alves A., Burgess, T., Barber, P., & Groenewald, J. Z. (2006). Phylogenetic lineages in the Botryosphaeriaceae. *Studies in Mycology*, 55: 235-253.

Alves, A., Correia, A., Luque, J., & Phillips, A. J. L. (2004). Botryosphaeria corticola *sp*. nov. on Quercus species, with notes and description of Botryosphaeria stevensii and its anamorph Diplodia mutila. *Mycologia,* 96: 598–613.

Alves, A., Phillips, A. J. L., Henriques, I., & Correia, A. (2005). Evaluation of amplified ribosomal DNA restriction analysis as a method for the identification of Botryosphaeria species. *FEMS Microbiology Letters,* 245 (2): 221-229.

Amponsah, N. T., Jones E. E., Ridgway H. J., & Jaspers, M. V. (2008). Production of Botryosphaeria species conidia using grapevine green shoots. *New Zealand Plant Protection*, 61: 301–305.

Amponsah, N. T., Jones, E. E., Ridgway, H. J., & Jaspers, M. V. (2009a). First report of Neofusicoccum australe [Botryosphaeria australis], a cause of grapevine dieback in New Zealand. *Australasian Plant Disease Note,* 4: 6–8.

Amponsah, N. T., Jones, E. E., Ridgway, H. J., & Jaspers, M. V. (2009b). Rainwater dispersal of Botryosphaeria conidia from infected grapevines. *New Zealand Plant Protection*, 62: 228-233.

Amponsah, N. T., Jones, E. E., Ridgway, H. J., & Jaspers, M. V. (2010). Effects of solar radiation and relative humidity on germination of Botryosphaeriaceae species conidia. *New Zealand Plant Protection,* 63: 28-32.

Amponsah, N. T., Jones, E. E., Ridgway, H. J., & Jaspers, M. V. (2011). Identification, potential inoculum sources and pathogenicity of botryosphaeriaceous species associated with grapevine dieback disease in New Zealand. *European Journal of Plant Pathology,* 131: 467–482.

Amponsah, N. T., Jones, E. E., Ridgway, H. J., & Jaspers, M. V. (2012a). Susceptibility of grapevine tissues to Neofusicoccum luteum conidial infection. *Plant Pathology*, 61 (4): 719-729.

Amponsah, N. T., Jones, E. E., Ridgway, H. J., & Jaspers M. V. (2012b). Evaluation of fungicides for the management of botryosphaeriaceous dieback diseases of grapevines in New Zealand. *Pest Management Science,* 68: 676-683.

Amponsah, N. T., Jones, E. E., Ridgway, H. J., & Jaspers, M. V. (2014). Factors affecting Neofusicoccum luteum infection and disease progression in grapevines. *Australasian Plant Patholology*, 43: 547–556.

Arauz, L. F., & Sutton, T. B. (1989). Influence of temperature and moisture on germination of ascospore and conidia of Botryosphaeria obtusa. *Phytopathology,* 79: 667-674.

Arauz, L. F., & Sutton, T. B. (1990). Effect of interrupted wetness periods on spore germination and apple infection by Botryosphaeria obtusa. *Phytopathology,* 80 (11): 1218-1220.

Arauz, L. F., & Sutton, T. B. (1991). Influence of temperature and moisture on germination of ascospores and conidia of Botryosphaeria dothidea. *Plant Disease,* 75: 1146-1149.

Aroca, A., García-Figueres, F., Bracamonte, L., Luque, J., & Raposo, R. (2006). A survey of trunk disease pathogens within rootstocks of grapevines in Spain. *European Journal of Plant Pathology*, 115 (2): 195-202.

Ayres, M. R., & Sosnowski, M. R. (2013). Optimizing management of Eutypa dieback. Final report to the Grape and Wine Research and Development Corporation, SAR 1001. South Australian Research and Development Institute, Adelaide. 52p.

Azouaoui-Idjer, G., Della Rocca, G., Pecchioli, A., Bouznad Z., Bouznad & Danti R. (2012). First report of Botryosphaeria iberica associated with dieback and tree mortality of Monterey cypress (Cupressus macrocarpa) in Algeria. *Plant Disease,* 96 (7): 1073-1073.

Babadoost, M. (2005). Botryosphaeria canker and white rot: University of Illinois extension. (Report on plant disease No: 813).

Balci, Y., Balci, S., Blair, J. E., Park, S.Y., Kang, S., & MacDonald, W. L. (2008). Phytophthora quercetorum sp. nov., a novel species isolated from eastern and north-central U.S.A oak forest soils. *Mycological Research,* 112 (8): 906-916.

Baskarathevan, J. (2011). *Botryosphaeriaceous infection in New Zealand vineyards: identification, population structure and genetic diversity* (Doctoral dissertation, Lincoln University).

Baskarathevan, J., Jaspers, M. V., Jones, E. E., & Ridgway, H. J. (2012). Identification, incidence and distribution of botryosphaeriaceous species in New Zealand vineyards. *European Journal of Plant Pathology*, 132: 549-560.

Baskarathevan, J., Jaspers, M. V., Jones, E. E., & Ridgway, H. J. (2013). Development of isolate-specific markers for Neofusicoccum parvum and N. luteum and their use to study rainwater splash dispersal in the vineyard. *Plant Pathology*, 62 (3): 501-509.

Bester, W., Crous, P. W., & Fourie, P. H. (2007). Evaluation of fungicides as potential grapevine pruning wound protectants against Botryosphaeria species. *Australasian Plant Pathology*, 36 (1): 73-77.

Biggs, A. R. (1986). Wound age and infection of peach bark by Cytospora leucostoma. *Canadian journal of botany*, 64 (10): 2319-2321.

Biggs, A. R. (1987). Occurrence and location of suberin in wound reaction zones in xylem of 17 tree species. *Phytopathology*, 77 (5): 718-725.

Biggs, A. R. (1990). Managing wound-associated diseases by understanding wound healing in the bark of woody plants. Journal of Arboriculture, 16: 108–112.

Biggs, A. R. (2004). Effect of inoculum concentration and calcium salts on infection of apple fruit by Botryosphaeria dothidea. *Plant disease*, 88 (2): 147-151.

Bihon, W., Burgess, T., Slippers, B., Wingfield, M. J., Wingfield, B. D. (2011). Distribution of Diplodia pinea and its genotypic diversity within asymptomatic Pinus patula trees. *Australasian Plant Pathology*, 40: 540–548.

Bihon, W., Wingfield, M. J., Slippers, B., Duong, T. A., & Wingfield, B. D. (2014). MAT gene idiomorphs suggest a heterothallic sexual cycle in a predominantly asexual and important pine pathogen. *Fungal Genetics and Biology*, 62: 55-61.

Billones, R. G., Ridgway, H. J., Jones, E. E., & Jaspers, M. V. (2010). First report of Neofusicoccum macroclavatum as a canker pathogen of grapevine in New Zealand. *Plant Disease*, 94 (12): 1504-1504.

Baaijens Billones, R. (2011). *Botryosphaeria infections in New Zealand grapevine nurseries: Sources of inoculum and infection pathways* (Doctoral dissertation, Lincoln University).

Billones-Baaijens, R. G., Ridgway, H. J., Jones, E. E., & Jaspers, M. V. (2015b). Spatial distribution of Neofusicoccum species within a rootstock mothervine indicates potential infection pathways. *European Journal of Plant Pathology,* 141: 267-279.

Billones-Baaijens, R., Jaspers, M., Allard, A., Hong, Y., Ridgway, H., & Jones, E. (2015a). Management of Botryosphaeriaceae species infection in grapevine propagation materials. *Phytopathologia Mediterranea*, 54 (2): 355-367.

Billones-Baaijens, R., Jones, E. E., Ridgway, H. J., & Jaspers, M. V. (2013). Virulence affected by assay parameters during grapevine pathogenicity studies with Botryosphaeriaceae nursery isolates. *Plant pathology*, 62 (6): 1214-1225.

Billones-Baaijens, R., Ridgway, H. J., Jones, E. E., & Jaspers, M. V. (2014). Susceptibility of common rootstock and scion varieties of grapevines to Botryosphaeriaceae species. *Australasian Plant Pathology*, 43: 25-31.

Billones-Baaijens, R., Ridgway, H. J., Jones, E. E., Cruickshank, R. H., & Jaspers, M. V. (2013). Prevalence and distribution of Botryosphaeriaceae species in New Zealand grapevine nurseries. *European Journal of Plant Pathology*, 135: 175-185.

Billones-Baaijens, R. G., Ridgway, H. J., Jones, E. E., & Jaspers, M. V. (2012). Pathogenicity of a New Zealand grapevine isolate of Neofusicoccum macroclavatum on Eucalyptus globulus. *New Zealand Plant Protection*, 65: 262-266.

Biswas, G., & Pan, S. (2011). Bioefficacy of commercial adjuvants against some plant pathogens. *Journal of Mycopathological Research*, 49 (2): 291-294.

Brown Rytlewski, D. E., & McManus, P. S. (1999). Canker formation on apple by Botryosphaeria spp. and control with wound treatment. Fruit Pathology, University of Wisconsin, Madison. htpp.

Burgess, T. I., Barber, P. A., & Hardy, G. S. J. (2005). Botryosphaeria spp. associated with eucalypts in Western Australia, including the description of Fusicoccum macroclavatum sp. nov. *Australasian Plant Pathology*, 34 (4): 557-567.

Burgess, T. I., Sakalidis, M. L., & Hardy, G. E. (2006). Gene flow of the canker pathogen Botryosphaeria australis between Eucalyptus globulus plantations and native eucalypt forests in Western Australia. *Austral Ecology*, 31 (5): 559-566.

Carter, M. V. (1998). Eutypa dieback. In: Compendium of Grape Diseases. R. C. Pearson and A. C. Goheen (Eds), (pp. 32-34). *American Phytopathological Society Press, Saint Paul, Minnesota, USA*.

Carter, M. V., & Price, T. V. (1974). Biological control of Eutypa armeniacae. 2 Studies of the interaction between E. armeniacae and Fusarium lateritium and their relative sensitivities to benzimidazole chemicals. *Australian Journal of Agricultural Research,* 25: 105–119.

Carter, M. V., Bolay, A., & Rappaz, F. (1983). An annotated host list and bibliography of Eutypa armeniacae. Station fédérale de recherches agronomiques de Changins. *Review of Plant Pathology,* 62: 251-258.

Castillo-Pando, M., Somers, A., Green, C. D., Priest, M., & Sriskanthades, M. (2001). Fungi associated with dieback of Semillon grapevines in the Hunter Valley of New South Wales. *Australasian Plant Pathology,* 30 (1): 59-63.

Cesati, V., & De Notaris, G. (*1863).* Schema di classificazione degli sferiacei italici aschigeri piu' o meno appartenenti al genere Sphaeria nell'antico significato attribuitoglide Persoon. *Erbario crittogamico italiana,* 4*:* 177-240.

Chamberlain, G. C.*,* Willison, R. S.*,* Townshend, D. J., & de Ronde, J. H. *(*1964). Two fungi associated with dead-arm disease of grapes. *Canadian Journal of Botany*, 42*:* 351–*355*.

Chapuis, L., Richard, L., & Dubos, B. (1998). Variation in susceptibility of grapevine pruning wound to infection by *Eutypa lata* in south-western France. *Plant Pathology*, 47 (4): 463-472.

Chen, W. Q., Morgan, D. P. Felts D., & Michailides, T. J. (2003). Antagonism of Paenibacillus lentimorbus to Botryosphaeria dothidea and biological control of panicle and shoot blight of pistachio. *Plant Disease,* 87: 359–365.

Connor, S. R. (1968). Canker formation on apple bark by Botryosphaeria ribis. Ph.D. thesis University of Delaware, Newark.157 pp.

Copes, W. E., & Hendrix, F. F. (2004). Effect of temperature on sporulation of Botryosphaeria dothidea, B. obtusa and B. rhodina. *Plant Disease*, 88 (3): 292-296.

Cristinzio, G. (1978). Gravi attacchi di Botryosphaeria obtusa su vite in provincia di Isernia. *Informatore fitopatologico*.

Crous, P. W., Groenewald, J. Z., Wingfield, M. J., & Phillips, A. J. L. (2007). Neofusicoccum mediterraneum. *Fungal Planet*, 19.

Crous, P. W., Slippers, B., Wingfield, M. J., Rheeder, J., Marasas, W. F., Phillips, A. J., & Groenewald, J. Z. (2006). Phylogenetic lineages in the Botryosphaeriaceae. *Studies in mycology*, 55: 235-253.

De Notaris, G. (1845). Micromycetes italici novi vel minus cogniti. Decas 4. *Memorie della Reale Accademia delle Scienze di Torino*, 7: 17–30.

Denman, S., Crous, P. W., Sadie, A., & Wingfield, M. J. (2004). Evaluation of fungicides for the control of Botryosphaeria protearum on Protea magnifica in the Western Cape Province of South Africa. *Australasian Plant Pathology*, 33 (1): 97-102.

Denman, S., Crous, P. W., Taylor, J. E., Kang, J-C., Pascoe, I., & Wingfield, M. J. (2000). An overview of the taxonomic history of Botryosphaeria, and a re-evaluation of its anamorphs based on morphology and ITS rDNA phylogeny. In: Molecules, morphology and classification: Towards monophyletic genera in the Ascomycetes. *Studies in Mycology* 45: 129–140.

Dhingra, O. D., & Sinclair, J. B. (1985). *Basic plant pathology methods*: CRC Press Inc., Boca Raton, Florida, USA.

Di Marco, S., Osti, F., & Cesari, A. (2004). Experiments on the control of esca by Trichoderma. *Phytopathologia Mediterranea*, 43 (1): 108-115.

Díaz, G. A., & Latorre, B. A. (2013). Efficacy of paste and liquid fungicide formulations to protect pruning wounds against pathogens associated with grapevine trunk diseases. *Crop Protection,* 46: 106-112.

Elena, G., Garcia-Figueres, F., Reigada, S., & Luque, J. (2015). Intraspecific variation in Diplodia seriata isolates occurring on grapevines in Spain. *Plant Pathology*, 64 (3): 680-689.

Elena, G., Sosnowski, M. R., Ayres, M. R., Lecomte, P., Benetreau, C., Garcia-Figueres, F., & Luque, J. (2015). Effect of the inoculum dose of three grapevine trunk pathogens on the infection of artificially inoculated pruning wounds. *Phytopathologia Mediterranea*, 54 (2): 345-354.

El-Goorani, M. A., & El-Meleigi, M. A. (1972). Dieback of grapevine by Botryodiplodia theobromae Pat. in Egypt. *Phytopathologia Mediterranea,* 11: 210-211.

Emmett, R. W., Nair, N. G., & Wicks, T. J. (1998). Phomopsis. In P. Nicholas, P. A. Magarey and M. Wachtel (Eds.), *Diseases and Pests'. Grape Production Series* (pp. 22-24). Winetitles, Adelaide.

Epstein, L., Kaur, S., & Vander-Gheynst, J. S. (2008). Botryosphaeria-related dieback and control investigated in non-coastal California grapevines. *California Agriculture*, 62 (4): 161-166.

Ernincik, O., & Madden, L. V. (2001). Effect of growth stage on susceptibility of grape berry and rachis tissues to infection by Phomopsis viticola. *Plant Diseases,* 85: 517-520.

Eskalen, A. Feliciano, A. J., & Gubler, W. D. (2007). Susceptibility of grapevine pruning wounds and symptoms development in response to infection by Phaeoacremonium aleophilum and Phaeomoniella chlamydospora. *Plant Diseases,* 91 (9): 1100-1104.

Eskalen, A., Faber, B., & Bianchi, M. (2013). Spore trapping and pathogenicity of fungi in the Botryosphaeriaceae and Diaporthaceae associated with avocado branch canker in California. *Plant Diseases*, 97: 329-332.

Farr, D. F., & Rossman, A. Y. (2011). Fungal Databases, Systematic Mycology and Microbiology Laboratory, ARS, USDA. Retrieved November 26, 2011.

Ferreira, J. H. S., Matthee, F. N., & Thomas, A. C. (1991). Biological control of Eutypa lata on grapevine by an antagonistic strain of Bacillus subtilis. Phytopathology, 81: 283–287.

Ferreira, P. M., Maia, H. L., & Monteiro, L. S. (1999). High yielding synthesis of heterocyclic β-substituted alanine derivatives. *Tetrahedron letters*, 40 (21): 4099-4102.

Flowers, E., Nuckles, E., Hartman, J., & Vaillancourt, L. (2001). Latent infection of Austrian and Scots pine tissues by Sphaeropsis sapinea. *Plant Disease,* 85: 1107–1112.

Fourie, P. H., & Halleen, F. (2004b). Proactive control of Petri disease of grapevine through treatment of propagation material. *Plant Disease*, 88 (11): 1241-1245.

Garcia, P. C., Rivero, R. M., López-Lefebre, L. R., Sánchez, E., Ruiz, J. M., & Romero, L. (2001). Direct action of the biocide carbendazim on phenolic metabolism in tobacco plants. *Journal of agricultural and food chemistry*, 49 (1): 131-137.

Gough, F. J., & Lee, T. S. (1985). Moisture effects on the discharge and survival of conidia of Septoria tritici. *Phytopathology*, 75 (2): 180-182.

Griffon, W. M., & Maublanc, A. (1909). Sur une maladie du cacaoyer. *Bulletin de la Society Mycologoque de France,* 25: 51-58.

Guarro, J., Gene, J., & Stchigel, A. M. (1999). Developments in fungal taxonomy. *Clinical Microbiology Review*, 12 (3): 454-500.

Gubler, W. D., Rolshausen P. E., Trouillase, F. P. Úrbez- Torres, J. R., & Voegel, T. (2005). Grapevine trunk diseases in California. Practical Winery and Vineyard Magazine, 6–25.

Haidar, R., Deschamps, A., Roudet, J., Calvo-Garrido, C., Bruez, E., Rey, P., & Fermaud, M. (2016). Multi-organ screening of efficient bacterial control agents against two major pathogens of grapevine. *Biological Control*, 92: 55-65.

Hartill, W. F. T., & Everett, K. R. (2002). Inoculum sources and infection pathways of pathogens causing stem-end rots of 'Hass' avocados. *New Zealand Journal of Crop and Horticultural Science,* 30: 249-260.

Helgason, T., Daniell, T. J., Husband, R., Fitter, A. H., & Young, J. P. W. (1998). Ploughing up the wood-wide web? *Nature,* 394:431.

Hewitt, W. B. (1994). Diplodia cane dieback and bunch rot. In: Compendium of Grape Diseases (pp. 25-26). *American Phytopathological Society Press, Saint Paul, Minnesota, USA.*

Hewitt, W. B. (1998). Berry rots and raisin molds. In: Compendium of Grape Diseases. R. C. Pearson and A. C. Goheen (Eds.), (pp. 26-27). *American Phytopathological Society Press*, *Saint Paul, Minnesota, USA.*

Holmes, J., & Rich, A. E. (1970). Factors affecting release and dissemination of Physalospora obtusa spores in New Hampsphire in apple orchard. *Phytopathology*, 60: 1052-1054.

Jackson, D., & Schuster, D. (1987). *Production of grapes & wine in cool climates*. Butterworths of New Zealand.

John, S., Wicks, T. J., Hunt, J. S., & Scott, E. S. (2008). Colonisation of grapevine wood by Trichoderma harzianum and Eutypa lata. *Australian journal of grape and wine research*, 14 (1): 18-24.

John, S., Wicks, T. J., Hunt, J. S., Lorimer, M. F., Oakey, H., & Scott, E. S. (2005). Protection of grapevine pruning wounds from infection by Eutypa lata using Trichoderma harzianum and Fusarium lateritium. Australasian Plant Pathology, 34: 569–575.

Johnson, G. I., Wells, I. A., & Cooke, A. W. (1992). Effect of brushing on mango quality. *Horticulture Postharvest Group Biennial Review*, 31.

Johnston, P. R., Cooper, J. A., Park, D., Southernland, P. W., & Wood. P. N. (2014). Sexual stage of grape powdery mildew (Erysiphe necator) newly recorded for New Zealand. (Abstract only).

Kaczmarek, J., Jędryczka, M., Fitt, B. D., Lucas, J. A., & Latunde-Dada, A. O. (2009). Analyses of air samples for ascospores ofLeptosphaeria maculans andL. biglobosa by light microscopy and molecular techniques. *Journal of Applied Genetics*, *50*(4), 411-419.

Kexiang, G., Xiaoguang, L., Runfang, G., Baojian, G., & tianbo, Z. (2002). Mycoparasitism of Trichoderma spp. on five plant pathogenic fungi. *Journal of Shandong Agricultural University,* 33:42.

Kexiang, G., Xiaoguang, L., Yonghong, L., Tianbo, Z., & Shuliang, W. (2002). Potential of Trichoderma harzianum and T. atroviride to control Botryosphaeria berengeriana f. sp. piricola*,* the cause of apple ring rot. *Journal of Phytopathology*, 150 (4): 271-276.

Kim, K. W., Park, E. W., & Ahn, K. K. (1999). Pre-penetration behavior of Botryosphaeria dothidea on apple fruits. *The Plant Pathology Journal*, 15 (4): 223-227.

Kotze, C., Van Niekerk, J., Halleen, F., Mostert, L., & Fourie, P. (2011). Evaluation of biocontrol agents for grapevine pruning wound protection against trunk pathogen infection. *Phytopathologia Mediterranea*, 50 (4): 247-263.

Kredics, L., Antal, Z., Manczinger, L., Szekeres, A., Kevei, F., & Nagy, E. (2003). Influence of environmental parameters on Trichoderma strains with biocontrol potential. *Food Technology and Biotechnology*, 41 (1): 37-42.

Kumeda, Y., & Asao, T. (1996). Single-strand conformation polymorphism analysis of PCR-amplified ribosomal DNA internal transcribed spacers to differentiate species of Aspergillus section Flavi. *Applied and Environmental Microbiology*, 62 (8): 2947-2952.

Kummuang, N., Smith, B. J., Diehl, S. V., & Graves Jr, C. H. (1996a). Muscadine grape berry rot diseases in Mississippi: disease identification and incidence. *Plant disease*, 80 (3): 238-243.

Kummuang N., Smith, B. J., Diehl, S. V., & Graves, Jr., C. H. (1996b). Muscadine grape berry rot diseases in Mississippi: Disease, epidemiology and crop reduction. *Plant Disease*, 80: 244–247.

Kuntzmann, P., Villaume, S., & Bertsch, C. (2009). Conidia dispersal of Diplodia species in a French vineyard. *Phytopathologia Mediterranea,* 48 (1): 150-154.

Larignon, P., & Dubos, B. (2001). The villainy of Black Dead Arm. *Wines & Vines,* 82: 86–89.

Larignon, P., Fulchic, R., Cere, L., & Dubos, B. (2001). Observations on black dead arm in French vineyards. *Phytopathologia Mediterranea*, 40: 336-342.

Latorre, B. A., Torres, R., Silva, T., & Elfar, K. (2013). Evaluation of the use of wound-protectant fungicides and biological control agents against stem canker (Neofusicoccum parvum) of blueberry. *Ciencia e Investigación Agraria*, 40 (3): 537-545.

Latorre, B. A., X. Besoaín, & Flores, V. (1986). Botryosphaeria canker of table grapes. *Phytopathology*, 76: 11-12.

Leavitt, G. (2003). The occurrence, distribution, effects and control of Botryodiplodia theobromae on Vitis vinifera in California, Arizona and Northern Mexico. *Phytopathologia Mediterranea,* 43:155 (abstract only).

Lehoczky, J. (1974). Black dead-arm disease of grapevine caused by Botryosphaeria stevensii infection. *Acta Phytopathologica Academiae Scientiarum Hungaricae*, 9 (3/4): 319-327.

Lehoczky, J. (1988). Black Dead Arm. In: Compendium of Grape Diseases. R. C. Pearson and A. C, Goheen (Eds.), (pp. 35). *American Phytopathological Society Press, Saint Paul, Minnesota, USA*.

Lo, C. T., Nelson, E. B., Hayes, C. K., & Harman, G. E. (1998). Ecological studies of transformed Trichoderma harzianum strain 1295-22 in the rhizosphere and on the phylloplane of creeping bentgrass. *Phytopathology*, 88 (2): 129-136.

Lorenzini, M., Cappello, M. S., & Zapparoli, G. (2015). Isolation of Neofusicoccum parvum from withered grapes: strain characterization, pathogenicity and its detrimental effects on passito wine aroma. *Journal of applied microbiology*, 119 (5): 1335-1344.

Luque, J., Martos, S., & Phillips, A. J. L. (2005). Botryosphaeria viticolas p. nov. on grapevines: a new species with a Dothiorella anamorph. *Mycologia,* 97 (5): 1111-1121.

Luque, J., Pera, J., & Parlade, J. (2008). Evaluation of fungicides for the control of Botryosphaeria corticola on cork oak in Catalonia (NE Spain). *Forest Pathology,* 38: 147-155.

Luttrell, E. S. (1950). Botryosphaeria stem canker of elm. *Plant Diseases Reporter*, 34: 138-139.

Ma, Z., Morgan, D. P., & Michailides, T. J. (2001). Effects of water stress on Botryosphaeria blight of pistachio caused by Botryosphaeria dothidea. *Plant Disease*, 85 (7): 745-749.

McCue, T., Carruthers, E., Dawe, J., Liu, S., Robar, A., & Johnson, K. (2008). Evaluation of generalized linear model assumptions using randomization. *Unpublished manuscript. Retrieved from http://www. mun. ca/biology/dschneider/b7932/B7932Final10Dec2008. pdf*.

Michailides T. J. (1991). Pathogenicity, distribution, source of inoculum and infection courts of Botryosphaeria dothidea on pistachio. *Phtopathology*, 81 (5): 566-573.

Michailides T. J., & Morgan, D. P. (1992). Effect of temperature and wetness duration on infection of pistachio by Botryosphaeria dothidea and management of disease by reducing duration of irrigation. *Phytopathology,* 82: 1399-1406.

Michailides, T. J. (1991). Susceptibility of pistachio male cultivars to botrytis blossom and shoot blight caused by Botrytis cinerea*. Plant Disease,* 75: 410-415.

Michailides T. J., & Morgan, D. P. (1993). Spore release of Botryosphaeria dothidea in Pistachio orchards and disease control by altering the trajectory angle of sprinklers. *Phytopathology,* 83: 145-152.

Michelmore, R. W., & Hulbert, S. H. (1987). Molecular markers for genetic analysis of phytopathogenic fungi. *Annual Review of Phytopathology*, 25 (1): 383-404.

Milholland, R. D. (1972). Histopathology and pathogenicity of Botryosphaeria dothidea on blueberry stems. *Phytopathology*, 62 (6): 654-660.

Milholland, R. D. (1988). Macrophoma Rot. In: Compendium of Grape Diseases. R. C. Pearson and A. C, Goheen (Eds.), (pp. 24). *American Phytopathological Society Press, Saint Paul, Minnesota, USA.*

Milholland, R. D. (1991). Muscadine grapes: some important diseases and their control. *Plant Disease,* 75: 113–117.

Moller, W. J., & Kasimatis, A. N. (1980). Protection of Grapevine pruning wounds from Eutypa dieback. *Plant Disease,* 64: 278-280.

Montagne, J. F. C. (1834). Notice sur les plantes cryptogames récemment découvertes en France contenant aussi l'indication précis des localités de quelques espéces les plus rares de la flore française. *Annales des Sciences Naturelles; Botanique*, 1: 295–307.

Morales, A., Bernardo, A., Latorre, Piontelli, E., & Besoain, X. (2012). Botryosphaeriaceae species affecting table grape vineyards in Chile and cultivar susceptibility. Ciencia e Investigación Agraria*,* 39 (3): 445-458.

Moyo, P. (2013). The role of arthropods in the dispersal of trunk disease pathogens associated with Petri disease and Esca.

Mungai, L., Graniti, A., & Surico, G. (1999). Esca (black Measles) and brown wood streaking: two old and elusive diseases of grapevines. *Plant Diseases,* 83: 404-407.

Munkvold, G. P., & Marois, J. J. (1995). Factors associated with variation in susceptibility of grapevine pruning wounds to infection by Eutypa lata. Phytopathology, 85: 249–256.

Mutawila, C., Fourie, P., Halleen, F., & Mostert, L. (2011). Grapevine cultivar variation to pruning wound protection by Trichoderma species against trunk pathogens. *Phytopathologia Mediterranea*, 50 (4): 264-276.

Navi, S. S., Bandyopadhyay, R., Reddy, R. K., Thakur, R. P., & Yang, X. B. (2005). Effects of wetness duration and grain development stages on sorghum grain mold infection. *Plant Disease*, 89 (8): 872-878.

New Zealand Winegrowers Annual Report. (2014). New Zealand.

New Zealand Winegrowers Annual Report. (2015). New Zealand.

New Zealand Winegrowers Fact sheet. (2013). New Zealand.

Ntahimpera, N., Driever, G. F., Felts, D., Morgan, D. P., & Michailides, T. J. (2002). Dynamics and pattern of latent infection caused by Botryosphaeria dothidea on pistachio buds. *Plant disease*, 86 (3): 282-287.

Paradela Filho, O., Ribeiro, I. J. A., & Kuniyuki, H. (1995). Podrido do tronco da videira (Vitis vinifera) causada por Dothiorella sp., forma anamórfica de Botryosphaeria dothidea. *Summa Phytopathologia*, 21: 40-42.

Parker, K. C., & Sutton, T. B. (1993). Susceptibility of apple fruit to Botryosphaeria dothidea and isolate variation. *Plant Disease,*77: 385-389.

Pascoe I. (1998). Trunk diseases of grapevines- perspectives from a tour of California. *The Australian Grape grower & Winemaker,* 417: 68–71.

Pavlic, D., Slippers, B., Coutinho, T. A., & Wingfield, M. J. (2009). Molecular and phenotypic characterization of three phylogenetic species discovered within the Neofusicoccum parvum/N. ribis complex. *Mycologia*, 101 (5): 636-647.

Pearson, R. C., & Goheen, A. C. (1988). Compendium of Grape Diseases. *American Phytopathological Society.*

Pennycook, S. R., & Samuels, G. J. (1985). Botryosphaeria and Fusicoccum species associated with ripe fruit rot of Actinidia deliciosa (kiwifruit) in New Zealand. *Mycotaxon*, 24: 445-458.

Petit, P., Lucas, E. M., Abreu, L. M., Pfenning, L. H., & Takahashi, J. A. (2009). Novel antimicrobial secondary metabolites from a Penicillium sp. isolated from Brazilian cerrado soil. *Electronic Journal of Biotechnology*, 12 (4): 8-9.

Petzoldt, C. H., Moller, W. J., & Sall, M. A. (1981). Eutypa dieback of grapevine: seasonal differences in infection and duration of susceptibility of pruning wounds. *Phytopathology,* 71: 540-543.

Phillips, A. J. L. (1998). Botryosphaeria dothidea and other fungi associated with excoriose and dieback of grapevines in Portugal. *Journal of Phytopathology*, 146 (7): 372-332.

Phillips, A. J. L. (2000). Excoriose, cane blight and related diseases of grapevine: a taxonomic review of the pathogens. *Phytopathology Mediterranea*, 39: 341-356.

Phillips, A. J. L. (2002). Botryosphaeria species associated with diseases of grapevines in Portugal. *Phytopathology Mediterranean,* 41: 3-18.

Phillips, A. J. L., Alves, A., Correia, A., & Luque, J. (2005). Two new species of Botryosphaeria with brown, 1-septate ascospores and Dothiorella anamorphs. *Mycologia,* 97: 513–529.

Phillips, A. J. L., Alves, A., Pennycook, S. R., Johnston, P. R., Ramaley, A., Akulov, A., & Crous, P. W. (2008). Resolving the phylogenetic and taxonomic status of dark-spored teleomorph genera in the Botryosphaeriaceae. *Persoonia-Molecular Phylogeny and Evolution of Fungi*, 21 (1): 29-55.

Phillips, A. J. L., Crous, P. W., & Alves, A. (2007). Diplodia seriata, the anamorph of "Botryosphaeria" obtusa. *Fungal Diversity*, 25: 141–155.

Phillips, A. J. L., Fonseca, F., Povoa, V., Castilho, R., & Nolasco, G. (2002). A reassessment of the anamorphic fungus Fusicoccum luteum and description of its teleomorph Botryosphaeria lutea sp. Nov. *Sydowia*, 54: 59-77.

Pitt, W. M., Huang, R., Steel, C. C., & Savocchia, S. (2010a). Identification, distribution and current taxonomy of Botryosphaeriaceae species associated with grapevine decline in New South Wales and South Australia. *Australian Journal of Grape and Wine Research,* 16: 258-271.

Pitt, W. M., Huang, R., Steel, C. C., & Savocchia, S. (2013). Pathogenicity and epidemiology of Botryosphaeriaceae species isolated from grapevines in Australia. *Australasian Plant Pathology*, 42 (5): 573-582.

Pitt, W. M., Sosnowski, M. R., Huang, R., Qiu, Y., Steel, C. C., & Savocchia, S. (2012). Evaluation of fungicides for the management of Botryosphaeria canker of grapevines. *Plant Diseases*, 96: 1303-1308.

Pitt, W. M., Sosnowski, M. R., Taylor, A., RuJuan, H., Quirk, L., Hackett, S., & Savocchia, S. (2010b). Management of Botryosphaeria canker of grapevines. *Australian Viticulture-Practical Vineyard Management*, 14 (2): 52-56.

Price, T. V., & Carter, M. V. (1975). Persistence of benomyl in pruned apricot sapwood. *Crop and Pasture Science*, 26 (3): 529-536.

Prusky, D., Fuchs, Y., & Yanko, U. (1983). Assessment of latent infections as a basis for control of postharvest disease of mango. *Plant disease*, 67 (7): 816-818.

Punithalingam, E. (1976). Botryodiplodia theobromae. *CMI Descriptions of Pathogenic Fungi and Bacteria,* 519: 1-2.

Pusey, P. L. (1989). Availability and dispersal of ascospores and conidia of Botryosphaeria in peach orchards. *Phytopathology,* 79: 635-639.

Pusey, P. L. (1993). Role of *Botryosphaeria* species in peach tree gummosis on the basis of differential isolation from outer and inner bark. *Plant Disease,* 77: 170–4.

Pusey, P. L., & Bertrand, P. F. (1993). Seasonal infection of non-wounded peach bark by Botryosphaeria dothidea. *Phytopathology*, 83 (8): 825-829.

Qiu, Y., Savocchia, S., Steel, C. C., & Ash, G. J. (2008). Botryosphaeria dothidea associated with grapevine trunk diseases in south-eastern Australia. *Australasian Plant Pathology,* 37: 482-485.

Ramos, D. E., Moller, W. J., & English, H. (1975). Production and dispersal of ascospores of Eutypa armeniacae in California. *Phytopathology*, 65 (12): 1364-1371.

Rasmus, K., & Rosendahl, S. (2000). Detection of arbuscular mycorrhizal fungi (Glomales) in roots by nested PCR and SSCP (Single Stranded Conformation Polymorphism) *Plant and Soil,* 226 (2): 189-196.

Ridgway, H. J., Amponsah, N. T., Brown, D. S., Baskarathevan, J., Jones, E. E., & Jaspers, M. V. (2011). Detection of botryosphaeriaceous species in environmental samples using a multi-species primer pair. *Plant Pathology,* 60 (6): 1118-1127.

Ridgway, H. J., Sleight, B. E., & Stewart, A. (2002). Molecular evidence for the presence of Phaeomoniella chlamydospora in New Zealand vineyards and its detection in grapevine wood using species specific PCR. *Australasian Plant Pathology*, 31: 267–271.

Ridgway, H. J., Steyaert, J. M., Pottinger, B. M., Carpenter, M., Nicol, D., & Stewart, A. (2005). Development of an isolate-specific marker for tracking Phaeomoniella chlamydospora infection in grapevines. *Mycologia*, 97 (5): 1093-1101.

Robin, C., Desprez-Loustan, M. L., & Delattour, C. (1992). Spatial and temporal enlargement of trunk cankers of Phytophthora cinnamomi in red oak. *Canadian Journal Forestry Research,* 22: 362-366.

Rolshausen, P. E., & Gubler, W. D. (2005). Use of boron for the control of Eutypa dieback of grapevines. *Plant Diseases,* 89: 734-738.

Saldanha, R. L., Garcia, J. E., Dekker, R. F., Vilas-Boas, L. A., & Barbosa, A. M. (2007). Genetic diversity among Botryosphaeria isolates and their correlation with cell wall-lytic enzyme production. *Brazilian Journal of Microbiology*, 38 (2): 259-264.

Sammonds, J., Jaspers, M. V., & Jones, E. E. (2016). Pre-infection processes of *Botryosphaeria* spp. Conidia: adhesion to different substrata. *Plant Pathology* In Press.

Savocchia, S., Laurent, E. N., Stodart, B. J., & Steel, C. (2005). Botryosphaeria canker and sensitivity to fungicides in vitro. Paper presented at the 43rd annual Congress of the South African. *Society for Plant Pathology*, South Africa.

Savocchia, S., Steel, C. C., Stodart, B. J., & Somers, A. (2007). Pathogenicity of Botryosphaeria species isolated from declining grapevines in sub-tropical regions of Eastern Australia. *Vitis Journal for Grapevine Research,* 46: 27-32.

Schmidt, C. S., Lorenz, D. Wolf, G. A., & Jager, J. (2001). Biological control of grapevine dieback fungus Eutypa lata II: Influence of formulation additives and transposon mutagenesis on the antagonistic activity of Bacillus subtilis and Erwinia herbicola. Phytopathology, 149: 437–445.

Serra, S., Mannoni, M. A., & Ligios, V. (2008). Studies on the susceptibility of pruning wounds to infection by fungi involved in grapevine wood diseases in Italy. Phytopathologia Mediterranea, 47: 234–246.

Shoemaker, R. A. (1964). Conidial stages of some Botryosphaeria species on Vitis and Quercus. *Canadian Journal of Botany,* 42: 1298-1301.

Siebert, J. B. (2001). Eutypa: the economic toll on vineyards. *Wines Vines April*, 50: 56.

Slippers, B., Fourie, G., Crous, P. W., Coutinho, T. A., Wingfield, B. D., & Wingfield, M. J. (2004). Multiple gene sequences delimit Botryosphaeria australis sp. nov. from B. lutea. *Mycologia*, 96 (5): 1030-1041.

Slippers, B., Fourie, G., Crous, P. W., Coutinho, T. A., Wingfield, B. D., Carnegie, A. J., & Wingfield, M. J. (2004). Speciation and distribution of Botryosphaeria spp. on native and introduced Eucalyptus trees in Australia and South Africa. *Studies in Mycology,* 50: 343–358.

Smith, B., & Hendrix, F. F. (1984). Primary infection of apple buds by Botryosphaeria obtusa. *Plant disease*, 68 (8): 707-709.

Smith, D., & Stanosz, G. (2001). Molecular and morphological differentiation of Botryosphaeria dothidea (anamorph Fusicoccum aesculi) from some other fungi with Fusicoccum anamorphs. *Mycologia*, 93: 505-515.

Smith, H., Crous, P. W., Wingfield, M. J., Coutinho, T. A., & Wingfield, B. D. (2001). Botryosphaeria eucalyptorum sp. nov., a New Species in the *B. Dothidea*-Complex on Eucalyptus in South Africa. *Mycologia,* 93 (2): 277-285.

Smith, H., Kemp, G. H. K., & Wingfield, M. J. (1994). Canker and die-back of Eucalyptus in South Africa caused by Botryosphaeria dothidea. *Plant Patholology,* 43: 1031–1034.

Smith, H., Wingfield, M. J., Crous, P. W., & Coutinho, T. A. (1996). Sphaeropsis sapinea and Botryosphaeria dothidea endophytic in Pinus spp. and Eucalyptus spp. in South Africa. *South African Journal of Botany,* 62: 86-88.

Sosnowski, M. R., Creaser, M. L., Wicks, T. J., Lardner, R., & Scott, E. S. (2008). Protection of grapevine pruning wounds from infection by Eutypa lata. *Australian Journal of Grape Wine Research,* 14: 134-142.

Sosnowski, M. R., Shtienberg, D., Creaser, M. L., Wicks, T. J., Lardner, R., & Scott, E. S. (2007). The influence of climate on foliar symptoms of Eutypa dieback in grapevines. *Phytopathology*, 97: 1284-1289.

Sosnowski, M., Loschiavo, A., Wicks, T., & Scott, E. (2009). Managing eutypa dieback in grapevines. *The Australian and New Zealand Grape grower and Winemaker Annual Technical Issue*, 13-16.

Sosnowski, M., Loschiavo, A., Wicks, T., & Scott, E. (2010). Final Report to Grape and Wine Research & Development Corporation Sustaining grapevines infected with eutypa dieback.

Spagnolo, A., Marchi, G., Peduto, F., Phillips, A. J. L., & Surico, G. (2011). Detection of Botryosphaeriaceae species within grapevine woody tissues by nested PCR, with

particular emphasis on the Neofusicoccum parvum/N. ribis complex. *European Journal of Plant Pathology,* 129 (3): 485-500.

Srivastava, P., Andersen, P. C., Marois, J. J., Wright, D. L., Srivastava, M., & Harmon, P. F. (2013). Effect of phenolic compounds on growth and ligninolytic enzyme production in Botryosphaeria isolates. *Crop Protection*, 43: 146-156.

Steel, C. C., Greer, L. A., & Savocchia, S. (2007). Studies on Colletotrichum acutatum and Greeneria uvicola: Two fungi associated with bunch rot of grapes in sub-tropical Australia. *Australian Journal of Grape and Wine Research*, 13 (1): 23-29.

Sun, Q., Rost, T. L., & Matthews, M. A. (2006). Pruning-induced tylose development in stems of current-year shoots of Vitis vinifera (Vitaceae). *American Journal of Botany*, 93 (11): 1567-1576.

Sutton, T. B. (1981). Production and dispersal of ascospores and conidia by Physalospora obtusa and Botryosphaeria dothidea in apple orchards. *Phytopathology,* 71: 584-589.

Sutton, T. B. (1991). White Rot. In: Compendium of Apple and Pear Diseases. A. L. Jones and H. Aldwinckle, (Eds.), (pp. 16-18). *American Phytopathological Society Press, Saint Paul, Minnesota, USA.*

Sutton, T. B., & Arauz, L. F. (1991). Influence of temperature and moisture on germination of ascospores and conidia of Botryosphaeria dothidea. *Plant Disease*, 75: 1146-1149.

Tabacchi, R., Fkyerat, A., Poliart, C., & Dubin, G. M. (2000). Phytotoxins from fungi of esca of grapevine. *Phytopathologia Mediterranea*, 34: 156-161.

Taylor, A., Hardy, G. S. J., Wood, P., & Burgess, T. (2005). Identification and pathogenicity of Botryosphaeria species associated with grapevine decline in Western Australia. *Australasian Plant Pathology*, 34 (2): 187-195.

Toh, R., Galea, V. J., Diplock, N., & van Klinken, R. D. (2008). Evaluation of fungal isolates for potential use as mycoherbicides for seed bank reduction of Parkinsonia aculeata. In *Proceedings of the 16th Australian Weeds Conference, eds RD van Klinken, VA Osten, FD Panetta and JC Scanlan* (pp. 284-6).

Tort, N., & Turkyilmaz, B. (2003). Physiological effects of captan fungicide on pepper (Capsicum annuum L.) plant. *Pakistan Journal of Biological Sciences*, 6 (24): 2026-2029.

Trapero-Casas, A., & Kaiser, W. J. (1992). Influence of temperature, wetness period, plant age, and inoculum concentration on infection and development of Ascochyta blight of chickpea. *Phytopathology*, 82 (5): 589-596.

Trese, A. T., Burton, C. L., & Ramsdell, D. C. (1980). Eutypa armeniacae in Michigan vineyards: ascospore production and survival, host infection, and fungal growth at low temperatures. *Phytopathology,* 70: 788-793.

Trouillas, F. P., Urbez-Torres, J. R., & Gubler, W. D. (2010). Diversity of diatrypaceous fungi associated with grapevine canker diseases in California. *Mycologia,* 102: 319-336.

Urbez-Torres, J. R. (2011). The status of Botryosphaeriaceae species infecting grapevines. *Phytopathologia Mediterranea*, 50 (4): 5-45.

Úrbez-Torres, J. R., & Gubler, W. D. (2009). Pathogenicity of Botryosphaeriaceae species isolated from grapevine cankers in California. *Plant Disease,* 93: 584–592.

Úrbez-Torres, J. R., & Gubler, W. D. (2008). Double pruning, a potential method to control Bot canker disease of grapes, and susceptibility of grapevine pruning wounds to infection by Botryosphaeriaceae. *Phytophathology Mediterranean,* 48: 185 (Abstract only).

Úrbez-Torres, J. R., & Gubler, W. D. (2011). Susceptibility of grapevine pruning wounds to infection by Lasiodiplodia theobromae and Neofusicoccum parvum. *Plant Pathology*, 60 (2): 261-270.

Úrbez-Torres, J. R., Battany, M., Bettiga, L. J., Gispert, C., McGourty, G., Roncoroni, J., Smith, R. J., Verdegaal, P., & Gubler, W. D. (2010b). Botryosphaeriaceae species spore-trapping studies in Californian vineyards. *Plant Disease,* 94 (6): 717-724.

Úrbez-Torres, J. R., Bruez, E., Hurtado, J., & Gubler, W. D. (2010). Effect of temperature on conidial germination of Botryosphaeriaceae species infecting grapevines. *Plant Disease*, 94 (12): 1476-1484.

Úrbez-Torres, J. R., Leavitt, G. M., Voegel, T. M., & Gubler, W. D. (2006a). Identification and distribution of Botryosphaeria spp. associated with grapevine cankers in California. *Plant Disease,* 90: 1490–1503.

Urbez-Torres, J. R., Peduto, F., Striegler, R. K., Urrea-Romero, K. E., Rupe, J. C., Cartwright, R. D., & Gubler, W. D. (2012). Characterization of fungal pathogens

associated with grapevine trunk diseases in Arkansas and Missouri. *Fungal diversity*, 52 (1): 169-189.

Úrbez-Torres, J. R., Peduto, F., Smith, R. J., and Gubler, W. D. (2013). Phomopsis dieback: A grapevine trunk disease caused by Phomopsis viticola in California. *Plant Disease*, 97: 1571-1579

Uys, D. C. (1981). *Utilisation of nutrient reserves in the vegetative propagation of the grapevine* (Doctoral dissertation, University of Stellenbosch).

Valencia, D., Torres, C., Camps, R., López, E., Celis-Diez, J. L., & Besoain, X. (2015). Dissemination of Botryosphaeriaceae conidia in vineyards in the semiarid Mediterranean climate of the Valparaíso Region of Chile. *Phytopathologia Mediterranea,* 54 (2): 394−402.

van Niekerk J. M., Calitz, F. J., Halleen, F., & Fourie, P. H. (2010a). Temporal spore dispersal patterns of grapevine trunk pathogens in South Africa. European Journal of Plant Pathology, 127: 375-390.

van Niekerk J. M., Fourie, P. H., Halleen, F., & Crous, P. W. (2006). Botryosphaeria spp. as grapevine trunk disease pathogens. *Phytopathologia Mediterranea,* 45: S43–S54.

van Niekerk, J. M., Crous, P. W., Groenewald, J. Z., Fourie, P. H., & Halleen, F. (2004). DNA phylogeny, morphology and pathogenicity of Botryosphaeria species on grapevines. *Mycologia,* 96 (4): 781-798.

van Niekerk, J. M., Groenewald, J. Z., Farr, D. F., Fourie, P. H., Halleer, F., & Crous, P. W. (2005). Reassessment of Phomopsis species on grapevines. *Australasian Plant Pathology*, 34 (1): 27-39.

Viala, P., & Ravaz, L. (1892). Sur la dénomination botanique (Guignardia bidwellii) du black-rot Bulletin de la Société. *Mycologique de France*, 8: 63.

Vilgalys, R., & Hester, H. (1990). Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several Cryptococcus species. *Journal of Bacteriology*, 172: 4238–4246.

Weaver, D. J. (1979). Role of conidia of Botryosphaeria dothidea in the natural spread of peach tree gummosis. *Phytopathology,* 69: 330-334.

Williamson, B., Cooke, D. E. L., Duncan, J. M., Leifert, C., Breese, W. A., & Shattock, R. C. (1998). Fungal infections of micropropagated plants at weaning: a problem exemplified by downy mildews in Rubus and Rosa. *Plant cell, tissue and organ culture*, 52 (1): 89-96.

Wood, P. M., & Wood, C. E. (2005). Cane dieback of Dawn Seedless table grapevines (Vitis vinifera) in Western Australia caused by Botryosphaeria rhodina. *Australasian Plant Pathology,* 34 (3) : 393–395.

Wunderlich, N., Ash, G. J., Steel, C. C., Raman, H., & Savocchia, S. (2015). Association of Botryosphaeriaceae grapevine trunk disease fungi with the reproductive structures of Vitis vinifera. *VITIS-Journal of Grapevine Research*, 50 (2): 89.

Wunderlich, N., Ash, G. J., Steel, C. C., Raman, H., Cowling, A., & Savocchia, S. (2011). Refining the biological factors affecting virulence of Botryosphaeriaceae on grapevines. *Annals of Applied Biology*, 159 (3): 467-477.

Xu, X. M., & Ridout, M. S. (1998). The effects of inoculum dose, duration of wet period, temperature and wound age on infection by Nectria galligena of pruning wounds on apple. *European journal of plant pathology*, 104 (5): 511-519.

Yan, J. Y., Xie, Y., Zhang, W., Wang, Y., Liu, J. K., Hyde, K. D., Seem, R. C., Zhang, G. Z., Yao, S. W., Bai, X. J., Dissanayake, A. J., Peng, Y. L., Li, X. H. (2013). Species of Botryosphaeriaceae involved in grapevine dieback in China. *Fungal Diversity,* 61: 221–236.

Personal communications

Mr. Andrew Naylor (2014) Pernod-Ricard, Marlborough, New Zealand.

Publications and conference presentations

Shafi, A., Jaspers, M. V., Ridgway H. J., Jones E. E. (2015). Splash dispersal of *Botryosphaeriaceae* species in Marlborough vineyards. New Zealand Plant Protection Conference11-13th August, Christchurch., New Zealand.

Shafi, A., Jaspers, M. V., Ridgway H. J., Jones E. E. (2015). Variation between isolates of *Neofusicoccum* species in pathogenicity and sporulation on grapevine shoots. Australasian Plant Pathology Conference, 16-18th Sept, Fremantle, Australia.

Appendix A - Recipes

A.1 Potting mix composition (8-9 months)

Potting mix

60% Horticultural bark,

30% Premier bark chips,

10% Screened peat,

Fertilisers were added to the mix, with the following amounts per m^3:

2 Kg Basacote® plus 6 M (Compo),

4 Kg Nutricote® 8/9month 13N (Yates),

1 Kg Penetraide®, (Hort-fert)

2.5 KgDolomite,

1 Kg, Agricultural Lime

1.5 Kg Gypsum (screened)

0.5 Kg Magri-Trace granular (Hort-fert)

1 Kg Tribon® (Hort-fert)

A.2 All recipes

A.2.1 loading dye

40% (w/v) Sucrose (Sigma Chemical Co., USA)

0.25% (w/v) Bromophenol blue

0.25% (w/v) Xylene cyanol

Preparation: All ingredients were added to 8 mL of sterile nanopure water and mixed on a magnetic stirrer until dissolved. The final volume was made up to 10 mL, mixed, dispensed as 1 mL aliquots and store at -20°C.

A.2.2 TAE

242g Tris (invitrogen life technologies, CA, USA)

57.1 mL Glacial acetic acid

100 mL 0.5 M Ethylenediaminetetra-acetic acid (pH 8.0)

Preparation: In 1 L bottle all ingredients were added with 800 mL of ROW, dissolved on magnetic stirrer, the final volume was made up to 1000 mL, autoclaved for 15 min at 121°C and 15 Psi. Prior to use the resulting 10x stock was diluted to 1x.

A.2.3 CTAB buffer

2 mL of 1 M Tris (pH 8)

4 mL of 0.5 M EDTA

16 mL of 5 M NaCl

2 g of CTAB

Millipore water added to make up to a total volume of 100 mL.

A.2.4 MDE Gel Mix

12.5 ml of 2xMDE

8.0 ml of 5xTBE

19.5 ml of Millipore water

Total of 40 mL per gel

To the above mix, add the catalysts:

+ 30 µL TEMED (N,N,N',N'-tetra-methyl-ethylenediamine)

+280 µL 10% w/v APS (ammonium persulfate) - 0.05 g/0.5 ml

A.2.5 5x TBE (Tris-borate)

54 g Tris Base (J.C Baker)

27.5 g Boric Acid

20 mL of 0.5M EDTA pH8

Millipore water to make up to 1 litre

Appendix B – Analyses for Chapter 2

B. 1 Analysis of variance (ANOVA) for detached grapevine shoots of Sauvignon blanc

B.1.1 Lesion lengths on soft green shoots for blocks and species

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Block	5	40.364	8.073	7.24	<0.001
Species	1	1.937	1.937	1.74	0.192
Total	83	128.215			

B.1.2 lesion lengths on semi-hard shoots for blocks and species

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Block	5	128.079	25.616	14.36	<0.001
Species	1	3.984	3.984	2.23	0.139
Total	83	269.426			

B.1.3 Lesion lengths on soft green shoots for blocks and isolates

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Block	5	1.823	0.364	7.76	<0.001
Isolates	13	12.784	0.983	20.92	<0.001
Total	83	17.662			

B.1.4 Conidial numbers from soft green shoots for blocks and isolates

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Block	5	1.823	0.36468	7.76	<0.001
Isolates	13	12.784	0.938335	20.92	<0.001
Species	2	1.249	0.6244	3.08	0.051
Total	83	17.662			

B.1.5 Lesion lengths on semi-hard shoots for blocks and isolates

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Block	5	128.08	25.616	22.38	<0.001
Isolates	13	66.94	5.149	4.50	<0.001
Total	83	269.426			

B.1.6 Conidial numbers from semi-hard shoots for blocks, species and isolates

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Block	5	2.512	0.502	3.05	0.370
Isolates	13	15.804	1.215	21.89	<0.001
Species	2	0.6300	0.315	1.27	0.285
Total	83	21.926			

B. 2 ANOVA for attached grapevine shoots

B.2.1 Lesion lengths on attached soft green shoots for isolates

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Isolates	7	3.240	0.462	6.29	<0.001
Error	40	2.945	0.073		
Total	47	6.185			

B.2.2 Conidial numbers from soft green shoots for isolates

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Vale
Isolates	7	7.125	1.017	78.81	<0.001
Error	40	0.516	0.012		
Total	47	7.642			

B.2.3 Lesion lengths on soft green shoots for grapevine varieties and species

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Variety	1	0.001	0.001	0.03	0.859
Species	1	0.206	0.206	4.98	0.032
Variety*Species	1	0.035	0.035	0.86	0.361
Total	39	1.737			

B.2.4 Conidial numbers from soft green shoots for grapevine varieties and species

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Variety	1	0.712	0.712	7.89	0.008
Species	1	0.275	0.275	30.48	<0.001
Variety*Species	1	0.006	0.006	0.67	0.418
Total	39	0.667			

B.2.5 Lesion lengths on semi-hard shoots for grapevine varieties and species

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Variety	1	0.065	0.065	6.42	0.016
Species	1	0.026	0.026	2.62	0.114
Variety*Species	1	0.008	0.008	0.80	0.377
Total	39	0.469			

B.2.6 Conidial numbers from semi-hard shoots for grapevine varieties and species

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Variety	1	0.003	0.003	0.28	0.600
Species	1	0.213	0.213	17.51	<0.001
Variety*Species	1	0.004	0.004	0.37	0.548
Total	39	0.660			

B.2.7 Lesion lengths on trunks for grapevine variety and species

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Variety	1	0.176	0.176	4.27	0.046
Species	1	1.414	1.414	34.27	<0.001
Variety*Species	1	0.000	0.000	0.00	0.963
Total	39	3.075			

B.2.8 Lesion lengths on semi-hard shoots for grapevine varieties and species

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Variety	1	5.091	5.091	11.51	<0.001
Species	1	18.695	18.695	42.27	<0.001
Variety*Species	1	1.013	1.013	2.29	0.136
Total	63				

B.2.9 Conidial numbers from semi-hard shoots for grapevine varieties and species

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Variety	1	4.532	4.532	123.19	<0.001
Species	1	0.023	0.023	0.64	0.428
Variety*Species	1	0.001	0.001	0.03	0.863
Total	63	6.76			

B.2.10 Lesion lengths on grapevine canes for varieties and species

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Variety	1	0.165	0.165	5.96	0.021
Species	1	0.877	0.877	31.66	<0.001
Variety*Species	1	0.037	0.037	1.36	0.253
Total	31				

B.3 ANOVA of numbers of conidia produced from grapevine shoots for blocks, species, isolates, temperatures and relative humidities

B.3.1 Conidial numbers from detached semi-hard shoots

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Block	5	0.868	0.173	3.35	0.006
Isolates	5	9.659	1.931	37.32	<0.001
Temperature	3	29.90	9.968	192.55	<0.001
RH	2	13.63	6.816	131.67	<0.001
Block*Isolates	25	1.81	0.072	1.40	0.099
Block*Temperature	15	1.61	0.107	2.08	0.011
Block*RH	10	0.65	0.065	1.26	0.250
Isolates*Temperature	15	1.62	0.108	2.10	0.010
Isolates*RH	10	1.48	0.148	2.86	0.002
Temperature*RH	6	0.67	0.112	2.17	0.046
Isolates*Temperature*RH	30	2.15	0.071	1.45	0.006
Species*Temperature	3	2.5311	8.43721	4.55	0.004
Species*RH	2	2.22311	1.11155	6.00	0.003
Species*Temperature*RH	6	1.661	2.76900	1.49	0.179
Total	414	78.505			

B.3.2 Conidial numbers from attached semi-hard shoots

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Isolates	5	3.207	0.654	123.68	<0.001
Species	1	12.46	12.46	0.84	0.362
Temperature	1	0.30	0.304	57.51	<0.001
RH	1	0.809	0.809	153.06	<0.001
Isolates*Temperature	5	0.073	0.014	2.80	0.020
Isolates*RH	5	0.045	0.009	1.71	0.137
Temperature*RH	1	0.025	0.025	4.76	0.031
Isolates*Temperature*RH	5	0.064	0.012	2.42	0.039
Total	143	5.22	0.005		

Appendix C – Analyses for Chapter 3

C.2 ANOVA of conidial numbers in rain water samples for vineyards, grapevine varieties and months in 2015

C.2.1 *Neofusicoccum* spp. conidia in rain water samples

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Months	3	0.10747	0.03582	14.36	0.004
Vineyard	2	0.02035	0.01017	4.08	0.076
Variety	1	0.01839	0.01839	7.37	0.035
Months*Vineyard	6	0.022404	0.003734	1.50	0.318
Months*Variety	3	0.008434	0.002811	1.13	0.410
Vineyard*Variety	2	0.159055	0.079528	31.87	<0.001
Error	6	0.014972	0.002495		
Total	23	0.35108			

C.2.2 *Diplodia* spp. conidia in rain water samples

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Months	3	0.057915	0.019305	4.85	0.048
Vineyards	2	0.158644	0.079322	19.94	0.002
Variety	1	0.000219	0.000219	0.06	0.822
Months*Vineyard	6	0.112224	0.018704	4.70	0.041
Months*Variety	3	0.006196	0.002065	0.52	0.684
Vineyard*Variety	2	0.33918	0.016959	4.26	0.070
Error	6	0.023863	0.003977		
Total	23	0.392981			

Appendix D – Analyses for Chapter 4

D.1 GLM analyses of logit incidences for different wound ages and species

D.1.1 Green shoots

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Wound age	29.94	6	4.99	65.0	<0.001
Species	0.40	1	0.40	65.0	0.531
Wound age*Species	2.05	6	0.34	65.0	0.912

D.1.2 Wood of trunks

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Wound age	36.61	6	6.10	65.0	<0.001
Species	0.00	1	0.00	65.0	1.000
Wound age*Species	0.00	6	0.00	65.0	1.000

D.1.3 Bark of trunks

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Wound age	33.06	6	5.51	65.0	<0.001
Species	0.64	1	0.64	65.0	0.425
Wound age*Species	0.91	6	0.15	65.0	0.998

D.2 ANOVA of colonisation distances for wound age and species

D.2.1 Green shoots

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Wound age	6	50.2762	8.3794	52.49	<0.001
Species	1	0.3344	0.3344	2.09	0.153
Wound age*Species	6	0.8814	0.1469	0.92	0.486
Error	65	10.3758	0.1596		
Total	83	62.9470			

D.2.2 Wood of trunks

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Wound age	6	11.8095	1.96825	17.00	<0.001
Species	1	0.0000	0.00000	0.00	1.000
Wound age*Species	6	0.6667	0.11111	0.96	0.459
Error	65	7.5238	0.11575		
Total	83	20.8095			

D.2.3 Bark of trunks

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Wound age	6	72.8424	12.1404	40.47	< 0.001
Species	1	0.4876	0.4876	1.63	0.207
Wound age*Species	6	0.4157	0.0693	0.23	0.965
Error	65	19.4986	0.3000		
Total	83	94.5657			

D.3 ANOVA of infection incidences in grapevine tissues for wound ages and seasons

D.3.1 Green shoots

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Season	3	1.3333	0.4444	3.51	0.018
Wound age	4	12.3000	3.0750	24.28	<0.001
Season*Wound age	12	0.8333	0.0694	0.55	0.877
Residual	95	12.0333	0.1267		
Total	119	28.8000			

D.3.2 Trunks

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Season	3	1.2250	0.4083	3.28	0.024
Wound age	4	15.1333	3.7833	30.39	<0.001
Season*Wound age	12	1.0667	0.0889	0.71	0.734
Residual	95	11.8250	0.1245		
Total	119	29.9250			

D.4 ANOVA of colonisation distances in grapevine tissues for wound ages and seasons

D.4.1 Green shoots

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Seasons	3	26.48	8.8276	11.18	<0.001
Wound age	4	138.26	34.5643	43.79	<0.001
Seasons*Wound age	12	13.36	1.1135	1.41	0.173
Error	100	78.93	0.7893		
Total	119	257.03			

D.4.2 Trunks

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Seasons	3	9.210	3.0700	9.19	<0.001
Wound age	4	67.689	16.9223	50.66	<0.001
Seasons*Wound age	12	3.884	0.3237	0.97	0.173
Error	100	33.403	0.3340		
Total	119	114.187			

D.5 GLM analyses of logit incidences for wound ages and inoculum concentrations

D.5.1 Green shoots

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Wound age	29.63	3	9.88	95.0	<0.001
Inoculum conc.	1.80	4	0.45	95.0	0.772
Wound age*Inoculum conc.	6.46	12	0.54	95.0	0.885

D.5.2 Trunks

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Wound age	25.72	3	8.57	95.0	<0.001
Inoculum conc.	8.21	4	2.05	95.0	0.093
Wound age*Inoculum conc.	5.59	12	0.47	95.0	0.930

D.6 ANOVA of colonised distances in tissues for tissues and inoculum concentrations

D.6.1 Green shoots

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Inoculum conc.	4	6.105	1.5263	3.06	0.020
Wound age	3	89.292	29.7639	59.74	<0.001
Inoculum conc.*Wound age	12	9.535	0.7946	1.59	0.105
Error	100	49.820	0.4982		
Total	119	154.752			

D.6.2 Trunks

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Inoculum conc.	4	11.249	2.8122	7.10	<0.001
Wound age	3	78.051	26.0169	65.72	<0.001
Inoculum conc.*Wound age	12	9.738	0.8115	2.05	0.027
Error	100	39.588	0.3959		
Total	119	138.625			

D.7 GLM analyses of logit incidences after saprophytic infection of bark, for infection positions/treatments, species and grapevine varieties

D.7.1 Canes

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Species	0.40	1	0.40	55.0	0.529
Treatment	6.22	2	3.11	55.0	0.050
Variety	0.05	1	0.05	55.0	0.826
Species*Treatment	0.31	2	0.16	55.0	0.856
Species*Variety	0.04	1	0.04	55.0	0.834
Treatment*Variety	0.07	2	0.03	55.0	0.966
Species*Treatment*Variety	0.06	2	0.03	55.0	0.969

D.7.2 Wood of trunk

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Species	0.03	1	0.03	54.8	0.871
Treatment	10.35	2	5.18	54.9	0.009
Variety	0.07	1	0.07	54.8	0.791
Species*Treatment	4.56	2	2.28	54.8	0.112
Species*Variety	0.07	1	0.07	54.8	0.788
Treatment*Variety	0.07	2	0.03	54.8	0.966
Species*Treatment*Variety	0.07	2	0.03	54.8	0.968

D.7.3 Bark of trunk

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Species	0.00	1	0.00	54.8	0.946
Treatment	6.61	2	3.31	54.8	0.044
Variety	0.83	1	0.83	54.8	0.365
Species*Treatment	1.00	2	0.50	54.8	0.611
Species*Variety	0.23	1	0.23	54.8	0.636
Treatment*Variety	0.78	2	0.39	54.8	0.680
Species*Treatment*Variety	0.23	2	0.12	54.8	0.889

D.8 GLM analyses of logit incidences after saprophytic infection of Pinot noir bark for species and infection positions/treatments

D.8.1 Wood of trunk

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Species	0.67	1	0.67	24.9	0.422
Treatment	7.28	2	3.64	24.9	0.041
Species*Treatment	0.57	2	0.29	24.9	0.753

D.8.2 Bark of trunk

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Species	1.03	1	1.03	30.0	0.318
Treatment	8.62	2	4.31	30.0	0.023
Species*Treatment	0.22	2	0.11	30.0	0.896

D.9 ANOVA of infection incidences after inoculation next to lenticels for segment positions, tissue types, species and grapevine varieties

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Variety	1	0.4885	0.48846	2.61	0.111
Species	1	0.0561	0.05612	0.30	0.586
Tissue types	2	1.9237	0.96184	5.13	0.008
Segments types	2	3.6404	1.82020	9.71	<0.001
Variety*Species	1	0.0561	0.05612	0.30	0.586
Variety*Tissue types	2	0.0267	0.01337	0.07	0.931
Error	70	13.1162	0.18737		
Total	107	28.6644			

D.10 ANOVA of infection incidences in leaf buds for inoculation at different stages of development for species and grapevine varieties

D.10.1 All wounded bud stages together

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	59.85	59.853	6.59	0.037
Variety	1	133.33	133.333	14.68	0.006
Stages	2	286.78	143.391	15.78	0.003
Error	17	63.60	9.085		
Total	11	543.57			

D.10.2 Dormant wounded buds

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	0.03258	0.03258	1.51	0.238
Variety	1	0.03258	0.03258	1.51	0.238
Species*Variety	1	0.02944	0.02944	1.36	0.261
Error	15	0.32376	0.02158		
Total	23	0.56425			

D.10.3 Woolly wounded buds

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	0.034210	0.034210	1.95	0.182
Variety	1	0.008145	0.008145	0.47	0.506
Species*Variety	1	0.008145	0.008145	0.47	0.506
Error	15	0.262630	0.017509		
Total	23	0.381611			

D.10.4 Green wounded buds

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	0.05600	0.05600	0.50	0.491
Variety	1	0.10979	0.10979	0.98	0.339
Species*Variety	1	0.02275	0.02275	0.20	0.659
Error	15	1.68866	0.11258		
Total	23	1.94145			

D.10.5 Non-wounded green buds

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	0.008145	0.008145	0.37	0.552
Variety	1	0.000020	0.000020	0.00	0.976
Species*Variety	1	0.000020	0.000020	0.00	0.976
Error	15	0.329575	0.021972		
Total	23	0.562878			

D.11 ANOVA of colonised distances in green shoots and canes for buds infected at different stages, species and grapevine varieties

D.11.1 All bud stages together progression into green shoots

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	0.90750	0.907500	162.19	<0.001
Variety	1	0.30083	0.300833	53.77	<0.001
Stages	2	0.81500	0.407500	72.83	<0.001
Error	7	0.03917	0.005595		
Total	11	2.06250			

D.11.2 All bud stages together progression into canes

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	0.96333	0.963333	238.00	<0.001
Variety	1	0.33333	0.333333	82.35	<0.001
Stages	2	0.87167	0.435833	107.68	<0.001
Error	7	0.02833	0.004048		
Total	11	2.19667			

D.11.3 Dormant buds progression into green shoots

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	2.92053	2.92053	26.63	<0.001
Variety	1	0.91853	0.91853	8.38	0.006
Species*Variety	1	0.01920	0.01920	0.18	0.678
Error	44	4.82533	0.10967		
Total	47	8.68360			

D.11.4 Dormant buds progression into canes

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	3.43470	3.43470	29.67	<0.001
Variety	1	1.21603	1.21603	10.50	0.002
Species*Variety	1	0.08003	0.08003	0.69	0.410
Error	44	5.09400	0.11577		
Total	47	9.82477			

D.11.5 Woolly buds progression into green shoots

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	3.25521	3.25521	34.55	<0.001
Variety	1	0.82688	0.82688	8.78	0.005
Species*Variety	1	0.02521	0.02521	0.27	0.608
Error	44	4.14583	0.09422		
Total	47	8.25313			

D.11.6 Woolly buds progression into canes

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	3.41333	3.41333	31.83	<0.001
Variety	1	0.90750	0.90750	8.46	0.006
Species*Variety	1	0.04083	0.04083	0.38	0.540
Error	44	4.71833	0.10723		
Total	47	9.08000			

D.11.7 Green tip buds progression into green shoots

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	5.1352	5.13521	31.10	<0.001
Variety	1	1.4352	1.43521	8.69	0.005
Species*Variety	1	0.0752	0.07521	0.46	0.503
Error	44	7.2642	0.16509		
Total	47	13.9098			

D.11.8 Green tip buds progression into canes

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	5.2669	5.26687	30.11	<0.001
Variety	1	1.6502	1.65021	9.43	0.004
Species*Variety	1	0.0919	0.09187	0.53	0.472
Error	44	7.6958	0.17491		
Total	47	14.7048			

D.12 ANOVA of data for berries inoculated at different developmental stages for species and grapevine varieties

D.12.1 Infection incidences in all berry stages

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	60.75	60.75	4.84	0.064
Variety	1	252.08	252.08	20.07	0.003
Stages	2	7789.50	3894.75	310.10	<0.001
Error	7	87.92	12.56		
Total	11	8190.25			

D.12.2 Infection incidences in berries at pre-bunch closure

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Variety	1	0.065412	0.065412	6.33	0.017
Species	1	0.064127	0.064127	6.21	0.018
Variety*Species	1	0.001818	0.001818	0.18	0.678
Error	33	0.340976	0.010333		
Total	47	0.881282			

D.12.3 Infection incidences in rachii of berries at pre-bunch closure

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Variety	1	0.08859	0.08859	5.79	0.022
Species	1	0.10723	0.10723	7.01	0.012
Variety*Species	1	0.01296	0.01296	0.85	0.364
Error	33	0.50466	0.01529		
Total	47	1.09667			

D.12.4 Infection incidences in berries at veraison

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Variety	1	0.47864	0.478643	14.73	<0.001
Species	1	0.00631	0.006310	0.19	0.662
Variety*Species	1	0.00007	0.000069	0.00	0.964
Error	33	1.07262	0.032504		
Total	47	1.97594			

D.12.5 Infection incidences in rachii of berries at verasion

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Variety	1	0.41075	0.410753	12.12	<0.001
Species	1	0.00905	0.009054	0.27	0.609
Variety*Species	1	0.00003	0.000031	0.00	0.976
Error	33	1.11811	0.033882		
Total	47	2.42647	0.410753		

D.12.6 Infection incidences in berries at harvest

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Variety	1	0.31057	0.310570	6.07	0.019
Species	1	0.33219	0.332195	6.49	0.016
Variety*Species	1	0.00557	0.005571	0.11	0.743
Error	33	1.68790	0.051149		
Total	47	3.05885			

D.12.7 Infection incidences in rachii of berries at harvest

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Variety	1	0.36499	0.364991	10.68	0.003
Species	1	0.22462	0.224623	6.57	0.015
Variety*Species	1	0.00695	0.006946	0.20	0.655
Error	33	1.12780	0.034176		
Total	47	2.49044			

D.12.8 Incidences of pycnidia in infected berries inoculated at different developmental stages for species and grapevine varieties

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Stages	2	7.9657	3.98287	23.93	<0.001
Variety	1	0.0305	0.03046	0.18	0.670
Species	1	0.4874	0.48739	2.93	0.090
Stages*Variety	2	0.0152	0.00762	0.05	0.955
Stages*Species	2	0.1980	0.09900	0.59	0.553
Variety*Species	1	0.0000	0.00000	0.00	1.000
Stages*Variety*Species	2	0.0457	0.02285	0.14	0.872
Error	121	20.1352	0.16641		
Total	143	30.6750			

D.12.9 Distances of colonisation upwards in canes from berries inoculated at harvest

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Variety	1	16.333	16.333	5.74	0.021
Species	1	8.333	8.333	2.93	0.094
Variety*Species	1	4.083	4.083	1.44	0.237
Error	44	125.167	2.845		
Total	47	153.917			

D.12.10 Distances of colonisation downwards in canes from berries inoculated at harvest

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Variety	1	14.083	14.083	6.72	0.013
Species	1	5.333	5.333	2.55	0.118
Variety*Species	1	4.083	4.083	1.95	0.170
Error	44	92.167	2.095		
Total	47	115.667			

D.13 ANOVA of infection incidences of berries for species and development stages in the vineyard

D.13.1 Incidences of pycnidia in wounded berries at all development stages

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	8.9073	4.45366	95.66	<0.001
Stages	2	0.2425	0.24252	5.21	0.027
Species*Stages	2	0.0888	0.04440	0.95	0.392
Error	50	2.3279	0.04656		
Total	65	12.1044			

D.13.2 Incidences in wounded berries at all stages

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	1.1437	1.14371	18.23	<0.001
Stages	2	5.6057	2.80285	44.67	<0.001
Error	260	16.3147	0.06275		
Total	263	23.0641			

D.13.3 Incidences in wounded berries at pre-bunch closure

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	0.05189	0.05189	2.60	0.110
Error	86	1.71586	0.01995		
Total	87	1.76775			

D.13.4 Incidences in rachii of wounded berries at pre-bunch closure

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	0.5290	0.52901	29.74	<0.001
Error	86	1.5298	0.01779		
Total	87	2.0588			

D.13.5 Incidences in wounded berries at veraison

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	0.0698	0.069799	5.17	0.026
Error	86	1.022621	0.013503		
Total	87	1.16016			

D.13.6 Incidences in rachii of wounded berries at veraison

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	1.670	1.6697	12.85	<0.001
Error	86	11.174	0.1299		
Total	87	12.844			

D.13.7 Incidences in wounded berries at harvest

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	3.571	3.5711	29.23	<0.001
Error	86	10.508	0.1222		
Total	87	14.079			

D.13.8 Incidences in rachii of berries at harvest

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	4.291	4.2913	36.35	<0.001
Error	86	10.154	0.1181		
Total	87	14.445			

D.13.9 Distances of colonisation downwards in canes from wounded berries inoculated at harvest

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	13.14	13.136	6.78	0.026
Error	10	19.36	1.936		
Total	21	68.59			

D.13.10 Distances of colonisation upwards in canes from wounded berries inoculated at harvest

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	16.41	16.409	6.54	0.029
Error	10	25.09	2.509		
Total	21	89.50			

D.13.11 Incidences in non-wounded berries at harvest

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Species	1	0.5707	0.5707	4.55	0.036
Error	68	8.5216	0.1253		
Total	69	9.0922			

Appendix E - Analyses and information for Chapter 5

E.1 Fungicides and biocontrol agents tested

Treatment	Active ingredient	Fungicide group	Field rate	Supplier
Folicur®	430 g/L tebuconazole	Demethylation Inhibitor (DMI)	30 mL/100L	Bayer CropScience
Chief®	500 g/L carbendazim	Methyl Benzimadazole Carbamate	100 mL/100L	Agronica
Gem®	500 g/L fluazinam	2,6-dinitro-aniline	100 mL/100L	Agronica
Dithane™	750 g/kg mancozeb	Dithiocarbamate	210 g/100L	Dow Agrosciences
Megastar™	200 g/kg flusilazole	Demethylation Inhibitor (DMI)	20 g/100L	Grochem
TRI D25	*Trichoderma* spp.	Biocontrol	100 g/100L	Bayer CropScience
Serenade	*Bacillus subtilis*	Biocontrol	400g/100L	Bayer CropScience

E.2 GLM analyses of logit incidences for inoculation of potted grapevines at different times, species and treatments

E.2.1 Hard green shoots

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Fungicides	47.26	7	6.75	235.0	<0.001
Species	0.56	1	0.56	235.0	0.456
Time	0.53	2	0.26	235.0	0.769
Fungicides*Species	4.60	7	0.66	235.0	0.708
Fungicides*Time	11.13	14	0.79	235.0	0.674
Species*Time	0.51	2	0.26	235.0	0.774
Fungicides*Species*Time	3.70	14	0.26	235.0	0.997

E.2.2 Wood of trunks

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Fungicides	22.53	7	3.22	234.9	0.003
Species	0.16	1	0.16	234.9	0.690
Time	1.55	2	0.77	234.9	0.462
Fungicides*Species	8.35	7	1.19	234.9	0.308
Fungicides*Time	5.67	14	0.41	234.9	0.973
Species*Time	0.26	2	0.13	234.9	0.876
Fungicides*Species*Time	9.47	14	0.68	234.9	0.796

E.2.3 Bark of trunks

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Fungicides	27.63	7	3.95	234.9	<0.001
Species	0.27	1	0.27	234.9	0.606
Time	0.97	2	0.48	234.9	0.617
Fungicides*Species	1.41	7	0.20	234.9	0.985
Fungicides*Time	10.55	14	0.75	234.9	0.719
Species*Time	0.50	2	0.25	234.9	0.777

E.3 ANOVA of colonised lengths for inoculation of potted grapevines at different times, species and treatments

E.3.1 Green shoots

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Fungicides	6	109.719	18.2864	16.61	<0.001
Species	1	0.373	0.3734	0.34	0.561
Wound age	2	67.86	33.93	30.18	<0.001
Fungicides*Species	6	3.392	0.5653	0.51	0.798
Fungicides*Wound age	12	101.44	8.45	7.68	<0.001
Species*Wound age	2	0.656	0.328	0,30	0.743
Fungicides* Species* Wound age	12	1.823	0.1519	0.14	1.000
Error	210	231.262	1.10		
Total	251	516.537			

E.3.2 Wood of trunks

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Fungicides	6	63.563	10.5939	6.46	<0.001
Species	1	0.477	0.47	1.95	0.259
Wound age	2	10.469	5.234	3.19	0.043
Fungicides*Species	6	6.596	1.099	0.67	0.674
Fungicides*Wound age	12	44.335	3.696	2.25	0.011
Species*Wound age	2	1.777	0.8886	0.54	0.582
Fungicides* Species* Wound age	12	11.188	0.9324	0.57	1.866
Error	210	344.26	1.6394		
Total	251	488.693			

E.3.3 Bark of trunks

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Fungicides	6	105.642	17.6070	14.60	<0.001
Species	1	5.833	5.8332	4.84	0.029
Wound age	2	50.898	25.449	21.10	<0.001
Fungicides*Species	6	8.616	1.43	1.19	0.312
Fungicides*Wound age	12	27.187	2.265	1.88	0.038
Species*Wound age	2	0.504	0.2521	0.21	0.811
Fungicides* Species* Wound age	12	18.921	1.5767	1.31	1.216
Error	210	253.242	1.2059		
Total	251	470.843			

E.4 GLM analyses of logit incidences for inoculation of potted grapevines at different times, species and treatments

E.4.1 Green shoots

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Fungicides	35.33	7	5.05	160.0	<0.001
Species	1.11	1	1.11	160.0	0.295
Time	4.02	1	4.02	160.0	0.047
Fungicides*Species	1.03	7	0.15	160.0	0.994
Fungicides*Time	3.94	7	0.56	160.0	0.785
Species*Time	1.69	1	1.69	160.0	0.196
Fungicides*Species*Time	1.42	7	0.20	160.0	0.984

E.4.2 Wood of trunks

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Fungicides	35.31	7	5.04	154.9	<0.001
Species	0.10	1	0.10	154.9	0.757
Time	0.95	1	0.95	154.9	0.332
Fungicides*Species	0.41	7	0.06	154.9	1.000
Fungicides*Time	6.25	7	0.89	154.9	0.513
Species*Time	0.00	1	0.00	154.9	0.989
Fungicides*Species*Time	1.30	7	0.19	154.9	0.988

E.4.3 Bark of trunks

Fixed term	Wald statistic	d.f.	Wald/d.f.	chi pr
Fungicides	26.15	7	3.74	<0.001
Species	0.07	1	0.07	0.785
Time	0.74	1	0.74	0.390
Fungicides*Species	3.18	7	0.45	0.868
Fungicides*Time	6.58	7	0.94	0.474
Species*Time	0.08	1	0.08	0.783
Fungicides*Species*Time	5.00	8	0.62	0.758

E.5 GLM analyses of logit incidence of *N. luteum* on potted grapevine plants for inoculum concentrations, re-inoculation times and treatments

E.5.1 Green shoots

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Inoculum conc.	4.09	1	4.09	353.0	0.044
Treatment	10.76	4	2.69	350.3	0.031
Fungicides	52.14	5	10.43	295.8	<0.001
Fungicides*Treatment	6.19	20	0.31	295.3	0.998
Fungicides*Inoculum conc.	1.89	5	0.38	295.0	0.864
Treatment*Inoculum conc.	2.77	4	0.69	296.0	0.597

E.5.2 Wood of trunks

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Fungicides	37.52	5	7.50	295.6	<0.001
Treatment	3.74	4	0.94	295.1	0.443
Inoculum conc.	0.46	1	0.46	294.9	0.498
Fungicides*Treatment	3.16	20	0.16	295.2	1.000
Fungicides*Inoculum conc.	3.39	5	0.68	294.9	0.641
Treatment*Inoculum conc.	0.82	4	0.20	295.5	0.936
Fungicides*Treatment*Inoculum conc.	3.28	20	0.16		

E.5.3 Bark of trunks

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Fungicides	24.27	5	4.85	295.4	<0.001
Inoculum conc.	0.30	1	0.30	294.9	0.586
Treatment	4.46	4	1.11	295.0	0.350
Fungicide*Inoculum conc.	11.25	5	2.25	294.9	0.049
Fungicide*Treatment	4.39	20	0.22	295.1	1.000
Inoculum conc.*Treatment	0.94	4	0.23	295.3	0.919
Fungicide*Inoculum conc.*Treatment	7.71	20	0.39	295.3	

E.6 ANOVA of colonised lengths of tissues on potted grapevine plants, for inoculum concentrations, re-inoculation times (treatments) and fungicides (plus biocontrol products)

E.6.1 Green shoots

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Fungicides	4	52.905	13.2261	18.46	<0.001
Inoculum conc.	1	4.613	4.6128	6.44	0.012
Treatment	4	13.913	3.4782	4.85	<0.001
Fungicides*Inoculum conc.	4	0.427	0.1068	0.15	0.963
Fungicides*Treatment	16	7.132	0.4458	0.62	0.865
Inoculum conc.*Treatment	4	1.550	0.3876	0.54	0.706
Fungicides* Inoculum conc.*Treatment	16	2.150	0.1344	0.19	1.000
Error	245	175.575	0.7166		
Total	299	268.570			

E.6.2 Wood of trunks

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Fungicides	4	25.032	6.25798	13.92	<0.001
Inoculum conc.	1	2.022	2.022212	4.50	0.035
Treatment	4	5.119	1.27969	2.85	0.025
Fungicides*Inoculum conc.	4	1.247	0.31165	0.69	0.597
Fungicides*Treatment	16	3.379	0.21117	0.47	0.960
Inoculum conc.*Treatment	4	3.998	0.99946	2.22	0..067
Fungicides* Inoculum conc.*Treatment	16	1.362	0.08503	0.19	1.000
Error	245	110.128	0.44950		
Total	299	161.075			

E.6.3 Bark of trunks

Source of variation	d.f.	s.s.	m.s.	F-Value	P-Value
Fungicides	4	26.393	6.5983	10.49	<0.001
Inoculum conc.	1	2.613	2.6133	4.15	0.043
Treatment	4	27.402	6.8505	10.89	<0.001
Fungicides*Inoculum conc.	4	4.159	1.0397	1.65	0.162
Fungicides*Treatment	16	3.251	0.2032	0.32	0.994
Inoculum conc.*Treatment	4	2.153	0.5383	0.86	0.491
Fungicides* Inoculum conc.*Treatment	16	3.723	0.2327	0.37	0.998
Error	245	154.163	0.6292		
Total	299	246.245			

E.7. GLM analysis of logit incidences for species, inoculation times after treatments and fungicides (plus biocontrol products) in the vineyard

E.7.1 Wood of canes

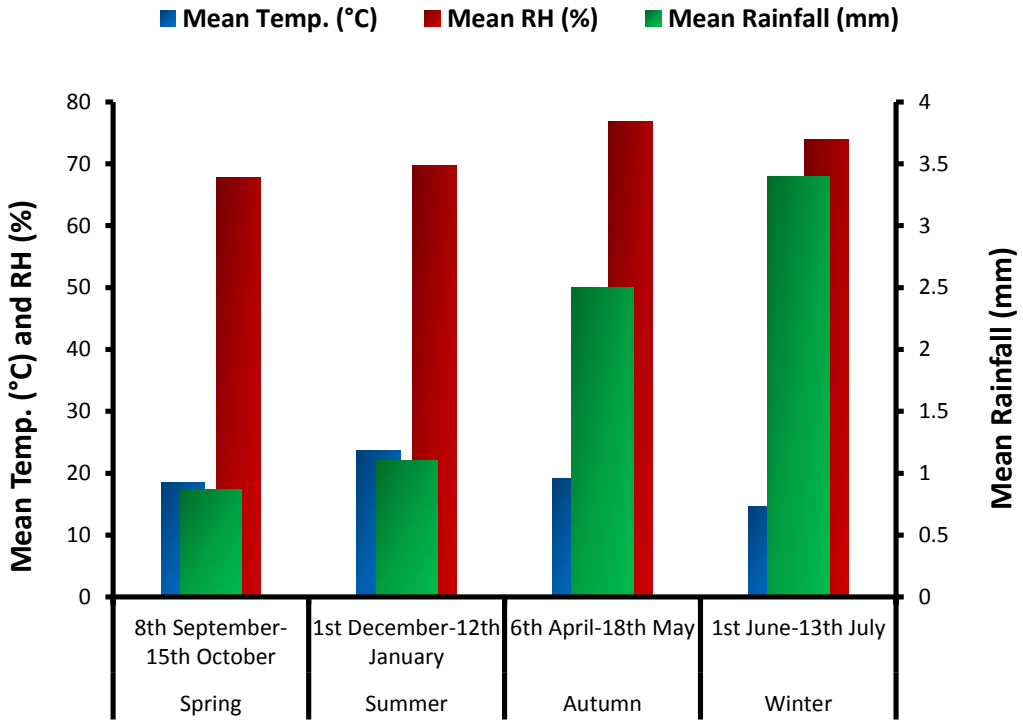
Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Fungicides	781.20	7	111.60	1626.9	<0.001
Species	0.08	1	0.08	1626.0	0.776
Time	3.28	2	1.64	1626.1	0.194
Fungicides*Species	7.64	7	1.09	1626.0	0.366
Fungicides*Time	102.97	14	7.35	1626.0	<0.001
Species*Time	1.52	2	0.66	1626.0	0.568
Fungicides*Species*Time	2.93	14	0.21	1626.0	0.999

E.7.2 Bark of canes

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Fungicides	666.42	7	95.20	1627.4	<0.001
Species	0.01	1	0.01	1626.6	0.940
Time	1.00	2	0.50	1626.7	0.608
Fungicides*Species	5.16	7	0.74	1626.6	0.640
Fungicides*Time	98.02	14	7.00	1626.6	<0.001
Species*Time	0.52	2	0.26	1626.6	0.772
Fungicides*Species*Time	6.16	14	0.44	1626.6	0.962

Appendix F - Additional information and results

F.1 Mean temperature, RH and rainfall for Blenheim (http://hortplus.metwatch.co.nz/ for the duration of the experiment with weather conditions (Chapter 4).



F.2 Mean lengths of colonised tissues of *Neofusicoccum luteum* from different grapevine inoculates with 2 and 40 conidia/wound after fungicides application. Fungicides were applied and wounds inoculated after ~2 hours (Treatment 1) or after 2 weeks (Treatment 2), 4 weeks (Treatment 3), 8 weeks (Treatment 4) or at 10 weeks (Treatment 5) after wounding.