Epidemiology of botryosphaeriaceous species associated with grapevines in New Zealand

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A thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

at

Lincoln University

by

Nicholas Tabi Amponsah

Lincoln University

2010

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Botryosphaericeous species affect most grapevines tissues leading to dieback and wood decay. During sampling of vineyards across New Zealand, botryosphaeriaceous species were found in brown necrotic lesions on grapevine trunks (42%), green shoots (20%), canes (17%), plant debris on the ground (7%), weak buds (8%), leaves (3%) and shrivelled flowers (3%). The isolates, identified by morphological and molecular methods as *Neofusicoccum australe*, *N. luteum, N. parvum, Diplodia mutila* and *D. seriata,* were also found in dieback on twigs and branches of non-grapevine woody hosts living around the vineyards. All isolates were pathogenic on wounded grapevine green shoots, except *D. seriata*. On wounded trunks of potted vines of scion varieties Cabernet Sauvignon, Chardonnay, Pinot noir, Riesling, and Sauvignon blanc, with mycelial or conidial inoculum, the disease developed to a similar extent ($P>0.05$) with all species except *D. seriata*, and *N. luteum* was the most pathogenic.

A microscopy investigation of conidium infection showed that 24 h after inoculation, conidia of *N. luteum* had not germinated and was shed from attached non-wounded leaves and shoots but had germinated and developed mycelium quickly on detached or wounded green leaves and shoots. Longitudinal stem sections showed mycelium within xylem vessels. Buds, green shoots, canes and trunks were susceptible to infection. In trunks of young vines, *N. luteum* progressed more quickly upward than downward, while bud and shoot infection could progress downward to infect adjacent tissues. As little as two conidia per wound on detached green shoots led to infection.

A 12 month investigation of spore dispersal, in a Canterbury vineyard detected conidia of *Neofusicoccum* spp. (59.8%) and *Diplodia* spp. (40.2%) in rainwater traps throughout the year. The conidia were identified by molecular methods as *N. australe, N. luteum and N. parvum,* (the *Neofusicoccum* spp.) which were most abundant and *D. africana, D. olivarum,*

D. cupressi or *D. mutila* (the *Diplodia* spp). No botryosphaeriaceous species conidia were collected on Vaseline®-coated slides set up to trap air-borne spores.

When effects of environmental factors on conidial development and viability were investigated, they demonstrated that the conidia were well-adapted to the vineyard environment. The temperature ranges for conidial germination, growth and development varied between *N. luteum, N. australe, N. parvum* and *D. mutila* ($P<0.001$), however all four species germinated after 3 h and grew quickly on PDA at 20-30°C. Exposure to different levels of solar radiation showed no significant difference in germination between *N. luteum, N. australe* and *D. mutila.* Conidia exposed to non-filtered sunlight (+UV) had 35% germination after 7 h which reduced to 0% by 56 h, whereas conidia exposed to filtered sunlight (-UV) had 57% germination after 7 h which reduced to 21% after 70 h. However, conidia put under shade had 81% germination after 7 h and 65% after 70 h. Dry conidia placed at 100% RH showed 98% germination in 3 h and had developed into a mycelium by 24 h, however, at 93% RH germination was about 46% and took 24 h, with no mycelium being formed by 48 h. No conidial germination had occurred after 48 h at 84% RH. Survival was also affected by low RH. Conidia incubated at 68% RH and then re-incubated at 100% RH showed levels of mortality that increased with the time of the low RH exposure, reducing from 88% after 7 h and to 26% germination after 70 h at 68% RH. *Neofusicoccum luteum* conidial suspensions could infect wounds with no continuing surface wetness, although infection and subsequent progression through the plant was significantly greater at 95% than 78% RH. Wounds on trunks of potted vines were susceptible to infection for the first two days and then incidence decreased to 0% by 14 days for conidia and 40% by 30 days for mycelium inoculum. Stress factors such as water stress of 15% or 100% field capacity (FC) had a delayed effect, with significantly increased dieback and more dead buds visible when new growth occurred after winter pruning.

In vitro fungicide experiments with 16 products and three isolates each of *N. australe, N. luteum* and *D. mutila* showed differences in mycelial growth and conidial germination between isolates and species, and between products with *N. luteum* being the least sensitive. *In vivo* evaluations on cane pruning wounds made on potted and field grapevines, subsequently inoculated with *N. luteum* conidia, showed that flusilazole and carbendazim, were the most effective at protecting pruning wounds from infection.

Keywords: *Diplodia, Neofusicoccum,* conidia, mycelium, fungicides, humidity, pathogenicity, splash dispersal, ultraviolet and temperature.

Acknowledgements

I give thanks and praise to the Almighty God for His guidance and protection throughout my doctorial program. I have quite a number of people to thank. First of all, I extend my deepest gratitude to Dr. Marlene Jaspers and Dr. Erian Jones for supervising and directing the whole dissertation process and also to Dr. Hayley Ridgway for supervision of all the molecular work. This dissertation was your brain child and you saw it through to a successful end. You were very patient and listened to me with all the needed attention. Your inputs were second to none and I really enjoy working with you three.

I also thank all staff and colleagues for their support in one way or the other. I will also like to thank Ms. Candice Barclay for providing help with all the technical support, Mr. Neil Andrews at Canterbury University for help with scanning electron microscopy and Mr. Brent Richards for taking good care of my potted grapevines plants in the nursery.

I will also like to thank the New Zealand Winegrowers association for sponsoring my research work, and DuPont (New Zealand) Ltd, Syngenta Corp., and Nufarm LTD, for providing me with fungicides that were used for the sensitivity studies against the botroyosphaeriaceous species. I am grateful to ICPP-New Zealand for the travel grants to attend conferences and workshops in and out of New Zealand, and also to the New Zealand Plant Pathology Society for the 2009 research scholarship award.

I dedicate this dissertation to my family. To my dear wife, Juliet Tabi Amponsah I thank you for your support and encouragement and also to my three daughters, Dansoa Tabi-Amponsah, Agyeiwaa Tabi-Amponsah and Amponsaa Tabi-Amponsah for their love and support that occupy my heart during all this time.

To my pastor, Rev. Dugald and his wife Janet and to Alan Hayward and his wife Vivienne, I say thank you very much for your dedicated love and care to me and my family. I also extend my sincere gratitude to the entire family of St Marks Presbyterian church for their care and concerns and for accepting us into their community. It is my wish that the good Lord shall reward each and every one of you. My very sincere thanks go to my parents who encouraged and motivated me throughout my education and to all my siblings, nephews and nieces, I say thank you all for being there for me.

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Chapter 1
Introduction

1.1 Worldwide Distribution of Grapevines

The wine grape originated from the temperate climatic regions of the Caucasus, a region in Eurasia between the Black Sea and the Caspian Sea and just north of Turkey and Iran. Due to the economic significance of grapes they spread successfully throughout the Middle East and the Mediterranean regions, and were then taken to France and Western Europe by the Romans. At present, grapes are grown in all the temperate and cool climatic regions of the world, with different varieties having been developed for the different climatic conditions (Jackson and Schuster, 2001).

1.2 Grape production in New Zealand

Grapes for wine production are one of the most economically important horticultural crops in New Zealand. Although much of the country has a temperate to cool climate with moderately high rainfall and sufficient sunshine hours that suit grapevine production, some areas are less suitable, such as the sub-tropical north of the North Island and the cool region in the south of the South Island. The areas known to be suitable for grape production have average daily summer maxima of 20-30ºC, winter maxima of 10-15ºC and have annual rainfall of 650-1050 mm. Soil types are not critical since grapevines can be grown in soils that range from fertile clay loam to stony alluvial deposits (Jackson and Schuster, 2001).

Presently, grape production in New Zealand extends from Auckland to central Otago. It has been increasing annually over the past decade, with the New Zealand grape producing area having increased from 7,400 ha to over 25,000 ha of land. The annual wine production reached 113 million litres by June 2009, of which the annual value of wine exports reached $1 billion (Smith and Green, 2009).

With the increase in grapevine production within New Zealand, different cultural management procedures have been put in place to ensure greater fruit quality that meets both export and domestic demands for wine production. In view of this, improved planting materials have been introduced to growers *via* regional Vine Improvement Groups. To avoid the damage caused by phylloxera, vineyards are usually established with grafted plants, in which resistant rootstock varieties are grafted to the desired

fruiting varieties. These rootstock varieties are also selected for growth characteristics that compliment the vigour of the fruiting variety with respect to the site. Most of the fruiting varieties grown for wine-grape production are cultivars of *Vitis vinifera*, which in its natural habitat is a vigorous, climbing vine that produces many small bunches of fruit (Mullins *et al.*, 1992). At present, more than 18 different fruiting varieties of grapevines are cultivated in New Zealand, with the most widely grown being Sauvignon blanc followed by Chardonnay and Pinot noir. These early-ripening grapes are well suited to the conditions found in the three principal grape-growing districts of Gisborne, Hawke's Bay and Marlborough (Jackson and Schuster, 2001).

For ease of maintenance and harvesting, commercial crops are grown on support systems consisting of post and wire trellises that compliment various training systems. The two most commonly used training systems in New Zealand are vertical shoot positioning (VSP) and Scott Henry (Jackson and Schuster, 2001). Annual pruning and trimming of the crop, is carried out to ensure optimum fruit yield per vine, which is often relatively low because it is believed to equate to improved wine quality. Winter pruning removes about 90% of the previous season's growth with the aim being to provide an optimum number of shoots, but also enough space among the shoots for optimum photosynthesis and air circulation, which reduces humidity and disease levels (Jackson and Schuster, 2001). Pruning also ensures that only healthy new wood is retained, thereby extending the productive life of the vine (Mullins *et al.*, 1992). Trimming of shoots is done 2-4 times during summer to improve aeration and light penetration. Although all the above-mentioned management practices aim to achieve optimum yields of high quality fruit, the artificially created wounds arising out of such practices do serve as entry points for opportunistic pathogens such as the botryosphaeriaceous species that initiate woody trunk diseases within the vineyard.

1.3 Major trunk diseases associated with grapevine production worldwide

Grapevine trunk diseases (GTD) may be caused by one or a combination of several fungi, which result in decline and/or death (Mugnai *et al.*, 1999; Mostert *et al.*, 2005; Christen *et al.*, 2007). During the last 10-15 years, these diseases have been of increasing significance worldwide. This is believed to be associated with the increased use of grafted vines with Phylloxera resistant rootstocks, which may be more susceptible to some trunk pathogens than the scion varieties of *Vitis vinifera* that were initially grown on their own

roots (Gubler *et al.*, 2004). The causal agents of GTD are generally xylem-inhabiting fungal pathogens, which grow primarily in the mature wood (Pascoe, 1998), where they may cause degradation of the cells associated with water and nutrient transport systems. For this reason, the external symptoms of the diseases often resemble those of water and nutrient deficiencies, including stunted shoots, chlorotic leaves and dieback, sometimes followed by death of the vine (Pascoe and Cottral, 2000). In California, Munkvold *et al.* (1994) estimated that yield reductions due to GTDs ranged from 30 to 62% in vineyards depending on the severity of the disease. However, the pathogens responsible for these diseases have also been isolated from apparently healthy plants (Aroca *et al.*, 2006), which supports the suggestions that the fungi may exist as latent infections and become pathogenic when the vines become stressed (Gubler *et al.*, 2005). The main diseases considered to be GTDs are described below:

1.3.1 Petri disease

Petri disease, which has also been called 'black goo' or 'young vine decline', is a vascular disease associated with decline of young grapevines. It is caused by *Phaeomoniella chlamydospora* and several *Phaeoacremonium* spp. (Fourie and Halleen, 2004b). The disease primarily affects young vines and can be a significant problem in the establishment of new vineyards (Edwards and Pascoe, 2004; Gubler *et al.*, 2004). It has been diagnosed in declining vines from many countries around the world (Ferreira *et al.*, 1994; Mugnai *et al.*, 1999; Pascoe and Cottral, 2000).

External symptoms include stunted growth, shortened internodes, interveinal chlorosis/ necrosis of leaves (Figure 1.1a), grapevine decline, and possibly shoot dieback, as well as graft failure (Scheck *et al*., 1998; Fourie and Halleen, 2002; Edwards and Pascoe, 2004). Wood symptoms include black streaks in the longitudinal sections of the heartwood that also appear as dark dots in cross section, and are associated with individual xylem vessels or groups of vessels (Figure 1.1b). After 5-20 minutes of being cut, vessels usually exude a glistening, black tarry substance (Pascoe and Van Der Walter, 1999; Gubler *et al*., 2004).

Conidia produced on the surfaces of infected tissues are thought to be splashed or blown onto pruning or other wounds to cause infections. The pathogen can remain as an endophyte, with the ability to cause disease when vines are stressed (Gubler *et al.*, 2001). Primary infection is also believed to occur in propagation nurseries, most likely through the use of infected propagation material from infected mother-vines (Mugnai *et al.*, 1999;

Larignon and Dubos, 2000), which Whiteman (2004) demonstrated were frequently from the rootstock mother vines. Whiteman *et al.* (2007) also found *P. chlamydospora* on grafting tools and in the propagating materials, such as hydration tanks, from which they were shown to infect the young grafted vines.

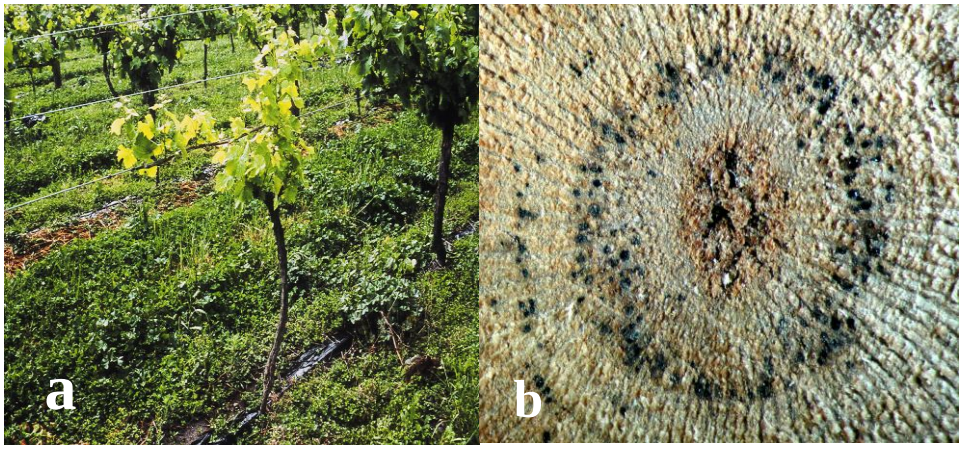

Figure 1.1: (a) Leaves showing symptoms of chlorosis (b) cross section through grapevine trunk showing Petri disease symptoms (Photographs: Marlene Jaspers and Sonia Whiteman, respectively).

1.3.2 Esca

Esca is a fungal disease of the grapevine trunk that is believed to be caused by a succession of two fungi. Initial infection by *P. chlamydospora* or *Phaeoacremonium* species, leading to Petri disease, is followed with secondary infection by specific basidiomycete fungi, of which *Fomitiporia mediterranea* and *F. punctata* are the most predominant species (Chiarappa, 1997; Mugnai *et al.*, 1999; Tabacchi *et al.*, 2000; Mostert *et al.*, 2006; McLean *et al.*, 2009). Although esca is most common in mature vines, young vines have been found to be infected, with symptoms of stunted growth, reduced yield and vine death (Mugnai *et al.*, 1999; Edwards *et al.*, 2001).

The leaf symptoms of esca first appear at the base of canes and are associated with structural and biochemical changes, which lead to chlorotic spots between the leaf veins, which gradually expand and turn yellow brown or red brown, leaving only a narrow strip of green tissue along the main veins (Figure 1.2a). In California, purple to black blotches often form on the surface of fruit and is referred to as ‘black measles’. The fruit may also be small and cracked. Black measles is very common in the USA and is believed to result

from the toxins associated with *P. chlamydospora* wood infection having a systemic effect on berries, forming spots on them (Gubler *et al.*, 2004).

The xylem-inhabiting basidiomycete pathogens infect the plants through large wounds and they initially produce cankers or rotting woody tissue that is cream in colour and spongy (Figure 1.2b), surrounded by a brown/red or black border from which the pathogens are often isolated (Mugnai *et al.*, 1999). The severe form, called 'apoplexy', is a sudden wilting of the entire plant or of part of it (Petit *et al.*, 2006) followed by death of the vine sections. The methods of disease spread are not yet fully understood, however spore trapping in infected vineyards found air-borne conidia of the primary pathogens, with large numbers of spores trapped during rainfall (Eskalen *et al.*, 2007).

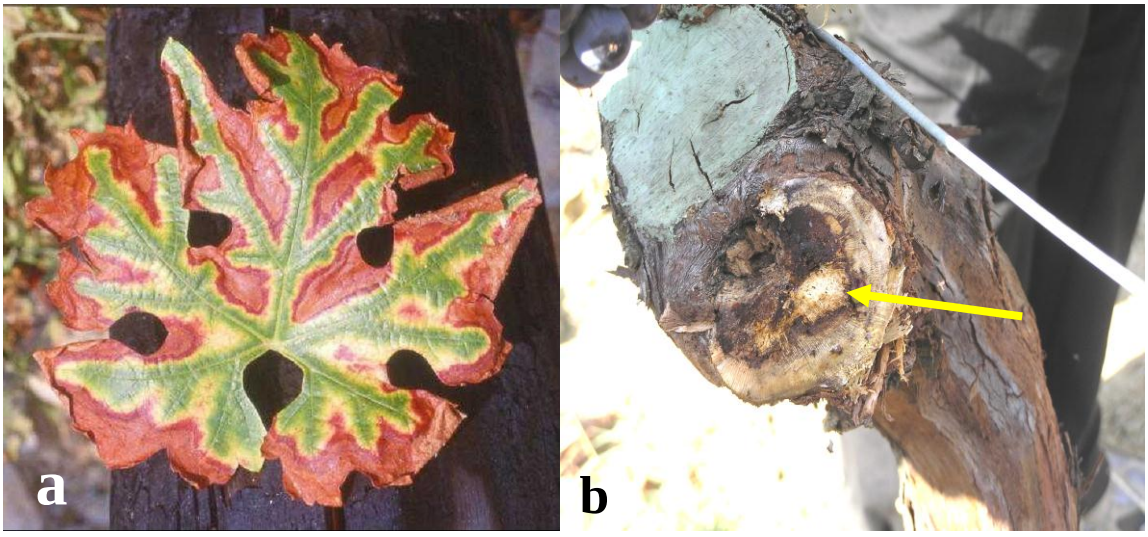

Figure 1.2: (a) Esca symptoms on leaves and (b) esca wood decay symptoms (yellow arrow showing white rot) in an old vine. (Photographs: Marlene Jaspers).

1.3.3 Phomopsis cane and leaf spot

Phomopsis cane and leaf spot, caused by *Phomopsis viticola* and other *Phomopsis* spp. is an important disease of grapes worldwide (Lal and Arya, 1982; Phillips, 1998; Mostert and Crous, 2000; Scheper *et al.*, 2000; Aroca *et al.*, 2006). The disease was formally known as 'dead arm'. However, the term 'dead arm' has also been applied to diseases caused by *Eutypa lata* and other fungi (Sosnowski *et al.*, 2007a). The fungus attacks all green parts of the vine, including canes, leaves, flowers, rachides and berries.

When the pathogen infects grape berry tissue a light brown fruit rot develops, on which raised black pycnidia are produced (Erincik and Madden, 2001). On shoots, pedicels and

rachides, symptoms appear as small brownish black spots that may expand into oval-shaped lesions. When numerous lesions occur at the base of a shoot, they often coalesce and blacken that portion of the shoot, causing girdling and death, or weakened stems that break during windy conditions (Phillips, 2000). This symptom is believed to be the major cause of yield loss in New Zealand (M. Jaspers, pers. comm., 2009), and the occurrence of Phomopsis fruit rot has been reported to be the major cause of yield loss in southern Ohio vineyards, where it is estimated to be as great as 30%. However this disease is not generally believed to be of great significance to vine health (Pscheidt and Pearson, 1989; Erincik and Madden, 2001).

In winter, infected canes can become bleached, particularly around nodes, and they produce black pycnidia (Figure 1.3a). This symptom is often used in the diagnosis of Phomopsis cane and leaf spot, however Phillips (1998; 2000) has shown that in Portugal the pathogen responsible for the bleached canes was *Fusicoccum aesculi* (*Botryosphaeria dothidea*) and not *Phomopsis viticola*. This was confirmed by Rawnsley *et al.* (2006) who stated that the diagnosis of the disease based on bleached canes may be misleading because bleaching may be induced by *Diaporthe perjuncta*, *P. viticola* and other fungi that produce pycnidia, such as the botryosphaeriaceous species. Because *D. perjuncta* and *P. viticola* cannot be visually distinguished on bleached canes during winter, reliable diagnosis of Phomopsis cane and leaf spot is based on symptom expression on leaves (Figure 1.3b) and shoots early in the growing season, when *P. viticola* causes distinct small dark brown to black leaf spots surrounded by a yellow halo (Hewitt and Pearson, 1990; Emmett *et al.*, 1998).

The pathogen overwinters in the outer bark of shoots and bud spurs infected the previous growing season. As temperatures increase in late winter, conidia are formed in the pycnidia that develop in the bark tissue. During spring rains, these conidia are exuded from pycnidia in tendril-like ooze and are subsequently dispersed by splashing rain droplets onto the young shoots, where infection occurs (Emmett *et al.*, 1998).

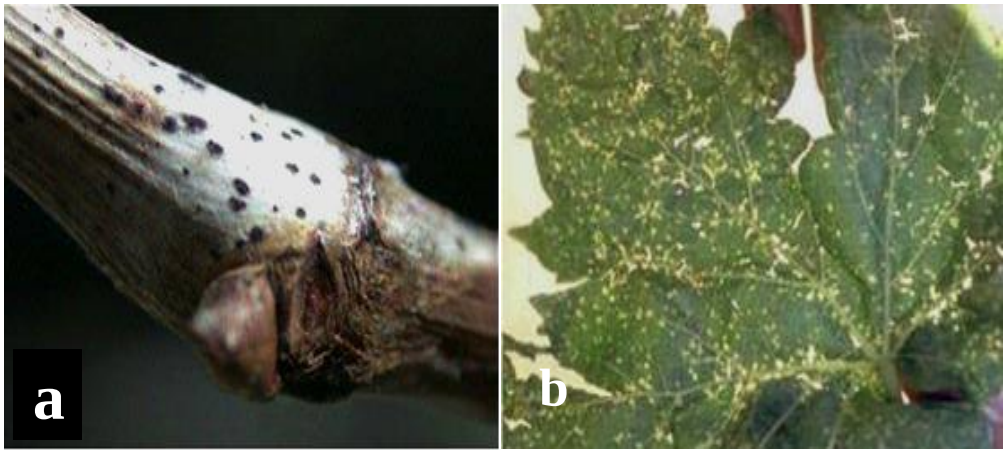

Figure 1.3: (a) Bleach cane with pycnidia grapevine and (b) yellow leaf spots caused by *Phomopsis viticola*. Photographs from Rawnsley (2008).

1.3.4 Eutypa dieback

Eutypa dieback, caused by diatrypaceaous species, of which *E. lata* is most common, infects the trunks and cordons of mature vines (John *et al.*, 2005; Octave *et al.*, 2006). For many years Eutypa dieback has been attributed to *Eutypa armeniaceae/ Eutypa lata* but more recently other diatrypaceaous species have been implicated in the dieback symptoms (Trouillas and Gubler 2010; Trouillas *et al*., 2010).

The pathogen overwinters by producing perithecia in wood that was killed by the pathogen at least 1-2 years earlier (Marlene Jaspers, pers. comm. 2008). Perithecia reach maturity in early spring, and ascospores are subsequently released during moist condition. Ascospores are the main methods of disease dissemination, and they are produced in areas with at least 350 mm average rainfall (carter et al., 1983; carter, 1991). The ascospores infect vines *via* fresh pruining wounds (up to 4 weeks old) which usually result in the formation of cankers and death of the infected cordons or vines after a number of years (Emmett and Magarey, 1994). However, grapevines are only one of the many hosts to E. lata since reports from Australia, Europe and North America in the past 25 years have revealed 88 additional host species in 27 families (Carter et al., 1985). It has been reported in the major grapevine growing areas around the world, including United States, Australia, Europe and South Africa, where it causes significant yield losses through destruction of the wood and also shortened productive life of vineyards (Carter, 1991; Munkvold *et al.*, 1994; Lardner *et al.*, 2007). The disease is prevalent in regions where winters are severe, such as central Europe and the eastern United States, and in

temperate regions, such as coastal California, south eastern Australia, southern France, and the Cape Province of South Africa (Carter, 1988).

The first external symptoms of the disease usually appear 3-10 years after infection, showing cankers typically around infection sites such as large pruning wounds. Cross-sections through the cankers reveal dark-brown, often wedge-shaped necroses (Figure 1.4a) within the wood of grapevine trunks and cordons (Carter, 1988; Highet and Wicks, 1998; Rudelle *et al.*, 2005). The pathogen also produces toxic metabolites that cause new shoots to have shortened internodes as well as deformed and chlorotic leaves in spring (Figure 1.4b) (Rudelle *et al.*, 2005).

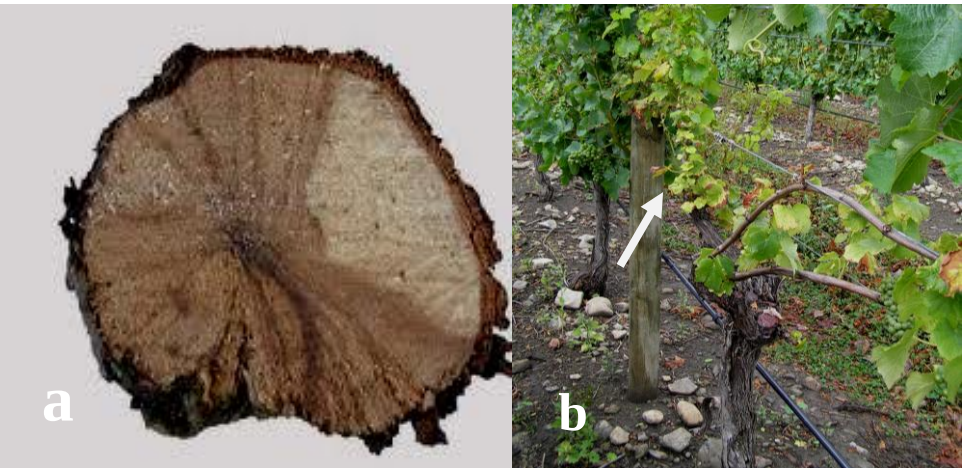

Figure 1.4: (a) A typical wedge of necrosis in wood that has expanded annually and (b) stunted growth and leaf discolouration (arrowed) caused by *E. lata*.(Photographs: Sosnowski and Wicks, 2010 and Marlene Jaspers, respectively).

The pathogen overwinters by producing perithecia in wood that was killed by the pathogen at least 1-2 years earlier (Marlene Jaspers, pers. comm.) Perithecia reach maturity in early spring, and ascospores are subsequently released during moist conditions. Ascospores are the main method of disease dissemination, and as they are produced in areas with at least 350 mm average annual rainfall (Carter *et al.*, 1983; Carter, 1991). The ascospores infect vines *via* fresh pruning wounds, (upto 4 weeks old) which usually result in the formation of cankers and death of the infected cordons or vines after a number of years (Emmett and Magarey, 1994). However, grapevines are only one of the many hosts to *E. lata* since reports from Australia, Europe and North America in the past 25 years have revealed 88 additional host species in 27 families (Carter *et al.*, 1985).

1.4 Botryosphaeriaceous diseases and symptoms associated with grapevines

Botryosphaeriaceous species have recently gained importance as pathogens of grapevines worldwide (Taylor *et al.*, 2005). In the past, their presence in woody trees and shrubs, including grapevines, was often overlooked because of their reputation as saprophytic or endophytic organisms (Castillo-Pando *et al.*, 2001, Phillips, 2002). Worldwide, 13 botryosphaeriaceous species have been identified as pathogens of grapevines, although the prevalence of the species reported differs between countries (Table 1.1). They have been reported to cause dieback of shoots, spurs and arms as well as severe internal wood necrosis (Larignon *et al.*, 2001; Fourie and Halleen, 2004a). They usually cause cankers and decline as well as a general loss of vigour, termed 'grapevine decline syndrome' (Von Arx, 1987; Denman *et al.*, 2000; Castillo-Pando *et al.*, 2001; Taylor *et al.*, 2005), as well as being associated with stunted growth, bud necrosis and mortality, delayed bud burst, bleached canes, incomplete graft unions and bunch rot (Lehoczky, 1974; Phillips, 1998; 2000; Larignon and Dubos, 2001; Castillo-Pando *et al.*, 2001; van Niekerk *et al.*, 2004; Taylor *et al.*, 2005; Úrbez-Torres *et al.*, 2007). However, as with some other woody trunk pathogens, their significance in grapevines was not fully appreciated until the last decade (Phillips, 2002; van Niekerk *et al.*, 2004; Taylor *et al.*, 2005).

1.4.1 Grapevine decline syndrome

Grapevine decline syndrome, caused by the major botryosphaeriaceous species (Table 1.1), is generally considered the most important symptom. It usually progresses slowly within an affected vineyard, which may account for the observations by Larignon and Dubos (2001) and van Niekerk *et al.* (2006) that severe symptoms due to Botryosphaeria diseases do not usually occur in vines that are less than 8 years old. Pearson and Goheen (1994) also observed that the disease does not spread rapidly, but builds up progressively in a vineyard over a number of years, leading to a general decline in vigour and yield of the vineyard. Recent studies have identified the botryosphaeriaceous species as being responsible for such decline, with typical symptoms being stunted growth and bud necrosis (Figure 1.5a and b), graft failure, dead spurs, cane bleaching in winter and die-back (Phillips, 2000; Larignon *et al.*, 2001; van Niekerk *et al.*, 2004; Taylor *et al.*, 2005; Úrbez-Torres *et al.*, 2007). Leaves of vines infected by the botryosphaeriaceous species were also found to show small necrotic spots with wrinkled surfaces (Bonfiglioli and McGregor, 2006).

Table 1.1: Botryosphaeriaceous species associated with major disease symptoms in grapevine, their country of origin and the relevant references.

Species	Symptoms				Country	References
	Grapevine decline syndrome	Fruit rot	Cane dieback	Wood canker		
[a]***N. australe*** (*B australis*)	✓		✓	✓	Australia, USA, New Zealand	2, 12,14
N. luteum (*B. lutea*)	✓			✓	Portugal USA, New Zealand, Australia	1, 12, 14, 16
N. parvum (*B. parva*)	✓		✓	✓	Portugal USA, New Zealand, Australia	1, 8, 12, 14, 15,17
N. ribis (*B. ribis*)		✓	✓		USA, S. Africa	5, 9
N. vitifusiformes	✓				S. Africa	13
N. viticlavatum	✓				S. Africa	13
[b]***D. viticola*** (*B. viticola*)	✓			✓	Spain, USA	8, 12
N. vitis (*B. vitis*)	✓				S. Africa	13
[c]***Do. sarmentorum*** (*B. sarmentorum*)				✓	USA	12
D. seriata (*B. obtusa*)	✓		✓	✓	Portugal, USA, Australia, S. Africa	1, 2, 9, 12, 15
[d]***L. theobromae*** (*B. rhodina*)	✓		✓	✓	USA Australia	7, 12, 18
D. mutila (*B. stevensii*)	✓			✓	Portugal, USA, Australia, New Zealand, Hungary,	2, 3, 4, 6, 14, 15
D. porosum	✓				S. Africa	13
[e]***F. aesculi*** (*B. dothidea*)	✓	✓	✓	✓	Portugal, USA, Brazil, S. Africa	1, 5, 7, 9, 10,12

* Previous scientific names in parenthesis

References: **1**. Phillips, 2002; **2**, Taylor *et al.*, 2005; **3**. Lehoczky, 1974; **4**. Choueiri and Jreijiri, 2005; **5**. Phillips, 1998; **6**. Hewitt, 1994; **7**. Phillips, 2000; **8**. Luque *et al.*, 2005; **9**. Crous *et al.*, 2001; **10**. Millholland, 1991, **11**. Filho *et al.*, 1995; **12**. Úrbez-Torres *et al.*, 2007; 2006 **13**. van Niekerk *et al.*, 2004; **14**. Amponsah *et al.*, 2007; **15**. Castillo-Pando *et al.*, 2001 ; **16.** Savocchia *et al.*, 2007; **17**. Cunnington *et al.*, 2007.

a. *Neofusicoccum,* b. *Diplodia,* c. *Dothiorella,* d. *Lasiodiplodia, e. Fusicoccum*

Bleaching of dormant canes during winter was found to be caused by *Neofusicoccum parvum, N. luteum, D. mutila* and *D. seriata*, while the consistent isolations of *D. mutila* and sometimes *N. parvum* from failed graft unions and bud necroses suggested that these species were responsible (Castillo-Pando *et al.*, 2001). Oliveira *et al.* (2004) reported that in Portugal several botryosphaeriaceous species were responsible for the decline of young vines and young vine death, as well as loss of productivity, and Phillips (2002) reported that *N. parvum, D. mutila, N, luteum,* and *D. seriata* were associated with grapevine decline in Portugal. In New South Wales and South Australia, eight Botryosphaeriaceae species have been found to be associated with the decline of grapevines in the major viticultural regions (Pitt *et al.*, 2010a).

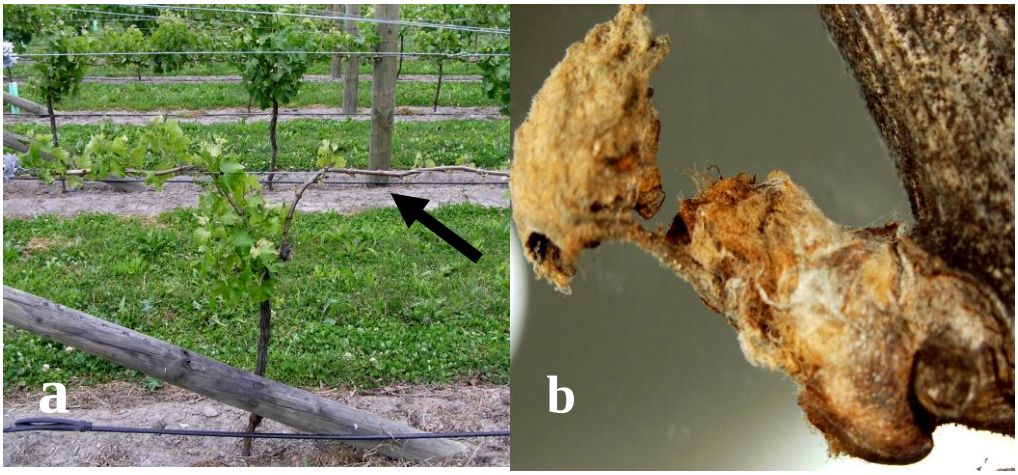

Figure 1.5: Some symptoms associated with grapevine decline syndrome caused by botryosphaeriaceous species (a) vine showing stunted growth (arrowed), (b) bud necrosis.

1.4.2 Shoot and cane dieback

The symptom of shoots dying back from the tip towards the base (Figure 1.6a) is common in vineyards. Such symptoms usually occur in summer and are most severe in areas with warm weather and high relative humidity or occasional summer rain (Hewitt, 1994). Symptoms of cankers are most commonly seen on shoots (Figure 1.6b), but also on spurs, canes, branches, cordons or trunks, with subsequent grapevine dieback and bleaching of the matured canes, where pycnidia may be produced on the bark of the infected parts. The disease is also associated with large pruning wounds which can lead to death of canes and eventually extend to the trunk. In California, the most common fungi isolated from grapevine plants showing cane dieback symptoms were *L. theobromae* and *N. parvum* (Úrbez-Torres and Gubler, 2009).

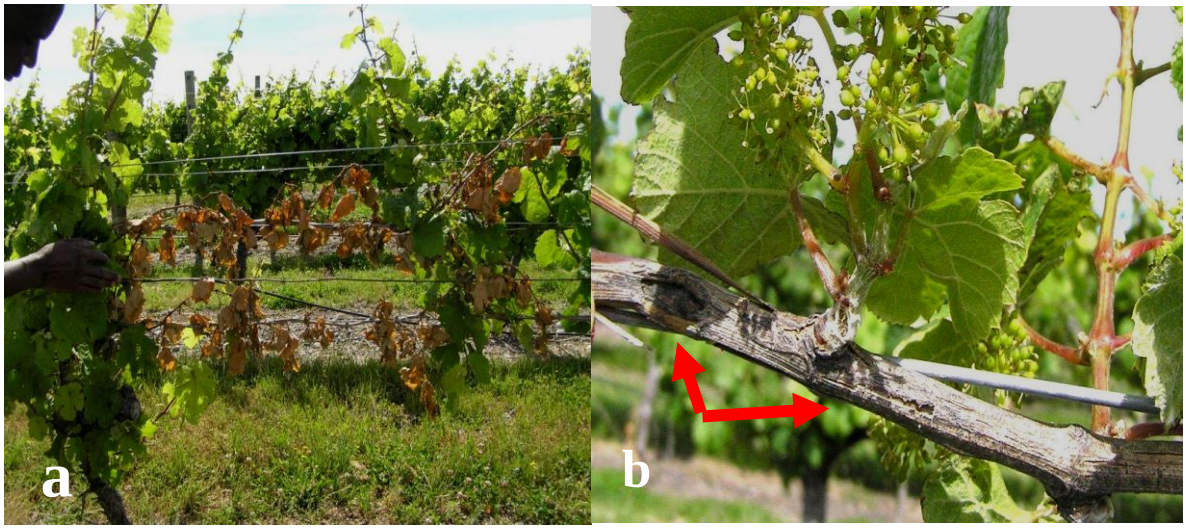

Figure 1.6: Grapevine cane dieback caused by botryosphaeriaceous species (a) shoot dieback, (b) canker developing on shoots (arrows).

1.4.3 Botryosphaeriaceous cankers and wood decay

The botryosphaeriaceous species have been reported to cause grapevine cankers in North and South America (Úrbez-Torres *et al.*, 2008; Úrbez-Torres and Gubler, 2009), Australia (Castillo-Pando *et al.*, 2001; Taylor *et al.*, 2005), South Africa (van Niekerk *et al.*, 2004) and Europe (Dubos *et al.*, 2001; Phillips, 2002; Úrbez-Torres *et al.*, 2006). A field survey conducted in 166 vineyards in California showed that seven botryosphaeriaceous species (*N. australe, B. dothidea*, *N. luteum*, *D. seriata*, *N. parvum*, *L. theobromae*, and *D. mutila*) were consistently isolated from cankers in all grape-growing regions surveyed (Úrbez-Torres *et al.*, 2006), while in Mexico, *L. theobromae* and *D. seriata* were also isolated (Úrbez-Torres *et al.*, 2008). Experiments confirmed these species to be pathogenic and responsible for the cankers (Úrbez-Torres and Gubler, 2009). The cankers often begin with small areas of discoloured bark, which can extend to girdle branches and cause extensive cambial death along the trunks. Cankers often appear sunken and/or darkened and the bark may peel and drop from a cankered area. When the bark around a canker is removed, the wood beneath shows discolored brown to reddish-brown lesions (Figure 1.7a). It often shows a wedge shaped necrotic lesion when cut across (Figure 1.7b). In vineyards, cankers may be seen around large pruning wounds, typically at spur positions, from where the pathogen grows into adjacent cells and vascular elements. When cankers girdle the branches, the leaves may wilt and die but often remain attached to the branch (Babadoost, 2005). Cankered twigs and branches are often unnoticeable

until wilting and dieback occurs. Large cankers may cause death of arms, cordons or entire vines, often so suddenly that there are no early foliar symptoms (Gubler *et al.*, 2005).

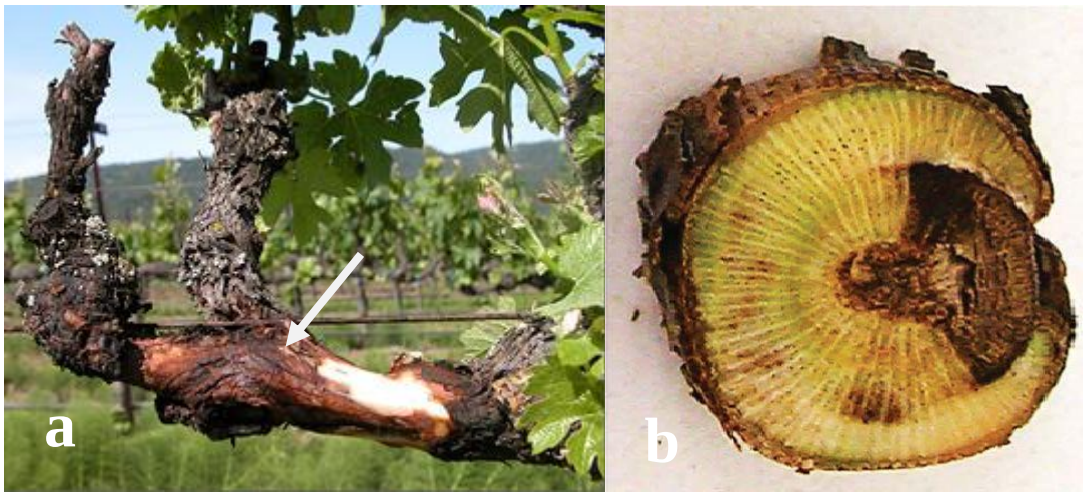

Figure 1.7: Brown necrosis of botryosphaeriaceous cankers on grapevine (a) around pruning wound (arrow) and (b) cross section through cankered section showin wedge shaped wood decay (Photographs: Úrbez-Torres *et al.*, 2006 and Epstein *et al.*, 2008, respectively).

1.4.4 Bunch rot and macrophoma rot

Mature berries, may develop lesions that first appear to be sunken and water-soaked, developing into a brown rot on which the skin cracks. Berries may then become covered with white mycelium or shrivel, eventually becoming mummified with black pycnidia emerging on the surface (Hewitt, 1994). On berries of white varieties small (1-4 mm), flat lesions may develop. They have small brownish centers, in which the fruiting bodies (pycnidia) of the fungus are embedded. In red grapes, colour change due to infection is barely noticeable and the infected berries may mummify and drop from the vine (Anonymous, 2005).

In the USA, *B. dothidea* is regarded as the causal organism of macrophoma rot of Muscadine grapes. This disease is characterized by round, sunken lesions which develop on infected berries as they mature. Infected berries later drop from the vine and dry up with abundant pyncnidia covering their entire surfaces (Millholland, 1991; Kummuang *et al.*, 1996). In South Africa, *L. theobromae* is regarded as a major causal organism of bunch rot in grapes (van Niekerk *et al.*, 2000). In Australia, Nicola Wunderlich (pers.

comm.) inoculated grape berries with *D. seriata* and observed black, shrivelled, and sticky symptoms (Figure 1.8).

Figure 1.8: Bunch rot caused by *D. seriata*. (Photograph: Nicola Wunderlich, CSU).

1.5 Worldwide distribution of the botryosphaeriaceous species

As shown previously in Table 1.1, not all botryosphaeriaceous species are equally widely distributed in vineyards across the globe. Human mediated movement of the botryosphaeriaceous species has probably been responsible for the apparently random differential distribution (Slippers and Wingfield, 2007), but there is also some evidence that the different species have different temperature optima (Úrbez-Torres *et al.*, 2006), which may limit their establishment in some regions. For example the predominance of *L. theobromae* in the southern part of California, particularly in the desert area of Coachella Valley (Riverside County), and its absence from the northern part of the state could be a consequence of climate differences (Úrbez-Torres *et al.*, 2006). This is consistent with reports of it being the dominant taxon in tropical and subtropical regions, where it was reported to cause dieback diseases on a very wide range of plants (Punithalingam, 1976; Sandlin and Ferrin, 1992; Roux *et al.*, 2001). Domsch *et al*. (1980) also observed *L. theobromae* to be a common soil-borne saprophyte or wound parasite distributed throughout the tropics and subtropics, where it was usually associated with different decline syndromes. In Western Australia, Taylor *et al*. (2005) also observed differences in regional distribution of the botryosphaeriaceous species. For example *N. australe* was found to be present in the Margaret River and Pemberton/ Manjimup regions but never

isolated from the Swan region probably due to temperature differences. In South Africa, *L. theobromae, N. luteum, N. parvum, N. australe, Diplodia* sp. (resembling *D. sarmentorum*), *D. porosum, N. viticlavatum* and *N. vitifusiforme* were also isolated from grapevines (van Niekerk *et al.*, 2004) but none of the isolates collected in South Africa were representative of *B. dothidea* or *B. ribis*, which are some of the common species found in Europe (Pascoe, 1998; Phillips, 2002).

Another factor which may affect the distribution of species is their capacity to adapt to new hosts after being introduced into a new environment (Slippers *et al.*, 2005). For example, the presence of *F. aesculi* in cankers and dieback in numerous plant species in regions of the world with temperate climates has been reported by Barnard *et al.* (1987), Fisher *et al.* (1993), Smith *et al.* (1994) and Slippers *et al.* (2004c). Knowledge of the species diversity and distribution is therefore useful in understanding the host-pathogen relationships and for developing disease management strategies (Begoude *et al.*, 2009). In New Zealand, which has a temperate climate, the botryosphaeriaceous species most commonly found to be associated with graft failure, cane dieback and trunk disease of grapevines were *N. luteum, N. parvum, D. seriata, D. mutila* and *F. aesculi* (Bonfiglioli and McGregor, 2006), which are similar to those found elsewhere in temperate climates (Taylor *et al.*, 2005; Úrbez-Torres *et al.*, 2006; Savocchia *et al.*, 2007).

1.6 Botryosphaeriaceous species that occur on non-grapevine hosts

Botryosphaeriaceous species are known to be very cosmopolitan, inhabiting the wood of many monocotyledonous, dicotyledonous and gymnosperm hosts (Barr, 1972; Burgess *et al.*, 2005; van Niekerk *et al.*, 2004; Wingfield *et al.*, 2001). They may be saprophytes of woody debris or endophytes, where they cause no visible disease symptoms in the healthy plant or its seeds (Burgess *et al.*, 2001; Flowers *et al.*, 2001; Danti *et al.*, 2002). However, they are also reported to be pathogenic, causing shoot blights, leaf spots, fruit and seed rots, and witches' brooms in several host plants (Barr, 1972; Smith *et al.*, 1994; Denman *et al.*, 2000; Phillips, 2002; Sanchez *et al.*, 2003). As canker pathogens, botryosphaeriaceous species have been isolated from numerous hosts in many parts of the world and have been implicated in the decline of *Eucalyptus gomphocephala* (Taylor *et al.*, 2009). Seven new species (*Dothiorella longicollis, Fusicoccum ramosum, Lasiodiplodia margaritacea, Neoscytalidium novaehollandiae, Pseudofusicoccum adansoniae, P. ardesiacum* and *P. kimberleyense*) have recently been found to be associated with the dying branches of the baobab tree (*Adansonia gibbosa*) in Western

Australia (Pavlic *et al.*, 2008). *Neofusicoccum australe* and *F. macroclavatum* have been isolated from eucalypts (Old and Davison, 2000; Burgess *et al.*, 2005) and *D. mutila*, *F. aesculi*, *B. corticola* and *D. sarmentorum* from oak, (mainly in the western Mediterranean area) (Alves *et al.*, 2004). Table 1.2 shows some of the botryosphaeriaceous species found to be responsible for cankers and dieback on a diverse range of valuable woody hosts of economic importance worldwide.

The many reports of botryosphaeriaceous species on a range of woody hosts have often conflicted, with a wide range of species being reported for individual host types. However, the difficulties associated with identification mean that some of the identifications made are likely to be incorrect. The reports of the botryosphaeriaceous species found have not always been accompanied by detailed morphological descriptions. In addition, more than one species may occur on the same host and same part of the plant, which in turn may complicate identification (Slippers *et al.*, 2004a). Furthermore colony characteristics of the botryosphaeriaceous species can be strongly influenced by the substrate on which they are grown (Sutton, 1990). In addition, the general mycelial growth pattern, speed and colour of a colony on agar may differ with temperature (Pennycook and Samuels, 1985; Phillips *et al.*, 2002; Slippers *et al.*, 2004a). While some members of this genus are difficult to morphologically identify to species level due to similarities in colony and conidium characteristics, some can be distinguished based solely on characteristics of colonies or conidia (Pennycook and Samuels, 1985; Slippers *et al.*, 2004a).

Table 1.2: Botryosphaeriaceous species associated with diseases of some fruit trees of economic importance, their country of origin and the relevant references.

Host	Botryosphaeriaceous fungi	Country	Reference*
Pyrus communis (Pear)	*Diplodia seriata, Neofusicoccum parvum, N. ribis, N. parvum, N. australe, Fusicoccum aesculi, D. mutila, D. seriata*	USA, New Zealand, South Africa	2, 3, 4, 5, 12,10
Malus domestica (Apple)	*D. seriata, N. luteum, N. parvum, N. ribis, N. parvum, N. australe, F. aesculi, D. mutila, D. seriata*	USA, New Zealand, Holland, Belgium, Germany, South Africa	1, 2 ,3, 4, 5, 13, 10
Prunus persica (Peaches)	*F. aesculi, N. ribis, N. parvum, N. australe F. aesculi, D. mutila, D. seriata, N. vitifusiforme*	USA, South Africa	9, 10, 16
Prunus dulcis (Almond)	*N. australe, D. seriata*	South Africa	10
Pistacia vera (Pistachios)	*F. aesculi*	USA	6, 7, 8
Vaccinium sp. (Blueberry)	*D. seriata, N. luteum, N. parvum, N. australe*	New Zealand	14
Mangifera indica (Mango)	*D. mangiferae , N. mangiferae, N. ribis*	South Africa	10, 11
Olea europaea (olive plant)	*D. seriata,*	Spain	15
Actinidia deliciosa (Kiwi fruit)	*N. luteum, N. parvum*	New Zealand	1

*__1__.Pennycook and Samuels, 1985; **2**. Parker and Sutton, 1993; **3**. Atkinson and Shearer, 1971; **4**. Jones and Aldwinkle, 1991; **5**. Brown and Britton, 1986; **6.** Ma and Michailides, 2002; **7**. Ma *et al.*, 2001; **8**. Swart and Blodgett 1998; **9**. Pusey, 1989; **10**. Slippers *et al.*, 2005; 2007; **11**. Ramos *et al.*, 1991; **12**. Hartill and Everett, 2002; **13**. Trapman *et al.*, 2007;. **14.** Sammonds *et al.*, 2009; **15**. Moral *et al.*, 2008; **16.** Damm *et al.*, 2007**.**

1.7 Taxonomy of the botryosphaeriaceous species

The taxonomy of botryosphaeriaceous genera has been confused for a long time (Slippers and Wingfield, 2007). The teleomorphs of many of these ascomycete fungi have not been found and although some do produce a teleomorph stage in nature, they have rarely been produced in culture (Denman *et al.*, 2000; Crous *et al.*, 2006). The ascospores obtained were usually hyaline, aseptate, fusoid to ellipsoid or ovoid, bi- to triseriate, mostly without a mucoid sheath or appendages (Slippers *et al.*, 2004a; Crous *et al.*, 2006).

The genus, Botryosphaeria, was originally used exclusively to represent the teleomorph stages of these fungi (Slippers *et al*., 2004b). However, Slippers *et al*. (2004b) reported that teleomorph characters differed on different hosts and were often not distinctive at species level. The taxonomic confusion has been exacerbated by the diversity and overlap between anamorph states of the botryosphaeriaceous species (van Niekerk *et al*., 2004), as there have been at least 18 anamorph genera associated with the Botryosphaeriaceae (Phillips *et al*., 2008). Only two genera were recognised by Denman *et al*. (2000) and Alves *et al*. (2004), according to a combination of morphological and molecular

characteristics. These genera are *Diplodia* (dark, mostly >10 μm wide, thick-walled conidia), and *Neofusicoccum* (hyaline, mostly <10 μm wide, thin-walled conidia). However, Pavlic *et al*. (2009) recognised *Lasiodiplodia* as a genus distinct from *Diplodia* because of the distinct sequence of some genes (rDNA or EF-1α) and morphological characteristics. The genus *Dothiorella*, which differs from *Diplodia* by having conidia that are brown and 1-septate early in their development, while they are still attached to the conidiogenous cells, was also re-introduced as a distinct botryosphaeriaceous anamorph (Phillips *et al*., 2005). Another genus, *Dichomera*, was also found to be linked to the Botryosphaeriaceae because it had *Fusicoccum* anamorphs (Barber *et al*., 2005). The anamorph species that occur on grapevines are currently placed in the genera *Diplodia*, *Dothiorella*, *Fusicoccum*, *Neofusicoccum*, and *Lasiodiplodia* (Denman *et al*., 2000; Phillips *et al*., 2005; Crous *et al*., 2006). The overall phylogenetic associations between many of these genera and the Botryosphaeriaceae remain untested and so phylogenetic understanding of the groups within the Botryosphaeriaceae remains poor (Crous *et al.*, 2006).

1.8 Morphological identification of the botryosphaeriaceous species associated with grapevines

The anamorph stages display a wide range of morphologies. To avoid the unnecessary introduction of new generic names, taxonomists have opted to use existing anamorph generic names for most of the lineages (Phillips *et al.*, 2008). In view of that, the botryosphaeriaceous pathogens will be referred to throughout this thesis by their anamorph names, wherever it is possible to do so. In the subsequent sections the teleomorph names will be put in brackets. The following sections provide some descriptions on morphological characteristics from the literature that can be used for species identification for the major pathogens, whereas the minor ones are described in Appendix A1. An important feature of these descriptions is the size ranges of the conidia, from which the lengths to width ratios were calculated. However, although a discrete figure is often given in the different references, they differ because of the ranges of sizes of the conidia in each species.

1.8.1 *Neofusicoccum australe* (*B. australis*)

Cultures on potato dextrose agar (PDA) are buff to light primrose, with light yellowish pigment diffusing into the medium, most noticeably after 3 days growth in the dark. After 1 week, they become olivaceous buff to olivaceous gray, with a sparse to moderately

dense, appressed mycelial mat in the colony centres and sparse tufts of aerial mycelium around edges. The pycnidia formed after 21 days on water agar (WA) embedded with pine needles are superficial, globose and mostly solitary, and are covered by mycelium. The conidia are fusiform and have subtruncated bases (Figure 1.9a) with size ranges of (18–) 23–26 (–30) × 5–6 (–7.5) µm and a mean length to width ratio of 4.8. They are hyaline, unicellular, rarely forming a septum before germination. In nature, the dark walled perithecia of about 1.2 mm diameter are formed on and protrude through the bark. The ascospores formed are fusoid to ovoid (Figure 1.9b), with size ranges of 20–23 (–25) × 7–8 (–9) mm and a mean length to width ratio of 2.9. They are unicellular, hyaline and smooth with granular contents (Slippers *et al.*, 2004c).

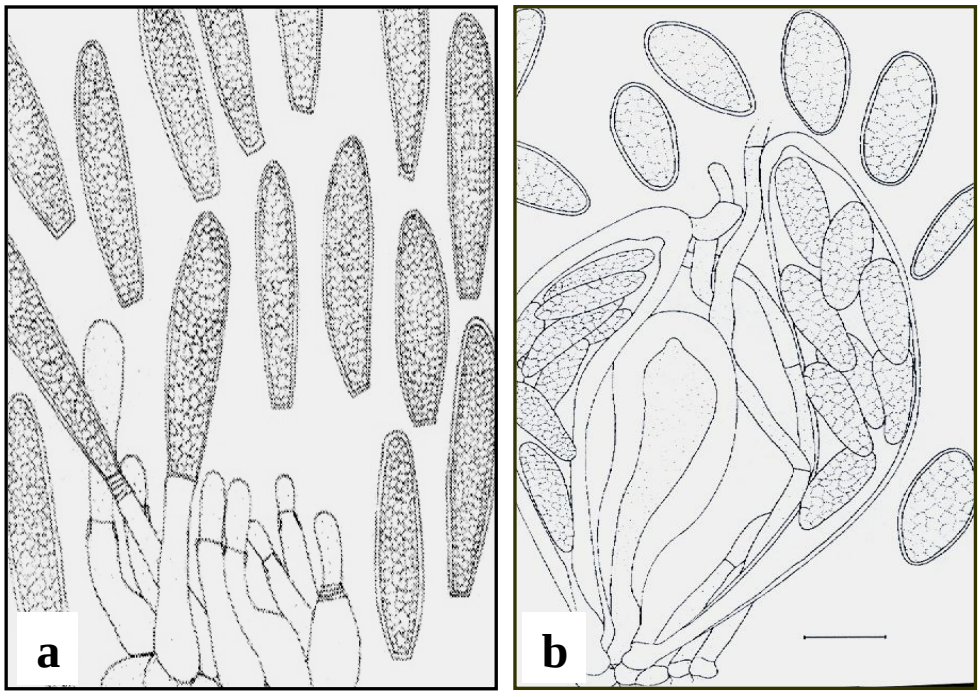

Figure 1.9: ***Neofusicoccum australe.*** **Reproductive structures of (a) conidia and conidiogenous cells (b) ascospores with asci (holotype). Bar=10 µm (Source*:* Slippers *et al.*, 2004c).**

1.8.2 *Neofusicoccum luteum* (*B. lutea*)

Cultures on PDA are buff with yellow colouration being observed on PDA after 3 days growth in diffuse light or darkness. They develop rope-like hyphal strands mostly at the periphery and after 1 week, the colour gradually changes to violaceous, becoming grey to dark grey with age and generally lacking aerial mycelium at the center. This distinctive production of a yellow pigment is a characteristic feature of *N. luteum* and *N. australe* species (Pennycook and Samuels, 1985; Burgess *et al.*, 2005). The pycnidia that form on

PDA cultures within 9 days are dark-walled and numerous. They are superficial or partially immersed and scattered over the surface, tending to form an almost continuous layer with blackening of the colony in some areas. The conidia formed are ellipsoidal to fusiform (Figure 1.10a), with a truncated or round base and size ranges of (14-) 20-24 (-32) × (5-) 6-7 (-9) µm and a mean length to width ratio of 3.2 (Pennycook and Samuels, 1985; Phillips *et al.*, 2002). The microconidia which are sometimes produced by some isolates are rod-shaped (Figure 1.10a) with either truncated or rounded ends and size ranges of 3–5 × 1–2 µm (Phillips *et al.*, 2002). In nature, the dark walled perithecia of about 0.5 mm in diameter are formed in the bark, and are initially immersed but later become erumpent. The ascospores formed are irregularly biseriate, oval to broadly fusiform (Figure 1.10b), widest in the middle or upper third, tapering to the obtuse base and apex, and with size ranges of (18-22.5 (-24) × 7.5-12 µm and mean length to width ratio of about 1.8. They are hyaline, guttulate, smooth and aseptate (Phillips *et al.*, 2002).

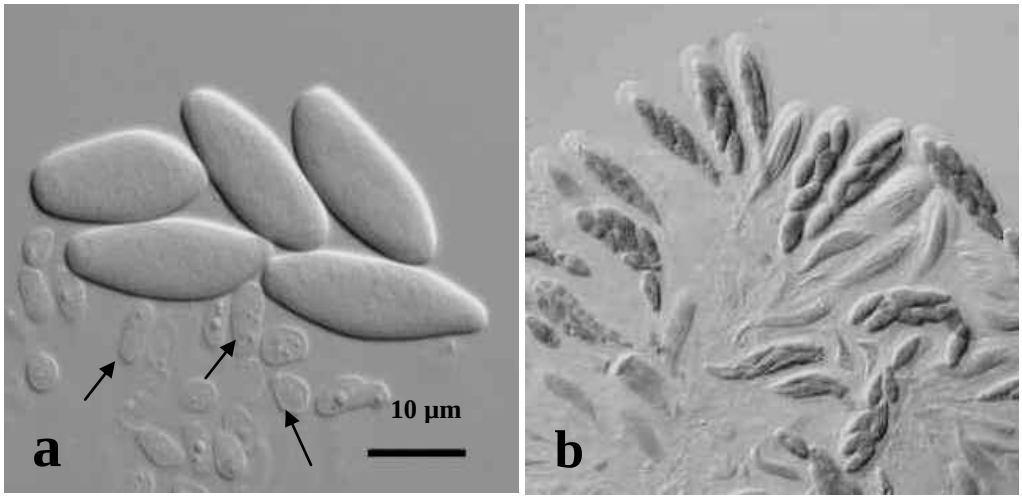

Figure 1.10: *Neofusicoccum luteum* (a) conidia and microconidia (arrowed) and (b) ascospores in ascus (Source: http://*www.crem.fct.pt/Botryosphaeria_site*) (accessed 5/11/09).

1.8.3 *Neofusicoccum parvum* (*B. parva*)

In PDA culture, the colonies produce aerial mycelium which appears similar to colonies of *F. aesculi*. However, they differ in that colonies of most *N. parvum* isolates change from white through greenish white to blackish brown on the upper surface, while the reverse side begins as faintly yellowish in colour which persists for up to 1 week and finally turns bluish black (Pennycook and Samuels, 1985; Gure *et al.*, 2005). Pycnidia may be formed on PDA cultures within 9 days after 12 h under near–ultraviolet and visible illumination. Pennycook and Samuels (1985) reported that they are largely

immersed in the medium, dark-walled, globose, non-papillate and about 0.5 mm in diameter. Their distribution is mainly peripheral, solitary or in aggregates, and they are sparsely covered in mycelium. Conidia are formed rarely and in only small quantities after several days. They are ellipsoidal with round apices and truncate bases, with size ranges of (11-) 14-18 (-23) × 5-7 (-10) and a mean length to width ratio of 2.7. They are unicellular, hyaline, smooth and thin-walled, but become light brown and 1-2 septate with age (Figure 1.11a and b). In nature, the dark walled perithecia formed on bark are morphologically indistinguishable from those of *F. aesculi* (Phillips *et al.*, 2002). The asci are bitunicate with a thick endotunica, and contain eight stipitate ascospores. The ascospores are broadly ellipsoid to fusoid, widest in the middle to upper third, with a size range of (15-) 18-27 (-29) × (6-) 8-11 µm and a mean length to width ratio of 2.4. They are hyaline, smooth, thin-walled, biseriate and aseptate, becoming light brown and 1 or 2 septate with age.

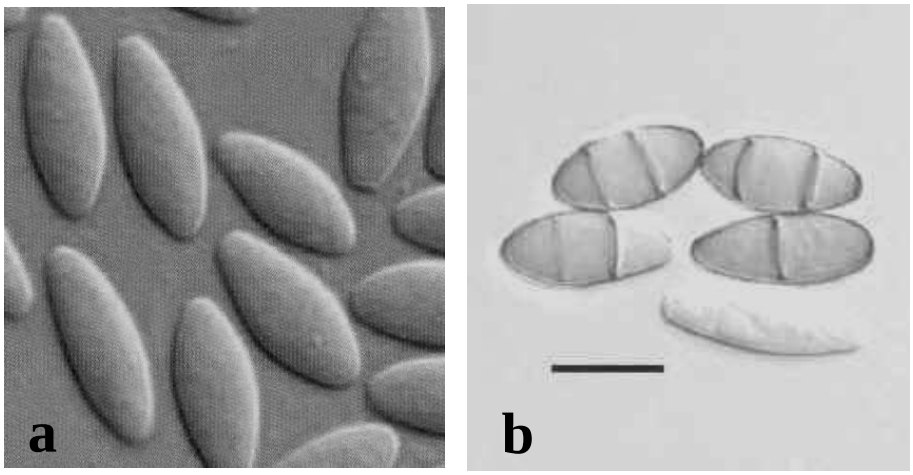

Figure 1.11: ***Neofusicoccum parvum*** **(a) young conidia and (b) aged conidia with brown walls and one or two septa. Bar = 10 µm (Source: http://*www.crem.fct.pt/Botryosphaeria_site*) (accessed 5/11/09).**

1.8.4 *Neofusicoccum ribis* (*B. ribis*)

In PDA culture, colonies are white to olivaceous buff, turning to violaceous black, often becoming rapidly darker with age. The mycelium becomes very thick and felty, extending from the agar surface to the lid of the Petri dish. The pycnidia that can be formed within 14 days on sterilised pine needles embedded in WA are superficial, globose and solitary but sometimes in aggregates. The conidia are similar to those of *N. parvum*, being ellipsoidal with round apices and truncate bases, with size ranges of (16) 19–23 (–24) × 5–6 (–7) µm and a length to width ratio of 3.8. They are unicellular, hyaline, smooth and

thin-walled, becoming light brown and 1-2 septate with age (Figure 1.12) (Slippers *et al.*, 2004b). In nature, dark-walled perithecia which emerge through the bark are 100 to 400 µm in diameter and occur in aggregations of 5–50. The ascospores are fusoid to ellipsoid, often rounded at the ends, with size ranges of (14–) 18–23 (–27) × 6–8 (–10) µm, with a mean length to width ratio of 2.8. They are unicellular, hyaline and have smooth surfaces with granular contents, becoming light brown and 1-2 septate with age (Slippers *et al.*, 2004b; Crous *et al.*, 2006).

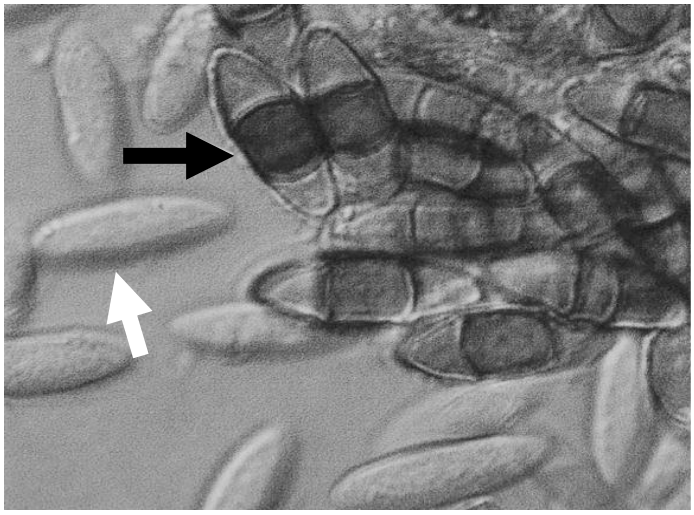

Figure 1.12: *Neofusicoccum ribis.* Mixture of young conidia (white arrow) with some aged conidia becoming dark coloured (black arrow) (Source: Slippers *et al.*, 2004b).

1.8.5 *Diplodia seriata* (*B. obtusa*)

In PDA culture, colonies appear grey-brown with dense aerial mycelium on the top whilst the underside becomes black. Pycnidia, which are formed on oat meal agar within 7 days, have dark thick walls, are small in size (0.2-1.0 mm diameter), numerous and scattered over the entire medium surface. Conidia are initially hyaline, becoming dark brown and moderately thick-walled (Figure 1.13). They are aseptate, ovoid, with obtuse apices, and truncated or rounded bases. Their size ranges are (21.5-) 22-27 (-28) × (11-) 11.5-14.5 (-15.5) µm with a mean length to width ratio of 1.9. They have smooth external walls that are roughened on the inner surface (Phillips *et al.*, 2007). In nature, the perithecia that protrude through the bark have thick dark walls and occur separately or in aggregates. The ascospores are irregularly biseriate with size ranges of (23-) 26-34 (-38) × 7-12 (-13) µm and a mean length to width ratio of 2.8. They are

hyaline, unicellular, fusoid and wider around the mid region (The Botryosphaeria website: http://www.crem.fct.unl.pt/botryosphaeria_site/).

Figure 1.13: *Diplodia seriata.* Mixture of young (hyaline) and mature (brown) conidia. Bar = 10 µm (Source: http://*www.crem.fct.pt/Botryosphaeria_site*) (accessed 5/11/09).

1.8.6 *Lasiodiplodia theobromae* (*B. rhodina*)

In oatmeal agar culture, colonies are grey-brown to black with dense fluffy aerial mycelia on the media, while the underside becomes black. Pycnidia formed on the media occur separately or in aggregations, are dark brown, and thick- or thin-walled, being 250–399 µm in diameter. Conidia are granulose, subovoid to ellipsoid-oblong and thick-walled, with truncated bases and size ranges of (18–) 20–30 × 10–15 µm and a mean length to width ratio of 2.3. They are initially hyaline (Figure 1.14a) but turn brown with age (Figure 1.14b), becoming dark brown, with longitudinal striations and 1-septate when mature (Figure 1.14c) (Fu *et al.*, 2007). In nature, perithecia formed on bark are dark brown to black with thick-walls that become thinner and hyaline towards the inner layers. Their size ranges are from 250 to 399 µm in diameter and they are usually found in aggregations. Ascospores are fusiform with size ranges of (24-) 30-35 (-42) × (7-) 11-14 (-17) µm.

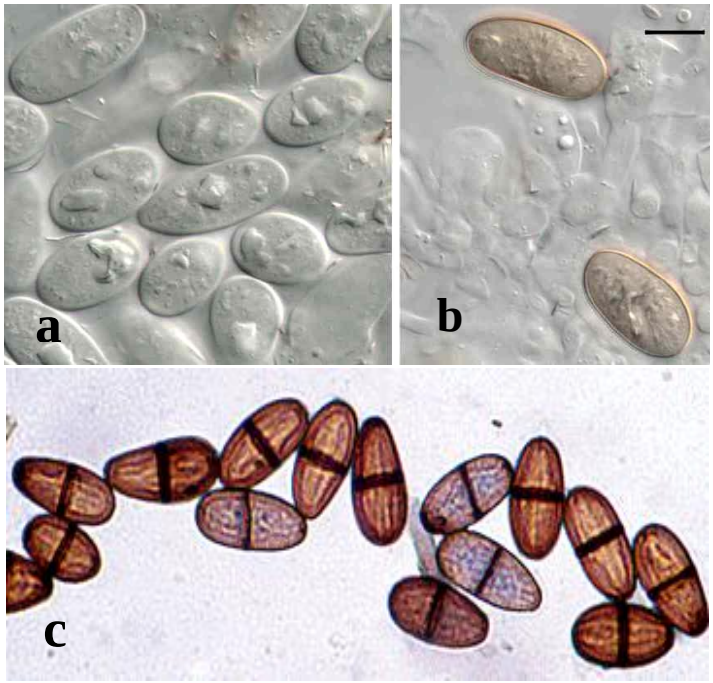

Figure 1.14: ***Lasiodiplodia theobromae.*** **(a) Immature hyaline conidia (b) conidia turning brown (Source: http://*www.crem.fct.pt/Botryosphaeria_site*) and (c) mature two-celled dark brown conidia with typical striations. (Source: *http://www.mycology.adelaide.edu.au/Fungal*) (accessed 5/11/09).**

1.8.7 *Diplodia mutila* (*B. stevensii*)

In PDA culture, colonies have abundant aerial mycelium which is initially white but turns dark to olivaceous after 5–6 days at 25^{o} C. Pycnidia formed on PDA after 21 days under daylight are thick-walled, about 600 µm in diameter and dark brown to black with an inner layer of thin-walled hyaline cells. They are immersed or partially erumpent and may be solitary or in clusters. Conidia are oblong to ovoid, straight, with both ends broadly rounded (Figure 1.15a), having size ranges of (23.5–) 25.1–25.7 (–27.4) × (12.4–) 13.2–13.5 (–14.3) µm and a mean length to width ratio of 1.9. They are hyaline, aseptate, smooth, thick-walled and rarely become pale brown (Figure 1.15b) and 1-septate with age (Figure 1.15c). In nature, perithecia formed on bark are thick-walled and dark brown to black with a size of about 300 µm. Ascospores are fusiform, widest in the middle with both ends being obtuse and with size ranges of (24.8–) 30.8–32.1 (–36.2) × (9.5–) 11.2–11.7 (–13.4) µm and a length to width ratio of 2.8. They are hyaline, thin-walled, smooth, aseptate, rarely becoming light brown and 1- or 2-septate with age (Mohali and Encinas, 2001; Alves *et al.*, 2004).

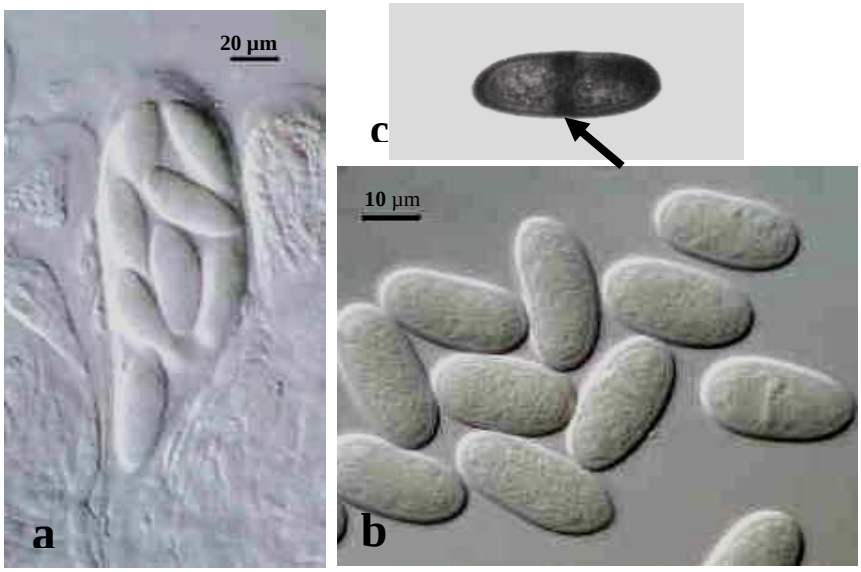

Figure 1.15: *Diplodia mutila.* (a) Ascospores in ascus (Source: Mohali and Encinas *(*2001) and (b) hyaline and unicellular conidia and (c) mature dark conidia with septation (arrow) (Source: http://*www.crem.fct.pt/Botryosphaeria_site*) (accessed 5/11/09).

1.9 Difficulties associated with morphological identification of the botryosphaeriaceous species

The identification of botryosphaeriaceous species and associated anamorphic fungi therefore, relied heavily on the characters of conidial morphology such as size, colour, septation and surface texture (Pennycook and Samuels, 1985; Denman *et al.*, 2000; Zhou *et al.*, 2001). However, production of pycnidia and conidia in culture is unreliable in many botryosphaeriaceous species. The overlapping of conidial characteristics in some species has also caused difficulties, for example the conidia of *N. ribis* and *N. parvum* are morphologically similar (Slippers *et al.*, 2004a; Crous *et al.*, 2006), as are those of *N. vitifusiforme*, *N. australe* and *N. luteum.* Given the lack of distinctive, morphological anamorph characters, molecular sequence analysis of the nuclear rDNA and ITS sequence has been used to clarify the taxonomy and phylogenetic relationships of the botryosphaeriaceous species (Denman *et al.*, 2000; Alves *et al.*, 2004; Slippers *et al.*, 2004c).

1.9.1 DNA-based identification of the botryosphaeriaceous species

The various DNA based techniques to distinguish between the botryosphaeriaceous species include use of dominant and codominant molecular markers such as randomly amplified polymorphic DNA (RAPDs), the inter simple or short sequence repeats

(ISSRs) and microsatellites (Burgess *et al.*, 2001; Zhou *et al.*, 2001). Ribosomal sequence data has also been used for a number of gene regions (Denman *et al.*, 2000; Phillips *et al.*, 2002; Slippers *et al.*, 2004b). Phylogenetic re-evaluation of the botryosphaeriaceous anamorphs has confirmed that there are two separate groups, those with dark conidia and *Diplodia*-like anamorphs and those with hyaline conidia with *Neofusicoccum*-like anamorphs (Denman *et al.*, 2000). Slippers *et al*. (2004a; 2004c) showed that a combined analysis of sequences from different gene regions namely ß-tubulin, translation elongation factor 1-α (EF1- α) and rDNA (ITS1, 5.8S, and ITS2), can effectively differentiate closely related species such as in the *N. ribis* and *N. parvum* group, and the *N. luteum* and *N. australe* group.

In a more recent and broadly based phylogenetic study, 10 lineages were identified for the Botryosphaeriaceae and these were shown to represent several newly described genera (Crous *et al.*, 2006). The teleomorph name is no longer available for the species previously referred to as *'Botryosphaeria'* and where the taxonomy remains uncertain the name '*Botryosphaeria'* should only be used in the broad sense (Slippers *et al.*, 2007). The new genera are *Neofusicoccum, Pseudofusicoccum, Neoscytalidium, Macrophomina, Dothiorella, Saccharat, Tiarosporella*, *Diplodia* and *Lasiodiplodia*, with *Botryosphaeria* only retained for *B. dothidea, B. mamane* and *B. corticis* (Crous *et al.*, 2006; Taylor *et al.*, 2009). Although there has been a new clarity to the taxonomy of the botryosphaeriaceous species, there are still some taxonomic problems in the identity and phylogenetic relationships regarding the genera with *Diplodia*-like anamorphs that belong to *Diplodia, Dothiorella* and *Lasiodiplodia* (Crous *et al.*, 2006).

1.10 Disease cycle and pathogenicity of botryosphaeriaceous diseases in grapevines

In grapevines, many authors have reported isolating botryosphaeriaceous species from declining or dead woody tissues. They have also been isolated from 1-year-old rootstock canes and certified rootstock mother vines in South Africa (Fourie and Halleen, 2004a). *Diplodia seriata* and other botryosphaeriaceous species were detected in rootstock and scion cuttings in grapevine nurseries in Spain (Giménez-Jaime *et al.*, 2006) and France (Larignon and Dubos, 2001). In New Zealand, Billones *et al.* (2009) isolated *N. luteum, N. parvum* and *D. mutila* from grafted nursery plants and from the rootstock and scion cuttings used in grafting of plants. Taylor *et al*. (2005) collected both perithecia and pycnidia from grapevines in Western Australia, indicating that ascospores and conidia may have roles in the spread of the disease.

The pathogenicity of these species has been demonstrated by infection studies, which usually relied on the use of mycelial plugs for inoculations (Castillo-Pando *et al.*, 2001; Taylor *et al.*, 2005; van Niekerk *et al.*, 2004; Wood and Wood, 2005), although it is likely conidia or ascospores are the primary infection propagule in the field. The studies which used mycelial plugs for infection of wounds made on trunks, cordons, canes and shoots of vines, demonstrated the pathogenicity of *N. australe, D. seriata, N. parvum, N. ribis, L. theobromae* and *D. mutila* isolates, with subsequent lesion formation. However, the different researchers from a range of countries, who all used local isolates, have observed differences in virulence between species. These differences may have been due to variations in susceptibility of the local grapevine varieties used or prevailing environmental conditions. Ascospores and conidia of *F. aesculi, D. seriata* and *L. theobromae* were considered to comprise a large percentage of the primary inoculum in cooler areas of the United States, such as the Upper Piedmont and mountains of North Carolina and further north, but a small percentage of the primary inoculum in most of the south-eastern United States, including the mountains of Georgia (Sutton, 1981; Brown and Britton, 1986). Further studies are needed to fully investigate the cycle of conidium germination, infection, disease progression, sporulation and dispersal within vineyards, for the major species reported worldwide. Investigations conducted on the botryosphaeriaceous pathogens of apple and peach have provided potential models to follow.

1.10.1 Disease cycles of botryosphaeriaceous diseases on non-grapevine hosts

Botryosphaeriaceous species have been found in living or dead branches, twigs and in living pedicels of many woody hosts (Wingfield *et al.*, 2001; Damm *et al.*, 2007; Slippers and Wingfield, 2007). The disease cycle on apple, which is caused by several botryosphaeriaceous species, has been more fully investigated than for other crops. Brown-Rytlewski and McManus (1999) described with a diagram (Figure 1.16) the disease cycle of *D. seriata* on apple trees. The cycle begins with winter survival as mycelium within infected tissues and as pycnidia and perithecia produced on fruit mummies, dead or blighted twigs, and living or dead cankered limbs. During favourable conditions, such as during late spring and summer rainfall, ascospores are released and infect young fruits and leaves through wounds, leading to production of lesions. The abundant pycnidia formed on the lesions release conidia for subsequent infections through natural openings and wounds, causing development of further lesions and

pycnidia throughout the growing season. Infection requires moisture and temperatures of 15-37ºC for germination and 5-37ºC for growth (Anonymous, 2005). Spread of these fungi usually occurs through air movement or splash dispersal, with vast numbers of conidia or ascospores being dispersed during spring rains (Sutton, 1981; Brown-Rytlewski and McManus, 1999; Babadoost, 2005). Spread by insects, birds, vegetative propagation and the use of contaminated pruning shears has also been reported (Michailides and Morgan, 1992; Hartill and Everett, 2002; Epstein *et al.*, 2008). Sutton (1981) observed that ascospores and conidia of *F. aesculi* and *D. seriata* were produced throughout an apple growing season from the naturally colonized apple prunings that remained in the orchard.

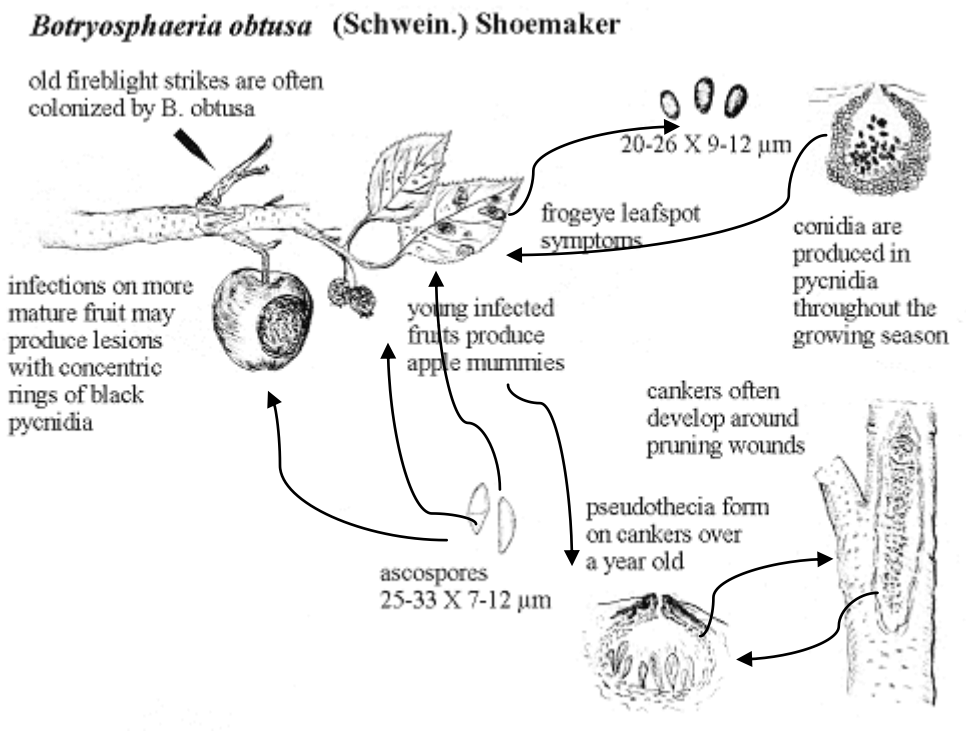

Figure 1.16: The disease cycle of *Diplodia seriata* (*B. obtusa*) as formed on apple tree (Source: Brown-Rytlewski and McManus (1999). Slightly modified.

On peach trees, the pathogens survive as perithecia in cankers which have been found to remain active for more than 1 year. These lead to infections on new shoots, blossoms, leaves, and fruit during spring when moisture is available and temperatures are 12-39ºC for *F. aesculi* and 12-32ºC for *D. seriata* (Pusey and Bertrand, 1993; Pusey, 1993; Copes and Hendrix, 2004). Weaver (1979) and Pusey and Bertrand (1993) also reported that the botryosphaeriaceous pathogens overwinter in diseased bark and in dead and dying wood

of peach, where it produces an abundance of spores, mainly conidia, which spread within the orchard in rain water, particularly during warm weather.

In pistachio, Michailides (1991) found *F. aesculi* to overwinter in rachides and shrivelled fruits that were retained on the tree as well as in perennial shoot cankers, which all provided inoculum (conidia) for winter and spring infections. In addition, pycnidia that formed on current-season's infected rachides, fruit, blighted shoots, petioles and leaf lesions provided conidia that were disseminated in windblown rain for summer and autumn infections (Epstein *et al.*, 2008).

In New Zealand avocado orchards, Hartill and Everett (2002) reported that *N. luteum* pycnidia oozed conidia throughout the season, but *N. parvum* was observed on only a few occasions. However, both species were found on dead twigs and branches. Virtually all plant parts have been reported as hosts to latent botryosphaeriaceous species, expressing symptoms such as seed capsule abortion; lesions on leaves and cankers on stems and branches, (Smith *et al.*, 1996; Slippers and Wingfield, 2007), as well as flower and fruit rot (Johnson *et al.*, 1992). However, these disease expressions are often associated with some form of stress or non-optimal growth conditions of trees (Smith *et al.*, 1994; Ma *et al.*, 2001).

1.11 Control of grapevine trunk diseases

Sanitary practices in the nursery are considered the best approach to reduce infections by many GTD pathogens (Surico *et al.*, 2008). Management of *P. chlamydospora* and several *Phaeoacremonium* spp. that cause Petri disease has been achieved by drenching rootstock cuttings in suspensions of chemical formulations to limit superficial growth on propagation material during storage and callusing stages rather than eliminating internal pathogens (Fourie and Halleen, 2004a). Sporekill (a product patented as a didecyldimethylammonium chloride formulation) at 1.5 ml/L and captan at 10 ml/L were found to be effective treatments, reducing pathogen incidences in basal ends and graft unions of uprooted plants, and did not negatively influence plant growth parameters (Fourie and Halleen, 2004a). The use of hot water treatment (at 50^{o}C for 30 minutes) prior to grafting has also been used to achieve disinfection of canes during the propagation process (Crous *et al.*, 2001; Fourie and Halleen, 2004a).

1.11.1 Preventive management practices for botryosphaeriaceous species

Wound protection by cultural (timing of pruning), chemical and biological control agents such as different bacterial and *Trichoderma* spp. have been used for some GTDs (Di Marco *et al.*, 2004; Fourie and Halleen, 2004b; Sosnowski *et al.*, 2008). Research conducted in the Cooperative Research Centre for Viticulture in Australia has identified some alternative chemicals, paints and pastes that are able to protect wounds during the period of greatest susceptibility (Sosnowski *et al*. 2004). Incidence of Eutypa dieback disease can be reduced by removing the infected wood from vines and destroying it, as well as by applying protective wound dressings on large pruning cuts. Sentinel® and Vinevax™ are biological pruning wound dressings that have also been shown to be effective in greenhouse and field studies (John *et al.*, 2005). Phomopsis cane dieback can also be controlled through good cultural practices that increase air circulation, light penetration and good soil drainage; proper timing of early-season fungicide sprays is also important for control (Ellis and Erincik, 2008).

In hosts other than grapevines, where research into aspects of the disease cycle has provided information about mechanisms of spread and infection, cultural and chemical practices have been used for control of botryosphaeriaceous diseases. For example the development of stem cankers on Proteaceae, caused by *B. protearum* can be prevented through sanitation and application of fungicides such as tebuconazole, benomyl, prochloraz, iprodione or fenarimol (Denman *et al.*, 2004). In apple orchards, pruning out of any dead wood, and removal of prunings and mummified fruits reduced the pathogen inoculum sources (Babadoost, 2005). In avocado, frequent sterilization of the clippers used to harvest the fruit reduced the incidence of stem-end rot infections, in particular those caused by *N. parvum* and *N. luteum* (Hartill and Everett, 2002). Apple limb and branch infections caused by *B. dothidea* were also found to be reduced by irrigation during hot, dry periods (Momol *et al.*, 2007). Research on the use of fungicides on pruning wounds to control the botryosphaeriaceous species has been reported for cork oak in Spain and Portugal (Luque *et al.*, 2008), for Japanese apricot and peach in China (Li *et al.*, 1995) and for pistachios in the USA (Ma *et al*., 2002).

In grapevines, research on pruning wound protection has focused mainly on *E. lata* and has been achieved by applying fungicides or biological control products (Munkvold and Marois, 1995; Sosnowski *et al.*, 2008). However, for prevention of infection by Botryosphaeriaceous species Epstein *et al.* (2008) recommended cutting out dead wood

from vines and painting the wounds with Duration (Sherwin Williams K34T154) paint, which forms an impenetrable and stable physical barrier on grapevine surgical cuts and pruning wounds, as well as removing pruned materials from the vine floor and burying them. An investigation into the use of fungicides to protect grapevine pruning wounds against infection by botryosphaeriaceous species was reported from South Africa (Bester *et al.*, 2007), while similar fungicide evaluations have been done in Australia (Pitt *et al*., 2010b). No such studies have been done on botryosphaeriaceous species associated with grapevines in New Zealand, which will be investigated in this research program.

1.12 Aims of the Ph.D

The overall aim of this study was to investigate the identity and epidemiology of the botryosphaeriaceous species associated with grapevines in New Zealand, with the following specific objectives:

1. Identify the sources of inoculum commonly available in or around vineyards from different climatic zones.

2. Identify to species level the different isolates obtained from the field sampling using both the morphological characteristics and molecular technology.

3. Develop protocols for conidium production, infection and re-isolation using the most prevalent and pathogenic species found in New Zealand vineyards.

4. Investigate routes and rates of disease progression of the pathogenic botryosphaeriaceous species on a range of grapevine tissues.

5. Identify the cultural and environmental factors that can affect disease development and spread.

6. Investigate the effectiveness of some fungicides for use as wound protectants in the control of the botryosphaeriaceous diseases in grapevines.

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Chapter 2
Distribution, identification and inoculum sources of pathogenic botryosphaeriaceous species associated with grapevine decline in New Zealand

2.1 Introduction

The grapevine trunk diseases, which have been found throughout New Zealand, can cause dieback and death, with incidence likely to increase, as the national vineyard becomes more susceptible with age (Mundy, 2008). Botryosphaeriaceous species recently gained recognition as major pathogens of grapevines worldwide (Taylor *et al.*, 2005) and the identification to species level of the main botryosphaeriaceous pathogens has been of major interest (Crous *et al.*, 2006). Some researchers have relied on a combination of cultural, morphology and molecular techniques for identification (Crous *et al.*, 2006; Phillips *et al.*, 2008). However, the use of mycelial growth characteristics of isolates at the optimum temperature was also able to assist with species identification (Slippers *et al.*, 2004b; Alves *et al.*, 2006).

In most cases, severe dieback symptoms became visible only when vines were eight or more years old, or had been subjected to stress (Larignon and Dubos, 2001). Dieback symptoms due to botryosphaeriaceous species have been reported in most grape-growing regions of the world such as Hungary (Lehoczky, 1974), Italy (Rovesti and Montermini, 1987), California (Leavitt and Munnecke, 1987; Gubler *et al.*, 2005), France (Larignon and Dubos, 2001), Australia (Castillo-Pando *et al.*, 2001; Taylor *et al.*, 2005), South Africa (van Niekerk *et al.*, 2004) and Spain (Luque *et al.*, 2005; Úrbez-Torres *et al.*, 2006).

Many of the 13 botryosphaeriaceous species reported to-date on grapevines have also been isolated from a range of other angiosperm and gymnosperm woody hosts (Phillips, 2002; van Niekerk *et al.*, 2004; Luque *et al.*, 2005). On these, they may be saprophytes or endophytes, sometimes existing for long periods of time in the absence of symptoms (Denman *et al.*, 2000; Slippers and Wingfield, 2007), but often associated with dieback and wood cankers. However, the diseases caused by botryosphaeriaceous species on Eucalyptus were almost always associated with stress or wounding (Mohali *et al.*, 2009).

Earlier reports in New Zealand had identified botryosphaeriaceous species as pathogens of blueberries (Johnston and McKenzie, 1982; Young and Fletcher, 1997), kiwi fruit, apple and poplar (Pennycook and Samuels, 1985).

Most researchers working on diseases caused by botryosphaeriaceous species have used mycelium-colonised agar plugs for pathogenicity studies (Sánchez-Hernández *et al.*, 2002; Maloney *et al.*, 2004; Taylor *et al.*, 2005), which does not reflect the natural situation in which conidia and ascospores are likely to play a major role in infection. Mycelium was probably used for inoculation because the researchers had difficulty producing sufficient conidia in culture. The various types of agar media commonly used to induce sporulation, include half-strength potato dextrose agar (½PDA) (Alves *et al.*, 2004), prune agar (PA) (Leung *et al.*, 1988), oat meal agar (OMA) (Phillips *et al.*, 2005), malt extract agar (MEA) (Gure *et al.*, 2005) and water agar (WA) embedded with sterile pine needles (van Niekerk *et al.*, 2004).

The aim of this study was to identify common sources of infection in and around vineyards, to identify the botryosphaeriaceous species and to develop infection protocols that could be used for future experiments. This involved two series of experiments which has been divided into two sections.

Section 1 (Sampling of grapevine tissues and species identification)

(i) Collection of necrotic tissues from a range of non-grapevine plants and different grapevine cultivars, as well as from woody detritus on the vineyard floor for isolation of botryosphaeriaceous species, (ii) identification of the isolates using morphological characteristics and molecular methods (iii) development of a reliable method for producing large quantities of conidia for this and subsequent infection studies.

Section 2 (Test for pathogenicity)

(iv) Testing the pathogenicity on grapevines of isolates that had been recovered from both grapevines and non-grapevine woody hosts, (v) determining the susceptibility of some of the commonly used grapevine varieties to infection by selected isolates, (vi) determining *in vivo* pathogenic effects of mycelium and conidium inoculum and (vii) use of molecular techniques to confirm the presence of the pathogen in grapevine wood tissues.

2.2 Materials and Methods

2.2.1 Vineyard sampling

The 20 vineyards from which samples were collected in November 2006 and April 2007 comprised six in Canterbury, six in Marlborough, four in Nelson and four in Auckland (Appendix B.1). From each vineyard, 10 grapevines that exhibited characteristic symptoms, such as stunted growth, trunk, shoot or cane dieback, or bleached canes, were selected for sample removal. The samples collected comprised the wood showing dieback and other necrotic tissues, such as weak buds, flower buds and leaves, as well as woody vine debris (Figure 2.1). In addition, necrotic tissues were collected from within and around a grapevine propagation nursery in Gisborne (Appendix B.1), courtesy of Dr. Roderick Bonfiglioli. The samples were taken from stumps and cordons of nursery rootstock mother vines, varieties 101-14, 3309, Schwarzmann and Richter and other non-grapevine plants.

Non-grapevine woody hosts and woody weeds growing around vineyards were also inspected for symptoms of dieback and necrotic wood lesions (Figure 2.2). The symptomatic woody tissues were collected for isolation. In addition, symptomatic woody tissues from three blueberry bushes growing near a vineyard were also collected by Dr. Marlene Jaspers in Hastings.

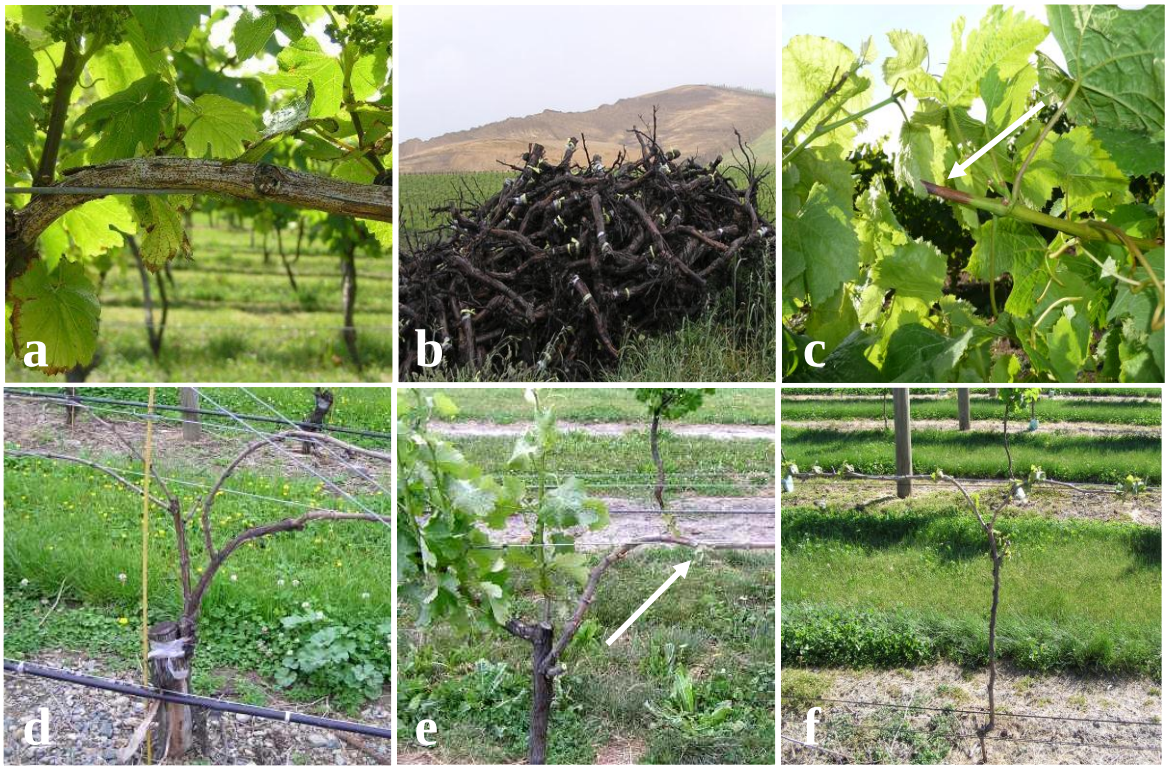

Figure 2.1: Examples of the diseased grapevines from which samples were collected for pathogen isolation (a) bleached cane, (b) trunks removed from vineyards, (c) green shoots that had dieback after pruning, (d) grapevine canes with dieback, (e) weak grapevine buds and (f) vines with general growth decline.

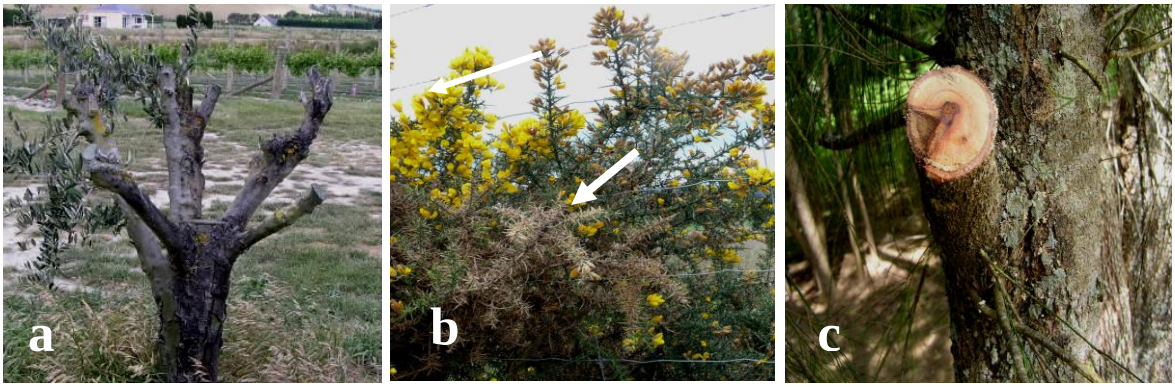

Figure 2.2: Examples of diseased non-grapevine woody hosts found close to vineyards from which samples were collected for pathogen isolation (a) olive tree with dieback symptoms, (b) broom (woody weed) showing tip dieback and (c) pine branch with necrotic canker revealing a wedge shaped lesion.

2.2.2 Isolation of fungal pathogens from the plant tissues

The plant samples were washed under tap water and initially observed under a stereo microscope (Olympus® BX51, Olympus Optical Co. Ltd. Japan) for characteristic necrotic scars and cankers. One half of each sample was incubated under high relative humidity (within sealed containers with moist paper towels) for 3 days at room temperature (18-24^{o}C) to try to induce pycnidium production and conidium exudation. The other half was surface-sterilized by immersing the sample in 70% ethanol for 30 s, then in 1% sodium hypochlorite (NaOCl) for 1 min and again in 70% ethanol for 30 s (van Niekerk *et al.*, 2004), then rinsed twice in sterile tap water and finally dried under sterile air flow in the laminar hood (Airpure™ Westinghouse Pty Ltd Inc. NSW). From each of these plant samples, 10 pieces (3-5 mm^2) of tissue were aseptically excised from the edges of the necrosis and placed cut surface down onto half-strength potato dextrose agar (PDA; Difco™ Becton, Dickinson and Company, Maryland, USA) amended with 50 µg/ mL chloramphenicol (Sigma-Aldrich™ Co. St. Louis, MO USA) (½ PDA-Cph) to suppress bacterial growth (Cao *et al.*, 2002). The plates were incubated at room temperature (18-24^{o}C) for 3 to 4 days until fungal colonies were observed. The fungal colonies emerging from the plant pieces that were characteristic of botryosphaeriaceous species, as shown on the Botryosphaeria website (http://www.crem.fct.unl.pt/botryosphaeria_site/), were aseptically subcultured onto individual Petri dishes containing PDA for pure culture isolation and identification. The individual isolates were assigned culture collection numbers (Appendix B.2).

2.2.3 Morphological characterization of botryosphaeriaceous species by colony and conidium characteristics

For morphological description of colony colour and growth characteristics, all the subcultured fungal colonies characteristic of the botryosphaeriaceous species were incubated at 24.5°C in the dark. After 3, 7 and 30 days' incubation, the cultures were observed morphologically and allocated to groups according to colony growth and colour characteristics and the production of pycnidia. After 30 days, the plates containing pycnidia were flooded with 10 mL of sterile water containing 0.01% of Polyoxyethylene (20) sorbitan mono-oleate (Tween 80; BDH Chemicals Ltd, Poole, England), and the pycnidia dislodged from the agar with a sterile scalpel, causing them to float in the water. The pycnidia and wash water were aseptically placed into a mortar

and crushed with a pestle to release the conidia into the water. The conidium suspension was then centrifuged for 5 min at 2000 × *g* and some of the supernatant discarded. The remaining 5 mL of suspension was vortexed to resuspend the conidium pellet so that the conidia could be examined and the isolates assigned to groups based on the observed colony and conidium characteristics.

2.2.3.1 Induction of sporulation using a range of media

Since not all isolates were able produce numerous pycnidia and conidia on PDA, a range of media were tested with three representative isolates each from Groups 1, 2, 3, 4 and 5, as determined initially by colony type and later confirmed with molecular technology to represent *N. luteum*, *N. australe*, *D. mutila*, *N. parvum* and *D. seriata*, respectively (Appendix B.3). Group 6 was not tested as it consisted of only one isolate. The origin and identity of the isolates are given in Appendix B.2. These isolates were subcultured for 3 days on PDA and mycelia disc from the edge of colonies used to inoculate the test agars. The media used included half-strength PDA, oat meal agar (OMA), prune agar (PA), and malt extract agar (MEA) (Appendix B.4). The two replicate cultures were randomly allocated to positions within a 25ºC dark incubator and grown for 4-5 weeks. The pycnidia were counted under ×40 magnification using stereo a microscope and then dislodged and conidia harvested as before, except that the resulting conidium slurry was filtered through a double layer of cheesecloth before being centrifuged and vortexed. The concentrations of conidia in the suspensions obtained from the different media were determined using a haemocytometer.

2.2.3.2 Induction of sporulation on grapevine green shoots tissues and on sterilised pine needles in water agar

Green grapevine shoots were inoculated to induce lesions and pycnidia. Grapevine shoots 20-25 cm long were cut from field-grown Pinot noir vines in summer. The base of each shoot was inserted into a Universal bottle filled with water and the top wrapped with Parafilm® (Pechiney Plastic Packaging Co., Chicago, IL) to support it and to prevent evaporation. Mycelium colonised agar discs (3 mm) cut from the growing edges of 3-day-old PDA cultures of the same isolates as above were used to inoculate superficial fresh wounds (~4 mm in diameter) immediately after they had been made in the centre of the shoot using a sterilised scalpel, with the mycelium surface of the disc facing the wound. The six replicate shoots per isolate were randomly allocated to positions within an enclosed transparent chamber at room temperature (18-24^{o}C), with frequent misting for the first 3 days to ensure high humidity for pathogen infection and

lesion development. After 10 days incubation, the shoots were removed from the chamber, and the 6-10 cm lesion sections were surface sterilised and air dried within a laminar hood for 6 h. The dried stem lesions were placed on sterilised moist filter paper in a Petri dish, sprayed with a fine mist of sterile water and incubated for 24-36 h at 25^{o}C to allow pycnidium development and release of conidia. Shoot sections with pycnidia that oozed conidia were each placed into a 45 mL centrifuge tube containing 20 mL sterile water with 0.01% Tween 80 and shaken vigourously by hand for 2 min to disperse the conidia into the water. The liquid suspension from each tube was filtered through two layers of Miracloth® (CalBiochem, EMD Biosciences Incorporated, CA, USA) and centrifuged at 1295 × *g* for 15 min. The top 10 mL of the supernatant was carefully poured out and the remaining 10 mL of the suspension was vortexed at maximum speed for 30 s to re-suspend the conidia.

Five pieces of autoclaved pine needles (30 mm long) were placed onto the 2% molten water agar (WA) poured into each Petri dish. After being allowed to solidify, the agar plates were inoculated centrally with mycelium colonised agar discs (3 mm diameter) cut from the growing edges of 3-day-old PDA cultures of the same isolates as used previously. Three replicate plates per isolate were then incubated in the dark as described for the above media, and the pycnidia removed and crushed to release conidia, as before in Section 2.2.3.

Isolates from all groups produced pycnidia and conidia except for those of Group 4, for which several attempts were made. However, when shoots were finally inoculated with a mycelium mixture made from three isolates belonging to Group 4, pycnidia and conidia were produced. These conidia allowed for completion of the conidium descriptions.

The concentrations of conidia in the suspensions obtained from both the pine needles embedded in the water agar and the grapevine shoots were determined using a haemocytometer. (Sections 2.2.3.1 and 2.2.3.2 have been modified and published in New Zealand Plant Protection, 61: 301-305).

2.2.3.3 Morphological characterization of conidia

The concentrated conidium suspensions were mounted onto microscope slides and observed at 100 × magnification with a light microscope for characteristic shape, colour, size (length and width) and other distinguishing features. Digital images were captured using a digital camera mounted on the light microscope. The lengths and widths of 50 conidia from each of the isolates were measured using the AnalySIS® imaging software,

which also calculated the means and standard deviations. The length and width ratios were analysed using GenStat, which also calculated the 95% confidence intervals.

In some cases, the morphological characteristics of the conidia caused some isolates to be reassigned to different groups. This occurred more commonly with Groups 1 and 2 which had similar colony characteristics. In most cases, the conidial characteristics confirmed the groups assigned by colony and mycelium appearance. The group characteristics were used to assign different species names to them. This was done by comparing the mycelium colony growth and colour characteristics and the conidium characteristics to those published on the Botryosphaeria website (http://www.crem.fct.unl.pt/botryosphaeria_site/). All the pure culture isolates from both grapevines and non grapevine woody hosts were stored for a few days at 4^{o}C on PDA plates prior to further subculturing onto PDA and incubation at different temperatures to determine whether their ranges and optima could assist with presumptive identification.

2.2.4 Temperature effect on mycelium growth characteristics

Three isolates were selected from each morphological group, based on the initial presumptive identification made in Section 2.2.3.3. Groups with less than two isolates assigned to them were not included. A total of 15 isolates (Appendix B.3) were tested from five of the groups. The isolates were selected to represent the variability in colony morphology within each group. The isolates were inoculated centrally onto PDA using mycelium colonised agar discs of 5 mm diameter cut from the leading edges of 3-day-old cultures. For each isolate, three replicate plates were incubated at each of six temperatures: 10, 15, 20, 25, 30 and 35 ºC (±1ºC) in continuous darkness for 48 h. One incubator was used for all temperature settings, with temperature order selected at random and replicates and isolates placed in a completely randomised design. After 48 h incubation, colony diameters were measured at two positions at right angles to each other, using a digital calliper (Mitutoyo, U.K Ltd). The mean diameters from the two perpendicular measurements (less the diameter of the inoculation disc) were recorded as the mycelial growth for that isolate. The experiment was repeated once.

2.2.5 Statistical analysis

Pycnidium numbers and conidium counts obtained with the haemocytometer in Sections 2.2.3.1 and 2.2.3.2 from different media and on green shoots or pine needles, respectively, were then analysed with analysis of variance (ANOVA) using GenStat

version 11[Lawes Agricultural Trust (Rothamsted Experimental Station)]. Because Group 4 (*N. parvum*) isolates did not sporulate on any of the above media tested and the delay in getting them to sporulate on the green shoots, it was eliminated from the analysis. The colony diameters after growth at different temperatures for 48 h (Section 2.2.4) were also analysed with ANOVA. Where there was a significant difference, the means were separated by Fisher's protected least significant difference (LSD) at $P \leq 0.05$. The mean diameter growth for the individual isolates was plotted against the temperature and the curve transformed into quadratic function of the form $Y=-AX^2+BX-C$, where Y is the optimum growth and X is the optimum temperature.

2.2.6 Confirmation by molecular technology of the presumptive identifications of species

For confirmation of the presumptive morphological identifications, 7, 6, 4 and 3 isolates, from Groups 1, 2, 3 and 4, respectively, a total of 20 isolates were randomly selected for verification using molecular techniques. The number of isolates selected per group depended upon the total number of isolates assigned to each group, according to their colony and conidium characteristics.

2.2.6.1 Genomic DNA extraction

A tuft of mycelium taken from the growing edge of each 5-day-old culture growing on 2% WA was used to inoculate 20 mL of potato dextrose broth (PDB, Difco™ Becton, Dickinson and Company, Maryland, USA). After 7 days incubation at approximately 24°C in continuous darkness, the mycelia were harvested, squashed between two layers of sterile Miracloth to remove the broth, wrapped in aluminium foil, snap frozen in liquid nitrogen and stored at -80ºC until use. The frozen mycelium from each sample was finely ground with a heat-sterilised mortar and pestle in liquid nitrogen prior to DNA extraction. The genomic DNA was extracted using the PureGene genomic isolation kit (Gentra systems, Minneapolis, MN). Typically, approximately 20 mg of finely ground, frozen mycelium was put into a 1.5 mL tube followed by addition of 500 µL cell lysis solution and then incubated at 65^{o}C for 60 min. The tube was inverted 10 times to mix and cooled to room temperature (approx. 21^{o}C), and then 167 µL of protein precipitation solution was added and the mixture vortexed at high speed for 20 s. Each tube was centrifuged at 13,000 × g for 1 min to pellet all proteins and the supernatant containing the DNA was poured into a clean 1.5 mL tube containing 500 µL of 100% isopropanol. The sample was mixed by inversion for 20 s and then centrifuged at 13,000

× g for 1 min to pellet the DNA. The supernatant was discarded and the pellet washed in 300 µL of 70% ethanol. Samples were then centrifuged at 13,000 × g for 1 min after which the pellet was air- dried by inverting the tube on clean absorbent paper for 10-15 min. After air drying, 100 µL DNA hydration solution (TE) was added to each sample tube and the DNA left overnight to resuspend. On the next day, the tube was incubated at 65^{o}C for 60 min to aid re-suspension and centrifuged at 13,000 × g for 5 min. The supernatant containing the DNA was then transferred into a clean 1.7 mL tube and its concentration determined by absorbance using a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Delaware, USA).

2.2.6.2 PCR amplification

The ITS1, 5.8S and ITS2 regions of the ribosomal DNA were amplified using the universal primers ITS1 ($^{5}\prime$-TCCGTAGGTGAACCTGCGG-$^{3}\prime$) and ITS4 ($^{5}\prime$-TCCGTAGGTGAACCTGCGG-$^{3}\prime$) (White *et al.*, 1990) (Invitrogen Technologies, New Zealand). Each polymerase chain reaction (PCR) contained 1 × buffer (40 mM Tris HCl pH 8.5, 2 mM $MgCl_2$ and 25 mM KCl), 200 µM of each dNTP, 5 µM of each primer, 1.25 U of Hotstart Taq polymerase (MBI Fermentas, Vilnius, Lithuania) and 20 ng of template DNA in a total volume of 25 µL per isolate sample. The non-template control included sterile water instead of the template DNA. Amplification using a Bio-Rad iCycler Thermal cycler (Hercules, California, USA) was achieved by the following temperature regime: 2 min initial hot start at 94°C, followed by 35 cycles of 30 s at 94°C (denaturing), 30 s at 55°C (annealing) and 30 s at 68°C (elongation), with a final elongation period of 7 min at 68°C. After the amplification, each PCR product (5 µL) was separated by electrophoresis in a 1% agarose gel in 1 × TAE buffer (40 mM Tris acetate, 2 mM Na_2EDTA, pH 8.5). Each PCR product was prepared by combining it with 2 µL of 6 × loading buffer (0.025% bromophenol blue, 0.025% xylene cyanol FF, 40% (w/v) sucrose in water). The molecular marker was prepared in a similar manner, except that the 5 µL PCR product was replaced by 5 µL of 1 Kb plus DNA Mass^{TM} Ladder (Invitrogen). The samples were loaded into individual wells in the prepared gel and separated by electrophoresis at 10 V/cm for approximately 45 min. The gel was then stained with 0.5 µg/mL ethidium bromide (Amresco®, OH, USA) for 15 min, destained with tap water for 10 min and visualised with ultraviolet light, using the Versa Doc™ imaging system (Biorad). This ensured that only a single band of the expected size was produced, thus indicating the purity of amplified DNA prior to sequencing.

2.2.6.3 DNA sequencing

Directly after amplification, the PCR products were sequenced by the Lincoln University DNA sequencing facility using an ABI PRISM® 310 Genetic Analyzer (PE Applied Biosystems, Foster City, California, USA). The sequences of the rRNA gene region (partial 18S, ITS1, 5.8S gene, ITS2, and partial 28S sequences) were checked using Sequencher™ (Gene Codes Corporation, Michigan, USA) and sequences compared with those of known origin using the Basic Local Alignment Search Tool (BLAST) search nucleotide database programme from the website (http://www.ncbi.nlm.nih.gov). These results were used to confirm the identities of the remaining 43 isolates, according to their morphological group, that matched the groups of the 20 isolates identified.

2.3 Results

2.3.1 Vineyard sampling

Some of the symptomatic tissues, collected for isolation from a range of grapevine varieties, are shown in Figure 2.3. The regions from where the botryosphaeriaceous species were isolated, the grapevine varieties and plant ages are listed in Table 2.1.

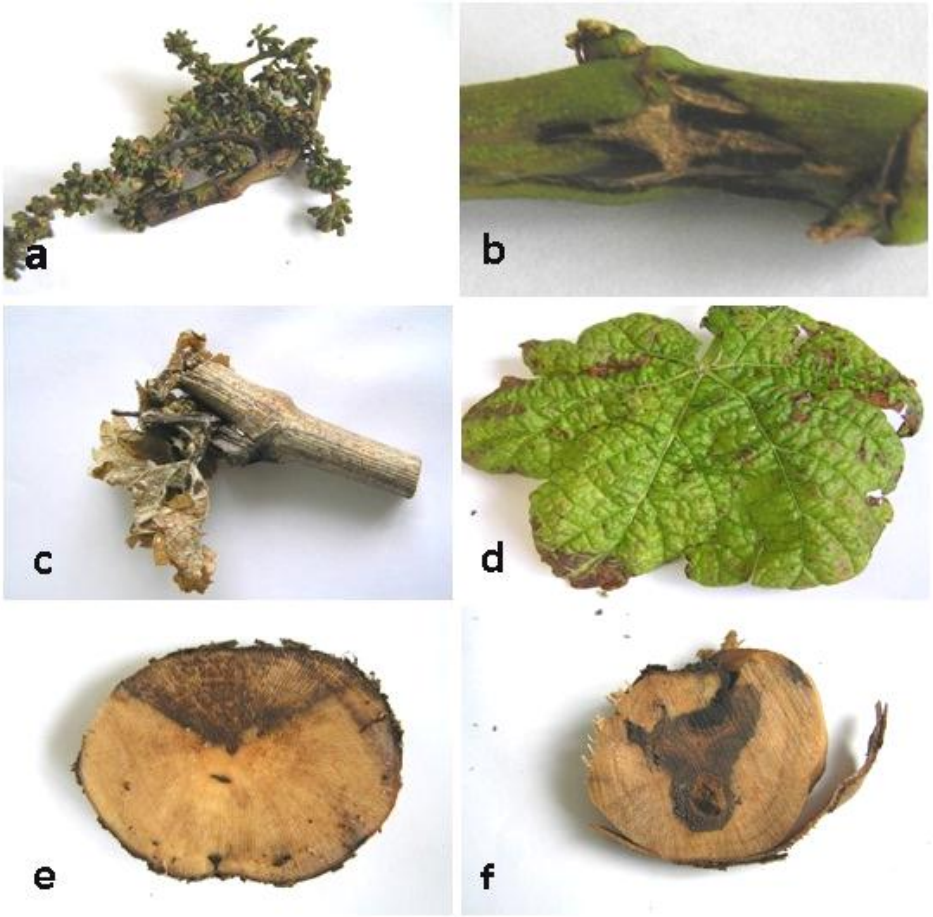

Figure 2.3: Tissues with different grapevine symptoms from which the botryosphaeriaceous species were isolated (a) dead or shrivelled flowers (b) shoot lesion, (c) weak bud with dead shoot (d) leaf spots, (e) wedge-shaped trunk necrosis and (f) internal necrosis in trunk.

Table 2.1: Vine age, variety, tissue type and botryosphaeriaceous species isolates obtained from vineyards in five regions sampled in either November 2006 or April 2007***

Region	Vineyard code for isolates	No. of isolates	No. of species	Age of grapevines during sampling	Varieties	Presence (+) or absence (-) of botryosphaeriaceous species						
						Trunks/ cordons	Canes	Plant Debris	Weak Buds	Leaves	Flowers	Green shoots
Canterbury	1. La*	3	2	25	Beidecker	+	+	-	-	-	-	+
	2. Ts**	0	0	1	Reisling GM 239	-	-	-	-	-	-	-
	3. Rd**	1	1	20	Pinot noir	+	-	-	-	-	-	-
	4. Kat*	2	2	23	Pinot noir	+	+	+	+	-	-	+
	5. Mel*	4	1	20	Pinot noir	+	+	+	+	-	-	+
	6. San*	1	1	No records	Chardonnay	+	-	-	-	-	-	-
Nelson	1. H**	4	3	15	Sauvignon blanc	+	-	-	-	-	-	-
	2. I**	3	3	33	SO4	+	-	-	-	-	-	+
	3. J or K	3	2	20	Chardonnay, Pinot noir	+	-	-	+	-	-	+
	4. L**	5	3	2	Pinot noir, Reisling	+	+	-	-	-	-	-
Marlborough	1. C**	0	0	4	Sauvignon blanc	-	-	-	-	-	-	-
	2. D**	1	1	5	Sauvignon blanc	-	+	-	-	-	-	-
	3. G**	1	1	3	Sauvignon blanc	-	-	+	-	-	-	-
	4. F**	2	2	No records	No records	+	-	-	-	-	-	-
	5. Q or Q(s)**	4	3	22	Sauvignon blanc, Chardonnay	+	+	-	-	-	-	+
	6. R(s)**	1	1	23	Reisling	+	-	-	-	-	-	-
Auckland	1. .M**	4	2	7	Chardonnay (clone 95)	+	+	-	-	-	+	+
	2. N**	3	2	23	Cabernet sauvignon	+	-	-	-	+	-	+
	3. O**	1	1	30	Chardonnay	+	-	-	-	-	-	-
	4. Cob**	2	2	No records	Rootstock varieties †	+	-	-	-	-	-	-
Gisborne	1. RGSC, Sch, G(s)-1*	4	2	No records	Rootstock varieties †	+	-	-	-	-	-	-

† Rootstock varieties 3309, 101-14, Richter and Schwarzmann

2.3.2 Isolations of fungal pathogens from plant tissues

The moist incubated tissues produced no pycnidia or conidia in 3 days. Botryosphaeriaceous fungi were isolated from trunks (42%), green shoots (20%), canes (17%), plant debris (7%), weak buds (8%) leaf spots (3%) and shrivelled flowers (3%) (Figure 2.4). From the grapevine tissues, 49 isolates were characteristic of the botryosphaeriaceous species (Appendix B.2). Out of the 20 vineyards sampled, species characteristic of the botryosphaeriaceous fungi were isolated from seven scion cultivars with ages ranging from 2 to 33 years (Table 2.1). Botryosphaeriaceous species were also isolated from trunks and cordons of the four rootstock varieties, 3309, Richter, 101-14 and Schwarzmann that were sent from Gisborne (Figure 2.5). Multiple botryosphaeriaceous species were isolated from most vineyards and in all the major grapevine growing regions.

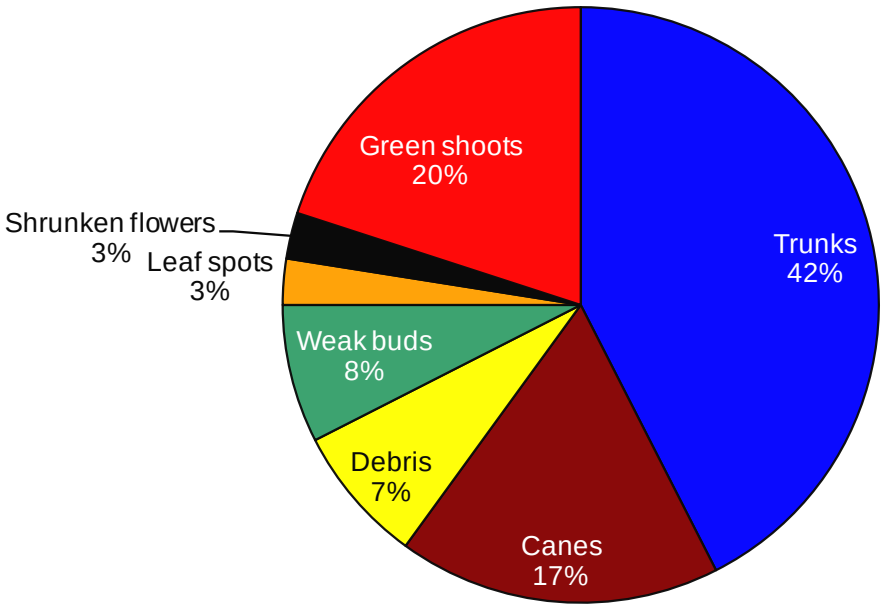

Figure 2.4: Incidence of botryosphaeriaceous isolates with respect to the collected grapevine tissues.

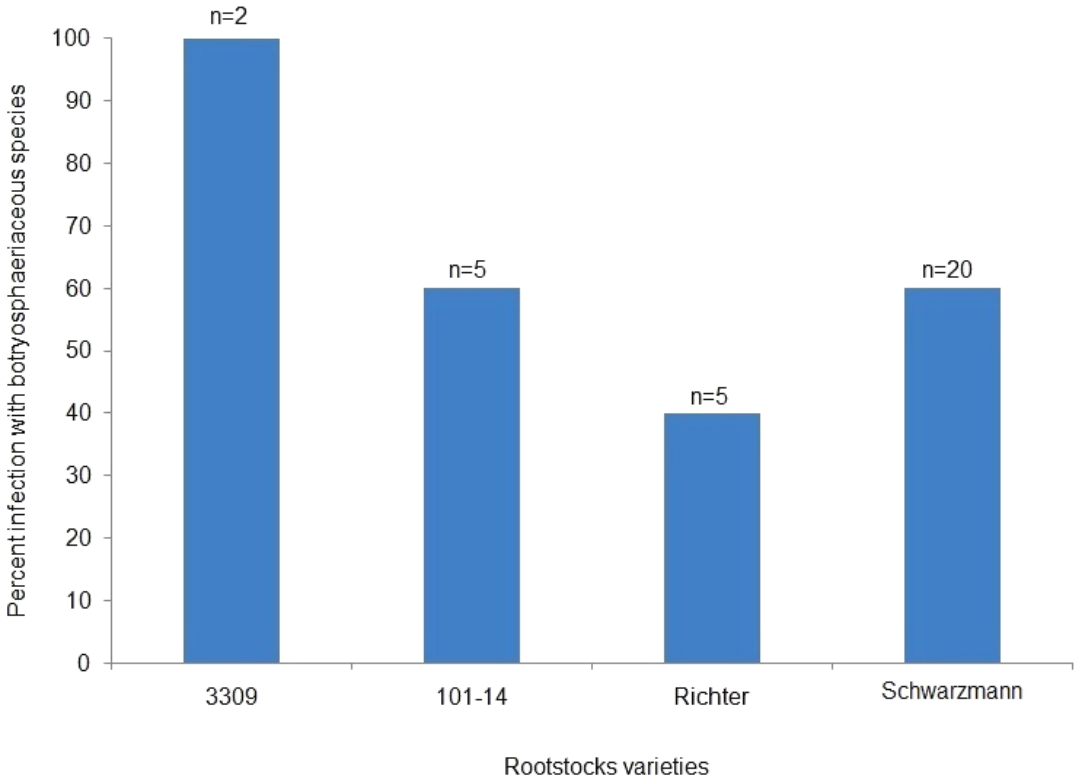

Figure 2.5: Percent botryosphaeriaceous species isolated from the different rootstock varieties sent from a grapevine nursery in Gisborne (n represents the total number of samples).

Another 14 isolates characteristic of botryosphaeriaceous species were also isolated from non-grapevine woody hosts growing close to some vineyards, which showed signs of wood decay or dieback (Table 2.2).

Table 2.2: Identity and origin of botryosphaeriaceous species isolates obtained from non-grapevine woody hosts growing close to the vineyards.

Isolate	Identity	Host	Property	Region
MJ-1	*N. luteum*	Blueberries	Gourmet Blueberries	Hastings
MJ-2	*N. luteum*	Blueberries	Gourmet Blueberries	Hastings
MJ-3	*N. luteum*	Blueberries	Gourmet Blueberries	Hastings
F-1	*D. mutila*	Willow	Brancott	Blenheim
C-4	*D. mutila*	Olive	Dracquoh	Blenheim
Iso-2	*D. mutila*	Native Ngaio	Linnaeus	Gisborne
I-1	*D. seriata*	Lemon wood	Kahurangi	Nelson
O-1	*N. parvum*	Pine	Matua Valley	Auckland
La-1	*N. luteum*	Pine	Larcombs	West Melton
O-3	*N. parvum*	Cherries	Matua Valley	Auckland
A-3	*D. mutila*	Plum	Mc Kendry Park	Blenheim
A-2	*D. mutila*	Apple	Mc Kendry Park	Blenheim
J-3	*N. australe*	Broom	Neudorf	Nelson
J-4	*D. mutila*	Oak	Neudorf	Nelson

2.3.3 Presumptive identifications

2.3.3.1 Morphological characterizaion of botryosphaeriaceous species by colony and conidium characteristics

The assignment of isolates into groups was determined by colony characteristics (Section 2.3.3.3). Mycelium colour and colony growth characteristics on PDA after 3, 7 and 30 days incubation at 25^{o}C in continuous darkness showed consistent differences between groups of isolates. In all, six groups were identified among all the isolates from grapevines and non-grapevine woody hosts. Groups 1 and 2 colonies were similar at day 3 but the colours of the upper surfaces and the reverse sides of colonies gradually changed with time. Conidia were required for morphological characterisation by conidium colour, shape and size which allowed differentiation to species level.

2.3.3.2 Induction of sporulation using a range of media

There was no significant interaction between the groups and media ($P=0.43$ and $P=0.36$, respectively) for pycnidium or conidium production (Appendices B.5.1 and B.5.2). Group 4 isolates did not produce pycnidia on any of the medium types tested. However, the Group 6 (one isolate only) produced sufficient pycnidia which oozed conidia on PDA and so it was not included in the experiment. The effect of the media type on numbers of pycnidia was highly significant ($P<0.001$; Appendix B.5.1), with OMA and PA having higher numbers of pycnidia than ½PDA and MEA for isolates from all the groups (Table 2.3). Pycnidium and conidium production differed between groups ($P<0.001$), with Group 5 isolates producing significantly more conidia than Group 3 isolates, which produced more conidia than the Group 1 and Group 2 isolates.

Table 2.3: Mean numbers of pycnidia (P) per plate and conidia (C) (x10^4) /mL produced after 4 weeks incubation for the four botryosphaeriaceous groups (presumptive species) on malt extract agar (MEA), oatmeal agar (OMA), prune agar (PA) and half strength potato dextrose agar (½PDA).

Media type		Group 1	Group 2	Group 3	Group 5	Media effect
MEA	**(P)**	48.0	18.7	32.0	186.7	**71.3 b**[1]
	(C)	3.0	1.3	4.3	16.0	**6.2**
OMA	**(P)**	157.3	186.7	208.0	360.0	**228.0 a**
	(C)	1.7	1.7	7.3	18.3	**7.3**
PA	**(P)**	266.7	152.0	98.7	328.0	**211.3 a**
	(C)	3.7	1.3	6.0	21.0	**8.0**
½PDA	**(P)**	93.3	18.7	80.0	226.7	**104.7 b**
	(C)	1.7	1.3	5.7	23.3	**8.0**
Groups effect	**(P)**	**104.7 f**[1]	**94.0 f**	**141.3 f**	**275.3 g**	
	(C)	**2.5 c**[1]	**1.4 c**	**5.8 d**	**19.7 e**	

[1]Values within the rows or columns followed by the same letters are not significantly different according to Fisher's protected LSD at $P \leq 0.05$. Media effect (a-b). For pycnidium production (f-g), the main effect of both media and species was highly significant ($P<0.001$; LSD=57.51). For conidial production (c-e), only the main effect of species was highly significant ($P<0.001$; LSD=2.41). Table has been slightly modified from Amponsah *et al*., 2008.

2.3.3.3 Induction of sporulation on grapevine green tissue and on sterilised pine needles in water agar

Inoculated grapevine green shoots clearly produced more pycnidia and conidia than on pine needles (Table 2.4). Pycnidium and conidium production on the green grapevine shoots also differed significantly between botryosphaereaceous groups ($P<0.001$ for both; Appendix B.5.3). Pycnidium and conidium production was highest for Group 1, followed by Group 2 and Group 3 which were similar, and Group 5 produced the least (Table 2.4).

On pine needles embedded in water agar, numbers of pycnidia and conidia differed significantly between botryosphaeriaceous groups ($P<0.001$ for both; Appendix B.5.4) Group 1 isolates had significantly higher numbers of pycnidia and conidia followed by Group 3 and Group 5, with the least pycnidia produced by Group 2 (Table 2.4). Conidium production from Group 4 isolates was only achieved 3-4 months later after inoculating wounds created on green grapevine shoots (Section 2.2.3.2) with a mixture of isolates.

Table 2.4: Mean numbers of botryosphaeriaceous species pycnidia and conidia (no./mL x 10^4) produced per 30 mm length of autoclaved pine needle embedded in water agar and on 15 mm lesion lengths of green Pinot noir grapevine shoots.

Botryosphaeriaceous Species	Pine needle in water agar		Green grapevine shoot	
	Pycnidia	Conidia	Pycnidia	Conidia
Group 1	21.3 a[1]	20.0 a	165.7 a[1]	186.0 a
Group 2	6.7 c	15.0 b	143.7 b	161.7 b
Group 3	11.7 b	15.7 b	137.3 b	147.0 b
Group 5	10.0 b	13.3 b	5.0 c	14.7 c
LSD	**2.49**	**3.35**	**16.46**	**15.86**

[1]Means followed by the same letters within each column are not significantly different according to Fisher's protected LSD at $P \leq 0.05$. Table published in Amponsah *et al.*, (2008).

The methods above allowed production of conidia from isolates in Groups 1-6 and these were used for identification of the groups, originally assigned by their similar colony characteristics, to species level. The identifications were made from previously published descriptions and from the Botryosphaeria website (http://www.crem.fct.unl.pt/botryosphaeria_site/) based on colour, size and shape of conidia and characteristics of the colonies, grown on PDA at 25ºC in continuous darkness.

Group 1

The mycelia of the Group 1 isolates showed deep yellow pigmentation after 3 days incubation (Figure 2.7). Upon reaching the edge of the Petri dish, the colony margin became more fluffy and dense than at the centre. The yellow pigmentation visible on the surface and reverse side of the plate at day 3 gradually changed to a violaceous colour by day 7 and then darkened, with the reverse side becoming black after 30 days. The aerial mycelium became grey and flat at the centre of the colony by day 7, and by day 30 the entire colony surface then became grey to dark grey with production of pycnidia covered with mycelium. The conidia produced were hyaline and non-septate when young. The characteristics of this group's mycelia and conidia were very similar to those of Group 2 although the conidia were slightly smaller in Group 1 (Table 2.5; Figure 2.6). This group of isolates was subsequently identified as being *Neofusicoccum luteum*, based on the similar description given by Taylor *et al*. (2005) and the mean conidium L/W ratio of 3.6, which matched the range published for *N. luteum* on the Botryosphaeria website.

Group 2

The Group 2 isolates initially grew in a similar manner to the Group 1 isolates. Their mycelia also showed yellow pigmentation after 3 days, however, unlike those of Group 1, the yellow colouration of the colony surface and reverse side did not change to violaceous after 7 days but rather to a brownish colour (Figure 2.7). Upon reaching the edge of the Petri dish, each colony margin became more fluffy and dense than at the centre. The surface of each colony gradually became greyish in colour, and the reverse became black after day 30 but with no pycnidia produced. Conidia obtained from the grapevine green shoots had a mean L/W ratio of 3.9 which, together with the colony and conidium characteristics (Table 2.5). Group 1 was differentiated from Group 2 by the violaceous colour of colonies seen after 7 days in Group 1 and not in Group 2 and the larger size of conidia observed in the Group 2 isolates compared with the Group 1 isolates (Slippers *et al.*, 2004c). Another observation made was that, the colour intensity of the Group 1 and 2 colonies (chromogenesis) depended on the medium type as it was more obvious in PDA and OMA cultures than in PA and MEA cultures. The colour was more intense on thicker agar plates and when incubated without white light in the incubator.

Group 3

Colonies of Group 3 isolates were initially white and smaller than colonies of Groups 1, 2, 4 and 5, whose colonies filled the plates by 4 days at 25^{o}C whereas the Group 3 isolates did not fill the plates until 7 days. The Group 3 isolates also had shorter aerial mycelium after 3 days incubation and had smoother, more distinct margins than for Groups 5 and 6 isolates (Figure 2.7). After 7 days the colony surface of the Group 3 isolates began changing from grey to dark grey starting from the centre, and by day 30, the colonies were black with many large pycnidia. The reverse sides of these colonies were initially white and turned black by day 30. Conidial and colony characteristics (Table 2.5; Figure 2.6) were consistent with those reported for *Diplodia mutila* (Alan Phillips, pers. comm. 2006).

Group 4

Colonies of the Group 4 isolates were characteristically whitish but with a slight yellowish or brownish hue at day 3 (Figure 2.7). This had changed to a light brown

centre with fluffy mycelium at the margins by day 7, and by day 30 the entire colony was dark brown in colour with much aerial mycelium. The reverse sides of the colonies were initially yellowish / brownish and had turned dark grey/brown by day 30. There was no pycnidium production on agar, however the conidia that were finally produced on green shoots after many attempts were aseptate and initially hyaline, but turned light brown with age (Table 2.5; Figure 2.6). They were consistent with the description reported for *N. parvum* (Alan Phillips, pers. comm. 2006).

Group 5

The mycelia of the Group 5 isolates were whitish in colour like the Group 4 isolates, but after 3 days incubation, the mycelium was less fluffy and their growing edges were irregular (undulating) (Figure 2.7). At day 7, the surface colour was greyish brown with little or no aerial mycelium. By 30 days, the colony colour had turned black with no visible aerial mycelium. Numerous small pycnidia were produced with white tufts of mycelium emerging from them, but they had few conidia (Figure 2.7). The reverse sides of the colonies which were initially whitish and became black by day 30. The characteristics of the aseptate, brown conidia (Table 2.5; Figure 2.6) were consistent with the description for *D. seriata* (Alan Phillips, pers. comm. 2006).

Group 6

The mycelium of the single Group 6 isolate was initially white in colour at day 3, had turned grey by day 7 and by day 30, had turned dark grey to black, with little or no aerial mycelium. By day 30, a few, small pycnidia were produced (Figure 2.7). The reverse side of the colony became grey by day 7 and dark grey by day 30. The conidia produced after 4 weeks growth on OMA were hyaline when young and became brown and septate by day 35, while still attached to the conidiogenous cell. The conidium characteristics (Table 2.5; Figure 2.6) were similar to those described for *Dothiorella* sp. in the Botryosphaeria website.

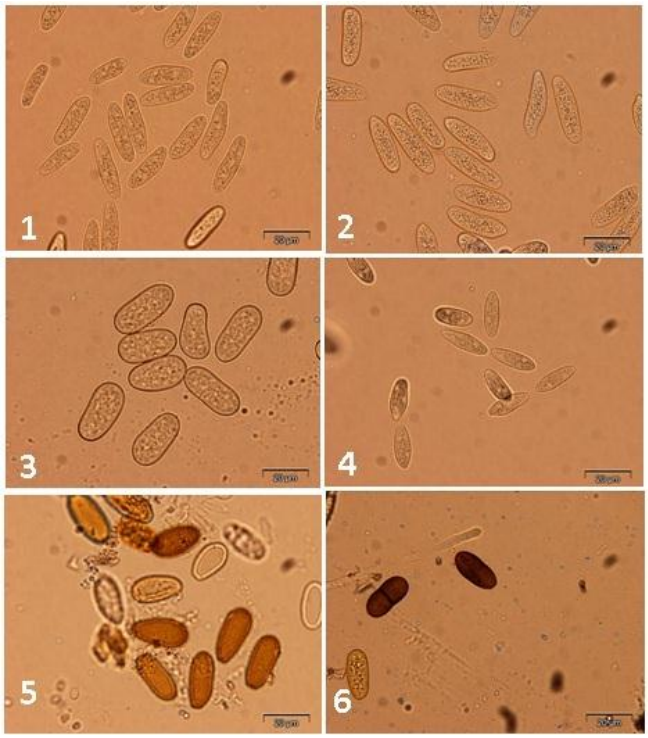

Figure 2.6: Characteristics of conidia from representative groups of the botryosphaeriaceous isolates that were recovered from grapevine tissues. They were identified after comparison with the Botryosphaeria website (http://www.crem.fct.unl.pt/botryosphaeria_site/) as (1) *N. luteum,* (2) *N. australe,* (3) *D. mutila,* (4) *N. parvum,* (5) *D. seriata* and (6) *Dothiorella* sp.

Table 2.5: Conidium characteristics, mean measurements, length/width ratios (L/W) and 95% confidence intervals (CI) from three isolates of each morphological group of botryosphaeriaceous species.

Groups	Conidia characteristics	Length & width (µm)	L/W ratio	95% CI
1	Hyaline, aseptate thin walled, septate when germinating, fusiform, base subtruncate.	18.9-26.0 x 4.0-8.1	3.6	3.45-3.80
2	Hyaline, aseptate thin walled smooth with granular contents, rarely forming a septum before germination.	22.1-32.0 x 5.0-8.4	3.9	3.72-4.09
3	Hyaline, aseptate with a thick wall, rarely becoming pale brown and 1-septate, apex and base broadly rounded.	20.5- 28.4 x 12.3-16.1	1.9	1.86-1.94
4	Hyaline, aseptate, thin walled becoming darker and 1-or 2-septate before germination.	13.2-20.9 x 3.9-7.3	3.2	2.87-3.24
5	Brown, aseptate, inner surface, of wall finely roughened, of wall finely roughened, apex broadly rounded, and base truncate.	21.2 -26.7x 10.5-13.4	2.1	2.02-2.16
6	Dark brown, 1- septate, ovoid, apex rounded, base truncate, smooth external wall.	18.7- 28.1-x 6.6-11.7	2.5	2.39-2.63

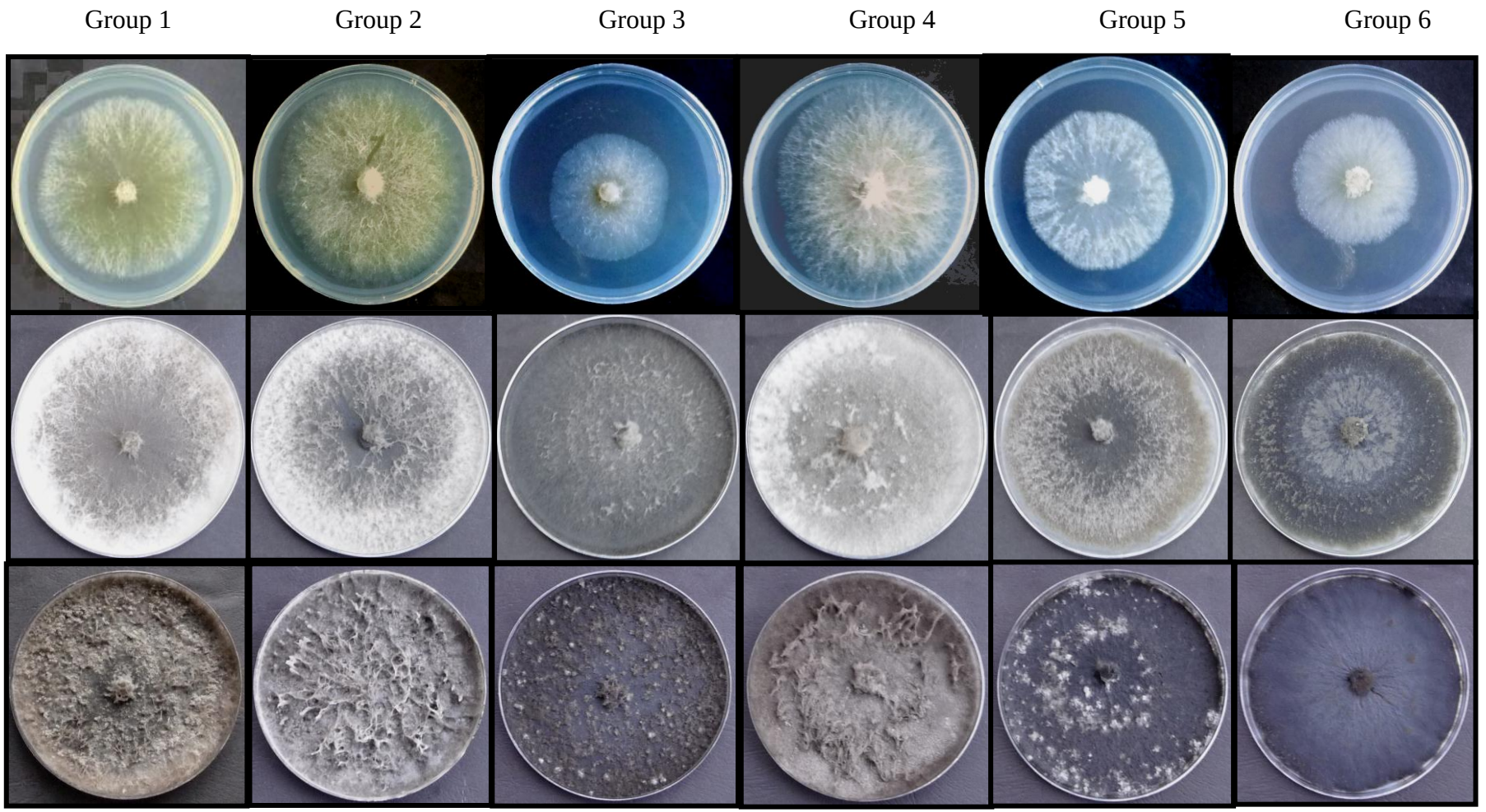

Figure 2.7: Culture morphology of the botryosphaeriaceous groups on PDA based on colour and growth characteristics after 3, 7 and 30 days, respectively, of incubation at 25°C in continuous darkness.

2.3.4 Temperature effects on mycelium growth characteristics

After 48 h the temperature ranges of the 15 botryosphaeriaceous isolates selected to represent the five morphological groups (except Group 6), differed significantly (P<0.001; Appendix B.6.1) between individual isolates within the groups. No mycelial growth occurred at 10°C for any of the isolates. For all isolates, 25°C gave the greatest mean mycelium diameter (54.5 mm) while the lowest mean mycelium diameter of 7.8 at 15°C was significantly lower than all other temperatures other than 10 °C (Table 2.6).

Table 2.6: Mean mycelial growth (mm) of three species from each of the botryosphaeriaceous groups after 48 h growth on PDA at six different temperatures.

Groups		Temperature (°C)						Mean isolate
	Isolates	10	15	20	25	30	35	/group effect
Group1	M(13)2	0	8.2	40.0	65.1	54.6	5.6	28.9 n[1]
	N(12)2	0	11.3	34.9	68.3	54.2	7.4	29.4 n
	G(s)-1	0	8.6	42.5	63.5	68.3	12.4	32.6 o
Group 2	J-3	0	6.0	28.2	55.1	42.8	11.9	24.0 jk
	Kat-1	0	6.8	27.8	53.6	36.4	18.1	23.8 j
	Mel-2	0	7.5	31.9	53.8	41.3	14.2	24.8 k
Group 3	F (12)-2	0	9.3	35.2	64.1	51.8	5.8	27.7 m
	Iso-2	0	9.9	42.7	58.2	45.9	5.4	27.0 m
	Q	0	8.1	33.2	60.7	58.4	6.5	27.8 m
Group 4	I(15)2	0	9.6	17.5	43.9	44.8	8.1	20.7 i
	I(15)3	0	5.5	16.3	41.2	43.3	8.2	19.1 h
	Q-2	0	0.7	21.0	45.4	34.3	6.6	18.0 g
Group 5	L(16)2	0	8.3	37.1	47.3	50.7	11.9	25.9 l
	L-1	0	10.1	34.4	54.4	42.8	14.2	26.0 l
	I-1	0	7.5	33.9	43.1	44.9	13.4	23.8 j
Mean Temperature Effect		0a[1]	7.8b	31.8d	54.5f	47.7e	9.9c	

[1]Values within the rows or columns followed by the same letter are not significantly different at P≤0.05 according to Fisher's protected LSD at P≤0.05. For the main effect of temperature (a-f) was significant (P<0.001; LSD =0.56) and isolates/group effect (g-o) was significant (P<0.001; LSD=0.88), and Temperature x isolates (P<0.001; LSD=2.18), For Groups analysis P=0.100; LSD 8.16.

The statistical differentiations of isolates within a group were different when the 48 h growth diameters were analysed over all temperature and at 25ºC. Although the differences between isolates were statistically significant (P<0.05), the actual differences between diameters were minimal (Table 2.6 and Figure 2.8). For example in Group 1, the overall temperature means differentiated (P≤0.05) isolate G(s)-1 (32.6 mm) from isolates M (13)2 and N (12)2 (29.4 and 28.9 mm, respectively) whereas at 25ºC isolate N (12)2 had a greater (P<0.05) diameter (68.3 mm) than the similar isolates, G(s)-1 and

M (13)2 (63.5 and 65.1 mm, respectively). The overall trends after 48 h growth were for isolates of Groups 1 and 3 to have the largest mycelia, especially at 25 and 30 ºC. However at 35ºC, the isolates of Groups 2 and 5 had the largest mycelia. The Group 4 isolates were the smallest overall, especially at 15-25ºC. These effects illustrate the significance temperature and isolate interaction (P<0.001; Appendix B.6.1).

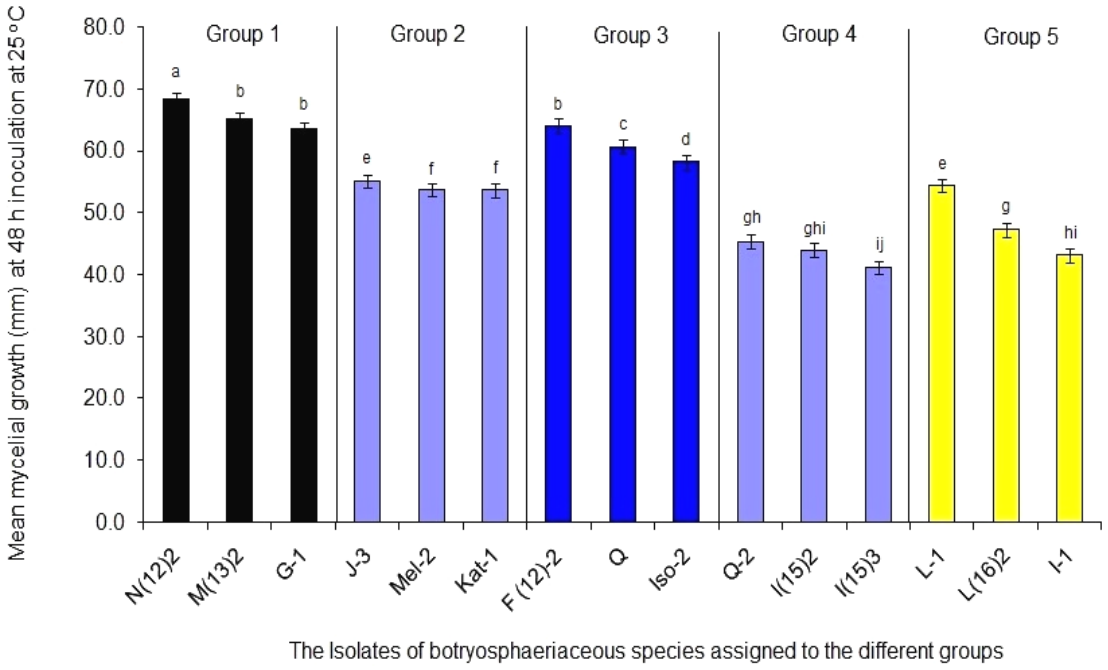

Figure 2.8: Mean mycelial growths (mm) of the individual botryosphaeriaceous isolates after 48 h of incubation at 25°C. Bars with the same letters are not significantly different according to Fisher's protected LSD at $P \leq 0.05$. Error bars represent standard errors of the means.

Models of the temperature data as quadratic curves showed close temperature ranges for optimum growth of the isolates. The ranges were 24.4-25.6°C for Group 1 isolates, 25.4-25.9°C for Group 2 isolates, 24.1-24.5°C for Group 3 isolates, 25.3-25.7°C for Group 4 isolates and 24.4-25.3°C for the Group 5 isolates (Appendix B.6.2).

2.3.5 Confirmation of the presumptive identifications using molecular technology

Molecular identification of 20 randomly selected isolates, representative of the first four morphological groups, confirmed the Group 1 isolates as being *N. luteum* (AY259091.1, DQ233609.1), Group 2 as *N. australe* (FJ150697.1, FJ150696.1), Group 3 as *D. mutila* (AY259093.2, DQ458886.1) and Group 4 as *N. parvum* (EU339545.1) based on the similarity of sequence results to those obtained from the BLAST search tool in the

GenBank. Group 5 isolates, whose conidia exactly matched the descriptions of Allan Phillips (pers. comm. 2006) and the pictures and description published on the Botryosphaeriaceae website (http://www.crem.fct.unl.pt/botryosphaeria_site/) were identified as *D. seriata*. This was later confirmed using molecular technology (Baskarathevan, pers. comm. 2009), however the single Group 6 isolate, which had conidia that were hyaline when young but turned brown and septate with age, was identified based on pictures and description on the Botryosphaeria website as *Dothiorella* sp.

All six isolates of *N. australe* showed 100% identity for similar isolates deposited in the GenBank and 98-99% for the isolates of *N. luteum* according to the GenBank BLAST query (Figure 2.9; Table 2.7). The New Zealand *N. australe* isolate Mel-2 showed four polymorphisms with *N. luteum* reference cultures from South Africa (AY343416, AF452554) and USA (DQ233609) and one polymorphism with other published *N. australe* strains e.g. EF638778 (Appendix B.7). Isolates identified as *N. luteum*, *N. parvum* and *D. mutila* also showed 99-100% identity for similar isolates deposited in the GenBank (Table 2.7). All the sequences from the 20 isolates confirmed by molecular techniques were very clear (Table 2.7; Appendix B.8).

The 63 botryosphaeriaceous isolates obtained from both grapevines and non- grapevine woody hosts were identified using the ITS4 ($^{5\prime}$-TCCGTAGGTGAACCTGCGG-$^{3\prime}$) primer (White *et al.*, 1990) followed by sequence analysis of the PCR product. This confirmed five botryosphaeriaceous species of which *N. parvum*, *N. luteum,* and *D. mutila,* were most common on grapevines (27, 17 and 14%, respectively) and on non-grapevine woody hosts (3, 6 and 10%, respectively). This was followed by *N. australe* with 10 and 2%, respectively, on grapevines and non-grapevines and *D. seriata* (8 and 2%, respectively) *Dothiorella* sp. was the least from grapevine only (2.5%) (Figure 2.10).

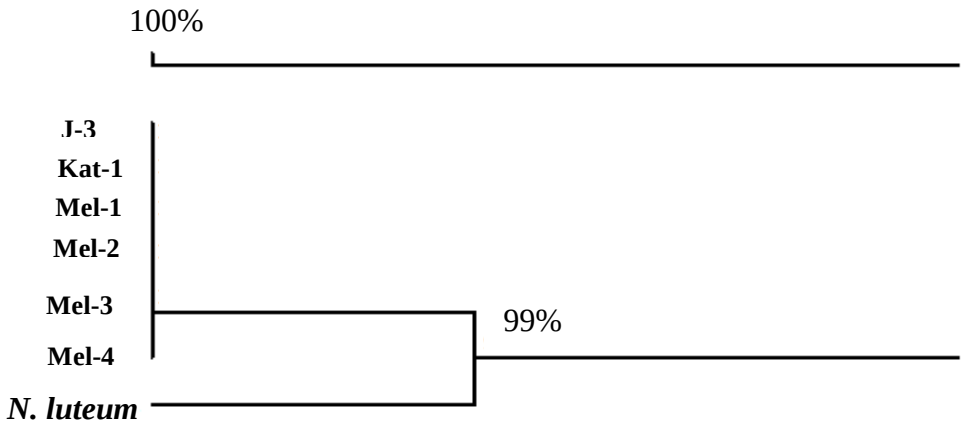

Figure 2.9: Similarity tree showing sequence similarity between six New Zealand isolates of *N. australe* and *N. luteum* (AY343416).

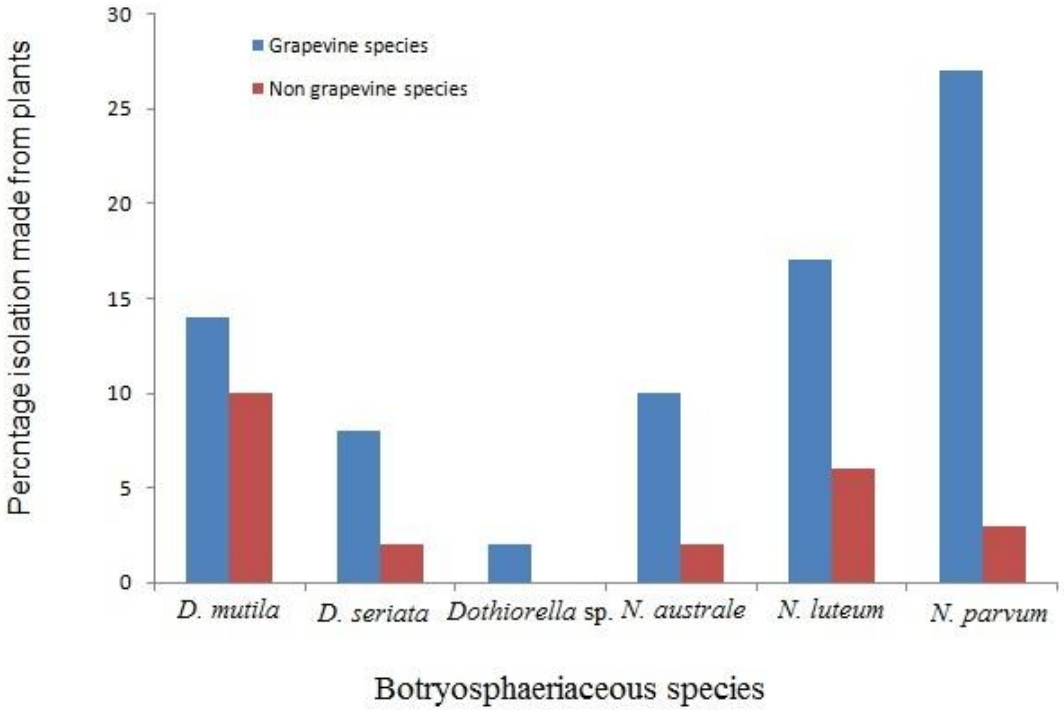

Figure 2.10: Percentage of botryosphaeriaceous species in samples isolated from grapevine and non grapevine woody hosts across New Zealand.

Table 2.7: Sequencing results for botryosphaeriaceous species isolated from grapevines.

Isolate no.	Species	ITS GenBank accession no.	E Value	% identity	Product size	Reference*
N-1	***N. luteum***	AY259091.1	0.0	99	522	2
M (8)-4		AY259091.1	0.0	99	522	2
N(12)2		AY259091.1	0.0	100	512	2
San-1		DQ233609.1	0.0	99	520	3
G(s)-1		DQ233609.1	0.0	100	522	3
Q (s)		DQ233609.1	0.0	99	537	3
M(13)2		DQ233609.1	0.0	99	539	3
Kat-1	***N. australe***	FJ150697.1	0.0	100	520	1
Mel-3		FJ150697.1	0.0	100	537	1
Mel-1		FJ150697.1	0.0	100	520	1
Mel-2		FJ150697.1	0.0	100	524	1
Mel-4		FJ150697.1	0.0	100	524	1
J-3		FJ150696.1	0.0	100	526	1
F(12)2	***D. mutila***	AY259093.2	0.0	99	520	2
Q		AY259093.2	0.0	99	507	2
Iso-2		AY259093.2	0.0	100	516	2
A-3		DQ458886.1	0.0	99	519	2
Q-2	***N. parvum***	EU339545.1	0.0	99	505	4
I (15)-3		GQ857660.1	0.0	100	340	5
I (15)-2		DQ008328.1	0.0	100	394	3

* **1**. Marincowitz *et al.* (2008), **2**. Alves *et al*. (2004), **3**. Úrbez-Torres *et al*. (2006), **4**. Burgess *et al*. (2001), and **5**. McDonald *et al*. (2009).

Section 2

2.4 Materials and Methods

2.4.1 Pathogenicity of grapevine and non-grapevine botryosphaeriaceous isolates on detached green grapevine shoots

In this study, the pathogenicity of five botryophaeriaceous species was determined using detached green grapevine shoots. Fifteen botryosphaeriaceous isolates detected from grapevines comprising five species found in New Zealand (Appendix B.3) and 11 isolates from non-grapevine woody hosts were used. The grapevine isolates were selected randomly from each of the groups representing the five species. All selected isolates, which had been stored on PDA at 4^{o}C, were subcultured onto PDA plates and incubated at 25^{o}C in continuous darkness for 3 days before being used for inoculation.

The detached green shoots for *in vitro* pathogenicity studies were taken from the Lincoln University vineyard where previous vine sampling had not yielded any fungal cultures characteristic of botryosphaeriaceous species. The soft green shoots (20-25 cm long from the tip) were cut from Pinot noir vines in early summer and their basal ends immediately inserted into tap water. For the experimental set-up, they were individually placed into Universal bottles filled with sterile water, the top of each bottle being wrapped with Parafilm® as described previously in Section 2.2.3.2, to support the shoot and to prevent evaporation. Superficial wounds (~4 mm in diameter) were made in the centres of the shoots using a sterilised scalpel.

Mycelium agar discs (3 mm diameter) cut from the growing edges of the 3-day-old cultures of the grapevine isolates were used to inoculate onto the wounds or onto similar positions on the non-wounded shoots (Figure 2.11a). For non-wounded treatments, the agar discs were placed directly onto the shoot tissue. Control shoots were inoculated using sterile PDA discs. The six replicates of both wounded and non-wounded shoots for each grapevine isolate were arranged in a completely randomised design (CRD) in a transparent, humid chamber, as described in Section 2.2.3.2. Ten days after inoculation, shoots were removed and lesion lengths (Figure 2.11b) measured using a digital calliper. The infected tissue was surface sterilised in 70% alcohol for 30 s, rinsed once with clean tap water and air-dried under a laminar hood for 30 min. Stem pieces cut at 1 cm intervals from above and below the inoculation point were placed onto PDA-Cph followed by presumptive identification of colonies, as described in Section 2.3.3. These

colonies were used to repeat the inoculation onto three fresh green wounded shoots per treatment to demonstrate the similarity of symptoms and confirm Koch's postulates. Subsequent isolations were made from the edges of 3 day-old lesions as before.

An identical experiment was also conducted to assess the pathogenicity of the non-grapevine isolates on grapevine plants, using five replicates. In addition, the lesions formed on the shoots after 10 days were put under moist incubation to induce production of pycnidia and conidia as described previously in Section 2.2.3.2. This was done to determine if the non-grapevine isolates could provide conidia as sources of inoculum for further infections.

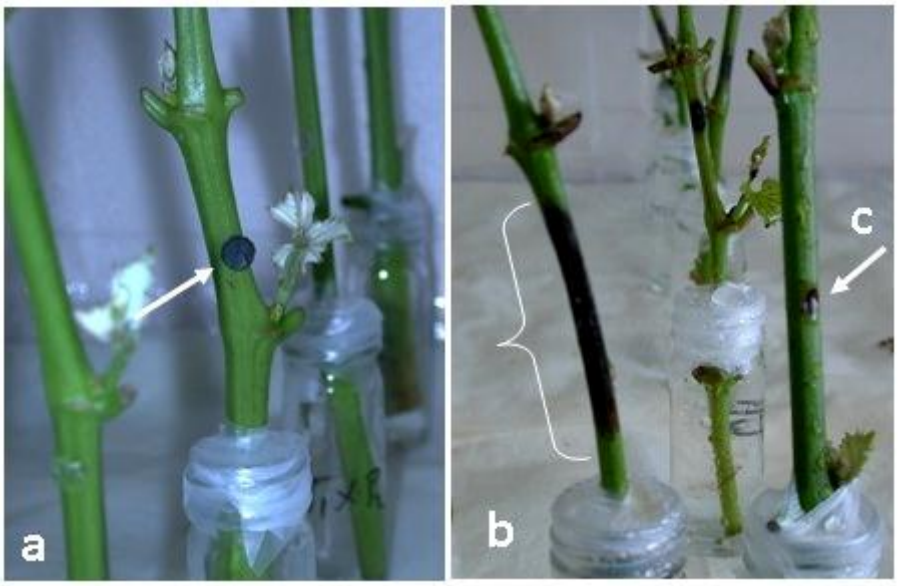

Figure 2.11: *In vitro* inoculation of grapevine shoots with mycelium colonised agar plugs (a) mycelium agar plug inoculated onto wounded shoot denoted by the arrow, (b) green shoots 10 days after inoculation; brackets denote lesion area and (c) the arrow showing a control shoot without lesion development.

2.4.2 Statistical analysis

Lesion lengths measured with a digital calliper and pathogen isolation distances were analysed by general analysis of variance (ANOVA) using GenStat 11 to determine treatment effects on lesion lengths. Separation of means was by Fisher's protected LSD at $P \leq 0.05$.

2.4.3 Suceptibility of detached grapevines of five scion varieties to botryosphaeriaceous infection

Detached green shoots of varieties Cabernet Sauvignon, Chardonnay, Pinot noir, Riesling, and Sauvignon blanc were cut from the Lincoln University vineyard during the summer and prepared for inoculation as described in Section 2.2.3.2. The botryosphaeriaceous species inoculum comprised mycelium colonised agar discs from one isolate each of *N. australe* (Kat-1), *N. luteum* [M (13)2], *N. parvum* [I (15)2] and *D. mutila* [F (12)2]. The shoots were wounded or not wounded and immediately inoculated as described previously. Controls were inoculated with sterile PDA plugs and there were five replicates per isolate. The experiment was set up as before, with CRD with similar incubation conditions, assessment and data analysed as described in Sections 2.4.1 and 2.4.2.

2.4.4 Susceptibility of potted grapevines of five scion varieties to botryosphaeriaceous infection

2.4.4.1 Production of rooted grapevine plants and their management

Mature canes, each containing 5-7 nodes, were cut from dormant vines in the Lincoln University vineyard during early winter 2007 for growing potted grapevines of the same varieties listed in Section 2.4.3. The canes were stored in new polythene bags (120 cm × 60 cm) in a cold room at 4^{o}C for one month prior to rooting. Cuts were made through the basal bud of each cutting (cane) which were then inserted into trays filled with 1-4 mm pumice granules (Atiamuri sand and pumice Co., New Zealand) to a depth of 10 cm. Trays were placed on a heat pad set at 25^{o}C for 5-6 weeks in an outdoor shade house (covered with 50% Sarlon shadecloth (R.J Reid, New Zealand, Ltd) at Lincoln to facilitate the formation of adventitious roots from the basal bud. Cuttings that had developed healthy roots were grown in 2 L pots with potting mix [80% composted bark, 20% pumice, 2 kg/m^3 Osmocote® Exact® Standard 12-14 months gradual release fertilizer (15:3.9: 9.1; N: P: K, respectively, plus trace elements), 1 kg/m^3 agricultural lime and Hydraflo® 2 (granular wetting agent, Scott Product New Zealand Ltd)] as the growing medium and grown in a shade house from October 2007 to July 2009. Plants were watered daily and weeds controlled by hand removal every month. After 15 months, each pot received approximately 10 g of an additional top dressing with Triabon® (BASF New Zealand Ltd complex fertilizer containing 16: 3.5: 10; N: P: K, respectively, plus 9% sulphur and 2.4% magnesium and trace elements).

2.4.4.2 Pathogenicity of conidia on attached green shoots

A mixed isolate conidium suspension (10^6/ mL) for inoculation was made from three isolates each for *N. australe*, *N. luteum*, *N. parvum* and *D. mutila* (Appendix B.3), as described in Section 2.2.3.2. The Pinot noir vines to be inoculated had trunks approximately 450 mm in height and 9-10 mm in diameter, and were growing in 2 L pots with potting mix as previously described in Section 2.4.4. The green shoots (~6-8 mm diameter) emerging from the main stem were wounded at 2 cm from their bases by cutting out a core (~2.5 mm diameter) of the tissue with the tip of a sterile scalpel to expose the cambium. Each hole was immediately inoculated with a 50 µL drop of the conidium suspension (10^5/ mL) and control plants were inoculated with sterile water. Each plant was covered with a transparent plastic bag for 24 h to prevent the immediate evaporation of the conidium suspensions. Thirty-five replicate plants were arranged in a completely randomised block design (CRBD) in an open area similar to field conditions, and managed as described in Section 2.4.4. Five replicate plants for each treatment were assessed for lesion development at 10, 20, 30, 40, 50 and 60 days after inoculations. At each assessment time, the shoots were sliced through lengthwise and the internal lesions visually observed, but at 60 days after inoculations, lesion lengths were also measured using a digital calliper. Pathogen isolations were carried out from the lesion edges as described in Section 2.2.2. The data were analysed as described in Section 2.4.2.

2.4.5 Pathogenicity of conidia and mycelium on woody trunks of different grapevine varieties

The main stems of 18-month-old potted grapevines of the varieties, Chardonnay, Pinot noir, Riesling, Cabernet Sauvignon and Sauvignon blanc were wounded and inoculated with either conidia or mycelia. The same isolates were used to make mixed isolate conidium suspensions (10^6/ mL) of the same species (Section 2.2.3.2) as described in Section 2.4.5.1. The mixed isolate mycelial inoculum was made for each species by chopping and mixing mycelial discs cut from the 3-day-old cultures of the same three representative isolates for each species. Each main stem was wounded by drilling a 3.5 mm diameter and 2-3 mm deep hole into it. Each wound was inoculated with 100 µL of 10^6 conidia /mL or with sufficient mixed mycelium/agar to fill the wound. The inoculated wounds were wrapped with Parafilm® to prevent drying of the mycelium plug and run off or evaporation of the conidium suspension. Control plants were inoculated with sterile agar or sterile water, respectively.

The 20 replicate plants of each treatment combination were arranged in a CRBD and grown from November 2008 until March 2009 in an open area at the Lincoln University Nursery that simulated field conditions. Plants were watered daily and weeds controlled by hand removal every month. The plants were sprayed once at 3 months after inoculation with Saprol™ fungicide (190 g/L Triforine) to control powdery mildew infection. Four months after inoculation, five plants per treatment and variety were randomly selected and assessed for lesion development by scraping off the bark and measuring the lesion length with a digital calliper. However, lesions were sometimes difficult to identify clearly and so the tissues were surface sterilised as described in Section 2.2.2 and pathogen isolation carried out by cutting the tissues at 10 mm intervals above and below the inoculation point and sequentially putting them onto PDA. After and 3 days incubation at 18-21^{o}C, the emergence of fungal colonies characteristic of the botryosphaeriaceous species used for the inoculation was used to assess infection progression. The maximum distance colonised by each species was used as a measure of its pathogenicity.

At 7 months after inoculation, five plants per treatment were randomly selected and the bark removed from the entire trunk to expose the extent of damage. Lesions formed by *N. luteum*, *N. australe*, *N. parvum* and *D. mutila* were observed and described. For all species, pathogen isolation was conducted at 10 mm intervals to determine distance colonised as described before. The data were analysed as described in Section 2.4.2.

Five replicate plants were taken for assessement at 7 months after inoculation for pathogen presence in the wood using molecular techniques. The remaining five replicate plants for each treatment combination were left to grow till the end of the growing season (winter) and following senescence the plants were pruned to 4-5 buds per cane. After 1-2 months of complete dormancy, plants were put into a greenhouse (12-20ºC) under high-pressure sodium lights (400W SON-T-AGRO, Phillips, Belgium) set to turn on from 4 am to 12 pm and again from 4 pm to 8 pm through the winter period. Bud development, shoot growth and dieback were observed and recorded after bud break.

2.4.6 Molecular confirmation of the botryosphaeriaceous species in woody trunks of young vines

2.4.6.1 DNA extraction from mycelium

The five replicate grapevines for each treatment and variety combination from Section 2.4.5.2 were surface sterilised as described in Section 2.2.2. Wood sections (~12-14 mm

in diameter and 10 mm thick) were cut across the 2-year-old trunk pieces from the edges of lesions that had developed. Each piece was divided into two, of which one was plated onto PDA-Cph for 3 days to allow mycelial growth and the other piece was stored at -80^{o}C. A small amount (~10 mg) of the mycelium was taken from the growing edge of each colony for DNA extraction using the REDExtract-N-Amp™ Plant PCR Kit (Sigma-Aldrich Corp. St. Louis, MO, USA). The mycelium was put into a 2 mL tube containing 100 µL of the extraction solution and vortexed briefly, after which it was incubated at 95^{o}C for 10 min. After that, 100 µL of the dilution solution was added and mixed with a vortex. This solution was stored at 4^{o}C prior to amplification by PCR.

2.4.6.2 DNA extraction from infected wood pieces

A PowerSoil^{TM} DNA isolation kit (MO BIO laboratories, CA, USA) was used for direct DNA extraction from infected wood tissues. The wood pieces stored at -80^{o}C that matched those which had produced mycelium for the above DNA extraction were taken from storage and ground to powder in liquid nitrogen using heat sterilized mortars and pestles. About 0.25 g of each ground wood sample was added to a 2 mL PowerBead™ tube which contained small beads and an aqueous solution of acetate and salts (to protect the nucleic acid from degradation) and gently mixed with a vortex. Sixty microlitres of solution C1containing sodium dodecyl sulphate (SDS) (responsible for cell lysis and break down of fatty acids and lipids associated with the cell wall membrane) was added to the contents of each PowerBead™ tube and vortexed briefly. The tubes were then secured horizontally using the vortex adapter tube holder (MO BIO) and vortexed at maximum speed for 10 min, after which they were centrifuged at 10,000 × *g* for 30 s at room temperature. Each supernatant (~500 µL) was then transferred to a clean 2 mL collection tube, 250 µL of solution C2 was added and the tube was vortexed for 5 s to precipitate the non-DNA organic and inorganic material, including humic acids, cell debris and proteins. Each tube was then incubated at 4^{o}C for 5 min and centrifuged at 10,000 × *g* at room temperature for 1 min. About 600 µL of the resulting supernatant was again transferred into a clean 2 mL collection tube and 200 µL of solution C3 was added to precipitate non DNA materials. Each tube was vortexed briefly, incubated at 4^{o}C for 5 min and centrifuged at 10,000 × *g* for 1 min at room temperature. Each supernatant (750 µL) was transferred to a clean 2 mL collection tube followed by addition of 1200 µL of solution C4 (a solution with high concentration of salts which allows the binding of DNA) and vortexed for 5 s. The resulting solution was then filtered through a spin filter by centrifugation at 10,000 × *g* for 1 min at room temperature and the flow through solution discarded, leaving the DNA bound to the

silica membrane in the filter. Aliquots (500 µL) of solution C5 (ethanol based wash solution) was added onto the spin filter and it was centrifuged at room temperature (18-21^{o}C) at 10, 000 × *g* for 30 s and the wash solution that flowed through was discarded. The spin filter was then again centrifuged at room temperature for 1 min, discarding the flow through solution again. The spin filters were then transferred to 2 mL clean tubes and 100 µL of solution C6 (10 mM Tris pH 8) was pipetted onto the centre of each filter membrane to release the DNA into the solution during centrifugation at 10, 000 × *g* for 30 s at room temperature. The resulting DNA suspension was stored at -20^{o}C prior to PCR aplification.

2.4.6.3 PCR Amplification

The PCR reactions were carried out in a Bio-Rad iCycler Thermal Cycler (Hercules, CA, USA). The reactions were performed in 20 µL reaction mixtures which were made up of 10 µL REDExtract-N-Amp PCR ReadyMix (which contained buffer, salts, dNTPs and Taq polymerase,) 4 µL water, 4 µL DNA extract, 1 µL each of forward and reverse primers. The Botryosphaeriaceae genus specific primers, (BOT100) AAACTCCAGTCAGTRAAC (forward primer) and (BOT472) TCCGAGGTCAMCCTTGAG (reverse primer) developed by Dr. Hayley Ridgway (Lincoln University) and synthesised by Invitrogen technologies (New Zealand) were used to amplify part of the rDNA region (partial ITS1, 5.8S and, partial ITS2). The amplification conditions were: 94^{o}C for 3 min initial denaturation, then 35 cycles of 94^{o}C for 30 s, 58^{o}C for 30 s, 72^{o}C for 30 s and finally 72^{o}C for 10 min for the last extension. The amplified DNA samples were then loaded onto 1% agarose gels and DNA separated by electrophoresis as described previously in Section 2.2.6.2. Sequencing was carried out and sequence alignment confirmed with BLAST as described previously in Section 2.2.6.3.

2.5 Results

2.5.1 Pathogenicity of grapevine Botryosphaeriaceae isolate on detached grapevine shoots

Tiny dark spots (Figure 2.12) were observed on the inoculated areas of all non-wounded shoots inoculated with botryosphaeriaceous species. However, attempts to reisolate the pathogens from these dark spots after surface sterilization yielded no colonies

characteristic of botryosphaeriaceous species. The control plants (inoculated with sterile agar) produced no symptoms.

Figure 2.12: Section of a non-wounded grapevine green shoot previously inoculated with a botryosphaeriaceous species mycelium agar plug showing tiny black spots after 10 days incubation.

On wounded, inoculated shoots, brown to dark brown lesions developed lengthwise along the stems (Figure 2.13). There were significant differences ($P<0.001$; Appendix B.9.1) in the lesion lengths produced by the different botryosphaeriaceous isolates 10 days after inoculation (Table 2.8; Figure 2.13), although those within a species were generally similar. Overall, the *N. luteum* isolates were most pathogenic with mean lesion lengths of 67.2 to 78.6 mm, followed by *N. australe*, which caused lesions of 63.9 to 68.4 mm (Table 2.8). The lesions caused by *N. parvum* isolates were also not significantly different, mean lengths ranging from 59.0 to 61.6 mm. In *D. mutila*, one isolate [M (30)3] caused a mean lesion length of 57.8 mm which was similar to the *N. parvum* isolates, and significantly longer than the lesions caused by the other two *D. mutila* isolates [Q and F (12)2], which caused mean lesion lengths of 31.9 and 27.0, respectively. The isolates of *D. seriata* caused significantly smaller lesions (5.2-6.0 mm) than all other species. In all cases, isolations from the lesion edges produced similar colonies to those used for inoculation. A repeated inoculation caused similar lesions, and subsequent isolations made from the edges of the lesions after 3 days incubation again produced identical colonies, therefore confirming Koch's postulates. No botryosphaeriaceous species colonies were recovered from the control plants.

Figure 2.13: Lesions that developed 10 days after inoculating wounded green shoots of grapevine (Pinot noir) with mycelium colonised agar plugs of isolates (a) *N. luteum* M(13)2, (b) *N. australe* Kat-1, (c) *N. parvum* I(15)2 and (d) *D. mutila* F (12)2.

Table 2.8: Mean lesion lengths caused by botryosphaeriaceous isolates from grapevines 10 days after inoculation with mycelium colonised agar plugs onto wounded detached green grapevine shoots var. Pinot noir.

Species name	Isolate	Mean lesion length (mm)
Neofusicoccum luteum	N(12)2	78.6 a[1]
N. luteum	G(s)-1	69.5 b
N. luteum	M(13)2	67.2 bc
N. australe	K(18)1	68.4 bc
N. australe	Kat-1	67.9 bc
N. australe	Mel-2	63.9 bcd
N. parvum	I(15)3	61.6 cd
N. parvum	Q-2	59.3 d
N. parvum	I(15)2	59.0 d
Diplodia mutila	M(30)3	57.8 d
D. mutila	Q	31.9 e
D. mutila	F(12)2	27.0 e
D. seriata	L-1	5.9 f
D. seriata	L(17)4	5.6 f
D. seriata	L(16)2	5.2 f
LSD ($P \leq 0.05$)		**6.80**

[1]Means followed by the same letter do not differ significantly at $P \leq 0.05$ according to Fisher's protected LSD. Controls inoculated with sterile agar had no lesions and were excluded from ANOVA.

2.5.2 Pathogenicity of the non-grapevine Botryosphaeriacous isolates

All of the botryosphaeriaceous isolates recovered from the non-grapevine hosts, except *D. seriata* produced substantial lesions 10 days after inoculation onto wounded grapevine green shoots, but no lesions were produced for non-wounded or control treatments. There were significant differences ($P<0.001$; Appendix B.9.2) in the mean lesion lengths produced by the different botryosphaeriaceous species isolates (Table 2.9), with the *N. luteum* and *N. australe* isolates being most pathogenic, followed by *N. parvum* as in the previous experiment. However, there was a great deal of variation in the mean lengths of the lesions caused by the *D. mutila* isolates, being 37.4 to 58.6 mm. These mean lengths fell into three significance groups, with isolates [Iso-2] and [J-4] having significantly longer lesions than isolates [C-4] and [A-3], which had longer lesions than isolates [F-1] and [A-2] ($P \leq 0.05$). The mean lesion length of 5.7 mm caused by the single isolate of *D. seriata* showed that this species was less pathogenic. In all cases, isolations from the lesion edges produced similar colonies to those used for

inoculation, which when reinoculated onto green shoots again caused similar lesions, and subsequent isolations produced identical colonies to initial isolations. No botryosphaeriaceous species colonies were recovered from non-wounded or control plants. In addition, all the isolates with significant lesions were able to produce pycnidia with large amounts of conidium ooze.

Table 2.9: Mean lesion lengths caused by botryosphaeriaceous isolates from non-grapevine hosts 10 days after inoculation with mycelium colonised agar plugs onto wounded detached green grapevine shoots var. Pinot noir.

Species name	Isolates	Source hosts	Lesion length (mm)
Neofusicoccum australe	J-3	Broom	65.1 a[1]
N. luteum	MJ3	Blueberry	69.2 a
N. parvum	O-1	Willow	43.4 c
N. parvum	O-3-1	Cherry	43.1 cd
Diplodia mutila	Iso-2	Native ngaio	58.6 b
D. mutila	J-4	Oak	51.1 b
D. mutila	C-4	Plum	44.0 c
D. mutila	A-3	Apple	43.2 c
D. mutila	F-1	Pine	42.9 d
D. mutila	A-2	Olive	37.4 d
D. seriata	I-1	Lemon wood (*P. genioidoes*)	5.2 e
LSD (*P*≤0.05)			**5.77**

[1]Means followed by the same letter do not differ significantly at $P \leq 0.05$ according to Fisher's protected LSD. Controls inoculated with sterile agar had no lesions and were excluded from ANOVA.

2.5.3 Susceptibility of detached green shoots of different grapevine varieties to botryosphaeriaceous species

The wounded green shoots of the five grapevine varieties tested were susceptible to infection by the isolates of the four botryosphaeriaceous species tested. There was no significant cultivar and isolate interaction ($P>0.378$) (Table 2.10). Mean lesion lengths differed significantly ($P<0.001$; Appendix B.10.1) between the four isolates (Table 2.10), also reflecting the species trend shown in Table 2.8. However the five grapevine varieties tested did not differ significantly ($P>0.141$) in the mean lengths of lesions produced. Fungal colonies characteristic of the inoculated isolates were recovered from the lesion edges of all the inoculated detached wounded shoots. No fungi characteristic of botryosphaeriaceous species were isolated from the control shoots.

Table 2.10: Mean lesion lengths caused by isolates of four botryosphaeriaceous species 10 days after inoculation with mycelium colonised agar plugs onto wounded detached green shoots of five grapevine varieties.

Species	Isolates	Lesion lengths (mm) on grapevine varieties					
		Cabernet Sauvignon	Chardonnay	Pinot noir	Riesling	Sauvignon blanc	Isolate means (mm)
N. luteum	M (13)2	28.3	25.9	34.9	22.1	25.9	**27.4 a**[1]
N. australe	Kat-1	25.7	23.5	27.4	21.9	24.8	**24.7 a**
N. parvum	I (15)2	18.9	20.3	16.3	17.6	21.5	**18.9 b**
D. mutila	F (12)2	14.8	11.9	14.2	13.4	16.0	**14.0 c**
Variety means (mm)		**21.9**	**20.4**	**23.1**	**18.7**	**22.1**	

[1]Values within column followed by the same letters are not significantly different according to Fisher's protected LSD at $P \leq 0.05$. The main effects of isolates was significant ($P<0.001$, LSD=3.24). Controls inoculated with sterile agar had no lesions and were excluded from ANOVA.

2.5.4 Pathogenicity of conidia on attached green shoots

The conidia of all botryosphaeriaceous species caused internal lesions whose lengths differed significantly between species ($P<0.001$; Appendix B.10.2). At 10 days after inoculation, the lesion lengths that had developed were found to be very small compared with lesion lengths obtained for the detached shoots inoculated for the same period. However, at 60 days after inoculations, internal lesion lengths were clearly visible (Figure 2.14), although the lesion colours differed for the species; they were light brown for *N. luteum* and dark brown or black for *N. parvum, N. australe* and *D. mutila*. As a general observation, shoots inoculated with *N. australe, N. parvum* and *D. mutila* appeared to be stunted, with short internodes, whereas those inoculated with *N. luteum* did not. However, internode lengths were not measured. The pathogenicity of species after 60 days reflected earlier trends with the mean lesion lengths being 73.2, 56.5, 57.9 and 40.3 mm for *N. luteum, N. parvum, N. australe* and *D. mutila,* respectively, and mean lesion lengths again being similar for *N. australe* and *N. parvum* ($P>0.05$). Fungal colonies characteristic of the inoculated isolates were recovered from the lesion edges of all the wounded inoculated shoots. No fungi characteristic of the botryosphaeriaceous species were isolated from the control shoots.

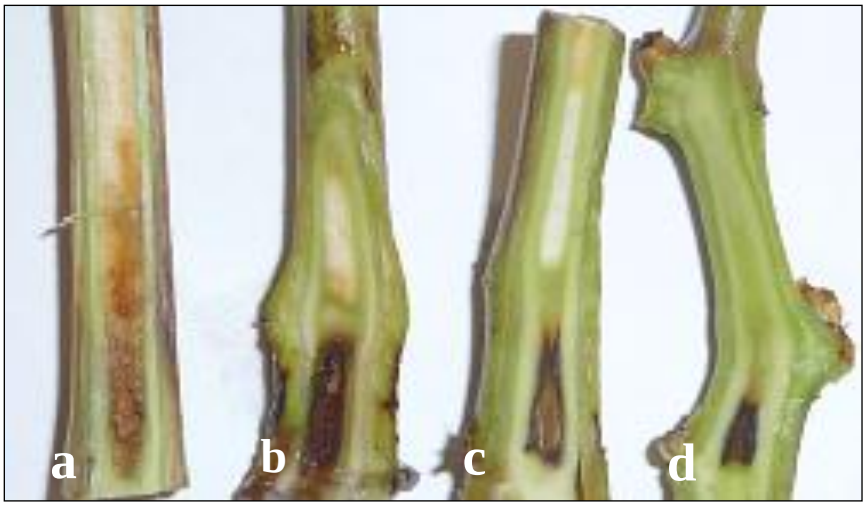

Figure 2.14: Typical internal lesions that developed 60 days after inoculating attached green shoots on one-year-old potted Pinot noir grapevines with conidia of (a) *N. luteum*, (b) *N. parvum*, (c) *N. australe* and (d) *D. mutila* were grown in an open area similar to field conditions.

2.5.5 Pathogenicity of conidia and mycelium on woody trunks of different grapevine varieties to botryosphaeriaceous species

There was no significant interaction (P=0.691) between variety and pathogen species when inoculated with either conidia or mycelium (Appendix B.10.3). At 4 months after inoculations, pathogen isolation at 10 mm intervals from the trunks of the grapevine varieties inoculated with either conidia or mycelium showed significant differences (P<0.001; Appendix B.10.3) in the distances colonised by the four botryosphaeriaceous species and between mycelium and conidium inocula, but not between grapevine varieties (P=0.398) (Table 2.11). The mean distances colonised from the inoculation sites generally reflected similar species effects to those observed on green shoots, with *N. luteum* having the longest lesions (102.0 and 121.6 mm, respectively, for conidium and mycelium inoculations) although the mean distances colonised by *N. parvum* (88.0 and 109.2 mm, respectively), were similar to those colonised by *N. australe* (86.4 and 107.2 mm, respectively). The least movement of 54.8 and 76.0 mm for conidia and mycelium, respectively, was for *D. mutila.*

Table 2.11: Mean total distances (mm) of pathogen recovery (combined from above and below the trunk inoculation points) on 1-2 year old plants of five grapevine varieties, 4 months after inoculating them with mycelium (M) or conidia (C) of four botryosphaeriaceous species.

Species		Isolation distances (mm) on grapevine varieties					
		Cabernet Sauvignon	**Chardonnay**	**Pinot noir**	**Riesling**	**Sauvignon blanc**	**Inoculum means (mm)**
N. luteum	***(C)***	98	102	106	102	102	**102.0 c**[1]
	(M)	118	122	126	122	120	**121.6 a**
N. parvum	***(C)***	84	90	88	88	90	**88.0 d**
	(M)	110	110	108	108	110	**109.2 b**
N. australe	***(C)***	86	86	90	84	86	**86.4 d**
	(M)	106	106	114	104	106	**107.2 b**
D. mutila	***(C)***	54	54	52	54	60	**54.8 f**
	(M)	74	76	74	76	80	**76.0 e**
Variety means		**91.3**	**93.3**	**94.8**	**93.2**	**94.3**	

[1]Values followed by the same letters, within columns, are not significantly different according to Fisher's protected LSD at $P \leq 0.05$. The main effects of species and inoculum type were highly significant ($P<0.001$; LSD=2.50), Controls were not inoculated and were excluded from ANOVA.

At 7 months after the inoculations, pathogen isolations at 10 mm intervals again showed a significant difference between species ($P<0.001$; Appendix B.10.4). The distances of pathogen recovery (combined from above and below the trunk inoculation points) were greater than was observed at 4 months. The relative distances colonised by the botryosphaeriaceous species (Table 2.12) were greater but in a similar order to the assessment at 4 months. There was no significant difference between varieties with respect to the distances colonised and there was no significant interaction ($P=0.277$) between varieties and pathogen species when inoculated with either conidia or mycelium (Table 2.12).

Table 2.12: Mean total distances (mm) of pathogen recovery (combined from above and below the trunk inoculation points) on 1-2 year old plants of five grapevine varieties, 7 months after inoculating them with mycelium (M) or conidia (C) of four botryosphaeriaceous species.

Species		Isolation distances (mm) on grapevine varieties					
		Cabernet Sauvignon	**Chardonnay**	**Pinot noir**	**Riesling**	**Sauvignon blanc**	**Inoculum means**
N. luteum	***(C)***	142	146	152	144	144	**145.6 b**[1]
	(M)	150	154	158	154	146	**152.4 a**
N. parvum	***(C)***	136	136	140	138	148	**139.6 cd**
	(M)	146	142	144	142	150	**144.8 bc**
N. australe	***(C)***	134	136	144	138	134	**137.2 d**
	(M)	138	144	148	142	132	**140.8 bcd**
D. mutila	***(C)***	118	120	122	118	118	**119.2 f**
	(M)	126	130	132	128	128	**128.8 e**
Variety means		**136.3**	**138.5**	**142.5**	**138.0**	**137.5**	

[1] Value followed by the same letters, within columns, are not significantly different according to Fisher's protected LSD at $P \leq 0.05$. The main effects of species and inoculum type ($P < 0.001$; LSD=5.62), Controls were not inoculated and were excluded from ANOVA.

Necrotic lesions were observed under the bark of trunks (Figure 2.15) following inoculation with all four species although the exterior of the trunks only showed obvious external lesions when inoculated with *N. parvum*. These had cankers of sunken, dark, decaying dead wood along one side of the stem close to the inoculation point. Cross sections through the stems also revealed wedge shaped lesions similar to some of the symptoms observed from the field sampling. The lesions caused by *N. australe*, *N. luteum* and *D. mutila* were observed to be much more restricted to the inoculation point and there appeared to be no varietal differences to the lesion development. Pathogen re-isolations from these tissues yielded colonies characteristic of the species that were used for the inoculations, with none being recovered from the control plants.

Figure 2.15: Typical lesions observed on de-barked trunks of 2-year-old vines 7 months after inoculations with *N. australe*, *N. luteum*, control, *N. parvum* or *D. mutila* onto wounded trunks of five different grapevine cultivars grown in pots (from left to right: Chardonnay, Cabernet Sauvignon, Pinot noir, Riesling, and Sauvignon blanc).

After the onset of dormancy in early winter, many pycnidia were observed with a stereo microscope (with 100 × magnification) underneath the bark of the trunk on the necrotic *N. parvum* lesions. They were about 5–10 mm from the inoculation site (insert Figure 2.16a) but no conidia were seen oozing from the pycnidia after incubation at 100% RH for 36 h. When the remaining vines became dormant in early winter, most of those inoculated with *N. australe*, *D. mutila* and *N. luteum* showed bud death and dieback from the shoot tips (Figure 2.16). Some of the dieback tips felt rough to the finger tips and so were removed for observation with the stereo microscope. They showed many pycnidia that oozed conidia after incubation at 100% RH for 36 h.

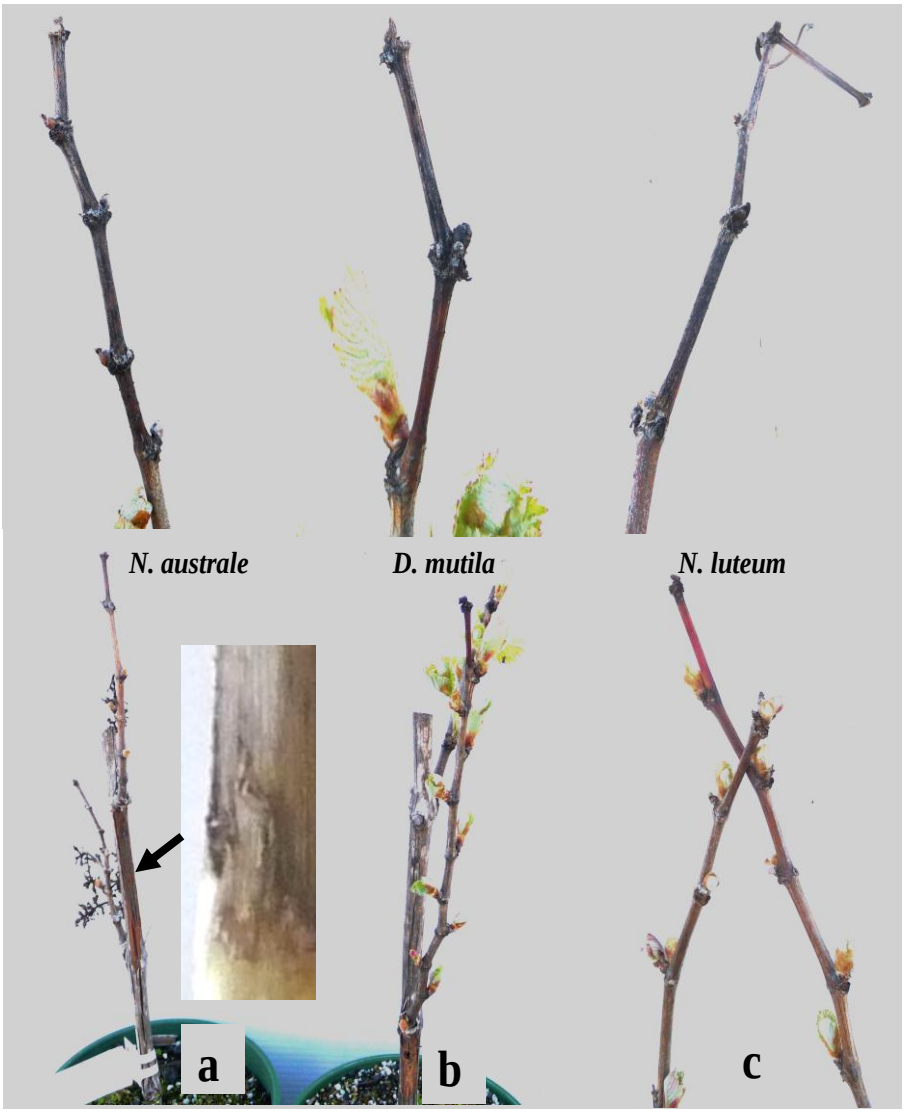

Figure 2.16: Above, grapevine plants showing severe tip dieback symptoms caused by *N. australe D. mutila* and *N. luteum* infection. Below *N. parvum* infected plants showing (a) canker (bracket) on which pycnidia were produced (arrow to insert) (b) little dieback and (c) control plants breaking bud with no dieback.

2.5.6 Molecular confirmation of botryosphaeriaceous species in woody trunk of young vines

Sequences of the amplified DNA which was extracted from the isolated colonies and directly from the wood of the grapevines from each botryosphaeriaceous species inoculation treatment confirmed all the replicates as the *N. luteum, N. australe, N. parvum* and *D. mutila* used for inoculation. The sequences showed 100% similarity to the original isolates (Appendix B.10.5).

2.6 Discussion

In this study, 63 isolates of six different botryosphaeriaceous species were isolated from grapevine plant materials, mostly from symptomatic trunks and canes. The six botryosphaeriaceous species found here in New Zealand have also been reported as dieback pathogens of grapes in other countries (Crous *et al*., 2001; Phillips 2002; Luque *et al*., 2005; Taylor *et al*., 2005; Úrbez-Torres *et al.*, 2006).

Although 13 different botryosphaeriaceous species have been reported to be associated with grapevines worldwide (Lehoczky, 1974; Larignon and Dubos, 2001; Phillips 2002; Phillips *et al.*, 2002; van Niekerk *et al.*, 2004; Úrbez-Torres *et al.*, 2006), this study found only five of them (*N. australe, N. luteum, N. parvum, D. mutila* and *D. seriata*.) were common. These species were also isolated by Úrbez-Torres *et al.* (2006; 2007) from grapevines in California, Mexico and Spain and Taylor *et al*. (2005) in Australia. However, in both reports, *L. theobromae* was also isolated. The absence of that one species in this study could reflect the limited nature of the survey or the climatic influences as *L. theobromae* appears to occur most commonly in consistently warmer areas than New Zealand (Taylor *et al.*, 2005; Úrbez-Torres *et al.*, 2008).

Identification of the isolates was challenging as most of the isolates produced no spore-bearing structures on PDA and their colonies showed similar mycelial growth characteristics and colours. However, further careful observation of colony colours within the first 3 days of growth showed differences between some isolates. For example, the characteristic yellow pigmentation of colonies was observed to be a typical characteristic of *N. luteum* and *N. australe*, which agreed with reports by Pennycook and Samuels (1985), Phillips (2000) and Slippers *et al*. (2004b). However this did not always

differentiate them from some isolates of Group 4 (*N. parvum*), which also exhibited colour formation that was slightly yellowish to brownish. Colony characteristics are not generally considered as reliable clues to identification. For example Slippers *et al*. (2004b) noted that isolates of *N. luteum* from South Africa produced a much brighter yellow pigment in culture at 25^{o}C than the Australian isolates. Variation in mycelial growth among isolates of *N. parvum* on PDA at the optimum temperature (25^{o}C) was also reported by Stevens (1926) as cited in Alves *et al*. (2008) and for some *Diplodia* cultures which differed with respect to growth rate and colour formation on PDA at 36-37ºC.

In this study, six groupings were finally obtained after careful observation of colony colours and growth form. However, even a presumptive identification could not be completed unless a way of producing the more characteristic conidia was found. Most of the isolates did not sporulate readily on the different medium types used, and the pycnidia produced by some isolates often did not contain enough conidia for reliable observation of shapes, sizes and other characteristics. Denman *et al.* (2000) also reported that many isolates of the botryosphaeriaceous species did not readily form fruiting structures in artificial media under laboratory conditions. However, the novel green shoot inoculation method developed in this study and already reported (Amponsah *et al.*, 2008) provided sufficient sporulation for description of conidium characteristics, which were used for the presumptive identification, and pathogenicity experiments.

The conidium characters (size, shape, colour and septation) were useful in the identification of some species. The *Diplodia* /*Dothiorella* species could be differentiated from the *Neofusicoccum* species by having thick-walled, rounded conidia, whereas *Neofusicoccum* conidia were mostly thin-walled, hyaline and slender. Luque *et al.* (2005) and Phillips *et al.* (2005) also used the cell wall thickness, dark colour and smaller length to width ratio to differentiate between *Neofusicoccum* groups and *Diplodia* groups. However, the overlap in characteristics of the *Neofusicoccum* species made them difficult to differentiate. Denman *et al.* (2000) also reported that the overlap in conidium characteristics of the botryosphaeriaceous species, which made them notoriously difficult to identify had led to the taxonomy of this group being confused for many years.

The mycelium growth recorded at their optimum temperatures was not a successful tool for separating the isolates into species, although mycelial growth characters at optimum temperature had been reported to be useful for distinguishing closely related species

within "*Botryosphaeria*" anamorphs (Phillips *et al.*, 2002; Slippers *et al.*, 2004a; Alves *et al.*, 2006). The estimated optimum temperature across all the isolates tested ranged from 24.1 to 25.8^{o}C with the optimum temperature within some isolates also overlapping one another (Appendix B.6.2). The initial concept of using differences in the optimum temperatures between the isolates was therefore not possible due to the small differences in the temperatures recorded for optimum growth. All the isolates were able to grow at the maximum temperature of 35^{o}C and produced no growth at the minimum temperature of 10^{o}C. This findings was in agreement with Úrbez-Torres *et al*. (2006), who reported that, *N. luteum, N. parvum,* and *D. mutila* grew at 35ºC, but contradicts reports by Pennycook and Samuels (1985) that *N. luteum* and *N. parvum* failed to grow at this temperature, and by Jacobs and Rehner (1998) who made a similar observation for *D. mutila*. However, the optimum temperatures recorded for *N. luteum, N. australe, D. mutila, N. parvum* and *D. seriata* by Úrbez-Torres *et al*. (2006) in California were 29.4, 27.8, 24.8, 28.8 and 26.8^{o}C, respectively, which are slightly higher than those recorded in this study. This could be due to differences in geographical location of isolate sources, California having a Mediterranean climate characterized by warm to hot, dry summers and mild, wet winters, whereas New Zealand has a temperate climate that varies from warm subtropical in the far north to cool temperate in the far south.

Differences in optimal mycelial growth temperatures were observed between some isolates of the same species in this study; for example *N. luteum* isolates G(s)-1 from Gisborne and N (12) 2 from Auckland had optimum growth temperatures of 25.2 and 24.6^{o}C, respectively (Appendix B.6.3), which could have reflected the temperatures in their regions of origin. There was also significant unexplained variation between some isolates of the same species, for example the three *D. seriata* isolates (all from Nelson) and two *D. mutila* isolates (from Blenhiem) differed significantly in their mycelial growth rate when incubated at the generally optimum temperature of 25^{o}C. This observation disagrees with Úrbez-Torres *et al*. (2006) who reported that there was no significant variation between isolates of the same species at each specific growth temperature.

In this study, the use of mycelial growth characteristics at optimum temperature was not useful for species discrimination. However, since mycelium growth for all the species was optimum at 24.1 to 25.9^{o}C, this may indicate similar temperature adaptation in the field. This is in agreement with Lehoczky, (1974) who observed that optimum infection

with *D. mutila* occurred through wounds and infections were most successful at 23 to 26^{o}C in the field. Ploetz *et al*. (2009) also observed in incubator studies, that *N. parvum* was able to cause significant external symptoms, vascular discoloration, and mortality at 25 to 30°C; whereas only vascular symptoms developed at 20°C and no symptoms developed at 15°C. Thus, temperature may be associated with disease development.

The morphology of colonies and conidia of most isolates provided characteristics that were useful in determining their species identification, but DNA sequence analysis of the ITS regions of the rRNA gene region, was necessary to discriminate among the groups of closely related isolates. This allowed the identity of four species, namely *N. australe, N. luteum, N. parvum* and *D. mutila,* to be confirmed after comparison with sequences lodged in GenBank whereas the *D. seriata* and *Dothiorella* sp. were identified based on mophological characteristics of conidia and due to their distinctive appearance.

In this study, the species prevalence of isolates, from most to least prevalent, were *N. parvum, N. luteum, D. mutila, N. australe* and *D. seriata*. In contrast to this study, *D. seriata* was the most frequently isolated species in New South Wales (Castillo-Pando *et al.*, 2001), France (Larignon and Dubos, 2001), Spain 2001(Armengol *et al.*, 2001), Western Australian (Taylor *et al.*, 2005) and California (Úrbez-Torres *et al.*, 2006). It was also the dominant species on fruit trees in the Western Cape Province of South Africa (Slippers *et al.*, 2007).

In this study, the isolates were found to be associated with trunk cankers, cane and shoot dieback, internal trunk necrosis, shoot lesions, leaf spots, poor bud development and bud necrosis, which was supported by Phillips (2002) who reported a wide variety of grapevine disease symptoms associated with these pathogens in Portugal. Similar work carried out in Western Australia (Taylor *et al.*, 2005), USA (Úrbez-Torres *et al.*, 2006), South Africa (van Niekerk *et al.*, 2004), and Spain (Luque *et al.*, 2005) also reported a wide range of grapevine symptoms associated with botryosphaeriaceous species. In New Zealand, the botryosphaeriaceous species were isolated from naturally infected grapevines of different ages (2-33 years old) although most of the severe trunk symptoms were observed in older vines. Larignon and Dubos (2001) reported that in a survey of French vineyards, the excoriose disease caused by botryosphaeriaceous species usually developed slowly and so severe symptoms became visible only in grapevines that were eight or more years old.

In this present study, five botryosphaeriaceous species were isolated from 11 non-grapevine woody hosts, with *D. mutila* being the most common followed by *N. luteum.* The isolation of similar botryosphaeriaceous species from woody debris and symptomatic non-grapevine woody hosts growing in close proximity to the vineyards in New Zealand also confirms reports from the United States, where Stanosz *et al.* (1998) and McDonald *et al.* (2009) isolated *D. mutila* on *Juniperus* species and *N. luteum, N. parvum* and *N. australe* from avocado branches. In Victoria and New South Wales, Cunnington *et al.* (2007) also isolated *N. australe, D. seriata* and *N. parvum* from some horticultural plants such as *Vaccinium corymbosum, Phoenix canariensis, Olea africana,* and *Persea americana*. In New Zealand, Pennycook and Samuels (1985) and Young and Fletcher (1997) isolated *N. luteum* and *N. parvum* from kiwifruit (*Actinidia deliciosa*) and apple (*Malus x domestica*) whilst Sammonds *et al.*, (2009) also isolated *N. luteum, N. parvum* and *D. seriata* from blueberries.

The isolations of *N. australe* from *Vitis vinifera* (grapevines) and *Cytisus scoparius* (broom) in this study represented a first report of this fungus in New Zealand. It was initially isolated from native *Acacia* in Australia where the new epithet *B. australis* (*N. australe*) was proposed by Slippers *et al.* (2004b). It has since been reported on fruit trees in South Africa (Slippers *et al.,* 2007) and on *Eucalyptus* and *Pistachio* in Spain (Armengol *et al.,* 2008). *Neofusicoccum australe* has also been isolated from grapevine in California (Úrbez-Torres *et al.,* 2006), South Africa (van Niekerk *et al.,* 2004) and Western Australia (Taylor *et al.,* 2005). It was concluded to be a frequent pathogen of native and introduced hosts in South Africa and Australia (Slippers *et al.*, 2004d; Burgess *et al.*, 2006; Cunnington *et al.*, 2007).

The botryosphaeriaceous species used in this study were unable to infect non-wounded grapevine tissues, as they did with wounded tissues. This confirms them as wound pathogens, which is consistent with other reports (Smith *et al.*, 1994; Taylor *et al.*, 2005; van Niekerk *et al.,* 2006). Phillips (1998) also observed that *D. seriata* and *D. mutila* could cause symptoms only on wounded grapevine tissues. However, on apple and pear, Sutton (1990) also recognized lenticels as common entrance sites for *B. dothidea*, while Brown and Hendrix (1981) and Michailides (1991) observed that *B. dothidea* isolates can also infect directly through stomata openings on healthy apple and pistachio plants. Kim *et al.* (1999) reported observing the formation of appressoria at the tip of *B. dothidea* germ tubes developing on an apple fruit surface which evidently altered the surface, indicating the possibility of direct penetration of the fungus by enzymatic

degradation of the cuticle layers. Since direct infection by botryosphaeriaceous species was reported for *B. dothidea* on apple fruit surfaces and pistachio plants, further studies by microscopy should be done on other botryosphaeriaceous species to investigate their mode of infection.

In this study, 3-day-old mycelial discs were used for all the inoculations to ensure that invading hyphae were young and active, which was similar to that used by Phillips (1998). By contrast, van Niekerk *et al.* (2004) and Taylor *et al.* (2005) used 7-day-old mycelial discs and Castillo-Pando *et al.* (2001) also used 7-day-old hyphal inoculum or conidium suspensions for pathogenicity studies. In a preliminary study, lesion development was found to be faster when wounded tissues were inoculated with mycelium plugs from a 3-day-old culture than with a 7-day-old culture (Appendix B. 10.6). The difference in culture age and inoculum type (conidia or mycelium) may therefore influence the development of lesions produced by the pathogen and may account for some differences in pathogenicity reported in the literature.

In the pathogenicity assays using detached green grapevine shoots and isolates taken from grapevines, isolates of *N. luteum* and *N. australe* were found to be the most pathogenic followed by *N. parvum*, with the least pathogenic being *D. mutila*. However, variation in pathogenicity was observed between isolates of the same species. This has been observed elsewhere for *N. australe* (van Niekerk *et al.*, 2004; Taylor *et al.*, 2005) and *D. seriata* (Larignon *et al.*, 2001; van Niekerk *et al.*, 2004), which might indicate that isolates within a species can be divided into different virulence groups. Larignon *et al.* (2001) were of the view that the differences in pathogenicity could be due to differences in virulence among pathogen strains after finding out that two of their *D. seriata* isolates were not pathogenic. They went on to identify four distinct virulence groups within their 10 isolates of *D. seriata* that had originally been isolated from five different grapevine varieties (Syrah, Merlot, Clairette, Pinot meunier and Cabernet sauvignon), which were from six different geographic regions (Perpignan, Medoc, Die, Champagne, Entre-2-Mers and Graves). However, they could not link the level of virulence to the geographic origins of the isolates.

Variation amongst isolates of other grapevine pathogens has also been reported. The *Cylindrocarpon* species, which causes black foot disease, have demonstrated variation in symptoms on grapevines roots (Alaniz *et al.*, 2007). In addition, isolates of *E. lata* collected from wine regions throughout Australia and tested for their pathogenicity on

grapevines were found to vary in their ability to induce wood and foliar symptoms of grapevines, thus reflecting their levels of virulence (Sosnowski *et al.*, 2007a).

Diplodia seriata isolates produced minor lesions that did not extend beyond the inoculation point. This finding was in agreement with Taylor *et al.* (2005) and Phillips (1998) who reported that although *D. seriata* was shown to be a wound pathogen, the lesions produced were small and caused very little damage to the host. In California, *D. seriata* was also found to be the least virulent of all nine botryosphaeriaceous species tested (Úrbez-Torres *et al*., 2006). However, in contrast to these findings, other authors have reported this species to be very pathogenic on detached green shoots of several scion varieties (Savocchia *et al.*, 2007), and detached mature canes (Larignon *et al.*, 2001; van Niekerk *et al.*, 2004). Epstein *et al.* (2008) also demonstrated in the non-coastal areas of California, that conidia of *D. seriata* were the most abundant species collected in rain water traps in grapevines. They demonstrated its pathogenicity in greenhouse studies, conducted by inoculating wounded stems of own-rooted Cabernet Sauvignon vines with mycelium discs. In various regions of Italy *D. seriata* has also been recorded as causing black dead arm in grapevines (Cristinzio, 1978; Rovesti and Montermini, 1987).

The discrepancy in pathogenicity reports of *D. seriata* from different grapevine growing areas worldwide could be due to the physiology of plants, especially those factors that may lead to changes in the xylem sap content. Host-pathogen responses, cell wall metabolism, and cell death have also been reported to be triggered by specific proteins such as peroxidases and chitinases present in xylem sap, although their specific roles are not clear (Aguero *et al.*, 2008). In field vines, xylem sap composition was reported to be influenced by the availability of soil nutrients and the soil water potential (Dambrine *et al.*, 1995). Martos *et al.* (2008) observed that phytotoxic activity for *D. seriata* was lowest at low sap pH values, whilst that of *N. luteum* and *N. parvum* remained consistently high across a range of sap pH levels. Further to that, Larbi *et al.* (2003) reported that soil elements such as iron (Fe) caused changes in the chemical composition of xylem sap in pear and in peach, including concentrations of organic anions, Fe concentrations and pH. Similar effects may occur in the xylem of grapevines, affecting the activity of the botryosphaeriaceous pathogens, which were shown to be present within the xylem vessels (Rayachhetry *et al.*, 1996; Amponsah *et al.*, 2009b).

All the isolates except those of *D. seriata* obtained from the non-grapevine hosts in this study were found to be pathogenic on wounded, detached green shoots of grapevine var

Pinot noir, on which they caused long lesions within 10 days, which ultimately produced pycnidia and conidia. This indicated their potential as important sources of inoculum for vineyard infection. Denman *et al*. (2000), Rubini *et al*. (2005) and Mohali *et al*. (2005) reported that botryosphaeriaceous species are pathogens of many woody hosts, and can be pathogenic or saprophytic. Botryosphaeriaceous species that have in recent years been confirmed to infect a wide range of hosts ('host neutral') include *B. dothidea, D. seriata, N. parvum, N. australe* and *L. theobromae* (Slippers *et al.*, 2004d; Burgess *et al.*, 2005). Pavlic *et al.* (2007) also demonstrated that species such as *N. ribis, N. parvum* and *L. theobromae,* that were cosmopolitan and cross-infected different hosts, were amongst the most pathogenic on *Eucalyptus* species and concluded that botryosphaeriaceous species from native hosts could pose a threat to the introduced *Eucalyptus* species and *vice versa*. In this study, samples were taken from a narrow range of host genera or plant families because they were considered to be common in the vicinity of the vineyard. Further studies should be done to determine the breadth of host species in New Zealand, and the botryosphaeriaceous species that may infect them and their pathogenicity on grapevines.

The botryosphaeriaceous isolates recovered in the initial sampling study of 20 vineyards were from eight common grapevine varieties and from four common rootstock varieties, which indicated wide susceptibility to a range of botryosphaeriaceous species. The pathogenicity experiments that used mixed isolate inoculum for each of four species for inoculating wounded detached green shoots or trunks of five different potted grapevine varieties showed that all varieties were equally susceptible. Other authors have reported the susceptibility of a range of grapevine scion varieties to various botryosphaeriaceous species, such as Red Globe to *L. theobromae, D. mutila*, *D. seriata* and *N. australe* (Taylor *et al.*, 2005), Periquita, Chardonnay and Cabernet sauvignon to *N. australe, N. parvum, L. theobromae, F. vitifusiforme, F. viticlavatum, D. seriata, Diplodia* sp. *D. porosum, B. dothidea, N, ribis,* and *N. luteum* (van Niekerk *et al.*, 2004), Espadeiro to *B. dothidea, D. mutila* and *D. seriata* (Phillips, 1998), Sauvignon blanc, Cabernet Sauvignon and Cabernet Franc to *B. dothidea, D. mutila* and *D. seriata* (Larignon and Dubos, 2001) and *L. theobromae* and *D. seriata* to Thompson seedless and Chardonnay (Úrbez-Torres *et al.*, 2008)**.** The susceptibility of rootstock varieties has not been so well investigated. In this study, *N. parvum* and *N. luteum* were found to be associated with 101-14, 3309, Richter and Schwarzmann. Aroca *et al.* (2006) reported isolation of several botryosphaeriaceous species (*N. parvum, B. dothidea, D. seriata*), from grapevine rootstock mother plants (110 R, 140 Ru, 161–49, SO4 and 41 B). These

examples worldwide therefore support the conclusion that most grapevine varieties are susceptible to infection by the botryosphaeriaceous species.

It was observed in this study that the grapevine green shoots infected with *N. parvum, N. australe* and *D. mutila* developed shorter internodes than normal, which restricted the development of the internal lesions. However the shoots inoculated with *N. luteum* did not show any development of short internodes. The observations of shortened internodes in this study is in agreement with reports by Larignon *et al.* (2001), Phillips (2002) and Taylor *et al.* (2005) who described them as characteristic symptoms of growth decline in grapevines. Gardner (1997) also observed that branches of *Sophora chrysophylla* infected with *Botryosphaeria mamane* became thickened with short internodes. Several isolates of botryosphaeriaceous fungi obtained from non-grapevine hosts are known for producing bio-active toxic metabolites, which are probably involved in the disease symptoms they cause (Barbosa *et al*. 2003; Crognale *et al*. 2003). However, Martos *et al*. (2008) working on the production of phytotoxic metabolites by five botryosphaeriaceous fungi (*F. aesculi, D. seriata, N. luteum, N. parvum* and *Dothiorella viticola*) observed that all the species caused phytotoxicity on grapevine leaves. However the tested isolates showed variable levels of toxin production and the toxins were determined from gas-chromatography activity analysis to be acetylated *O*-methyl glycosides of phytotoxic exopolysaccharides. In another phytotoxicity assay on 13 strains of *N. parvum* isolated from vines showing decline symptoms in Portugal and France, Abou-Mansour *et al*. (2010) identified a novel compound along with mellein, *cis* hydroxymellein and *trans* hydroxymellein through chemical studies of fermentation extracts of *N. parvum.* Further studies should investigate the relationship between toxin production by isolates and symptoms caused.

Toxin production has been demonstrated for other grapevine pathogens including *E. lata* and *Pa. chlamydospora.* Andolfi *et al*. (2009) used a flow cytometry technique to detect toxic metabolites (polysaccharides) produced by *Pa. chlamydospora*, in symptomatic leaves of esca-affected grapevines, and concluded that the method could be used to develop a simple kit to study the mechanisms underlying the development of esca foliar symptoms. Defranq *et al*. (1993) also postulated that fruiting spurs and stems of grapevines are damaged when the *E. lata* toxins are translocated in spring and Mahoney *et al*. (2005) observed that the presence of metabolites such as eutypinol and eutypine correlated with visible evidence of pathogenic *E. lata* in infected tissues. In California, Lardner *et al*. (2007) identified six phytotoxic metabolites, namely eutypinol, methyl

eutypinol, eulatachromene, eutypine, 2-*iso*-propenyl-5-formylbenzofuran and eulatinol, which were detected in culture filtrates of *E. lata* isolates. When a crude culture filtrate was inoculated onto micropropagated grapevine shoots and platelets they yielded symptoms similar to those inoculated with mycelium of *E. lata*, thus confirming the symptoms to be due to the phytotoxicity of the metabolites produced by the fungus. In Australia, Sosnowski *et al*. (2007b) hypothesised that disease prevalence and the incidence of vines with severe disease symptoms was influenced by the environment, and went ahead to hypothesize that toxin accumulated in the vine during favourable winter and spring conditions.

In this study, conidia were as pathogenic as mycelium plugs when inoculated onto wounded woody trunks of five different grapevine varieties but mycelium infections appeared to develop faster than conidium infections for all botryosphaeriaceous species and the grapevine varieties. This was probably due to a lag phase as conidia needed to first attach, develop germ tubes, penetrate and develop within the internal tissues. Prusky and Plumbley (1992) also observed that the rate of conidium germination of some fungi including botryosphaeriaceous species has been found to be influenced by the presence of nutrients and the chemical nature of the host surfaces. Mercure *et al.* (1994) also demonstrated that the ability of many fungal spores to germinate and to produce infection structures was associated with the ability of a spore to attach to the tissue surface prior to infection. It is therefore possible that the initial delay in symptom expression in the grapevine plants inoculated with conidia might have been due to one or a combination of such factors. The faster symptom expression with mycelial inoculations could also be due to sap nutrients released by wounding being available for absorption by the mycelium, leading to rapid growth into the plant tissue.

The rate of symptom development differed for detached and attached green shoots and also for woody trunks. On the detached grapevine green shoots, dark lesions were quickly caused by *N. australe, N. luteum* and *N. parvum* inoculation and they spread along the shoot reaching 50-70 mm within 10 days. Such lesions were not restricted by the nodes. However, on attached green shoots it took 2 months for lesions of similar size to develop. These lesions could only be observed within shoots after they were longitudinally split through the inoculation points. The internal lesions differed in colour intensities and lengths, with respect to the inoculated species. Lesions were dark brown for *N. parvum*, *N. australe* and *D. mutila* infected shoots, while for *N. luteum* they were light brown. In contrast, the lesions produced on the *in vitro* (detached) inoculated

shoots were dark brown for all species and were visible on shoot surfaces. Symptoms that developed on the woody trunks of 2-year-old grapevines 7 months after inoculations differed between species. Inoculations with *N. australe, N. luteum* and *D. mutila* caused no external lesions, except at the inoculation point where wounding occurred. However, inoculation with *N. parvum* caused external wood necrosis. On all the five cultivars inoculated with *N. parvum*, the trunks showed much greater external symptoms of dead wood (canker), that were usually restricted to one-half of the stem, which made it appear more virulent than *N. luteum.* When the plants infected by botryosphaeriaceous species were pruned after 7 months in winter and put under lights to break bud dormancy, dieback was observed in those plants inoculated with *N. luteum, N. australe* and *D. mutila* but not with *N. parvum*. This observation suggests that such species are dieback pathogens and that *N. parvum* is a canker pathogen in New Zealand although this needs further confirmation.

Cross sections cut through the trunks of 2-year-old vines revealed wedge-shaped symptoms, which were similar to symptoms reported by (Taylor *et al.*, (2005) and Úrbez-Torres *et al.*, (2006). The wedge-shaped symptoms have been confused with those of other pathogens, mainly *E. lata* and *P. viticola*, therefore making the diagnosis of the diseases in the field difficult (Phillips 1998; Castillo-Pando *et al.*, 2001; van Niekerk *et al.*, 2006). In California, Gubler *et al.* (2005) reported that *L. theobromae* was associated with wedge shaped wood staining and canker symptoms in grapevines, a disease usually referred to as "Bot canker".

Overall, the limited sampling in this study showed that *N. luteum, N. parvum, N. australe* and *D. mutila* were the most prevalent botryosphaeriaceous species in New Zealand vineyards and the pathogenicity studies indicated that they were able to infect mature woody tissues, causing cankers, vascular discoloration, and/or dark streaking of the internal tissues, as well as dieback on new vegetative growth. However, virulence varied among the species and between isolates. This finding was in agreement with work done in California by Úrbez-Torres and Gubler (2009) who also indicated *L. theobromae* was the most virulent species followed by *N. luteum, N. parvum*, and *N. australe*, which were all considered highly virulent. Savocchia *et al.* (2007) in their pathogenicity tests on detached 1-year-old canes from glasshouse-grown grapevines found *N. luteum* and *D. seriata* to be the most pathogenic species. Pathogenicity studies conducted by van Niekerk *et al*. (2004) also revealed that, *N. australe, N. parvum, N. ribis* and *D. mutila* were the most virulent species in South African vineyards. Since pathogenicity studies

conducted globally have indicated that the level of virulence varies between pathogens and countries, none of the 13 botryosphaeriaceous species identified on grapevines could be considered a lesser pathogen. However, there are many factors which affect pathogenicity and disease progression and the next chapter of this thesis will focus on such factors.

Chapter 3
Factors affecting infection and disease progression of the botryosphaeriaceae species in grapevines

3.1 Introduction

The pathogenicity tests initially conducted on detached green shoots with the botryosphaeriaceous species isolated from New Zealand vineyards (Chapter 2) showed that *N. luteum* was the most pathogenic on all the five grapevine varieties tested and *D. seriata* was not pathogenic. However, on the inoculated living grapevine tissue the four pathogenic species caused only internal discolouration of green tissue.When inoculated onto woody trunks, most species caused no visible external symptoms, with only *N. parvum* causing a canker-like necrosis. For the vines inoculated with *N. australe*, *N. luteum* and *D. mutila*, it was only when the plants were pruned in winter that dieback symptoms appeared. This type of response was described by Baker *et al*. (1973) who observed that most stem canker and dieback fungi are non aggressive or weak parasites, and after entering host plants through wounding, often remain inactive as long as the host plant remains vigourous. Swart and Wingfield (1991) also reported that development of botryosphaeriaceous diseases mostly follows the onset of stress.

Smith *et al.* (1996) observed that some of the botryosphaeriaceous species have a latent phase and do not become pathogenic until environmental or physiological conditions are favourable. However Slippers *et al.* (2007) reported that some members of the botryosphaeriaceous species are among the most aggressive pathogens, often killing large parts of their host, following infection through wounds. Virtually all plant parts such as seeds (Cilliers *et al.*, 1995), fruit, inflorescences and pedicels (Johnson *et al.*, 1992; Kim *et al.*, 2001), trunks, branches and leaves (Smith *et al.*, 1996) have been reported as hosts to latent botryosphaeriaceous species. On grapevines, Castillo-Pando *et al*. (2001), who investigated infection within different tissues, also confirmed botryosphaeriaceous species to be frequently isolated from grapevine buds including dead buds, from which they concluded that botryosphaeriaceous species were associated with budburst failure.

In nature, the spores (sexual and asexual) that may become airborne or splash-borne during and following rain can infect and colonize exposed wood vessels (Eskalen *et al.*,

2007; Úrbez-Torres and Gubler, 2008). Wounds, especially pruning wounds, are regarded as primary infection sites for these pathogens (Larignon and Dubos, 2000; Mostert *et al.*, 2005; van Niekerk *et al.*, 2004), but their susceptibility may vary depending on the age of the pruning wound and the time of pruning, as has been reported for *Eutypa lata* infection of grapevines. Usually, the susceptibility of wounds to *E. lata* infection decreases over time after pruning, decreasing faster during higher degree days (Munkvold and Marois, 1995). Rolshausen *et al.* (2010) also observed less wound colonization by *E. lata* in grapevine spurs that were artificially inoculated with the fungal pathogen at 1 week versus 1 day after pruning. Conidia are likely to be the most common natural means of grapevine wound infection by the botryosphaeriaceous species however there is little information on the factors affecting their infection success.

The factors contributing to botryosphaeriaceous infections on grapevines have also not been investigated, and are important for understanding the potential effectiveness of different management strategies. In this chapter, *N. luteum* was the species principally selected for investigating a range of such factors however *N. australe, N. parvum* and *D. mutila* were also included for further verification of some aspects of pathogen progression. The objectives of this research were to investigate (i) the optimum conidial concentration required for infection by *N. luteum*, (ii) the susceptibility of different wounded parts of the grapevine to infection by *N. luteum,* (iii) infection development by *N. luteum* on attached and detached grapevine plant materials, (iv) disease progression by the four pathogenic botryosphaeriaceous species through the grapevine tissue, (v) the effect of stem tissue age and wound age on infection and progression by *N. luteum* and (vi) the effect of water stress on *N. luteum* progression and symptom development.

3.2 Materials and Methods

3.2.1 Fungal isolates, inoculum preparation and isolation

All experiments described here were conducted with mixed isolates of the botryosphaeriaceous species previously used for pathogenicity studies in Chapter 2, Table 2.8. Conidia for inoculation were produced by inoculating green shoots with mycelium colonised PDA plugs to produce lesions and pycnidia that oozed conidia. Conidia were collected by shaking the sporulating infected shoots in 20 mL of sterile water, and filtering the suspension through sterile Miracloth as described previously in

Section 2.2.3.2. The conidia were counted using a haemocytometer; and equal numbers from each isolate were mixed together before adjusting to the required concentration in sterile distilled water. Conidial viability was confirmed by spreading the conidium suspensions on PDA and examining their germination with a stereo microscope after 3 h at room temperature. A fresh conidium suspension was used for each inoculation event onto wounded surfaces. The methods of growing mature Pinot noir canes into potted vines and their overall management were similar to those described previously in Section 2.4.4. All inoculations were carried out in late spring 2008 (October-December) after the plants has been grown in the 2 L pots with potting mix for at least 12-18 months.

Isolation methods were similar to those used before. Woody and root tissues were surface sterilised as described in Section 2.2.2. Soft green tissues such as leaves, shoots and buds were surface sterilised by dipping in 70% ethanol for 30 s and rinsing twice in sterile tap water. The tissue pieces were isolated directly onto PDA. The plates were incubated in continuous darkness at 25^{o}C and the botryosphaeriaceous isolates identified by colony appearance. Data were analysed by ANOVA to determine significance of the above treatments. The separation of means was done using Fisher's protected LSD at $P \leq 0.05$.

3.2.2 Effects of *N. luteum* inoculum dose on disease incidence and symptom development

The effect of *N. luteum* conidium concentration on infection success was determined in a detached green shoot experiment. A 10^6/mL conidial suspension was made from each isolate as above and they were mixed before making a dilution series containing 10^2, 10^3, 10^4, 10^5 and 10^6 conidia/mL. Fresh and healthy Pinot noir green shoot tips of about 20 cm in length, with at least two terminal leaves and a diameter of about 10 mm were taken from field grapevines in December 2008. Three milimeter diameter wounds were made in shoot mid-sections and immediately inoculated with 20 µL droplets of each of the different conidium concentrations and control shoots were inoculated with sterile water. The 10 replicate detached green shoots per treatment were arranged in a CRD in an enclosed transparent plastic chamber at 18-21^{o}C and maintained as described in Section 2.2.3.2. At 10 days after inoculation, lesion lengths were measured using a digital calliper followed by pathogen isolation onto PDA. Data were analysed as described in Section 3.2.1.

3.2.2.1 *Botryosphaeriaceous species progression and shoot lesion development*

An experiment was setup to check that a concentration of 10^4 conidia/mL was equally effective for other botryosphaeriaceous species on attached green shoots as on detached green shoots for *N. luteum*. On each of the Pinot noir vines aged 18 months, a green shoot was selected and wounded with the tip of a sterile scalpel, creating a depression, prior to inoculation as described previously in Section 2.4.1. The wounds were drop-inoculated immediately with 25 µL of a mixed isolate conidial suspension (10^4/mL) from each of *N. luteum, N. australe, N. parvum* or *D. mutila* obtained as described in Section 3.2.1 or sterile water (control shoots). The 30 replicate plants per inoculated treatment were arranged in CRD and maintained as described in Section 2.4.4 except that the plants were placed in an open area that simulated field conditions for 9 months (Figure 3.2).

Figure 3.1: Two-year-old potted vines (var. Pinot noir) growing in an open area with the canes stretched out and tied onto a wire to simulate vineyard trellis systems with the green shoots growing vertical prior to inoculation with *N. luteum* conidia.

To track disease development and pathogen movement, five randomly selected replicates per treatment were assessed at 1 month after inoculations by measuring the

internal lesions with a digital calliper after slicing lengthwise through each inoculated green shoot. Another five replicates were assessed by pathogen isolation at 10 mm intervals by surface sterilising the green shoots as described in Section 2.2.2, then cutting them into 10 mm pieces and putting then sequentially onto PDA for incubation at 18-21^{o}C for 3 days. At 2, 3, 4 and 9 months after the inoculation, natural browning of the bark and the pith meant that lesions became less visible and so the pathogen progression along the shoots was assessed only by pathogen isolation at 10 mm intervals. The maximum distance colonised by each species was used as a measure of its pathogenicity and data were analysed as described in Section 3.2.1.

3.2.3 Internal progression of botryosphaeriaceous species through grapevine tissues

The four different species (*N. australe, N. luteum, N. parvum* and *D. mutila*) were used to inoculate wounds created on fresh green shoots of potted Pinot noir grapevines in November 2008 as described in Section 2.4.1. There were 18 replicate plants for each of the botryosphaeriaceous species (nine inoculated with a mycelium mixture of isolates made as described in Section 2.4.5.2 and nine inoculated with a conidial mixture of isolates made as described in Section 2.4.5.1). A higher concentration of 10^5 conidia/mL was used to improve the chances of observing them in the sap. Control plants were drop-inoculated with sterile water or sterile agar discs. All plants were arranged in a CRBD except plants were placed in an open area that simulated field conditions and plants maintained as described in Section 2.4.4.

3.2.3.1 Plant assessment to determine fungal propagule in plant tissue sap

The first assessment of three randomly selected plants per treatment was carried out after 2 months by observing the inoculation sites for symptoms, and by examining the fungal content of sap, through microscopy and isolation. A cut was made across the shoot at healthy sections immediately above and below the inoculation area. The sap that oozed from each freshly cut wound was collected into a 25 mL centrifuge tube, vortexed briefly and 10 drops (20 µL per drop) from each treated plant sample were mounted onto 10 glass slides for observation at ×100 magnification with a light microscope for presence of any botryosphaeriaceous propagules. Ten drops per plant sample were spread onto 10 PDA plates and incubated at 25^{o}C in continuous darkness for 3 days to allow growth of any fungal propagules. In addition, the same three plants cut for sap

extrusion were used to make isolations from six sections (5 mm intervals) along the shoot area above the inoculation point and plated onto PDA.

3.2.3.2 Microscopy to determine fungal structures in plant tissue

A second assessment of three plants randomly selected per treatment was carried out 3 months after inoculation with the botryosphaeriaceous species. Microscopic sections were cut by hand-slicing thin sections from non-lesion portions immediately above or below the lesions. From each plant, a total of 24 sections (12 transverse and 12 longitudinal) were cut, of which six of each type were immediately fixed by a standard fixation method (Appendix C.1) for 6 h, followed by 3 or 4 times washing in phosphate buffered saline (PBS) solution for 1 h. A stock solution of 1 mg wheat germ agglutinin (WGA) labelled with fluorescein isothiocyanate (FITC) (Sigma life sciences) was prepared in 1 mL phosphate buffer saline (PBS) after which it was diluted 1:30 in PBS for staining the fungal structures (Stephens *et al.*, 2008). The sections were soaked in the solution for 6 h at room temperature to label any fungal structures present, and then the sections washed briefly in distilled water and examined under a fluorescent microscope (Olympus® BX51) using a blue filter cube with a 405 nm wavelength. The remaining six-hand-sectioned shoots were immediately taken to the Department of Biological Sciences, Canterbury University where each sample was mounted onto an aluminium stub using carbon glue tabs and sputter-coated with gold. The sections were examined with a Philips XL 20 scanning electron microscope (SEM) at 15kV for conidial numbers, germ tube development and penetration into host tissue. The experiment was repeated with new plants grown for the purpose.

3.2.3.3 Assessment for dieback and budburst failure

A third assessment of the remaining three plants per treatment was carried out after 8 months, at the end of the growing season when the apparently healthy plants became dormant (winter). After pruning them to two canes with 3-4 buds per plant, the plants that exhibited dieback from the pruned shoots were then placed under sodium lights in a greenhouse to break dormancy, as described in Section 2.4.5.2. They were then visually examined for extent of bud break and the dieback measured. Pathogen isolations were carried out from the dieback lesion edges. This part of the assessment was carried out to confirm results previously observed in Section 2.5.5.

3.2.4 *Neofusicoccum luteum* conidial infections on attached and detached grapevine tissue

Since previous experiments had shown different rates of lesion development between attached and detached green shoots, the behaviour of conidia on these tissues was investigated using the green shoots and leaves growing within the mid sections of the shoots on Pinot noir grapevines growing in pots. The tissues were selected to investigate infection development. Six replicate shoots (two per plant) and six replicate leaves (two mid section leaves per shoot) provided the attached green shoots and leaves for inoculation in November 2008 with a mixed isolate conidial suspension (10^5/mL), made as described in Section 2.4.5.1. Leaf surfaces on the replicate plants were wounded by lightly scraping the surface layer with a sterilised scalpel. Shoots were wounded by cutting off a small surface section (3 mm^2) mid-way up the shoot (~75 mm from the base) with a sterilised scalpel.

The attached shoots and leaf surfaces (wounded and non-wounded) were drop-inoculated with 20 µL of a *N. luteum* conidial suspension, covered with transparent polyethylene bags for 2 h and the potted vines placed under a tree canopy in a shaded area during the early hours of a clear sunny morning. Six replicate detached shoots (~50 mm long) and six replicate leaf surfaces (wounded and non-wounded) of 1-year-old Pinot noir vines were put into small transparent polyethylene bags and immediately taken to the plant pathology laboratory, where they were drop-inoculated onto the wounded and non-wounded tissues as before and incubated on moist sterile tissue paper at room temperature (18-21^{o}C).

At 3, 6, 12 , 18 and 24 h after inoculation, three disc segments of about 5 mm diameter were cut from inoculated areas on all replicate samples of each tissue and treatment type (both wounded and non-wounded, attached and detached). The segments were immediately taken to the Department of Biological Sciences, Canterbury University, prepared for SEM and prepared as described in Section 3.2.3.2. The experiment was repeated.

3.2.5 Susceptibility of different grapevine tissues to *N. luteum* infection

The susceptibility to infection of six grapevine tissues (pruned cane ends, buds, leaf surfaces, shoots, trunks and roots) (Figure 3.2) was determined with *N. luteum* conidia (10^5/mL) prepared as described in Section 2.4.5.1. The plants used were 1-year-old Pinot noir vines growing in 2 L pots with potting mix and maintained as described previously

in Section 2.4.4. Separate plants were used for each grapevine tissue treatment. Plants were arranged in CRD with six replicate vines assigned to each tissue type (one tissue point inoculated on each vine). Assessment for susceptibility was done by observing all inoculated tissues for necrosis followed by pathogen isolation onto PDA to observe the presence of fungal colonies characteristic of *N. luteum* and the incidence data were analysed as described in Section 3.2.1.

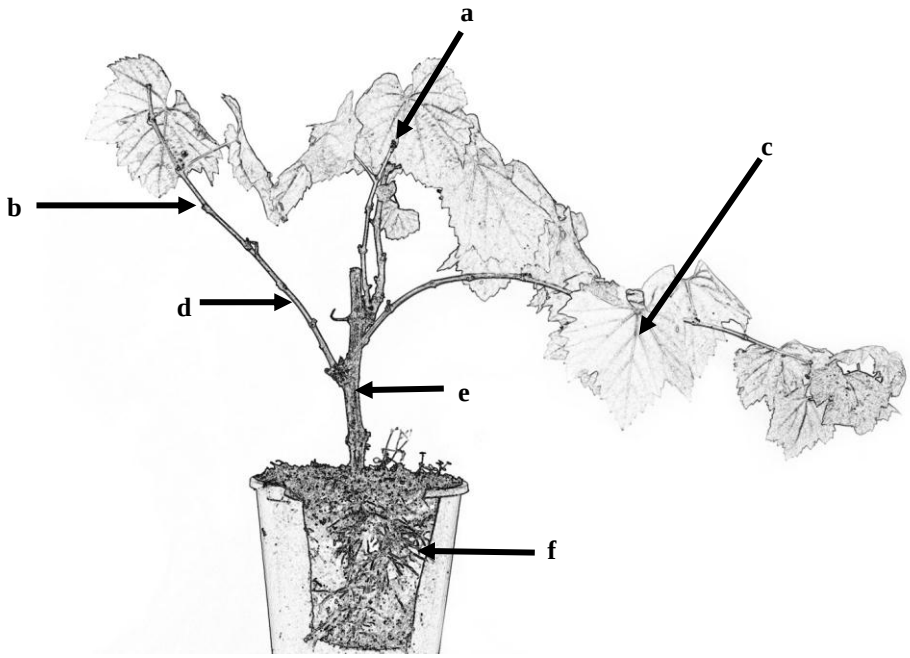

Figure 3.2: Diagram of potted Pinot noir vine plant showing the different wounded entry sites for infections with *N. luteum* conidia (a) pruned cane end, (b) bud, (c) leaf, (d) shoot, (e) trunk and (f) root infection.

3.2.5.1 Pruned cane ends, shoot and trunk tissues

Wounding of the trunks and shoots was done by cutting out portions approximately 2-3 mm in diameter and 1-2 mm deep with the tip of a sterile scalpel to expose the cambium. The pruning wounds of cane ends were made by a transverse cut using an alcohol sterilised secateur. The wounds on these tissues were each drop-inoculated with 20 µL of a mixed isolate *N. luteum* conidium suspension (10^5/mL) as described in Section 2.4.5.1 and control tissues were inoculated with 20 µL of sterile water. Each inoculated tissue per plant was covered with a transparent polyethylene bag for 24 h to provide high humidity for infection. The plants were incubated for 4 weeks then assessed as described in Section 3.2.5 and the incidence data were analysed as described in Section 3.2.1.

3.2.5.2 Leaf surfaces and buds

On each replicate vine, four buds or four leaves from the mid sections of shoots (two shoots per plant) assigned to each tissue type were tagged for wounding and inoculation. The surfaces of leaves and buds were wounded by scratching lightly with a sterile scalpel and drop-inoculated with 20 µL of a mixed isolate *N. luteum* conidium suspension (10^5/mL) as described in Section 2.4.5.1, and control tissues were inoculated with 20 µL of sterile water. Each vine with inoculated tissue was covered with a transparent polyethylene bag for 24 h to provide high humidity for infection. Leaves and dead buds were assessed as described in Section 3.2.5 at 1 and 2 weeks after inoculations, respectively, and the incidence data were analysed as described in Section 3.2.1. Buds that burst and developed into shoots were monitored for symptom development. If they displayed any necrosis during the following 4 weeks, isolations were made from lesion edges as before.

3.2.5.3 Roots

Experiment 1

For root inoculation, the potting mix in one half of a potted vine was removed to expose the roots which were then trimmed using a sterile scalpel and immediately inoculated by trickling 10 mL of the *N. luteum* mixed isolate conidial suspension (10^5/mL) over them before replanting and covering the roots with potting mix. Control plants were wounded in the same way and inoculated with sterile distilled water and the five replicate plants placed in an open area that simulated field conditions. Three months after inoculation all base stem pieces with roots were removed, washed twice under tap water to remove all potting mix and observed under a stereo microscope for lesion development. Pathogen isolation was carried out at 5 mm intervals from the tips of 10 randomly selected roots from the inoculated half of each pot and from all stems base pieces and observed for the presence of colonies characteristic of *N. luteum*.

Experiment 2

Since the first attempt at inoculating roots using *N. luteum* conidia was unsuccessful, a second experiment was conducted using *N. australe, N. luteum, N. parvum* and *D. mutila*. The roots of Pinot noir vines growing in pots in the shade house were exposed by removing the potting mix in one half of each pot and the root tips were wounded by trimming them with a sterile scalpel as before. The wounded roots tips were inoculated with a mixture of three isolates for each species, (Appendix B.3). There were five

replicate plants per treatment combination. Inoculation was with 10 mL of a mixed isolate conidial suspension (10^5/mL) applied as before or by spreading over them a mixed isolates mycelium from agar colonies plus chopped infected green shoot tissue. The infected green shoot fragments prepared as in Section 2.4.1 capable of exuding conidia and mycelium were chopped up (~10 mm pieces) and mixed in approximately equal amounts with 3-day-old cultures on agar for inoculation. Controls were treated as above except with either sterile water or a mixture of non-infected shoots and sterile agar. After inoculating the roots they were covered up with the potting mix and arranged in a CRD and grown in an outdoor area as before. The roots were assessed 3 months after inoculation as described above.

3.2.6 Progression of *N. luteum* through different grapevine tissues

To check the infection efficacy of a lower concentration of *N. luteum* conidia (10^4/mL), infection studies on trunks and attached buds were repeated and pathogen progression also observed through trunks, buds and shoots. Pinot noir grapevine plants, produced and maintained as described in Section 2.4.4 were wounded and immediately drop-inoculated with the mixed conidial suspension prepared as described in Section 2.4.5.1 and 2.4.5.2. To ensure infection so that the pathogen progression could be tracked, 3-day-old mycelium colonised PDA plugs were also used to inoculate plants by placing the colonised plugs onto wounds. All inoculations in this section were carried out in October 2008.

3.2.6.1 Progression of N. luteum through trunks

The trunks (~10-12 mm in diameter) of 20 potted Pinot noir vines were wounded by drilling 3.5 mm diameter holes 2-3 mm deep into the wood as described in Section 2.4.5.2. The wounds were inoculated by pipetting 50 µL aliquots of a mixed isolate conidial suspension (10^4/mL), made from the same *N. luteum* isolates as described previously, into the fresh wounds. A second set of 20 plants with wounded trunks were inoculated with a mixture of 3-day-old mycelium colonised agar plugs of the same three isolates as described in Section 2.4.5.2. Control trunks were inoculated with sterile water and sterile agar, respectively. The plants were arranged in a CRD in an open area that simulated field conditions. Five plants were randomly selected for assessment at 1, 2, 3 and 4 months after inoculation. At each monthly assessment, the maximum upward and downward distance moved by *N. luteum* was assessed by surface sterilizing the trunk tissues as described in Section 2.2.2, after which the trunk tissue was dissected at 10 mm intervals from the inoculation points and these pieces placed sequentially onto PDA

plates as described in Section 2.4.5.2. At 3 and 4 months after inoculation, isolation was also done at 10 mm intervals along the side shoots closest to and above the inoculation points on the trunks. The *N. luteum* colonies growing from the tissue indicated the distances moved by the pathogen which provided incidence data that were analysed as described in Section 3.2.1.

3.2.6.2 Progression of N. luteum *through buds and into shoots*

Twenty five mature detached Pinot noir canes, each with two apparently healthy swollen buds were taken from the Lincoln University vineyard in late September 2009 for bud inoculations within 1-3 days. Each cane was immediately inserted into a Universal bottle filled with sterile water and the top of the bottle wrapped with Parafilm™ to prevent evaporation. Each bud on a cane was inoculated by injecting into its centre 20 µL of a mixed *N. luteum* conidial suspension (10^4/mL) made as described in Section 2.4.5.1. Ten additional canes with two buds each served as the controls being inoculated by injecting the buds with sterile water. The canes were then arranged in a CRD in a transparent plastic chamber and maintained as described in Section 2.2.3.2. After 1 week, the treated buds were assessed for percentage budburst and shoot emergence. All symptomatic emerged shoots and dead buds were split lengthwise and observed for the presence of internal lesions. One half of each split shoot and dead bud was used for isolation, the shoots being cut into 10 mm pieces before plating cut surface down onto PDA. Fungal colonies characteristic of *N. luteum* indicated the distances moved by the pathogen and provided the incidence data that were analysed as described in Section 3.2.1.

3.2.7 Environmental factors affecting *N. luteum* infection and disease progression in grapevine

3.2.7.1 Effects of tissue wetness duration after inoculation

This experiment was set up to determine the effect of tissue wetness duration and relative humidity (RH) on disease development in late spring 2008. The potted 13-months-old Pinot noir plants used for it were grown in a shade house and maintained as described in Section 2.4.4. On each plant, one mature shoot approximately 30 cm in height and approximately 6-8 mm in diameter was wounded near the base (~2 cm) as described in Section 2.4.1 and drop-inoculated with 25 µL of a mixed isolate conidial suspension of *N. luteum* (10^4/mL) or sterile water for control plants as before. There were 10 replicate plants assigned to each of the wetness periods: 0, 3, 6, 9, 12, 18 and 24 h. After the inoculations, the plants were put into a growth chamber (Conviron® PGV36, Controlled Environments Ltd, Winnipeg MB, Canada) set at 95% RH and 24^{o}C.

The area around the vines was constantly sprayed with a fine mist to maintain wetness for the specified wetness duration. After each wetness period, 10 inoculated plants were removed, air dried for 1 h under a laminar flow hood and then transferred to another growth chamber set at 95% RH for a period calculated to make the total incubation period up to 24 h. After 24 h, five replicate plants per treatment were transferred to a plastic chamber with approximately 95% RH for 3 months. At the same time the other remaining five plants per treatment were transferred to a shade house where the mean daily maximum temperature was 24.5^{o}C and the RH was 78.1% for 3 months. The plants in both humidity treatments were arranged in CRBD and maintained as described in Section 2.4.4. Three months after inoculations, the shoots were surface sterilised and dissected into 10 mm segments for isolation onto PDA plates as described in Section 2.4.5.2. Incidence data which indicated the distances moved by the pathogen were analysed as described in Section 3.2.1.

3.2.7.2 The effect of plant age on susceptibility to infection

To determine the effect of age of stem tissues on the susceptibility to *N. luteum* infection, Pinot noir grapevine plants were used. The plants were grown in a shade house, being maintained as described in Section 2.4.4 and then transferred to an open area that simulated field conditions in late winter. The dormant plants were pruned to two canes and five buds per cane, with the plants being tied onto supporting wires to simulate vineyard trellis systems.

Two months after budburst, the plants were randomly assigned to the different treatment ages [trunks (with 2-year wood), canes (with 1-year wood) and shoots (with current growth)]. Wounds were made in the shoots and canes as described in Section 2.2.3.2 and in the trunks as described in Section 2.4.5.2. Each wound was inoculated with a 25 µL drop of a mixed isolate conidial suspension (10^4/mL) made from the same three isolates as before. Tissues of control plants were inoculated with 25 µL drops of sterile water. The 10 replicate plants per treatment were arranged in a CRBD in an open area that simulated field conditions and maintained as described in Section 2.4.4. After 5 months growth, the lengths of any lesions developing from the inoculation points were measured with a digital calliper. The relevant tissues on each plant were surface sterilised as before and pathogen isolations made from the wound edges. The presence of colonies characteristic of *N. luteum* was used to confirm pathogenicity. The incidence data were analysed as described in Section 3.2.2.

3.2.7.3 *The effect of wound age on susceptibility to* N. luteum *conidial or mycelial infection and disease progression*

To determine the effect of wound age on susceptibility to infection, the Pinot noir potted vines were wounded by drilling a hole, approximately 3.5 mm diameter and 2-3 mm deep into the trunk as described in Section 2.4.4.2. Inoculation was done with mycelium from a mixture of isolates as described in Section 2.4.5.2, or with a 25 µL drop of a mixed isolates conidial suspension (10^4/mL) both made from the same three *N. luteum* isolates as described in Section 2.4.5.1. Recent studies have shown that when isolations were made from naturally infected current year's canes, the pathogen was found to have resided in the bark and not the wood in the majority of cane samples (Billones, pers. comm., 2010). Since it is likely that such pathogens can grow into nearby wounds, it was considered relevant to also use mycelium as inoculum. Inoculations were performed at 0, 1, 2, 7, 14 and 30 days after wounding and the control plants had 30 day old wounds inoculated with sterile agar discs or sterile water. The inoculated wounds were wrapped with Parafilm™ for 3 days to prevent drying out of the inoculum. There were five replicate plants per treatment and the plants were arranged in a CRBD. Two months after inoculation, pathogen incidence and colonization was assessed by isolation from surface sterilised tissues at 10 mm intervals below and above the wound area. The presence of colonies characteristics of *N. luteum* emerging from the trunk pieces indicated infection and the incidence data were analysed as described in Section 3.2.1.

3.2.7.4 *The effect of water stress on disease symptoms and* N. luteum *progression*

Experiment 1

A preliminary water stress experiment was set up in November 2008 to determine the effect of soil moisture stress on the susceptibility of 13-month-old non-grafted own rooted Pinot noir plants to botryosphaeriaceous infection. Plants were grown in potting mix and maintained as described in Section 2.4.4. Four different levels of water availability were established by daily monitoring of water use and watering the potting mix (in 2 L pots) to 25, 50, 75 and 100% field capacity (FC). The amount of water content taken up by the dry potting mix, after excess water had drained away, was determined by weight as field capacity (Appendix C.2). The water treatments were applied from 10 days prior to inoculation and continued for another 2 months after

inoculation. Thereafter, the plants were each given 25% FC watering daily for another 2 months.

The trunks of the vines were wounded and inoculated using a mixed isolate conidial suspension (10^4/mL) of *N. luteum* as described Section 2.4.5.1. Control plants inoculated with sterile water were also assigned to each moisture level. There were six replicate plants for each inoculation treatment and moisture level and the plants were arranged in a CRD in a greenhouse. In winter when the plants became dormant they were put under high pressure sodium lights set to be on from 4 am to 12 pm and on again from 4 pm to 8 pm throughout the winter period for 2 months, to break the vine dormancy as described in Section 2.4.5.2. Shoot tip dieback was assessed after bud break, at 8 months after inoculation by measuring dieback lesion lengths with a digital calliper and data were analysed with ANOVA as described in Section 3.2.1.

Experiment 2

In December 2009, the experiment was repeated using 1-year-old vines of Pinot noir grafted onto 101-14 rootstock that were obtained from Corbans Viticulture, (Whenuapai, Auckland, New Zealand). The soil medium consisted of a fragic pallic silt loam which was taken from the Lincoln University vineyard (Wakanui series) and mixed thoroughly with river sand (2:1 by volume). The grafted grapevines plants were then transplanted into the soil medium in 2.5 L pots and were grown for 2 months. The plants were watered to field capacity (FC) and the volumetric water availability measured using the HydroSense™ Soil Moisture System (Campbell scientific Inc. Logan, Utah). To set the soil water range for study, the volumetric soil water content was monitored for some sample plants, given different amounts of water and measured on a daily basis. The minimum water level was set to be when some basal leaf yellowing was evident or shoot tip growth was minimal, which indicated that the plants were under mild stress but above permanent wilting (Neal and Dryden 2003). This was determined with the HydroSense™ to correspond to 15% FC. Thereafter, the test plants were assigned three different watering levels [100, 25 and 15% soil water (field capacity), respectively]. Each day the HydroSense™ was used to determine the volumetric water available in each pot and only enough water provided to raise it to the specified moisture level.

One week after subjecting the plants to the different watering regimes, rootstock sections close to the graft union were wounded by drilling a hole approximately 3.5 mm diameter and 2-3 mm deep into the trunk as described in Section 2.4.5.2. Inoculation was done

using a mixed isolate conidial suspension (10^4/ mL) of *N. luteum* as described in Section 2.4.5.1. Control plants inoculated with sterile water were also assigned to each moisture level. The four replicate plants of each treatment combination were arranged in a CRBD in the greenhouse. However, in each block there was only one uninoculated control plant for each of the assigned moisture levels because there were insufficient plants available at the time of the experiment. All the plants were maintained in a shadehouse as described in Section 2.4.4. After 6 months (in winter), during dormancy, the canes were pruned to 5-10 buds per plant depending on shoot height and immediately put under high pressure sodium lights as before to break dormancy. The plants were assessed after 2 months by determining the proportion of dead buds and measuring the lengths of shoot growth. Shoots and buds were also divided lengthways and examined. To verify infection, five pieces (~5 mm long) of the canes at 5-10 cm away from the inoculation point and buds that failed to burst were used for isolation onto PDA. Pathogen identification was based on culture morphology. Incidence of buds infected with *N. luteum* and other naturally occurring fungal pathogens was recorded. Data were analysed as described in Section 3.2.1.

3.3 Results

3.3.1 Effect of *N. luteum* inoculum dose on infection incidence and symptom development

Incidence of *N. luteum* in lesion edges of the inoculated plants was 100% and all the inoculated shoots developed lesions irrespective of the conidial concentrations. The two repeated experiments did not differ and data were pooled together for analysis. The different *N. luteum* concentrations caused significantly different (P<0.001; Appendix C.3) lesion lengths (Figure 3.3). The greatest mean lesion length of 41.7 mm with 10^4 conidial/mL was significantly greater than lesion lengths of 28.6 and 27.8 mm produced with 10^5 and 10^6 conidia/mL, respectively which were similar, and they were significantly greater than the lesion lengths produced with 10^3 and 10^2 conidia/mL. Control plants inoculated with sterile water did not produce any lesions and pathogen isolation yielded no colonies characteristics of *N. luteum*.

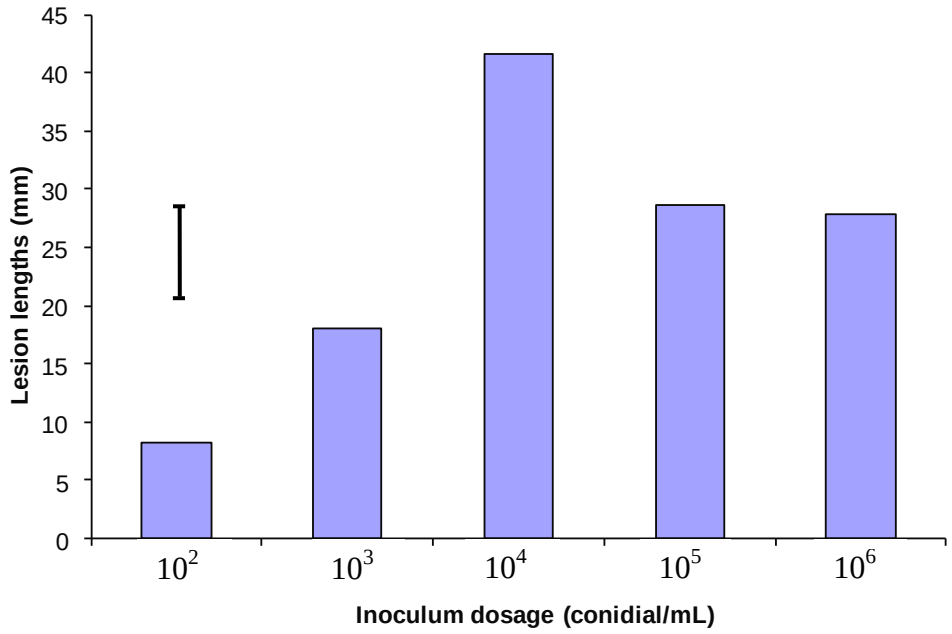

Figure 3.3: Effect of different inoculum doses on mean lengths of lesions developed on grapevine shoots. Error bar represent Fisher's protected LSD at $P \leq 0.05$.

3.3.1.1 Mycelial colonization and shoot lesion development

At 1 month after inoculation when the green shoots were sliced through lengthwise, internal lesions were clearly visible upwards from and around the inoculation points and their lengths differed for the four botryosphaeriaceous species (Table 3.1). Pathogen isolation at 10 mm intervals also showed significant differences ($P<0.001$; Appendix C.4) between species, mean lengths being 93.4, 87.2, 80.6 and 57.8 mm for *N. luteum*, *N. australe*, *N. parvum* and *D. mutila*, respectively, which covered longer distances than the mean lesion lengths (Table 3.1). This demonstrated that pathogen colonization exceeded the lengths of the dark lesions formed.

Over the incubation periods, the progression of all four botryosphaeriaceous species increased, with mean distances that differed significantly ($P \leq 0.05$) between all assessments, mean lengths being 18.8, 68.7, 106.2, 116.3 and 153.6 mm for 1, 2, 3, 4 and 9 months, respectively (Table 3.1). The significant interaction ($P<0.001$; Appendix C.4) between the incubation periods and botryosphaeriaceous species was associated with the different rates at which the species colonized through the tissues. At each assessment, *D. mutila* had colonized significantly shorter distances than the other species ($P \leq 0.05$). For the other species, there was a trend for greatest colonization by *N. luteum*

followed by *N. australe* and *N. parvum*. The differences in pathogen colonization between the species increased over time (Table 3.1).

Table 3.1: Mean progression distances (mm) of *N. australe, N. luteum. N. parvum* and *D. mutila* inoculated onto wounded grapevine shoots and monitored over nine months [IL@1*represents mean internal lesion lengths (mm) after one month and PG represents mean internal pathogen progression distances through isolation at 10 mm intervals at different times after inoculation].

Months of Assessment	Botryosphaeriaceous species				Mean of assessments
	N. luteum	*N. australe*	*N. parvum*	*D. mutila*	
IL @ 1*	20.9	15.8	16.2	7.1	14.9
PG @ 1	25.0	20.0	20.1	10.0	18.8 i[1]
PG @ 2	82.1	75.0	70.1	47.6	68.7 h
PG @ 3	129.9	119.8	102.5	72.6	106.2 g
PG @ 4	135.0	125.0	112.5	92.6	116.3 f
PG @ 9	167.6	167.5	162.5	117.4	153.6 e
Mean of species	93.4 a[1]	87.2 b	80.6 c	57.8 d	

[1]Values within the rows or columns followed by the same letters are not significantly different according to Fisher's protected LSD at $P \leq 0.05$. For species and months interaction, means were significantly different ($P<0.001$; LSD = 7.46). Means of species (a-d) were significantly different ($P<0.001$; LSD = 3.05); Means for months of assessment (e-i) were significantly different ($P<0.001$; LSD = 3.73)

3.3.2 Internal progression of botryosphaeriaceous species through grapevine tissues

3.3.2.1 Plant assessment for presence of fungal propagules in plant tissue sap

There were no propagules observed on any slide mounts of sap taken from lesion edges on green shoots, which had been inoculated 2 months earlier with conidia or mycelial discs of *N. luteum, N. australe, N. parvum* and *D. mutila*. Plating of the sap onto PDA resulted in no fungal colonies, although a few colonies characteristic of bacteria and yeasts grew from the sap of plants inoculated with the botryosphaeriaceous species, water or sterile agar (controls).

3.3.2.2 Microscopy to determine fungal structures in plant tissue

Longitudinal and transverse hand sections of green shoots taken 3 months after the inoculation and stained with a fluorescent dye (WGA lectin) showed much auto fluorescence from plant tissues making observation of fungal structures difficult. However, observations made with normal white light showed mycelium growth within the vessels (Figure 3.4) both above and below the inoculation point. The amount of

fungal growth and its structural characteristics within the vessels appeared to be similar for the four species inoculated. No fungal structures were observed in control plants.

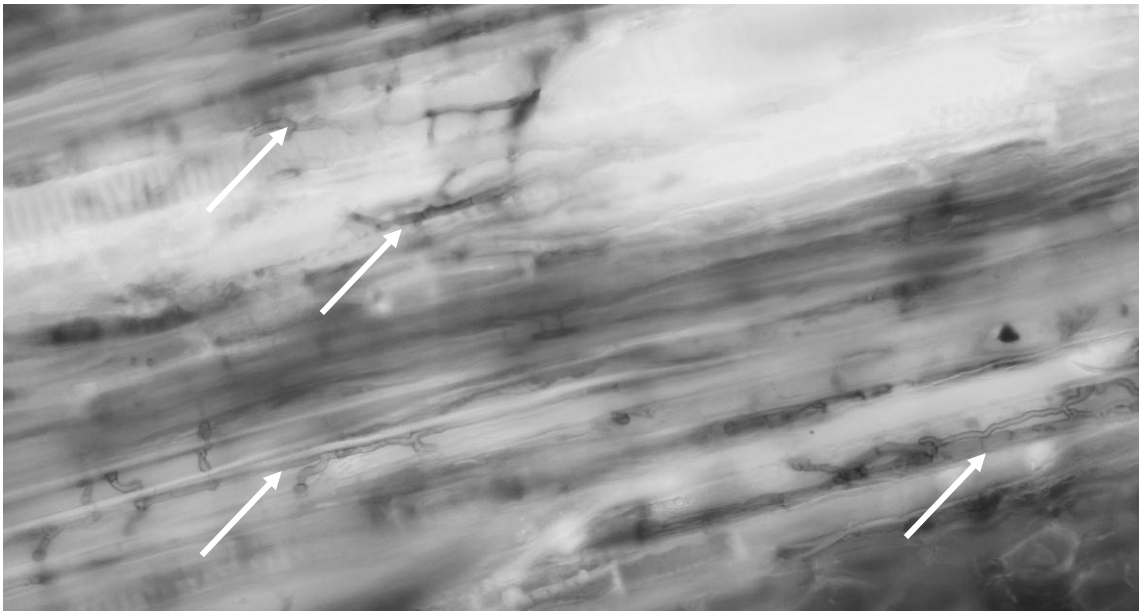

Figure 3.4: Microscopic observation (×400) of grapevine plant tissue colonised by mycelium of botryosphaeriaceous species (shown by arrows) growing through xylem vessels at 3 months after inoculation.

Hyphae were also observed in xylem vessels using SEM. These observations also showed vertical growth of the hyphae of all four species and they appeared to be attached to the walls of the vessels (Figure 3.5a). No fungal structures were observed in tissues of any control plants (Figure 3.5b).

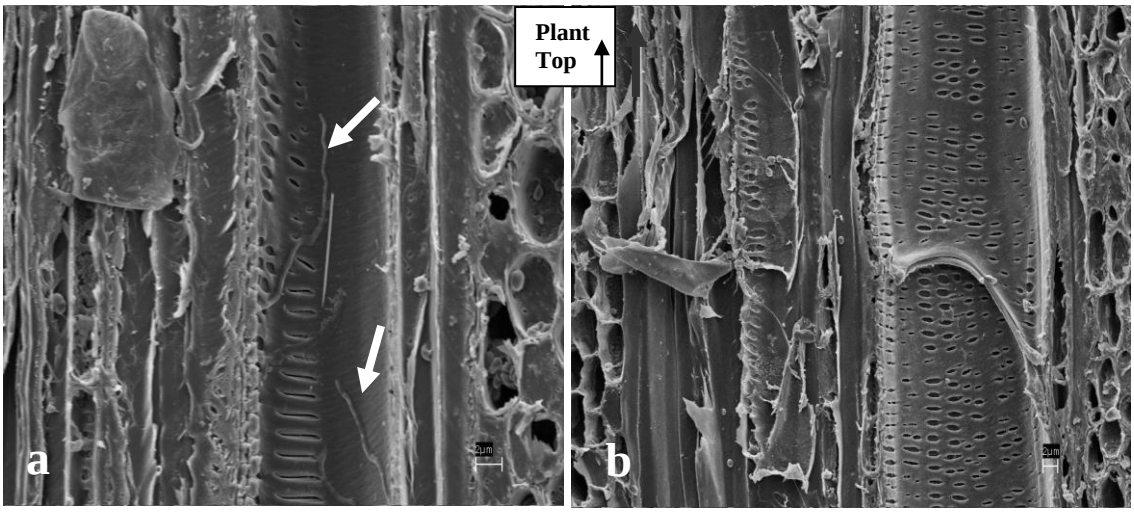

Figure 3.5: Scanning electron micrographs (a) showing longitudinal section images of *N luteum* mycelium (shown in arrows) with tips moving upwards in the xylem vessels at 3 months after inoculation (b) control plant with no mycelium observed.

3.3.2.3 Assessment for dieback and budburst failure

All three plants of the *N. luteum*, *N. australe* and *N. parvum* treatments that were pruned in late May after becoming dormant showed dieback symptoms in early July although only two out the three plants inoculated with *D. mutila* showed dieback. Lesion lengths were not measured as the plants were just observed to confirm previous results (Chapter 2). However, when the vines were put under high pressure sodium lights in a greenhouse, buds below the dieback developed into vigourous symptomless shoots and the dieback extension stopped in plants of all the treatments (Figure 3.6a). No control plants showed any dieback after pruning (Figure 3.6b). Isolation of pathogens from the edges of dieback lesions onto PDA yielded colonies characteristics of the four inoculated botryosphaeriaceous species from the respective plants. Isolations from control plants yielded no fungal colonies characteristic of the botryosphaeriaceous species.

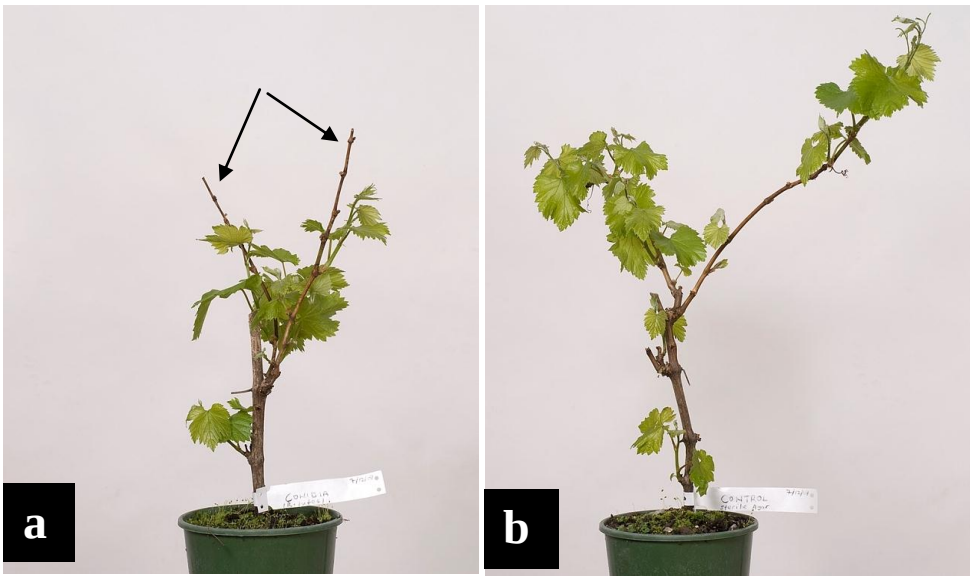

Figure 3.6: (a) Effects of *N. luteum* infection on winter dieback (arrows) which stopped when spring growth resumed compared to (b) control plant showing no dieback symptoms.

3.3.3 *Neofusicoccum luteum* conidial infections on attached and detached grapevine tissues

3.3.3.1 Leaf inoculations

On the attached, non-wounded leaf surfaces at 3 h after inoculation, the SEM images of the 12 leaf discs revealed the presence of many non-germinated conidia on all of them

(Figure 3.7a). Between 3 and 18 h after the inoculation, there was still no germination visible; however the conidia seemed to have reduced in numbers during the time interval (Figure 3.7b), and by 24 h no conidia were seen on the leaf surfaces (Figure 3.8a). When the leaf surfaces were observed at a much higher magnification, many wax platelets were seen (Figure 3.8b).

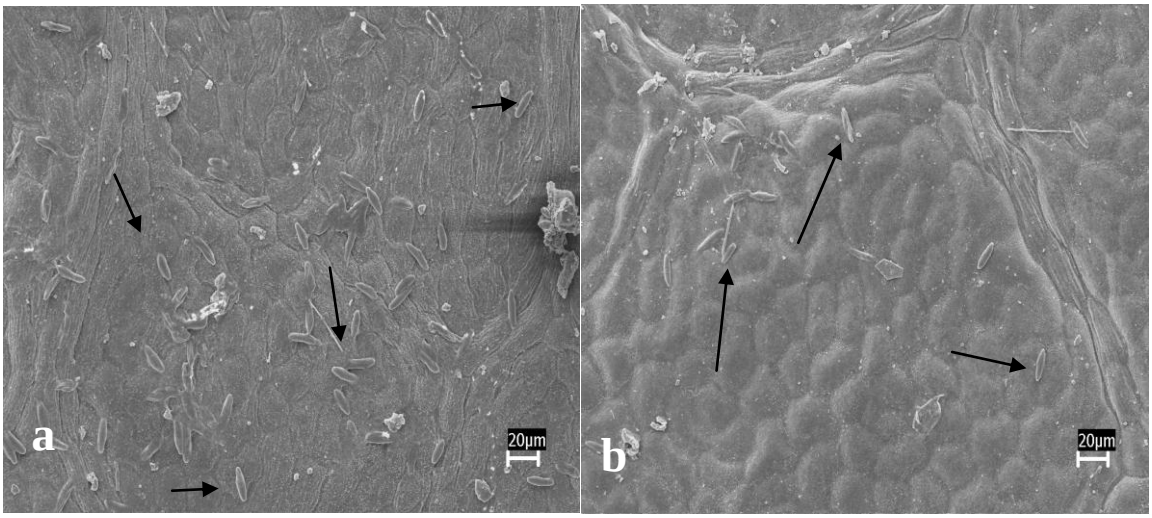

Figure 3.7: Arrows show conidia of *N. luteum* inoculated onto a non-wounded Pinot noir grapevine leaf surface (a) leaf surface with conidia at 3 h, and (b) reduced numbers of conidia at 18 h after inoculation.

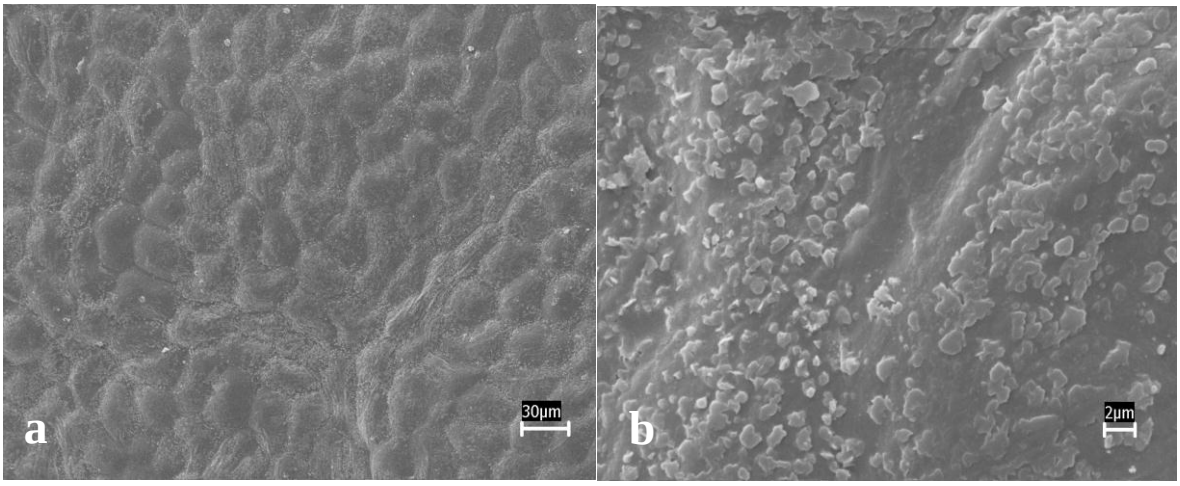

Figure 3.8: Non-wounded leaf surface of Pinot noir grapevine showing none of the inoculated *N. luteum* conidia (a) after 24 h at low magnification and (b) same leaf surface at 24 h after inoculation, a much higher magnification showing lots of wax platelets.

However on the attached and wounded leaf surfaces, many conidia were uniformly present between 3 and 24 h after inoculation, with many of them starting to germinate by 3 h. By 24 h after inoculation, most conidia observed had developed germ tubes and many had penetrated into the host tissue without formation of appressoria (Figure 3.9a). On wounded and detached leaf surfaces, most conidia were uniformly present between 3 and 18 h after inoculation with most of them starting to germinate by 3 h after inoculation. By 24 h after inoculation, all visible conidia had germinated and a network of mycelium was seen above and penetrating the tissue (Figure 3.9b). A repeat of the experiment revealed the same trends in results. Overall, there was a faster rate of conidial germination on detached and wounded leaf surfaces compared with attached and wounded leaf surfaces.

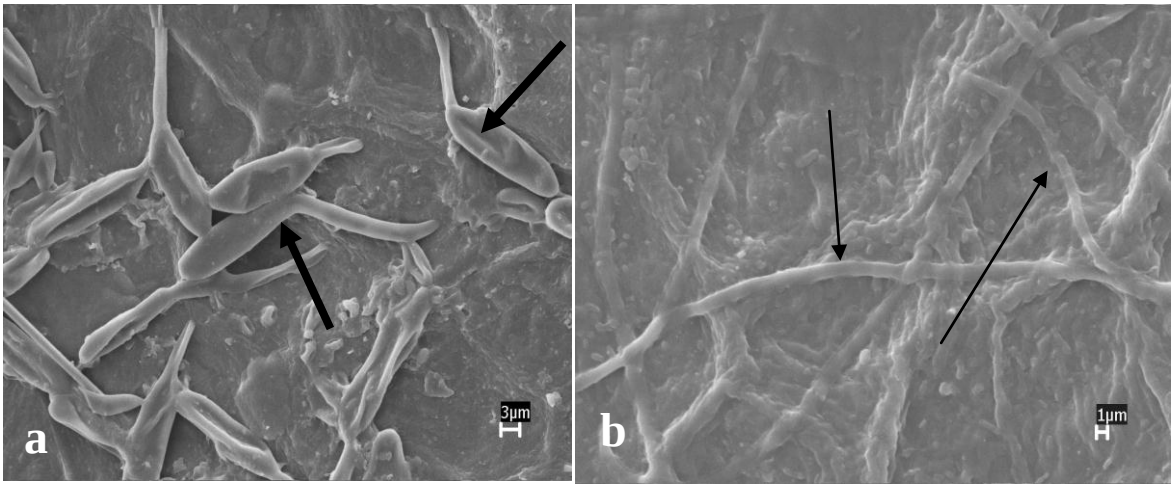

Figure 3.9: Arrows show development of *N. luteum* conidia on a wounded grapevine leaf at 24 h after inoculation (a) conidia germinating on attached leaf surface (b) network of mycelium on detached leaf surface.

3.3.3.2 Shoot inoculations

On the inoculated attached, non-wounded shoot surfaces, the SEM images of the 12 shoots did not revealed any conidia present at all incubation times. However on the attached and wounded shoot surfaces, many conidia were uniformly present between 3 and 24 h after inoculation with many of them starting to germinate by 3 h. By 24 h after inoculation, most conidia observed had developed germ tubes and many had penetrated into the host tissue (Figure 3.10a). On the wounded and detached shoot surfaces, most conidia were uniformly present between 3 and 18 h after inoculation with most of them starting to germinate by 3 h after inoculation. By 24 h after inoculation all visible conidia had germinated and a network of mycelium was seen above and penetrating the

tissue (Figure 3.10b). A repeat of the experiment revealed the same trends in results. Overall, there was a faster rate of conidial germination on detached and wounded shoots compared with attached and wounded shoots.

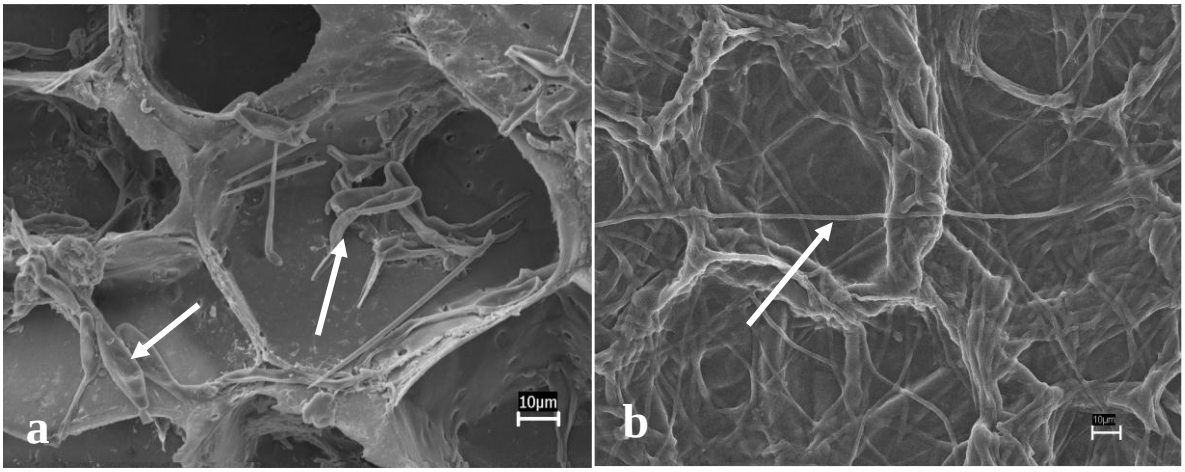

Figure 3.10: Arrows show development of *N. luteum* on a wounded grapevine stem 24 h after inoculation (a) conidia germinating on attached stem (b) network of mycelium on detached stem.

3.3.4 Susceptibility of different grapevine tissues to *N. luteum* infection

The incidence of infection was significantly (P<0.001; Appendix C.5) affected by the plant tissue type inoculated by *N. luteum.* Mean incidence was 100% on pruned cane ends and trunks, and was not significantly different to the 91.7 and 87.5% recorded for wounded shoots and buds, respectively (Figure 3.11). Mean infection incidences for these four tissue types were significantly higher than for the leaves (29.2%) and for roots (0%).

On the leaves, lesions extended only about 0.5 mm beyond the wounded inoculated area, and after 1 week they developed into holes. No further extension of the lesions was observed thereafter although isolations from the lesion edges yielded *N. luteum* colonies. Pathogen isolation from the petioles also did not yield any fungal colonies characteristic of *N. luteum*. On the roots, some dieback was observed from the pruned inoculated tips of three out of the six potted grapevine plants in Experiment 1, but no colonies characteristic of *N. luteum* were isolated. In Experiment 2, none of the inoculation attempts using any of the inocula of the four botryosphaeriaceous species (*N. luteum*, *N. australe*, *N. parvum* and *D. mutila*) were able to cause infections of the grapevine roots. A few root tips (2-4 per plant) out of the 20 inoculated roots sampled per plant had

visible dieback at 3 months after inoculations but none of the isolation attempts yielded colonies characteristic of the botryosphaeriaceous species.

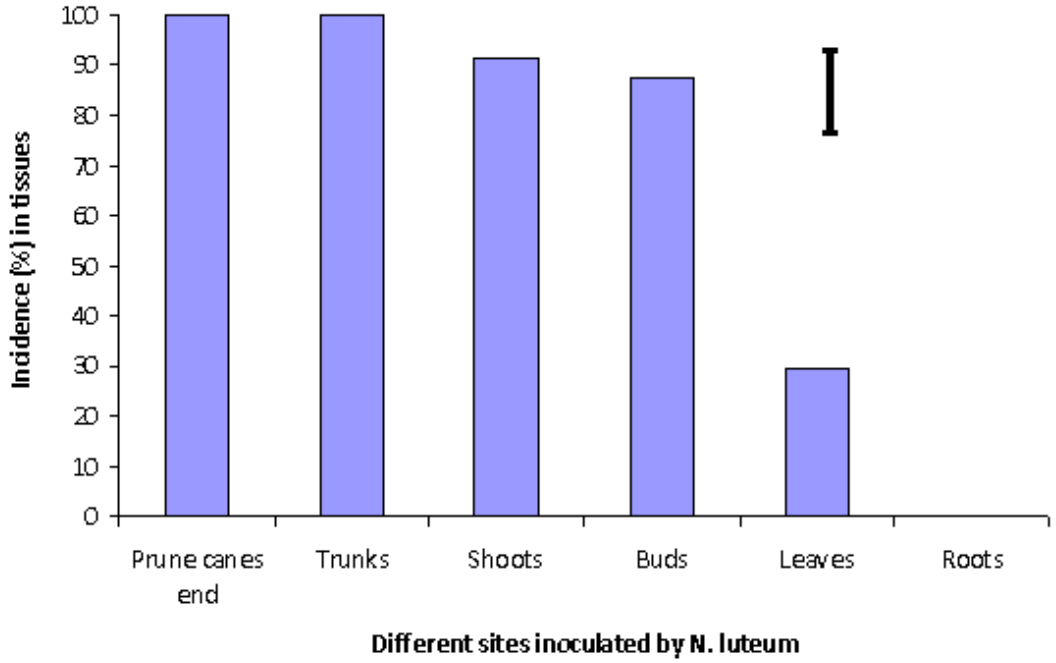

Figure 3.11: Mean percent incidences by *N. luteum* obtained from inoculated grapevine tissues of different types. Error bar represent Fisher's protected LSD at $P \leq 0.05$.

3.3.5 Disease progression of *N. luteum* through different grapevine tissues

3.3.5.1 Progression of N. luteum through trunks

The vines did not show any visible symptoms externally. However, isolations showed that the rate of *N. luteum* movement through the trunks differed with time, direction and inoculum type for both upward and downward movement. The distance progressed was significantly affected by time ($P<0.001$; Appendix C.6). The mean pathogen progression through the trunks differed significantly between monthly assessments being 17.8, 32.8, 40.3 and 47.2 mm, respectively for 1, 2, 3 and 4 months (Table 3.2). Upwards movement by *N. luteum* also differed significantly ($P<0.001$; Appendix C.6) from the downward movement, the mean distances being 53.4 and 15.6 mm, respectively. There was an interaction between direction and time with overall upward movement being significantly greater ($P \leq 0.05$) each time than the downwards movement (Table 3.2). There was also a significant interaction between inoculum type and direction ($P<0.001$; Appendix C.6) with movement of *N. luteum* in the upward direction being significantly

greater for mycelium than for conidial inoculum, means being 63.8 and 43.1 mm, respectively over all monthly assessments.

Table 3.2: The upwards and downwards progression (mm) of *N. luteum* using conidia and mycelium discs for inoculations onto trunks of Pinot noir grapevines.

Time (months)	Direction and inocula type						Overall mean for times
	Upward movement			Downward movement			
	Mycelium	Conidia	↑	Mycelium	Conidia	↓	
1	35.0	16.3	25.6c	10.0	10.0	10a	17.8g[1]
2	65.0	36.3	50.6d	15.0	15.0	15ab	32.8h
3	70.2	55.0	62.5e	20.0	16.3	18.1b	40.3i
4	85.0	65.0	75.0f	21.3	17.5	19.4b	47.2j
Inoculum x direction effect	63.8m[1]	43.1n		16.6	14.7		
Direction effect	53.4p			15.6q			

[1]Values within the rows and columns followed by the same letters are not significantly different according to Fisher's protected LSD at $P \leq 0.05$. Time effect (f-i) was significant ($P<0.001$; LSD=4.10); Inoculum x direction interaction (m-n) was significant ($P<0.001$; LSD=2.90); Time and direction interaction (a-f) was significant ($P<0.005$; LSD=5.80); Direction effect (p-q) was significant ($P<0.001$; LSD=2.90).

At 3 and 4 months after inoculations, sections cut across the bases of shoots closest to the inoculation point on the trunks of all five replicate plants, which were either inoculated with mycelial discs or conidial suspensions, showed lesions progressing into the shoots (Figure 3.12). Isolations made at 10 mm intervals from the bases of these shoots showed that *N. luteum* had progressed into shoots on all inoculated trunks, with mean distances progressed being 17.5 and 25.0 mm for conidial and mycelial inoculation, respectively at 3 months after inoculation and 37.5 and 55 mm, respectively at 4 months after inoculations. There were no visible lesions in control plants and pathogen isolations yielded no fungal colonies characteristic of *N. luteum*.

Figure 3.12: Cross section through an infected grapevine trunk and the attached side shoot showing the brown lesion (arrow) progressing into the shoot 4 months after inoculating the trunk with conidia of *N. luteum*.

3.3.5.2 Progression of N. luteum through buds

Inoculation of buds caused a significant increase ($P<0.001$; Appendix C.7) in bud infection incidence. Of the inoculated grapevine buds, 68% did not survive the swelling stage, which was significantly different ($P<0.05$) from the 32% of buds that did burst after inoculation. Of the 32% buds that burst, 10% were able to develop into shoots without showing any external symptoms but 22% that developed into asymptomatic shoots withered and died within 1 month of inoculation.

Most of the inoculated buds showed visible symptoms while the control buds showed no visible symptoms (Figure 3.13). Longitudinal hand sections made through all the infected buds that did not survive the bud burst stage showed necrosis of tissues (Figure 3.14a) that extended down into the cane. Longitudinal sections through the young emerging shoots that were able to develop from the inoculated buds showed no external symptoms but showed internal necrosis forward into the shoots and development of necrotic areas back into the cane (Figure 3.14b). However, longitudinal sections through the control buds, that were inoculated with sterile water, did not show any necrosis in the buds or the young emerging shoots (Figure 3.14c and d) and pathogen isolation did not yield any fungal colonies characteristic of *N. luteum*. Pathogen isolations from all inoculated necrotic buds and the young shoots (both weak and apparently healthy ones) that developed from inoculated buds all yielded colonies characteristic of *N. luteum*.

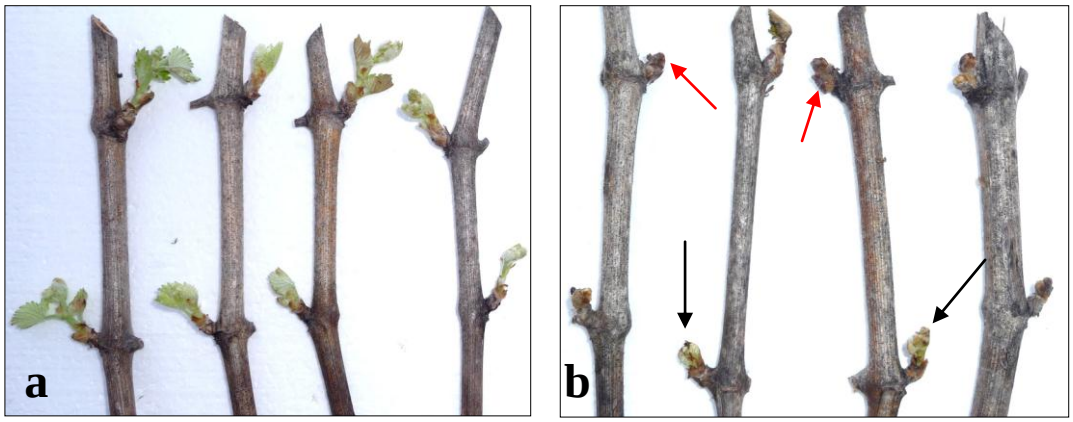

Figure 3.13: Bud transmissions studies (a) buds infected with sterile water serving as controls and (b) buds inoculated with conidia of *N. luteum* showing complete bud failure (red arrows) and retarded growths with necrosis (black arrows).

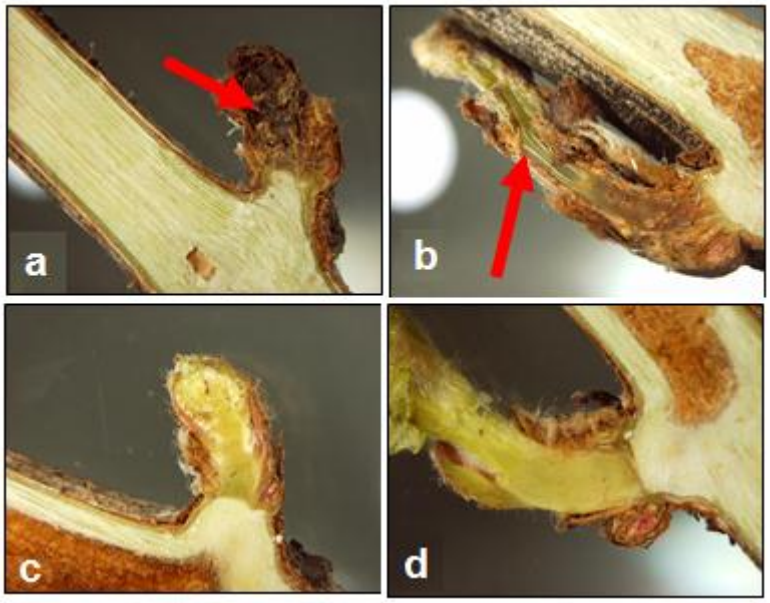

Figure 3.14: Longitudinal sections through grapevine buds and developing shoots (a) swelling stage with necrosis (arrowed) after inoculation with *N. luteum* conidia, (b) inoculated bud developed into young shoot stage with necrosis (arrowed) advancing towards the shoot tip and in adjoining shoot, (c) the swelling bud stage with no necrosis and (d) young shoot stage with no necrosis.

3.3.6 Environmental factors affecting *N. luteum* infection and disease progression in grapevines

3.3.6.1 The effect of tissue wetness duration on disease progression

Pathogen isolation from all the inoculated shoots yielded 100% colonies characteristic of *N. luteum* and none from the control plants. The different wetness durations did not significantly (P=0.128; Appendix C8) affect *N. luteum* incidence or infection progression (Table 3.3), however the effect of RH on pathogen progression was significant (P<0.001; Appendix C.8) with a mean 100.6 mm progression in 95% RH, which was significantly higher than the 77.0 mm mean recorded for 78% RH. There was a significant (P=0.002; Appendix C.8) interaction between RH and wetness duration which was associated with increasing lesion size with longer wetness at 95% RH but no increases with increasing wetness duration at 78% RH (Table 3.3).

Table 3.3: The effect of wetness duration (h) on *N. luteum* infection progression (mm) in mature shoots of Pinot noir subsequently incubated at 78 and 95% relative humidity (RH) for three months.

Wetness duration (h)	RH (%)		Mean for wetness durations
	78	95	
0	76.0 efg	96.7 bcd	86.3
3	82.7 defg	90.7 bcde	86.7
6	82.0 defg	88.0 cdef	85.0
9	70.7 g	99.3 bc	85.0
18	76.7 efg	104.7 b	90.7
21	76.7 efg	102.0 bc	89.3
24	74.7 fg	122.7 a	98.7
Mean for RH	77.0 x[1]	100.6 y	

[1]Values within the rows and columns followed by the same letter are not significantly different according to Fisher's protected LSD at $P \leq 0.05$. The main effect for RH (x-y) was significant (P<0.001; LSD=5.57); Interaction between RH and wetness duration (a-g) was significant (P=0.002; LSD=14.74); Wetness duration was not significant (P=0.128; LSD=10.42). Control plants were not included in the analysis.

3.3.6.2 The effect of plant tissue age on susceptibility to infection

Pathogen isolations from lesion edges onto PDA yielded colonies characteristic of *N. luteum* from all the inoculated tissues and not from the control tissues. Mean lesion lengths at 3 months after *N. luteum* inoculation were significantly (P<0.001; Appendix C.9) affected by ages of stem tissues. The mean lesion lengths of 38.9, 32.3 and 23.2

mm, for trunks, cane and current green shoots, respectively, differed significantly ($P \leq 0.05$). No lesions were seen on the non-inoculated control plants.

3.3.6.3 The effect of wound age on susceptibility to N. luteum conidial or mycelium infection and disease progression

Wound age at the time of inoculation significantly affected ($P<0.001$; Appendix C.10) infection by *N. luteum* for both conidial and mycelial inoculation which differed ($P=0.013$; Appendix C.10). Mean infection incidence caused by conidia and mycelium were 50 and 80%, respectively at 2 months after the inoculation (Table 3.4). Pathogen incidence was 100% for wounds inoculated at 0, 1 and 2 days with mycelium inoculation, and at 0 and 1 days with conidia. Incidence in plants inoculated with conidia onto 2-day-old wounds was reduced to 80% and reduced to 40% for 7-day-old, and to 0% for 14 and 30-day-old wounds. For mycelium inoculation, the incidence was 60% for wounds inoculated at 7 and 14 days and 40% incidence on 30-day-old wounds. Isolations from wounds on control plants did not yield any colonies characteristic of *N. luteum*.

Table 3.4: Incidence (%) of *N. luteum* infection after inoculation with conidia or mycelium on different wound ages made on trunks of potted Pinot noir grapevines after 2 months incubation.

	Inocula type		
Wound age (days)	**Conidia**	**Mycelium**	**Age effect**
0	100	100	100a[1]
1	100	100	100a
2	80	100	90a
7	40	60	50b
14	0	60	30b
30	0	40	20b
Inoculum effect	50c[1]	80d	

[1]Values within the rows and columns followed by the same letter are not significantly different according to Fisher's protected LSD at $P \leq 0.05$. The main effect of wound age (a-b) was highly significant ($P<0.001$; LSD=0.31); and of inoculum type (c-d) ($P=0.013$; LS =0.18); the inocula type x wound age interaction was not significant ($P=0.36$; LSD=0.41). Control plants were not included in the analysis.

3.3.6.4 The effect of soil water stress on disease progression by N. luteum

In the first water stress experiment, soil water level significantly affected ($P<0.001$ Appendix C.11.1) the degree of shoot dieback in inoculated plants during dormancy. The mean lengths of dieback lesions were proportional to the moisture supplied, with the least (2.3 mm) at 25% FC, being significantly less than the similar lesions at 50 and 75%

FC (4.1 and 3.9 mm, respectively), which were significantly less than 6.2 mm lesions at 100% FC (Table 3.5). Isolations from lesion edges on all inoculated plants yielded fungal colonies characteristic of *N. luteum*. The uninoculated controls had minimal levels of dieback, which did not differ between the moisture levels and isolations yielded no fungal colonies characteristic of *N. luteum*. There was significant interaction between inoculation and soil water level ($P<0.001$; Appendix C. 11.1) probably due to the moisture only treatments causing similar lengths of dieback, whereas the inoculated treatments showed different levels of dieback (Table 3.5).

Table 3.5: The effect of different soil water levels and inoculations with *N. luteum* on shoot dieback lesion (mm) development on Pinot noir vines during dormancy in winter.

Soil water level (%)	Treatments		Soil water level effect on shoot dieback
	Inoculated	Control	
100	11.9e[1]	0.5	6.2a[1]
75	7.3f	0.6	3.9b
50	7.7f	0.5	4.1b
25	4.6g	0.1	2.3c
Inoculation effect	7.9h	0.4i	

[1]Values within the rows and columns followed by the same letter are not significantly different according to Fisher's protected LSD at $P\leq0.05$. The main effect of inoculation (h-i) was highly significant ($P<0.001$; LSD=0.44) and soil water level (a-c) was significant ($P<0.001$; LSD=0.62), and treatment x soil water level interaction (e-g) was also significant ($P<0.001$; LSD=0.87).

In the second experiment, various bud symptoms were significantly affected by soil water levels, inoculation and the interaction between these treatments (Appendix C.11.2A-F). The mean total number of buds per cane were affected by the water level ($P<0.05$) but not by inoculation ($P=0.232$) although the effect of the interaction ($P=0.043$) was evident in the greater effect of inoculation at 100% FC. The number of dead buds were significantly greater in the inoculated plants ($P<0.001$) and were consistently more numerous in inoculated plants held at soil water levels of 100 and 15% FC ($P=0.002$), but there was no interaction between these factors (P=0.465) Appendix C.11.2B). The same factors had significant effects ($P<0.001$, $P=0.006$, and $P<0.001$), respectively, (Appendix C.11.2 C) on numbers of buds that developed into healthy shoots (Table 3.6). The number of dead buds that were found to be infected with *N. luteum* was also affected by the same factors ($P=0.001$, $P<0.001$and $P=0.089$,

respectively (Appendix C.11.2E) (Table 3.6). No fungal colonies characteristic of *N. luteum* was isolated from the control plants. Although *N. luteum* infection did not account for all the dead buds observed, infections from other fungal pathogen such as the *Fusarium* spp. isolated from infected and dead buds also did not account for the dead buds (P>0.05; Appendix C.11.2F).

Table 3.6: Mean effects of *N. luteum* inculation and different soil water levels at 2 months after bud break on mean number of buds per cane to develop or be infected, in potted grapevines (Pinot noir grafted onto 101-14) growing in soil.

Soil water level (%) (SWL)	Inoculation	Mean buds per cane	Dead buds per cane	Buds to healthy shoots	Shoots dead	Buds infected with *N. luteum*
100	Cont.	9.0	2.5	5.3	1.3	0.0
	Inoc.	7.8	5.6	0.8	1.3	6.8
25	Cont.	6.5	1.5	3.8	1.3	0.0
	Inoc.	6.3	3.8	1.1	1.3	5.4
15	Cont.	5.3	2.5	2.0	1.0	0.0
	Inoc.	5.8	4.4	0.6	1.0	5.0
***P*-Value (Water levels)**		<0.001	<0.001	<0.001	> 0.05	<0.001
$LSD_{0.05}$ (Inoculation)		0.55(NS)	0.78	0.59	0.38 (NS)	0.68
$LSD_{0.05}$ (Soil water)		0.58	0.82	0.63	0.40 (NS)	0.73
$LSD_{0.05}$ (Interaction)		0.96	1.35(NS)	1.03	0.65 (NS)	1.19 (NS)

[1]The above effects were all significant (P<0.05) except where the $LSD_{0.05}$ shows 'NS'.

Shoot growth after bud break was also significantly affected by soil water levels (P<0.001; Appendix C.11.3) with plants in 25% FC having longer shoots than in 100 and 15% FC soil, and by inoculation (P<0.001; Appendix C.11.3) which resulted in shorter shoots (Table 3.7). The significant soil water and inoculation interaction (P<0.001; Appendix C.11.3) was due to inoculation reducing shoot length in 15 and 100% FC soil, mean lengths being 56.1 and 198.1 mm, respectively, compared with 25% FC, the mean length being 442.8 mm (Table 3.7; Figure 3. 15). This indicated that the reduction in shoot lengths in grapevines grown under 15 and 100% FC was partly due to water stress. Dry matter weights of leaves and shoots could not be measured because severe winds after the shoot assessment toppled the pots causing breakages of leaves and shoots.

Table 3.7: Mean effects of *N. luteum* and soil water levels at 2 months after bud break on mean growth of shoots (mm), in potted grapevines (Pinot noir grafted onto 101-14) growing in soil.

Soil water level (%)	Inoculation treatment		Soil water level
	Control	Inoculated	effect
100	368 b	142 cd	198.1 f
25	992a	260 bc	442.8 e
15	85 cd	46 d	56.1 g[1]
Inoculation effect	482 h[1]	149 i	

[1]Values within the rows and columns followed by the same letter are not significantly different according to Fisher's protected LSD at $P \leq 0.05$. The main effect of inoculation (h-i) was highly significant ($P<0.001$; LSD=123.4); and soil water level (e-g) was also significant ($P<0.001$; LSD =130.9); and inoculation x soil water level interaction (a-d) was significant ($P<0.001$; LSD=213.7).

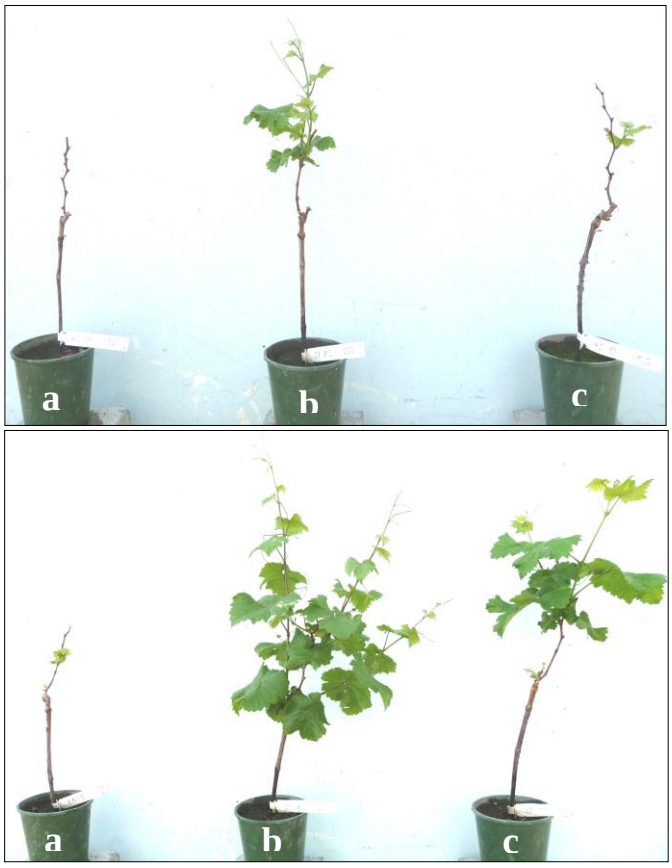

Figure 3.15: New spring growth on grapevine plants that has been inoculated with *N. luteum* and supplied with different amounts of water to induce stress during the previous season. Inoculated plants above: (a) 15%, (b) 25% and (c) 100% FC and below: control plants (a) 15%, (b) 25% and (c) 100% FC.

3.4 Discussion

Adhesion of fungal spores to the plant surface, which prevents their displacement from plant surfaces, is considered an essential first step in the infection process (Elad *et al.*, 1990). In this study, SEM studies showed that *N. luteum* conidia placed on wounded attached shoot pieces and green leaves remained in place and germinated by 24 h after inoculation, whereas those placed on non-wounded attached green leaf tissues were shed from the surfaces 24 h after inoculation. This indicated that the conidia were unable to adhere strongly to the intact attached tissue possibly because their germination responses were not activated. When the leaf surfaces were observed at high magnification many wax platelets were seen which appeared to be superficial and loosely aggregated. This observation was consistent with the work of Bensalem-fnayou *et al*. (2009) who reported that high magnification observations with SEM showed that mature leaf surfaces of grapevines were covered with wax layers of loosely crystalline granules or platelets. Eigenbrode and Jetter (2002) also reported that waxes on plant surfaces have anti-adhesive properties inherent in their chemical constituents and Juniper *et al*. (1989) also reported that the wax crystals on plant surfaces are easily detachable structures. These reports could explain the mechanism for disappearance of the *N. luteum* conidia from the non-wounded plant surfaces. It is also possible that the the wax and conidia were blown from the tissue surface or that the conidia might have been removed during sample preparation for SEM (Neil Andrews, pers. comm. 2009). Fluctuations in temperature can also influence respiration and metabolic rate, which could thus impair spore adhesion (Tucker and Talbot, 2001). Since the wounding or detachment of the tissues can affect plant metabolism this may also affect the pathogen's ability to recognise susceptible host tissues.

Wounding of plant surfaces may have direct effects on conidium adhesion and germination. Filonow (2004) reported that a water-insoluble compound (butyl acetate) on the wound surface played a role in adhesion of *Botrytis cinerea* and *Penicillium expansum* conidia on apple fruit and Amiri *et al*. (2005) also observed increased attachment of waterborne conidia of *P. expansum* when wounds were made on apple fruit surfaces, with greater emergence of the germ tubes in the 12 to 24 h post inoculation compared to their development on non-wounded fruits. The availability of plant nutrients on wounds has been found to positively influence the attachment of spores of *P. expansum* (Hamer *et al.*, 1988; Amiri *et al.*, 2005). In this study, conidia on the wounded grapevine tissues germinated and produced germ tubes from one or both

ends within 3 h but no appressoria were visible. The attachment and germination of the conidia was probably due to the nutrients provided in the sap oozing from the wound. This observation is consistent with other reports that showed wound pathogens such as *Alternaria* and *Fusarium* species penetrating wounded tissues directly without appressoria or cell wall-degrading enzymes (Mendgen *et al.*, 1996; Gupta *et al.*, 1998).

Fungal spores of some plant pathogens such as *Nectria haematococca, Uromyces viciae* and *Manaporthe grisea* normally adhere to hydrophobic surfaces such as the leaf cuticle prior to germination (Jones and Epstein, 1989; Beckett *et al.*, 1990; Hedge and Kolattukudy, 1997). The spores of rust fungi for example use physical or chemical signals from the plant surfaces to trigger germination and differentiation into appressoria that facilitate penetration into the host tissue (Collins *et al.*, 2001). However, Amiri *et al.* (2005) also noted that the attachment of conidia is more complex than simple attractions between hydrophobic surfaces, with the whole process not being well understood for most fungi, and that environmental conditions encountered by the spore when it lands on a plant surface are often pivotal in triggering its attachment and germination process.

The rapid formation of germ tubes on wounded tissues, which was observed within 3 h of inoculation, could be due to the stimulation of germination by plant compounds released by the wounds, thus making penetration fast enough to overcome the need for adhesion. This observation of rapid germ tube growth on wounded tissues was similar to reports made about the germ tube growth and penetration of *Alternaria* species which cause leaf spots on bean and rough lemon leaves (Ruehle, 1964; Saad and Hagedorn, 1969). Yamoah *et al*. (2008) also reported that the increased infection of wounded gorse tissue by *Fusarium tumidum* was due to nutrients released by wounded cells, since they showed enhanced conidial germination and hyphal growth in response to gorse extract.

The development of a network of mycelia observed on wounded detached tissues of both the leaf surfaces and shoot tissues, as opposed to only germ tube growth on wounded attached tissues at 24 h after the conidial inoculations, was an indication that the presence of the conidia on the wounded attached grapevine tissue may have induced a defence response leading to the slower growth of the germ tubes than with the wounded but detached tissues. This hypothesis is supported by the findings of Kortekamp (2006) who reported that fungal development on attached tissues may be reduced or retarded due to development of active defence mechanisms, such as the accumulation of phytoalexins and pathogen related proteins.

In this study, botryosphaeriaceous species were able to successfully infect the wounded leaves, buds, trunks, canes and shoots. This finding was supported by the isolations of some botryosphaeriaceous species from necrotic grapevine leaves, buds, shoots, canes, trunks and cordons during the initial vineyard sampling (Chapter 2). This is the first report of botryosphaeriaceous species being able to infect grapevine leaves. However, Marincowitz *et al.* (2008) who isolated some botryosphaeriaceous species directly from leaf spots on plants in the Proteaceae and Pérez *et al.* (2008) also observed that these fungi could exist as endophytes in healthy *Eucalyptus* leaves, typically causing disease after the onset of stress. In this study, leaf lesions did not extend far and the pathogen was not isolated from the petioles. The necrotic tissues on leaves abscised quickly and were not incubated to observe for sporulation. However, Crous *et al*. (2006) observed pycnidia from a *Stenocarpella* sp., which belongs to the Botryosphaeriaceae, on the upper leaf surface of *Zea mays*. Leaf infection could therefore play a role in the disease cycle and future research needs to investigate this subject.

In this study, pruned canes, wounded trunks, shoots and buds were susceptible to *N. luteum* infection and disease development. However, the higher incidence of the pathogen in wounded trunks, pruned canes and shoots confirm it to be primarily a wood and stem pathogen. Grapevine husbandry requires the plants to be supported on trellis and to be pruned or trimmed several times per annum. This inevitably leads to frequent wounding *via* pruning, rubbing against the wire by wind action and insect damage, which provide sites for botryosphaeriaceous species entry into grapevines. Bud infection was also shown to cause significant effects, because they failed to grow into healthy shoots and because the pathogen could move endophytically into the supporting stems, with subsequent stem dieback. Although most of the inoculated buds failed to burst, probably because they were killed by *N. luteum*, some were able to grow into shoots without any external symptoms but internal necrosis from the inoculation point. The presence of lesions observed inside emerged shoots when they were sectioned at 7 days after bud inoculation confirms that the pathogen can move from buds to the shoots without necessarily killing them. Castillo-Pando *et al.* (2001) also concluded that buds of the grapevine could be entry points for the botryosphaeriaceous species, since they were associated with budburst failure, but they did not demonstrate disease transmission.

Wounded roots did not become infected in this study, which showed that the New Zealand isolates of *N. luteum, N. australe, N. parvum* and *D. mutila* were not root pathogens. However, this finding contradicts the reports of Whitelaw-Weckert *et al*.

(2006), who grew 3-year-old Pinot noir grapevines in a potting mixture of coarse river sand, loam and Canadian peat moss, in which a 5 cm core portion was replaced with a mixture of *D. mutila* mycelium and pycnidia in infected wheat bran. After 6 months, they isolated the pathogen from the shoots of two out of the six grapevines planted in the infected soil and concluded that the fungus had moved from the soil *via* the roots and the trunk into the shoots. However they did not isolate the pathogen from the roots or trunk base and so the shoot infections could have come about through splash dispersal from the soil during watering and not from soil-roots transmission upwards as suggested. Since they had no control plants, which they could demonstrate were disease-free, it is also possible that the source plants were infected before the beginning of the experiment. Castillo-Pando *et al.* (2001) also found during a survey that *D. seriata* was isolated from stems and roots showing dieback in one out of 11 sites sampled in the Hunter Valley of New South Wales, but they did not try to determine whether the root infections were from stem infections that progressed downwards and did not carry out any pathogenicity studies to confirm soil-root transmission. Since the botryosphaeriaceous pathogens were found to spread in both directions in this study, it is possible that isolation from the shoots as in the case of Whitelaw-Weckert *et al.* (2006) or even in the stems and roots as observed by Castillo-Pando *et al.* (2001) was caused by the pathogen moving downwards from a stem or shoot infection and not upwards from a root infection. Further research with external inoculation of the stem base could be done in an attempt to track the pathogen to the roots.

In this study propagules (mycelial fragments or conidia) of *N. luteum* were not seen or isolated from sap indicating that they were not translocated freely through the sap of the infected grapevine shoots as reported for *Verticillium albo-atrum* conidia which had been observed within xylem vessels of cotton plants and moving acropetally within the transpiration stream (Garber and Houston 1966). In xylem vessels of the cotton plants, conidia of *Verticillium albo-atrum* have been observed to be trapped in the pit border membranes between the vessels, germinating and penetrating through to the neighbouring vessels, where they produced more conidia to repeat the process (Klosterman *et al.*, 2009). In this study however, the *N. luteum* conidia inoculated onto wounded grapevine shoots grew into mycelium which was observed by light microscopy and SEM growing within, and apparently adhering to, the inner walls of the xylem vessels. In grapevine shoots and trunks, the xylem vessels are open and continuous (Thorne *et al.*, 2006), which can allow unimpaired pathogen movement in either direction as observed in this study. When the botryosphaeriaceous species were

inoculated onto trunks of vines as mycelium or conidia, 100% incidence was achieved and the pathogen progressed from the inoculation point, more quickly in the upward than downward direction. The internal lesions produced by *N. luteum* were also seen to move across from the inoculated portion of the trunks to the attached shoots. However the infected plants did not show any symptoms after inoculation and during the incubation period, which supports the evidence that the botryosphaeriaceous species can be latent pathogens, only causing visible dieback when the plants were pruned after onset of winter dormancy during the winter season or stressed in other ways.

The effect of available soil water content was investigated to determine whether it could affect the host-pathogen relationship. Pinot noir grapevine plants which were growing in potting mix and inoculated with *N. luteum* on their trunks showed no symptoms during the growing season, however after winter dormancy, the plants that had received 100% FC showed significantly greater dieback symptoms than those that had received 50 and 75% FC, with the least dieback in the 25% FC treatment. The reason for the greater dieback in the initially well-watered treatments could be due to their greater shoot and canopy growth not being supported when the available water was reduced to 25% FC, leading to water deficit in plants and severe dieback. This is in agreement with Lu *et al*. (2003) who also observed that if water inputs in grapevines are drastically reduced there is a risk of inducing undesirable water deficits in the crop, although they did not relate it to disease incidence as observed in this study. The plants that received a constant supply of 25% FC throughout the growing period had normal growth throughout and that could also explain why dieback was relatively low.

In the repeated experiment using soil and Pinot noir grafted onto 101-14 rootstock that included inoculation with *N. luteum* on trunks, water availability affected the number of buds infected and subsequent shoot development. Overall, more buds developed into healthy shoots from the grapevine plants that were grown in soils with 25% FC compared with grapevine plants grown in soils with 15 and 100% FC, indicating that stress might have contributed to the failure of buds to develop into shoots. At 2 months after bud break, the shoot growth was also highest for grapevine plants growing in soils at 25% FC and least for those growing at 15% FC, indicating that the water stress of 100 and 15% FC also contributed to stunted growth. This finding is in agreement with van Niekerk *et al*. (2004) who reported that botryosphaeriaceous species are opportunistic pathogens more able to cause disease in hosts that are predisposed by stress. The effects of stress on xylem sap was investigated by Agüero *et al*. (2008), who reported that sap

from water stressed grapevines enhanced the *in vitro* growth of *P. chlamydospora* and *Phaeoacremonium* species compared with non-water stressed grapevines, which indicates that water deficit may predisposes grapevine plants to infection. Stoll *et al*. (2008), who investigated the effects of drought stress on infection after inoculating leaves of potted grapevine (cv Riesling), observed a higher percentage of leaves infected with *P. viticola* in non-irrigated vines compared with leaves of vines in well-irrigated grapevines and concluded that the pathogen had an increased effect on drought stressed leaves.

When different concentrations of *N. luteum* conidia were used to inoculate detached green shoots, all concentrations were found to cause infection on fresh wounds, with 100% incidence but varying lesion lengths. The high incidence observed by inoculating wounds with 10^2 conidia/ mL was surprising as that equated to about two conidia per wound based on the drop application of 20 µL. The ability of such a few conidia to germinate and penetrate the tissue and cause lesion formation within 10 days confirmed *N. luteum* as being highly pathogenic. However, further research needs to be carried out to determine if such a few conidia can equally infect green shoot tissue or woody stems *in vivo* and cause lesion formation, as found with the detached green shoot tissue which probably lack active host defence mechanisms. Since the experiment was conducted on a detached shoot at room temperature (15-22^{o}C), and under high RH, it is also possible that ideal temperature and RH for infection could have caused improved germination and subsequent invasion through the tissues. A study by Foster (1937) observed that temperature was an important factor that influenced conidial germination of *D. seriata* on apple twigs, although approximately 90% of the conidia were able to germinate at temperatures between 12 and 32^{o}C.

Infection by botryosphaeriaceous species has been observed to occur *via* ascospores or conidia (Slippers *et al.*, 2007) although these authors did not report on the minimum number of spores required. However many researchers have reported that increased inoculum concentration caused increased incidence or severity of other diseases. For example Nao (2008) observed that leaf spot infection caused by *Septoria lactucae* on lettuce leaf was more severe, with 100% incidence, when the sprayed inoculum concentration exceeded 10^2 conidia/ mL, and severity increased with increasing concentrations. Carter and Moller (1971) also reported 25% and 60% dieback incidence on almond (cvs Strouts and Nonpareil) at 6 months after inoculating wounds with 10 and 100 *E. lata* ascospores, respectively. Petzoldt *et al.* (1981) also observed that more

infection resulted when grapevine pruning wounds were inoculated with 10^3 ascospores/mL of *E. lata* than, with 10^2 ascospores/mL. In this study, longer lesion lengths with 10^4 conidial/mL than with 10^6 and 10^5 conidial/mL could have been due to competition for resources on the small inoculation site (3 mm diameter).

In grapevines, pruning wounds are the most obvious points of entry for the botryosphaeriaceous pathogens. However, as wounds age they may become less susceptible. In this study, the aging of wounds made in 2-year-old grapevine wood decreased their susceptibility to infection by *N. luteum* conidia applied at 10^4 conidia/mL, with 0% infection by 14 days although the wounds were susceptible to mycelium infection after 30 days. Xu *et al*. (1998) also observed that the incidence of canker lesions on apple trees was greater when microconidial suspensions of *N. galligena* were applied to fresh pruning cuts than older cuts. However, Gubler *et al.* (2002) reported that inoculation with *Phaeoacremonium aleophilum* or *Phaeomoniella chlamydospora* at approximately 10^6 spores/ mL caused successful infection of fresh and up to 10-month-old pruning wounds on grapevine varieties Thompson Seedless and Cabernet Sauvignon.

The investigation into the effects of wetness duration after inoculation showed that inoculation with a *N. luteum* conidial suspension caused lesion development on grapevine shoots without the need for further wetness periods and therefore surface wetness cannot be considered as an important factor in *N. luteum* infection. A study by Navi *et al*. (2005) on grain mould fungi such as *Curvularia lunata, Cladosporium oxysporum, Bipolaris australiensis, Fusarium moniliforme, F. pallidoroseum,* and *Phoma sorghina* showed that these fungi were able to cause infections of the sorghum plant at different grain development stages when inoculation was done under zero wetness duration. Xu *et al*. (1998) also observed that wetness duration had no effect on development of canker lesions caused by *N. galligena* on apple trees. Although many pathogens are known to cause increasing disease incidence and severity with increasing wetness duration following inoculation, such information is not available for infection of grapevines by the botryosphaeriaceous fungi. However, Parker and Sutton (1993) reported that apple fruit infection caused by *B. dothidea* increases with increasing wetness period and Aranuz and Sutton (1989) also reported that apple fruit and leaf infection caused by *D. seriata* increases with increasing wetness period. However, Sosnowski *et al*. (2009) reported that the risk of grapevine infection by *E. lata* can be reduced if pruning is avoided in wet weather and vines pruned at least 36 h after rainfall.

In this study, the higher relative humidity provided for 3 months after inoculation with *N. luteum* conidia increased pathogen progression, possibly due to increased rates of germination and early mycelial growth within the vessels. Amiri *et al.* (2005) observed that a saturated atmosphere (relative humidity >90%) stimulated the attachment of *Penicillium expansum* conidia through hydration and extrusion of spore tip mucilage, which improved the conidium attachment and germination. Aranuz and Sutton (1989) also reported that RH higher than 95% was critical for *D. seriata* conidial germination and infection to occur. This provides similar examples of higher disease progression in a moist atmosphere, as found in this study for 95% RH as compared with 78% RH.

The physiological age of the attached grapevine stem tissues was observed in this study to have a significant effect on lesion development which was more rapid in older tissues than younger tissues. Van Niekerk *et al.* (2004) also demonstrated pathogenicity of different botryosphaeriaceous species on wounded grapevine tissue at different stages of phenological development (green shoots, mature canes and mature wood) and showed that all the tissues were susceptible, but they did not compare the susceptibility among the different aged tissues. Many researchers working on different pathogens and hosts have also made similar observations. For example the lesions formed by *Phytophthora* spp. on 20-year-old oak tree stems were considerably larger than the stem lesions on 1 and 2-year-old seedlings of the same hosts, indicating that susceptibility of stem tissue to damage may increase with age (Balci *et al.*, 2008). Increased susceptibility with tree trunk age was also reported for *Phytophthora cinnamom*i-infected *Quercus rubra* plants (Robin *et al.*, 1992). Moller and Kasimatis (1980) and Trese *et al*. (1980) also observed 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, their reports contradict another by Munkvold and Marois (1995) who observed that age of wood at the time of wounding did not significantly affect the susceptibility of the wood to infection from *E. lata*. However, Sosnowski *et al*. (2007a) also observed that as the wood ages, differences in susceptibility to *E. lata* infection become more apparent in 18-month-old grapevines varieties Cabernet Sauvignon and Merlot with the younger vines being more susceptible than older vines.

In conclusion all parts of the grapevines were susceptible to *N. luteum* conidial infection except the roots. The susceptibility of fresh wounds which are made through routine trimming and pruning in vineyards and the availability of the botryosphaeriaceous species conidia throughout the growing season as observed in the spore-trapping studies

(Chapter 4) can make management of botryosphaeriaceous dieback extremely difficult. Susceptibility of 7-day-old wounds to conidia and 30-day-old wounds to mycelium, indicate the significant risk they present in a vineyard. Very few *N. luteum* conidia were necessary to cause infection on detached green shoots therefore conidial infection is likely to be the major cause of field infection since wounding is inevitable in vineyards. Chapter 4 of this thesis is therefore focused on rain splash dispersal and the effects of temperature, relative humidity and sunlight exposure on the viability of botryosphaeriaceous species conidia.

Chapter 4
Effects of environmental factors on dispersal and viability of botryosphaeriaceous species conidia

4.1 Introduction

The dispersal of fungal pathogens is critical in epidemic development of plant diseases (Madden, 1997). Spore release in fungi has also been a subject of classical biological research for more than a century (Meredith, 1973). Wind, rain-splash, insect feeding, and contaminated pruning tools may also contribute to dispersal of the released spores, which can then infect plants through wounds, growth cracks, insect feeding damage, and natural openings. Pusey (1989) detected waterborne ascospores and conidia of *B. dothidea* (*Fusicoccum aesculi*) and *B. obtusa* (*Diplodia seriata*) in rain water runoff from prunings and diseased scaffold limbs of peach trees throughout most of the year. Sutton (1981) found that ascospores of *F. aesculi* were usually more abundant at the beginning of a rain period and Britton and Hendrix (1986) showed a seasonal succession of botryosphaeriaceous species in peach with F. *aesculi* spores predominating in summer and autumn and *D. seriata* in spring.

The factors affecting spore release and dispersal of botryosphaeriaceous species in vineyards have not been fully investigated. Úrbez-Torres *et al.* (2010b) studied the seasonal release of botryosphaeriaceous species spores in Californian vineyards using glass microscope slides covered with petroleum jelly, which were placed on grapevine cordons, and Burkard volumetric spore traps. They trapped spores from the first rainfall in autumn through to the last spring rains, being September to April in the northern hemisphere. Van Niekerk *et al*. (2010) also reported that timing and amount of spores released by the botryosphaeriaceous species were governed by rainfall, relative humidity, temperature and wind speed prior to and during the 'spore' release events. Although the above reports focussed on aerial trapping, conidia may also be trapped in rain water, as has also been reported for *Leucostoma cinctum* (Grove and Biggs, 2006), *Valsa leucostoma* (Bertrand and English, 1976), *Colletotrichum musae* (De Lapeyre de Bellaire and Mourichon, 1998), *Gibberella zeae* (Paul *et al.*, 2004) and *Sclerotinia sclerotiorum* (Jenkins, 1965).

Environmental factors such as temperature, relative humidity and sunlight are also likely to affect the viability of conidia dispersed into the atmosphere, as reported by Parnell *et al.* (1998). Most of the UV studies on the *in vitro* germicidal effect of ultraviolet radiations on conidial germination have been done with UV lamps under laboratory conditions (Alves *et al.*, 1998; Boyd-Wilson *et al.*, 1998; Ghajar *et al.*, 2006). However radiation from the sun is also considered a limiting factor on the development of several aerial fungi, the effect being mainly caused by the spectral part corresponding to the UV-B and the increasing surface temperature caused by the radiation (Rotem *et al.*, 1985; Stevenson and Pennypacker, 1988).). Caesar and Pearson (1983) and Rotem *et al.* (1985) also reported that sunlight, greatly affects spore survival, and that the short wavelength UV-B component (250-270 nm) is the main fungicidal element. Other studies have shown that UV radiation is highly mutagenic, affecting a variety of biochemical processes in the fungi (Klein, 1978).

The spores of fungi have temperature maxima for germination and different maxima for survival, however temperature effects interact with available moisture in the environment. The botryosphaeriaceous species conidia have generally been reported to germinate between 8 and 39^{o}C with no growth beyond 40^{o}C for most species (Copes and Hendrix, 2004), although the effect of temperature on their mortality has not been reported. Relative humidity (RH) in general has also been shown to significantly affect germinability of conidia. For example, maximum germination of conidia of *D. seriata* occurred in free water and declined as relative humidity (RH) was reduced from 100 to 92% while no germination was observed at 88.5% RH (Arauz and Sutton, 1989). However the effects of RH and UV light on the ability of conidia to survive in the environment has not been reported for botryosphaeriaceous species conidia. The purpose of this study was to determine (i) the effects of some environmental influences on availability and /or dispersal of botryosphaeriaceous species conidia in vineyards, (ii) the effect of temperature on *in vitro* conidial germination of the four most important species, (iii) the effect of different sunlight periods and screening treatments on survival of conidia, as measured by their germination and (iv) the effect of different relative humidities on conidial germination and survival.

4.2 Materials and Methods

4.2.1 Seasonal availability of conidia

Trapping of botryosphaeriaceous species air-borne spores was attempted in March 2008 in three vineyards in the Marlborough region. From May 2008 to April 2009, trapping of botryosphaeriaceous species water-borne spores was also attempted in a 20-year-old Pinot noir vineyard in the Canterbury region. In both regions, previous grapevine sampling had yielded isolates which were characteristic of the botryosphaeriaceous species and their identity had been confirmed by PCR of rDNA followed by DNA sequencing as *N. luteum, N. australe, N. parvum, D. seriata* and *D. mutila* (Chapter 2).

4.2.1.1 Airborne dispersal

To determine the presence of wind dispersed ascospores/conidia under periods of little or no rain, microscope slides coated with Vaseline® were fixed using fold-back stationery clips onto wooden posts (Figure 4.1a) within the grapevine canopy at a height of 50 cm above the floor, at three vineyards in Marlborough in March 2008 and then one in Canterbury, from July 2008 to February 2009. A horizontal and vertical Vaseline®-coated slide was fastened to each of the 10 randomly selected wooden posts to ensure that wind-borne spores and those settling through gravity could be trapped. During the sampling periods, the slides were collected and replaced weekly, and observed under a light microscope (Olympus® BX51) for characteristic botryosphaeriaceous species conidia or ascospores, as distinguished by their morphology and colour (Pennycook and Samuels, 1985; Crous *et al.,* 2006; http://www.crem.fct.unl.pt/botryosphaeria_site/). The Vaseline® was then washed off each slide by agitating it for 2 min in 10 mL of 30ºC water and 100 µL aliquots of each suspension plated onto a Petri dish containing ½ PDA-Cph. After incubation in darkness at 25ºC for 3-7 days, the plates were assessed visually for colonies characteristics of botryosphaeriaceous species.

4.2.1.2 Waterborne dispersal

Water trapping for conidia was carried out in the Canterbury vineyard only, from May 2008 to April 2009. The rainwater runoff traps consisted of 210 × 297 mm transparent acetate sheets folded and stapled at one end to create funnels, whilst the opposite ends were stapled onto the canes that were wrapped to the left and right of the trunk (Figure 4.1b). Each funnel base was placed into a 500 mL container fixed to the trunk at 20 cm above the ground. There were 10 traps attached to 10 randomly selected grapevines. After every significant rainfall event (>2 mm), the water was collected and the trap

replaced. The collected water was then filtered through a 50 µm mesh sieve lined with a double layer of sterile cheese cloth. The filtrate was centrifuged at 2952 × *g* for 15 min, the supernatant discarded and the pellet resuspended in 20 mL sterile distilled water. A drop (20 µL) was mounted on a glass slide and observed under a light microscope for any conidia characteristic of the botryosphaeriaceous species, as described earlier in Section 2.2.3.3. The total number of conidia in these suspensions, which were characteristic of *Diplodia* spp. and *Neofusicoccum* spp., were counted on 10 replicate slides, the total number calculated and data analysed with ANOVA as described in Section 4.2.4.1. Aliquots (100 µL) of a dilution (10^4/ mL) of the spore suspensions from two rain trap samples per month were also plated onto PDA, incubated in the dark at 25^{o}C for 3 days and the colonies identified based on morphological characteristics. The remaining samples were combined for each collection date and were stored at -80^{o}C for molecular identification.

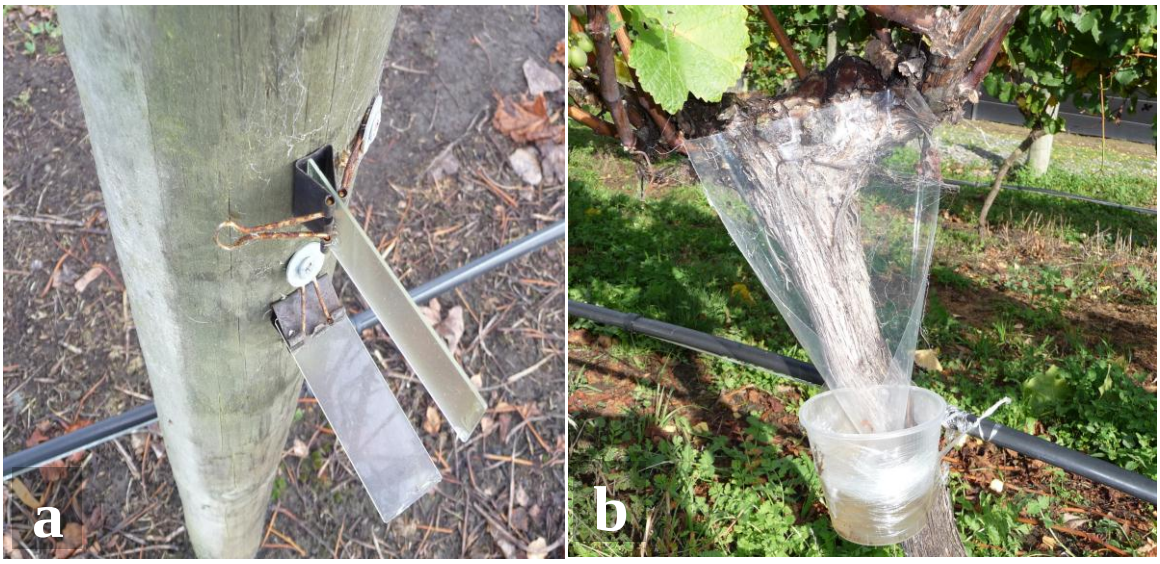

Figure 4.1: Two spore trapping methods used for catching spores of botryosphaeriaceous species in a Canterbury vineyard (a) Vaseline®-coated slides and (b) rain water trap.

Between 12 and 15 canes that were pruned off in winter and lying on the vineyard floor were also collected and washed in water. The washings obtained were centrifuged at 2952 × *g* for 15 min, the supernatant discarded and the pellet resuspended in 20 mL sterile distilled water so that any characteristic conidia in the pellet could be observed under a light microscope. The shoots that developed dieback within 2-3 weeks after summer trimming were also incubated in closed containers with moist paper towels at room temperature for 36 h to check for conidium production.

4.2.2 Molecular identification of conidia collected in rain water

4.2.2.1 Isolation of genomic DNA of botryosphaeriaceous conidia collected in rain water

The combined, frozen samples were analysed. A PowerSoil™ DNA isolation kit (MO BIO laboratories, CA, USA) was used to extract genomic DNA from the conidia obtained from the rain water traps. The above spore suspensions were thawed, centrifuged at 2952 × *g* for 15 min and the supernatant discarded. For each sample, approximately 0.25 g of the pellet (or all if whole pellet <0.25 g) was added to a 2 mL PowerBead™ tube containing an aqueous solution of acetate and salts (to protect nucleic acid from degradation) and the extraction done as described in Section 2.4.6.2. The undiluted DNA was stored at -20°C until a PCR could be conducted.

4.2.2.2 PCR amplification using botryosphaeriaceous multi-species primers

The PCR was conducted in a Bio-Rad iCycler Thermal Cycler (Hercules, CA, USA). The DNA extracted from the rain water pellet was amplified in a total volume of 25 µL which contained 200 µM of each dNTP, 1 U Taq DNA polymerase (FastStart®, Roche), 1 × buffer, 5 mole of each primer and 1 µL of the DNA template. The primers used were BOT100F ($^{5'}$AAACTCCAGTCAGTRAAC$^{3'}$) and BOT472R ($^{5'}$TCCGAGGTCAMCCTTGAG$^{3'}$), which were primers designed to amplify multiple botryosphaeriaceous species .They were developed by Dr. Hayley Ridgway (Lincoln University) and synthesised by Invitrogen (New Zealand). These primers were used to amplify portions of the internally transcribed spacers (ITS1 and ITS2) and the 5.8S subunit rDNA region. The amplification conditions were: one cycle of 94°C for 2 min, followed by 45 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s and one cycle of 72°C for 7 min. The PCR products were sequenced by the Lincoln University Sequencing Facility and the sequence identified using the dideoxy chain termination method with an ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and AmpliTAQ DNA Polymerase (PE Applied Biosystems, Foster City, California) in a Bio-Rad iCycler Thermal Cycler as described in Section 2.2.6.3.

4.2.2.3 Single stranded conformational polymorphism (SSCP) detection of botryosphaeriaceous species

The overlapping sequences of the 350 bp amplimers from the PCR that used the genus specific primers and the presence of several areas of consensus suggested that multiple botryosphaeriaceous species were present. Single stranded conformational polymorphism (SSCP) was used to distinguish the amplimers of the brighter bands

(representing 20 samples) in the PCR gel. A series of experiments were conducted by Dr H. Ridgway to optimize the SSCP protocol by modifying factors such as the amount of PCR product, acrylamide concentration, voltage, running time and temperature used (Ridgway *et al*. in prep.). Under optimised conditions 2 µL of each PCR product was denatured by heating the sample in SSCP loading dye (95% formamide, 20 mM EDTA, 10 mM NaOH, 0.05% bromophenol blue and 0.05% xylene cyanol) at 99^{o}C for 5 min. The reaction tube was then immediately placed on ice for up to 5 min prior to loading onto a 0.625 × MDE™ acrylamide gel (Appendix D.1). The gel was run at 22^{o}C for 17 h at 350V in a BioRad Protean II xi vertical electrophoresis unit using 1× TBE buffer. Gels were silver stained using the method of Bassam and Caetano-Anollés (1993). This method involved fixing the gel in a fixative solution of 10% (v/v) ethanol and 0.5% (v/v) acetic acid for 3 min and then staining in fixative solution which contained 0.002% (w/v) silver nitrate for 5 min. The gel was briefly rinsed in water for 2 min prior to developing in a solution of 3% (w/v) NaOH and 0.001% (v/v) formaldehyde for 20 min, after which it was rinsed in tap water. The wet gel was scanned with a photocopier prior to drying using a Biorad Model 583 gel dryer set at 70^{o}C for 45 min for permanent storage. Profiles were compared to bands from PCR products generated from DNA standards made with type of *N. luteum* (A243), *N. australe* (Kat-1), *N. parvum* (G141), *D. mutila* (M213) and *D. seriata* (A (1)2) whose identity had been confirmed through colony and conidial characteristic and DNA sequencing (Baskarathevan, pers. comm. 2009). Identification of the spore samples was made on the basis of similarities between band positions. Band profiles that did not match with any of the isolate band profiles were sequenced and identified by sequence comparison as before.

4.2.3 Effects of environmental parameters on germination of conidia

Three isolates each of *N. luteum* [N (12)2, M(13)2 and G(s)-1], *N. australe* [Kat-1, Mel-2 and J-3], *N. parvum* [Q-2, I(15)3 and I(15)2] and *D. mutila* [(F(12)2, Q and Iso-2] (Appendix B.8) were induced to sporulate on green shoots (Section 2.2.3.2), and an equal amount of a mixed isolate conidial suspension (10^6 conidia/mL) was prepared for each species in 2 mL sterile water as described in Section 2.2.3.2. The mixed conidial suspension which was serially diluted to final concentrations of 10^4 conidia /mL was used for the subsequent experiments, but freshly made each time. After each treatment and incubation period, the slide containers were placed above a layer of ice to suspend development during assessment. Slides were mounted and observed at ×100 magnification with a light microscope and conidia assessed as being germinated when

their germ tube lengths were at least half the length of the conidium. Percent germinated conidia was calculated from the total number observed, being at least 120 but up to 200 each time. Data were analysed using ANOVA as described in Section 4.2.4.2.

4.2.3.1 Effect of light on conidial germination

Two drops (10 µL aliquots each) of each spore suspension (Section 4.2.3) were placed onto a thin layer of PDA (~3 mm thick) that coated a glass slide. There were three replicate PDA slides per species and each slide was placed in an individual Petri dish wrapped with cling film. Slides were arranged in a CRD under continuous white light or dark for 3 h at 25^{o}C and then assessed for germination and data analysed.

4.2.3.2 Effect of temperature on conidial germination

Three drops (10 µL aliquots each) of each spore suspension (Section 4.2.3) were placed at three equidistant points onto a thin layer of PDA (~3 mm thick) that coated a glass slide as before. There were three replicate PDA slides per species for each temperature and incubation period, which were placed in individual Petri dishes wrapped with cling film and arranged in a CRD as before. The conidia were incubated for 3, 12 and 24 h at 5, 10, 15, 20, 25, 30, 35 and 40^{o}C (±1^{o}C) in continuous darkness. After each incubation period per temperature, the slides were assessed for germination of conidia and data analysed.

4.2.3.3 Effect of relative humidity on conidial germination

Mycelium agar discs from three isolates each of *N. luteum*, *N. australe*, *N. parvum* and *D. mutila*, as listed in Section 4.2.3, were mixed for each species and used to inoculate one green shoot per species to induce sporulation as described in Section 2.2.3.2. Conidial tendrils which oozed from inoculated green grapevine shoots were each removed with the tip of a sterilized scalpel and mixed thoroughly into a 20 µL drop of sterile water on a sterile glass slide. The conidial suspension was then spread to cover an area of approximately 1 cm^2 and air dried immediately within a laminar flow cabinet. The green shoots inoculated with *N. parvum* isolates failed to produce conidium tendrils and so were not included in the final experimental design.

The different RH levels were prepared in square Petri dishes (100 mm^2) lined with five layers of moist facial tissue which soaked up the saturated salt solution and prevented spillage (Figure 4.2). The saturated salt solutions used to create the different RH levels in the air above contained potassium chloride (KCl), potassium nitrate (KNO_3), potassium sulphate (K_2SO_4) and water, providing approximate RH levels of 84, 93, 97

and 100%, respectively, at 25°C (Greenspan, 1977). The salt solutions were poured into the square Petri dishes (100 mm^2) after inserting candle wax supports. The lids of the square Petri dishes were replaced and sealed with Parafilm™. The containers were left to equilibrate for 1 h at 25°C, after which the air dried conidium-laden slides of *N. luteum*, *N. australe* and *D. mutila*, were quickly placed inside the equilibrated Petri dishes and incubated at 25°C for 3, 6, 24 or 48 h. Three replicates of conidium-laden slides per treatment were set out in a CRD. After the incubation period, the slides were assessed for germination of conidia and data analysed.

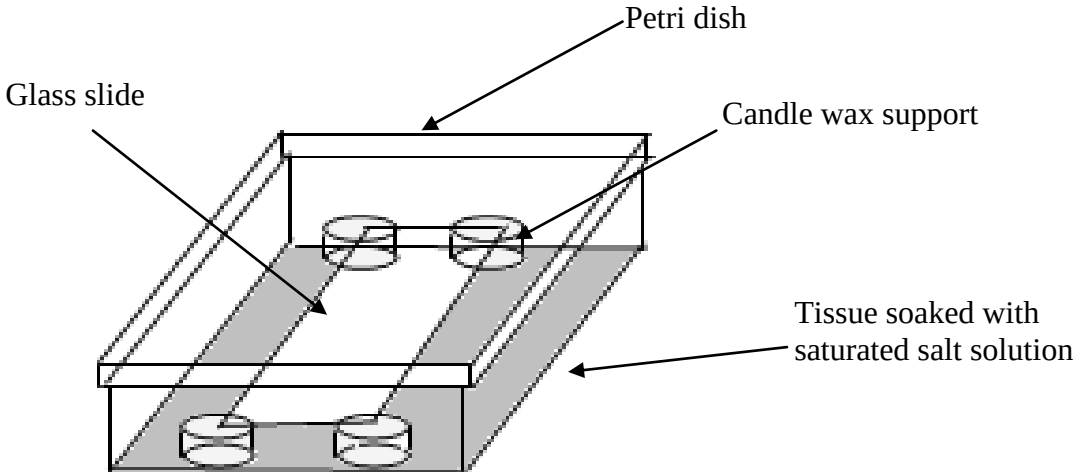

Figure 4.2: Diagrammatic representation of the RH chamber containing saturated salt solutions to maintain four different levels of RH and a conidium-laden glass slide.

4.2.3.4 Effect of solar radiation on viability of conidia

Conidium-laden slides of *N. luteum*, *N. australe* and *D. mutila* were prepared as described in the previous section. The conidia were then exposed to three levels of solar radiation by covering the open square Petri dishes with different materials supported on wooden blocks so as to create an approximately 50 mm high ventilation gap. The radiation levels were: (1) filtered sunlight (–UV) achieved with 1.5 mm thick Makrolon® UV Polycarbonate (Otto-Hesse-Straße, Darmstadt, Germany) that allowed penetration of visible light only, (2) non-filtered sunlight (+UV) achieved with a 1.5 mm Acrylic sheet, ΛA-cast™ (Asia Poly Industrial Sdn Bhd, Malaysia) which allowed penetration of both UV and visible light and (3) shade which was achieved with a 1.5 mm thick sheet of brown cardboard. The temperature within each square Petri dish in each treatment was recorded every 30 min with a TinyTag Talk 2 temperature logger (Gemini Data Logger UK Ltd). The three replicate slides per species were arranged in a CRD and exposed to

7, 14, 28, 42, 56 or 70 h of sunlight. The light exposure times were achieved over continuous days during sunny periods from 10 to 28 February 2010 when daylight length for the period lasted 13.5–14.5 h each day (from sunrise to sunset) with daily UV index ranging from moderate to very high (Appendix D.2). After each period of exposure, three replicate slides per treatment were assessed with a light microscope (dry mounted) for frequency of germinated conidia, and then they were incubated under 100% RH at 25^{o}C in continuous darkness for 12–24 h and assessed again for germination frequency. For every solar radiation treatment of each species, an extra slide of conidia was made, and incubated at 100% RH in continuous darkness for germination assessment and to serve as a preset control (no exposure). The data of percent germinated conidia were analysed with ANOVA as described in Section 4.2.4.2. This experiment was repeated, although temperature measurements were only made in the second experiment. When slides had zero germination, they were washed with a drop of sterile water, which was then spread onto PDA and incubated in the dark at 25 ^{o}C for 48 h, followed by observation of germination. This confirmed mortality of conidia rather than temporary inhibition by the exposure to sunlight. The last step was not considered sufficiently quantitative and so data were not analysed

4.2.3.5 Effect of prolonged periods of dryness on viability of conidia

To determine if conidial viability could also be affected by the prolonged periods of dryness likely in a sunny situation, a second parallel experiment was carried out with the same conidial source. Conidium-laden slides were prepared with the same three botryosphaeriaceous species using the same methods as in Section 4.2.3.4. They were placed in square Petri dish chambers of 68% RH, prepared with a saturated solution of potassium iodide (KI) as described in Section 4.2.3.3. Eighteen replicate conidium-laden slides per species were arranged in a CRD in an incubator set at 25^{o}C for 7, 14, 28, 42, 56 or 70 h in continuous darkness. After each period of exposure, three replicate slides per treatment were removed for germination assessment (dry mounted) after which they were transferred to a 100% RH chamber also prepared as before and incubated for a further 24-72 h at 25^{o}C and in continuous darkness. After this incubation period, germination was again assessed and the data analysed with ANOVA as described in Section 4.2.4.2. Conidia were washed from slides with zero germination to test for viability on PDA plates as before.

4.2.4 Statistical analysis

4.2.4.1 Spore trap and weather data

The total numbers of botryosphaeriaceous conidia per trap and per month were $\log_{10}$ (x+1) transformed prior to analysis by a regression suitable for an unbalanced design using GenStat version 11, with means being separated by Fisher's protected least significant differences at $P \leq 0.05$. Data of total rainfall and means of maximum daily temperature and relative humidity for each month were based on the Templeton weather station report, which was approximately 6 km away from the Canterbury vineyard, and accessed from the National Institute of Water and Atmospheric Research, New Zealand (http//www.niwa.co.nz/ncc/data).

4.2.4.2 Temperature, relative humidity, and exposure to sunlight

For each experiment, the data of percent germination, with species and treatments as separate factors, were analysed separately with ANOVA using GenStat version 12. Treatment means were compared by Fisher's protected LSD tests at $P \leq 0.05$.

4.3 Results

4.3.1 Seasonal production of conidia

Many spores were trapped on the Vaseline®-coated slides, but none were characteristic of the ascospores or conidia of *Neofusicoccum* and *Diplodia* spp. (Figure 4.3), and no colonies characteristic of these botryosphaeriaceous species grew on agar. In contrast, the rain water traps caught conidia of botryosphaeriaceous species (black and white arrows) and those of other fungal species (yellow arrows) (Figure 4.4). Rain water suspensions plated on PDA also resulted in colonies characteristic of botryosphaeriaceous species (Figure 4.5).

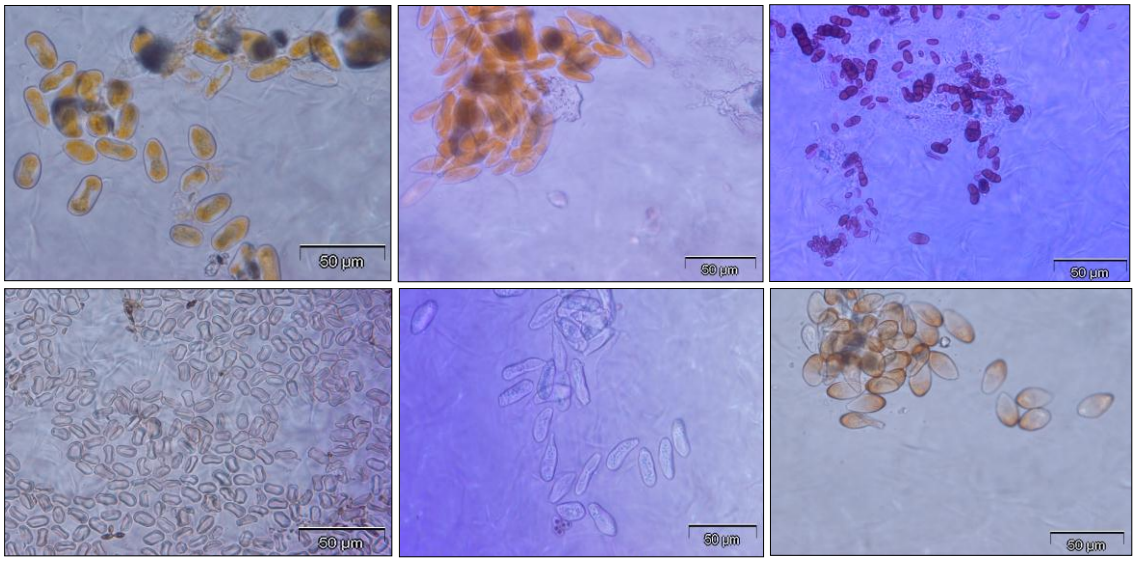

Figure 4.3: Spores of different fungi caught on the Vaseline®-coated slides set up in vineyards in the Marlborough and Canterbury regions.

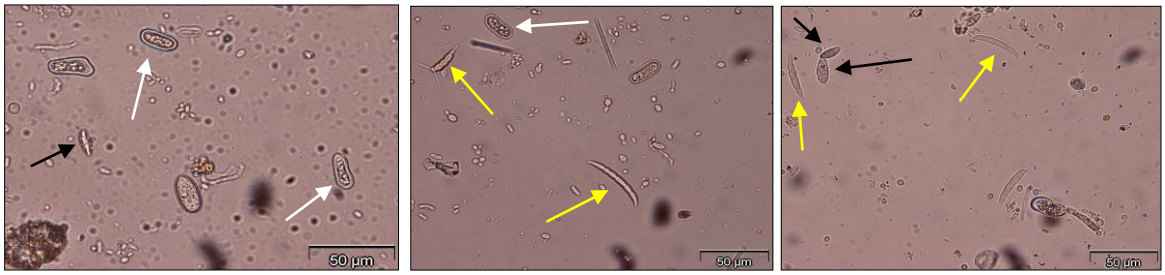

Figure 4.4: Light microscope view of *Neofusicoccum* spp. conidia indicated by black arrows and *Diplodia* spp. indicated by white arrows and yellow arrows indicating other fungi, which were present in a rain water sample.

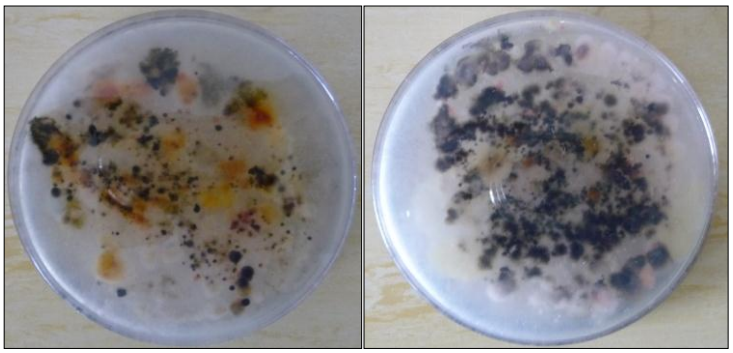

Figure 4.5: Mycelial growth of conidial suspensions from rain water traps growing on PDA incubated at 24.5°C for 3-7 days. All the black and grey mycelial colony colours were characteristic of some botryosphaeriaceous species.

Of the botryosphaeriaceous-like spores collected in the 10 traps during the year, 59.8% were characteristic of *Neofusicoccum* spp. and 40.2% of *Diplodia* spp. Mean numbers of *Neofusicoccum* spp. conidia trapped differed significantly over time ($P<0.001$; Appendix D.3). Numbers were significantly higher in the summer months, especially January and February, than in March, April, June and August (Figure 4.6c). Mean numbers of *Diplodia* spp. conidia trapped differed significantly over time ($P<0.001$; Appendix D.3). Numbers were highest in January, April and May (Figure 4.6b). Relative humidity was almost constant and did not vary significantly throughout the year (Figure 4.6a). Although the maximum period of rainfall occurred in July and August (Figure 4.6a), the conidium numbers were relatively low then. At this time, the winter pruning had reduced the wood in the canopy from more than 10 canes to 2 canes. Overall, the monthly conidium counts of both *Diplodia* and *Neofusicoccum* species did not appear to reflect patterns in monthly rainfall or mean monthly relative humidity, although they were greater during warmer temperatures (Figure 4.6a).

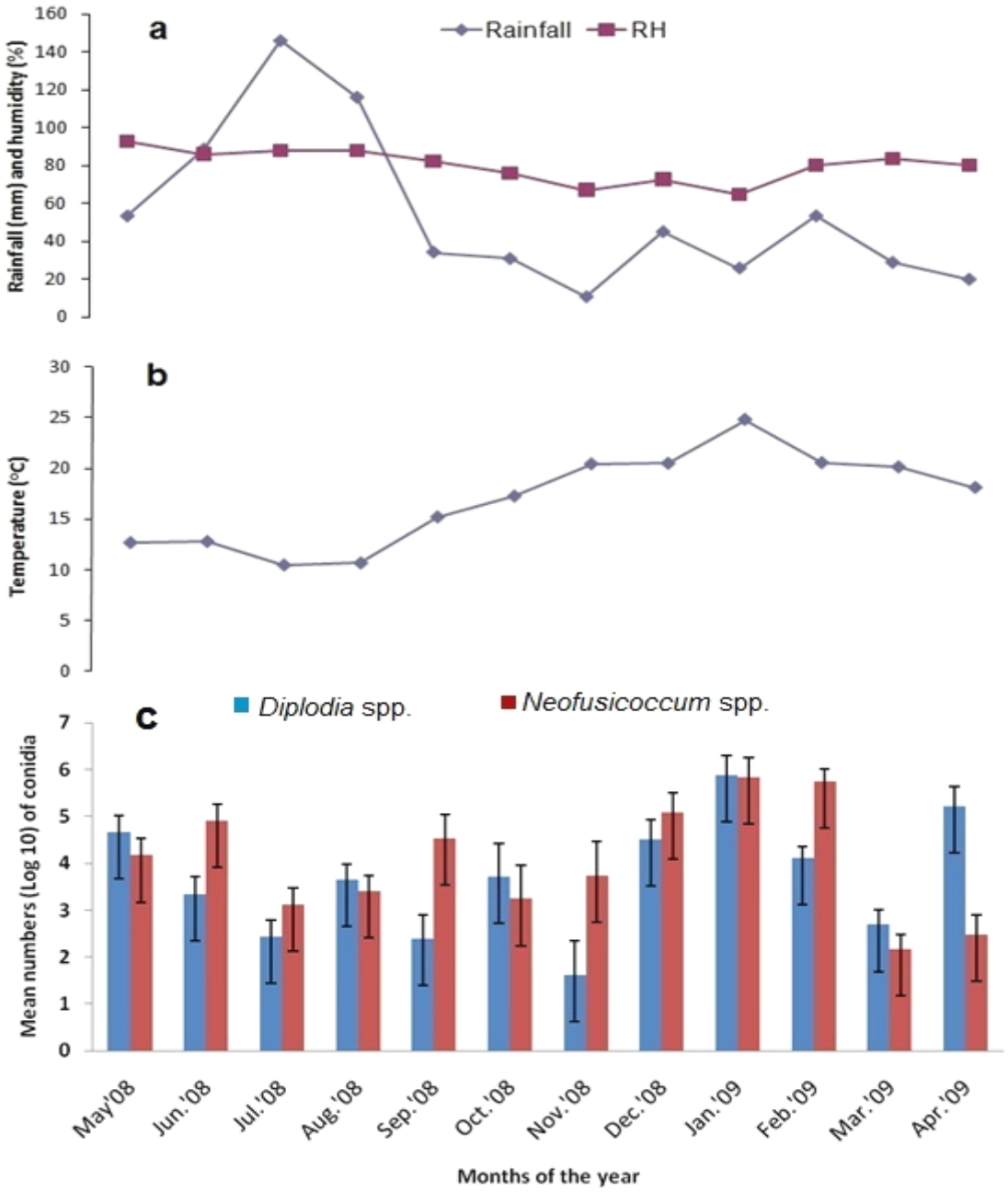

Figure 4.6: (a) Total monthly rainfall (mm) and mean relative humidity (%) over an annual sampling period. (b) Mean daily maximum temperatures (°C) for each month during the sampling period. (c) Mean numbers (log_{10}) of conidia characteristic of *Neofusicoccum* spp. and *Diplodia* spp. collected each month in 10 water traps within a Canterbury grape canopy during May 2008 to April 2009. Bars represent standard errors of the monthly mean conidium numbers.

Washings of the canes collected from the vineyard floor had abundant botryosphaeriaceous species conidia similar to those collected in the rain traps. The green shoots with dieback symptoms (Figure 4.7a), that were incubated under high humidity produced pycnidia that oozed conidia (Figure 4.7b). When examined with a light microscope, the conidia were similar to those of the *Neofusicoccum* and *Diplodia* species observed in the rain water traps.

Figure 4.7: (a) Grapevine shoot showing botryosphaeriaceous dieback after summer pruning (February 2009) in a Canterbury vineyard where the conidium trapping experiment was carried out (b) pycnidia oozing conidia in paste tendrils after incubation of the shoots under high humidity.

4.3.2 Molecular identification of conidia collected in rain water

4.3.2.1 PCR amplication using botryosphaeriaceous multi-species primers

As shown in Figure 4.8, the PCR for all samples, except lane 13, resulted in a single band with a molecular weight of approximately 350 bp. Multiple bands were not present and each sample contained an amplimer of the same size. There was some variation in band intensity between the samples. Submission of regions of the sequence data to GenBank (website) using the basic local alignment search tool (BLAST) confirmed that the rain water samples contained botryosphaeriaceous fungi. However the isolates could not be unambiguously identified because the relatively short pieces of DNA (350 bp) amplified by the genus specific primer were specific for several botryosphaeriaceous species. The primers successfully used for species identification in Section 2.3.5 amplified much longer sections of DNA.

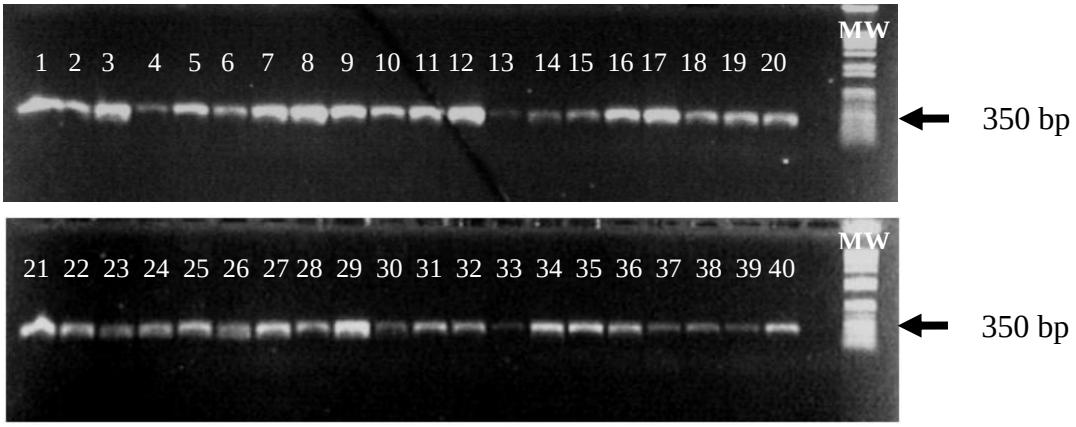

Figure 4.8: PCR products amplified from rain water samples with botryosphaeriaceous species specific primers BOT100F and BOT472R (Lanes represent dates of sampling. In 2008, 1-4 May; 5-7 June; 9-12 July; 13-17 Aug.; 18-19 Sept.; 20-22 Oct.; 23-24 Nov.; 24-27 Dec.; In 2009, 28-30Jan.; 31-35 Feb. 36-39 March and 40 for April].

4.3.2.2 Single stranded conformational polymorphism (SSCP) detection of botryosphaeriaceous fungi

The SSCP gels (Figure 4.9) showed that up to three species were present in any one sample although they could not discriminate between *N. parvum* and *N. ribis*. The bands on the gel had a slight curvature due to uneven heat distribution which was taken into account when identifying the species present. Results from the SSCP indicated that *N. parvum/N. ribis, N. luteum, N. australe* and *Diplodia* species were present. Bands corresponding to those produced by *N. parvum/ N. ribis* were observed in 13 out of the 20 samples during the months of May, June, July, August, September, October, December and January indicating that they were the most predominant species occurring throughout most of the sampling period. The next most common species were *N. luteum*, which occurred in 3 out of 20 samples in July and October, and *N. australe*, which occurred in 3 out of 20 samples in January and February. In addition, some bands that did not correspond to those produced by the five type botryosphaeriaceous species were observed faintly in several samples (lanes 34 and 38 in Figure 4.9) and strongly in two samples (lanes 40 and 42 in Figure 4.9) where they were the only bands present. The sequencing of the PCR product from samples 40 and 42 showed that they were 100% identical and that the sequence matched sequences from *D. olivarum* (EU392295-97, EU392301-2), *D. africana* (EF445343-45), two *D. mutila* isolates (AF243403 and AY259095) and *D. cupressi* (DQ458893-94, DQ846775-79 and EU220433). However, *Diplodia olivarum, D. africana* and *D. cupressi* were not isolated during the vineyard sampling in 2007 (Chapter 2).

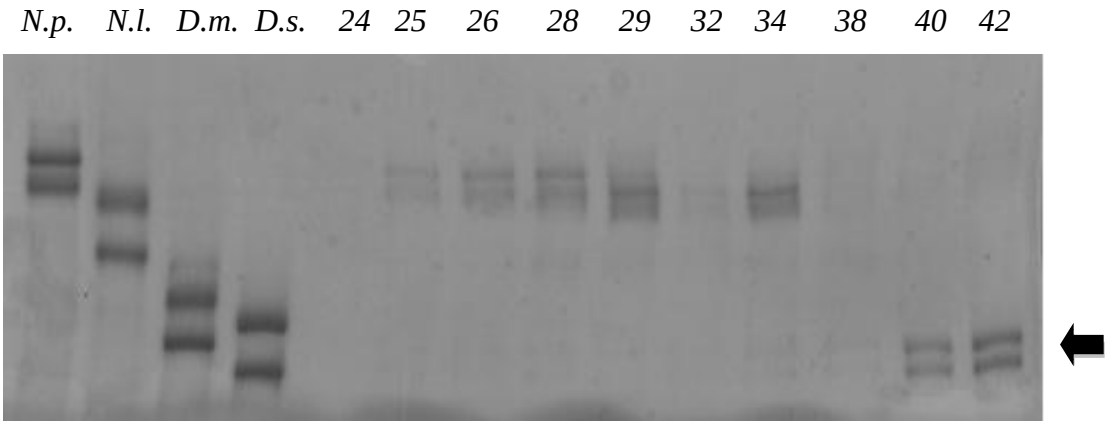

Figure 4.9: An example of the SSCP pattern of the botryosphaericeous species after PCR amplification with Botryosphaeria specific primers BOT100 and BOT 472 [In 2008, lanes 24: Nov.; 25 and 26: Dec.; In 2009, lanes 28 and 29: Jan.; 32 and 34: Feb.; 38: March; 40 and 42: April] *N.p=N. parvum*, *N.l=N. luteum*, *D.m=D. mutila* and *D.s=D. seriata*]. The bands corresponding to the unidentified *Diplodia* species are denoted by the black arrow.

4.3.3 Effects of environmental parameters on germination of conidia

4.3.3.1 Effect of light on conidial germination

The initial preliminary germination test done under continuous darkness or continuous white had no significant effect ($P>0.05$) on germnation rates, which were 52 and 55%, respectively, with similar germ tube growth. This showed that conidial germination of all four botryosphaeriaceous species was independent of light.

4.3.3.2 Effect of temperature on conidial germination

The effect of the different temperatures on conidial germination was significant ($P<0.001$; Appendix D.4) for all four species. At 5^{o}C after 3 h, *N. luteum* showed some germination (0.3%), whilst the small germ tubes produced by *N. australe*, *N. parvum* and *D. mutila* had not developed sufficiently to reach the length specified for recognition as being germinated. However by 24 h, some isolates of all species had germinated but with low percentages (1.8-2.6%). At 10^{o}C after 3 h, no germination had occurred in *D. mutila* but the other species had a few conidia germinating, and by 24 h some had developed mycelial networks. The absolute temperature range for conidial germination after 24 h was therefore from 5 to 35^{o}C for all species, although they only exceeded a 10% germination threshold when at 20 to 30^{o}C (Figure 4.10).

The overall mean maximum germination frequency of 100%, occurred for all species at 25^{o}C after 24 h, which differed significantly from the 77.5 and 59.4% recorded at 12 h

and 3 h, respectively for all the species (Figure 4.10). After 12 h at the optimum temperature of 25°C, the highest mean germination percentage of 88.7% was recorded for *N. luteum*, which was significantly higher than for *N. australe* (84.1%), *N. parvum* (73.2%) and *D. mutila* (70.1%). At the optimum temperature of 25°C, there was a significant (P<0.001; Appendix D.4) interaction between the species and temperatures tested, which was mainly associated with the higher temperature adaptation of *N. luteum*. At 30°C, the 54.9% germination of *N. luteum* was significantly higher than the germination frequencies of *N. australe*, *N. parvum* and *D. mutila* (46.1, 40.6 and 33.9%, respectively). There was no conidial germination at 40°C and so this data was excluded from the analysis.

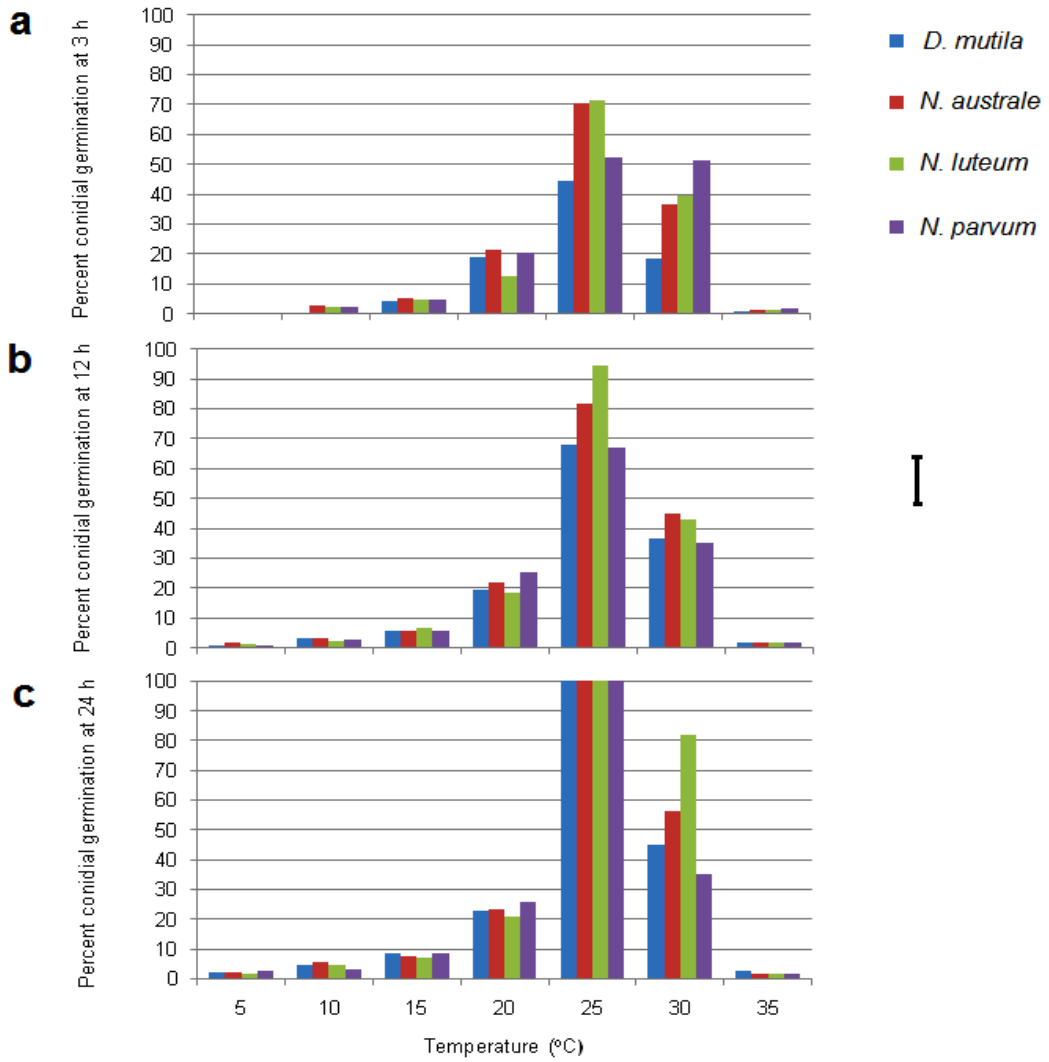

Figure 4.10: Mean percent germination (%) of conidia of four different botryosphaeriaceous species on PDA incubated at seven different temperatures for (a) 3, (b) 12 and (c) 24 h. Error bar represent Fisher's protected LSD at $P \leq 0.05$.

4.3.3.3 *Effect of relative humidity on conidial germination*

The effects of RH on percent germination did not differ (P=0.98) between *N. australe*, *N. luteum* and *D. mutila* and so these data were combined in a second analysis. The effects of RH and incubation time on percent conidial germination were highly significant (P<0.001 for both; Appendix D.5) (Figure 4.11) and the interaction between RH and incubation time was also significant (P<0.001; Appendix D.5). None of the conidia of the three species incubated at 84% RH germinated even after 48 h incubation at the optimum temperature. The RH of 100% resulted in the quickest and highest germination frequency of 91.8% within 3 h and did not increase significantly (P>0.05) over time. In 97% RH the conidial germination was 62.8% after 3 h which did not differ significantly (P>0.05) from the maximum of 67.9% after 48 h. In 93% RH, no conidia had germinated after 3 and 6 h but germination reached 43.9% after 24 h, and 48.9% after 48 h (Figure 4.11).

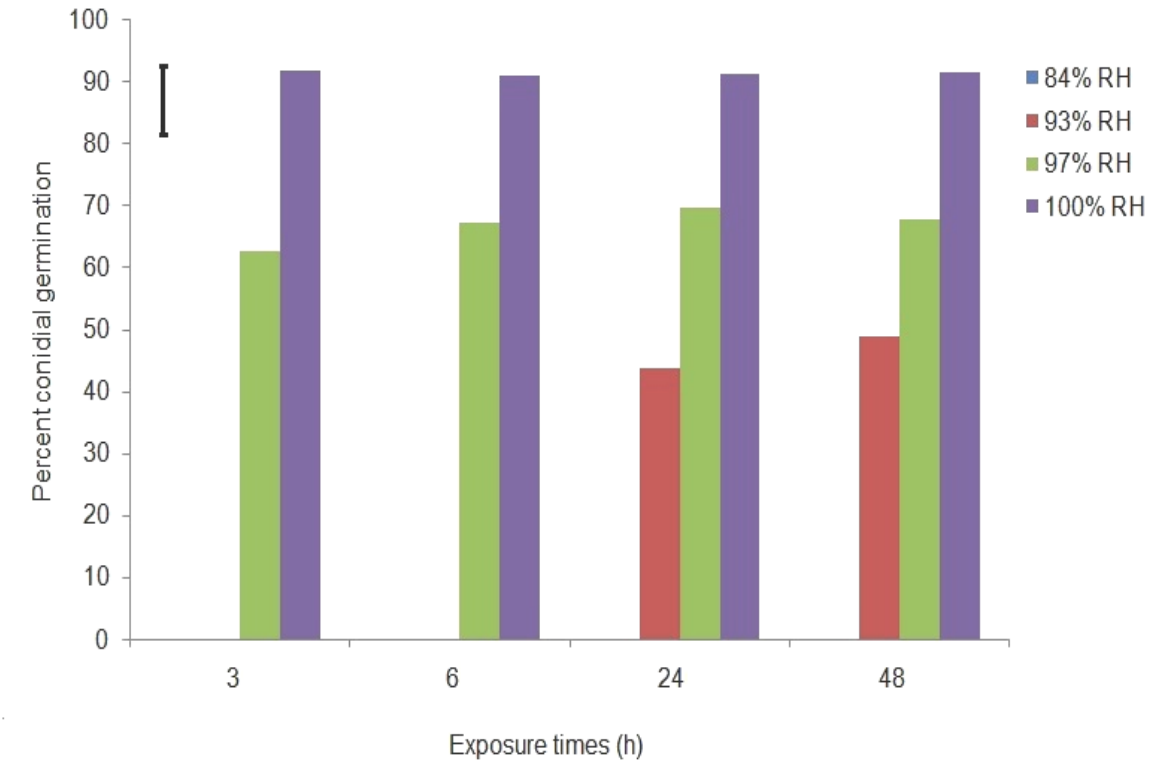

Figure 4.11 Mean percent germination of botryosphaeriaceous species conidia during exposure to different relative humidities (RH) at 25°C for 3, 6, 24 or 48 h. Error bar represent Fishers protected LSD at $P \leq 0.05$.

The development of mycelia was affected by the RH levels. Conidia placed in 100 and 97% RH formed mycelial networks by 24 h, although the hyphae in 97% RH were less developed than at 100% RH. In 93% RH, the germinating conidia had not developed mycelium networks, even by 48 h, when the germ tubes were about 7-8 times the lengths of the conidia (Figure 4.12).

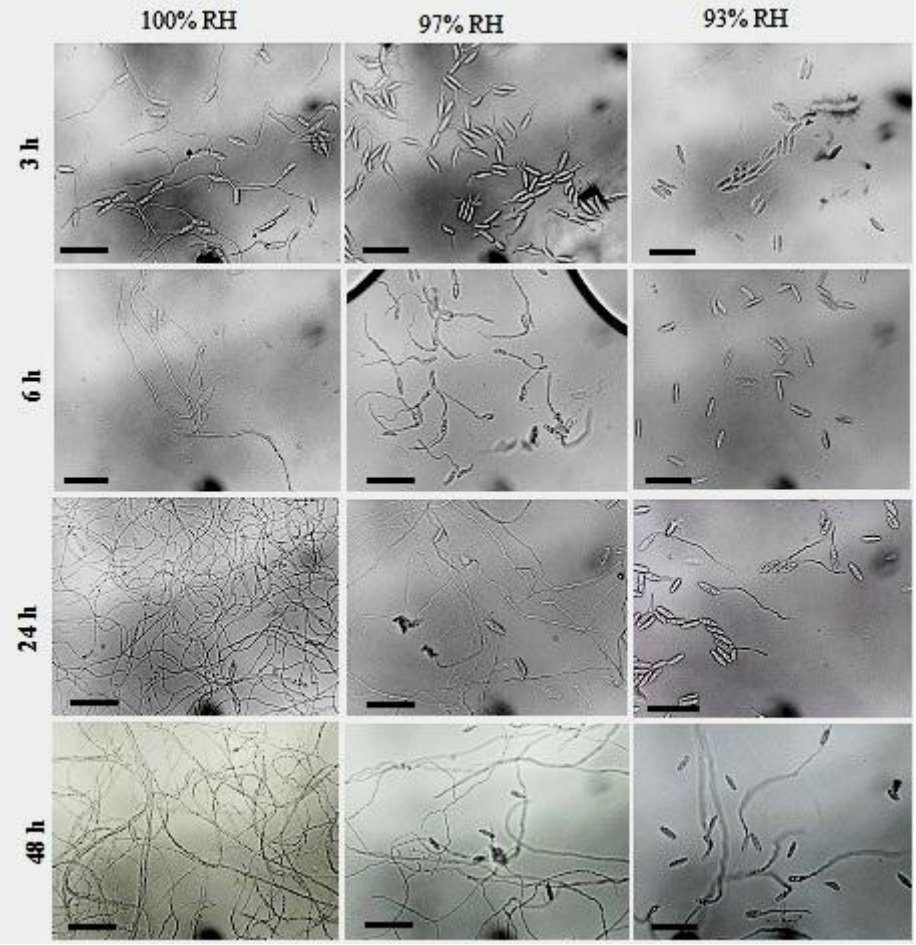

Figure 4.12: The germ tube growth and mycelium development of *N. luteum* over a period of time at the three different relative humidities. (Bar=50µm).

4.3.3.4 Effect of solar radiation on viability of conidia

The percent germination from the repeated experiments did not differ ($P>0.05$) with respect to the effects of solar radiation exposure, nor did the different species, *N. australe, N. luteum* and *D. mutila*, so the replicate and species data were combined for a second analysis. No conidia of any of the three species (*N. luteum, N. australe* and *D. mutila)* had germinated when initially removed from any of the different exposures to solar radiation levels. After incubation at 100% RH for 12-24 h, final germination of these conidia was found to be significantly affected by the sunlight levels ($P<0.001$;

Appendix D.6.1), with conidia exposed to shade, filtered solar radiation (-UV) and non-filtered solar radiation (+UV) having mean germination frequencies of 75.2, 28.1 and 12.2%, respectively across the exposure times, compared to 96% in the untreated (preset) controls. Increasing exposure times caused a decrease in germination ($P<0.001$; Appendix D.6.1), with an overall mean germination of 58.0% after 7 h exposure which was significantly higher than that of 28.6% recorded after 70 h exposure (Figure 4.13).

There was a significant ($P<0.001$; Appendix D.6.1) interaction between the exposure times and the radiation levels, which was associated with the greater effects of the +UV and –UV than the shade treatments during the 7-28 h exposure times (Figure 4.13). Non-filtered solar radiation (+UV) caused the germination rate to decline significantly and killed all conidia after exposure of 56 h or more. However, germination of those placed in shade did not reduce significantly until after 42 h exposure. Conidia collected from the shade, filtered radiation (-UV) and non filtered (+UV) solar radiation treatments after 70 h that were put onto PDA and incubated at 25^{o}C in continuous darkness for 3 days showed different levels of conidial viability compared with the preset control (no exposure). The non-germinated conidia from the slides exposed to non filtered sunlight (+UV) failed to germinate after incubating on PDA for 3 days at 25°C in continuous darkness. However 28% of conidia from the filtered radiation (-UV) germinated while 75% of conidia from the shade treatments germinated.

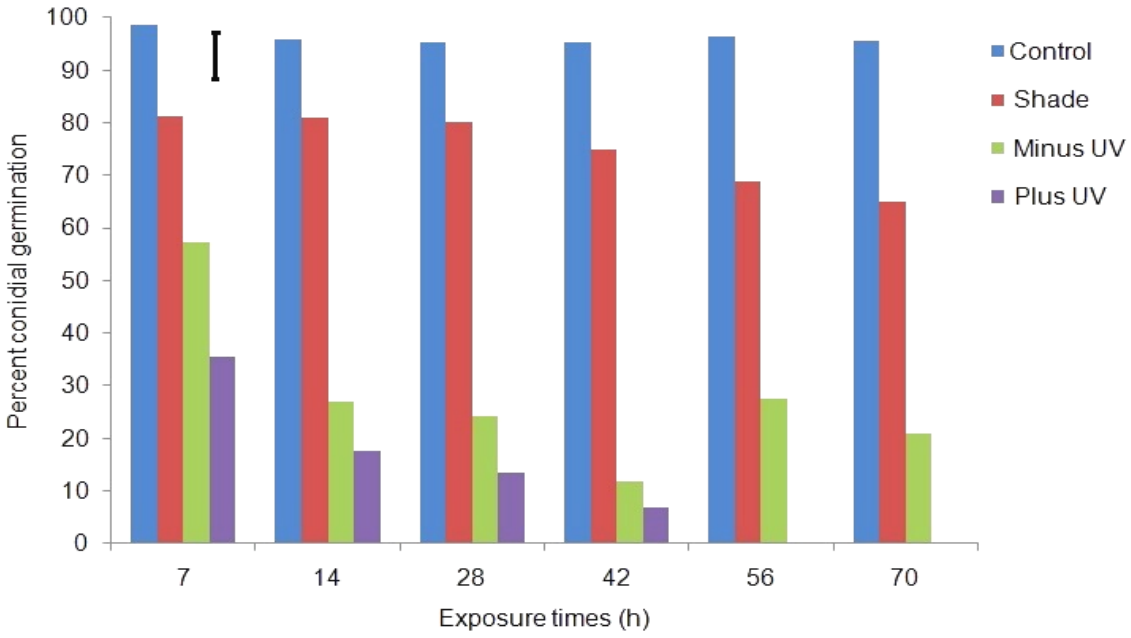

Figure 4.13: Mean percent germination of botryosphaeriaceous species conidia after different exposure times and levels of solar radiation (shade, filtered sunlight= -UV, non-filtered sunlight = +UV) followed by incubation at 100% RH for 7-70 h. Error bar represents Fishers protected LSD at *P*≤0.05.

The mean temperatures recorded during the different exposure times under the three different radiation levels were 23^{o}C for shade, 26^{o}C for filtered solar radiation (-UV) and 30^{o}C for non-filtered solar radiation (+UV) The highest temperature of 41^{o}C was recorded under all the radiation treatments but for different periods of time (Table 4.1).

Table 4.1: The temperatures recorded within the square Petri dish housing the botryosphaeriaceous species conidia-laden slide during exposure to three different levels of solar radiations (shade, -UV and +UV) in February 2010.

Exposure time (h)	Exposure level	Temperature readings from sunrise to sunset (°C)			Total time exposed to maximum Temp. (h)
		Maximum	Minimum	Average	
7	Shade	35	12	18	0.5[A]
	-UV	41	8	21	4
	+UV	41	20	33	1
14	Shade	39	8	21	1
	-UV	41	12	24	5
	+UV	41	14	29	3.5
28	Shade	41	8	23	2
	-UV	41	8	27	10.5
	+UV	41	11	29	8
42	Shade	41	8	25	2
	-UV	41	8	27	16.5
	+UV	41	8	29	13.5
56	Shade	41	8	26	6
	-UV	41	8	28	19
	+UV	41	8	29	21
70	Shade	41	8	26	10
	-UV	41	8	28	22
	+UV	41	8	28	24

NB: [A] The total times recorded under the maximum temperatures are cumulative

4.3.3.5 Effect of prolonged periods of dryness on viability of conidia

When the conidia were exposed to 68% RH, the time of the exposure significantly (P<0.001; Appendix D.6.2) affected the conidium viability of all three species which did not differ so the data were combined in a second analysis. No conidia of the three species germinated during the exposure to 68% RH. However, after re-incubating the conidia under 100% RH at 25°C, only those that were exposed to shorter durations of 7, 14 and 28 h were able to germinate after 24 h incubation, with germination rates of 87.8, 81.8 and 64.0%, respectively (Figure 4.14). Conidial germination rates after 7 and 14 h exposure to 68% were not significantly different but were significantly higher than those of conidia exposed for 28 h to 68% RH. However, the conidia that were initially exposed to the 68% RH environment for 42 to 70 h were only able to germinate after 72 h when re-incubated at 100% RH, and with significantly lower rates of 35.7, 30.3 and 25.9% germination for the times of 42, 56 and 70 h, respectively.

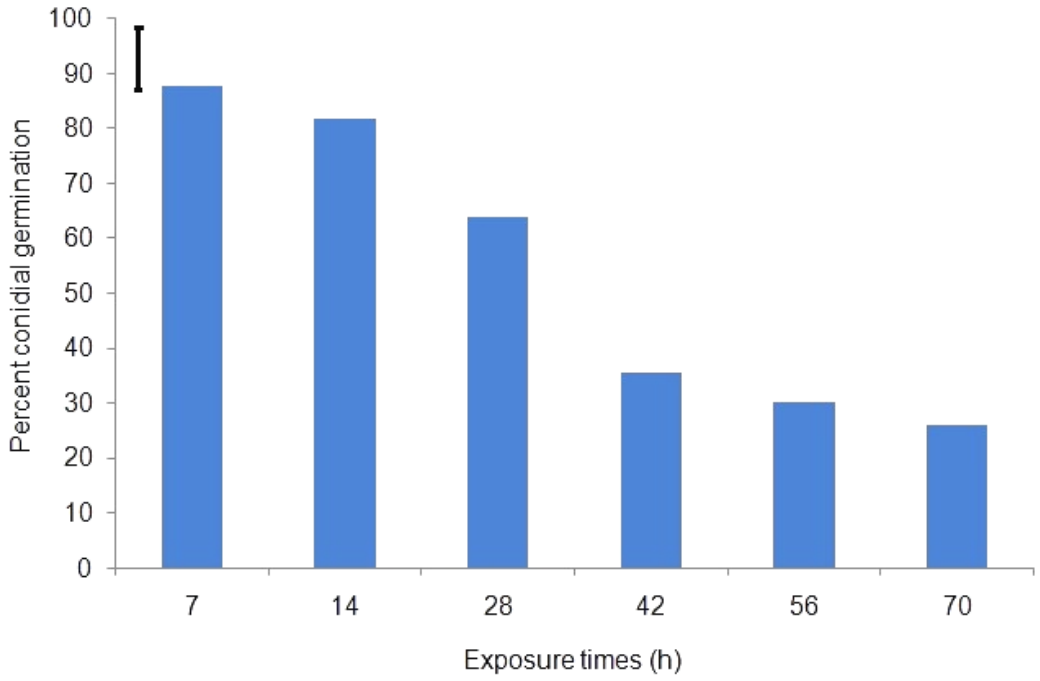

Figure 4.14: The effect of different times of exposure to 68% RH and subsequent percent germination of botryosphaeriaceous species conidia after re-incubation at 25°C and in 100% RH for 7-70 h. Error bar represent Fisher's protected LSD at $P \leq 0.05$.

4.4 Discussion

This study investigated the rainwater dispersal of four species of botryosphaeriaceous fungi commonly found in symptomatic grapevine wood with the aim of identifying temporal dispersal patterns. Microscopic observation of rainwater run-off collected from a Canterbury vineyard showed that high numbers of conidia characteristic of botryosphaeriaceous fungi (*Neofusicoccum* spp. and *Diplodia* spp.) were present throughout the year. The additional observation, that when trimmed canes with dieback symptoms were collected from the field and incubated under high relative humidity they developed pycnidia which oozed conidia in a water soluble paste, indicated that rainfall provides the mechanism for conidium release, dispersal and infection. Since conidia were produced on dieback that occurred in summer, this suggests that they may provide important sources of primary inoculum for secondary infection during the season.

The release of the botryosphaeriaceous species conidia seemed to have occurred during or immediately after rainfall but was not affected by the amount of the rainfall. Van Niekerk *et al.* (2010) also trapped 'spores' of botryosphaeriaceous pathogens using a

Quest volumetric spore trap (Interlock Systems, Pretoria, South Africa) and observed the presence of spores after the occurrence of as little as 0.25 mm rainfall, but with higher numbers of spores trapped during periods of higher rainfall. However, they observed that in some cases where sufficient rainfall did occur, no spores were trapped. The release of conidia after rainfall appears to be a general feature of botryosphaeriaceous pathogens as Pusey (1989) and Sutton (1981) identified abundant conidia and ascospores of *D. seriata* and *F. aesculi,* respectively, in the air of peach orchards during rainy periods. They found that the amount and duration of rainfall were the most important factors in waterborne dispersal of ascospores and conidia.

In addition to rainfall events and warm temperatures that drive the release of botryosphaeriaceous species conidia, the abundance of conidia as observed from this present study appeared to be also influenced by the number of grapevine canes. The numbers of conidia from botryosphaeriaceous fungi were observed to be somewhat reduced in July (mid-winter) through to November (late-spring). The reason for this was likely to be that the number of canes (8-10) per grapevine had contributed greatly to the number of spores trapped in rainwater in June, followed by a reduction in conidium numbers in July after pruning. This finding was verified by washing dropped pruned canes, which yielded solutions containing high concentrations of conidia for *Neofusicoccum* and *Diplodia* species. Reports from other researches also support this conclusion. Van Niekerk *et al*. (2010) reported seeing abundant mature pycnidia that oozed conidia typical of botryosphaeriaceous anamorph genera, *Dothiorella* and *Diplodia,* on the pruning debris collected around spore trap locations. Ahimera *et al.* (2004) demonstrated that in a simulated precipitation experiment in the field, the dispersal of *F. aesculi* conidia by single droplets from a nozzle at 138 kPa pressure resulted in up to 65% of the conidia being removed from infected pistachio nuts, and they were collected as far as 1 m from the tree canopy edge. This indicated that rain splash is an important mechanism for local dispersal, which may enable some long distance dispersal by rebounding of the rain-water drops bearing conidia that can also be wind driven.

In this study the spores were trapped throughout the year, except that numbers were lower in winter and spring. The greater numbers of conidia trapped during periods of high temperature in this study is supported by the report of Pusey (1989) who detected waterborne conidia of *F. aesculi* in peach orchards in Georgia, USA for most of the year, but not in January and February (winter in North America). However they also

detected conidia of *F. aesculi, D. seriata,* and *L. theobromae* in April (spring in North America). Infection within other woody host plants growing close to the vineyard may have also contributed conidia to the rainwater traps.

In this study, no conidia or ascospores characteristic of botryosphaeriaceous fungi were captured on the Vaseline® -coated slides and this led to the termination of the airborne trapping after 32 weeks. However, contrary to this and previous studies, Urbez-Torres *et al*. (2010b) observed in Californian vineyards that microscope slides coated with white petroleum jelly detected the greatest number of botryosphaeriaceous 'spores' (68.9%) during winter months of December, January and December and very few or no spores (1%) were trapped in late spring and summer (May through September). The difference in the findings is related to the environmental conditions, with mild, wet conditions occurring in winter and early spring in California, and could be due to their experimental setup. In this study, the Vaseline®-coated slides were fastened onto wooden posts at a height of 50 cm above the vineyard floor (Figure 4.1) whereas in the study conducted by Urbez-Torres *et al*. (2010b), they placed the horizontal slides onto the vines (cordons). It could be that the spores captured on the slides were splashed from the vines onto the slides where they remained after evaporation. In volumetric spore trapping conducted by Urbez-Torres *et al*. (2010b), they could not trap any botryosphaeriaceaous spores beyond 2 h following the termination of rainfall which also supports the current conclusion about the conidia being only splash dispersed. In contrast, *E. lata* ascospores are capable of continuous release for up to about 36 h after rainfall has ceased (Carter 1991; Gubler *et al*., 2005). This led Urbez-Torres *et al*. (2010b) to conclude that what they trapped on the petroleum jelly coated glass slides were conidia oozed from pycnidia rather than being ascospores. However the spores trapped in the volumetric spore trap were not identified as being conidia or ascospores although the shapes should be different.

The absence of air-borne ascospores in this study was not unexpected since perithecia were not observed on any of the diseased grapevine materials collected in vineyards during the earlier sampling (Chapter 2). This was consistent with Smith *et al.* (2001) who reported from their study with eucalyptus that the teleomorphs of botryosphaeriaceous fungi were rarely encountered in nature. Michailides and Morgan (1992) also reported that perithecia of botryosphaeriaceous species have been rarely observed in perennial crops, including grapevines. However in apple and peach orchards, Sutton (1981) and Pusey (1989) respectively, reported that airborne ascospores

of *D. seriata*, *F. aesculi* and *L. theobromae* were observed during periods of wetness. Their studies used a Burkard volumetric spore trap, which may have been more effective than the Vaseline® -coated slides used in this study. However it is generally believed that conidia are the important and usually dominant form of dispersal and infection by the botryosphaeriaceous fungi (Michailides 1991; Ma *et al*., 2004; Burgess *et al*., 2005). In this study, the greater abundance of botryosphaeriaceous species conidia from December to February (summer) was probably related to the rise in temperature as well as there being a large canopy of mature canes at that time. Copes and Hendrix (2004) also observed that botryosphaeriaceous species were able to produce conidia over a wide temperature range of 6-30^{o}C, with optimum production occurring around 18-24^{o}C. The ambient daytime temperature in Canterbury is generally within the former range and this could explain why conidia were present throughout the year in Canterbury, including winter periods.

Amplification of a 350 bp product from DNA extracted from rainwater samples using a genus specific PCR primer pair indicated that botryosphaeriaceous species were present in the rainwater. Sequencing of these amplimers confirmed that they contained overlapping sequences of botryosphaeriaceous species. This is the first report of using SSCP to separate amplimers of botryosphaeriaceous species. The comparison with known standards indicated that three different botryosphaeriaceous species known to infect grapevines (*N. luteum, N. parvum N. australe* and *Diplodia* species*)* were present in the water samples throughout the year. In the spore trapping studies in California, Urbez-Torres *et al*. (2010b) identified five species using morphological methods (*B. dothidea, D. seriata, D. mutila, L. theobromae* and *N. parvum*) with the most abundant species that was trapped in all the vineyards being *D. seriata* (59%) and the least being *D. mutila* and *N. parvum* (1 and 5.6%, respectively).

The use of SSCP to distinguish fungal species present in mixed PCR amplimers has been demonstrated previously. Kumeda and Asao (1996) used the SSCP technique to differentiate species of *Aspergillus* section *flavi* and Szentiványi *et al*. (2005) also used it to show that the *Ampelomyces* mycoparasites collected from apple powdery mildew fell into a distinct SSCP group of genetically homogeneous isolates. The application of this technology to differentiate between botryosphaeriaceous species has provided a simple mechanism to distinguish species without the cumbersome and costly process of cloning and sequencing the PCR product from each sample. However, The SSCP was not able to discriminate between *N. parvum* and *N. ribis* which are closely related.

The *N. parvum* /*N. ribis* group appeared to be most numerous and present throughout most of the year in this study. Pérez *et al*. (2008) reported that *N. parvum* was found to have sporulated well on *Eucalyptus grandis* debris (dead tissue) and on stem cankers on *E. globulus*. Among all the botryosphaeriaceous species, *N. parvum* is also the only species found to have world-wide distribution on a wide range of woody hosts (Barber *et al.*, 2005; Burgess *et al.*, 2005; Gure *et al.*, 2005; Mohali *et al.*, 2007; Pavlic *et al*., 2009). It is also the only species with significant DNA sequence variation observed amongst isolates, which raises questions about population differentiation or even speciation in this group (Pavlic *et al*., 2009).

Microscopic analysis had indicated that both *Neofusicoccum* and *Diplodia* type conidia were present in the rainwater samples, however, the SSCP analysis had only identified the *Neofusicoccum* species known to infect grapevine. The unidentified *Diplodia* species were present in the water samples throughout the whole sampling period being represented by the unidentified novel band, whose sequence matched *D. olivarum, D. africana, D. mutila* and *D. cupressi*. These species have all been identified as trunk pathogens, with *D. africana* reported on *Prunus* species (plum, peach, nectarine and apricot) in South Africa (Damm *et al*. 2007), *D. olivarium* on olive in southern Italy (Lazzizera *et al.* 2008) and *D. cupressi* on *Cupressus sempervirens* (cypress) in Iran (Abdollahzadeh *et al*. 2009). However, *D. olivarum, D. africana* and *D. cupressi* were not found during the vineyard sampling of grapevine tissues (Chapter 2) and they have not been reported in New Zealand, although the above hosts are common in the same district as the vineyard where the rainwater samples were collected. Further molecular studies, as described by Alves *et al*. (2006), would need to be conducted to clarify the identity of these species.

With respect to the environmental parameters, factors such as temperature, humidity, light intensity, rainfall and wind are the critical climatic factors that can affect the propagation, dispersal and survival of fungi (Doohan *et al.*, 2003). Temperature and moisture (free water and RH), are the most frequently investigated parameters affecting conidial germination and have also been reported to affect conidial survival (Arauz and Sutton, 1989; Casals *et al.*, 2009). Findings from this study showed that the conidial germination of botryosphaeriaceous species was independent of light, since the germination rates under continuous darkness or light did not differ significantly. This could mean that the timing of conidial release is immaterial, as long as humidity levels are high enough for germination and wounds are present for infection. Although many

fungi require darkness for germination, a similar study by Kothari and Verma (1972) also observed that light and darkness did not affect germination of a powdery mildew fungus on poppy plants.

In this study conidia of the botryosphaeriaceous species germinated at a temperature range of 5-35^{o}C, which matched the ranges found for mycelial growth (Section 2.3.4), with an optimum of 25^{o}C. The very few (0.3%) *N. luteum* conidia germinating at 5^{o}C within 3 h and the 1.8-2.6% germination of all species by 24 h indicated that there could be some infection risks during winter from *N. luteum* when pruning provides wounds for infection. The normal maximum daily temperature during winter (10-15 ^{o}C) would also enhance germination. The observed temperature range for conidial germination was consistent with a report by Úrbez-Torres *et al*. (2010a) who studied 10 different botryosphaeriaceous species (*Botryosphaeria*, *Lasiodiplodia*, *Diplodia*, *Dothiorella*, *Spencermartinsia* and *Neofusicoccum* species) from the United States and Mexico, and reported germination occurring from 10 to 35^{o}C. None of the New Zealand isolates used in this study germinated at 40^{o}C; however Úrbez-Torres *et al.* (2010a) reported that conidia of *L. theobromae*, a species prevalent in warm and dry grape growing areas of California, could reach germination frequency of over 80% at 40^{o}C. In this study, the maximum time required for low or high temperature to be detrimental to conidial germination was not considered and should be considered in future experiments.

The survival of conidia exposed to sunlight is important in the build-up of viable inoculum, which can be transported repeatedly by rain or wind. In the present study, the exposure of *N. luteum*, *N. australe* and *D. mutila* conidia to sunlight reduced the viable proportion. Direct sunlight had a significant effect on all three species, as direct exposure of 7 h resulted in less than 35% germination and no germination after 56 h exposure (equivalent to 4 sunny days in summer). Conidia exposed to shade and light without UV radiation for 56 h had 82.2 and 57.3%, respectively, indicating that the losses in germinability of conidia were mainly due to the lethal effects of sunlight. The damaging effect of sunlight UV on *N. luteum, N. australe* and *D. mutila* conidia in this study is a first report for these species. Similar observations had also been made on the lethal effects of UV in sunlight on germination rates of *Puccinia striiformis* uredospores (Maddison and Manners, 1972), conidia of *Pyrenophora graminea* (Arabi *et al.*, 2009), *Mycosphaerella fijiensis* (Parnell *et al,.* 1998), *Alternaria solani*, *Alternaria macrospora, Peronospora tabaci*, *Uromyces phaseoli*, *Sclerotium rolfsii* and *Botrytis cinerea* (Rotem *et al.* 1985; Rotem and Aust 1991). The role of pigmentation in survival

spores, sclerotia, and pycnidia of fungi was discussed by Houssaini-Iraqui *et al*. (2001) who reported that pigmentation provided some tolerance to UV radiation by protecting them against its the damaging effects. In this study however, the conidia of *N. luteum N. australe* and *D mutila* were all hyaline.

The greater sunlight levels were also associated with longer periods of temperatures above 41°C, which is likely to have contributed to reduced conidium viability. For *Uncinula necator,* a grape powdery mildew with hyaline exposed structures, Willocquet *et al*. (1996) observed that, under two different temperature ranges 20-24°C and 26-31°C, solar radiation caused lower germination rates of conidia and mycelial growth at the highest temperature range 26-31°C. In contrast, Aylor and Sanogo (1997) observed that for *V. inaequalis* which has dark-coloured conidia, the difference in conidial germination of those exposed to sunlight (23.6–34.6°C) and the non-sunlight treatments (22 to 25°C) was not significant. Under outdoor sunny conditions, RH is also likely to be lower than in protected, shady areas. This study showed that 68% RH for 42-70 h greatly reduced conidial viability. The results from this study clearly indicate that after their release many conidia are likely to be killed by a combination of UV, high temperature and dry air although the conidia that land within shady areas or crevices may be long lived. Further studies should use an experimental set-up which also manages RH and temperature while monitoring conidial viability for combinations of these factors.

In this study, moisture was necessary for conidium exudation and germination. Maximum germination occurred at 100% RH and was much reduced at lower RH levels, being 48.9% after 24 h at 93% RH, with no germination at 84% RH even after 72 h at the optimum growth temperature of 25°C. Moisture availability is therefore important for release, survival and germination of botryosphaeriaceous species. This finding was in agreement with Arauz and Sutton (1989) who also observed that the maximum germination of *D. seriata* conidia occurred in free water and declined as RH was reduced from 100 to 92%, with no germination at 88.5% RH. Arauz and Sutton (1990) also observed that for *F. aesculi* optimum conidial germination occurred after 12 h at 26.7 to 29.5°C and 95-100% RH and declined with a further decrease in RH and temperature, with few conidia germinating in the absence of free water. The conidia of *Potebniamyces pyri*, a woody trunk pathogen, were reported to have even more stringent requirements since no germination occurred at a relative humidity of 95%, but did occur at 98% RH (Liu and Xiao, 2005).

The RH levels also affected the extent of mycelial growth after germination. At 100% RH, conidia germinated within 3 h and by 24 h had developed into mycelial networks whilst those kept at 93% RH germinated after 24 h but the germ tubes had not grown any longer by 48 h which indicated that a higher moisture level was necessary for mycelium growth and development compared with conidial germination. These germinated conidia which remained in a state of suspended growth could have been rewetted to observe if they could resume normal growth into mycelium. However, Arauz and Sutton (1989) reported that germinating conidia of *D. seriata* were very sensitive to drying and in no instance did the rewetted germ tubes resume growth following dryness periods as short as 1 h. However, in this study, the conidia incubated at 84% RH did not germinate although those exposed to 68% RH germinated when later re-incubated at 100% RH. This may have been due to the provision of enough moisture for germination of those from the 68% RH treatment. Although conidia incubated under 84% RH were not re-wetted, the results obtained for the 68% RH treatment suggest botryosphaeriaceous conidia could germinate after a short period of dryness when re-wetted although the percent germination could be very low.

The information produced in these experiments has several practical implications for viticulture. During winter pruning of grapevines the weather is likely to be conducive for conidial germination. Conidia can germinate quite quickly during the 10-15^{o}C day time temperatures and are likely to survive well during the rainy or cloudy conditions that provide the condition for their release and dispersal. However, further studies should be done to investigate the frequency of conidial germination during long periods at cooler winter temperatures. In summer, some parts of the grapevine canopy may provide sufficient shade and high RH to ensure rapid germination of conidia that can infect through the wounds created by summer trimming, which is usually performed several times during the season. Since cut green stems usually exude sap for several hours after pruning they provide some of the necessary moisture. However, field experiments are needed to investigate this mechanism of infection.

In conclusion, the abundance of *N. parvum* and presence of *N. australe* and *N. luteum* from the sampling results (Chapter 2) in grapevines and other hosts was reflected by presence of their conidia trapped in rain water throughout the whole year. These pathogens are clearly well adapted to the climate and current management practices within vineyards. This has significant implications for vineyard management decisions. The major role played by temperature, UV and RH in the viability of

botryosphaeriaceous species conidia indicates the need for open canopies. The observation of dieback after summer pruning of grapevine canes and the susceptibility of all shoot tissue (Chapter 3) suggest the need to apply pruning wound treatment irrespective of the season. Therefore Chapter 5 will focus on investigating the efficacy of some selected fungicides on both mycelial growth and conidial germination *in vitro* and *in vivo* experiments on grapevines.

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Chapter 5
Evaluation of fungicides for the management of botryosphaeriaceous dieback diseases in grapevines

5.1 Introduction

Grapevine trunk diseases are caused by several species of *Phaeoacremonium Diatrypaceae, Eutypa lata, Phaeomoniella chlamydospora* and the botryosphaeriaceous species that infect through wounds resulting in canker formation and dieback. Protection of pruning wounds against these pathogens is therefore essential for effective prevention of these diseases (Bester *et al.*, 2007). Fungicides such as carbendazim, pyrimethanil and pyraclostrobin have been reported to provide wound protection against *E. lata* (Sosnowski *et al.*, 2004). Fungicides such as, fenarimol, tebuconazole, prochloraz and benomyl were reported by Groenewald *et al.* (2000) to inhibit mycelial growth of *P. chlamydospora* even when applied at low concentrations, while Jaspers (2001) also reported that pyrimethanil and a mixture of cyprodinil and fludioxonil were effective against mycelium growth but a high EC_{50} value of 34.1 mg/litre was required to effectively inhibit conidial germination of *Pa. chlamydospora.*

For the botryosphaeriaceous fungi, *D. seriata* and *N. luteum*, Savocchia *et al.* (2005) demonstrated the *in vitro* efficacy of tebuconazole, flusilazole, spiroxamine and fluazinam against mycelial growth. A more recent publication by Pitt *et al*. (2010b) reported that fungicides such as tebuconazole, flusilazole, carbendazim, fludioxonil, fluazinam, penconazole, procymidone and iprodione were able to inhibit *in vitro* mycelial growth of *D. seriata*, *N. parvum*, *L. theobromae* and *B. dothidea.* In a further field trial assessing the potential of these fungicides as spray fomulations for protecting pruning wounds in the Hunter Valley in Australia, they observed that carbendazim, flusilazole, cyprodinil+fludioxonil, fluazinam, iprodione and tebuconazole significantly reduced the mean percent recovery of *D. seriata* when applied at rates much higher than those recommended for use to control other grapevine diseases. However, in the Barossa Valley trial in Australia, Pitt *et al*. (2010b) reported that carbendazim, flusilazole, tebuconazole and fluazinam provided 30-55% protection of pruning wounds when applied at manufacturers' recommended label rates. In South Africa, Bester *et al*. (2007) also reported that the incidence of *D. seriata* in inoculated shoots declined from relatively high levels in water-treated shoots (39.4%) to low levels in those treated with

prochloraz, tebuconzole and benomyl, being 12, 7.7 and 1%, respectively. Rolshausen *et al*. (2010) also reported that thiophanate methyl was the most effective fungicide for protecting pruning wounds against infection by botryosphaeriaceous species such as *B. dothidea, D. seriata, Dothiorella viticola,* and *L. theobromae* which are known to cause grapevine trunk diseases in the United States. Although botryosphaeriaceous species are known to be major pathogens of grapevines worldwide, there are currently no registered fungicides for use against these pathogens (Pitt *et al*., 2010b).

In New Zealand, no fungicides have been evaluated against botryosphaeriaceous species. However, they have the potential to protect grapevines in a cost-effective way when applied directly or by spraying the canopies. In view of this, 16 fungicides were selected, which belonged to different chemical groups and were currently being used to control other fungal pathogens on grapevines and other fruit tree hosts. To provide a good understanding of their potential effects, the fungicides were evaluated for their ability to (i) reduce *in vitro* mycelial growth of the most common pathogenic species of botryosphaeriaceous species [*N. australe, N. luteum N. parvum* and *D. mutila*], (ii) *in vitro* conidial germination of the same species and (iii) for their ability to protect grapevine wounds against *N. luteum* infection.

5.2 Materials and Methods

5.2.1 Effect of fungicides on *in vitro* mycelial growth

Sixteen fungicides belonging to different chemical families (Table 5.1) were tested *in vitro* to evaluate their effect on mycelial growth inhibition of three pathogenic isolates each of *N. australe* [Kat-1, Mel-2 and J-3], *N. luteum* [G(s)-1, N (12)2 and M (13)8] and *D. mutila* [Iso-2, F (12)2 and Q]. *Neofusicoccum parvum* was excluded because the preliminary mycelial growth study showed it was the most sensitive to all the fungicides tested at the field rate and so would likely be controlled by the fungicides that would be effective against the other species. In addition, the difficulty in producing adequate *N. parvum* conidia was expected to be problematic.

Table 5.1: Fungicides tested against mycelial growth and conidial germination of *N. luteum*, *N. australe* and *D. mutila*.

Active ingredient	Trade name	Chemical class	Conc. Range (mg a.i/L)	Manufacturer
Azoxystrobin**	Amistar WG	Strobilurin	1 - 1000	Syngenta Corp Protection Ltd
Carbendazim*	Protek™	Benzimidazole	0.1 - 2	Tapuae partnership, NZ
Chlorothalonil**	Barrachlor™	Benzonitrile	1 - 50	Tapuae partnership, NZ
Copper hydroxide**	Champ™ DP	Inorganics	10 - 100	Nufarm Ltd
Dithianon**	Delan® WG	Nitrile	10 - 100	BASF New Zealand LTD
Fenarimol**	Rubigan® Flo	DMI -Pyrimidines	0.1 - 10	DuPont NZ
Flusilazole*	Nustar®	DMI-Triazole	0.001 - 1	DuPont NZ
Iprodione**	Rovral®Flo	Dicarboximides	0.1 - 5	Bayer NZ Ltd
Mancozeb **	Manzate® 200DF	Dithiocarbamates	2.5 - 50	DuPont NZ
Metalaxyl*	Speartek™	Phenylamides	15 - 60	Tapuae partnership, NZ
Prochloraz*	Mirage® 450	DMI-Imidazole	0.5 - 5	Agronica NZ Ltd
Procymidone**	Sumisclex® 500SC	Dicarboximides	0.1 - 5	Nufarm Ltd
Pyrimethanil**	Scala®	Anilopyrimidine	1 - 1000	Bayer NZ Ltd
Tebuconazole*	Hornet™ 430SC	DMI -Triazole	0.01 - 5	Nufarm Ltd
Thiophanate methyl**	Topsin M-4A	Benzimidazole	0.1 - 10	Dow Agro Science (Ltd) NZ
Triforine*	Saprol®	DMI-Piperazine	15 - 100	BASF New Zealand LTD

* Not registered for grapes in New Zealand
** Registered for grapes in New Zealand for controlling botrytis, powdery mildew, downy mildew or black spot diseases (Sunde, 2010).

Each of the fungicides tested was suspended in sterile water and added to molten PDA at a temperature of 50°C and poured into 90 mm Petri dishes. The test range of the active ingredient (a.i) concentrations varied from 0.001 to 1000 mg a.i. /L (Table 5.1) based on the reported activity range for each product. Each fungicide was tested at six different concentrations and control plates were composed of only PDA. Each plate was inoculated centrally with a 5 mm diameter mycelial disc. There were three replicate plates for each combination of fungicide concentration and isolate, and the plates were laid out in a CRD and incubated at 25°C in continuous darkness for 48 h, after which the mycelial growth were measured with a digital calliper across two perpendicular diameters of each colony and the mean mycelial growth inhibition calculated as a percentage of the control. The percent inhibition was calculated for each isolate and fungicide concentration and its EC_{50} determined using the probit as described in Section 5.2.4. The experiment was conducted twice.

5.2.2 Effect of fungicides on *in vitro* conidial germination

Based on the result of the *in vitro* mycelial inhibition study, 14 fungicides were selected and each mixed with sterile water to two times the designated concentrations. The same species and isolates as used in Section 5.2.1 were used to produce conidia on green grapevine shoots as described in Section 2.2.3.2 and their concentrations were adjusted to 10^4 conidial/mL using a haemocytometer. For each botryosphaeriaceous species the conidia from the three isolates were mixed in equal proportions. The conidium suspension (150 µL) was then mixed with 150 µL of each designated fungicide solution. For the control treatments (no fungicides) the conidium suspension was mixed with an equal amount of sterile water. Three separate drops, each containing 50 µL of fungicide/conidial suspension were placed separately onto three replicate glass slides. Each slide was then placed on a wet filter paper placed in a square plastic box (10 x 10 x 2 cm) and covered with its lid to maintain high RH. The slides in the plastic boxes were arranged in a CRD and were then incubated at 25^{o}C for 24 h in continuous darkness. Germination of 100 randomly selected conidia in each droplet was observed under a light microscope at ×40 and germination frequencies assessed. A conidium was considered germinated if the length of the germ tube exceeded half the length of the conidium. From the mean percent germination, relative to the no fungicide controls, the percent inhibition of germination was calculated for each fungicide/conidial suspension, and the EC_{50} determined as described in Section 5.2.4.

5.2.3 Evaluation of fungicides on *N. luteum* conidial infection of grapevine stem tissues

Based on the ability of these fungicides to inhibit conidial germination and mycelial growth, 12 were selected to evaluate their efficacy at preventing *N. luteum* infection of Pinot noir detached green shoots and pruned canes of 2-year-old potted Pinot noir grapevines. *Neofusicoccum luteum* was selected because it was generally the least sensitive to the fungicides. Eight fungicides (carbendazim, fenarimol, flusilazole, iprodione, prochloraz, procymidone, thiophanate methyl and tebuconazole) were selected based on their effectiveness at inhibiting mycelium growth (Section 5.2.1) and another four fungicides (chlorothalonil, copper hydroxide, dithianon and mancozeb) were selected based on their ability to only inhibit conidial germination (Section 5.2.2).

5.2.3.1 In vitro *detached green shoot assay*

Green shoots (var Pinot noir) were set up in Universal bottles filled with sterile water to prevent dehydration as described in Section 2.2.3.2. The top part of each shoot was cut

off using a sterile scalpel to mimic a trimming wound and immediately the wounds on five replicate shoots were sprayed with the selected fungicide applied at the recommended field rate and left to dry. After drying, the fungicide treated wounds were each inoculated with a drop (20 µL) of a mixed isolate *N. luteum* conidial suspension (10^4/mL), prepared with the isolates listed in Section 5.2.2 and by the method described in Section 2.2.3.2. Control shoots were initially sprayed with sterile water prior to inoculation with the conidial suspension. The shoots were arranged in a CRD and incubated at room temperature (18-21^{o}C) in a transparent chamber with frequent misting for the first 3 days as described in Section 2.2.3.2. The plants were then assessed by measuring the dieback length with a digital calliper at 10 days after inoculation and data were analysed with ANOVA. Pathogen isolation was also carried out in each shoot by surface sterilising with 70% alcohol for 1 min, rinsed twice in clean sterile tap water and air dried under a laminar flow hood for 30 min before cutting five discs at 10 mm intervals from the pruned end down to 50 mm, and placing each disc section sequentially onto PDA. Plates were incubated at room temperature (18-21^{o}C) for 3 days to allow for growth of fungal colonies characteristic of *N. luteum* and to determine incidence and the distance moved by the pathogen.

5.2.3.2 In vivo *potted grapevine plants assay*

Canes of 2-year-old potted Pinot noir grapevine plants growing in potting mix and maintained as described in Section 2.4.4 were pruned to two canes per plant using ethanol sterilised secateurs and immediately sprayed to run off with each of the selected fungicides at the manufacturer's recommended field rate (Appendix E.1). Twenty four hours after the fungicide applications, each pruning wound was drop-inoculated (40 µL) with a fresh conidium suspension of the same mixed isolates of *N. luteum* as before, but at 10^5 conidial/ mL. Two control treatments (positive and negative) were used. The positive control plants (no fungicide treatment) were sprayed to run off with sterile water before inoculating with *N. luteum*, and negative control plants (no fungicide and no *N. luteum* conidial inoculation) were drop inoculated with sterile water only. There were five replicate plants with two inoculated canes per plant for each fungicide treatment. The plants were arranged in a CRBD in an open environment that simulated field conditions.

At 3 months after inoculations, the canes were assessed by measuring the length of the dieback lesions with a calliper as described in Section 5.2.3.1 to determine the effects of the different fungicide treatments. After surface sterilization as described in Section

2.2.2, pathogen isolation was also carried out on all treated canes and the controls by cutting five discs at 10 mm intervals from each pruned end down to 50 mm and isolation conducted as described in Section 5.2.3.1, to determine the incidence of *N. luteum* and the distance moved through the canes, and data were analysed as described in Section 5.2.4.

5.2.3.3 Field evaluation of fungicides on mature vines

Due to the limited availability of field grown grapevines, only nine fungicides (carbendazim, fenarimol, flusilazole, iprodione, mancozeb, prochloraz, procymidone, tebuconazole and thiophanate methyl), which were effective in the previous experiments, were selected for pruning wound treatment in the field. The 1-year-old canes of grapevine var. Chardonnay were pruned with ethanol sterilised secateurs to 10-12 canes per plant in late winter of 2008. Six canes per grapevine plant, each 12-15 cm long with fresh pruning wounds, were tagged and immediately sprayed to run-off with 10 mL of each selected fungicide, applied at the recommended field rate (Appendix E.1). Twenty four hours after the fungicide applications, a mixed isolate *N. luteum* conidial suspension (10^5 /mL) obtained as described before, was used to drop-inoculate (100 µL per wound) the fungicide treated pruning wounds. Positive control plants with pruned canes were sprayed to run-off with sterile water (as the treatment) and subsequently drop-inoculated with the same *N. luteum* conidial suspension after 24 h. No negative control was used due to the limited number of the field grown plants available. There were five rows of grapevine plants representing the replication blocks and each row had 10 grapevine plants, one for each fungicide treatment, and treatments were randomly allocated.

Six months after inoculation, all six inoculated canes on each treated plant (including the controls) were cut off and observed for dieback symptoms in the laboratory. Pathogen isolation was then carried out on all six canes for each treatment and replicate. They were first surface sterilized in 70% alcohol for 1 min, followed by rinsing twice in sterile water and air drying under the laminar flow hood for 45 min, before cutting 5 mm thick segments from: (a) within the dieback lesion area close to the inoculation point, (b) the dieback lesion edge (2.5 mm above and below the visible lesion edge), (c) non-lesion area at 10 mm away from the lesion edge, (d) the closest side shoot, at 5 mm up from the cane junction and (e) lesion or non-lesion area 5 mm below the node of the closest side shoot of each cane (Figure 5.1). The pieces were placed cut surface down onto PDA and incubated at room temperature (18-21^{o}C) for 3 days as described before, and the

incidence of fungal colonies characteristic of *N. luteum* was recorded and data analysed as described in Section 5.2.4. Lesion lengths were not measured in this experiment because in the previous potted vine experiment, both the inoculated and non-inoculated canes (controls) had some dieback which was not necessarily due to *N. luteum* infection.

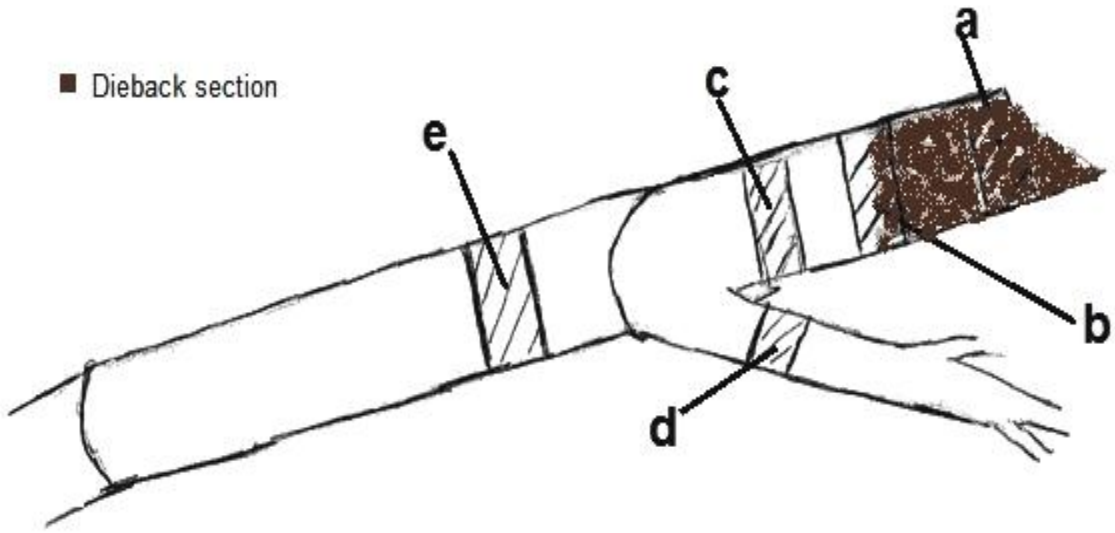

Figure 5.1: A hand sketch of a grapevine cane showing the different isolation sites (a) the dieback lesion area, (b) the dieback lesion edge, (c) the non-lesion area at 10 mm from the lesion edge, (d) the closest side shoot at 5 mm from the cane and (e) the lesion or non-lesion area 5 mm below the node of the closest side shoot.

5.2.4 Statistical analysis and determination of EC_{50} values for the selected fungicides

The dieback lengths measured were analysed with ANOVA and means separated with Fisher's protected LSD at $P \leq 0.05$. The EC_{50} values for both the conidial germination and mycelial growth were calculated for each fungicide/isolate combination using the probit analysis option within the Generalised Linear Model (GenStat version 12). The EC_{50} values obtained for the individual isolates and species were analysed with ANOVA (GenStat version 12) to determine fungicide and species effects, using isolate means as the replicate data, and means were separated using Fisher's protected LSD at $P \leq 0.05$. For the conidial germination, EC_{50} values for the species were obtained from mixed isolates and could not provide mean isolate replicates for analysis. No LSDs were

available for this method, so the data were presented with 95 percent confidence intervals.

5.3 Results

5.3.1 Effect of fungicide on *in vitro* mycelial growth

The two experiments did not differ significantly ($P>0.05$) so the data were combined for a second analysis. Table 5.2 shows the EC_{50} values (mg a.i/L) for mycelial growth inhibition caused by the various fungicides for the nine different isolates of the botryosphaeriaceous species. Because of the high sensitivity of some isolates to certain fungicides, it was not possible to determine their EC_{50} values as the minimum fungicide concentrations tested were highly effective at inhibiting mycelial growth, with no growth occurring in those cases (Table 5.2). On pyrimethanil and azoxystrobin amended PDA, the mycelial growth of all nine isolates, tested at the highest concentration were not different from the mycelial growth observed on control PDA plates and these fungicides were therefore excluded from the probit analysis which determined the EC_{50} values. They were also not used to test their effectiveness on conidial germination inhibition.

The EC_{50} values of the fungicides differed significantly ($P<0.001$; Appendix E.2) for all three botryosphaeriaceous species. The EC_{50} values for flusilazole, carbendazim, tebuconazole, prochloraz, procymidone, iprodione, fenarimol, thiophanate methyl, chlorothalonil and mancozeb did not differ significantly ($P>0.05$) from each other although the first eight were most effective on mycelial growth reduction, with EC_{50} values less than 1 mg/L active ingredient (Table 5.3). However the effects of triforine, dithianon, copper hydroxide and metalaxly differed significantly from each other, the least effective fungicide being metalaxyl. There was also a significant species effect ($P<0.001$; Appendix E.2) with *N. australe* and *N. luteum* being similar ($P>0.05$), and both differing significantly from *D. mutila*.

Table 5.2 The EC_{50} values of active ingredient concentrations (mg/L) obtained for fungicides tested *in vitro* for mycelial growth inhibition of nine botryosphaeriaceous species isolates.

Fungicides (a.i)	Isolates								
	Neofusicoccum luteum			*Neofusicoccum australe*			*Diplodia mutila*		
	G(s)-1	**N(12)2**	**M(13)2**	**Kat-1**	**Mel-2**	**J-3**	**F(12)2**	**Iso-2**	Q
Carbendazim	0.062	0.058	0.062	*	0.013	0.032	*	*	*
Chlorothalonil	10.21	4.53	8.88	5.29	3.52	2.73	0.243	0.003	0.529
Copper hydroxide	98.90	63.74	69.67	168.0	60.36	92.13	70.86	75.66	59.40
Dithianon	104.4	53.49	62.34	57.90	62.35	56.44	42.88	44.06	47.67
Fenarimol	0.393	0.409	0.396	0.339	0.674	0.511	0.039	0.067	0.123
Flusilazole	0.001	0.005	0.004	0.002	0.001	0.002	< 0.0001	< 0.0001	< 0.0001
Iprodione	0.390	0.312	0.239	0.317	0.224	0.277	0.207	0.277	0.257
Mancozeb	5.25	5.95	5.88	5.94	4.35	5.43	2.79	2.24	3.20
Metalaxyl	187.6	188.4	*	135.0	120.1	52.70	*	64.62	59.37
Prochloraz	*	0.058	0.049	0.087	0.116	0.091	0.001	*	*
Procymidone	0.256	0.219	0.214	0.194	0.176	0.184	0.256	0.170	0.254
Tebuconazole	0.069	0.061	0.099	0.083	0.115	0.096	0.011	0.008	0.011
Thiophanate methyl	1.169	1.333	1.210	0.807	1.225	1.204	0.522	0.442	0.491
Triforine	12.46	32.67	26.06	12.96	36.60	37.18	*	0.039	*

*EC_{50} unable to be determined as the minimum active ingredient concentration used resulted in no mycelial growth.

There was also a significant (P<0.001; Appendix E.2) interaction between the species and the fungicides which was mostly associated with the less effective fungicides and the different EC_{50} values of *D. mutila*, such as with triforine and metalaxyl, when its EC_{50} value was much lower, and with dithianon and copper hydroxide when it was similar to the EC_{50} of one of the other two species (Table 5.3).

Table 5.3: The mean EC_{50} (mg a.i/ L) values for different fungicides for *in vitro* inhibition of mycelial growth of three botryosphaeriaceous species.

Fungicides	**botryosphaeriaceous species**			**Fungicides**
	N. australe	***N. luteum***	***D. mutila***	**mean EC_{50}**
Flusilazole	0.0	0.0	0.0	0.0 a
Carbendazim	0.0	0.1	0.1	0.1 a
Tebuconazole	0.1	0.1	0.0	0.1 a
Prochloraz	0.1	0.1	0.1	0.1 a
Procymidone	0.2	0.2	0.2	0.2 a
Iprodione	0.3	0.3	0.2	0.3 a
Fenarimol	0.5	0.4	0.1	0.3 a
Thiophanate methyl	1.1	1.2	0.5	0.9 a
Chlorothalonil	3.8	7.9	0.3	4.0 a
Mancozeb	5.2	5.7	2.7	4.6 a
Triforine	28.9	23.7	0.1	17.6 b
Dithianon	58.9	73.4	44.9	59.1 c
Copper hydroxide	106.8	77.4	68.6	84.3 d
Metalaxyl	102.6	188.0	77.0	122.5 e
Species mean effect	**22.0 g**	**27.0 g**	**13.9 f**	

[1]Values within the rows and columns followed by the same letter are not significantly different according to Fisher's protected LSD at $P \leq 0.05$. Fungicide main effect (a-e) was significant (P<0.001; LSD=12.72). The main effect of species (f-g) was significant (P<0.001; LSD=5.89) and the species x fungicides effect was also significant (P<0.001; LSD=22.03).

5.3.2 Effect of fungicides on *in vitro* conidial germination

The EC_{50} values of the fungicides tested differed significantly (P<0.001; Appendix E.3) for all three botryosphaeriaceous species in reduction of conidial germination. Procymidone, carbendazim and iprodione were the most effective and did not differ significantly (P>0.05) from each other. This was followed by mancozeb and flusilazole (Table 5.4). The least effective fungicides were metalaxyl and triforine, which had similar (P>0.05) mean germination for the three botryosphaeriaceous species with mean EC_{50} values of 125.6 and 111.9 mg/L active ingredients, respectively (Table 5.4).

There was also a significant difference (P<0.001; Appendix E.3) between all three species with *N. luteum* being the least sensitive followed by *N. australe* and the most sensitive being *D. mutila.* The interaction between the fungicides tested and the three botryosphaeriaceous species was also significant (P< 0.001; Appendix E.3) the interaction mostly being associated with the less effective fungicides and *D. mutila.*

However *N. australe* had the lowest EC_{50} value of all species for prochloraz and the highest value for fenarimol (Table 5.4).

Table 5.4: The mean EC_{50} (mg a.i/ L) values for different fungicides for *in vitro* conidial germination inhibition of the three botryosphaeriaceous species.

Fungicides	Botryosphaeriaceous species		
	N. australe	***N. luteum***	***D. mutila***
Procymidone	0.01 (0.003-0.018)[1]	0.02 (0.005-0.043)[1]	0.00 (0.000-0.007)[1]
Carbendazim	0.01 (0.012-0.018)	0.04 (0.041-0.047)	0.01 (0.005-0.010)
Iprodione	0.05 (0.027-0.073)	0.04 (0.018-0.062)	0.03 (0.001-0.065)
Mancozeb	0.48 (0.287-0.670)	1.61 (1.137-2.087)	0.25 (0.072-0.433)
Flusilazole	1.34 (1.136-1.539)	1.23 (0.985-1.482)	0.56 (0.427-0.687)
Thiophanate methyl	5.09 (4.417-5.765)	2.41 (1.584-3.241)	0.29 (0.024-0.556)
Tebuconazole	3.38 (2.595-5.031)	6.23 (4.043-8.407)	0.60 (0.297-0.902)
Copper hydroxide	5.68 (5.272-6.083)	8.70 (8.303-9.096)	4.16 (3.433-4.894)
Chlorothalonil	10.79 (9.454-12.116)	11.15 (10.102-12.192)	1.35 (0.444-2.255)
Fenarimol	22.50 (11.539-33.471)	7.44 (5.270-9.620)	0.48 (0.326-0.633)
Dithianon	19.10 (15.251-22.939)	19.11 (18.625-19.587)	11.39 (7.934-14.848)
Prochloraz	13.23 (11.566-14.900)	46.86 (36.818-56.907)	22.97 (19.094-26.836)
Triforine	126.30 (105.646-146.947)	159.90 132.294-187.500)	49.59 (43.825-55.360)
Metalaxyl	113.11 (87.907-138.308)	218.01 (188.768-247.254)	45.72 (32.676-58.759)

[1]For each column, the confidence intervals for species and fungicides are provided in brackets.

5.3.3 Effect of fungicides on infection of grapevine stem tissues

5.3.3.1 In vitro *detached green shoot assay*

The *in vitro* fungicide effect on the detached green shoot wounds inoculated with *N. luteum* conidia was highly significant ($P<0.001$; Appendix E.4) with respect to dieback development. All fungicides tested significantly reduced the length of the dieback lesions compared with the untreated control, for which the mean lesion length was 57.7 mm (Table 5.5). Carbendazim, chlorothalonil, fenarimol, iprodione, mancozeb, procholraz and tebuconazole treated green shoot wounds showed no dieback (Table 5.5). Copper hydroxide was least effective, with a mean dieback length of 25.9 mm. The fungicides which totally prevented dieback also prevented *N. luteum* isolation completely. The fungicides also differed ($P<0.001$; Appendix E.5) with respect to percentage isolation of *N. luteum* from lesion edges. All inoculated control shoots, and those treated with dithianon and copper hydroxide yielded 100% fungal colonies characteristic of *N. luteum* although the lengths of the dieback lesions were much less than for the control (Table 5.5).

Table 5.5: Effect of fungicide application on mean lengths of dieback lesions (mm) and *N. luteum* incidence (%) 10 days after detached green shoots were inoculated with *N. luteum* conidia.

Fungicides	Dieback lesion length (mm)	*N. luteum* isolated from shoots (%)
Carbendazim	0.0 a	0 e
Chlorothalonil	0.0 a	0 e
Fenarimol	0.0 a	0 e
Iprodione	0.0 a	0 e
Mancozeb	0.0 a	0 e
Prochloraz	0.0 a	0 e
Tebuconazole	0.0 a	0 e
Procymidone	0.8 a	20 f
Thiophanate methyl	1.0 a	20 f
Flusilazole	1.2 a	20 f
Dithianon	4.9 b	100 g
Copper hydroxide	25.9 c	100 g
Control	57.7 d	100 g
LSD	**1.99**	**6.8**

[1]Values within the rows and columns followed by the same letter are not significantly different according to Fisher's protected LSD at $P \leq 0.05$.

5.3.3.2 In vivo *potted vine assay*

The effect of the fungicides on dieback development was also significant ($P<0.001$; Appendix E.6) when used for wound treatment of 2-year-old canes of potted grapevines (var Pinot noir). All fungicides significantly decreased the lengths of dieback lesions compared with both the negative and positive controls which were similar ($P>0.05$), with mean lesion lengths of 114.4 and 94.3 mm, respectively (Table 5.6). Although the most effective fungicides carbendazim, mancozeb, iprodione and flusilazole had lesion lengths similar to many of the other fungicide treatments, they differed significantly in percent pathogen isolations, ($P<0.001$; Appendix E.7) because they were 0% compared to ≥20% for the other fungicides. Saprophytic fungi, such as *Alternaria*, *Epicoccum*, *Fusarium* and *Ulocladium* species were isolated from the negative controls and from all fungicide treated canes, including the carbendazim, mancozeb, iprodione and flusilazole treatments.

Table 5.6: Effect of fungicide application on mean lengths of dieback lesions (mm) and *N. luteum* incidence (%) 3 months after pruned canes of 2-year-old Pinot noir grapevines were inoculated with *N. luteum* conidia.

Treatments	Dieback lesion length (mm)	*N. luteum* isolated from canes (%)
Carbendazim	33.2 e	0 f
Mancozeb	39.9 de	0 f
Iprodione	39.9 de	0 f
Flusilazole	41.4 cde	0 f
Copper hydroxide	41.9 cde	20 g
Procymidone	47.8 cd	20 g
Dithianon	49.6 bcd	30 g
Tebuconazole	50.2 bcd	20 g
Chlorothalonil	51.9 bcd	30 g
Fenarimol	52.8 bc	20 g
Thiophanate methyl	53.3 bc	30 g
Prochloraz	60.5 b	70 h
+ve control (+ *N. luteum*)	114.4 a	100 i
-ve control (- *N. luteum*)	94.3 a	0 f
LSD	**12.6**	**16.3**

[1]Values within the rows and columns followed by the same letter are not significantly different according to Fisher's protected LSD at $P \leq 0.05$.

5.3.3.3 Field evaluation of fungicides on mature vines

At 6 months after the treatments and inoculations, fungicide treatments had significantly affected *N. luteum* recovery from all the various sites at which isolation was attempted ($P < 0.001$ for all; Appendix E.8) except beyond the second node, at which the control only had 7% and other treatments had 0%. Isolation frequencies of *N. luteum* differed ($P<0.001$) between the different sections of the canes, with mean percent recovery being 58% from dieback lesions, 39% from the dieback lesion edges, 22% from the non-lesion areas and 5% from the closest side shoots (Table 5.7).

The mean isolation incidences of *N. luteum* from the control plants (52%) differed significantly ($P<0.001$) between fungicide treatments. The most effective fungicide was flusilazole, with 0% reisolation of *N. luteum.* This was followed by carbendazim and tebuconazole with 9 and 16% *N. luteum* recovery, respectively. There was also a significant interaction ($P<0.001$) between treatments and the different sites from which *N. luteum* was recovered (Table 5.7).

Table 5.7: The effects of fungicides on the mean incidence (%) of *N. luteum* isolations made from top-pruned 1-year-old canes and side shoots of mature grapevines 6 months after fungicide treatment and inoculation.

Treatments	Isolation sites					
	Dieback	**Dieback edges**	**Non lesion areas**	**Closest side shoot**	**Area beyond second node**	**Mean isolation incidence**
Flusilazole	0	0	0	0	0	0 g[1]
Carbendazim	27	10	7	3	0	9 f
Tebuconazole	37	27	13	3	0	16 ef
Thiophanate methyl	47	33	17	3	0	20 de
Mancozeb	47	37	20	3	0	21 de
Fenarimol	77	40	20	0	0	27 cd
Procymidone	70	40	20	7	0	27 cd
Iprodione	83	53	30	3	0	34 c
Prochloraz	97	67	40	13	0	43 b
Control	100	87	50	17	7	52 a
Isolation sites effect	58 h[1]	39 i	22 j	5 k	1 k	

[1]Values within the rows and columns followed by the same letter are not significantly different according to Fisher's protected LSD at $P \leq 0.05$. Treatment effect (a-g) was significant ($P<0.001$; LSD=8.0); individual isolation sites (h-k) were also significant ($P<0.001$; LSD=5.0) and treatment x isolation sites interaction was also significant ($P<0.001$; LSD=17.0).

5.4 Discussion

This study has provided an evaluation of a wide range of fungicides on conidial germination and mycelial growth reduction of different isolates of three botryosphaeriaceous species. In a preliminary study conducted before the commencement of the fungicide evaluation experiments, visual observation showed that mycelial growth of *N. parvum* was more sensitive to a range of fungicides such as fenarimol, procymidone, iprodione, tebuconazole, prochloraz, flusilazole, chlorothalonil and mancozeb compared with *N. australe*, *N. luteum* and *D. mutila*. This agrees with the report by Bester *et al.* (2007) who also observed that *N. parvum* was the most sensitive to the majority of the fungicides tested in their study. Due to the high sensitivity of *N. parvum* to most of the fungicides, it was not tested further in the subsequent experiments, as any fungicide which inhibited the less sensitive species (*N. luteum*, *N. australe* and *D. mutila*) were considered likely to inhibit *N. parvum* infection. In addition, sufficient conidia of *N. parvum* could not be produced after many attempts, so the effect of fungicides on the conidial germination on this pathogen could not be assessed.

This study presents the first report on the *in vitro* efficacy of selected fungicides belonging to different chemical classes to inhibit conidial germination and mycelial growth of different isolates of *N. australe*, *N. luteum* and *D. mutila.* Carbendazim, flusilazole, iprodione and procymidone were found to be equally effective at inhibiting both conidial germination and mycelial growth while, thiophanate methyl was slightly less effective but its effect on *D. mutila* did not differ from some of the more effective fungicides. Mancozeb was also consistently effective at inhibiting conidial germination of *N. australe*, *N. luteum* and *D. mutila*, with EC_{50} values not exceeding 2 mg/L active ingredient. Li *et al.* (1995) observed that carbendazim and thiophanate methyl consistently inhibited conidial germination of *B. dothidea* and *L. theobromae* and therefore recommended their use for controlling dieback disease development of apricot trees in Japan. The efficacy of benzimidazoles has also been reported for other fungal pathogens. Thiophanate methyl was reported as a potent inhibitor of both mycelial growth and conidial germination of *Dactylaria higginsii* (Yandoc *et al*., 2006), while Obanor *et al*. (2005) reported that carbendazim was very effective at inhibiting conidial germination of *Spilocaea oleagina* which causes olive leaf spot.

In this study, *in vitro* mycelial growth was significantly inhibited by nine out of the 14 fungicides tested (carbendazim, flusilazole, thiophanate methyl, tebuconazole, iprodione, fenarimol, procymidone, mancozeb, and chlorothalonil), which were equally effective against the three botryosphaeriaceous species. Denman *et al*. (2004) also reported that tebuconazole, prochloraz, iprodione and fenarimol were the most effective at inhibiting mycelial growth of *N. protearum*. In another study evaluating the effect of 14 fungicides belonging to different chemical classes, Luque *et al.* (2008) observed that carbendazim and thiophanate methyl were the most effective fungicides at inhibiting mycelial growth of *D. corticola* isolated from oak tree. Savocchia *et al*. (2005) also reported tebuconazole as being one of the most effective fungicides inhibiting *in vitro* mycelial growth of *D. seriata* and *N. luteum* isolated from grapevines in Australia. In Australia, Pitt *et al*. (2010b) also tested 17 technical-grade fungicides registered for use on grapevines *in vitro* for their ability to inhibit mycelial growth of *D. seriata*, *N. parvum*, *L. theobromae* and *B. dothidea.* In subsequent field trials, they reported that carbendazim, tebuconazole, procymidone, iprodione and flusilazole were the most effective fungicides for reducing *D. seriata* infection. All these findings by the various researchers are consistent with the results obtained for *N. luteum*, *N. australe* and *D. mutila* in this study.

Most fungicides used in this study had relatively consistent effects in reducing both mycelial growth and conidial germination of *N. luteum*, *N. australe* and *D. mutila*. However copper hydroxide, mancozeb and dithianon were much more effective at reducing conidial germination than mycelial growth. The higher efficacy of copper hydroxide on conidial germination than mycelial growth reduction as observed in this study also agrees with the results by Everett and Timudo-Torrevilla (2007) who observed that four different formulations of copper hydroxide (Kocide® DF 2000, Kocide® 3000, Cuprofix® Disperss® and Champ™ DP) were able to cause greater inhibition of conidial germination of *N. parvum* and *B. dothidea* than of mycelial growth of the same species. In New Zealand, the concentration of copper hydroxide recommended for field use for controlling postharvest rots of avocados is in the order of 1000 mg/L (Rolshausen *et al*., 2010). This concentration is well in excess of the concentration of copper hydroxide (Champ™ DP) that inhibited conidial germination of *N. luteum*, *N. australe* and *D. mutila* in this study.

Overall, the conidial germination or mycelial growth of *N. luteum* was found to be the least affected by all the fungicides tested, followed by *N. australe* and then *D. mutila*. However, for the less effective fungicides there were some clear differences between some species and isolates based on the EC_{50} values calculated for the *in vitro* studies (Table 5.2). The mycelial growth study, using a range of active ingredient concentrations, demonstrated that some isolates of the same species varied in their sensitivity to the same fungicide. For example it was observed that high concentrations of metalaxyl were required to inhibit the mycelial growth of two of the three *D. mutila* and *N. luteum* isolates however mycelial growth of the third isolate of each species was reduced by more than 50% by the lowest concentration of metalaxyl. The differences observed could be due to poor mixing of fungicides leading to low concentration in some plates (experimental error) or the fungicide application management histories of the various vineyards or nurseries from where the botryosphaeriaceous species were isolated may have led to the development of resistance. Byrde and Richmond (1976) observed that the efficiency or inefficiency of a particular fungicide was the result of emergence of insensitive strains leading to fungicide selectivity within a genus and species. In New Zealand, wide genetic variation has been observed among some botryosphaeriaceous species (Baskarathevan, pers. comm. 2009) and this could also have accounted for the variation in the sensitivity of some isolates of the same species to similar fungicides. The variation in botryosphaeriaceous species insensitivity to fungicides observed in this study has also been reported in South Africa, where Bester *et*

al. (2007) reported that in dormant 1-year-old Chenin Blanc vine cuttings, *L. theobromae* and *D. seriata* isolates were inhibited by low concentrations of fenarimol, except for two isolates which did not show good inhibition by the fungicide, but they could not link the differences in sensitivity to resistance developed in the vineyard of origin.

In this study, metalaxyl was the least effective fungicide at inhibiting mycelial growth and conidial germination of *N. luteum*, *N. australe* and *D. mutila*. This was not unexpected as this fungicide usually targets the oomycete group of fungi (Kerkernaar and Kaars-Sijpesteijn 1981). However pyrimethanil and azoxystrobin which are used to control diseases caused by ascomycetes, such as *Botrytis cinerea* and *Uncinula necator*, respectively (Sunde 2010) were ineffective at inhibiting mycelial growth of *N. australe*, *N. luteum*, and *D. mutila* isolates even at the highest concentration (1000 mg/L) tested and so they were not tested for conidial germination of the three botryosphaeriaceous species. Luque *et al.* (2008) also reported that azoxystrobin tested at 900 mg/L could not reduce mycelial growth of *B. corticola* isolated from oak tree. In contrast, Bester *et al*. (2007) reported iprodione to be ineffective at inhibiting the mycelial growth of *D. seriata*, *N. australe*, *N. parvum* and *L. theobromae*, whereas it was highly effective in this study, inhibiting both mycelial growth and conidial germination of *D. mutila*, *N. australe* and *N. luteum* at the 5 mg /L concentration.

In the infection trials on detached green shoots, potted vines and mature vines in a vineyard, the efficacy of the fungicides was only tested against *N. luteum*, because it was the least sensitive of the tested species to the fungicides. On the detached green shoots, carbendazim, chlorothalonil, fenarimol, iprodione, mancozeb and tebuconazole treatments were able to protect the wounds on shoots completely, with no dieback or pathogen isolation. These fungicides were clearly capable of preventing pathogen development within the tissue. Of these, carbendazim, tebuconazole and iprodione had also been very effective in the *in vitro* tests, whereas chlorothalonil, fenarimol and mancozeb had only moderate effects in the same tests.

However, procymidone, thiophanate methyl and flusilazole which had been very effective in the *in vitro* tests were less effective at protecting trimming wounds on the detached shoots, since some of the shoots showed minor dieback lesions with 20% pathogen isolation. When the same fungicides were applied to wounds on 1-year-old canes of vines growing in pots, carbendazim and iprodione again provided the best wound treatment, although mancozeb and flusilazole were also very effective. The

presence of lesions on all the canes, irrespective of fungicide treatment, and the isolations of other saprophytic fungal species such as *Epicoccum, Fusarium, Ulocladium* and *Alternaria* confirmed earlier reports by Rolshausen *et al*. (2010) about the difficulty of finding chemical treatments to control taxonomically unrelated species with the same efficacy. In another glasshouse experiment with 1-year-old dormant Chenin Blanc grapevine cuttings, Bester *et al.* (2007) observed tebuconazole and prochloraz to be more effective pruning wound protectants than flusilazole when plants were grown at a temperature of 27^{o}C for 3 months after inoculating with conidial suspensions of *D. seriata, N. australe*, *N. parvum* and *L. theobromae*. In contrast, this potted vine study showed prochloraz to be the least effective pruning wound protectant, although it resulted in a dieback lesion significantly shorter than the positive control. The differences between these studies and that of Bester *et al*. (2007) could be due to the difference in the botryosphaeriaceous species used rather than physiological age of the tissue or variety. The results obtained from the potted grapevine assay described in this study did not agree entirely with the field trial results, although the most effective three fungicides flusilazole, carbendazim and mancozeb were in the best five products in both assays. However, the potted grapevine assay was less labour intensive than the field assay and was relatively inexpensive to conduct, in terms of materials and the value of the vines infected.

In the field evaluation, flusilazole, carbendazim, tebuconazole, thiophanate methyl, mancozeb, fenarimol and procymidone were able to provide some level of control against *N. luteum* infection with ≤20% incidence of the pathogen in non-lesion areas 10 mm from the lesion edges. However flusilazole was the most effective with 0% pathogen isolation in these tissues followed by carbendazim (7%) and tebuconazole (13%). This finding was in agreement with Pitt *et al*. (2010b) who also reported that fungicides such as tebuconazole, flusilazole and carbendazim were the most effective protectants of pruning wounds from infection by several botryosphaeriaceous species excluding *N. luteum* which they did not test. Luque *et al.* (2008) also observed that carbendazim was the most effective fungicide found in field trials but they reported that thiophanate methyl was the next most effective at reducing surface lesions caused by *D. corticola* on oak trees in Spain. Sosnowski *et al.* (2008) also reported that, of 15 fungicides tested in the field, carbendazim was the most effective at reducing colonisation of pruning wounds by *E. lata* whereas other fungicides, such as fluazinam, pyrimethanil and pyraclostrobin, reduced colonisation of wounds by *E. lata* only when applied at much higher concentrations. Although prochloraz was highly effective at

inhibiting *in vitro* mycelial growth, it was less effective at inhibiting conidial germination or conidial infection of grapevine potted or field plants. Such conflicting results between *in vitro* and *in vivo* data has also been reported by Cole *et al*. (2005) who observed that copper hydroxide was effective at reducing anthracnose symptoms caused by *C. gloeosporioides* compared to water controls, but could not inhibit mycelial growth of the same pathogen on PDA. Evaluations of the fungicides in the field is important as it provides opportunities for assessing other factors, such as retention on plant surfaces (Gent *et al.*, 2003) and stability to UV light (Boudina *et al.*, 2003), which cannot be tested *in vitro* but can also influence fungicide effectiveness.

It was observed in this study that although fenarimol, tebuconazole, flusilazole and prochloraz are all demethylation inhibitors, there were differences in their efficacy against *N. luteum* infection in the grapevine potted and field plant assays probably because these fungicides belong to different chemical groups. Similarly, carbendazim and thiophanate methyl, which are known to affect mitosis and cell division, differed in their efficacy to *N. luteum* infection which could also be due to the fact that the fungicides have small differences although both are of the same chemical group (Table 5.1) and with different modes of action (Appendix E.10). Johnston *et al.* (2005) also reported that *B. dothidea*, *N. luteum*, *N. parvum* and *N. ribis* isolated from different host plants in New Zealand differed in their sensitivity to carbendazim and thiophanate methyl. However, procymidone and iprodione, which both affect osmotic signal transduction and belong to the chemical group dicarboximide, did not differ significantly in their efficacy against *N. luteum* infection. Similarly, flusilazole and tebuconazole, which both inhibit demethylation and belong to the chemical group triazoles, did not differ significantly in their efficacy against *N. luteum* infection. Scherm *et al.* (2009) however, observed that there was a wide range in efficacy among individual triazoles, with prothioconazole and tebuconazole performing best and fluquinconazole and difenoconazole being less effective on soybean rust caused by *Phakopsora pachyrhizi*.

The seven fungicides that were most effective in the field trial are not all available for use, since flusilazole and tebuconazole are not registered for grapevines in New Zealand. Although the other fungicides are registered for grapes, this is for controlling other fungal pathogens and not botryosphaeriaceous species. In addition, many of them are at risk for development of resistance in fungi, including fungicides belonging to the DMI groups such as flusilazole (Nustar), fenarimol (Rubigan), the benzimidazole chemicals such as carbendazim (Protek) and thiophanate methyl (Topsin), the dicarboximides

chemicals such as iprodione (Rovral) and procymidone (Sumisclex) (Beresford, 1994). For each fungicide, the applications should be few and they should be alternated with fungicides from different groups.

The use of three botryosphaeriaceous species (with three isolates each) in the *in vitro* experiments in this study has provided a robust evaluation of the fungicides that could potentially be used to control botryosphaeriaceous fungi in New Zealand vineyards if they can be registered. If several fungicides were registered, they could be used in succession to prevent development of resistance. Denman *et al*. (2004) observed that a significant reduction in the number of cankers caused by *N. protearum* on field grown *Protea mangifera* was achieved with applications of fenarimol and prochloraz alternated with mancozeb. Although carbendazim is found to be effective at controlling most pathogens by inhibiting fungal mitotic microtubule formation, thus affecting fungal cell division and eventually preventing fungal growth and spore germination, it was included in a biocide ban proposed by the Swedish Chemicals Agency (Anonymous, 2008). It was banned in the United States in 2001 and is currently banned in New Zealand and Australia (Ebono, 2009). However in the absence of carbendazim, thiophanate-methyl can be used since it has been approved until February 2016 (European Commission Directive 2005/53/EC) (Luque *et al.*, 2008).

This study considered the use of conventional fungicides for controlling botryosphaeriaceous species however John *et al.* (2003) reported the potential of *Trichoderma* species for the control of eutypa dieback in grapevines. In Australia and New Zealand, a biological control product, Vinevax^{TM}, has been registered for use on grapevine pruning wounds with the aim of controlling *E. lata*. Pitt *et al*. (2010b) reported that application of Vinevax^{TM} significantly reduced the mean percent recovery of *D. seriata* in a trial conducted in the Hunter Valley, Australia. Such biological control products could also be considered here in New Zealand in any future study aimed at managing grapevine trunk diseases caused by the various botryosphaeriaceous fungi.

In conclusion, development of botryosphaeriaceous disease incidence could be reduced in New Zealand vineyards by application of the four registered fungicides (mancozeb, fenarimol, procymidone, and thiophanate methyl) which can be applied in successions during pruning times. The importance of using such fungicides as part of the cultural practices, and avoiding pruning during and immediately after rainfall needs to be considered. Although the use of fungicide mixtures was not tested in this study, it could be considered in future research.

Chapter 6
Concluding discussion

The overall aim of this study was to identify and investigate the development and epidemiology of the botryosphaeriaceous species associated with grapevines in New Zealand and also to investigate the effectiveness of some control options such as fungicides for use as wound protectants. In this chapter, the key findings of this research are presented with a view to demonstrating improvements in the understanding of how these factors affect dispersal, pathogenicity and disease progression in the field.

A field sampling of grapevines and non-grapevine woody plants in 2006 and in 2007 showed that botryosphaeriaceous species are associated with wood necrosis, bud failure, shoot and cane dieback in grapevines, in wood necrosis of non-grapevine woody hosts and in grapevine woody debris left in the vineyard. The identification of the species that were isolated during the field sampling was confirmed through a combination of cultural characteristics and molecular technology. Five major botryosphaeriaceous species were identified, namely, *N. australe*, *N. luteum*, *N. parvum*, *D. mutila* and *D. seriata*, within the 63 isolates obtained from symptomatic grapevines and non-grapevine woody hosts.

Although the sampling did not cover all the grapevine growing areas in New Zealand, and the numbers of the botryosphaeriaceous isolates collected from the sampling were relatively small, the main species identified and their relative frequencies were consistent with the results from a much wider field survey (n=336 isolates) carried out later in vineyards throughout New Zealand (Baskarathevan, pers. comm., 2008). Subsequent sampling of nurseries (Billones *et al*., 2010a) also found the major botryosphaeriaceous species identified in this study although the relative frequencies found in nurseries were different, with *N. luteum* being more prevalent than *N. parvum* as found in vineyards. This sampling was able to verify that the economic damage associated with botryosphaeriaceous species in other grape growing regions worldwide was also occurring in New Zealand. The species recovered in vineyards were consistent with similar work done in California (Úrbez-Torres *et al.,* 2006), South Africa (Fourie and Halleen, 2004a) and Australia (Taylor *et al*., 2005). However, the number of the major species (five) found in this study was less than reported in other countries.

The five botryosphaeriaceous species isolated from grapevines were also isolated from 11 different non-grapevine woody hosts which were found to be pathogenic to

grapevines indicating a potential reservoir of inoculum. In New Zealand, vineyards are commonly grown close to orchards, shelter belts or other woody tree plantations, so sampling from non-grapevine woody plants was also important. It demonstrated their potential role as alternative hosts, able to provide the inocula for vineyard infection. Since not all non-grapevine woody plants were sampled, a more robust survey on woody plants including orchards and horticultural crops across New Zealand should be considered in the future to determine (i) the diversity of members of the botryosphaeriaceous species, (ii) their ability to infect multiple hosts and (iii) any difference in pathogenicity with different hosts. For example *N. parvum* was the most predominant species encountered in the sampling and these species have been reported to sporulate well on *Eucalyptus grandis* debris (dead tissue) and on stem cankers of *E. globulus* (Pérez *et al*. 2008). However, these tree species were not sampled during this study although they are present in New Zealand. Such a survey should also focus on the search for teleomorphs of the botryosphaeriaceous species on the alternative hosts, since they were not observed in this study. In the United States, Sutton (1981), Hewitt and Pearson (1990) and Pusey (1989) observed ascospores of *D. seriata*, *F. aesculi* and *L. theobromae* in peach orchards.

During the sampling, insects such as thrips, beetles, ants and wood lice, were frequently found to be present and observed moving from one plant to another, but these insects were not tested for the presence of any fungal propagules characteristic of botryosphaeriaceous species due to time constraints. However, transfer of fungal spores by insects has been reported in many wood diseases, such as Dutch elm disease which is caused by a beetle-transmitted fungus, *Ophiostoma ulmi*. The fungal spores of *O. ulmi* are sticky, so can become attached to the beetle which flies to other elms, where it introduces the spores into feeding wounds on small twigs (Carter 1973). Although there are no records of insects vectoring spores of botryosphaeriaceous species, Damm *et al.* (2007) isolated *D. africana* from insect galleries associated with pruning wound cankers of *Prunus persica* in South Africa. Since the conidia of botryosphaeriaceous species ooze from the pycnidia in a paste they may be sticky enough to become attached to insects and later transferred between vines or even onto wounds. Future studies should therefore consider investigating the species and behaviours of insects in vineyards to determine if they could act as vectors of inoculum for vineyard infection. Trapping of insects could be done by pheromone traps or by using coloured cards with sticky surfaces to catch the crawling and flying insects. The propagules could be washed off using sterile water and mounted for microscopic identification of botryosphaeriaceous

spores, followed by culturing and molecular identification. The use of quantitative PCR with the multi-genus primer pair would also allow quantitation of spores on a large number of insects.

This study was the first to isolate and confirm, through molecular identification, the presence of pathogenic *N. australe* in grapevines and non-grapevine woody hosts in New Zealand. *Neofusicoccum australe* was first described in 2004 from native *Acacia* species in Australia and later re-analyses of GenBank data showed that it also occurred on other native Australian hosts, namely *Banksia* sp. and *Eucalyptus* sp., as well as a native *Protea* sp. in South Africa and on Pistachio in Italy (Slippers *et al*., 2004c). Some of the *N. australe* isolates from this study and the evidence of their identity were submitted to Ministry of Agriculture and Forestry (MAF), New Zealand, which confirms and keeps records of all the pathogens reported to be present in the country, while the cultures are kept by Landcare Research, New Zealand. Recently Billones *et al*. (2010b) have also isolated *N. macroclavatum*, from shoots growing on grapevines rootstock and proved its pathogenicity on grapevine scions. This is the first report of it in New Zealand and the first record of it being a grapevine pathogen, since it has only been reported as a pathogen of *Eucalyptus globulus* in Western Australia (Burgess *et al*., 2005). It is likely that other botryosphaeriaceous species are present on the wide variety of endemic and exotic hosts, and a study which could determine the species present and their range of hosts will be of benefit to the management of fruit, forest and conservation species in New Zealand.

Trapping of conidia carried out separately in vineyards in the Marlborough and Canterbury regions for a period of one and 12 months, respectively, showed that conidia were not windborne (absent on Vaseline®-coated slides) but rather were splash-dispersed. The sequential collection of rainwater showed that the botryosphaeriaceous species conidia were dispersed throughout the year and the identification of collected conidia showed presence of the most common botryosphaeriaceous species. The practical implication of this is that any wounds created throughout the year can be pathways for infection by botryosphaeriaceous species. Although the distance moved by the botryosphaeriaceous species conidia was not considered in this study, Baskarathevan *et al*. (2010) in a similar study reported that conidia of *N. luteum* travelled 2 m in a single rainfall event, with the dispersal distance being influenced by wind. It is also possible that airborne ascospores, which are produced by the teleomorph stage, could be transported over even longer distances. Wind has been shown to carry ascospores of *E.*

lata or *E. armeniacae* for at least 50-60 km in California, after which they still remained viable and infectious (Ramos *et al*., 1975; Petzoldt *et al*., 1983). It is also possible that ascospores of botryosphaeriaceous species could be transported from Australia to New Zealand, as occurred for two species of poplar rust in 1973, probably *via* high trajectory wind currents (Close *et al*., 1978).

This work also demonstrated that infection in the field is likely to occur from spores landing on wounded plant surfaces during humid and shady conditions. Exposure to sunlight and low relative humidity (RH) was shown to decrease the conidial viability of three botryosphaeriaceous species (*N. luteum*, *N. australe* and *D. mutila*). The conidia of four botryosphaeriaceous species were also found in another study to tolerate low temperatures and germinated slowly, at 5^{o}C with relatively low numbers achieving germination after 24 h. However, rapid germination that is likely to be responsible for most field infections occurred at 20-30^{o}C. These results indicated that the botryosphaeriaceous species conidia are adapted to the environments that are common within grapevine canopies.

These pathogenicity studies which used conidia have improved on earlier pathogenicity studies which used mycelial plugs for inoculation (Taylor *et al*., 2005; van Niekerk *et al*., 2004). The improved methods in this study were possible due to the development of a novel method that used infected grapevine green shoots to provide abundant conidia for the inoculations (Amponsah *et al*., 2008). This study was unable to test the infectivity of ascospores as no perithecia were detected on any of the diseased grapevine materials collected during the sampling, which was not unexpected since teleomorphs of these fungi are reported to be rarely found in nature (Denman *et al*., 2000; Crous *et al*., 2006). However future research should examine different methods of inducing ascopores production on different host species.

Many researchers have determined pathogenicity by either measuring the visible lesion area or lesion length. However, in this study in addition to measuring lesion length, the movement of the pathogen beyond the visible lesion edge was also measured by isolation of the endophytic pathogens onto PDA at sequential 10 mm intervals. The total distance moved by the pathogen was then used as a measure of pathogenicity. This method of determining pathogenicity was used because of the browning of the vine stem tissues as they matured, which obscured the lesion edges. The results showed that *N. luteum, N. australe* and *D. mutila* could progress rapidly through the plant without visual symptoms except small lesions at the inoculation site. In contrast, isolates of *N. parvum*

often showed a large area of canker around the woody trunk which could ultimately kill the entire plant. Thus, these assessment methods were able to demonstrate a key difference in the pathogenicity between *N. parvum* (a canker causing pathogen) and *N. luteum, N. australe and D. mutila,* which are more endophytic.

The different growth patterns have implications for control measures. If the infected symptomless canes, are removed by winter pruning and left on the vineyard floor they may be able to release botryosphaeriaceous spores later in the season. Reworking of grapevines, by decapitating trunks below necrotic cankers and allowing the new trunk suckers to grow into a new vine canopy has been used to successfully control some trunk pathogens, such as *E. lata* in Australia (Sosnowski *et al.*, 2009). However, if the method were to be developed for control of the botryosphaeriaceous species, further research would need to be done as these species have been found to move endophytically upwards and downwards.

The use of this more labour intensive approach to determining pathogenicity uncovered a significant trait of *N. luteum* infection. If this study had considered visible lesion length measurement as the only means to determining pathogenicity, the conclusion would have been that *N. luteum* was not pathogenic during the assessment period which would have been false. In contrast, this study found it to be highly pathogenic, able to cause dieback and budburst failure, which often appeared only during the dormancy period, after cane pruning and/or changes to soil water availability.

In this study, water supply to the grapevines growing in pots in the glasshouse was found to be important as too much or too little imposed stress on the *N. luteum* infected vines that led to dieback during winter dormancy and/or budburst failure after winter pruning. The dormancy period and pruning of canes which also resulted in expression of symptoms could also be classed as stress factors since they caused adverse reactions in the plants. This work should be further investigated using a range of known stress factors to monitor disease expression by botryosphaeriaceous species on grapevines.

Due to rapid progression of *N. luteum* through the grapevine tissues, leading to dieback and budburst failure, it was considered the most pathogenic and so its conidia were used for further inoculation studies on different grapevine tissues. These studies showed that inoculation of detached, wounded green shoots with as little as two conidia (20 µL drop conidial suspension of 10^2 /mL) caused 100% incidence, although the highest rates of pathogen progression were caused when the detached shoots were inoculated with 200

conidia (20 µL drop conidial suspension of 10^4/mL). The low concentration of *N. luteum* able to cause infection could pose a great risk to the winegrape industry. However, since these experiments were conducted on detached green shoots, further studies are needed to determine the infection rates on potted vines, or field grown vines, which will be important to enable proper comparisons to be made with other grapevine wound pathogens.

Age of wounds on trunks of potted grapevines before conidium inoculation was also found to be an important factor in determining incidence of *N. luteum* infection, since it was 100% on fresh to 2-day-old pruning wounds, 40% on 7-day-old wounds and 0% on 14-day-old wounds. Wounds were found to remain susceptible to mycelial infection for up to 30 days which may prove to be significant since some recent experiments have shown that the pathogen frequently resides in the bark of canes sampled from mother vine blocks (Billones, pers. comm., 2010). Since these pathogens were shown to require wounds for infection, this is an area that requires future research. Wounds may differ in their susceptibility, according to the different varieties, tissue types and ages, seasons and environmental conditions. Since wounding of vines is inevitable due to wind movement and management practices, an understanding of the factors affecting susceptibility to the ubiquitous conidia could provide critical information for disease management.

This study also provided information on the preferred route by which the pathogen travelled once in the grapevine. Isolation studies showed that the pathogen progressed more quickly through the plants in an upwards than downward direction. The light microscopy and SEM observations of the movement of the mycelium through the xylem showed it appeared to adhere to the walls of the xylem vessels in plants with no visible external symptoms. It is therefore possible that progression of *N. luteum* through the xylem in the form of mycelium may be facilitated by nutrients absorbed directly from the sap. Development of symptoms may be associated with release of other enzymes or toxic metabolites. Although toxin production was not investigated in this study, future research should consider the production of any secondary metabolites in infected grapevine plants, as shown by Rolshausen *et al*. (2008) who detected secondary metabolites and hydrolytic enzymes in the xylem vessels, during wood degradation of grapevines infected by *E. lata*.

Inoculation studies to determine the susceptibility of different tissues demonstrated that pruned canes, wounded shoots, trunks and buds are highly susceptible to

botryosphaeriaceous species but that the pathogen is unable to infect roots. Botryosphaeriaceous species were also isolated from dead or shrivelled flowers (inflorescences) but time constraints prevented this from being pursued and future experiments should determine the susceptibility of flowers and fruit at different development stages.

These studies also demonstrated some key differences in conidial development between detached and attached tissues. Conidial inoculations onto detached wounded shoots or leaves showed rapid formation of mycelial growth within 24 h after inoculation. In contrast, the conidial development was much slower with inoculations onto wounded but attached shoots. The difference in conidial growth and development between attached (active) and detached tissues could be the result of the grapevine plant initiating a systemic defence mechanism in the attached tissue which prevented the germination and growth of the inoculated conidia. Since this study could not explore the defence response, future research should test for a systemic response. Breeding for blueberry resistance varieties has been found to reduce damage caused by Botryosphaeria stem blight in the USA. Through a breeding program, three southern high bush blueberries cultivars were found to be resistant when compared to the susceptible rabbit eye cultivar (Smith, 2009). A similar breeding program could be carried out on grapevines by inoculating different breeding lines with multiple botryosphaeriaceous species and then selecting those able to resist the pathogen infection and progression.

Although this study has demonstrated that most of the scion cultivars in New Zealand vineyards are susceptible to the major botryosphaeriaceous species and that they can cause severe symptoms that may affect yields, there are no records linking yield losses and fruit quality to botryosphaeriaceous species infection here in New Zealand. Future research on this aspect of the economic importance of the pathogen will reinforce the anecdotal evidence on the economic significance of this pathogen. Vineyard losses are likely to be caused by reduction of fruit yield and quality and the costs of managing the disease, such as replanting to replace weak or dead vines or pruning out infected wood. Samples of diseased trunks, canes and green shoots collected in this study showed 42, 17 and 20% infection by botryosphaeriaceous species, respectively, with most of the affected vines being in older vineyards. The outright removal of infected grapevine trunks (Figure 2.1b), leading to replanting of a whole vineyard in the Nelson region, was an indication of economic yield losses in New Zealand. In California, Siebert (2001) reported that losses of $260 million per annum in 1999 were due to grapevine trunk

diseases, which were found to be associated with nine different fungi of which four (*B. dothidea, D. seriata, L. theobromae* and *Dothiorella viticola*) were botryosphaeriaceous species, with infection rates up to 87%. Knowledge of the yield loss estimates in monetary terms is likely to add impetus to recommendations for disease assessment, use of control measures such as wound protectants and research into breeding for resistant cultivars.

The fungicide sensitivity study conducted on potted and field grown vines showed that carbendazim, mancozeb, flusilazole, tebuconazole, fenarimol, thiophanate methyl and procymidone were all effective at controlling *N. luteum* and therefore are also likely to control the other botryosphaeriaceous species. Pruning is likely to provide fresh wounds for infection and, therefore, all pruning wounds need to be protected with fungicide all year round. However, since other green tissues can be infected, overall spray cover is essential. However, if these fungicides were to be used for protecting the trimming/pruning wounds made several times each summer, they could cause residues in the fruits and should therefore be applied with caution. Such fungicides should only be applied during winter immediately after pruning to prevent infection and disease progression. Further research needs to be done to investigate ways to make the products more rain-fast and to increase their period of effectiveness so that they last until the wounds are no longer susceptible. The use of biological control products such as Vinevax^{TM} has been sucessfully used to control *E. lata* and botryosphaeriaceous species in in Australia. Similar evaluation studies can therefore be done here with New Zealand isolates.

This study has provided some basic information on the epidemiology of botryosphaeriaceous species and the possible sources of inoculum for vineyard infection, peak periods for inoculum release and how the environmental factors affect inoculum viability. It has demonstrated the infection and development processes and how they may be affected by some environmental and common host factors. This knowledge could help growers to develop cultural control strategies that may be used in conjuction with the fungicides identified to be effective, to reduce the damage caused by, and the spread of these pathogens.

Presentations and publications from thesis

Conference presentations

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Appendix A

A.1 Morphological description of some minor botryosphaeriaceous species associated with grapevines

A.1.1 Neofusicoccum vitifusiforme

In PDA culture, the colonies are effuse with even, smooth margins which are white on the surface and greenish olivaceous underneath, reaching a radius of 31 mm after 3 days at 25^{o} C. Pycnidia formed are solitary and globose with a size of up to 450 µm diameter. The pycnidia formed in culture on PDA have thick brown walls which become hyaline towards the inner region. The conidia are fusoid to ellipsoid (Fig. A1.1) being widest in the upper third with an obtuse apex and flattened truncated base, and with size ranges of (18–) 19–21 (–22) x (4.5–) 5.5–6.5 (–8) µm and a length to width ratio of 3.3. They are hyaline with granular contents (van Niekerk *et al.*, 2004). The conidia are similar to those of *N. australe* and *N. luteum* but colonies are distinguished by not producing yellow pigment in culture (Slippers *et al.*, 2004c; Lazzizera *et al.*, 2008).

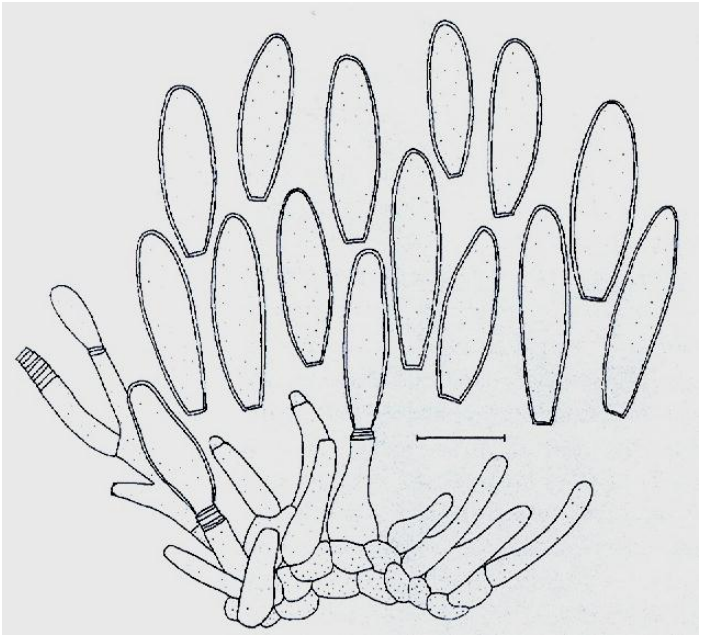


Figure A 1.1 ***Neofusicoccum vitifusiforme*** **conidia and conidiogenous cells (holotype). Bar=10 µm**. Source :(van Niekerk *et al*., 2004).

A.1.2 Diplodia porosum

In PDA culture, colonies are flat with undulating margins, appearing dark green on the surface and dull green underneath and reaching a radius of 32 mm after 3 days at 25^{o}C. Pycnidia formed in culture on PDA are solitary, globose to obpyriform and up to 400 µm wide having a thick dark brown wall that becomes hyaline towards the inner region. Conidia are ovoid to broadly ellipsoid with a bluntly rounded apices, and flattened bases with size ranges of (38–) 42–45 (–47) x (20–) 22–25 (–30) µm and a length to width ratio of 1.9. The conidia are initially hyaline while still in the pycnidia, have a pigmented thick walls, with pores and become pigmented with age (Fig. A 1.2a and b). Within the genus *Diplodia* this species is unique because of its large, thick-walled conidia with large pores that are clearly visible by light microscopy (van Niekerk *et al.* 2004).

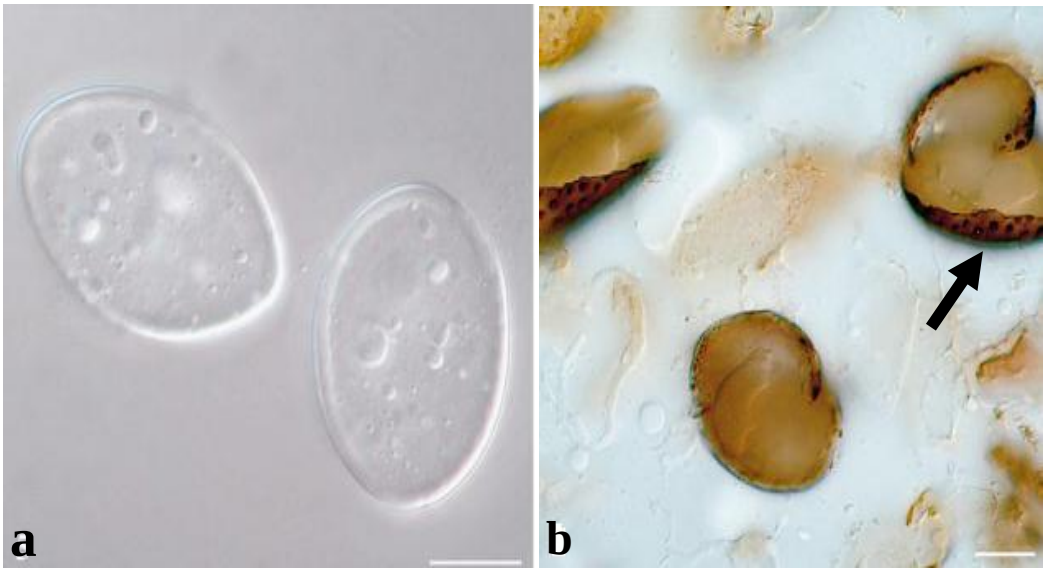


Figure A 1.2 *Diplodia porosum* (a) young hyaline conidia (b) broken, mature, pigmented conidia with dark pores (arrow) Bar = 10 µm. Source :(van Niekerk *et al*., 2004).

A.1.3 Neofusicoccum viticlavatum

In cultures, the colonies are umbonate with undulating margins. They appear olivaceous on the surface and dull green underneath, reaching a radius of 26 mm after 3 days at 25^{o}C. In nature, the usually solitary pycnidia are embedded in host tissue, are globose and up to 450 µm in diameter. The pycnidial wall is thick and brown, becoming hyaline toward the inner region. Conidia are ellipsoid to clavate, widest in the upper third, with obtuse apices and flattened, subtruncated bases, with size ranges of (15–)16–18(–20) x (6–)6.5–7.5(–8) µm and a length to width ratio of 2.4. They are hyaline and aseptate (Fig.A1.3) (van Niekerk *et al.*, 2004).

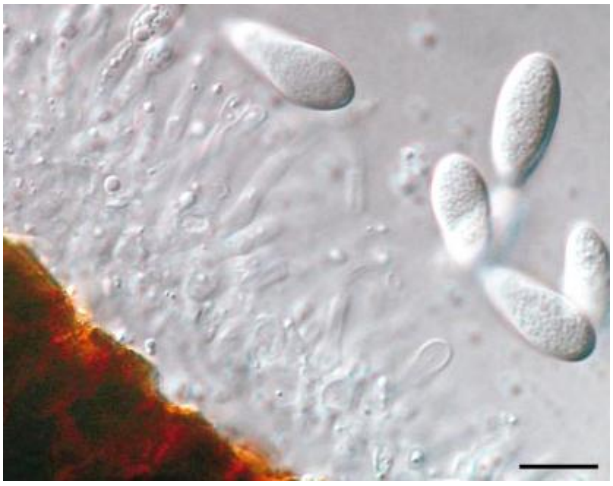


Figure A 1.3 *Neofusicoccum viticlavatum.* Conidiogenous cells giving rise to conidia Bar =10 µm. *Source* :(van Niekerk *et al*., 2004).

Appendix B

B.1 Vineyard sampling regions across New Zealand

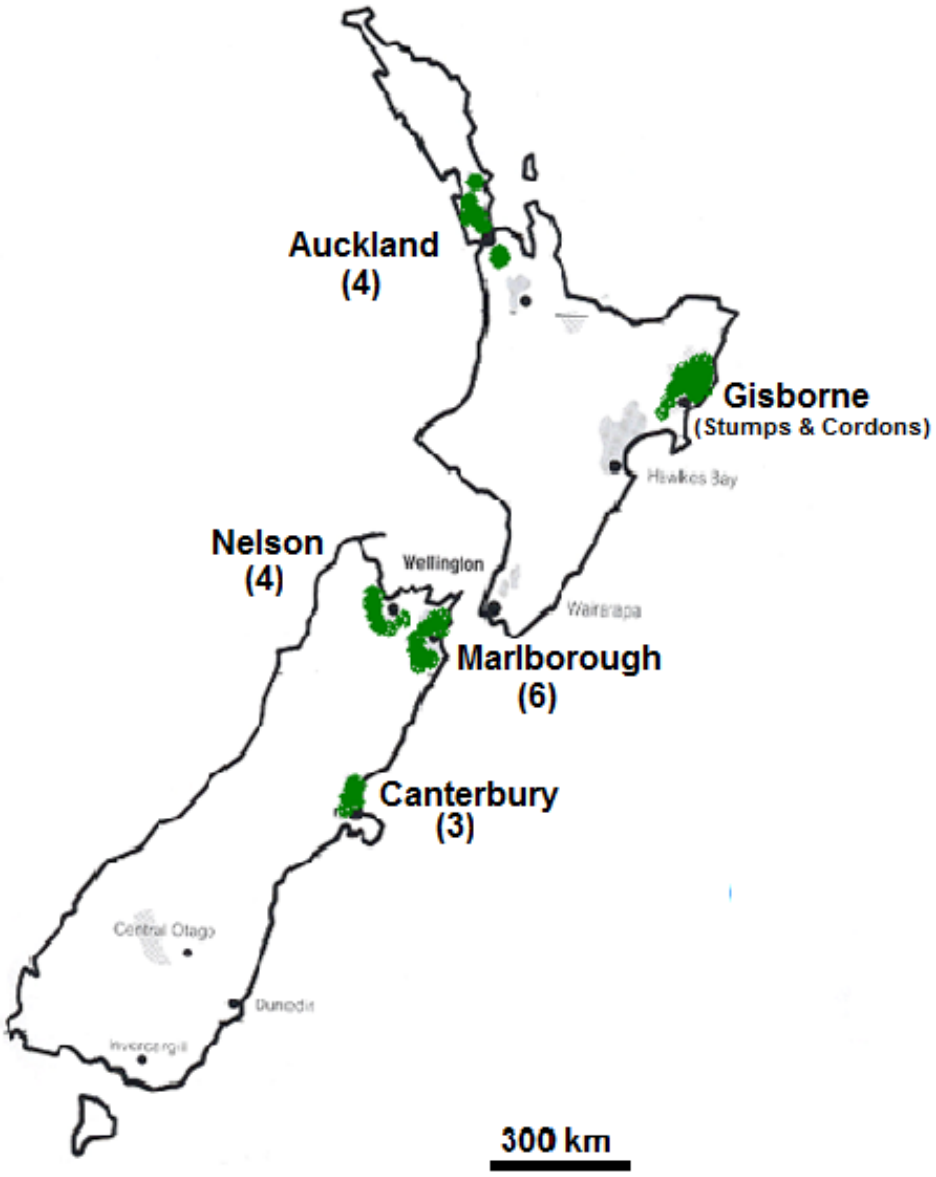


Figure A 2.1 A map of New Zealand showing the regions (green shadings) and number of vineyards from where grapevine materials and other non-grapevine host materials were sampled for the presence of botryosphaeriaceous species infection.

B.2 Identity and origin of pure culture botryosphaeriaceous species isolates obtained from grapevines in Marlborough, Nelson, Auckland and Canterbury regions of New Zealand.

Isolate	Identity	Host	Property	Region
Q	*Diplodia mutila*	Grapevine	Ashmora	Auckland
Q-2	*Neofusicoccum parvum*	Grapevine	Ashmora	Auckland
Q-1	*N. parvum*	Grapevine	Ashmora	Auckland
Q (s)	*N. luteum*	Grapevine	Ashmora	Auckland
F (20)-1	*D. mutila*	Grapevine	Brancott Estate	Blenheim
F (12)-2	*D. mutila*	Grapevine	Brancott Estate	Blenheim
R (s)	*D. seriata*	Grapevine	Framingham	Blenheim
L (17)-4	*D. seriata*	Grapevine	Greenhough	Nelson
L (17)-3	*N. parvum*	Grapevine	Greenhough	Nelson
L-1	*D. seriata*	Grapevine	Greenhough	Nelson
L-5	*N. luteum*	Grapevine	Greenhough	Nelson
L (16)-2	*D. seriata*	Grapevine	Greenhough	Nelson
H (1)-2	*D. seriata*	Grapevine	Herman Siefried	Nelson
H(2)-3	*N. parvum*	Grapevine	Herman Siefried	Nelson
H (1)-1	*N. parvum*	Grapevine	Herman Siefried	Nelson
H (2)-5	*D. mutila*	Grapevine	Herman Siefried	Nelson
I (15)-3	*N. parvum*	Grapevine	Kahurangi	Nelson
I-4	*N. parvum*	Grapevine	Kahurangi	Nelson
I (15)-2	*N. parvum*	Grapevine	Kahurangi	Nelson
G (26)-2	*N. luteum*	Grapevine	Kowhai	Auckland
M (13)-2	*N. luteum*	Grapevine	Kumea river	Auckland
M (8)-4	*N. luteum*	Grapevine	Kumea river	Auckland
M (30)-3	*D. mutila*	Grapevine	Kumea river	Auckland
M (7)-6	*D. mutila*	Grapevine	Kumea river	Auckland
Mel-1	*N. australe*	Grapevine	Melton Estate	Canterbury
Mel-3	*N. australe*	Grapevine	Melton Estate	Canterbury
Kat-2	*Dothiorella* sp.	Grapevine	Katuna Valley	Canterbury
Mel-2	*N. australe*	Grapevine	Melton Estate	Canterbury
O-3-1	*N. parvum*	Grapevine	Matua Valley	Auckland
J (22)-3	*N. parvum*	Grapevine	Neudorf	Nelson
J (12)-1	*N. parvum*	Grapevine	Neudorf	Nelson
K (18)-1	*N. australe*	Grapevine	Neudorf 2	Nelson
D (6)-1	*D. mutila*	Grapevine	Roger Rose	Blenheim
Rd3	*D. mutila*	Grapevine	Rossendales	Canterbury
La-(11)-2	*D. mutila*	Grapevine	Rossendales	Canterbury
La-12	*N. parvum*	Grapevine	Larcombs	Canterbury
La-13	*N. parvum*	Grapevine	Larcombs	Canterbury
Cob-1	*N. parvum*	Grapevine	Corbans	Auckland
Cob-2	*N. luteum*	Grapevine	Corbans	Auckland
N(13)-4	*N. parvum*	Grapevine	Rothesay	Auckland
N (12)-2	*N. luteum*	Grapevine	Rothesay	Auckland
N-1	*N. luteum*	Grapevine	Rothesay	Auckland
Kat-1	*N. australe*	Grapevine	Katuna Valley	Canterbury
Mel-4	*N. australe*	Grapevine	Melton Estate	Canterbury
San-1	*N. luteum*	Grapevine	Sandihurst	Canterbury
RGSC-3	*N. parvum*	Grapevine Rootstock	Linnaeus	Gisborne
G(s)-1	*N. luteum*	Grapevine Rootstock	Linnaeus	Gisborne
Sch	*N. luteum*	Grapevine Rootstock	Linnaeus	Gisborne
RGSC-1	*N. parvum*	Grapevine Rootstock	Linnaeus	Gisborne

B.3 Isolates of different botryosphaeriaceous species that were isolated from grapevines and other non-grapevine hosts in New Zealand that were used for pathogenicity studies.

Species	Isolate No.	Host	Region
N. luteum	N(12)2	Grapevine	Auckland
N. luteum	M(13)2	Grapevine	Auckland
N. luteum	G(s)-1	Grapevine	Gisborne
N. australe	Kat-1	Grapevine	Canterbury
N. australe	Mel-2	Grapevine	Canterbury
N. australe	K(18)1	Grapevine	Nelson
N. parvum	Q-2	Grapevine	Blenheim
N. parvum	I(15)2	Grapevine	Nelson
N. parvum	I(15)3	grapevine	Nelson
D. mutila	F(12)2	Grapevine	Blenheim
D. mutila	Q	Grapevine	Blenheim
D. mutila	M(30)3	Grapevine	Auckland
D. seriata	L(16)2	Grapevine	Nelson
D. seriata	L-1	Grapevine	Nelson
D. seriata	L(17)4	Grapevine	Nelson
D. mutila	Iso-2	Native ngaio	Gisborne
N. australe	J-3	Broom	Nelson
D. seriata	I-1	Lemon wood	Nelson
N. parvum	O-3-1	Cherries	Auckland
N. luteum	MJ3	Blue berries	Hastings
D. mutila	J-4	Oak	Nelson
D. mutila	C-4	Olive	Blenheim
D. mutila	F-1	Willow	Blenheim
N. parvum	O-1	Pine	Auckland
D. mutila	A-3	Plum	Blenheim
D. mutila	A-2	Apple	Blenheim

B.4 Recipes for media types used to induce sporulation

Malt yeast agar (MEA)
10 g light malt extract (obtained from supermarket)
20 g agar powder (Danisco® Bacteriological Agar)
1000 ml reverse osmosis water
Autoclave (121^{o}C) for 15 min

Oat meal agar (OMA)
40 g oatmeal
20 g agar powder (Danisco® Bacteriological Agar)
1000 ml reverse osmosis water
Autoclave (121^{o}C) for 15 min

Pine needle embedded in water agar
20 g agar powder (Danisco® Bacteriological Agar)
1000 mL reverse osmosis water
Autoclave (121^{o}C) for 15 min
5 autoclave-steriled pine needles placed on the agar of each plate before it set.

Prune Extract / Prune Extract Agar
Use 25 g prunes per 500 mL flask.
De-stone and chop up prunes. Fill flask with ¾ of distilled water and cap flask. Place in pressure cooker and boil in free steam for 30 minutes. Filter through Whatman filter paper no. 3 Autoclave and refrigerate.

To make agar:

	To make 1L
Sucrose	5 g
Yeast extract	1 g
Danisco® Bacteriological Agar	30 g
Prunes extract	100 mL
Distilled water	900 mL

Boil to dissolve before autoclaving at121^{o}C for 15 min

B.5 Analysis of variance on sporulation

B.5.1 ANOVA results on the effect of media type on pycnidia production of 4 different botryosphaeriaceous groups.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Groups (species)	3	250991	83664	17.5	<.001
Media	3	216367	72122	15.09	<.001
Groups x Media	9	44801	4978	1.04	0.43
Residual	32	152960	4780		
Total	47	665119			

B.5.2 ANOVA results on the effect of media type on conidia production of 4 different botryosphaeriaceous groups.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Groups (species)	3	2552.729	850.91	100.11	<.001
Media	3	27.062	9.021	1.06	0.379
Groups x Media	9	87.188	9.688	1.14	0.365
Residual	32	272	8.5		
Total	47	2938.979			

B.5.3 ANOVA results on conidia production from 4 different botryosphaeriaceous groups on grapevine green shoots

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Group (species)	3	53103.33	17701.11	249.6	<.001
Residual	8	567.33	70.92		
Total	11	53670.67			

B.5.4 ANOVA results on conidia production from 4 different species of botryosphaeriaceous groups on pine needle embedded in water agar.

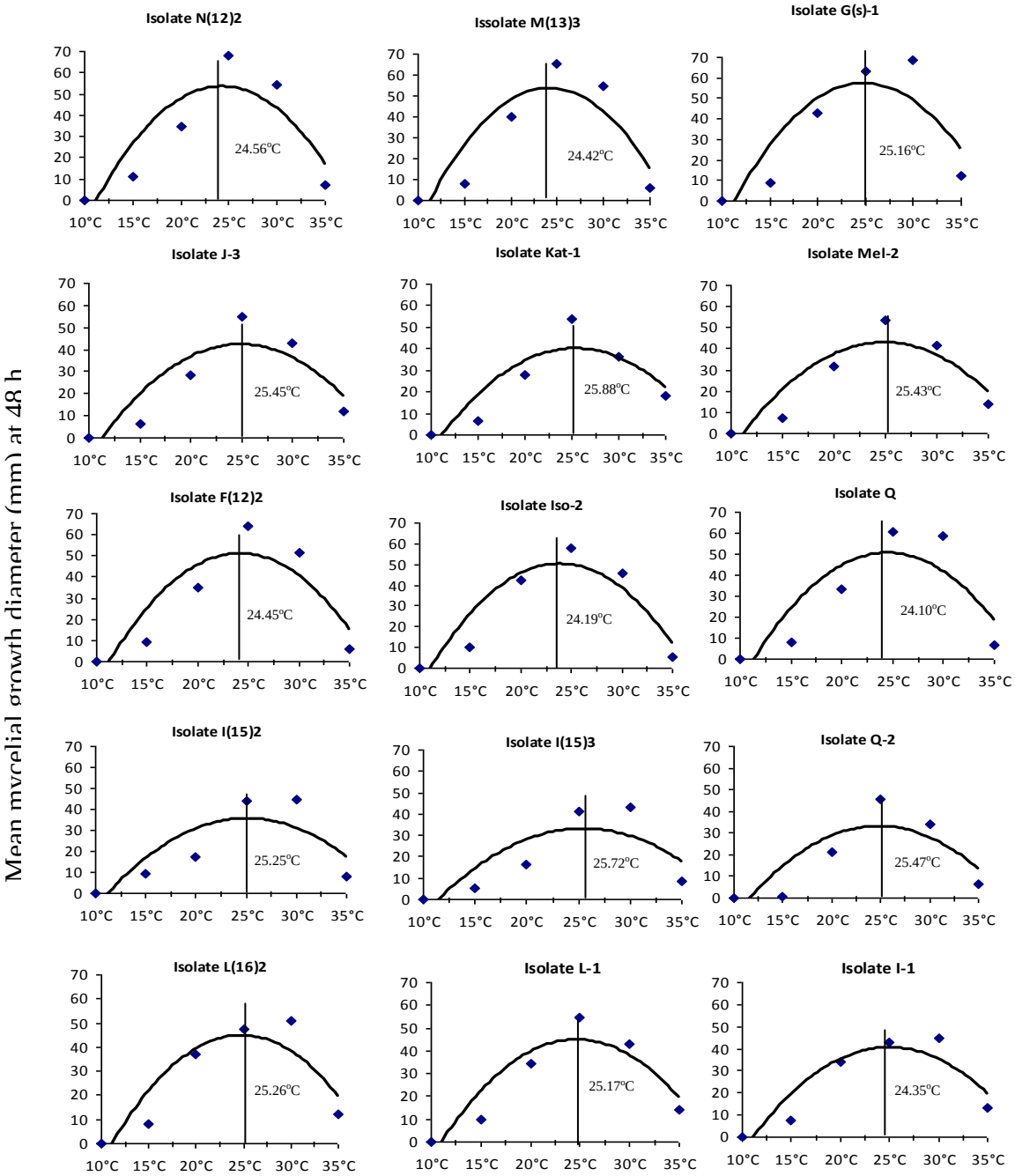
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Groups (species)	3	72.667	24.222	7.65	0.01
Residual	8	25.333	3.167		
Total	11	98			

B.6 Analysis of variance on the effect of temperature on mycelium growth

B.6.1 ANOVA results on the effect of different temperatures on mycelial growth (mm) of botryosphaeriaceous isolates after 48 h incubation.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	14	3930.613	280.758	154.01	<.001
Temp	5	115850.5	23170.09	12710.15	<.001
Isolate x Temp	70	6381.233	91.16	50.01	<.001
Residual	180	328.133	1.823		
Total	269	126490.5			

B.6.2 Optimum growths and temperatures for the individual isolates modelled using quadratic formula.



B.7 Mel-2 isolates' alignment with *N. luteum*

```
ORIGIN
Mel-2                ATCCGAGGTCAACCTTGAGAAAAATTCAAAGGTTCGTCCG     40
B. lutea             ----------------------------------------     40

Mel-2          GCGGGCGACGCCATGCGCTCCAAAGCGAGGTGTTTTCTAC     80
B. lutea             ------------g---------------------------     80

Mel-2          TACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTCGAGGC    120
B. lutea             ----------------------------------------    120

Mel-2          GCGTCCGCGGAGGACGGAGCCCAATACCAAGCAGAGCTTG    160
B. lutea             ------a-a-------------------------------    160

Mel-2          AGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAAT    200
B. lutea             ----------------------------------------    200

Mel-2          ACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCA    240
B. lutea             ----------------------------------------    240

Mel-2          CTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGC    280
B. lutea             ----------------------------------------    280

Mel-2          GTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAA    320
B. lutea             ----------------------------------------    320

Mel-2          AGTTTTAGTTTATTAACTTGTTTCTCAGACTGCGACGTTT    360
B. lutea             ----------------------------------------    360

Mel-2          ACTGACTGGAGTTTTGTGGTCCTCTGGCGGGCGCTGGCCG    400
B. lutea             ----------------------------------------    400

Mel-2          G.CCCCCGAACGGGGGTCGGTGCGGAGGACCGCGGCCCGC    439
B. lutea             -c--------------------------------------    440

Mel-2          CAAAGCAACAGAGGTAGGTACACATGGGGTGGGAGAGTCG    479
B. lutea             ----------------------------------------    480

Mel-2          AGCCGGAGCT                                  489
B. lutea     ----------                                  490
```

B.8 Nucleotide sequence of ITS region obtained from molecular identification of the representative botryosphaeriaceous species isolates selected

N. australe

Mel-4

TACCTGATCCGAGGTCAACCTTGAGAAAAATTCAAAGGTTCGTCCGGCGGGCGACGCCATGCGCTCCAAAGCGAGGT
GTTTTCTACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTCGAGGCGCGTCCGCGGAGGACGGAGCCCAATACC
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GAGATCCGTTGTTGAAAGTTTTAGTTTATTAACTTGTTTCTCAGACTGCGACGTTTACTGACTGGAGTTTTGTGGTCCTC
TGGCGGGCGCTGGCCGGCCCCCGAACGGGGGTCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTAC
ACATGGGGTGGGAGAGTCGAGCCGGAGCTCGAATCAACTCGGTAATGATCCTTCCGCA

Kat-1

GATCCGAGGTCACCTTCGAGAAAAATTCAAAGGTTCGTCCGGCGGGCGACGCCATCGCGCTCCAAAGCGAGGTGTTTT
CTACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTCGAGGCGCGTCCGCGGAGGACGGAGCCCAATACCAAGCA
GAGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATT
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CCGTTGTTGAAAGTTTTAGTTTATTAACTTGTTTCTCAGACTGCGACGTTTACTGACTGGAGTTTTGTGGTCCTCTGGCG
GGCGCTGGCCGGCCCCCGAACGGGGGTCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATG
GGGTGGGAGAGTCGAGCCGGAGCTCGAATCAACTCGGTAATGATCCATCCGCA

Mel-1

TGATCCGAGGTCACCTTGAGAAAAATTCAAAGGTTCGTCCGGCGGGCGACGCCATGCGCTCCAAAGCGAGGTGTTTTC
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CGTTGTTGAAAGTTTTAGTTTATTAACTTGTTTCTCAGACTGCGACGTTTACTGACTGGAGTTTTGTGGTCCTCTGGCGG
GCGCTGGCCGGCCCCCGAACGGGGGTCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATGG
GGTGGGAGAGTCGAGCCGGAGCTCGAATCAACTCGGTAATGATCCTTCCGCAG

Mel-2
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CATGGGGTGGGAGAGTCGAGCCGGAGCTCGAATCAACTCGGTAATGATCCTTCCGCAG

J-3
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GGCGGGCGCTGGCCGGCCCCCGAACGGGGGTCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACA
CATGGGGTGGGAGAGTCGAGCCGGAGCTCGAATCAACTCGGTAATGATCCTTCCGCAGAG

Mel-3
CCTGATCCGAGGTCAACCTTGAGAAAAATTCAAAGGTTCGTCCGGCGGGCGACGCCATGCGCTCCAAAGCGAGGTGT
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ATGGGGTGGGAGAGTCGAGCCGGAGCTCGAATCAACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAA

N. luteum

G (s)-1
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TCTACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTCGAGGCGCGTCCACAGAGGACGGAGCCCAATACCAAGC
AGAGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGAT
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Q(s)
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CGGGCGCTGGCCGGCCCCCCGAACGGGGGTCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACAC
ATGGGGTGGGAGAGTCGAGCCGGAGCTCGAATCAACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAA

M (13)2
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CATGGGGTGGGAGAGTCGAGCCGGAGCTCGAATCAACTCGGTAATGATCCTTCCGCAGGTTCCACCTACGGAA

San-1
TGATCCGAGGTCACCTTGAGAAAAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGTTTTC
TACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTCGAGGCGCGTCCACAGAGGACGGAGCCCAATACCAAGCAG
AGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTC
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GGGTGGGAGAGTCGAGCCGGAGCTCGAATCAACTCGGTAATGATCCTTCCGCA

N-1
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TCCGTTGTTGAAAGTTTTAGTTTATTAACTTGTTTCTCAGACTGCGACGTTTACTGACTGGAGTTTTGTGGTCCTCTGGC
GGGCGCTGGCCGGCCCCCCGAACGGGGGTCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACA
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M (8)4
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N (12)2
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D. mutila

A-3
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Iso-2
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Q
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N. parvum

Q-2
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I (15)-2
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B.9 Analysis of variance of botryosphaeriaceous species pathogenicity on Pinot noir

B.9.1 ANOVA results on lesion lengths caused by grapevine isolates on detached green shoots.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolates	14	69986.31	4665.75	142.3	<.001
Residual	80	2623	32.79		
Total	95	72609.31			

B.9.2 ANOVA results on lesion lengths caused by non-grapevine isolates on detached green shoots.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolates	10	23903.98	2173.09	105.42	<.001
Residual	48	989.44	20.61		
Total	59	24893.43			

B.10 Analysis of variance of botryosphaeriaceous species pathogenicity on different grapevine varieties

B.10.1 ANOVA results on lesion lengths caused by mycelium colonised agar plugs of four botryosphaeriaceous species on detached green shoots of five grapevine varieties.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Varieties	4	235.24	58.81	1.78	0.141
Isolate	3	2682.41	894.14	27.05	<.001
Varieties x Isolate	12	433.19	36.1	1.09	0.378
Residual	80	2644.43	33.06		
Total	99	5995.27			

B.10.2 ANOVA results on lesion lengths caused from four botryosphaeriaceous species on green shoots of five potted grapevine varieties.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	4	253.46	63.37	0.74	
Species	3	3461.15	1153.72	13.45	<.001
Residual	52	4459.64	85.76		
Total	59	8174.26			

B.10.3 ANOVA results on pathogen recovery distances (mm), below and above the trunk inoculation points on potted grapevines of five different varieties, 4 months after inoculating them with either conidia or mycelium of four botryosphaeriaceous species.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	4	713	178.25	2.22	
Varieties	4	328	82	1.02	0.398
Inoculum	1	21424.5	21424.5	266.8	<.001
Species	3	58045.5	19348.5	240.95	<.001
Varieties x Inoculum	4	28	7	0.09	0.986
Varieties x Species	12	732	61	0.76	0.691
Inoculum x Species	3	21.5	7.17	0.09	0.966
Varieties x Inoculum x Species	12	96	8	0.1	1.000
Residual	156	12527	80.3		
Total	199	93915.5			

B.10.4 ANOVA results on pathogen recovery distances (mm) below and above the trunk inoculation points on potted grapevines of five different grapevine varieties, 7 months after inoculating them with either conidia or mycelium of four botryosphaeriaceous species.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	4	2257	564.2	5.58	
Varieties	4	892	223	2.2	0.071
Inoculum	1	1984.5	1984.5	19.61	<.001
Species	3	16721.5	5573.8	55.09	<.001
Varieties x Inoculum	4	158	39.5	0.39	0.815
Varieties x Species	12	1476	123	1.22	0.277
Inoculum x Species	3	245.5	81.8	0.81	0.491
Varieties x Inoculum x Species	12	162	13.5	0.13	1.000
Residual	156	15783	101.2		
Total	199	39679.5			

B.10.5 Sequence confirmation of the ITS region of botryosphaeriaceous species re-isolated from grapevine woody trunks at 4 months after inoculation

N. australe

GCGTGCGGTAATAAACTAAACTTTCACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAA
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N. luteum

GACCTGACGATAATAACTAAACTTTCACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGAGAA
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N. parvum

TCGTACGTATAACTAAAACTTTCACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGT
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D. mutila

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B.10.6 Results of a preliminary pathogenicity assay with different aged cultures on PDA.

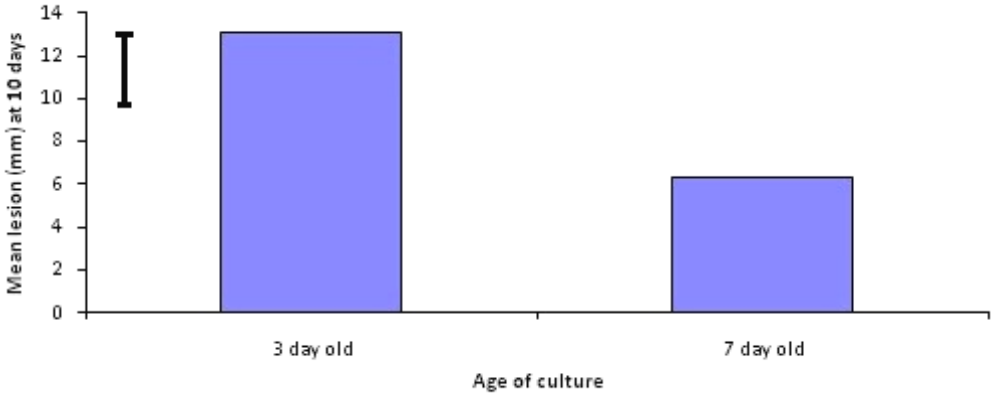


Figure B 1.1: Lesion development on grapevine green shoots inoculated with 3 or 7 days old *N. luteum* mycelial cultures. Bar represents LSD at $P \leq 0.05$.

Appendix C

C.1 Staining procedure

(a) Standard fixation mixture

50 mM Piperazine-N,N'-bis 2-ethanesulfonic acid (PIPES) pH 7.0, 2 mM MgSO4 and 2 mM supplement with 4% formaldehyde, 1.0% Dimethyl Sulfoxide (DMSO) and 0.1% triton X-100 made up in 1 L sterile water.

(b) Stain preparation

1 mg/ml stock solution of the wheat germ agglutinin-fluorescein (WGA-F) in phosphate buffer saline (PBS) (appx 0.06 mM using a mol wt of 17 kD for WGA). Store this solution at 4^{o}C when not being used.

For staining, dilute WGA-F (1/30 in PBS to give 2 uM) was used to label plant infected tissue that is soaked for 1 h. Wash briefly in PBS and mount for viewing by fluorescence microscope wave length of 405 or 488 (Stephens, *et al*., 2008).

C.2 Determination of potting mix moisture levels

The quantity of water in the potting mix was measured as the weight fraction of water compared to the weight of the potting mix in which it is contained. It was then expressed as percent water per 100 grams of the potting mix for which the different water levels were used to calculatemean percentage of water on a weight basis. The amount of the water loss at FC was measured and volume equated to 100%, from which 25, 50 and 75% were calculated and applied.

C.3 ANOVA results on lesion lengths on grapevine green shoots inoculated with 10^2, 10^3, 10^4, 10^5 and 10^6 conidial/mL.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Inoculum dose	4	6318.69	1579.67	27.34	<.001
Residual	45	2600.34	57.79		
Total	49	8919.03			

C.4 ANOVA results on distance of pathogen movement through the shoots from 1 to 4 months and at 9 months.

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Time	4	168091.4	42022.84	1290.84	<.001
Species	3	18361.26	6120.42	188.01	<.001
Time x Species	12	3479.27	289.94	8.91	<.001
Residual	60	1953.27	32.55		
Total	79	191885.2			

C.5 ANOVA results on infection incidence in canes, shoots, trunks, buds, leaf surface and roots.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Inoculation sites	5	54722.2	10944.4	68.52	<.001
Residual	30	4791.7	159.7		
Total	35	59513.9			

C.6 ANOVA results on distance of disease progression through trunks in upward and downward directions.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	3	7617.19	2539.1	76.17	<.001
Direction	1	22876.56	22877	686.3	<.001
Inoculum type	1	2025	2025	60.75	<.001
Time x Direction	3	3429.69	1143.2	34.3	<.001
Time x Inocula type	3	68.75	22.92	0.69	0.564
Direction x Inoculum type	1	1406.25	1406.3	42.19	<.001
Time x Direction x Inoculum type	3	162.5	54.17	1.62	0.196
Residual	48	1600	33.33		
Total	63	39185.94			

C.7 ANOVA results on incidence of *N. luteum* in inoculated grapevine buds.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Bud symptoms	3	10.175	3.3917	20.16	<.001
Residual	196	32.98	0.1683		
Total	199	43.155			

C.8 ANOVA results on the effect of wetness duration and relative humidity on lesion length.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	4	4095.2	1023.8	2.44	
Duration (hr)	6	4219	703.2	1.68	0.128
Humidity (%)	1	29051.9	29051.9	69.38	<.001
Duration (hr) x Humidity (%)	6	9038.1	1506.3	3.6	0.002
Residual	192	80398.1	418.7		
Total	209	126802.4			

C.9 ANOVA results on the effect of tissue age on *N. luteum* incidence.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	5	521.6	104.32	1.35	
Plant tissue age	2	2226.44	1113.22	14.41	<.001
Residual	46	3553.05	77.24		
Total	53	6301.09			

C.10 ANOVA results on the effect of wound age and inoculums type on *N. luteum* incidence.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	4	0.2333	0.0583	0.48	
Inoculum type (IT)	1	0.8167	0.8167	6.7	0.013
wound age (days)	5	6.55	1.31	10.74	<.001
IT x wound age (days)	5	0.6833	0.1367	1.12	0.364
Residual	44	5.3667	0.122		
Total	59	13.65			

C.11 Analysis of variance results for the effect of water stress

C.11.1 ANOVA results on dieback lengths caused by *N. luteum* on Pinot noir own-rooted vines grown in potting mix under different moisture levels.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Inoculation	1	670.2085	670.2085	1200.25	<.001
Soil water level (%)	3	89.5669	29.8556	53.47	<.001
Treatment x Soil water level (%)	3	74.4091	24.803	44.42	<.001
Residual	40	22.3356	0.5584		
Total	47	856.5202			

C.11.2 ANOVA results on number of buds developing from non inoculated and inoculated *N. luteum* trunks after winter pruning of the canes of Pinot noir scion grafted onto 101-14 rootstock grown in soil under different moisture levels.

(A) Total number of buds

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Inoculation	1	1	1	1.47	0.232
Water stress level (%)	2	50.5417	25.2708	37.24	<.001
Treatment x Water stress level (%)	2	4.625	2.3125	3.41	0.043
Residual	42	28.5	0.6786		
Total	47	84.6667			

(B) Total number of dead buds after dormancy break

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Inoculation	1	53.778	53.778	39.98	<.001
Water stress level (%)	2	19.625	9.812	7.29	0.002
Treatment x Water stress level (%)	2	2.097	1.049	0.78	0.465
Residual	42	56.5	1.345		
Total	47	132			

(C) Total number of buds developed into healthy shoots

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Inoculation	1	72.25	72.25	91.95	<.001
Water stress level (%)	2	9.0417	4.5208	5.75	0.006
Treatment x Water stress level (%)	2	13.625	6.8125	8.67	<.001
Residual	42	33	0.7857		
Total	47	127.9167			

(D) Total number of shoots that withered and died

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Inoculation	1	0.028	0.028	0.02	0.884
Water stress level (%)	2	3.375	1.688	1.32	0.279
Treatment x Water stress level (%)	2	0.014	0.007	0.01	0.995
Residual	42	53.833	1.282		
Total	47	57.25			

(E) Dead buds infected with *N. luteum*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Inoculation	1	291.84	291.84	278.05	<.001
Water stress level (%)	2	16.167	8.083	7.7	0.001
Treatment x Water stress level (%)	2	5.389	2.694	2.57	0.089
Residual	42	44.083	1.05		
Total	47	357.479			

(F) Bud death due to other pathogens

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Inoculation	1	1.3611	1.3611	2.81	0.101
Water stress level (%)	2	2.0417	1.0208	2.11	0.134
Treatment x Water stress level (%)	2	0.1806	0.0903	0.19	0.831
Residual	42	20.3333	0.4841		
Total	47	23.9167			

C.11.3 ANOVA results on shoot height (mm) development at 2 months after dormancy break of Pinot noir scion grafted onto 101-14 rootstock grown in soil under different moisture levels.

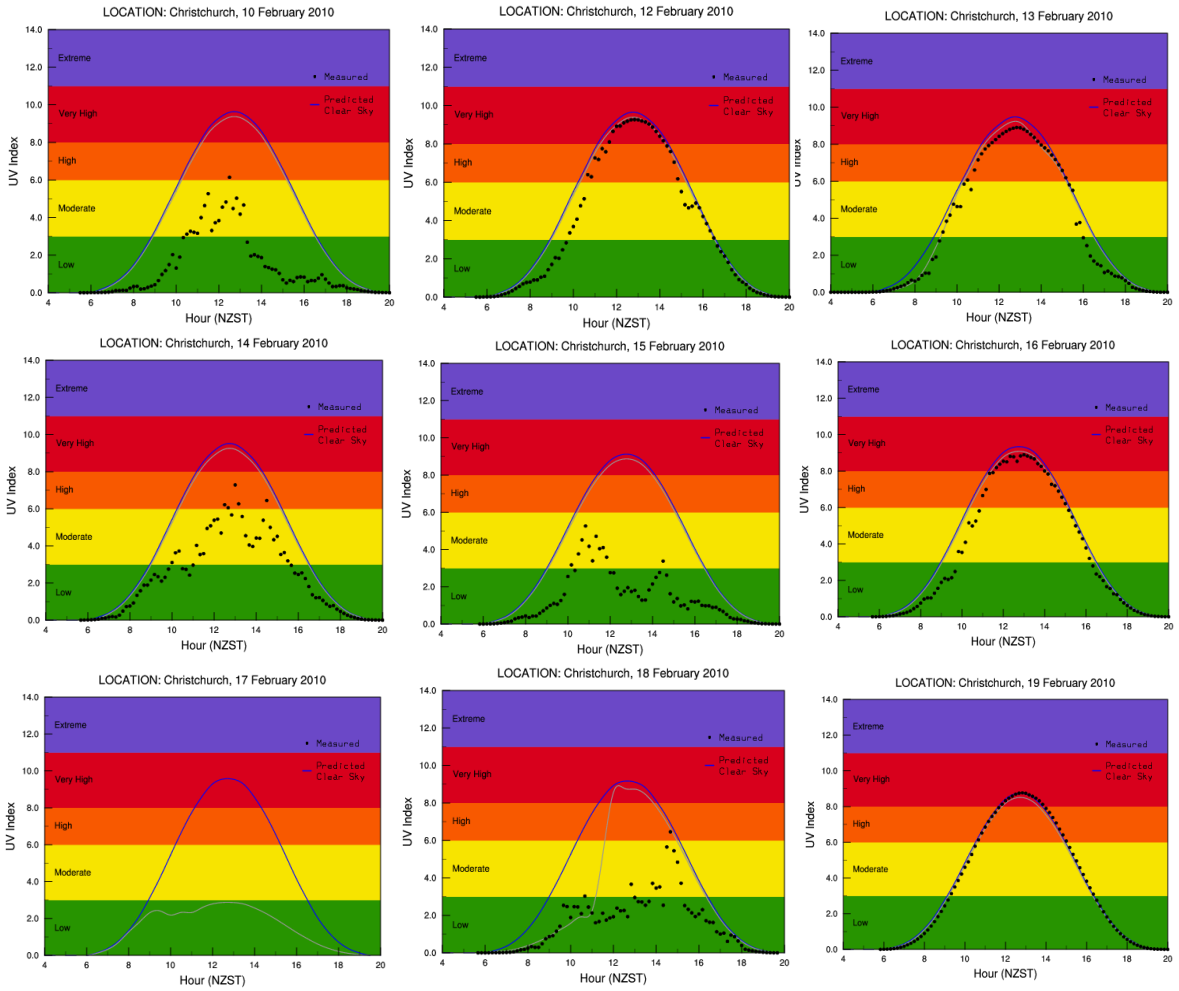
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	3	189363	63121	1.89	
Inoculation	1	994840	994840	29.71	<.001
Water stress level (%)	2	1224758	612379	18.29	<.001
Treatment x Water stress level (%)	2	774242	387121	11.56	<.001
Residual	39	1305838	33483		
Total	47	4489040			

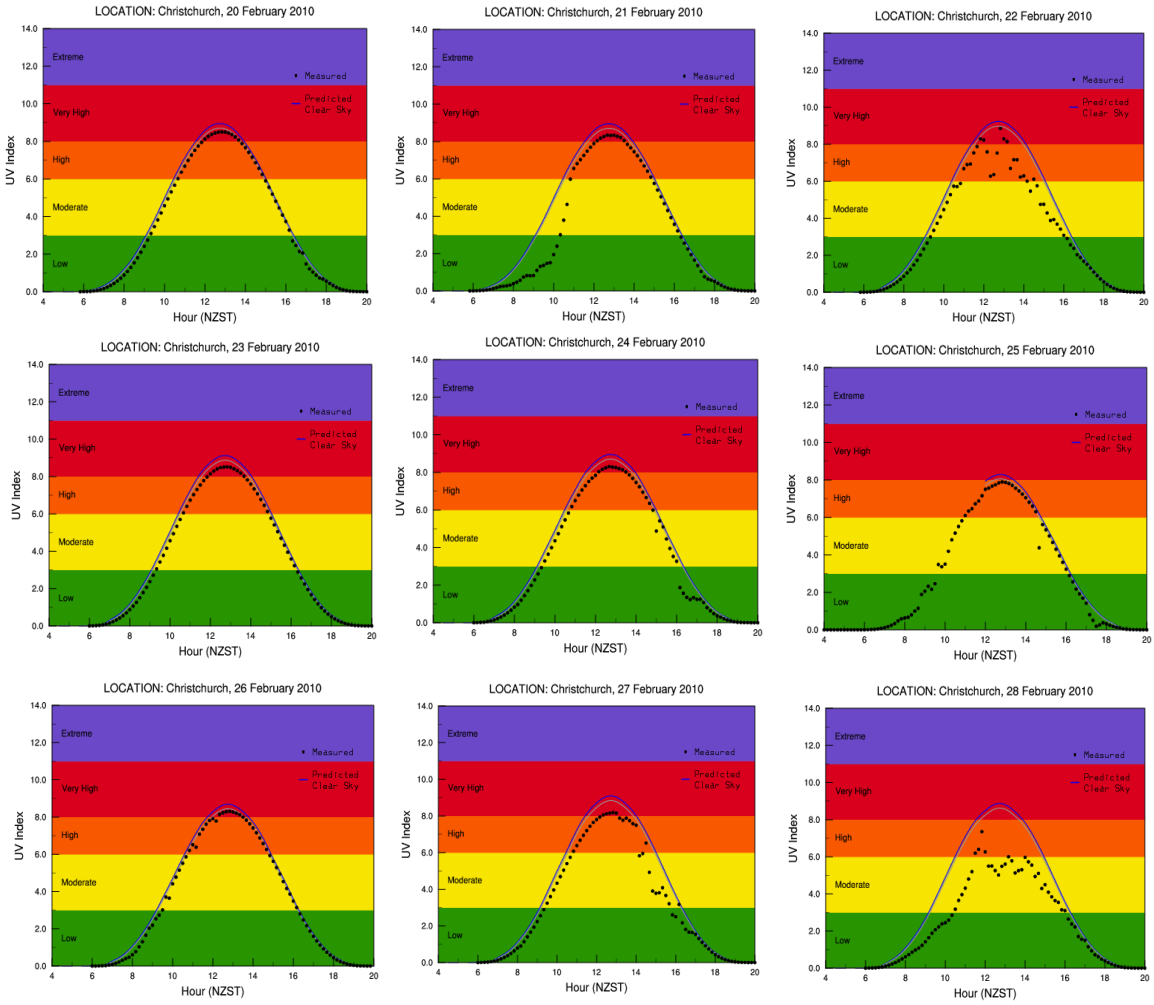
Appendix D

D.1 SSCP gel preparation

Gel Mix (2x)
30 mL Acrylamide (49:1), 3 mL 10x TBE, and 27 mL dH_2O. Mix thoroughly and add 60 µL TEMED and 300µL of 10% APS and pour into gel mould. Make sure that there is no bubble trapped within the gel. Let the gel polymerized for 15-20 minutes at room temperature.

D.2 UV index recorded for Christchurch from the NIWA website for the duration of the experiment on conidial exposure.





D.3 ANOVA results (unbalanced design) on conidial counts (Log_{10}) of botryosphaeriaceous species in rain water during 12 months of spore trapping.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Months	11	841.033	76.458	14.87	<.001
Species	1	28.492	28.492	5.54	0.019
Months x species	11	337.11	30.646	5.96	<.001
Residual	836	4299.774	5.143		
Total	859	5506.409	6.41		

D.4 ANOVA results on the effect of temperature on conidial germination of different botryosphaeriaceous species.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Pathogen	3	1041.15	347.05	24.79	<.001
Temperature (^{o}C)	6	186937.7	31156.28	2225.23	<.001
Time (h)	2	4489.41	2244.7	160.32	<.001
Pathogen x Temp	18	3418.49	189.92	13.56	<.001
Pathogen.Time (h)	6	420.95	70.16	5.01	<.001
Temp. x Time (h)	12	7793.19	649.43	46.38	<.001
Pathogen x Temp. X Time (h)	36	4080.33	113.34	8.1	<.001
Residual	168	2352.23	14		
Total	251	210533.4			

D.5 ANOVA results on the effect of relative humidity and duration on conidial germination of different botryosphaeriaceous species.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	5.9	2.9	0.02	0.977
Relative humidity (%)	3	184992.056	61664.019	19699.65	<.001
Duration (h)	3	7604.889	2534.963	607.38	<.001
Relative humidity x duration	6	12108.611	2018.102	483.54	<.001
Residual	96	400.667	4.174		
Total	107	106218.963			

D.6 Solar radiation and humidity effect on conidial viability

D.6.1 ANOVA results on the effect of exposure times on germination of botryophaeriaceous species conidia to filtered sunlight (-UV), non-filtered sunlight (+UV) and shade.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Exposure times	5	15652.85	3130.57	120.24	<.001
Radiation level	2	115645.6	57822.81	2220.86	<.001
Exposure times × Radiation level	10	5161.15	516.12	19.82	<.001
Residual	144	3749.21	26.04		
Total	161	140208.8			

D.6.2 ANOVA results on the germination of botryophaeriaceous species conidia previously exposed to low RH (68%) and then incubated under high RH (100%) at 25°C.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Exposure time	5	33349.25	6669.85	177.54	<.001
Species	2	24.85	12.43	0.33	0.721
Exposure time x Species	10	245.77	24.58	0.65	0.758
Residual	36	1352.46	37.57		
Total	53	34972.33			

Appendix E

E.1 Fungicides tested against botryosphaericeous species.

Active ingredient	Formulation	Action	Manufacturer	Field rate (/100L water)
Azoxystrobin**	500g/kg WG	Systemic	Syngenta Crop Protection Ltd	40 g
Carbendazim*	500g/L SC	Broad spectrum systemic	Tapuae partnership, NZ	50 mL
Chlorothalonil**	720g/L SC	Contact	Tapuae partnership, NZ	230 mL
Copper hydroxide**	375g/Kg WG	Contact	Nufarm Ltd	107-210 g
Dithianon**	700g/kg WG	Protectant	BASF New Zealand LTD	55 g
Fenarimol**	120g/L SC	Systemic	DuPont NZ	20 mL
Flusilazole*	200g/kg WG	Systemic	DuPont NZ	10-15 g
Iprodione**	250g/L SC	Broad spectrum contact	Bayer NZ Ltd	150-200 g
Mancozeb **	200g/kg WG	Contact	DuPont NZ	210 g
Metalaxyl*	250g/L EC	Systemic	Tapuae partnership, NZ	22-30 g
Prochloraz*	450g/L EC	Systemic	Agronica NZ Ltd	100 g
Procymidone**	500g/L SC	Contact	Nufarm Ltd	110 ml
Pyrimethanil**	400g/L SC	Curative and protectant	Bayer NZ Ltd	200 mL
Tebuconazole*	430g/L SC	Systemic	Nufarm Ltd	20 g
Thiophanate methyl**	400g/L SC	Systemic	Dow AgroScience (Ltd) NZ	100 mL
Triforine*	190g/L EC	Systemic	BASF New Zealand LTD	28.5 g

WG= Water Dispersible Granule, EC= Emulsifiable Concentrate, SC= Suspension Concentrate * Not registered for grapes in New Zealand

** Registered for grapes in New Zealand for control of Botrytis, Anthracnose, Powdery mildew, Downy mildew and Black spot diseases.

E.2 ANOVA results on EC_{50} values of fungicides on *in vitro* mycelial growth.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Fungicide	13	178073.7	13698	74.73	<.001
Species	2	3686.9	1843.5	10.06	<.001
Fungicide x Species	26	21717	835.3	4.56	<.001
Residual	74	13563.5	183.3		
Total	115	182489.8			

E.3 ANOVA results on EC_{50} values of fungicides on *in vitro* conidial germination.

Source of variation	df	SS (type 111)	m s	v.r	Fpr.
Corrected Model	42	416908.548[a]	9926.394	85.647	0.00
Intercept	1	873365.504	873365.504	7535.607	0.00
fungicides	13	301262.58	23174.045	199.951	0.00
species	2	22564.155	11282.078	97.344	0.00
Log-concentration	1	213809.773	213809.773	1844.802	0.00
fungicides x species	26	17824.498	685.558	5.915	0.00
Error	797	92371.1	115.898		
Total	840	3056294.028			
Corrected Total	839	509279.648			

NB: [a]R Squared=0.819 (Adjusted R Squared=0.809)

E.4 ANOVA results on the effects of fungicides on dieback on detached green shoots.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Fungicides	12	16931.05	1410.92	569.61	<.001
Residual	52	128.805	2.477		
Total	64	17059.85			

E.5 ANOVA results on the effects of fungicides on percent *N. luteum* isolation from lesions on detached green shoots.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	12	169.8462	14.15385	306.67	<.001
Residual	52	2.4	0.04615		
Total	64	172.2462			

E.6 ANOVA results on effect of fungicides on lesion lengths after conidial inoculation *in vivo* on potted grapevines.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	4	395.51	98.88	1.01	
Treatment	13	32527.17	2502.09	25.53	<.001
Residual	52	5096.55	98.01		
Total	69	38019.23			

E.7 ANOVA results on the effect of fungicides on percent *N. luteum* isolation from treated and inoculated canes on potted vines.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	13	87.5429	6.7341	25.22	<.001
Block	4	0.5143	0.1286	0.48	0.749
Residual	52	13.8857	0.267		
Total	69	101.943			

E.8 ANOVA results the effect of fungicides on percent *N. luteum* isolation from treated and inoculated canes on mature field vines.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	9	32.2027	3.5781	32.45	<.001
Isolation sites	4	69.196	17.299	156.9	<.001
Treatment x Isolation sites	36	20.484	0.569	5.16	<.001
Residual	1450	159.8667	0.1103		
Total	1499	281.7493			

E.9 Mode of action of some of the fungicides used for field study.

Chemical group	Example of fungicide	Mode of action
Benzimidazole	carbendazim†	Inhibition of mitosis and cell wall division
	thiophanate methyl†	
Dicarboximides	iprodione†	Inhibition of protein synthesis (signal transduction inhibitors
	procymidone†	
DeMethylation Inhibitors	tebuconazole†	Disruption of cell membrane
	prochloraz†	
	fenarimol†	
	flusilazole*	
Copper	mancozeb†	Multi site
	copper hydroxide†	

*Flusilazole is a new substituted triazole fungicide recently developed for the control of a variety of plant diseases and its mode of action is by inhibiting ergosterol (component of fungal cell membranes) biosynthesis through direct inhibition of the14α-demethylation (key step in sterol biosynthesis in eukaryotes) of ergosterol precursors (Henry 1990).

†http://www.dropdata.org/RPU/pesticides_MoA.htm#fungicides (10/12/09)