Botryosphaeria infections in New Zealand grapevine nurseries: Sources of inoculum and infection pathways

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A thesis

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Doctor of Philosophy in Plant Pathology

by

Regina Billones-Baaijens

Lincoln University

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The botryosphaeriaceous fungi can cause decline, dieback and death of grapevines. Anecdotal evidence has indicated that these pathogens might be present in the young vines sold by propagation nurseries, so this study investigated their role in spread of this disease. Sampling of grapevine nurseries across New Zealand showed that botryosphaeriaceous infections were present in eight out of nine nurseries with infection incidence ranging from 5 to 63%. Of the 311 propagation materials and plants received, 23% were positive for botryosphaeriaceous infection, with a total of 120 isolates recovered. The highest incidence was in failed grafted plants (33%), followed by Grade 1 plants (28%), rootstock cuttings (19%), scion cuttings (17%) and Grade 2 plants (7%). For grafted plants, the majority of botryosphaeriaceous species (49%) were isolated near the graft unions while infections on rootstocks and scion cuttings were mostly from the middle and basal parts. Identification of isolates by morphological and molecular methods showed that the six species commonly found in vineyards also occurred in nurseries, being *Neofusicoccum luteum* (57%), *N. parvum* (18%), *N. australe* (8%), *Diplodia mutila* (8%), *Botryosphaeria dothidea* (5%) and *D. seriata* (3%), with one novel isolate of *N. macroclavatum* and two unidentified botryosphaeriaceous isolates.

Pathogenicity tests using one-year-old Sauvignon blanc rooted canes and green shoots showed that the seven identified botryosphaeriaceous species from the nurseries were pathogenic but pathogenicity differed significantly between species and isolates within a species, with *N. parvum* being the most pathogenic in both assays ($P \leq 0.001$). Genetic variability analysis using UP-PCR showed that *N. luteum* isolates of different pathotypes were genetically diverse with intra- and inter-plant and nursery variability but no association between genotype and pathogenicity was observed. Susceptibility tests using three isolates each of *N. luteum*, *N. parvum* and *N. australe* against the most commonly used scion and rootstock varieties (six of each) found that all varieties were susceptible to the three species with 5C and SO4 being the most susceptible of the rootstock varieties, and Merlot and Pinot noir being the most susceptible of the scions.

Investigations into the sources of inoculum conducted in three nurseries in 2009 using conventional and molecular methods showed that the mothervines used to provide cuttings for propagation were the most likely source of botryosphaeriaceous infections. Propagules were detected on the surfaces of cuttings and dead grapevine materials, as well as in rain-water run-off, but not in soil samples collected from the mothervine blocks. The different botryosphaeriaceous isolates recovered from canes were mostly sited within the bark suggesting presence of latent infections. Since the isolates from mothervine trunk and canes were distributed in isolated patches and the UP-PCR assessment showed that they belonged to multiple genotypes and species, this indicated that they were spread from external sources. Microscopy and plating assays were not able to detect botryosphaeriaceous contamination from any of the nursery propagation stages but molecular methods using multi-species primers detected botryosphaeriaceous DNA in samples from a wash pit, pre-cold storage hydration tanks, post-grafting hydration tanks, grafting tools and callusing medium from the different nurseries.

An investigation into the probable survival of *N. luteum* conidia during the processing of cuttings showed that they adhered rapidly and could not be totally washed from the surfaces of cuttings after 0 h, with minimal recovery of conidia after 1, 2 and 4 h incubation at room temperature. During this time they germinated and colonised the periderm, phloem and xylem but not the pith of the cuttings. At storage temperatures of 2 and 8°C some conidia were able to germinate. At 2°C, conidial germination was 4% after 48 h, the number gradually increasing to 17% after 3 weeks. At 8°C, conidial germination was 23% after 24 h, the number gradually increasing to 34% after 72 h with no further increase observed up to 2 weeks. Cold storage did not kill all conidia, since conidia from 72 h and 3 weeks storage at 2°C had 84% and 33% viability, respectively, while those from 8°C storage had 72% and 39% viability after 72 h and 2 weeks storage, respectively. The *N. luteum* conidia stored at different temperatures were similarly pathogenic compared to freshly-harvested conidia but higher pathogenicity was observed on conidia stored at 2°C for 48 to 72 h.

Overall results suggest that botryosphaeriaceous infection in new vineyards may originate from latent infection that began in the nurseries, thus management of this disease should start at the nursery level to prevent this pathogen from being carried over into new vineyards.

Keywords: Botryosphaeriaceous species, *Diplodia*, *Neofusicoccum*, grapevine nurseries, inoculum sources, latent infections, pathogenicity, susceptibility, rootstocks, scions

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Chapter 1
General Introduction

1.1 Grapevine industry of New Zealand

The New Zealand grapevine industry started in the early nineteenth century when French settlers and religious missions introduced grapes for winemaking (Trought, 2006). The progress of viticulture in the country, however, was slow during this period especially in the North Island since the climate is not suitable for grape-growing. The high rainfall and high humidity around Auckland resulted in high mortality due to disease outbreaks (Jackson & Schuster, 1994). By 1906, about 220 hectares was planted with grapes but this declined to 72 hectares by 1923. The progress of the industry after that was slow with a total of 500 hectares being planted by 1965. During this period, the wine industry produced more fortified wine than table wine (Reeves, 2005). The new trend for production of table wine in New Zealand started in the 1970s when there was an increase in the area planted, and by 1984 production of fortified wine had been completely discontinued (Reeves, 2005).

At present, the grapevine industry is one of the major horticultural industries in New Zealand, since viticulture now occupies more land than kiwifruit or apples (Reeves, 2005). In 2010, the New Zealand grape industry occupied approximately 33,428 hectares of grapes (Figure 1.1) from which $1.04 billion worth of wine was exported *(*New Zealand Winegrowers Statistical Annual, 2010). The three main grape growing regions (Gisborne, Hawkes Bay and Marlborough) accounted for 90% of the total grapes in production *(*New Zealand Winegrowers Statistical Annual, 2010). Despite this, New Zealand is a tiny player in the world wine market with only about 1% of the total global wine production. However, the industry is rapidly expanding. The number of wineries has increased from 131 in 1990 to 672 in 2010 (New Zealand Winegrowers Statistical Annual, 2010). The producing vineyard area has significantly increased in the last decade and is projected to reach 33,600 hectares in 2012 (Figure 1.1; New Zealand Winegrowers Statistical Annual, 2010).

At present, approximately 54 varieties of grapevines are planted in 10 regions across New Zealand (New Zealand Winegrowers Statistical Annual, 2010). Sauvignon blanc and Chardonnay dominate the white wine grapes covering 50% and 12% of the total producing hectares, respectively. Pinot noir, on the other hand, is the dominant red variety (15%) followed by Merlot (4%).

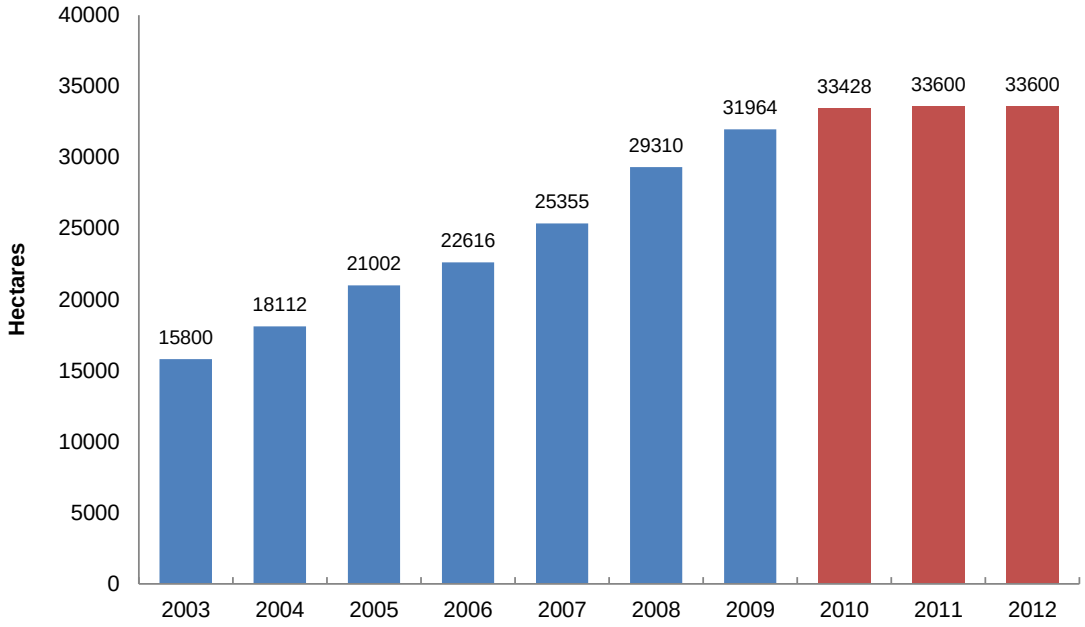

Figure 1.1 Increase in producing vineyard area from 2003 to 2009 and the projected total area to 2012 (New Zealand Winegrowers Statistical Annual, 2010).

1.2 Trunk diseases of grapevines

In the past, the main focus of pathology research in viticulture has been on external foliar and fruit pathogens, mainly because these diseases can cause obvious and significant crop losses which are directly attributed to the causal pathogens (Whiteman, 2004). However, trunk disease of grapevines have become a particular concern in New Zealand in the last decade because the national vineyard has aged, with 47% of the vineyard now being ≥ 8 years, so trunk diseases are likely to be more prevalent and cause significant losses (Larignon & Dubos, 2001). Unlike foliar diseases, they also persist within the affected vines from season to season causing long term effects which can reduce/damage the sustainability of a grapevine industry (Pascoe & Cottral, 2000). These diseases can significantly reduce yields and increase production costs as a result of the cultural and chemical controls to reduce infections (Urbez-Torres *et al.*, 2006a). Some of the most important trunk diseases of grapevines are described below:

1.2.1 Eutypa dieback

Eutypa lata is an ascomycete fungus that is the causal pathogen of Eutypa dieback, also called “dying arm disease”, of grapevines (Tey-Rulh *et al.*, 1991). This disease causes serious economic losses in many major grape-producing areas around the world (Mahoney *et al.*, 2003). Symptoms of Eutypa dieback include stunting of new shoots, cupping and marginal necrosis of leaves, dryness of inflorescences resulting in poorly developed fruit clusters and death of branches (Figure 1.2A). According to Tey-Rulh *et al.* (1991) cankers often develop around large pruning wounds on trunks and cordons, and cross-sections of the cankers demonstrate the characteristic wedge-shaped necrosis (Figure 1.2B). This pathogen is known to produce toxic compounds including eutypine that are involved in the expression of foliar and shoot symptoms such as ‘witches broom’, in which thin weak stems with small chlorotic leaves are clustered together (Amborabé *et al.*, 2001). Eutypa dieback was reported to have infected 20% of the mature grapevines in the Charentes vineyard and caused 80-90% of vines to be damaged in some vineyards in the Bordeaux area in France (Tey-Rulh *et al.*, 1991).

According to Pearson and Goheen (1998) the perithecia of *E. lata* develop in infected wood that has been dead for at least 2 years. The wind-borne ascospores are released in winter and spring after rainfall of more than 1 mm. These ascospores can infect pruning wounds for up to 4 weeks after pruning.

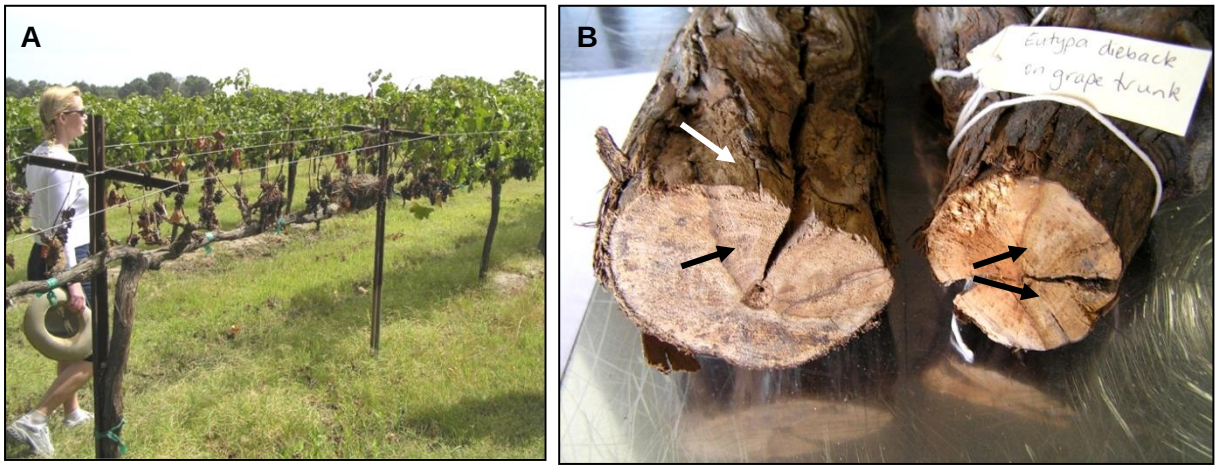

Figure 1.2 Symptoms of *Eutypa lata.* A) Death of vine and B) cross-section of a canker in the wood (white arrow) with the characterisitic wedge-shaped necrosis (black arrows) (Photographs by Marlene Jaspers)

1.2.2 Petri disease and Esca

Esca is a disease complex in grapevines that causes structural and physiological changes in the plants (Mugnai *et al.*, 1999; Feliciano *et al.*, 2004) and which often includes the Petri disease pathogens. Petri disease, formerly known as “black goo” decline, affects young grapevines, causing establishment problems in new vineyards and is associated with *Phaeomoniella chlamydospora* and *Phaeoacremomium* spp. (Feliciano *et al.*, 2004; Pascoe *et al.*, 2004). Its symptoms include graft failure, poor growth, shoot dieback, decline and gradual death of young vines with a characteristic wood streaking (Figure 1.3A) and production of black “goo” emerging from affected xylem vessels, which can be visible in cross-sections as black raised dots (Figure 1.3B; Edwards & Pascoe, 2004). The airborne-conidia were reported to infect pruning wounds (Larignon, 1998). The use of asymptomatic, but infected grapevine cuttings for grafting was considered a major means of dissemination of Petri disease (Retief *et al.*, 2006; Whiteman *et al.*, 2007; Aroca *et al.*, 2010;).

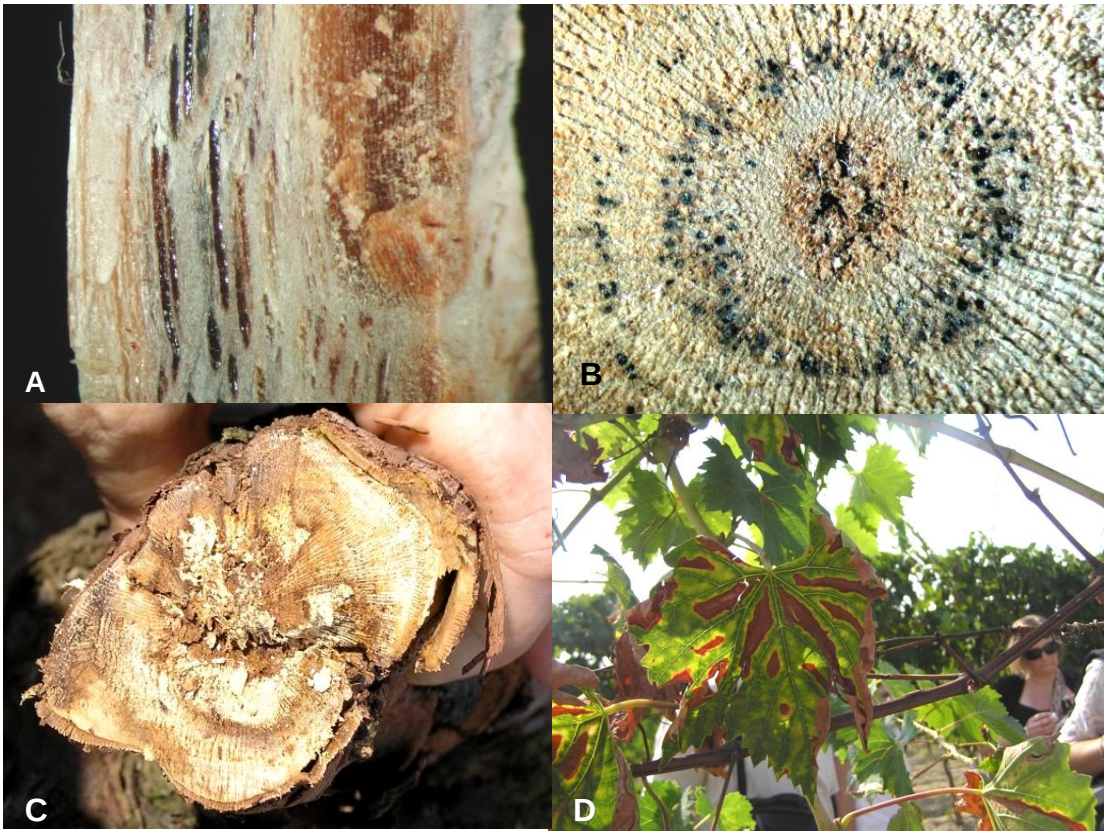

Figure 1.3 Symptoms of Petri disease. A and B) longitudinal and cross sections of wood showing the streaking and production of black “goo” in affected xylem vessels. Symptoms of Esca. C) White rot of wood; and D) leaves showing interveinal chlorosis and necrosis. (Photographs by Sonia Whiteman and Marlene Jaspers)

Esca affects mature vines, normally 10 years or older, causing apoplexy and death of vines in Europe (Mugnai *et al.*, 1999; Stamp, 2001) but it was also reported to affect young vines (Mugnai *et al.*, 1999). The most common internal symptom is white rot, which causes the hard wood to become cream-coloured or white, soft spongy mass (Figure 1.3C). In cross-sections, the white rot is often seen to be bordered by a dark line separating the decayed from the non-decayed wood, in which the black dots characteristic of Petri disease are often visible (Mugnai *et al.*, 1999). Leaves of infected vines exhibit interveinal chlorosis that appear as yellow to red and brown patterns (Figure 1.3D; Stamp, 2001). The aetiology of esca was believed to reflect the succession of infections, first by a Petri disease pathogen, *Ph. chlamydospora* or one of the *Phaeoacremomium* spp., followed by infection of the wood rotting basidiomycete *Fomitiporia punctata* (Sparapano *et al.*, 2000a). The Petri disease pathogens were believed to act as precursors to the wood decay (Larignon & Dubos, 1997), but recent reports have also shown that although these fungi can act as primary pathogens, *F. punctata* does not need prior colonisation by "precursor" species to invade and to degrade the woody tissue of grapevine (Sparapano *et al.*, 2000a, 2000b). However, it is generally believed that the fully-fledged esca syndrome, is caused by the combined or successive action of the Petri disease pathogens *Ph. chlamydospora* or *Phaeoacremomium* spp. accompanied by *F. punctata* (Graniti *et al.*, 2000). Mugnai et al. (1999) suggested that the Petri disease pathogens were frequently present in the propagation materials in a latent form when they were introduced in new vineyards while basidiospores of *F. punctata* could be disseminated by wind or spread through contaminated tools used for grafting and pruning.

1.2.3 Black foot disease

Black foot disease, which is associated with stunting and decline (Figure 1.4A) as well as dieback is caused by the soil-borne *Cylindrocarpon* spp. (Fourie & Halleen, 2006). In New Zealand, *C. macrodidymum*, *C. destructans*, and *C. liriodendri* were the main pathogens found causing black foot (Bleach *et al.*, 2007). Black foot disease is considered one of the most important trunk diseases affecting grapevines in nurseries and young vineyards as it ultimately causes their death (Halleen *et al.*, 2007). The soil-borne inoculum infects the vines via the roots and basal ends of the trunk (Bleach *et al.*, 2008b). Infected vines show sunken necrotic root lesions as well as reduced root biomass and root hairs and may develop a second tier of roots in compensation (Figure 1.4B; Alaniz *et al.*, 2007). When the rootstocks are cut in cross-section, they reveal black necrosis from the basal end of the rootstock (Figures 1.4C and D; Alaniz *et al.*, 2007; Bleach *et al.*, 2008b).

Figure 1.4 Symptoms of black foot disease. A) Stunting of vine; B) development of secondary roots (arrows); and C and D) black necroses from the basal end of the rootstocks. (Photographs by Carolyn Bleach and Marlene Jaspers).

1.3 Botryosphaeriaceous diseases of grapevines

Botryosphaeriaceous species are considered serious pathogens that cause cankers and dieback in many woody hosts. However, their importance in grapevines was not given much attention until recently, because they were generally regarded as saprophytes or weak pathogens (Phillips, 2002). The most important canker and dieback pathogen of grapevines for many years was thought to be *E. lata*, the causal agent of Eutypa dieback (Castillo-Pando *et al.*, 2001). Recently, however, the botryosphaeriaceous spp. have been found to be responsible for some of the cankers and dieback previously attributed to *E. lata* and so they have gained importance as pathogens of grapevines worldwide (Taylor *et al.*, 2005). Annual losses for botryosphaeriaceous disease are estimated to be 4-20% in Bordeaux vineyards in France (Larignon *et al.*, 2001) and 25-30% in South Africa (Pearson & Goheen, 1998).

1.3.1 Symptoms of botryosphaeriaceous infections

In spite of the range of symptoms associated with botryosphaeriaceous species, diagnoses are normally difficult because symptoms often resemble those caused by other pathogens such as *Phomopsis viticola* and *E. lata* (Castillo-Pando *et al.*, 2001). Botryosphaeriaceous diseases from different countries were also given different common names based on certain sets of symptoms, which often overlapped, creating confusion between diseases (van Niekerk *et al.*, 2004). The symptoms associated with different botryosphaeriaceous spp. as reported in published literature are listed in Table 1.1.

Although cane or arm dieback (Figure 1.5A) is one of the most obvious symptoms of botryosphaeriaceous infection, another common symptom associated with stem infection is bud necrosis, which occurs in shoots that are infected by the fungus. Infected shoots may have elongated brown lesions developing from wounds (Figure 1.5B). The base of the infected shoot may become swollen with a blackened cortex that ruptures (Figure 1.5B) making the branch fragile, so that it can collapse or die back. Infected canes may show internal browning (Figure 1.5C) and as they mature, their surfaces turn grey or whitish with small black spots that are the black fruiting bodies (Figure 1.5D) embedded in the host tissues (Phillips, 1998). The bleached appearance is similar to the symptoms of Phomopsis cane blight and leaf spot (Phillips, 2000).

Arm and trunk dieback often starts from a pruning wound (Figure 1.5E) and spreads down to the trunk. Small black spots can sometimes be observed in cross-sectioned trunks and arms. These may initially appear similar to the symptoms of Petri disease but the spots are more diffuse and do not ooze the black tarry exudate characteristic of that disease (Castillo-Pando *et al.*, 2001; Phillips, 2002). Wood necrosis is often associated with pruning wounds and when the wood is cut open different shapes of necrosis are visible that often reflect the position of entry wounds (Figure 1.5F; Table 1.1). External cankers may be visible (Larignon & Dubos, 2001; Larignon *et al.*, 2001), beneath which the wood appears necrotic. In longitudinal section, it appears as brown streaking beneath the bark, whereas in cross-section the common internal wood symptom is a wedge-shaped necrotic sector (Fig. 1.5G; van Niekerk *et al.*, 2004). This symptom is similar to the wood symptom of Eutypa dieback, but the infected vines do not show the characteristic shortened internodes and stunted, malformed chlorotic leaves (Castillo-Pando *et al.*, 2001). Another common symptom is the arch-shaped necrotic lesion which may originate from wound infections like severe pruning or girdling of trunks, suckering wounds and wounds from water shoot pruning (van Niekerk *et al.*, 2004).

Botryosphaeriaceous infection may also cause foliar symptoms as a result of wood infection. According to Larignon and Dubos (2001), the mild symptoms in red varieties include wine red patches at the margin of the leaves (Table 1.1). These patches may grow and coalesce to form large red areas between the veins and margins of the leaves. The discoloured areas often become necrotic, leaving a green zone along the main veins. The foliar symptoms are similar to those of Esca, however, unlike Esca disease, symptoms of botryosphaeriaceous disease usually appear early in the season while the Esca symptoms are observed in mid to late summer. Symptoms on white varieties include yellowish orange spots on leaves which coalesce, and do not resemble symptoms of Esca disease (Larignon & Dubos, 2001; Larignon *et al.*, 2001; van Niekerk *et al.*, 2004).

Shrivelling and drying of inflorescences and young fruit clusters may also be caused by botryosphaeriaceous infection (Table 1.1; Larignon & Dubos, 2001; Larignon *et al.*, 2001). Some species have been reported to directly infect the fruit, causing severe berry and cluster rot. Berries, which are usually infected near ripening, develop a brown rot, sometimes cracking and dripping juice, and may be covered with a white cottony mass of mycelium. They become dark brown, shrivelled and mummified (Pearson & Goheen, 1998).

In the nurseries, there are few reports on the symptoms of botryosphaeriaceous infections on nursery plants. However, some botryosphaeriaceous species were found to be associated with reduced vigour and decline of young plants (Gimenez-Jaime *et al.*, 2006) while some species were associated with incomplete graft unions (Phillips, 2002).

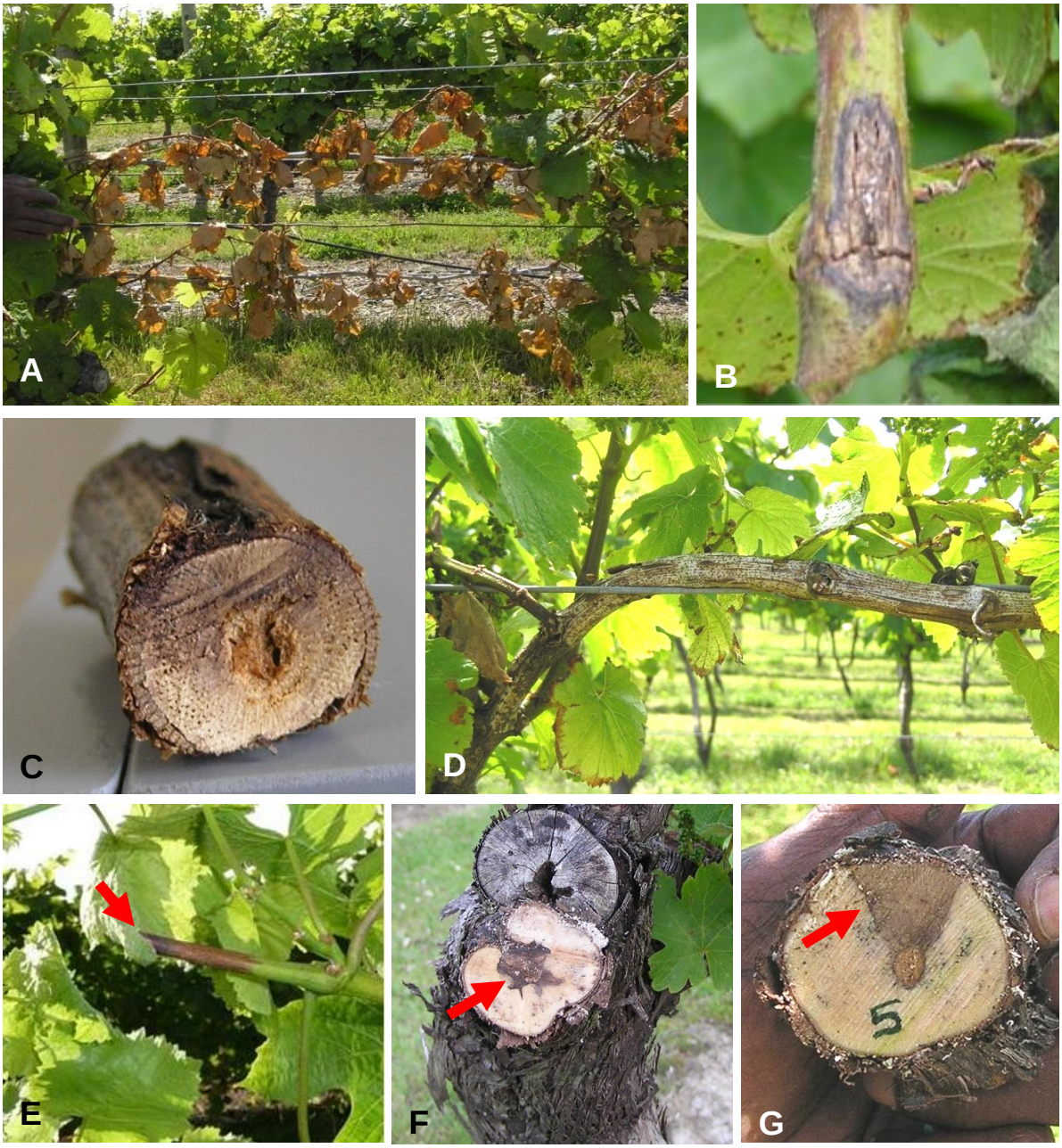

Figure 1.5 Symptoms of botryosphaeriaceous species infection. A) Dead arm; B) cane canker; C) internal cane staining; D) cane bleaching; E) dieback from pruning wound (arrow); F) internal wood necrosis (arrow) and G) wedge-shaped necroses (arrow) on wood similar to the symptom of *Eutypa lata.* (Photographs by Nicholas Amponsah and Marlene Jaspers).

Table 1.1 Symptoms associated with different botryosphaeriaceous species as reported in published literatures. [a]

Symptoms	*Botryosphaeria dothidea*	*Diplodia seriata*	*Neofusicoccum parvum*	*N. australe*	*Lasiodiplodia theobromae*	*N. luteum*	*Diplodia mutila*	Reference[b]
Country	Chile, France, New Zealand, Portugal, Spain	Australia, Chile, France, New Zealand, Mexico, Portugal, USA	New Zealand, Portugal, Spain, South Africa, USA	Australia, New Zealand, South Africa, USA	Australia, Mexico, Spain, USA	New Zealand, Spain, South Africa, USA	Australia, New Zealand, South Africa, USA	
External symptoms								
Necrotic buds	x	x	x			x	x	1; 2; 3; 5; 6;
Cane dieback	x	x	x		x	x	x	10
Cane bleaching	x	x	x			x	x	
Dead arm	x	x						
Chlorotic leaves, leaf necrosis	x	x						2; 5; 6
Red patches on leaf margins	x	x						6
Drying of influorescence and fruit clusters	x	x						5; 6; 8, 11
Berry rot	x	x	x		x		x	
Internal symptoms								
Wedge-shaped necrotic lesions	x	x	x	x	x	x	x	1; 3; 5; 6; 7;
Arch-shaped necrotic lesions				x				9
Brown streaking of wood	x	x	x		x	x	x	
Blackened cortex	x							
Nursery Symptoms								
Reduced vigour of nursery plants		x						1, 4
Reduce foliage		x						
Reduced leaf size		x						
Incomplete grafts		x	x			x		

[a] Table adapted from van Niekerk *et al.,* (2004)

[b] 1) Phillips, 2000; 2) Martin & Cobos, 2007; 3) van Niekerk *et al.,* 2006 ; 4) Gimenez-Jaime *et al.,* 2006 ; 5) Larignon *et al.,* 2001; 6) Larignon & Dubos, 2001; 7) Whitelaw-Weckert *et al.,* 2006; 8) Pearson & Goheen, 1998; 9) Castillo-Pando *et al.,* 2001; 10) Wood & Wood, 2005; 11) Wunderlich *et al.,* 2009.

1.4 The taxonomy of botryosphaeriaceous species

The fungal genus *Botryosphaeria* established by De Cesati and de Notaris in 1863 was a species-rich genus with cosmopolitan distribution (Crous *et al.*, 2006). Anamorphs of *Botryosphaeri*a species were not clearly established because the morphological features that separate them were poorly defined. This resulted in a multiplicity of names applied to similar fungi (Phillips, 2000). As many as 18 anamorph genera have been linked to *Botryosphaeria* but this number has been reduced to synonymy under *Diplodia* and *Fusicoccum* (Crous *et al.*, 2006). Those species with fusoid, hyaline, thin-walled conidia that may become septate and translucent brown prior to germination belong to the *Fusicoccum* group while those species with 0-1 euseptate conidia, that are mostly ovoid, thick-walled and opaque brown when mature, are members of *Diplodia*. These species normally have prominent deposits on their conidial walls that give impressions of striations, although their conidial walls are normally smooth (Denman *et al.*, 2000; Crous *et al.*, 2006). The *Lasiodiplodia* Ellis & Everh. spp. were considered as part of the *Diplodia* complex (Denman *et al.*, 2000) but the description of *L. gonubiensis* Pavlic, Slippers & M.J. Wingf. sp. nov. has shown that this group is morphologically distinct based on its unique conidial striations and should be considered a separate *Botryosphaeria* lineage (Pavlic *et al.*, 2004).

Recent taxonomic studies using the DNA sequence of 28S ribosomal DNA have identified twelve phylogenetic lineages under the botryosphaeriaceous sub-family (Crous *et al.*, 2006). The most common botryosphaeriaceous species occurring in grapevines are currently placed in the anamorphic genera *Neofusicoccum*, *Lasiodiploda/Diplodia* and *Botryosphaeria* (Denman *et al.*, 2000; Crous *et al.*, 2006).

Neofusicoccum parvum (Pennycook and Samuels, 1985) (syn. *B. parva*) produces hyaline, aseptate, thin-walled conidia that become darker and 1 or 2 septate before germination (Figure 1.6A; http://www.crem.fct.unl.pt/botryosphaeria_site). This species was first reported to be associated with grapevine decline in Portugal in 2002 (Phillips, 2002) but its pathogenicity on grapevines was first assessed in South Africa (van Niekerk *et al.*, 2004). Recent studies showed that this species is widespread, being found to infect grapevines worldwide (Table 1.1; Phillips, 2002; van Niekerk *et al.*, 2004; Martos, 2008; Urbez-Torres & Gubler, 2009). In New Zealand, *N. parvum* was first reported as pathogen of kiwifruit (*Actinidia deliciosa*) (Pennycook & Samuels, 1985) and was the most prevalent species found in symptomatic mature grapevines around the country (Baskarathevan *et al.*, 2008). Pathogenicity studies showed that this species can cause severe lesions and

cankers in different types of grapevine tissues and is considered one of the most pathogenic botryosphaeriaceous species in grapevines worldwide (van Niekerk *et al.*, 2004; Taylor *et al.*, 2005; Martos, 2008; Urbez-Torres & Gubler, 2009).

Neofusicoccum luteum (Pennycook & Samuels, 1985; *syn. B. lutea*) also produces hyaline, aseptate thin-walled conidia (Figure 1.6B; http://www.crem.fct.unl.pt/botryosphaeria_site). This species is most easily distinguished from other botryosphaeriaceous species by its yellow pigment in young cultures (Slippers *et al.*, 2004b). The origin of *N. luteum* is unknown although it is commonly found throughout Australasia and Portugal (Slippers *et al.*, 2004b). This species was also first reported as a pathogen of kiwifruit in New Zealand in 1985 (Pennycook & Samuels, 1985) and was reported in 2002 as pathogen of grapevines in Portugal (Phillips, 2002). Most recent studies showed that this species is widespread in major grapevine regions around the world (Table 1.1) including New Zealand (Amponsah *et al.*, 2008b; Baskarathevan *et al.*, 2008; Martos, 2008; Urbez-Torres & Gubler, 2009). Pathogenicity studies in South Africa found this species to be a weak pathogen although this study only used one isolate (van Niekerk *et al.*, 2004) but recent studies showed that this species was highly pathogenic to grapevines (Amponsah *et al.*, 2008b; Martos, 2008; Urbez-Torres & Gubler, 2009).

Neofusicoccum australe Slippers, Crous & Wingfield (syn. *B. australis*) produces conidia that are hyaline, aseptate and smooth with granular contents (Figure 1.6C; http://www.crem.fct.unl.pt/botryosphaeria_site). This species also produces yellow pigment in young cultures and is morphologically and genotypically similar to *N. luteum* (Slippers *et al.,* 2004b). This species was originally isolated from *Acacia* and *Sequoiadendron* species in Australia and was believed to be confined to the southern hemisphere (Slippers *et al.*, 2004b). It has since been reported as a grapevine pathogen around the world (Table 1.1; van Niekerk *et al.*, 2004; Taylor *et al.*, 2005; Urbez-Torres & Gubler, 2009), while its presence in New Zealand was only reported in 2009 (Amponsah *et al.*, 2009b). Pathogenicity studies around the world showed that *N. australe* is highly pathogenic to grapevines, similar to its close relative *N. luteum* (van Niekerk *et al.*, 2004; Taylor *et al.*, 2005; Amponsah *et al.*, 2008b; Urbez-Torres & Gubler, 2009).

Diplodia seriata Schwein (syn. *B. obtusa*) can be distinguished from other botryosphaeriaceous species by its large, dark brown, mostly aseptate conidia that are pigmented even before discharge from pycnidia (Figure 1.6D; http://www.crem.fct.unl.pt/botryosphaeria_site). It is commonly found on dead wood of a

wide range of hosts (http://www.crem.fct.unl.pt/botryosphaeria_site) but is widely associated with grapevines worldwide (Table 1.1; Phillips, 1998; Larignon *et al.*, 2001; Auger *et al.*, 2004; Taylor *et al.*, 2005; Urbez-Torres *et al.*, 2006a; Amponsah *et al.*, 2008b; Urbez-Torres *et al.*, 2008). However, its pathogenicity to grapevines remains unclear. Phillips (1998) found this species to be weakly pathogenic in grapevines and considered it a saprophyte. This species also did not cause any symptoms when inoculated onto grapevines rooted cuttings (Taylor *et al.*, 2005) and green shoots (Amponsah *et al.*, 2008b). In contrast, it was reported to be the primary pathogen causing "black dead arm" in Bordeaux vineyards and to cause lesions when artificially inoculated onto grapevine cuttings (Larignon *et al.*, 2001). This species was also reported to be associated with vine decline and dieback symptoms in Semillon grapevines in Hunter Valley, New South Wales, Australia and to cause internal dark wood streaking in inoculated 9-month old potted vines (Castillo-Pando *et al.*, 2001). Therefore, it is unclear whether this species is a pathogen or secondary coloniser of grapevines (http://www.crem.fct.unl.pt/botryosphaeria_site) or both, due to isolate specific abilities.

Diplodia mutila Shoemaker (syn. *B. stevensii*) can be differentiated from *D. seriata* by its conidia that remain hyaline long after release from pycnidia although they can become pigmented some time after discharge (Figure 1.6E; http://www.crem.fct.unl.pt/botryosphaeria_site). This species is widespread worldwide (Table 1.1; van Niekerk *et al.*, 2004; Whitelaw-Weckert *et al.*, 2006; Amponsah *et al.*, 2008b; Urbez-Torres & Gubler, 2009) and was reported to be moderate or weakly pathogenic when artificially inoculated onto grapevines (Phillips, 1998; Taylor *et al.*, 2005; Amponsah *et al.*, 2008b; Urbez-Torres & Gubler, 2009).

Lasiodiplodia theobromae (Pat.) Griffon & Maubl. (syn. *B. rhodina*) conidia are initially hyaline aseptate and become brown and one-septate with irregular longitudinal striations (http://www.crem.fct.unl.pt/botryosphaeria_site). This fungus is reported to be associated with approximately 500 host plants and can cause different symptoms including damping-off, wilt, dieback, root rot and fruit rots (http://www.crem.fct.unl.pt/botryosphaeria_site). In California, it was generally isolated from warm and dry grape-growing areas (Urbez-Torres & Gubler, 2009). This species was found to be one of the most pathogenic species in grapevines based on pathogenicity studies in Australia (Wood & Wood, 2005), Mexico (Urbez-Torres *et al.*, 2008), California (Urbez-Torres & Gubler, 2009) and Spain (Martin *et al.*, 2009), but has not been reported from grapevines in New Zealand (Baskarathevan *et al*., 2011)

Botryosphaeria dothidea (Moug. Ex Fr.) Ces. & De Not. produces conidia that are hyaline, aseptate and thin-walled that seldom become dark and 1-septate (http://www.crem.fct.unl.pt/botryosphaeria_site). This species was found to be the primary pathogen causing dieback of grapevines in Portugal (Phillips, 1998) and found to be associated with “black dead arm” disease in France (Larignon *et al.*, 2001), and was also reported in other grapevine growing regions around the world (Table 1.1; Urbez-Torres *et al.*, 2006a; Urbez-Torres *et al.*, 2006b; Baskarathevan *et al.*, 2008; Morales *et al.*, 2010;). This species was able to cause moderate lesions when artificially inoculated in different grapevine tissues (Larignon *et al.*, 2001; Urbez-Torres & Gubler, 2009).

Neofusicoccum ribis Grossenberg & Dugg. (syn. *B. ribis*) produces hyaline unicellular conidia, that are fusiform or irregular fusiform, smooth and rarely septate with age (http://www.crem.fct.unl.pt/botryosphaeria_site). A previous study had regarded *N. ribis* as a synonym of *B. dothidea* (Witcher & Clayton, 1963; Michailides, 1991), however, Slippers *et al.* (2004a) showed that *N. ribis* is distinct from *B. dothidea* but is closely related to *N. parvum* morphologically and phylogenetically; they can only be separated by their EF1-α gene sequences (Slippers *et al.*, 2004a; van Niekerk *et al.*, 2004). This species is commonly isolated from *Ribes* spp. (Slippers *et al.*, 2004a; van Niekerk *et al.*, 2004; Alves *et al.*, 2005). It has not been commonly isolated from grapevine, but was associated with discoloured wood (Spagnolo *et al.*, 2011) and bunch rots (Pascoe, 1998).

1.5 Identification of botryosphaeriaceous species

Identification of botryosphaeriaceous species by morphology alone has caused difficulties for many researchers (Slippers *et al.*, 2004a). Some botryosphaeriaceous species were unable to produce spore bearing structures in artificial media (Denman *et al.*, 2000; Amponsah *et al.*, 2008a). Some species like *N. luteum*, *N. australe* and *N. parvum* also showed similar mycelial growth characteristics and colour (Slippers *et al.*, 2004b; Amponsah, 2011). Furthermore size ranges and shapes of conidia overlap while age and maturity affect pigmentation and septation, making them difficult to identify at species level (Denman *et al.*, 2000; Alves *et al.*, 2005). These characteristics have been reported in full for the botryosphaeriaceous species most common in New Zealand (Amponsah, 2011).

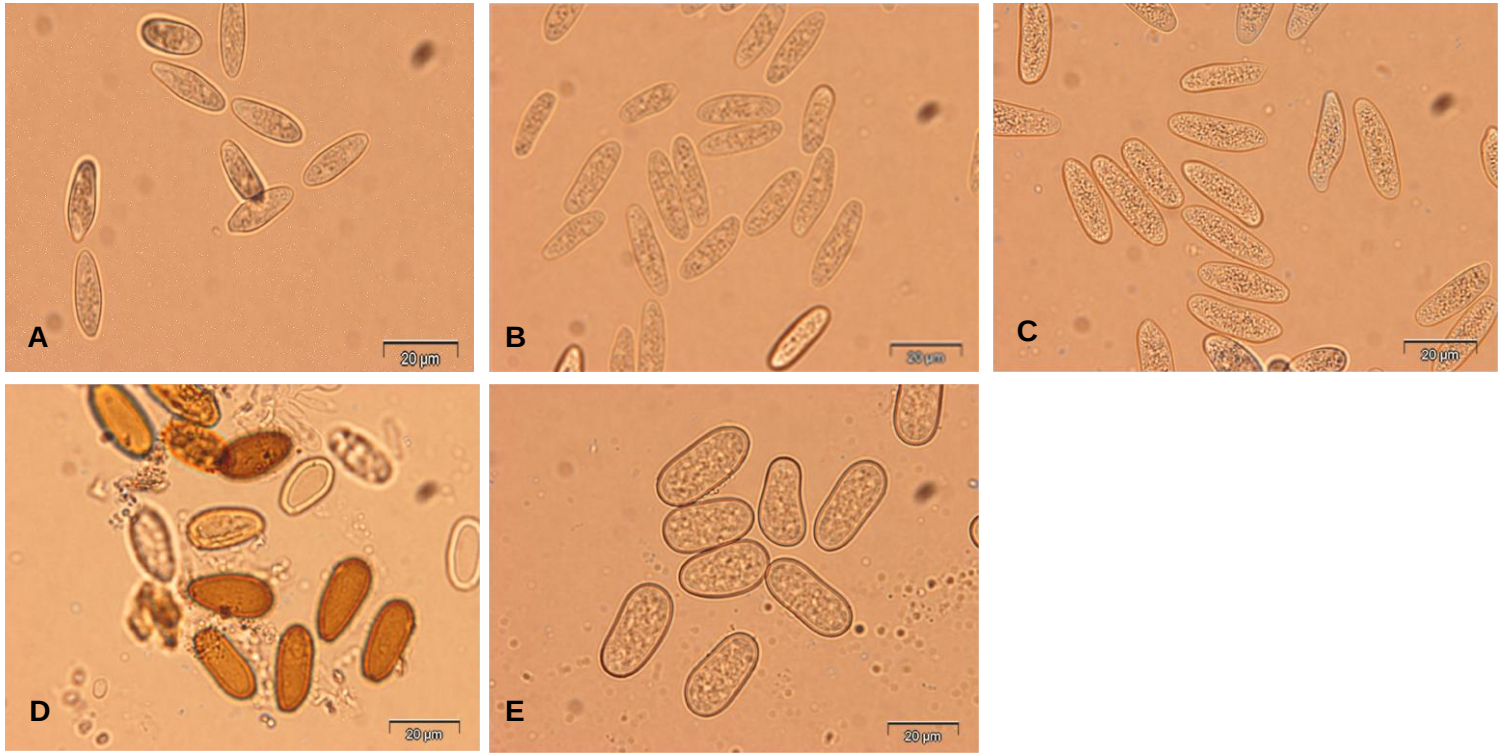

Figure 1.6 Conidial characteristics of the different botryosphaeriaceous species commonly isolated from grapevines in New Zealand. A) *N. parvum*; B) *N. luteum;* C) *N. australe*; D) *D. seriata* and E) *D. mutila.* (Photographs by Nicholas Amponsah).

1.6 Molecular identification of botryosphaeriaceous species

The recent advances in DNA-based molecular techniques have greatly contributed to the detection and identification of the botryosphaeriaceous species (Denman *et al.*, 2000; Slippers *et al.*, 2007). Ribosomal DNA (rDNA) is commonly used for phylogenetic studies of fungi because this gene is present in all organisms and parts of this gene are highly conserved (Guarro *et al.*, 1999). It has therefore, been used as reference point for some evolutionary studies (Guarro *et al.*, 1999). However, the internal transcribed spacer regions of this gene are more variable and are generally used for species differentiation (Guarro *et al.*, 1999). The variation of this gene can be exploited for identification; the rDNA is amplified by polymerase chain reaction (PCR) and then the amplified product can be digested with restriction enzymes which cut the DNA at specific sites that differ between species, producing different fragments (Guarro *et al.*, 1999). When the fragments are separated by gel electrophoresis the presence of products and patterns allow for identification (Guarro *et al.*, 1999; Olive & Bean, 1999). This technique is known as the amplified rDNA restriction analysis (ARDRA; Vaneechoutte *et al.*, 1992) and is now frequently used in inter- as well as intra-specific comparisons of many fungi (Guarro *et al.*, 1999; Olive & Bean, 1999).

In 2005, Alves *et al.* (2005) used ARDRA for rapidly identifying botryosphaeriaceous species. The amplification of ITS +D1/D2 variable domains of 28S rDNA was followed by the digestion of amplimers with restriction endonucleases to generate restriction fingerprints. This method could differentiate ten botryosphaeriaceous species including *N. luteum*, *N. ribis*, *N. parva*, *B. dothidea*, *D. mutila*, *D. seriata*, *D. corticola*, *Do. sarmentorum*, *Do. iberica* and *Lasiodiplodia theobromae* using endonuclease enzymes *Alu*I*, Asu*I*, Mbo*I*, Hae*III*, Nci*I*, Taq*I. Following the studies by Alves *et al.* (2005), the *Sac*II enzyme was further used to differentiate *N. luteum* from *N. australe* (Sammonds *et al.*, 2009; Baskarathevan et al., 2011).

While most taxonomic studies on botryosphaeriaceous species have used the sequence differences of the ITS region of rDNA, this gene may underestimate the diversity among closely related species (Slippers & Wingfield, 2007). Therefore, the sequences of β-tubulin and translation elongation factor 1-α genes have also been used in combination with ITS sequences to identify the different botryopshaeriaceous species (Slippers *et al.*, 2004a; Luque *et al.*, 2005; Urbez-Torres, *et al*., 2006a).

1.7 Detection of botryosphaeriaceous species and other pathogens by polymerase chain reaction (PCR)

Molecular techniques have also been developed for the detection and identification of fungal pathogens in different environmental sources. Polymerase chain reaction (PCR) using species-specific primers is a commonly used method for detection and diagnosis of trunk pathogens of grapevines. Nested-PCR, which uses universal fungal primers and then species-specific primers, was used to detect Petri disease pathogens *Ph. chlamydospora* and *Phaeoacremonium* spp. in infected young vines and in samples taken throughout the propagation system (Aroca *et al.*, 2006; Retief *et al.*, 2006; Aroca & Raposo, 2007; Whiteman *et al.*, 2007; Ridgway *et al.*, 2011). Different researchers reported that the method was very sensitive, being able to detect less than 1 pg (Ridgway *et al.*, 2002) and 1 fg (Retief *et al.*, 2006) of *Ph. chlamydospora* genomic DNA and 10 pg of *P. chlamydosporum* and *P. aleophilum* DNA (Tegli *et al.*, 2000). This method was also used to detect *Ph. chlamydospora* DNA from soil samples; it was sensitive enough to detect 10 fg (Retief *et al.*, 2006) and 5 fg of *Ph. chlamydospora* DNA (Whiteman *et al.*, 2007). Probst *et al.* (2009) was also able to detect 30 pg and 3 pg of *C. macrodidymum* and *C. liriodendri* DNA from inoculated soils using standard and nested PCR, respectively.

The development and use of PCR-based techniques for the detection of botryosphaeriaceous infections has been reported for the detection of *B. dothidea* (Ma *et al.*, 2003). The *B. dothidea* species-specific primers EBdF-EBdR and IBdF-IBdR that could amplify a 701- and a 627-bp fragment, respectively, could detect up to 1 fg of *B. dothidea* DNA from pistachio tissues using nested PCR. This EBdF-EBdR primer pair was also able to detect *B. dothidea* within young grapevine tissues (Romanazzi *et al.*, 2009). Recently, Spagnolo *et al.* (2011) developed *Botryosphaeriaceae*-specific primers to detect a closely related species complex *N.parvum/N. ribis* and a wider range of primers that could detect 17 botryosphaeriaceous species in wood.

Ridgway *et al.* (2011) also developed a botryosphaeriaceous multi-species primer pair Bot100F and Bot472F that could amplify six of the 12 lineages of the *Botryosphaeriaceae* that affect grapevines. The Bot100F primer was designed to anneal at nucleotide 131 of the ITS-1 region and was degenerate at nucleotide 15 where an A or G nucleotide (designated as R) was present. The Bot472R primer was designed to anneal at nucleotide 511 of the ITS-2 region and was degenerate at nucleotide 11 where a C or A nucleotide (designated M) was present. These primers were able detect *N. luteum*, *N. australe*, *D. mutila*, *D. seriata* and *N.parvum/N.ribis* complex propagules from soil, grapevine wood

and rainwater samples. These researchers further resolved the identities of botryosphaeriaceous species detected by these multi-species primers by analysing the PCR products using the single-strand conformation polymorphism (SSCP). SSCP is a molecular technique used for identifying sequence changes in amplified DNA (Fujita & Silver, 1994). This technique is based on the principle that single strands of the PCR product, which differ by as little as a single base, have different conformation under non-denaturing conditions and therefore migrate differently in polyacrylamide gels (Bastos *et al.*, 2001).

1.8 Genetic diversity among botryosphaeriaceous species

Teleomorph stages of botryosphaeriaceous species have rarely been observed in the field (Jacobs & Rehner, 1998; Phillips *et al.*, 2002; Slippers & Wingfield, 2007). Conidial spores were the most common propagules trapped in epidemiological studies by Swart *et al.* (1991), Amponsah *et al.* (2009a), and van Niekerk *et al.* (2010). These studies indicated that this fungal group is predominantly asexual.

Despite its highly asexual reproduction, genetic studies showed that some botryosphaeriaceous species have high genetic diversity. Baskarathevan *et al.* (2009) found that the *N. parvum* population from New Zealand was highly diverse with no clonal isolates being found among the 49 isolates analysed. Che-Omar (2009) also observed similar genetic diversity among *N. parvum* species isolated from blueberries in New Zealand orchards. These reports were further supported by pathogenicity studies showing variability in pathogenicity among isolates of the botryosphaeriaceous species. Urbez-Torres and Gubler (2009) found highly variable lesion sizes produced by isolates within the species *L. theobromae*, *N. parvum*, *N. luteum* and *N. australe* when they were inoculated onto different tissues of grapevines. Larignon *et al.* (2001) found four virulence groups among *D. seriata* isolates and Baskarathevan *et al.* (2009) further showed that *N. parvum* isolates from different genetic groups had different degrees of pathogenicity on grapevine green shoots, suggesting that variability in pathogenicity may be associated with genetic diversity.

1.8.1 Genetic diversity analysis

Understanding the genetic diversity of fungal pathogens can improve understanding of pathogen biology. Several PCR-based tools are used for studying the population genetics of fungi. The inter-simple sequence repeat (ISSR) method was used to investigate the genetic diversity of *Cylindrocarpon* spp. from Spain (Alaniz *et al.*, 2009) and *D. sapinea*

genotypes (Burgess *et al.*, 2004). Mostert *et al.* (2006) on the other hand used amplified fragment length polymorphism (AFLP) to study the genetic diversity among isolates of *Ph. chlamydospora*.

One of the most common methods for investigating genetic diversity of a fungus uses universal primers to amplify DNA from organisms with unknown DNA sequences and then generates fingerprints by gel electrophoresis (UP-PCR; Bulat *et al.*, 1998). This technique is similar to randomly amplified polymorphic DNA (RAPD; Williams *et al.*, 1990), except that the UP-PCR primers are relatively long (15-20 bp) and it uses higher annealing temperatures (52-56°C) which enhances reproducibility. The shorter primers and lower annealing temperatures of the RAPD method can cause non-specific amplifications (Bulat *et al.*, 1998; Lubeck *et al.*, 1999). The UP-PCR technique also results in higher numbers of bands than most RAPDs, making the identification and interpretation easier (Bulat *et al.*, 1998). This method was considered a highly robust fingerprint method for genetic studies of *Trichoderma harzianum* (Lubeck *et al.*, 1999) and was used to characterise *Trichoderma* isolates from Philippine rice fields (Cumagun *et al.*, 2000). It was further used to study geographical variations among *Peronospora farinosa* populations (Danielsen & Lubeck, 2010) and differentiate genetic groups of *Botrytis aclada* (Nielsen *et al.*, 2001). In New Zealand, UP-PCR was also used to study genetic diversity of *Spilocea oleagina* populations infecting olives (Obanor *et al.*, 2010), *N. parvum* populations from vineyards and blueberry farms (Baskarathevan *et al.*, 2009; Che Omar, 2009) and *Cylindrocarpon* species isolated from vineyards (Pathrose *et al.*, 2011). Pottinger *et al.* (2002) combined RAPD, randomly amplified microsatellites (RAM), AFLP and UP-PCR to determine the genetic diversity of *Ph. chlamydospora* populations in New Zealand.

1.8.2 Infection pathways of botryosphaeriaceous species

Botryosphaeriaceous species are generally known to infect their woody host through wounds. For example, *B. dothidea* and *D. seriata* were shown to invade peach and apple trees through wounds and other injury sites (Pusey, 1993; Brown-Rytlewski & McManus, 2000). However, other pathogenicity studies have also shown that some botryosphaeriaceous species pathogens could infect their woody hosts through stomata and lenticels (Brown & Hendrix, 1981; Michailides, 1991; Pusey, 1993), although infection occurred more rapidly when wounds were present (Brown & Hendrix, 1981).

In grapevines, pruning wounds are regarded as the major infection sites for botryosphaeriaceous species (Larignon *et al.*, 2001; Fourie & Halleen, 2004b). For example, frequent isolation from pruned canes was reported for *Dothiorella viticola* in Spain (Luque *et al.*, 2005) and *L. theobromae* in Australia (Wood & Wood, 2005). These pathogens were found to infect unprotected pruning wounds of rootstock mothervines in South African nurseries resulting in infected propagation materials (Fourie & Halleen, 2004b). Urbez-Torres and Gubler (2011) also showed that pruning wounds could be susceptible to botryosphaeriaceous infections for up to 13 weeks, but susceptibility decreased with age of pruning wounds, while Serra *et al.* (2008) found that spurs could still be infected for up to four months after pruning. Penetration through pruning wounds is aided by the production of plant sap that keeps the pruning wounds wet for a longer period of time (Pearson & Goheen, 1998). A histopathology study by Amponsah *et al.* (2009c) further demonstrated that *N. luteum* conidia were not capable of directly penetrating and infecting non-wounded tissues of attached and detached grapevine shoots and leaves. Wound protection is now considered an important measure to prevent infection by decline and dieback pathogens (Fourie & Halleen, 2006).

Previous studies suggested that botryosphaeriaceous species could infect through roots. In Australia, *D. seriata* was isolated from root dieback in a grapevine (Castillo-Pando *et al.*, 2001) while *D. mutila* was reported to infect grapevines through soil-root transmission (Whitelaw-Weckert *et al.*, 2006). However, a recent study by Amponsah (2011) contradicted these claims, since he showed that four botryosphaeriaceous species (*N. luteum*, *N. parvum*, *N. australe* and *D. mutila*) were not able to cause infections when inoculated on roots.

Rain plays an important role in dispersal and infection by botryosphaeriaceous propagules. Conidia of *D. seriata* and *D. mutila* were trapped throughout the year in a French vineyard (Kuntzmann *et al.*, 2009) and release of air-borne conidia was generally observed during raining periods when temperatures were above 10°C (Larignon & Dubos, 2001). High numbers of air-borne botryosphaeriaceous spores were also trapped following rain events in spring and winter months in California vineyards (Urbez-Torres *et al.*, 2010b) and during or after rainfall periods in South African vineyards (van Niekerk *et al.*, 2010). However, in New Zealand, botryosphaeriaceous conidia were only trapped in rain water run-off throughout the year with conidia being most abundant during summer periods (Amponsah *et al.*, 2009a). Furthermore, Baskarathevan *et al.* (2010) showed that these water-borne conidia could move up to 2 m from the source during a 2-3 day significant rainfall event and spore movement appeared to be influenced by wind direction.

Temperature and relative humidity are important factors involved in the sporulation and germination of botryosphaeriaceous spores. The optimum sporulation of botryosphaeriaceous species was observed at 18 to 30°C (Copes & Hendrix, 2004; Urbez-Torres *et al.*, 2010a; Amponsah, 2011). The conidia of several botryosphaeriaceous species were shown to germinate at a broad range of 5-40°C with optimum germination observed at 25 to 30°C (Sutton & Arauz, 1991; Urbez-Torres *et al.*, 2010a). However, Amponsah (2011) reported that New Zealand isolates of *N. parvum, N. luteum, N. australe* and *D. mutila* could germinate at 5-35°C, although germination frequencies were very low (<10%) below 15°C and above 30°C. High relative humidity (RH) favours germination, being highest at 95-100% and declining with RH, such that no conidia germinated at 84% RH (Amponsah, 2011; Sutton & Arauz, 1991). The role of relative humidity is further confirmed by studies showing that air drying for approximately 20 min can significantly reduce germination of *B. dothidea* (Sutton & Arauz, 1991) and interrupting wet periods significantly reduced infections of *D. seriata* conidia on apples (Arauz & Sutton, 1990).

Some studies also showed that botryosphaeriaceous species can survive and sporulate on dead plant materials. The species *B. dothidea*, *D. seriata* and *L. theobromae* were able to produce pycnidia and viable conidia when grown on autoclaved apple and peach stems (Copes & Hendrix, 2004). Pycnidia and viable conidia of *B. dothidea* were also observed on dead shoots of pistachio trees (Michailides, 1991). In grapevines, Amponsah *et al.,* (2008a) also recovered pycnidia and viable conidia from necrotic lesions on grapevine shoots infected with *N. parvum, N. luteum, N. australe* and *D. mutila,* which had been air-dried for up to 3 months and later exposed to high relative humidity. High numbers of botryosphaeriaceous pycnidia that produced conidia were also observed from pruning debris in South African vineyards (van Niekerk *et al.*, 2010).

1.8.3 Latent infections of botryosphaeriaceous species

Some members of the botryosphaeriaceous species are well known endophytes and latent pathogens of woody plants. A study by Sakalidis *et al.,* (2011) found that eleven botryosphaeriaceous species including *L. theobromae* were endophytes of boababs (*Adansoni gregorii*) but a pathogenicity test showed that *L. theobromae* could also cause necrotic lesions in this host. *Diplodia sapinea* (syn. *Sphaeropsis sapinea)* was also isolated from symptomatic and asymptomatic Austrian and Scots pine trees (Flowers *et al.*, 2001), on which it was primarily associated with the bark and phloem

tissues of asymptomatic infected shoots. This species was also found to persist in stems of asymptomatic red pine seedlings (*Pinus resinosa*) but after planting out it developed into a collar rot that caused mortality (Stanosz *et al.*, 2005). *Botryosphaeria dothidea,* which was associated with dieback and canker disease of various *Eucalyptus* species, was also commonly isolated from symptomless leaves of *E. grandis* and *E. nitens* (Smith *et al.*, 1996).

Latent pathogens generally do not cause symptoms for a part of their life cycles (endophytic stage) and symptom expression is only observed when the host becomes susceptible to infection. The latent pathogen then becomes aggressive and attacks the host (Slippers & Wingfield, 2007; Sakalidis *et al.*, 2011). Host susceptibility may be increased by stress. Infection severity of *B. dothidea* was significantly increased when peaches were water stressed (Pusey, 1989) while *B. dothidea* infections in Eucalyptus were also associated with environmental conditions such as droughts, hot winds and frost (Smith *et al.*, 1994).

In vineyards, botryosphaeriaceous disease development is normally slow but builds up progressively leading to general decline in vigour and yield (Pearson & Goheen, 1998). Botryosphaeriaceous species have also been associated with the decline of young vines on which there are no obvious symptoms of wood decay (Gimenez-Jaime *et al.*, 2006). Visible symptoms are usually only observed on vines 8 years or older (Larignon & Dubos, 2001; Martin & Cobos, 2007), which suggest presence of latent infection (Fourie & Halleen, 2004b). Endophytic colonisation has been demonstrated for some species on grapevines. Inoculation of *L. theobromae* showed that this species was present up to 5 cm beyond the external lesion edge of dawn seedless grapes (Wood & Wood, 2005). Amponsah (2011) also showed that *N. luteum*, *N. australe* and *D. mutila* were endophytic in trunks of potted young vines and dieback symptoms were only observed when plants were pruned in winter or after they were subjected to water stress.

1.9 Grafted grapevines

Vitis vinifera is the most commonly cultivated grape species, accounting for the majority of wine grape varieties (Hardie & Cirami, 1988; Keller, 2010b). However, this species is susceptible to phylloxera, a small sap-sucking insect that feeds on roots of grapevines (Jackson & Schuster, 1994; Keller, 2010b). To address this problem, plants are produced by grafting *V. vinifera* canes to rootstocks that are resistant to phylloxera, of which most are North American species (Jackson & Schuster, 1994; Keller, 2010b). Grafting involves joining a scion cultivar with desirable fruiting traits to a rootstock that confers resistance to

root pest and diseases (Whiteman *et al.*, 2007). Thus, for over 100 years, interspecific scion-rootstock grafting has been used in viticulture to impart pest resistance and to manipulate grapevine vigour, yield and fruit composition (Gambetta *et al.*, 2009).

Grafted grapevines were introduced to New Zealand in 1895 by Romeo Bragato when he found phylloxera in New Zealand soils (Anonymous - Romeo Bragato Conference, 2010). However, the value of grafting was only recognised in the early 1970s (Anonymous - Romeo Bragato Conference, 2010; Jackson & Schuster, 1994). Since then, it has become very popular; grafted vines now occupy 94% of the total vineyard area in New Zealand (New Zealand Winegrowers Statistical Annual, 2010).

1.9.1 Propagation of vines

Traditional grapevine propagation methods are generally similar around the world. However, some details of practices may vary between countries or individual nurseries.

The first stage of propagation normally involves the collection during winter of dormant cuttings, which represent the previous season's growth (Nicholas *et al.*, 2006). The rootstock shoots are generally cut to 30-38 cm long sections with 2 to 3 buds each, while scion shoots are cut in smaller sections with only one bud each (Zanzotto *et al.*, 2001). After harvest, these 'cuttings' are soaked in hydration tanks for up to 12 hours before storage at temperatures of 1-6^{o}C for 2 to 3 months (Fourie & Halleen, 2006; Waite & Morton, 2007; Aroca *et al.*, 2010). In some cases, cuttings are treated with fungicides like Chinosol® (8-hydroxyquinoline sulphate) to protect the cuttings from grey mould, caused by *Botrytis cinerea*, while in cold storage (Nicholas *et al.*, 2006). In spring, the cuttings are taken out of cold storage and may be soaked for a few hours or up to four days in hydration tanks, which may contain fungicides (Fourie & Halleen, 2006; Retief *et al.*, 2006; Aroca *et al.*, 2010). Rootstocks cuttings are disbudded, which involves manually removing all buds except the basal bud, using scissors, secateurs or a disbudding knife (Nicholas *et al.*, 2006; Aroca *et al.*, 2010). Rootstock and scion cuttings of similar diameters are then grafted by hand or mechanically, which uses a grafting machine that makes an omega- or v-cut on one piece and a cut of the inverse shape on the other, and joins the matching cuts together (Fourie & Halleen, 2006; Whiteman *et al.*, 2007; Aroca *et al.*, 2010). The graft unions are generally sealed with grafting tape or in molten wax which may contain a fungicide (Fourie & Halleen, 2006; Aroca *et al.*, 2010). The resulting grafted plants are packed upright in boxes and covered with callusing medium to above the graft union (Nicholas *et al.*, 2006). Different callusing media used in different countries include peat, pine sawdust, wood shavings, perlite and vermiculite (Whiteman, 2004 ; Fourie & Halleen,

2006; Nicholas *et al.*, 2006; Retief *et al.*, 2006; Aroca *et al.*, 2010). The boxes of grafted plants are then incubated in a hot house at 26-32°C with 70-90% relative humidity for 2 to 3 weeks to promote callus formation (Zanzotto *et al.*, 2001; Fourie & Halleen, 2006; Aroca *et al.*, 2010). Callusing boxes are then transferred to a shade house, where plants are allowed to acclimatise for 1-2 weeks (Fourie & Halleen, 2006). The resulting grafted plants are transplanted into outdoor nursery beds and allowed to grow until the following winter when the young dormant vines are uprooted, washed, graded and placed in cold storage until sold to vineyards (Whiteman, 2004; Fourie & Halleen, 2006; Aroca *et al.*, 2010).

1.9.2 Grapevine infection in nurseries

Although scion-rootstock grafting avoids damage by phylloxera (Keller, 2010b), this practice was also found to facilitate pathogen transmission (Gambetta *et al.*, 2009). For example, grapevine leaf roll virus spreads mainly by using infected scion or rootstock cuttings (Pearson & Goheen, 1998). The bacterial pathogen *Xyllela fastidiosa* was also reported to move internally through the graft union to cause systemic infections (Gambetta *et al.*, 2009).

Nurseries are also reported to be the main source of wood diseases of grapevines. For example, the major spread of infection of *Ph. chlamydospora* and *Phaeoacremonium* spp. is through the use of infected propagation materials, particularly rootstock material (Retief *et al.*, 2006; Whiteman *et al.*, 2007; Aroca *et al.*, 2010). Gramaje (2011) reported that in Spain grapevine mother plants are a major inoculum source of *Ph. chlamydospora* and *Phaeoacremonium* spp. Furthermore, the propagules of these pathogens were detected in hydration tanks, grafting tools and callusing media during grafting (Retief *et al.*, 2006; Whiteman *et al.*, 2007; Aroca *et al.*, 2010) and were reported to spread through the propagation process leading to grapevine infections (Whiteman *et al.*, 2007; Aroca *et al.*, 2010). Whiteman (2007) showed that when a batch of cuttings was processed through infested hydration tanks and callusing medium, the incidence of infection increased.

Black foot disease caused by *Cylindrocarpon* spp. is also known to start when the young grafted are planted out into the outdoor nursery fields (Halleen *et al.*, 2007) where the soil-borne pathogens infect the basal ends of the grafted plants. This disease has been reported to cause young vine death in new vineyards in South Africa, North and South America, Australia and New Zealand (Jaspers *et al.*, 2007). In California, both fungi, *Cylindrocarpon* spp. and *Phaeoacremonium* spp., have also been consistently isolated from young grapevines showing decline symptoms (Scheck *et al.*, 1998).

According to Waite (2005), infected nursery vines are a major source of trunk disease infections, causing serious effects on the health and long term productivity of vineyards. Although some infected vines may survive, they often develop poorly and require more care and maintenance than healthy vines. In some cases, the infected vines failed to establish and needed to be replanted which delayed cropping for at least one season (Waite & Morton, 2007).

1.10 Botryosphaeriaceous infection in the nurseries

Some botryosphaeriaceous species have been reported to be introduced through the use of infected nursery plants. For example, *D. sapinea* infections in red pine were reported to originate from the infected pine seedlings sold by nurseries (Palmer *et al.*, 1988). Since this pathogen can persist on seedlings without causing any symptoms, the seedlings may be unwittingly disseminated over wide areas (Stanosz *et al.*, 2005). Because of the endophytic nature of these pathogens, they can be easily introduced to new areas through the seeds or cuttings of a variety of hosts (Burgess *et al.*, 2005).

The infection pathways of botryosphaeriaceous infections of grapevines have not been thoroughly researched but there is evidence to show that infection may start from the nurseries. In New Zealand, botryosphaeriaceous species are frequently isolated from failed young grafts (Harvey, pers. comm., 2008). After an investigation of the health of one-year-old vines from five different New Zealand nurseries in Gisborne, Marlborough and Auckland, Graham (pers. comm., 2008) reported that the botryosphaeriaceous species were the most common pathogens isolated from the young vines. The species *D. seriata* and *B. dothidea* were also isolated from scion canes, rootstock canes and nursery cuttings in France (Larignon *et al.*, 2001) and Spain (Aroca *et al.*, 2006; Gimenez-Jaime *et al.*, 2006). Botryosphaeriaceous species were also associated with failed graft unions (Phillips, 2002). Six botryosphaeriaceous species were also isolated from rootstock mothervines from 14 mothervine fields in Spain (Aroca *et al.*, 2010). Recent studies by Spagnolo *et al.* (2011) showed that botryosphaeriaceous species were frequently detected in standing vines and propagation materials leading to the conclusions that infected plant materials can be an important source of primary inoculum of this disease.

1.11 Research objectives

The literature review presented in this chapter has shown a likely link between the presence of botryosphaeriaceous infections in the nurseries and the disease in the fields. Thus, the overall aim of this study was to investigate the prevalence of botryosphaeriaceous species occurring in New Zealand grapevine nurseries and their sources of inoculum, with the following specific objectives:

1. Conduct a survey for botryosphaeriaceous infection incidence within the propagation materials and young grafted vines from New Zealand nurseries in order to:
    a. Identify the important botryosphaeriaceous species present in the nurseries.
    b. Determine their pathogenicity on grapevines.
    c. Determine the genetic variability of the most prevalent species.
    d. Determine the susceptibility of the commonly used rootstock and scion varieties.
2. Investigate the sources of inoculum of botryosphaeriaceous species for nursery plants with respect to:
    a. The mothervine blocks, including plant debris and soil.
    b. The propagation system materials and environment.
3. Investigate the infection pathways of botryosphaeriaceous species with respect to:
    a. The mothervines as a source of infection for canes.
    b. The distribution and movement of botryosphaeriaceous fungi within the mothervine.
4. Investigate the mechanisms of persistence of the botryosphaeriaceous propagules that may be present in the nurseries:
    a. The persistence of conidia initially present on the surfaces of the propagation materials.
    b. The survival, viability and pathogenicity of conidia after being held at cold storage temperatures.

Chapter 2
Prevalence and distribution of botryosphaeriaceous species in New Zealand grapevine nurseries

2.1 INTRODUCTION

Botryosphaeriaceous species were first reported in New Zealand to be pathogens of kiwifruit (*Actinidia deliciosa*). These include *B. dothidea*, *N. parvum* and *N. luteum* (Pennycook & Samuels, 1985). Seven botryosphaeriaceous species (*N. parvum*, *N. luteum*, *N. australe*, *D. mutila*, *D. seriata, Do. iberica* and *B. dothidea*) were reported to infect grapevines (Amponsah *et al.*, 2011; Baskarathevan *et al.*, 2011). *Neofusicoccum parvum, N. luteum*, *N. australe* and *D. seriata* were also associated with dieback and crown rot of blueberries in New Zealand (Sammonds *et al.*, 2009).

The main period of infection of botryosphaeriaceous species and the ways they penetrate into the plant have not been thoroughly researched. However, it seems likely that vineyard infection may have originated from the nurseries that supplied the grapevine plants, since botryosphaeriaceous species have been isolated from canes of scion and rootstock varieties used as nursery cuttings (Larignon & Dubos, 2001; Gimenez-Jaime *et al.*, 2006), from failed graft unions of grapevine plants (Phillips, 2002), and symptomatic tissues of young vines (Gimenez-Jaime *et al.*, 2006). In New Zealand, botryosphaeriaceous species were frequently isolated from failed young grafts (Harvey, pers. comm., 2008) and from young vines sampled from five nurseries (Graham, pers. comm., 2008). These findings indicate that infected planting materials may be present in the propagation materials and processes but no in-depth studies have been done to verify the significance of this source to vineyard dieback diseases.

The aim of this study was to investigate the incidence of botryosphaeriaceous infection in plant materials from grapevine nurseries around New Zealand and to identify the most common botryosphaeriaceous species associated with the infection.

2.2 MATERIALS AND METHODS

2.2.1 Sampling

Samples were collected from grapevine nurseries from different regions of New Zealand from June to October 2008. A total of 14 commercial nurseries were contacted by email and then by a phone call 2 weeks later. Each nursery was asked to send 5 to 10 samples each of plants and propagation materials , which comprised apparently healthy grafted plants (Figure 2.1A), failed grafted plants (Figure 2.1C), scion and rootstock cuttings (Figure 2.1B) from a range of varieties. If failed grafted plants were not available, Grade 2 plants were requested. Grade 2 plants were defined as apparently-healthy plants that were downgraded because they did not meet the standard size, bud and root number set by the nurseries. Samples were sent by courier to the Lincoln University plant pathology laboratory and stored at 8°C until processed within 1-4 weeks. Samples were then examined and isolations conducted. Each nursery was assigned with a number for identification, and the locations for the nurseries were not specified for confidentiality reasons.

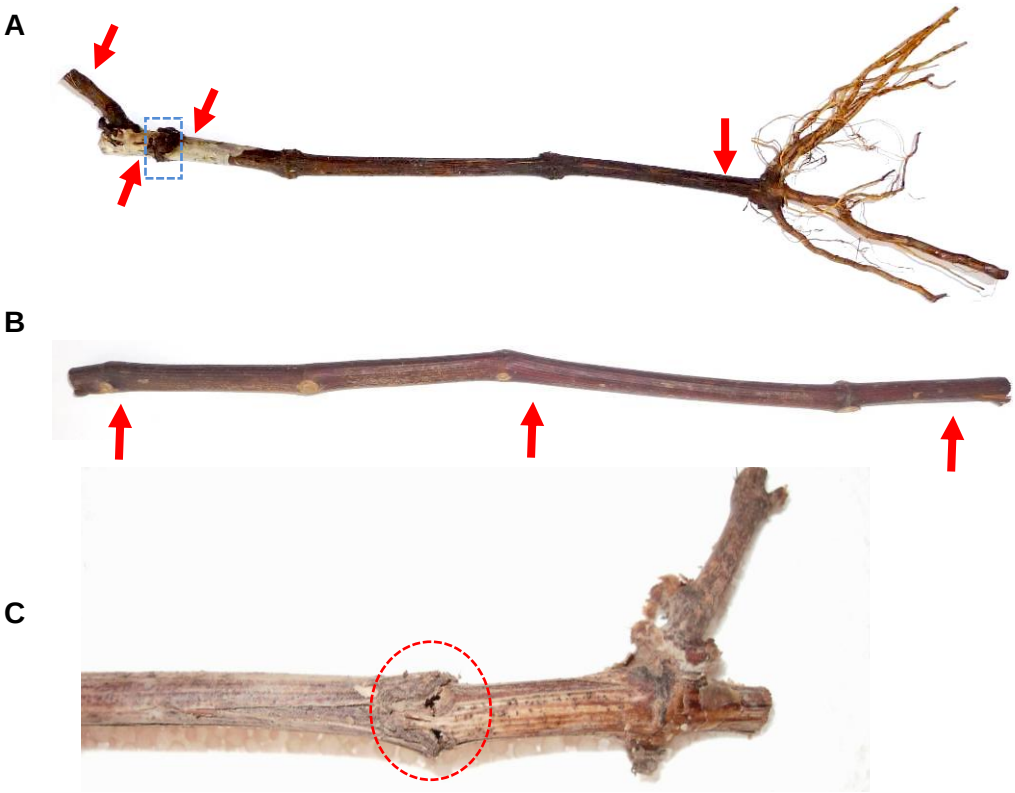

Figure 2.1 Nursery plants and cuttings collected for the survey. A) Apparently healthy grafted plant with graft union indicated by a box; B) rootstock cutting; C) failed grafted plant with partial separation of scion and rootstock indicated by a circle. Red arrows indicate the isolation points.

2.2.2 Evaluation of different surface sterilisation techniques for botryosphaeriaceous species isolation

Prior to isolation from the nursery samples collected, four surface sterilisation techniques were evaluated with five failed grafted plants each to find the fastest and most effective in eliminating contamination with highest recovery of botryosphaeriaceous isolates. The methods and results of the evaluation are presented in Appendix A.

2.2.3 Isolation of botryosphaeriaceous species from nursery plant samples

Following the evaluation of botryosphaeriaceous the surface sterilisation techniques, plant samples were surface-sterilised by dipping in 70% ethanol for 30 s and passed through the Bunsen flame to dry off the alcohol (Appendix A). For grafted plants, 0.5 cm sections were cut from 1 cm above the base, 1 cm above and below the graft union and 1 cm above the scion bud (Figure 2.1A). For rootstock and scion cuttings, 0.5 cm sections were cut from the top, middle and base of the cuttings (Figure 2.1B). Each tissue sample was further cut into quarters and placed onto PDA (Difco™, New Jersey, USA) supplemented with 0.05 g/L streptomycin sulphate (PDAS). Plates were incubated for 3 to 5 days at room temperature and examined for colonies with characteristics that resembled botryosphaeriaceous fungi.

2.2.4 Identification of botryosphaeriaceous isolates based on morphological characteristics

Of the 120 apparent botryosphaeriaceous isolates recovered, 118 were further examined for morphological characteristics while two isolates were excluded due to *Trichoderma* spp. contamination. All isolates were sub-cultured onto PDAS and after 4 and 15 days incubation at room temperature, colony colour and density and pycnidium formation at the base of the plates were recorded. The isolates were also sub-cultured onto prune extract agar (PEA; Appendix B.1) plates and incubated at 25°C under near UV light in a 12 h light-dark regime for 28 days to induce sporulation. All isolates were initially grouped based on mycelial characteristics and from each mycelial group, 10 representative isolates were selected for conidium characterisation. Groups with <10 isolates were all included in the characterisation. Pycnidia from the bases of the PEA plates of representative isolates were picked out with a sterile needle and each pycnidium was placed onto a sterile microscope slide with a drop of sterile reverse osmosis water (SROW). The conidia were released by pressing a cover slip firmly onto the top of each pycnidium. The conidial characteristics were compared to the descriptions given by Alan Phillips in a website for *Botryosphaeria* species

(http://www.crem.fct.unl.pt/botryosphaeria_site). The colony and conidium characteristics allowed presumptive identifications to be made of the isolate groups which were confirmed using molecular methods. Those isolates that produced sterile pycnidia or did not produce any pycnidia in culture were placed in a separate group and were identified using only molecular techniques.

2.2.5 Identification of botryosphaeriaceous isolates using molecular techniques

2.2.5.1 Amplified ribosomal DNA restriction analysis (ARDRA)

To confirm the identities of all 118 botryosphaeriaceous isolates recovered from the nurseries, the amplified ribosomal DNA restriction analysis (ARDRA) was conducted as described by Alves *et al.* (2005). DNA was extracted using the REDExtract-N-Amp^{TM} Plant PCR Kit (Sigma Aldrich, Missouri, USA). The isolates were grown on PDA at room temperature for 3 days and a sterile pipette tip was used to scrape up a tuft of mycelium (approximately 2 mm) from the edge of each culture and to add it to a 1.7 ml tube containing 100 µl of extraction buffer. The mixture was briefly vortexed and incubated at 95°C for 10 min. Then 100 ml of the dilution solution was added to the tube and briefly vortexed to neutralise inhibitory substances present in the extract. The mixture was centrifuged for 2 min at 3,220 x *g* and the supernatant was transferred to a new 0.6 ml tube and stored at -20°C until used for PCR.

The ribosomal DNA (rDNA) was amplified using the REDExtract-N-Amp™ PCR ready mix (Sigma Aldrich) using the ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3'; White *et al.,* 1990) and NL4 (5'-GGTCCGTGTTTCAAGACGG-3'; O'Donnell, 1992) primers following the manufacturer's recommendation. Each 20 µl amplification reaction contained 10 µl of the REDExtract-N-AmpTM PCR ready mix, 4 µl of the DNA extract, 1 µl each of each primer (5 µM) and 4 µl of sterile nanopure water (SNW). Negative controls using SNW instead of DNA template were included in every PCR reaction. Samples were briefly vortexed and centrifuged for 3,220 x *g* for 5 s before placing into the PCR machine (Biorad Laboratories, California, USA). The thermal cycle was performed with initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 30 s at 55°C and extension for 1 min at 72°C, followed by a final extension at 72°C for 7 min.

After amplification, 5 ul of every PCR product were separated by electrophoresis (10 V /cm for 45 min) in a 1% agarose gel (Bioline, London, UK) immersed in 1x TAE buffer (40 mM Tris acetate, 2 mM Na_2EDTA, pH 8.5). The 1 Kb Plus DNA Ladder™ (0.1 ng/µl) (Invitrogen, California) molecular weight marker was run in the first or last lane of each gel. The agarose gels were transferred to plastic containers containing ethidium bromide (0.5 µg/ml) and allowed to stain in a shaker for 15 min, then destained in water for 10 min. Stained gels were photographed under UV light using the Versa Doc^{TM} Imaging System Model 3000 (BIO-RAD Laboratories Inc.).

Once the ribosomal DNA was amplified, each ~1200 bp PCR product was digested separately with one or more restriction enzymes. The choice of restriction enzymes was determined in an iterative and sequential process (Figure 2.2). Restriction digestions using *Hae*III, *Sac*II and *Nci*I were done following the manufacturer's recommendations in a final volume of 20 µl containing 2 U of enzyme, 3 µl of 10x NE Buffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT), 12.8 µl of SNW and 5 µl of the PCR product. The *Hae*III and *Sac*II reactions were incubated at 37°C for 16 h using the PCR machine and inactivated by heating to 80°C and 65°C, respectively, for 20 min. The *Nci*I digest was incubated at 37°C for 14 h. For *Taq*I digestion, the reaction contained 2 units of enzyme, 3 µl of 10x NE Buffer 4, 0.3 µl of bovine serum albumin (BSA), 16.5 µl of SNW and 5 µl of the PCR product. Reactions were incubated at 65°C for 2 h and inactivated at 80°C for 30 min. DNA fragments were separated by electrophoresis in 1.5% agarose gels using 1X TAE buffer under constant voltage of 100 V for 1 h. The 1 Kb Plus DNA Ladder™ (0.1 ng/ul) molecular weight marker, was run in the first or last lane of each gel. The gel was stained and visualised as previously described. Species identity was assigned to each sample by comparing the DNA fragment patterns with those previously described (Alves *et al.*, 2005; Sammonds *et al.*, 2009; Baskarathevan *et al.,* 2011).

The initial digestion with *Taq*I gave a pattern that allowed the isolate to be placed into one of three groups (Alves *et al.*, 2005). Isolates with four bands of 364, 292, 189 and 53-92 bp were placed in Group A and were either *N. australe* or *N. luteum*. Those with three bands of 427-428, 291-292 and 53-117 bp were placed in Group B (*N. parvum or N. ribis)* and those with four bands of 426-432, 291-292, 173-189 and 51-63 bp were placed in Group C (*N. parvum, D. seriata, Do. sarmentorum* or *Do. Iberica;* Figure 2.2). The isolates in Group A were differentiated with the *Sac*II enzyme which resulted in two banding patterns, with *N. luteum* (Group D) having two bands of 1071 and 102 bp and *N. australe* (Group E) having three bands of 766, 304 and 102 bp (Sammonds *et al.*, 2009;

Baskarathevan *et al.,* 2011). The Group B and C isolates were further digested using the *Hae*III enzyme to differentiate *N. parvum* (Group G with five bands of 258, 254, 203, 157 and 58-83 bp) and *B. dothid*ea (Group H with three bands of 716, 157 and 58-83 bp) from *N. ribis, D. seriata* or *D. mutila* (Group F). The isolates belonging to Group F were further digested using the *Nci*I enzyme to differentiate *D. mutila* (Group K with two bands of 1079 and 93 bp)*, D. seriata* (Group J with three bands of 334, 747 and 93 bp) and *N. ribis* which was not cut by the enzyme (Group I with one band of 1173 bp). Those isolates that were not differentiated using ARDRA analysis (Group L) were subjected to DNA sequencing.

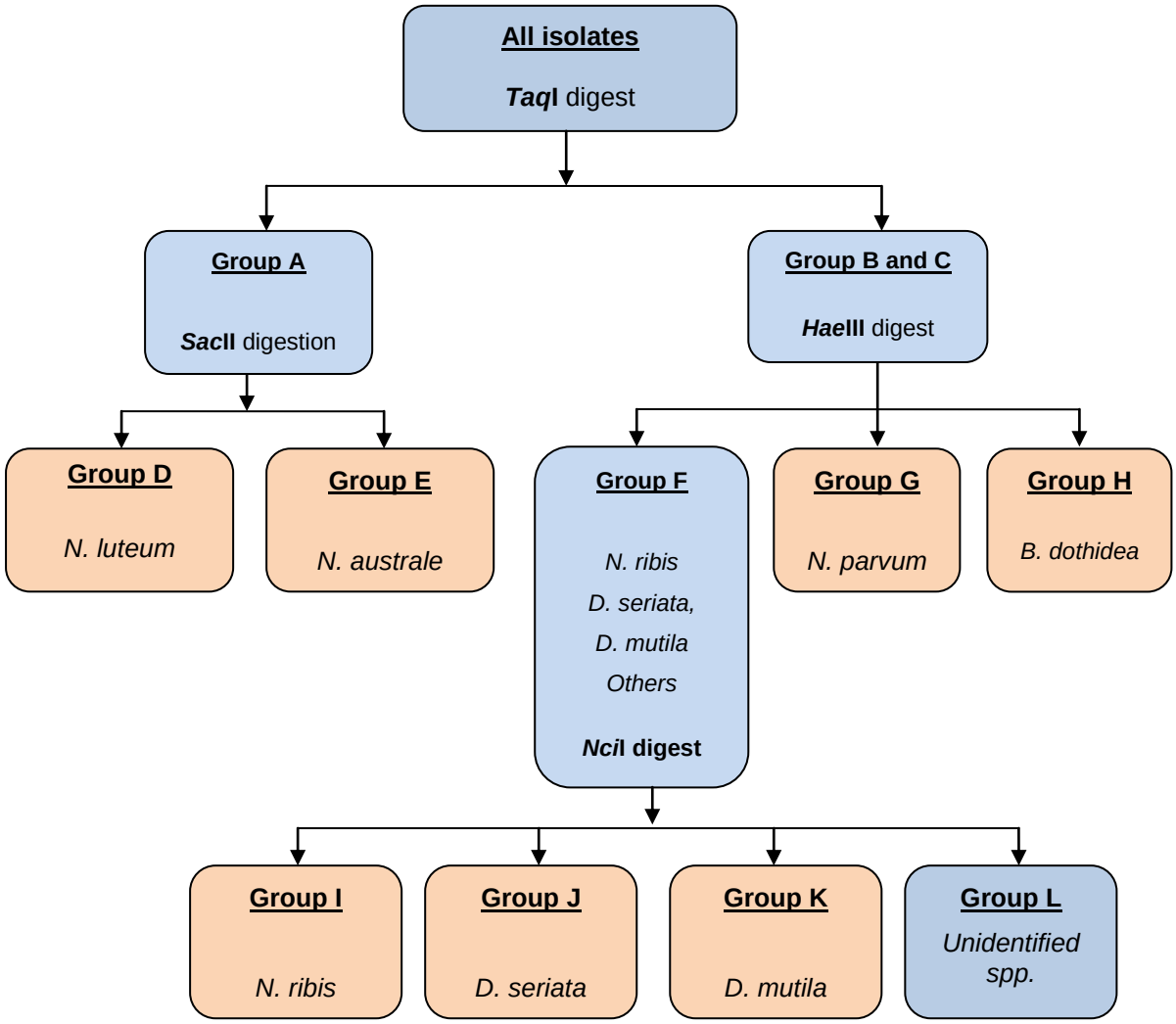

Figure 2.2 Diagram of the iterative restriction digestion analyses used for identifying the botryosphaeriaceous species isolated from grapevine nursery plants and propagation materials.

2.2.5.2 Sequencing of ribosomal DNA

To confirm the identity of the isolates of each species identified by ARDRA, four isolates of *N. luteum,* four of *N. parvum*, two of *N. ribis,* and one each of *N. australe*, *D. mutila, D. seriata* and *B. dothidea* were randomly selected for DNA sequencing of the internally transcribed spacer region (ITS) of the rDNA using the ITS1 primer as described in Section 2.2.5.1. The selected PCR products for DNA sequencing were sequenced at the Lincoln University Sequencing Facility using an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Foster City, California). The resulting sequences and chromatographs were analysed using the DNAMAN 5.2 (Lynnon Biosoft©) and Chromas Lite 2.1© (Technelysium PTY Ltd) softwares. For the eight botryosphaeriaceous species found in the nurseries, three to six published sequences of the ribosomal DNA, including the ITS region with at least 500 bp, were obtained from the NCBI database (www.ncbi.nlm.nih.gov) and aligned with the sequences of the above mentioned isolates using MEGA Version 4 (Tamura *et al.*, 2007). One rDNA sequence that included the ITS region of *Guignardia philoprina* (Berk. & M.A. Curtis) was further obtained from the NCBI database and included as an outgroup. This species was used as the outgroup for a similar phylogenetic study by Slippers *et al.* (2004a) because it was closely related to the botryosphaeriaceous group. The aligned sequences were tested for phylogeny and a neighbour joining tree was generated. The robustness of the trees obtained was evaluated by 1000 bootstrap replicates (Crous *et al.*, 2006).

2.2.5.3 Amplification and sequencing of β-tubulin and elongation factor α-1 genes

The novel species and the two isolates whose rDNA gene sequences were not 100% homologous with any of the accessions in Genbank were further identified by amplifying and sequencing the β-tubulin gene using primers: Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and Bt2b (5'-ACCCTCAGTGTAGTGACCCCTTGGC-3'; Glass & Donaldson, 1995) and the elongation factor α-1 gene primers: EF1-728F (5'-CATCGAGAAGTTCGAGAAGG-3') and EF1–986R (5'-TACTTGAAGGAACCCTTACC-3'; Carbone *et al.,* 1999).

The β-tubulin gene was amplified using the REDExtract-N-AmpTM PCR ready mix following the manufacturer's recommendation. PCR was conducted using the conditions described in Section 2.2.5.1.

For the amplification of elongation factor α-1 gene, each 25 µl PCR reaction contained 1.25 U of Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany), 1x PCR buffer (10 mM Tris-HCL, 1.5 mM $MgCl_2$, 50 mM KCl) (Roche Diagnostics, Basel, Switzerland), 200 µM each of dGTP, dCTP, dATP, dTTP, 1.0 mM $MgCl_2$, 20 pmoles of each primer, and 20-25 ng genomic DNA. Thermal cycling conditions were: denaturation at 94°C for 2 min, then 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s and 90 s primer extension at 72°C with a final extension at 72°C for 5 min (Slippers *et al.*, 2004a).

The resulting PCR products were analysed by 1% agarose gel electrophoresis before being sequenced. Although many other studies have used forward and reverse sequence of the nominated regions as the main method of identification, this study needed only confirmation of identity already determined by morphological characteristics, ARDRA techniques and sequence analyses of the ITS region. Therefore the sequencing of β-tubulin and elongation factor genes was only done for one strand of the amplicons using the Bt2a and EF1-728F α-1, respectively, with previously described methods. The returned sequences were analysed following the same methods as described in Section 2.4.2. A species was considered a match if it had 100% homology with a continuous section of at least 370 bp of the β-tubulin gene and 220 bp of the elongation factor α-1 gene of the reference species.

2.2.6 Data analyses

All statistical analyses were done using SPSS® Statistics 17, USA. All data collected from the sampling survey were categorical and in binomial form (presence or absence of pathogen), thus, analysis of variance (ANOVA) was not used. Instead of ANOVA, all variables were analysed using Pearson's Chi-square test of independence at $P \leq 0.05$. This method determined whether the paired observations of two variables (i.e. botryosphaeriaceous infection and nurseries) were independent or associated with each other. Effects of climatic regions on botryosphaeriaceous infection incidence were not determined since most regions had only one nursery participating in the survey.

2.3 RESULTS

2.3.1 Evaluation of different surface sterilisation techniques for botryosphaeriaceous isolation

The results of the surface sterilisation techniques are shown in Appendix A.2. The surface sterilisation technique that gave the highest botryosphaeriaceous species recovery (30%) was the 70% ethanol dip with flame or air-drying.

2.3.2 Nursery survey

Of the 14 nurseries contacted, nine nurseries responded to the survey. They were in Auckland (2), Gisborne (1), Hawkes Bay (1), Marlborough (2), Motueka (1), Otaki (1) and Otago (1) and submitted a total of 311 nursery plants and propagation materials. Sample types and varieties submitted by each nursery are summarised in Table 2.1.

All nine nurseries submitted Grade 1 plants (n=88) and rootstock cuttings (n=84) while 8 nurseries submitted scion cuttings (n=76). Nursery 4 did not provide failed grafted plants and scion cuttings while Nursery 9 did not provide failed grafted plants. Five nurseries submitted a total of 48 failed grafted plants while 2 nurseries submitted 15 Grade 2 plants *in lieu* of failed grafts. The failed grafted plants from Nursery 6 were all dead.

Of the 151 grafted plants submitted, the most common scion was Sauvignon blanc (40%), followed by Pinot noir (34%) and Pinot gris (26%). The rootstocks of these grafted plants were 101-14 (44%), Schwarzmann (40%), Riparia gloire (10%) and 5C (6%). Of the 84 rootstock cuttings collected, the majority were 101-14 (42%), followed by Schwarzmann (26%), 3309 (13%), 5C (12%) and Riparia gloire (7%). The 76 scion cuttings submitted included Sauvignon blanc (45%), Pinot gris (24%), Pinot noir (13%), Riesling (13%) and Chardonnay (5%). No visible external or internal symptoms were observed in any of the Grade 1 and 2 grafted plants, scion or rootstock cuttings collected. Failed grafted plants were frequently characterised by graft failure with partial or total separation of scion wood from rootstock and cracked or peeling bark around the graft union.

Table 2.1 Plants and propagation materials collected from different grapevine nurseries in New Zealand.

[A]Nursery	Variety		Sample type				
	Scion	Rootstock	Failed grafted plants	Grade 1 plants	Grade 2 plants	Rootstock cuttings	Scion cuttings
1	Sauvignon blanc	Riparia gloire		3	3		
	Sauvignon blanc	5C		2	2		
	Sauvignon blanc						5
		Riparia gloire				3	
		5C				2	
2	Sauvignon blanc	101-14		10	10		
	Sauvignon blanc					10	
		101-14					10
3	Sauvignon blanc	Riparia gloire		5			
	Pinot noir	101-14		5			
	Pinot noir	Riparia gloire	5				
	Sauvignon blanc	Schwarzmann	5				
	Sauvignon blanc						3
	Pinot noir						5
	Chardonnay						4
		101-14				3	
		Riparia gloire				3	
		Schwarzmann				3	
		3309				3	
4	Sauvignon blanc	5C		5			
	Sauvignon blanc	101-14		5			
		3309				5	
		101-14				5	
5	Pinot noir	Schwarzmann		10			
	Pinot gris	Schwarzmann	10				
	Riesling						10
		Schwarzmann				10	
6	Pinot noir	Schwarzmann		10			
	Sauvignon blanc	Schwarzmann	[B]10				
	Sauvignon blanc						11
		Schwarzmann				9	
7	Pinot gris	101-14	8	8			
	Pinot gris					8	
		101-14					8
8	Pinot noir	101-14	5	5			
	Pinot gris	101-14	5	5			
		5C				5	
		101-14				5	
	Pinot noir						5
	Pinot gris						5
9	Pinot gris	Schwarzmann		15			
	Pinot gris						5
	Sauvignon blanc						5
		5C				3	
		3309				3	
		101-14				4	
	TOTAL		48	88	15	84	76

[A] Nursery names and locations are not indicated for confidentiality reasons.
[B] Plants were all dead.

2.3.3 Botryosphaeriaceous infection incidence

2.3.3.1 Overall infection incidence in grapevine nurseries

Of the propagation materials and plants received (n=311), 71 (23%) were positive for botryosphaeriaceous infection with a total of 120 isolates recovered. Botryosphaeriaceous infections were present in some plant materials from every participating nursery except for Nursery 4 which only provided apparently healthy grafted plants and scion cuttings (Figure 2.3). The Pearson Chi-square tests indicated significant associations between botryosphaeriaceous infection incidence overall and nursery source (P<0.001; Appendix C.1.1). Nursery 5 had the highest infection incidence (63%) followed by Nursery 3 (41%), while Nurseries 2 and 6 had the lowest incidence of 5% each (Figure 2.3).

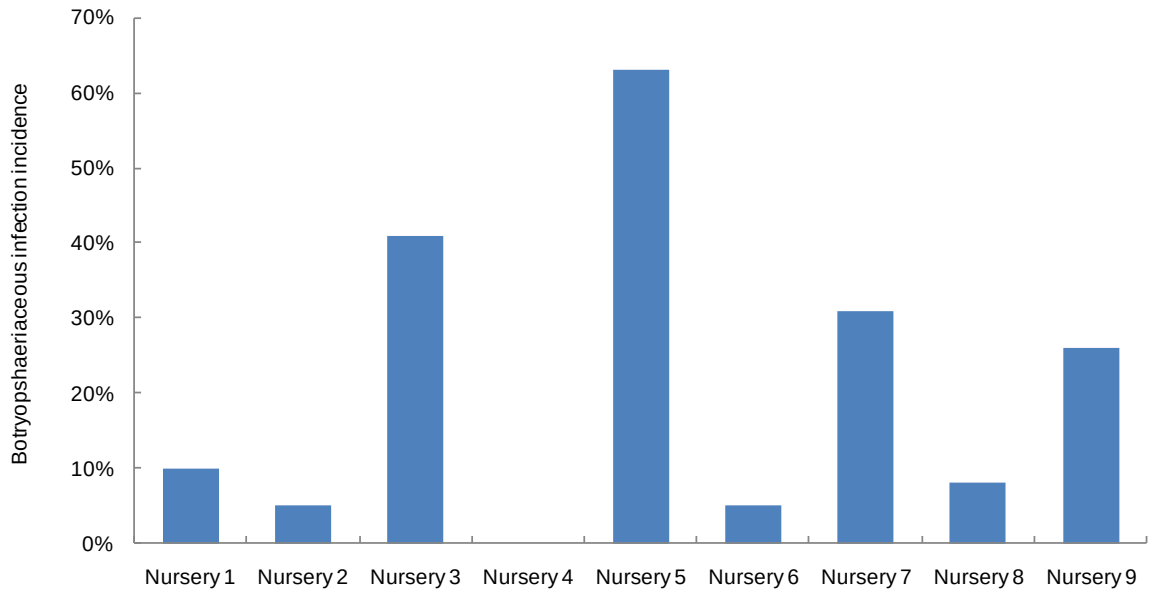

Figure 2.3 Incidence of botryosphaeriaceous infection in New Zealand grapevine nurseries.

2.3.3.2 Botryosphaeriaceous infection in plants and propagation materials from different nurseries

For botryosphaeriaceous infection incidence and number of isolates recovered, plant type was a significant factor (P=0.032 and P<0.001, respectively) with highest infection incidence in failed grafted plants (33%), followed by Grade 1 plants (28%), rootstock cuttings (19%), scion cuttings (17%) and Grade 2 plants (7%; Table 2.2; Appendix C.1.2). Grade 2 plants comprised only 5% of the total plant materials collected. Plant type was not a significant factor for multiple infections (P=0.067; Appendix C.1.3) with 11 and 9% of multiple infections found in rootstock and scion cuttings, respectively, and 23 and 13% for failed grafted and Grade 1 plants, respectively.

Table 2.2 Botryosphaeriaceous infections in different nursery plant materials.

Plant type	No. of samples	[A]No. of samples infected	% Incidence	Samples with multiple infections	% Multiple infections	[B] No. of isolates recovered (%)
Failed grafted plants	48	16	33%	11	23%	33 (28%)
Grade 1 plants	88	25	28%	11	13%	40 (33%)
Grade 2 plants	15	1	7%	0	0	1 (1%)
Rootstock cuttings	84	16	19%	9	11%	26 (22%)
Scion cuttings	76	13	17%	7	9%	20 (16%)
Total	311	71	23%			120

[A] Significant at P=0.032 using Pearson Chi-square test.
[B] Significant at P<0.001 using Pearson Chi-square test.

Nursery source was a significant factor (P<0.001) in the overall infection rates of failed grafted plants, Grade 1 plants and propagation cuttings, but had no significant effect on Grade 2 plants due to the low number of samples (Figure 2.4; Appendix C.1.4). For failed grafted plants, high infection was observed in Nursery 5 (58%), Nursery 3 (21%) and Nursery 7 (18%) and less in Nursery 8 (3%). Failed grafted plants from Nursery 6 were all dead and no botryosphaeriaceous isolates were recovered, while Nurseries 1, 2, 4 and 9 did not submit failed grafted plants. For Grade 1 plants, Nursery 5 also had the highest infection incidence of 30%, followed by Nursery 7 (28%) and Nursery 9 (25%) while Nurseries 8, 3 and 2 had 10%, 5% and 2% respectively. For the propagation cuttings, Nursery 3 had the highest infection incidence of 50% followed by Nursery 5 (33%), which were significantly lower than Nursery 6 (7%), Nursery 9 (4%) and 2% each for Nurseries 1, 2 and 7.

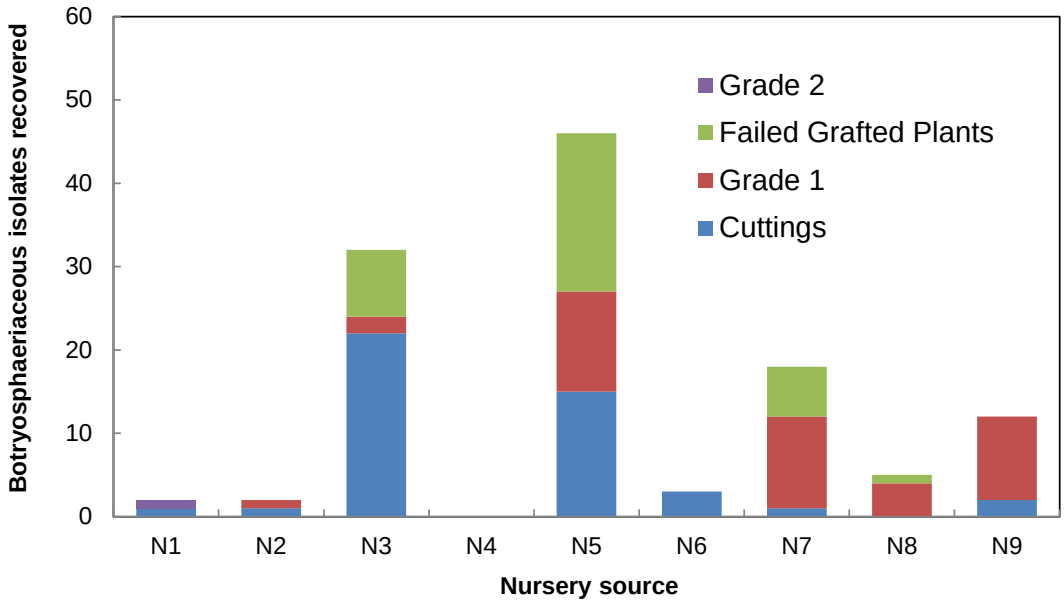

Figure 2.4 Incidence of botryosphaeriaceous infection in different types of plants and propagation materials from New Zealand grapevine nurseries.

The isolation position was also a significant factor in the overall infection rates of failed grafted plants (P=0.028) and Grade 1 plants (P<0.001), but had less effect on propagation cuttings (P=0.066; Appendix C.1.5). For failed grafted plants, infection incidence was higher at 1 cm above and below the graft union (43% and 33%, respectively) than the scion bud (18%) and stem base (6%). The same trend was observed in Grade 1 plants which had more infections at 1 cm above and below the graft union (43% and 40%, respectively) than near the scion bud (15%) and stem base (3%). For the propagation cuttings, infection was higher at the base (46%), than the middle (39%) and top (15%) of the cuttings.

2.3.3.3 Infection in different rootstock and scion varieties

Grapevine variety was also a significant factor for botryosphaeriaceous infection incidence in cuttings (P<0.001; Appendix C.1.6). All rootstock varieties were infected by botryosphaeriaceous species with highest recovery from Schwarzmann (24%) followed by 101-14 (13%), Riparia gloire (8%), and lesser infections from 3309 (2%) and 5C (1%). Scion varieties had similar significance (P<0.001), with higher incidence on Pinot gris (23%) compared to Riesling (13%), Sauvignon blanc (8%) and Pinot noir (8%). Chardonnay which consisted of only 4 cuttings had no infection.

2.3.4 Identification of nursery botryosphaeriaceous species

2.3.4.1 Morphological characteristics

Of the 120 botryosphaeriaceous isolates collected, 82 isolates produced dense aerial mycelium with light to bright yellow pigment after 3 to 5 days when grown on PDAS and after 7 days growth, this yellow colour disappeared as colonies turned dark purple to dark grey. When the ten randomly selected isolates of this group were grown in PEA for conidium characterisation, the yellow pigmentation was less intense and turned amber or brown after 2 to 3 weeks incubation. All PEA isolates produced small black pear-shaped pycnidia at the bases of the PEA plates, of which six isolates produced hyaline, thin-walled, aseptate, fusiform conidia and were assigned into Group 1 (*N. luteum* and *N. australe;* Figures 2.5A and 2.5B, respectively).

The second group (Group 2) included 15 isolates that produced pale yellow or cream pigment with white margins that developed into olivaceous green to dark grey after 7 days when grown in PDAS. When the ten representative isolates were grown in PEA, these isolates produced white dense aerial mycelia after 1 week and turned dark grey to black after 3 weeks. Eight of the PEA isolates produced a small number of pear-shaped pycnidia at the base of the plates while the other two isolates did not produce pycnidia. Of the eight isolates that produced pycnidia, only two produced a small number of hyaline, aseptate, fusiform conidia that were more round and smaller than the conidia observed in Group 1 (Figure 2.5C). This group was placed under the *N. parvum* group.

The third group (Group 3) consisted of six isolates which produced abundant white aerial mycelia on both PDAS and PEA after 1 week incubation. All six isolates grown on PEA turned amber to light brown after 3 weeks incubation and produced pear-shaped pycnidia with distinct long necks. The isolates also produced abundant conidia that were hyaline, thin-walled fusiform-shaped which were relatively longer than those from Group 1 and 2. This group was placed under the *B. dothidea* group (Figure 2.5D).

The fourth group (Group 4 – *Diplodia* spp.) consisted of 12 isolates with white dense aerial mycelia that developed into black colonies after a week on PDA. When the ten representative isolates were grown on PEA, they produced white dense mycelia that turned black after 2 weeks with seven isolates producing pycnidia that protruded on the surface of the plates. Of the seven that produced pycnidia, four produced cylindrical, hyaline, unicellular conidia with broad ends that were identified as *D. mutila* (Figure 2.5E) while the

three other isolates produced cylindrical to ellipsoid dark brown conidia which were rounded at both ends and were identified as *D. seriata* (Figure 2.5F).

Three isolates produced variable mycelial characteristics and had either no pycnidia or produced sterile pycnidia. These were placed under the botryosphaeriaceous spp. (Group 5). Two isolates were contaminated with *Trichoderma* spp. and could not be revived, thus, were not included in the identification.

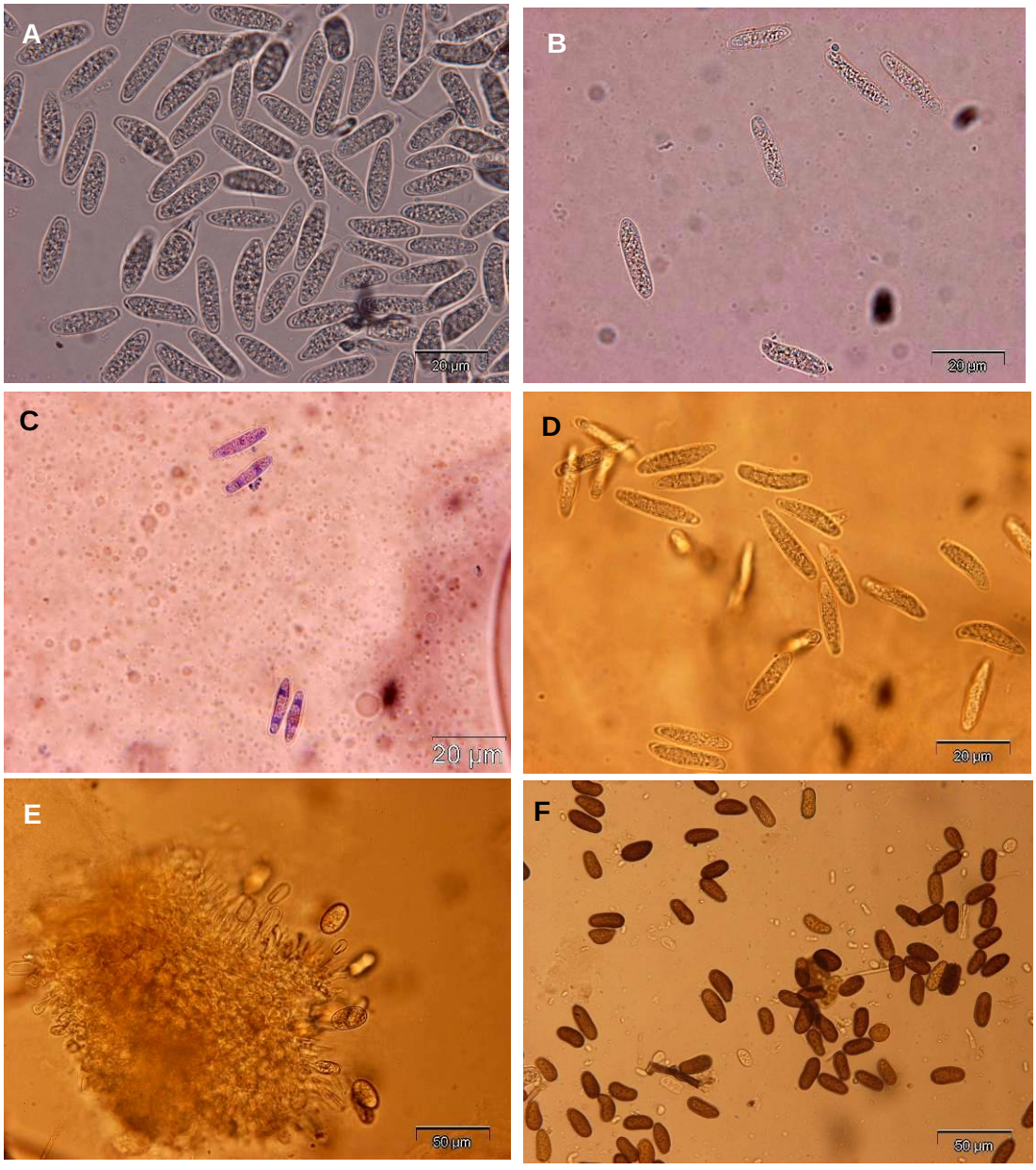

Figure 2.5 Conidial characteristics of representative botryosphaeriaceous species isolated from grapevine nursery plant materials. A) *N. luteum*; B) *N. australe*; C) *N. parvum*; D) *B. dothidea*; E) *D. mutila*; F) *D. seriata.*

2.3.4.2 Identification of botryosphaeriaceous isolates by ARDRA

Following the results of the morphological identification, in which colony characteristics were seen to overlap between species and many of the representative isolates did not produce conidia, all 118 isolates were included in the ARDRA. Identities of botryosphaeriaceous isolates from grapevine nursery plant materials in New Zealand using ARDRA are summarised in Table 2.3 and Appendix D.1.

Table 2.3 Incidence and distribution of botryosphaeriaceous species isolated from grapevine nursery plant materials in New Zealand based on ARDRA.

Species	No. of isolates	Percentage (%)	No. of nurseries infected
Neofusicoccum luteum	68	57	6
N. parvum	21	18	5
N. australe	8	7	6
Botryosphaeria dothidea	6	5	2
Diplodia mutila	8	7	4
D. seriata	4	3	2
N. ribis	2	1	1
Neofusicoccum sp.	1	1	1
Unidentified (contaminated)*	2	1	1

*Contaminated with *Trichoderma* spp. and could not be identified further.

PCR reactions using the ITS1 and NL4 primers yielded a product of approximately 1200 bp for each of the 118 isolates analysed (examples shown in Figure 2.6). All 118 isolates were initially digested using the *Taq*I enzyme from which 76 were further digested using *Sac*II, 42 with *Hae*III and 15 with *Nci*I (examples shown in Figures 2.7, 2.8; 2.9 and 2.10, respectively).

Of the 118 isolates digested using the *Taq*I enzyme, 76 (64%) gave banding patterns of Group A (*N. luteum*/*N. australe)*, 27 (23%) of Group B and 15 (13%) of Group C (examples shown in Figure 2.7). When Group A isolates (n=76) were further digested with the *Sac*II enzyme, 68 (89%) gave the banding pattern of Group D (*N. luteum)* while the remaining 8 isolates (11%) gave the banding pattern of Group E (*N. australe;* Figure 2.8). All 76 isolates under Group A belonged to morphology Group 1 (*N. luteum/N. australe)*.

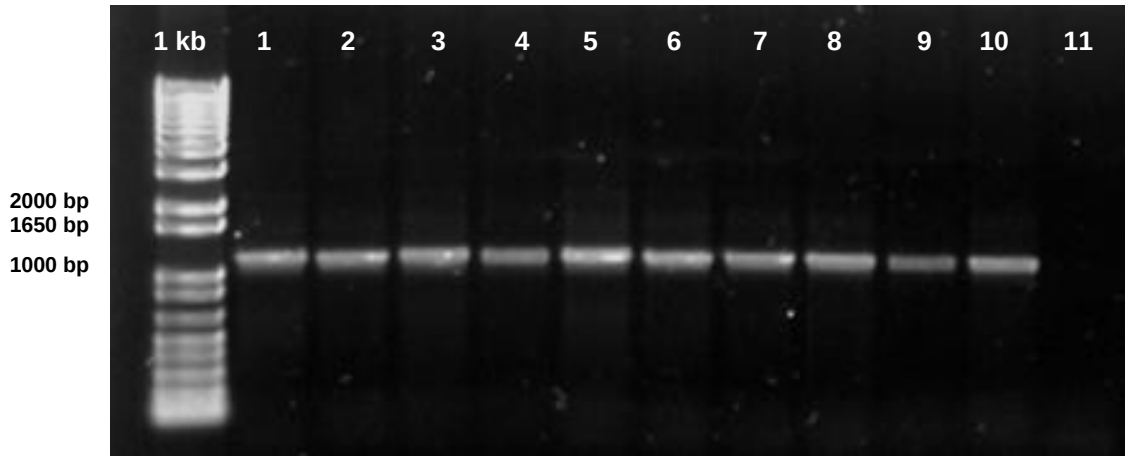

Figure 2.6 Representative photograph of the amplified 1200 bp fragment of the ribosomal DNA of different botryosphaeriaceous isolates using the ITS1-NL4 primers. Lanes 1-10 contain representative botryosphaeriaceous isolates; lane 11, negative control. The numbers on the far left denote the molecular weight of the 1 kb plus DNA ladder.

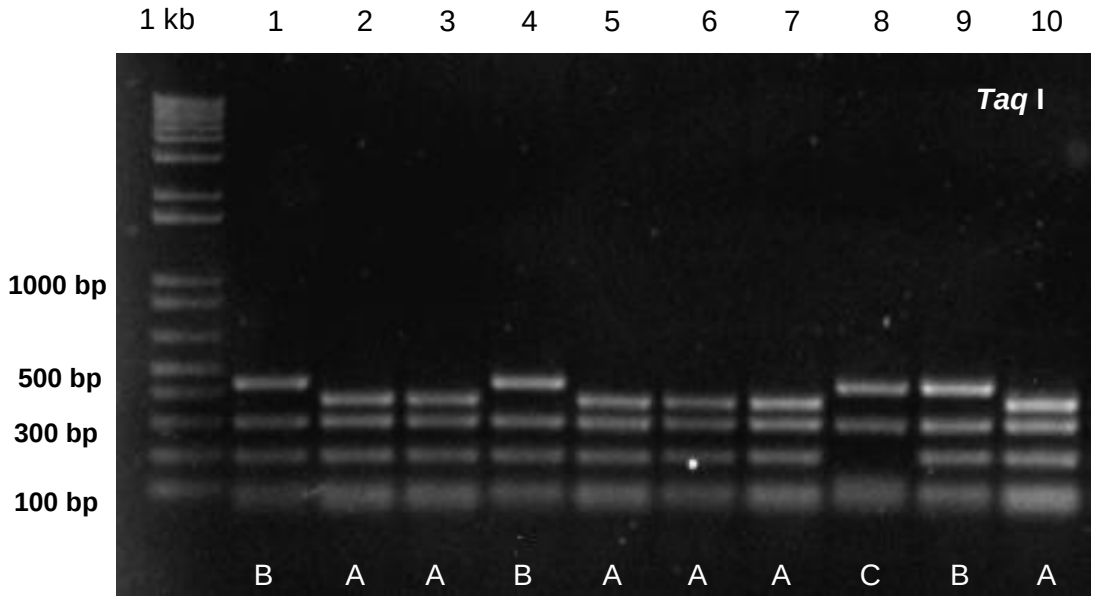

Figure 2.7 ARDRA of ITS1/NL4 PCR products using *Taq*I restriction enzyme. Lanes 1, 4 and 9 - banding pattern of Group B; lanes 2, 3, 5, 6, 7 and 10 - banding pattern of Group A (confirmed as *N. luteum* and *N. australe*); lane 8 - banding pattern of Group C. The numbers on the far left denote the molecular weight of the 1 kb plus DNA ladder.

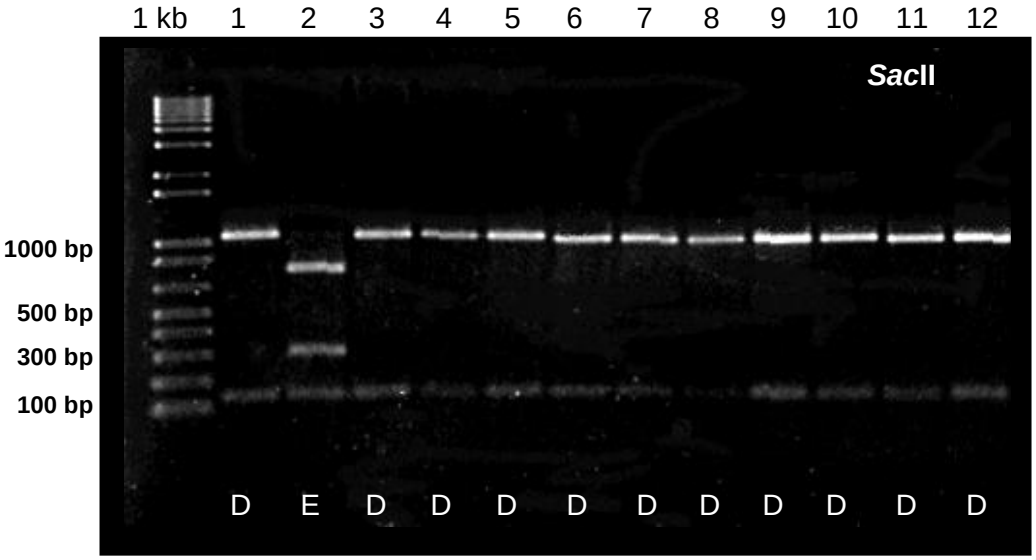

Figure 2.8 ARDRA of ITS1/NL4 PCR products using *Sac*II restriction enzyme. Lanes 1, 3 to 12 - banding pattern of Group D (*N. luteum*); lane 2 - banding pattern of Group E (*N. australe*). The numbers on the far left denote the molecular weight of the 1 kb plus DNA ladder.

When Groups B and C isolates (n=42) were digested using the *Hae*III enzyme, 21 isolates (50%) gave a restriction pattern of Group G (*N. parvum)*, 6 (14%) of Group H (*B. dothidea)* while the remaining isolates gave the banding pattern of Group F that could not be differentiated by *Hae*III (examples shown in Figure 2.9). The isolates under Group G included the 15 isolates from morphology group 2 (*N. parvum*) while the other six belonged to morphology Group 1 (*N. luteum/N. australe*). The six isolates from Group H were the same isolates from morphology Group 3 (*B. dothidea).*

The remaining 15 isolates from Group F were further digested using the *Nci*I enzyme (examples shown in Figure 2.10). Eight isolates gave banding patterns of Group K (*D. mutila)* and four of Group J *(D. seriata)*. These isolates belong to the morphology Group 4 (*Diplodia* group). Two isolates that were differentiated from *N. parvum* in the *Hae*III digest and *N.luteum/australe* using the *Taq*I enzyme gave a banding pattern typical of *N. ribis* using *Nci*I while one novel isolate gave two bands of approximately 1000 and 103 bp, making it distinct from all species (Figure 2.10) characterised by Alves *et al.* (2005). The *N. ribis* isolates and the novel species were the isolates placed in morphological Group 5 (botryosphaeriaceous spp.).

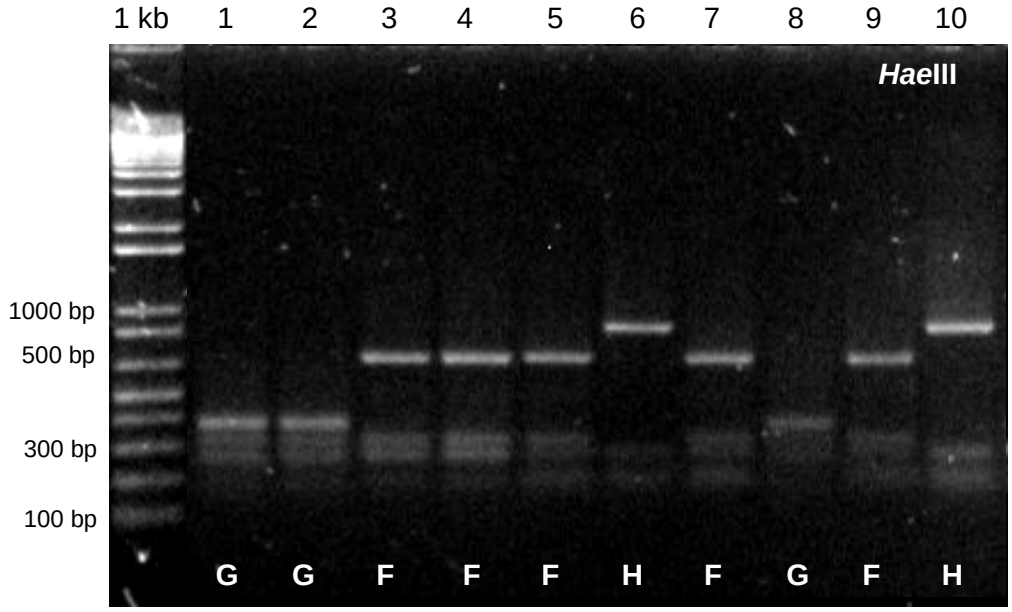

Figure 2.9 ARDRA of ITS1/NL4 PCR products using the *Hae*III restriction enzyme. Lanes 1 , 2 and 8 - Group G (*Neofusicoccum parvum*); lanes 3 to 5, 7 and 9 - Group F (*Diplodia seriata/mutila*; *N. ribis*); Lanes 6 and 10 – Group H (*Botryosphaeria dothidea*). The numbers on the far left denote the molecular weight of the 1 kb plus DNA ladder.

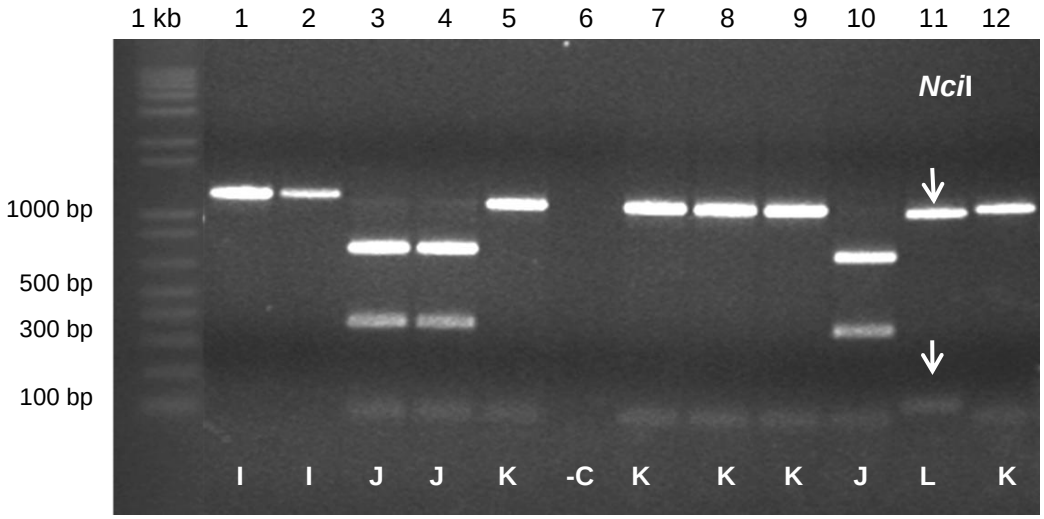

Figure 2.10 ARDRA of ITS1/NL4 PCR products using the *Nci*I restriction enzyme. Lanes 1 and 2, Group I (*N. ribis*); Lanes 3, 4 and 10, Group J (*Diplodia seriata*); Lanes 5, 7 to 9 and 12, Group K (*D. mutila*); Lane 6, Negative control (SNW); and Lane 11, Group L (a novel isolate with ~1000 and ~100 bp bands as indicated by arrows). The numbers on the far left denote the molecular weight of the 1 kb plus DNA ladder.

2.3.4.3 Sequence analyses of representative species

2.3.4.3.1 Sequence analyses of ribosomal DNA

The neighbour joining tree generated from the aligned sequences of representative isolates from nurseries and their matches from the NCBI database are presented in Figure 2.11 (Appendix E.1). The neighbour joining tree had two major branches which were further divided into different sub-branches (Figure 2.11). The first branch was occupied by *B. dothidea* while the second branch was divided into two sub-branches, being occupied by the *Neofusicoccum* and the *Diplodia* groups. The first sub-branch was further divided into more branches, with a cluster of *N. parvum, N. ribis* and *N. macroclavatum* and another with *N. luteum* and *N. australe*. The second sub-branch was also divided into two clusters with *D. seriata* occupying the first and *D. mutila* occupying the second. The representative isolates of *N. luteum*, *N. parvum, N. australe*, *B. dothidea*, *D. mutila* and *D. seriata* all clustered in the same branch with their matching accessions from the NCBI database confirming their initial identifications. The two isolates that were initially identified as *N. ribis* based on ARDRA were separated into two sub-branches of the *Neofusicoccum* group with R83a being found in the *N. parvum* branch while R83b in the *N. ribis* branch and their identity based on rDNA sequence was inconclusive (Appendix E.1.7). The novel isolate, M353, on the other hand was found in the same branch as the three *N. macroclavatum* accessions (Appendix E.2.1).

2.3.4.3.2 Sequence analyses of β-tubulin and the elongation factor 1α genes

Further attempts to identify isolates R83a and R83b by β-tubulin gene sequence analysis also gave inconclusive results with the two isolates achieving 99% homology with *N. ribis* and *N. parvum* (Table 2.4; Appendix E.2), differing by one base pair for each species. Amplifications of the elongation factor gene for these two isolates were also unsuccessful and sequence analysis for this gene was not done. Due to the cost of the analyses and time constraints, no further attempts were made to identify them and they were assigned as *Neofusicoccum* spp.

For the novel isolate M353, confirmation of its identity based on β-tubulin and elongation factor α1 gene sequence analyses showed that this isolate was 100% identical to reported sequences of *N. macroclavatum* (Table 2.4; Appendix E.3).

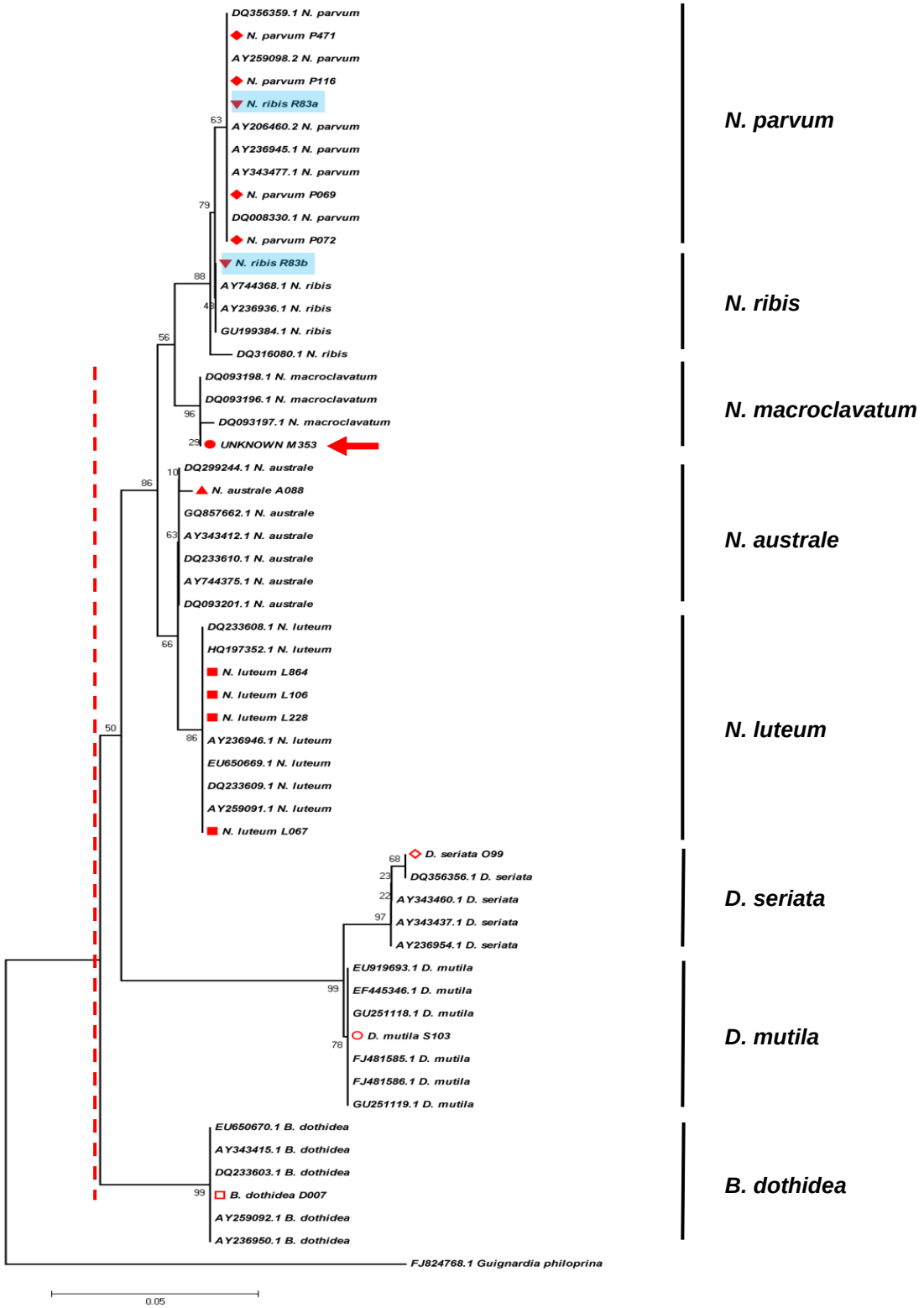

Figure 2.11 Neighbour joining tree obtained from the rDNA sequence data of representative isolates of botryosphaeriaceous species from New Zealand nurseries and representative sequences of each species from the NCBI database. The red dotted line indicates the main branches of the tree. Isolates with red symbols on the left are from the New Zealand nurseries and those obtained from the NCBI data base are indicated by their accession numbers. Isolates shaded in blue were identified as *N. ribis* by ARDRA but their DNA sequence analyses were inconclusive. The isolate indicated by an arrow is the novel isolate M353 that was not identified by ARDRA. Bootstrap values are given above the branches based on 1000 bootstrap replicates. The tree is rooted to the outgroup *Guignardia philoprina*.

Table 2.4 Sequence analyses for β-tubulin and the elongation factor 1α genes for unknown isolates (Appendices E.2 and E.3)

Isolate No.	Identity based on ARDRA	β-tubulin gene sequence	Genbank Accession No.	elongation factor 1α gene sequence	Genbank Accession no.
R83a	*N. ribis*	Inconclusive		*Not applicable	
R83b	*N. ribis*	inconclusive		*Not applicable	
M353	unknown	*N. macroclavatum*	DQ093207/206	*N. macroclavatum*	DQ093219/217

*Gene was not amplified

2.3.5 Botryosphaeriaceous species incidence and distribution

Nursery 3 had the highest number of botryosphaeriaceous species (n=7) followed by Nursery 9 (n=6), while only one species was found in Nursery 8 (Table 2.5). The novel species *N. macroclavatum* was found in Nursery 9. Although Nursery 5 had the highest botryosphaeriaceous infection incidence, it had only three species, most of which were *N. luteum*.

Table 2.5 Prevalence of different botryosphaeriaceous species in different nurseries

Species	Nursery									Total	Percent Incidence
	1	2	3	4	5	6	7	8	9		
N. luteum	0	1	12	0	42	2	6	5	0	68	57%
N. parvum	1	0	8	0	1	0	10	0	1	21	18%
N. australe	0	1	1	0	3	1	1	0	1	8	7%
B. dothidea	0	0	4	0	0	0	0	0	2	6	5%
Neofusicoccum spp.	0	0	2	0	0	0	0	0	0	2	1%
N. macroclavatum	0	0	0	0	0	0	0	0	1	1	1%
D. mutila	1	0	3	0	0	0	1	0	3	8	7%
D. seriata	0	0	2	0	0	0	0	0	2	4	3%
Unidentified*	0	0	0	0	0	0	0	0	2	2	1%
Total isolates	2	2	32	0	46	3	18	5	12	120	
Total species	2	2	7	0	3	2	4	1	6	8	

*Contaminated with *Trichoderma* spp. and could not be identified further.

The Pearson Chi-square tests were conducted to determine the relationships between nursery source, plant types, isolation position and varieties on incidence of only *N. luteum* and *N. parvum* infections, as these were the most predominant species found in the nurseries at 56 and 18% of all isolates, respectively.

2.3.5.1 Botryosphaeriaceous species in grapevine nurseries

For *N. luteum* infection incidence, nursery source was a significant factor (P<0.001; Appendix C.1.7). It was present in six out of seven nurseries, with most from Nursery 5 (62%), followed by Nursery 3 (18%) while the remaining 20% were from Nurseries 2, 6, 7 and 8. For *N. parvum* infection incidence, nursery source was also a significant factor (P<0.001; Appendix C.1.18). It was present in five nurseries with most isolates from Nursery 7 (48%) and Nursery 3 (38%). One isolate of *N. parvum* was recovered from each of Nurseries 1, 5 and 9, while Nurseries 2, 6 and 8 had no *N. parvum* infections.

2.3.5.2 Botryosphaeriaceous species in plants and propagation cuttings

Plant and propagation materials and isolation positions were also a significant factor for *N. luteum* and *N. parvum* infection incidence. For *N. luteum* infection incidence, plant type was a significant factor (P=0.003; Appendix C.1.7) with infections mostly being found in failed grafted plants (31%) and apparently healthy grafted plants (27%) and fewer in scion cuttings (22%) and rootstock cuttings (20%). Grade 2 plants, which represent only 5% of all samples collected, had no *N. luteum* infection. Plant type was also a significant factor for *N. parvum* infections (P=0.016; Appendix C.1.8), being mostly found from failed grafted plants (43%) and fewer from Grade 1 plants (24%), scion cuttings (14%), rootstock cuttings (14%) and Grade 2 plants (5%).

Isolation position was a significant factor for *N. luteum* infections in failed grafted plants (P=0.023) and Grade 1 plants (P=0.005; Appendix C.1.9). Most of the *N. luteum* infections in failed grafted plants were found at 1 cm above and below (45% and 36%, respectively), the graft unions, while 14 and 5% infections were from scion buds and stem bases, respectively. The same trend was observed in Grade 1 plants in which *N. luteum* infections were also mostly found 1 cm above or below the graft unions (47% and 40%, respectively) while only 10% were found in scion buds and 3% from stem bases. The isolation position in the rootstock and scion cuttings was not a significant factor for *N. luteum* infections (P=0.611; Appendix C.1.9) as this species was found in all parts of the cuttings. Isolation position was not a significant factor (P=0.134 to 0.526) for *N. parvum* infections in any plant type (Appendix C.1.10).

Of the 38 plants and propagation materials with multiple infections, ten of them were infected with two species while 28 were infected with only one species. Two failed grafted plants were infected with *N. luteum*/*N. parvum* while one was infected with *N. luteum*/*N. australe*. Three rootstock cuttings had multiple infections of either *N. luteum/N. australe*, *N. luteum/D. seriata* and *N. luteum/D. mutila* while four healthy grafted plants had multiple infections of either *N. parvum/D. mutila, D. mutila/B. dothidea* and *N. luteum/N. australe*

2.3.5.3 Botryosphaeriaceous species in different cultivars

Rootstock and scion varieties were also significant factors (P<0.001) for *N. luteum* infections (Appendix C.1.11). Three out of five rootstock varieties and four out of five scion varieties had *N. luteum* infections. Of the 68 *N. luteum* isolates, 57% were recovered from scion and 43% from rootstock cuttings. For rootstock varieties, Schwarzmann had the highest *N. luteum* infections (62%), followed by 101-14 (28%) and Riparia gloire (10%), while 3309 and 5C had no *N. luteum* infections. For scion varieties, Riesling had the highest *N. luteum* infection (38%), followed by Pinot gris (33%), Pinot noir (21%) and Sauvignon blanc (8%). The four samples of Chardonnay had no *N. luteum* infections.

Rootstock (P=0.008) and scion (P=0.01) varieties were significant factors for *N. parvum* overall infection incidence (Appendix C.1.12). For rootstock varieties, most *N. parvum* infections were found in 101-14 (56%) and 3309 (22%). One isolate (11%) each of *N. parvum* were recovered from Riparia gloire and Schwarzmann while none were recovered from 5C samples. For scion varieties, most *N. parvum* were from Pinot gris (67%), followed by Sauvignon blanc (33%), while Pinot noir, Riesling and Chardonnay had no infections.

2.4 DISCUSSION

This research is the first attempt to comprehensively sample the propagation materials used by grapevine nurseries and the grafted plants produced to assess the presence and diversity of botryosphaeriaceous species in New Zealand. It showed that infection by the botryosphaeriaceous species is widely spread, being present in eight out of the nine nurseries sampled. It is important to note, however, that this study was not a “true” survey since selection of nurseries and samples were not random and sample types and sizes were not uniform. Some nurseries also submitted only part of the plant sample types requested while others deliberately sent their problematic samples because they wanted some answers to their problems. Therefore, this research was purely exploratory in order to establish the presence or absence of botryosphaeriaceous infections in the nurseries. The use of the

convenient sampling system used here may have created bias in the results. The data generated from this study does not represent the actual disease situation in every nursery.

While randomised sampling would have been ideal for this research survey, there were some problems associated with this method, including: a) the cost and ease of sample collection since the nurseries were located in different regions around New Zealand and some were not easily accessible for field sampling; b) the costs of the analysis; and c) the value and availability of the nursery stocks, some of which were pre-ordered. In addition, some nurseries were hesitant to participate in the survey as they believed that it might affect the marketability of their products. However, this study has provided some relevant information about the botryosphaeriaceous infection incidence and species present in different plant and propagation materials from different nurseries.

Based on morphological characteristics and molecular analyses, seven species were accurately identified as infecting plants and propagation materials in eight major grapevine nurseries in different regions in New Zealand. *Neofusicoccum luteum* was the most predominant species, followed by *N. parvum*, *N. australe, D. mutila, B. dothidea, D. seriata,* and the novel isolate *N. macroclavatum.* This supports the anecdotal accounts of botryosphaeriaceous species being frequently isolated from failed grafts in young plants from New Zealand nurseries (Harvey, pers. comm., 2007) and from one-year old vines from five different nurseries in Gisborne, Marlborough and Auckland (Graham, pers. comm., 2008). In grapevine nurseries in Spain, *D. seriata* and an unidentified botryosphaeriaceous species were associated with the decline of the young plants (Gimenez-Jaime *et al.,* 2006). Botryosphaeriaceous species were also frequently isolated from basal internodes of one-year old rootstock canes (Fourie & Halleen, 2002) and 4 cm below and within the wounds of 2-year-old pruning stubs of the mothervines (Fourie & Halleen, 2004b). Apart from *D. seriata,* however, the other botryosphaeriaceous species were not reported or identified in these studies.

Accurate identification of species based on morphology has been problematic for many researches on botryosphaeriaceous species (Slippers *et al.*, 2004a; Taylor *et al.*, 2005; Amponsah, 2011). In this study, colony and conidium morphology overlapped, particularly in the *Neofusicoccum* species, making identification difficult. Both *N. luteum* and *N. australe* produced bright yellow pigment in young PDA cultures. Their conidium characteristics were also very similar and difficult to distinguish from each other resulting in these two species being placed together in one morphology group (Group A). Slippers *et al.* (2004b) also reported that it was difficult to distinguish *N. australe* and *N. luteum* as their general

appearance and conidial characteristics overlapped. For *N. parvum,* different isolates produced different colony pigment on PDA plates that were either pale yellow, cream or white. This variability in colony morphology resulted in placing those isolates with pale yellow pigment under Group A (*N. luteum*/*N. australe*) while the cream and white isolates were placed under Group B. Amponsah *et al.* (2011) also observed that some *N. parvum* isolates exhibited yellow pigmentation on PDA cultures that were difficult to differentiate from the *N. luteum*/*N. australe* group. In addition, many of the isolates did not produce pycnidia and conidia in artificial cultures.

The use of molecular tools like ARDRA, which employs the PCR - RFLP technique (Alves *et al.*, 2005) and DNA sequencing resolved the problem for identification at species level. DNA sequencing of rDNA also enabled the identification of the novel species *N. macroclavatum* that was not characterised by ARDRA. The identification of this novel isolate was further confirmed by sequence analyses of β-tubulin and elongation factor 1α genes. Despite the results of sequence analyses being clear and unambiguous, two isolates were not identified because they showed 99% homology to both *N. parvum* and *N. ribis* based on sequence analyses of rDNA and β-tubulin genes. These closely related *Neofusicoccum* species could only be differentiated based on their EF1- α gene sequences (van Niekerk *et al.*, 2004). However, the amplifications of EF1- α gene for these two isolates were unsuccessful maybe due to poor DNA quality and no further analyses was done due to cost and time constraints.

Of the seven species identified, *N. luteum, N. parvum* and *N. australe* were the most predominant as well as the most widespread species found in New Zealand nurseries. In a survey conducted by Baskarathevan *et al.* (2008) in 43 vineyards around New Zealand in 2007-2008, *N. parvum* (35%) was the most prevalent, followed by *D. mutila* (18%), *D. seriata* (17%), *N. luteum* (15%) and *N. australe* (12%). These results indicated that botryosphaeriaceous infections in the nurseries were caused by the same species that had been identified in vineyards and, thus, there was likely to be a connection to vineyard disease. However, it was clear that there was a major difference in prevalence as *N. parvum* dominated in vineyard and *N. luteum* was most common in nurseries. This may be explained by the types of symptoms caused by these species. In pathogenicity studies conducted by Amponsah (2011), he found that 7 months after inoculation with *N. parvum,* the grapevines had external cankers and internal wood necrosis while those inoculated with *N. australe*, *N. luteum* and *D. mutila* had only minor external symptoms around the inoculation wound. However, when the inoculated plants were pruned in winter and put under lights to break dormancy, plants inoculated with *N. luteum*, *N. australe* and *D. mutila*

had produced significant dieback while minor dieback was observed in plants inoculated with *N. parvum*. From this observation, Amponsah (2011) hypothesised that *N. parvum* is a canker pathogen causing external symptoms, while infections of *N. luteum*, *N. australe* and *D. mutila* are initially endophytic and symptomless but progress through the shoots but cause dieback symptoms when the hosts are stressed. Since the above survey by Baskarathevan *et al.* (2008) took samples from symptomatic trunk and cordons for the vineyard survey, it is therefore possible that more canker-producing species like *N. parvum* were obtained and fewer of the dieback species like *N. luteum* were intercepted in the survey. The high number of *N. luteum* infections in the plant and propagation materials found in this study may be due to its indistinct symptoms that are not detected during grading processes in nurseries, and so they were submitted as asymptomatic samples for this study. In contrast, *N. parvum* which produced external canker symptoms was most likely detected and discarded by nurseries, and so were not submitted for this study. This possibility was further investigated in Chapter 3, which followed the principles of Koch's postulates to demonstrate the pathogenicity of the isolates from nurseries. Any differences in pathogenic behaviour of the different botryosphaeriaceous species would be demonstrated during these tests. However, to clarify the differences in prevalence of different botryosphaeriaceous species in the two systems, a randomised parallel sampling of symptomatic and asymptomatic plants from vineyards and nurseries, with isolation and species identification, should be conducted.

Except for *N. macroclavatum*, all botryosphaeriaceous species reported in New Zealand vineyards and grapevine nurseries have been reported in vineyards in other grape-growing areas around the world, although the presence and predominance of species varied between countries. In Californian vineyards, *N. australe, B. dothidea, N. luteum, D. seriata, N. parvum, B. rhodina* and *D. mutila* were found, with *D. seriata* being the most common species (Urbez-Torres *et al.*, 2006a). In the Hunter Valley of New South Wales, Australia, *D. seriata* also dominated (Castillo-Pando *et al.*, 2001), while in Western Australia *N. australe, D. rhodina*, *D. seriata* and *D. mutila* were also associated with grapevine decline (Taylor *et al.*, 2005). The most prevalent species associated with excoriose and dieback of grapevines in Portugal was *B. dothidea* (Phillips, 1998) while *D. seriata* and *B. dothidea* were the fungi associated with black dead arm in French vineyards (Larignon *et al.*, 2001). In Spain, *D. seriata* and unidentified botryosphaeriaceous species were frequently isolated from grafted plants prior to planting in the nursery fields and caused the decline of young vines in grapevine nurseries (Gimenez-Jaime *et al.*, 2006). Studies by Phillips, (2002) also showed that in Portugal, *N. parvum* and *D. seriata* were frequently isolated from necrotic tissues associated with poor quality graft unions.

The novel species *N. macroclavatum* that was isolated from an apparently-healthy grafted plant from Nursery 9 was the first report of this species with grapevines and the first report of its occurrence in New Zealand (Billones *et al.*, 2010b). This species was first reported in Western Australia as a pathogen of *Eucalyptus globulus* after its introduction from eastern Australia where it was an endemic species (Burgess *et al.*, 2005). Despite its low infection incidence, it was found to be the most pathogenic of the four species infecting Eucalypts in Western Australia. The low infection incidence of *N. macroclavatum* was most likely due to the recent introduction of *E. globulus* from eastern states of Australia to Western Australia. The occurrence of *N. macroclavatum* in New Zealand may also have been through the introduction of *E. globulus* to the country, but this is the first report of it from grapevines. Botryosphaeriaceous species have a wide geographical distribution and occur on a wide range of woody hosts including monocotyledonous, dicotyledonous and gymnosperm hosts (van Niekerk *et al.*, 2004; Burgess *et al.*, 2005; Urbez-Torres *et al.*, 2006a). The association of *N. macroclavatum* on grapevine confirms the cosmopolitan nature of this fungal group. Amponsah (2011) found that the botryosphaeriaceous species commonly found in New Zealand vineyards were also infecting non-grapevine woody hosts growing close to some vineyards including pine trees, willow and oak used as shelter belts. However, *Eucalyptus* spp. were not included in that study. In New Zealand, *Eucalyptus* spp. are commonly used as shelter belts for orchards and other agricultural systems because they grow very quickly (Hosking, 1973; Sale, 1978). Since only one isolate of *N. macroclavatum* was obtained from this study, it is possible that it is not widely spread in grapevines; and that it may have originated from *Eucalyptus* spp. planted near the nursery where the infected sample was collected. This single isolate was tested for pathogenicity to grapevines in Chapter 3 of this study in order to determine its potential threat to grapevines. It is also important to investigate its prevalence on *Eucalyptus* spp. near vineyards and nurseries to determine its potential spread to grapevines.

This study showed that botryosphaeriaceous infection was common in all types of grapevine materials collected. In grafted plants, the infection incidence was higher in failed grafted plants (33%) than in healthy grafted ones (28%). Infection incidence was also relatively high in non-symptomatic rootstock and scion cuttings (19 and 17%, respectively) used for grafting. The high incidence of botryosphaeriaceous species on asymptomatic grapevine materials from New Zealand nurseries may be due to latent infection or endophytic development, which has been reported for this fungal group. The botryosphaeriaceous species have been reported to be endophytes of woody tissues in trees (Smith *et al.*, 1996; Flowers *et al.*, 2003; Burgess *et al.*, 2005; Sakalidis *et al.*, 2011). Fourie and Halleen (2004b)

also observed latent infections of botryosphaeriaceous species in rootstock mothervines. Due to their endophytic nature, this fungal group can be easily introduced to new areas in cuttings or seeds (Burgess *et al.*, 2005). The presence of the pathogens in non-symptomatic rootstock and scion cuttings and the lack of visible symptoms of infected Grade 1 plants from grapevine nurseries can, therefore, carry the infection to the new vineyards. Symptom development of botryosphaeriaceous infection in grapevines was also reported to be normally slow and visible symptoms were only observed after the plants had been stressed, leading to severe symptom development (Larignon *et al.*, 2001; Martin & Cobos, 2007). It is possible that young nursery plants with latent infections have been responsible for introducing botryosphaeriaceous infections into vineyards, where the young vines grew normally until they were subjected to stress. Since most botryosphaeriaceous isolates found in nursery plant materials were from asymptomatic materials, pathogenicity tests were done in Chapter 3 to demonstrate the infection potential of all the nursery isolates.

Botryosphaeriaceous species are known as wound pathogens. A study by Amponsah (2011) showed that no infection occurred in any non-wounded shoots and leaves when inoculated with *N. luteum* conidia while conidia germinated and colonised wounded shoots and leaves within 24 hours after inoculation. These species were also frequently isolated in pruning wound ends suggesting the wound as the point of entry of infections (Fourie & Halleen, 2004b). Botryosphaeriaceous species were also associated with poor quality graft unions (Phillips, 2002). In this study, infections in all types of grafted plants were mostly found near the graft union suggesting that the grafting wound might be a point of entry for infections that occurred during grafting. It is also possible that the grafting process renders the plants stressed and vulnerable to infections. Contamination by botryosphaeriaceous propagules in the grafting process may also contribute to the infection and spread of this disease. Therefore, investigation of the sources of botryosphaeriaceous inoculum in the nursery system and the mode of infection in the nursery plant materials was conducted in Chapter 5 and Chapter 6.

This study also showed that grapevine variety may be a significant factor for the overall botryosphaeriaceous infection incidence. Schwarzmann and 101-14 had the highest infection incidence among rootstock varieties while Pinot gris and Riesling had the highest infection incidence for the scion varieties. Variety also appeared to be a significant factor in the disease incidence of the different botryosphaeriaceous species. In rootstock varieties, *N. luteum* infections were mostly found in Schwarzmann and 101-14 while *N. parvum* were more frequently found in 101-14 and 3309 varieties. For scion varieties, *N. luteum* infections were mostly found in Riesling and Pinot gris while *N. parvum* was mostly found in Pinot gris

and Sauvignon blanc. However, since this study did not employ randomised sampling techniques and the sample size of the varieties were not uniform, the association between varieties on the overall infection and species incidence found may have reflected the sampling method rather than the true situation.

Past studies on the relative qualities of different grapevine rootstock varieties were focused on their relative susceptibilities to the insect pest phylloxera, nematodes, crown gall or Phytophthora root rot as well as their abilities to cope with factors such as drought, flooding and nutrient deficiencies (Marais, 1979; Sule, 1999; Bavaresco *et al.*, 2000; Keller, 2010a). In contrast, studies on scion cultivars were focused on resistance or susceptibility to fruit and foliar pathogens like downy mildew (Tian & Bertolini, 1996; Boso & Kassemeyer, 2008), powdery mildew, grey mould (Irimia *et al.,* 2009), viral diseases (Ramsdell *et al.*, 1995) and Pierce's disease (Krell *et al.*, 2008). Reports on relative resistance or susceptibility to trunk diseases have focussed mostly on Petri disease, caused by *Phaeocremonium* and *Phaeomoniella* spp. (Santos *et al.*, 2005; Borgo *et al.*, 2008), Eutypa dieback (Kobes *et al.*, 2005), Cylindrocarpon black foot disease (Alaniz *et al.*, 2007; Jaspers *et al.*, 2007) and *Phomopsis* spp. (Latinovic *et al.*, 2004).

For botryosphaeriaceous species, Martos (2008) observed differences in susceptibility of four rootstock and six scion varieties commonly used in Spain to *B. dothidea*, *D. seriata*, *N. luteum*, *N. parvum* and *Do. viticola*. Of the ten varieties tested in the above study only 5C and Cabernet Sauvignon are commonly found in New Zealand (New Zealand Winegrowers Statistical Annual, 2010). In New Zealand, Amponsah (2011) did not observe varietal differences in five scion varieties when inoculated with one isolate each of *N. luteum*, *N. australe*, *N. parvum*, and *D. mutila* using green shoot assays, but no studies were done on different rootstock varieties commonly used in the nurseries. Therefore, an investigation was conducted in Chapter 4 into the relative susceptibility of the different rootstock and scion cultivars commonly used in New Zealand to the most common botryosphaeriaceous species found in the grapevine nurseries. To date, control measures for botryosphaeriaceous infections have not been clearly identified, therefore, the identification of resistant grapevine varieties may contribute to its control.

Chapter 3
Pathogenicity of botryosphaeriaceous species from nurseries and genetic diversity of *N. luteum*

3.1 *Section 1.* Pathogenicity of botryosphaeriaceous species from grapevine nurseries

3.1.1 INTRODUCTION

Based on Koch's rule of proof of pathogenicity, the pathogen must be isolated from the diseased plant and grown in pure culture. It should then be inoculated on a healthy plant and produce the same symptoms, from which an identical isolate as the original one must be retrieved (Agrios, 2005). To confirm that the botryosphaeriaceous isolates collected during the survey of grapevine nurseries (Chapter 2) were not saprophytes or secondary colonisers, their pathogenicity needed to be investigated.

Previous studies on the pathogenicity of botryosphaeriaceous species in different countries have produced conflicting results, making it difficult to determine which species are most problematic to grapevines (van Niekerk *et al.*, 2004). These conflicting results may be due to the type of inoculum used, type of inoculated tissue, age and susceptibility of variety used, as well as different assessment periods and parameters. The most common inoculum used was mycelium plugs (Phillips, 1998; Larignon *et al.*, 2001; Taylor *et al.*, 2005; Amponsah *et al.*, 2008b) although some studies also used conidial suspensions (Castillo-Pando *et al.*, 2001; Wood & Wood, 2005) and hyphal suspensions (Castillo-Pando *et al.*, 2001). Other pathogenicity studies also differed in the type of inoculated tissue, age or varieties used. For example, Phillips (1998) used attached non-succulent green shoots of Espadiero while Amponsah *et al.* (2008a) used detached succulent Pinot noir green shoots and confirmed the pathogenicity of the isolates on rooted Pinot noir canes. Pathogenicity studies by van Niekerk *et al.* (2004) used detached green shoots, one-year-old cuttings and 15-year-old vines, while Urbez-Torres and Gubler (2009) used wounded one-year-old and two-year-old rooted canes, attached green shoots, spurs and cordons for inoculations.

Different assessment methods and parameters were also used in experiments. Most studies measured the length of the lesions (Wood & Wood, 2005; Urbez-Torres & Gubler, 2009)

while two studies measured the area of the lesion (Larignon & Dubos, 2001; Taylor *et al.*, 2005). Some studies also measured internal lesions (Larignon & Dubos, 2001; Urbez-Torres & Gubler, 2009) while Wood and Wood (2005) measured the external lesions of the canes. Other measures of pathogenicity included bud death incidence (Phillips, 1998) and plantlet mortality incidence (Castillo-Pando *et al.*, 2001).

The use of different inoculation techniques in different studies may result in different responses from the pathogens resulting in contradictory results. Using different plant materials of different ages and cultivars may also influence the pathogenicity of different botryosphaeriaceous species, making comparisons between species difficult. The use of mycelial plugs and conidial suspensions may also cause variability in the pathogenicity of different species and may affect the accuracy of the tests. Since the different botryosphaeriaceous species have been reported to cause different symptoms in the above tests and in the vineyard, one technique may not be enough to determine their pathogenicity.

The objective of this study was to evaluate the pathogenicity of different botryosphaeriaceous species isolated from grapevine nurseries on two different plant types and therefore determine whether all the nursery isolates were pathogenic and which species were important to grapevine nurseries.

3.1.2 MATERIALS AND METHODS

3.1.2.1 Fungal isolates

Of the 120 isolates recovered from the grapevine nurseries, the 115 clearly identified isolates representing the seven botryosphaeriaceous species (Section 2.3.4.2) were tested for their pathogenicity to grapevines using two inoculation methods. The two unidentified and one *N. luteum* isolates that were contaminated with *Trichoderma* spp. and two isolates, for which identification attempts were inconclusive, were excluded from the test. All isolates were maintained on PDAS at 4°C for 4 to 8 weeks until used.

3.1.2.2 Inoculum preparation

Since some isolates had not produced conidia in culture (Section 2.3.4.1), pathogenicity tests were conducted using mycelial plugs. The 115 isolates were randomly divided into nine manageable batches of 12 to 13 isolates per batch, with a negative control treatment consisting of sterile PDA. One *N. parvum* isolate (P069) which was in the first batch of isolates to be tested, was found to be highly pathogenic and was used as a positive control

for the succeeding batches. The selected isolates in each batch were taken out of cold storage, sub-cultured onto PDAS, incubated at ambient temperature (23±2^{o}C) for 4 days and mycelial plugs for inoculation were cut from the edge of actively growing mycelia.

3.1.2.3 Detached green shoots

3.1.2.3.1 Inoculation

The experiment was conducted from November to December 2008 using ~20 cm detached green shoots of one-year-old Sauvignon blanc potted vines grown inside the greenshouse. The green shoots were placed in Universal bottles containing pumice and 20 ml of tap water. The middle internode of each shoot was swabbed with a cotton bud dipped in 70% ethanol and a wound (~4 mm) was made in the area using a sterile scalpel. A 4 mm diameter mycelial plug from the 4-day-old PDAS culture was placed onto each stem wound with the mycelial surface facing the wound. Inoculated shoots were maintained in a humid transparent chamber at room temperature and plants were sprayed with water once a day to maintain the chamber's humidity. Four green shoots were used per isolate and control treatments and shoots in each batch were arranged in a randomised complete block design (RCBD).

3.1.2.3.2 Assessment

Some isolates produced lesions rapidly and killed the shoots before 7 days, so all lesions were measured with a digital calliper when the lesion on one of the positive control shoots in each batch had reached 50 mm in length, usually at 5 to 7 days. To complete Koch's postulates, one shoot from each of four test isolates and the control treatments was randomly selected from each batch for re-isolation of the pathogen. Re-isolation was done by cutting 5 mm tissue samples from the edges of the lesions and surface sterilised in 0.5% sodium hypochlorite for 30 s, rinsed twice with SROW and blot-dried in sterile tissue paper inside the laminar flow. The surface-sterilised tissues were placed onto PDAS, incubated for 5-7 days at room temperature and assessed for characteristic growth of the inoculated botryosphaeriaceous species.

3.1.2.4 One-year-old rooted canes

3.1.2.4.1 Production of rooted plants

The same isolates and replicates per treatment were used as in the green shoot trial (Section 3.1.2.1 and 3.1.2.2). The experiment was conducted from October 2008 to March 2009 using rooted one-year-old Sauvignon blanc canes. Dormant cuttings (~1 cm diameter) were collected from the Lincoln University vineyard in July (winter) 2008 and cut into similar three internode lengths. These cuttings were planted in plastic trays filled with pumice (Atiamuri Sand and Pumice Co., New Zealand) and the trays were placed on heat pads (27^{o}C) inside a shade house for 4-6 weeks to facilitate callusing and rooting. The rooted cuttings were transferred to 1.5 L pots containing potting mix (Appendix B.1) and allowed to grow inside the greenhouse for 8 weeks at ambient temperature and natural light prior to inoculation.

3.1.2.4.2 Inoculation

For inoculation, wounds were made by cutting off the 1 cm apical tip of the cane using ethanol-sterilised secateurs. Plants were inoculated with mycelium plugs (8 mm) cut from edges of 4-day-old isolates (Section 3.1.2.2) with the mycelial surface facing the wounds and the sides wrapped with Parafilm™ (Pechiney Plastic Packaging, Chicago, IL) leaving the top of the agar plugs exposed. To provide high humidity for infection, inoculated plants were sprayed with water before placing them individually into plastic bags that remained closed for 24 h. The inoculated plants were kept in a greenhouse for 28 days at ambient temperature (20±5^{o}C) under natural light and watered as needed.

3.1.2.4.3 Assessment

After 28 days incubation, plants were uprooted, foliage and roots removed and canes were washed. Canes were air-dried and the bark was peeled off to allow lesion measurements using a digital calliper. After lesion measurements, re-isolation was done on each test plant by surface sterilising the debarked stem using the methods described in Section 2.2.3. The 10 mm sections of the canes, that were cut from the inoculation point to 100 mm beyond each lesion, were plated onto PDAS. The plates were incubated for 72 h at room temperature and assessed for characteristic growth of inoculated botryosphaeriaceous species. Re-isolation distances were determined based on presence of the inoculated botryosphaeriaceous species in the sections.

3.1.2.5 Data Analyses

For green shoot inoculation, lesion measurements were adjusted by deducting the mean length of wound and necrotic edge on negative controls (4 mm) from the lesion lengths of each shoot. Since assessment periods for each batch differed, ratios of lesion lengths with respect to the mean lesion lengths shown by the positive control for each batch were calculated and included in the analysis.

For cane inoculation, the experiment ran for six months (spring to summer) and the environmental conditions differed in each month. Thus, ratios of lesion lengths with respect to positive control for each batch were also calculated and included in the analysis.

For the analysis, the adjusted data of green shoot lesion lengths, the cane lesions lengths, their calculated ratios with respect to the positive control, and corresponding re-isolation distances of canes were tested for homogeneity using Levene's test at $P \leq 0.05$. When necessary, $\log_{10}$ transformation was done to improve homogeneity of variance (Gomez & Gomez, 1984). Within each assay, isolate and botryosphaeriaceous species differences with respect to green shoot and cane lesion lengths and ratios, and re-isolation distances, were determined by one-way ANOVA using SPSS 17 Statistics. Means that were significant at $P \leq 0.05$ were separated by pairwise comparisons using least significant differences (LSD) at the 5% significance level. Lesion means of the positive controls from each batch were compared for each assay to determine variation between batches. Green shoot and cane lesion lengths, ratios relative to the positive controls and re-isolation distances of *N. luteum* and *N. parvum* isolates were separately analysed by one-way ANOVA to determine effects of nursery sources. Means were separated by LSD at $P \leq 0.05$. Differences in means between cane lesions and re-isolation distances for each species were determined using t-tests at $P \leq 0.05$ level.

Additionally, replication means for isolates of the same species, but only those with eight or more isolates, were graphed on scatter plots using Microsoft Excel 2010. This indicated the potential for linear relationships between cane lesion lengths and re-isolation distances, as well as between green shoot lesion lengths and cane lesion lengths. The extents of the linear relationships between the said variables were determined by coefficient of determination (R^2) values.

3.1.3 RESULTS

3.1.3.1 Detached green shoots

Five to seven days after inoculation, green shoots inoculated with all botryosphaeriaceous isolates, except for one isolate each of *B. dothidea* (D047) and *D. seriata* (O100), exhibited dark to light brown lesions that developed both upward and downward from the inoculation point (Figure 3.1). Some lesions, including those from positive controls, developed rapidly and encircled the stem leading to wilting leaves and eventually death of shoots within 5 days (Figure 3.1A). Other lesions only progressed on the inoculated side of the stem and the leaves remained normal for up to 7 days (Figure 3.1B and C). Negative control plants from all nine batches were free of lesions beyond the wound necrosis. Mean lesion lengths of the positive control differed significantly (P<0.001; Appendix C.2.1) between batches, with Batch 6 having the longest mean lesion of 135.75 mm while Batch 2 had the shortest mean lesion of 18.75 mm.

Colonies similar to the inoculated botryosphaeriaceous isolates were consistently isolated from the four randomly selected green shoots and positive control shoots, while no pathogens were isolated from negative control shoots for each batch.

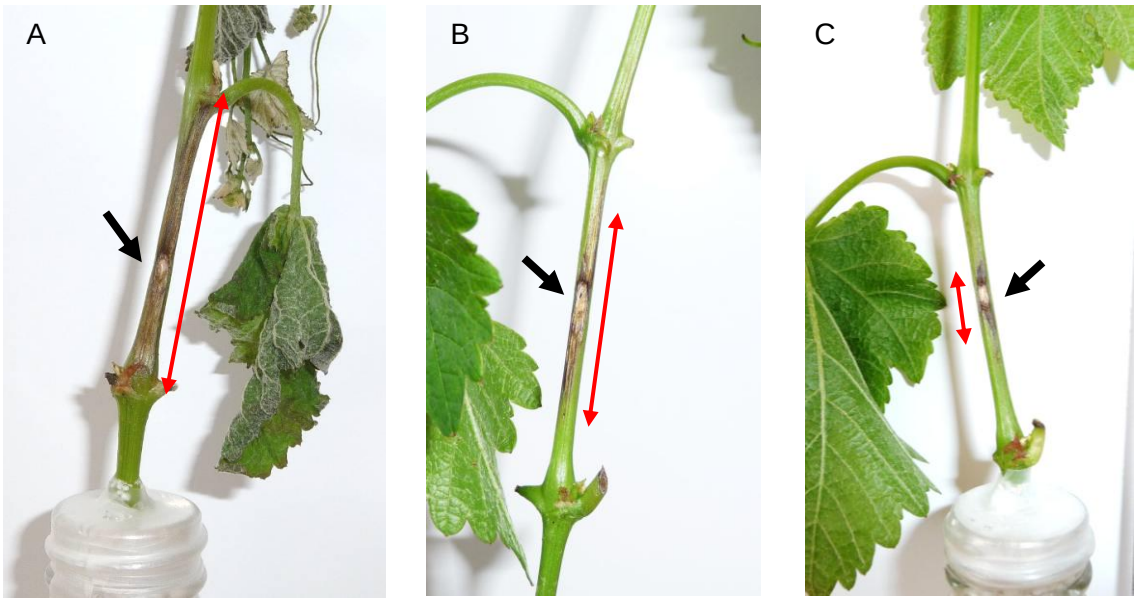

Figure 3.1 Green shoots inoculated with botryosphaeriaecous isolates exhibiting lesions that extended upward and downward (red arrows) from the inoculation point (black arrows).

Lengths of lesions were significantly different for the seven botryosphaeriaceous species and the controls (P<0.001; Table 3.1; Appendix C.2.2). Six of the seven botryosphaeriaceous species caused mean lesion lengths that were significantly longer (P≤0.05) than for the negative control (0.0 mm), while six out of seven species caused mean lesion lengths that were significantly shorter (P≤0.05) than for the positive control (mean 83.5 mm). Lesion lengths were greatest for *N. parvum* (P≤0.05), with a mean of 78 mm that was significantly (P≤0.05) longer than those of all other species and equally pathogenic to that of the positive control (*N. parvum* P069). *Neofusicoccum luteum* and *N. australe* were equally pathogenic with mean lesion lengths of 55 and 52 mm respectively, that were significantly longer than for *D. mutila* (29 mm) and *D. seriata* (9 mm). The mean lesion length for *D. seriata* was not significantly different from that of the negative control. The mean lesion length of the single *N. macroclavatum* isolate was not significantly different from those of other species except for *N. parvum*.

The ratios of shoot lesion lengths with respect to mean length of positive control lesions also varied significantly among species (P<0.001; Appendix C.2.2) and generally showed similar species effects to green shoot mean lesions, with *N. parvum* having the highest mean ratio (0.90) that differed significantly (P≤0.05) from those of all other species except for *N. australe* (0.85). The ratio means of *B. dothidea* (0.30), *D. mutila* (0.24) and *D. seriata* (0.12) were significantly lower than for the other three species. The ratio mean of the single *N. macroclavatum* isolate (0.37) was significantly different from that of *N. parvum* but not those of the other species.

Lesion lengths also varied among isolates of the six botryosphaeriaceous species (Appendices C.2.4 to C.2.9). Since there was only one isolate for *N. macroclavatum,* it was excluded from the analysis. Mean lesion lengths varied significantly between isolates of *N. luteum* (P<0.001), with means ranging from 3 to 115 mm. For *N. parvum*, mean lesion lengths also varied significantly (P=0.035) among isolates, with means ranging from 6 to 122 mm while the eight *N. australe* isolates also differed significantly (P<0.001), with means ranging from 10 to 104 mm. Mean lesion lengths among *D. mutila* and *B. dothidea* isolates also differed significantly (P<0.001), with means ranging from 3 to 85 mm and 0 to 104 mm, respectively. For *D. seriata*, mean lesion lengths of the four isolates also differed significantly (P=0.044), with means ranging from 0 to 26 mm.

The mean ratios of isolate lesion lengths relative to the positive control also varied significantly among isolates within species (P<0.001 to P=0.046) except for *D. seriata* (P=0.115) and generally showed similar isolate effects to green shoot mean lesion lengths.

Table 3.1 Mean lesion lengths and mean lesion length ratios relative to the positive control that were caused by isolates of botryosphaeriaceous species inoculated onto green shoots.

Species	No. of isolates	[A]Mean Lesion length (mm)	Range of lesion means (mm)	[A]Ratio with positive control	Range of ratio means
N. luteum	67	55.4 b	3-115	0.78 b	0.04-2.08
N. parvum	21	77.7 a	6-122	0.90 a	0.42-1.42
N. australe	8	52.2 bc	10-104	0.85 ab	0.14-2.18
D. mutila	8	28.8 de	3-85	0.24 c	0.40-0.64
B. dothidea	6	36.3 cd	0-104	0.30 c	0.00-0.78
D. seriata	4	9.1 ef	0-26	0.12 c	0.00-0.23
N. macroclavatum	1	40.5 bcde	2-58	0.37 bc	0.02-0.53
Positive control	1	83.5 a	19-136		
Negative control	1	0.0 f	0		

[A]Means within a column with different letters are significantly different at $P \leq 0.05$ LSD

3.1.3.2 One-year-old rooted canes

External lesions were not observed on inoculated canes, and all inoculated plants showed normal shoot development that did not differ from the negative controls 28 days after inoculation. However, when the bark was peeled from the trunks, symptoms which appeared as light streaking to dark brown lesions of varying lengths, were observed on all inoculated plants (Figure 3.2), while some negative control plants exhibited light brown streaking just below the inoculation point. Some lesions near the inoculation point had encircled the stem while some lesions only progressed on one side of the stem. Mean lesion lengths of the positive control differed significantly (P=0.002; Appendix C.2.1) between batches, with Batch 3 having the highest mean lesion of 157.5 mm while Batch 7 had the shortest mean lesion of 32.5 mm.

Figure 3.2 Canes inoculated with botryosphaeriaecous isolates exhibiting lesions (red arrow) of different lengths below the inoculation point (black arrow) with normal shoot development (blue arrow).

Lengths of cane lesions were significantly affected (P<0.001) by inoculation treatments as shown in Table 3.2 (Appendices C.2.2). Six of the seven species tested produced lesions that were significantly longer than for the negative control (P≤0.05), and *N. parvum* was the most pathogenic with the longest mean lesion length of 82 mm, which differed significantly (P≤0.05) from all other species and was equally pathogenic as the positive control (*N. parvum* P069). The mean lesion length caused by *D. mutila* (63 mm) was significantly (P≤0.05) longer than for *N. luteum* (41 mm) and *B. dothidea* (18 mm) but did not differ significantly from those caused by *N. australe* (43 mm) and *D. seriata* (43 mm). The mean lesion length caused by *N. luteum* did not differ significantly from those of *N. australe* and *D. seriata* but were significantly longer (P≤0.05) than for *B. dothidea* (18 mm), which produced the shortest mean lesion that did not differ significantly from that of the negative control plants (3 mm). The mean lesion length of the single isolate of *N. macroclavatum* (52 mm) was significantly shorter than for *N. parvum* (P≤0.05) but did not differ from those of the other botryosphaeriaceous species tested.

The ratio of cane lesions with respect to mean length of positive control lesions also varied significantly among species (P<0.001; Appendix C.2.2) and showed similar species effects to cane lesion lengths except that no significant differences were observed between *D. mutila N. luteum* and *N. australe* or between *D. seriata* and *B. dothidea*.

Table 3.2 Mean lesion lengths and mean lesion length ratios relative to the positive control caused by isolates of botryosphaeriaceous species inoculated onto one-year-old canes.

Species	No. of isolates	[A] Mean Lesion length (mm)	Range of lesion means (mm)	Ratio with positive control	[A] Re-isolation distances mean (mm)	Range of re-isolation distances (mm)
N. luteum	67	40.9 c	4-125	0.67 b	47.1 c	10-150
N. parvum	21	82.0 a	14-193	1.12 a	94.6 a	30-180
N. australe	8	43.3 bc	8-89	0.67 b	55.0 bc	32-85
D. mutila	8	62.8 b	25-138	0.77 b	59.4 b	33-105
B. dothidea	6	18.2 de	0-34	0.31 c	37.0 c	23-56
D. seriata	4	42.9 bc	30-55	0.53 bc	23.1 cd	18-30
N. macroclavatum	1	52.3 bcd	32-67	0.80 abc	67.5 bc	30-100
Positive control	1	75.4 a	33-158		85.3 a	33-65
Negative control	1	3.1 e	0-8		0.0 d	0

[A] Means within a column with different letters are significantly different at $P \leq 0.05$ LSD

Analyses of cane lesions caused by isolates of the six botryosphaeriaceous species are summarised in Appendices C.2.4 to C.2.15. Mean lesion lengths on canes varied significantly among isolates of *N. luteum* and *N. parvum* but not among the isolates of other species. For *N. luteum* isolates, the mean lengths of lesions varied significantly ($P<0.001$), with means ranging from 4 to 125 mm. For *N. parvum*, mean lesion lengths also differed significantly ($P<0.001$) among isolates, with replication means ranging from 14 to 193 mm. Variances of cane lesion means among *N. australe* isolates were not homogenous ($P=0.321$) and transformation did not improve homogeneity, thus, data were excluded from the ANOVA. However, means of lesion lengths among the eight *N. australe* isolates ranged widely from 8 to 89 mm. Mean lesion lengths among the eight *D. mutila* isolates did not differ significantly ($P=0.141$), with means ranging from 25 to 138 mm. Mean lesion lengths also did not differ significantly ($P=0.538$) among the six *B. dothidea* isolates, although isolate D047 produced no lesions while D445 caused a mean lesion length of 34 mm. For *D. seriata,* mean lesions lengths of the four isolates also did not differ significantly ($P=0.800$) with means ranging from 30 to 55 mm. There was only one isolate of *N. macroclavatum* and so it was excluded from the analysis.

The mean ratios also varied significantly among isolates for *N. luteum* and *N. parvum* ($P \leq 0.001$) and generally showed similar isolate effects to cane mean lesion lengths. The mean ratios among isolates of the four other species did not differ significantly (Appendices C.2.6 to C.2.9).

Similar to cane lesion lengths, re-isolation distances also differed significantly (P<0.001) between species, with *N. parvum* having the longest re-isolation distance mean at 95 mm that differed significantly (P≤0.05) from those of the other six species (Table 3.2; Appendix C.2.1). Mean re-isolation distances of *D. mutila* (59 mm), *N. australe* (55 mm), *N. luteum* (47 mm) and *B. dothidea* (37 mm) did not differ significantly between species. The mean re-isolation distance for *D. seriata* (23 mm) was significantly lower than for *N. parvum* and *D. mutila* (P≤0.05) but not for other species. For the positive control plants, the mean re-isolation distance (85 mm) differed significantly from those of all other species, except for *N. parvum,* while no pathogens were re-isolated from negative control plants. The mean re-isolation distance of the single isolate of *N. macroclavatum* was 68 mm and was significantly shorter than for *N. parvum*, but did not differ from those of the other botryosphaeriaceous species tested.

Means for re-isolation distances among isolates of the six botryosphaeriaceous species are summarised in Appendices C.2.10 to C.2.15. Significant differences in re-isolation distance means were observed among isolates of two out of six species tested. Re-isolation distances varied significantly among *N. luteum* isolates (P<0.001), with means ranging from 10 to 150 mm. For *N. parvum* isolates, re-isolation distances differed significantly (P<0.001) among isolates, with means ranging from 30 to 180 mm. For *N. australe*, variances of re-isolation distance means among isolates were not homogenous (P=0.061) and $\log_{10}$ transformation did not improve the homogeneity, thus, data were not analysed by ANOVA. Similar to cane lesions, no significant differences were observed for re-isolation distances among *D. mutila* (P=0.727), *B. dothidea* (P=0.280) and *D. seriata* (P=0.862). However, *B. dothidea* isolate B047 which did not produce any cane lesions was re-isolated up to 23 mm from the inoculation point. In contrast to the other five species, the four *D. seriata* isolates were only re-isolated from the older parts of the lesions and none were re-isolated from the younger parts or beyond the lesions.

3.1.3.3 Cane lesions vs. re-isolation distances

When the overall means of re-isolation distances were compared to overall cane lesion means using t-tests, the re-isolation distances were significantly longer than cane lesion lengths of *N. luteum* (P<0.001), *N. parvum* (P<0.001), *N. australe* (P=0.014) and *B. dothidea* (P<0.001) (Figure 3.3; Appendix C.2.3). The re-isolation distances of *D. mutila,* on the other hand, did not differ significantly from mean cane lesion lengths (P=0.551) while re-isolation distances for *D. seriata* were significantly shorter (P=0.023) than the cane lesion lengths.

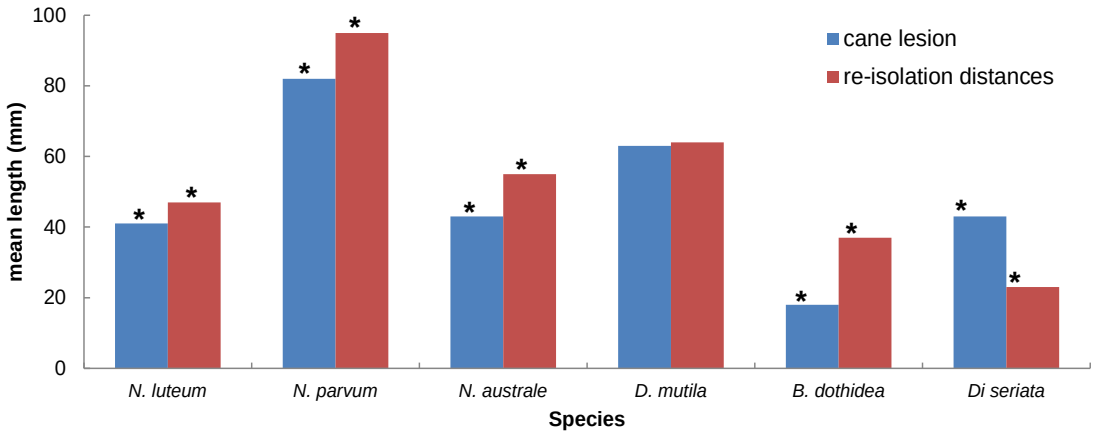

Figure 3.3 Means of cane lesion lengths and re-isolation distances among six botryosphaeriaceous species used to inoculate the pruned tips of one-year-old plants. Asterisks show significant differences between lesion lengths and reisolation distances of the same species at $P \leq 0.05$ using t-tests.

Positive linear relationships, as indicated by high coefficient of determination (R^2) values, were observed between cane lesion lengths and re-isolation distances (Figure 3.4) for *N. parvum* (R^2=0.90), *D. mutila* (R^2=0.87), *N. luteum* (R^2=0.85) and *N. australe* (R^2=0.68), which indicated that 90, 87, 85 and 68% of the variation in re-isolation distances, respectively, were attributed to the length of cane lesions (Figure 3.4). The numbers of isolates for the other species were less than eight and considered too low for analysis of this type. Means for cane lesions and re-isolation distances of different species are summarised in Appendices C.2.10 to C.2.15.

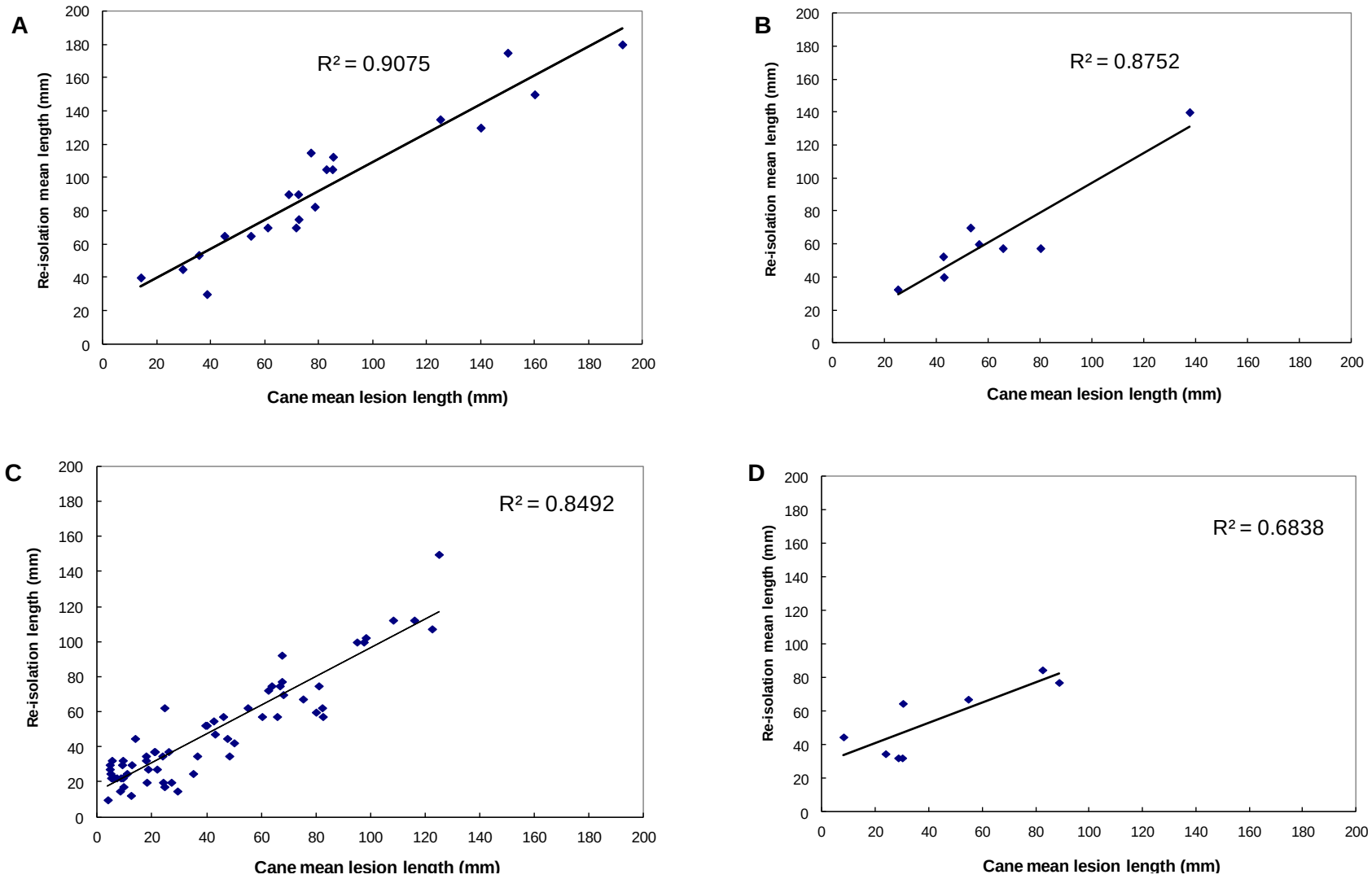

Figure 3.4 Linear relationships between mean lengths of cane lesions and re-isolation distances, with the corresponding coeficient of determination (R^2) values, for isolates of A) *N. parvum*; B) *D. mutila*; C) *N. luteum* and D) *N. australe.*

3.1.3.4 Comparison of cane lesions and green shoot lesions for individual isolates

For *N. luteum* isolates, no linear relationship was observed between mean lengths of cane and green shoot lesions (R^2=0.01; Figure 3.5). This indicated that isolates of this species did not demonstrate consistent relationships between their abilities to produce lesions in green shoots and lesions in canes. For example, isolates L106 and L105 were among the ten isolates that produced the longest cane lesions but were also among the ten isolates that produced the shortest lesions in green shoots. Isolates L228 and L456, on the other hand, consistently produced long lesions in both green shoots and canes. Means for *N. luteum* isolates are summarised in Appendix C.2.5.

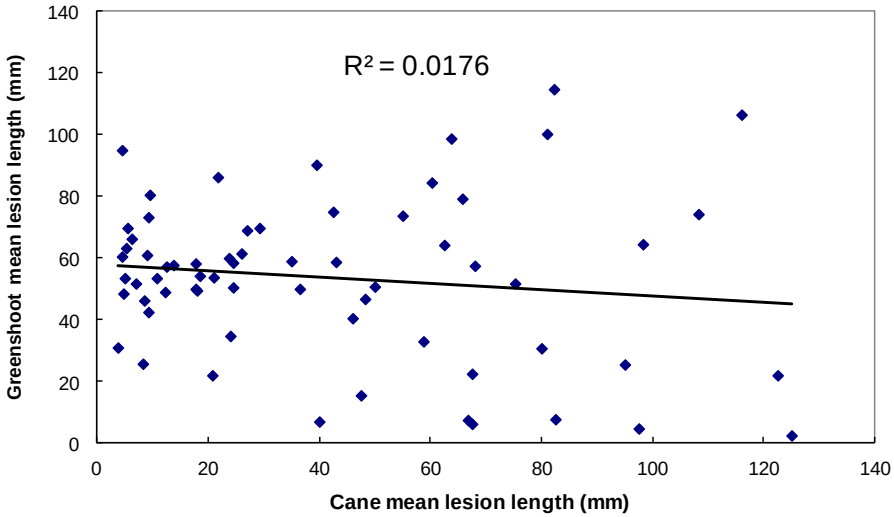

Figure 3.5 Relationship between mean lengths of green shoot and cane lesions of different *N. luteum* isolates (n=67).

For *N. parvum* isolates, a moderate linear relationship (R^2=0.51) was observed between mean lengths of green shoot and cane lesions. This indicated that the abilities of many isolates to produce lesions in green shoots were higher than their abilities to produce lesions in canes (Figure 3.6). However, some isolates displayed longer cane lesions than green shoot lesions (Appendix C.2.11). For example, the five isolates (P178, P94, P116, P225 and P098) that caused the longest cane lesions were the same isolates that caused the shortest lesions in green shoots (Appendix C.2.11).

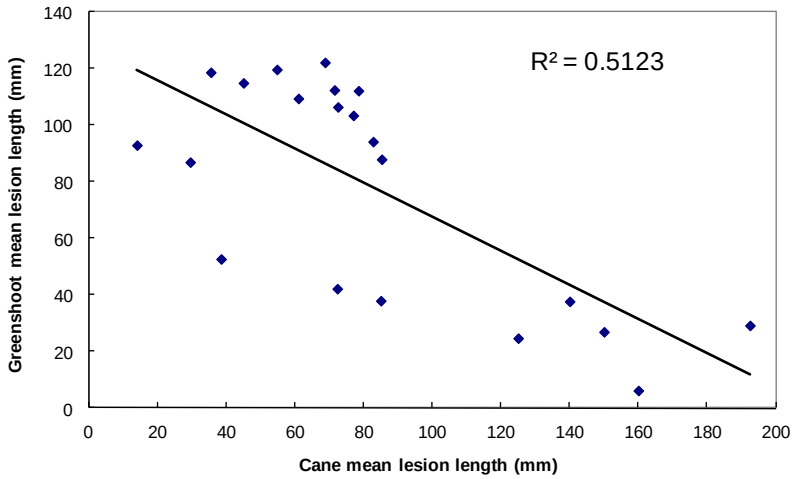

Figure 3.6 Relationship between mean lengths of cane lesions and green shoot lesions produced by *N. parvum* isolates (n=21).

No obvious linear relationships were observed between mean lengths of green shoot and cane lesions for *D. mutila* (R^2=0.17) and *N. australe* (R^2=0.22). However, some isolates within each species displayed differences in pathogenicity between the tissue types. For example, *D. mutila* isolates S109, S146 and S360 caused the shortest lesions on green shoots but longest lesions on canes. In contrast, *N. australe* isolate A826 and A480 consistently caused the shortest and longest lesions in green shoots and canes, respectively. The isolates of *D. seriata*, *B. dothidea* and *N. macroclavatum* were not included in the analysis because they had fewer than eight isolates, which was considered too low for this type of analysis.

3.1.3.5 Pathogenicity of N. luteum from different nurseries

For green shoots, pathogenicity of *N. luteum* isolates differed significantly (P<0.001; Appendix C.2.4) between nurseries. The isolates from Nurseries 7 and 2 (one isolate each) produced the longest lesions of 75 and 73 mm (means of replicates), respectively, that were significantly longer (P≤0.05) than the isolates from Nursery 5 (61 mm) and Nursery 3 (25 mm). The isolates from the rest of the nurseries had intermediate pathogenicity. The ratios of their means with respect to the positive control did not differ significantly between nurseries (P=0.072; Table 3.3).

Cane lesion lengths of *N. luteum* isolates also varied significantly between nurseries (P<0.001; Appendix C.2.4). The isolates from Nursery 7 and Nursery 3 were the most pathogenic giving the longest mean lesions (P≤0.05) of 85 and 77 mm, respectively. The ratios of their means

with respect to the positive control mean also differed significantly (P<0.001; Table 3.3; Appendix C.2.4) and generally showed similar nursery effects to the lesion lengths, except that Nursery 7 had a significantly higher mean ratio than Nursery 3. The mean re-isolation distances of the isolates varied among nurseries (P<0.001; Table 3.3; Appendix C.2.4) with similar nursery effects to the lengths of cane lesions.

Table 3.3 Pathogenicity of *N. luteum* isolates from different grapevine nurseries, based on mean lesion lengths and their ratios with the postive control on green shoots and cane lesions, as well as re-isolation distances on canes.

Nursery	No. of isolates	*Green shoot mean lesion (mm)	Ratio with positive control	*Cane mean lesion (mm)	*Cane ratio with positive control	*Re-isolation distances (mm)
2	1	73.3 a	1.18 a	9.3 b	0.15 c	32.5 b
3	12	25.1 c	0.53 a	76.9 a	0.72 b	80.0 a
5	42	60.9 b	0.77 a	29.4 b	0.37 c	35.9 b
6	2	61.5 ab	0.99 a	12.9 b	0.21 c	23.7 b
7	4	75.4 a	0.70 a	85.3 a	1.76 a	79.6 a
8	5	62.9 ab	1.01 a	24.9 b	0.40 c	45.0 b

* Means within a column with different letters are significantly different at P≤0.05 LSD.

3.1.3.6 Pathogenicity of N. parvum from different nurseries

For green shoots, the pathogenicity of *N. parvum* isolates also differed significantly (P=0.035) between nurseries (Table 3.4; Appendix C.2.5). The single isolate from Nursery 5 gave the longest mean lesion (112 mm) on green shoots, which was significantly longer than for the isolates from Nursery 1 (29 mm) and Nursery 9 (42 mm). However, the ratios of their means with respect to the positive control mean did not differ significantly (P=0.115; Table 3.4; Appendix C.2.5).

Cane lesion lengths of *N. parvum* isolates also varied significantly between nurseries (P<0.001) with the single isolate from Nursery 1 giving the longest mean lesion at 192.5 mm which was significantly different from all other nursery isolates. However, variances of the ratios of their means with respect to the positive control and re-isolation distances were not homogenous (P=0.283 and P=0.519, respectively) and were not tested for ANOVA (Table 3.4; Appendix C.2.5).

The numbers of isolates for *N. australe*, *D. mutila*, *B. dothidea*, *D. seriata* and *N. macroclavatum* were too low to be included in a similar analysis of nursery effects.

Table 3.4 Pathogenicity of *N. parvum* isolates from different grapevine nurseries, based on mean lesion lengths and their ratios with the postive control on green shoots and cane lesions, as well as re-isolation distances on canes.

Nursery	No. of isolates	[A]Green shoot mean lesion (mm)	[A]Ratio with positive control	[A]Cane mean lesion (mm)	[B]Cane ratio with positive control	[B]Re-isolation distances (mm)
1	1	29.3 c	0.39 a	192.5 a	1.22	180.0
3	8	72.3 ab	1.00 a	78.9 b	1.04	92.2
5	1	112.3 a	0.83 a	78.5 b	0.69	82.5
7	10	87.8 ab	0.86 a	76.5 b	1.22	89.2
9	1	42.3 b	0.42 a	54.3 b	0.83	90.0

[A] Means within a column with different letters are significantly different at $P \leq 0.05$ LSD.
[B] Data were not homogenous and were excluded in the ANOVA.

3.1.4 DISCUSSION

This is the first comprehensive study world-wide of the pathogenicity of different botryosphaeriaceous isolates from grapevine nurseries, which tested 115 isolates representing seven species. The other reported pathogenicity studies used a lesser number of vineyard isolates (72 isolates or fewer; van Niekerk *et al.*, 2004; Amponsah *et al.*, 2008b; Urbez-Torres & Gubler, 2009). Almost all isolates of the seven botryosphaeriaceous species from grapevine nursery materials were pathogenic and capable of causing lesions when inoculated into grapevine shoots or canes. This study included ratios with respect to positive control as a measure of pathogenicity to eliminate variability in different batches. Even with this attempt, significant differences in pathogenicity between species and isolates were evident.

The pathogenicity experiments showed that *N. parvum* consistently produced the longest mean lesions in both excised green shoots and the one-year-old trunks of plants, and the lesion length ratios with respect to the positive control gave similar effects confirming its high pathogenicity. This is consistent with earlier reports (Phillips, 2002; van Niekerk *et al.*, 2004; Luque *et al.*, 2007; Martos, 2008; Urbez-Torres & Gubler, 2009), where it was demonstrated to be one of the most virulent botryosphaeriaceous species on grapevines worldwide. In New Zealand, *N. parvum* was the most prevalent species found in vineyards (Baskarathevan *et al.*, 2011) and the second most prevalent species in grapevine nurseries (Chapter 2; Billones *et al.*, 2010a). Variability in pathogenicity among *N. parvum* isolates was observed in this study.

Similar differences in pathogenicity were also reported in South Africa (van Niekerk *et al.*, 2004), Spain (Martos, 2008), California (Urbez-Torres & Gubler, 2009) and New Zealand (Baskarathevan *et al.*, 2009). Since *N. parvum* was the second most prevalent species (Chapter 2) and this has shown that it is the most pathogenic, the species can be considered an important pathogen in the nursery system that can potentially affect the grapevine industry.

In this study, *N. luteum* followed *N. parvum* in level of pathogenicity. The first study on pathogenicity of *N. luteum* was in South Africa where they found this species to be weakly pathogenic although only one isolate was tested in their study (van Niekerk *et al.*, 2004). More recent pathogenicity studies have found *N. luteum* to be equally pathogenic (Luque *et al.*, 2007; Martos, 2008; Urbez-Torres & Gubler, 2009) or more pathogenic (Amponsah *et al.*, 2008b) than *N. parvum* on green shoots and different woody tissues of grapevines. A similar study in California also found *N. luteum* to have the highest variability in virulence in canes of potted one-year-old plants causing vascular lesions ranging from 73 to 314 mm (Urbez-Torres & Gubler, 2009). Its high pathogenicity and frequency as well as wide distribution among nurseries makes *N. luteum* the most important species in New Zealand grapevine nurseries.

Overall, the results in this study showed that *N. australe* was equally pathogenic to *N. luteum,* which confirmed previous pathogenicity studies using grapevine shoots and woody tissues (Amponsah *et al.*, 2008b; Urbez-Torres & Gubler, 2009). These two species are morphologically similar and closely related phylogenetically (Slippers *et al.*, 2004b; van Niekerk *et al.*, 2004). Other studies also found *N. australe* to be equally pathogenic (van Niekerk *et al.*, 2004; Urbez-Torres & Gubler, 2009) or more pathogenic than *N. parvum* (Amponsah *et al.*, 2008b) on green shoots and woody tissues. The wide range of lesion lengths between *N. australe* isolates observed in this study also indicated high variability within the species. However, differences between isolates were not statistically significant, probably due to the low number of isolates tested or number of replications used. Similarly, Urbez-Torres and Gubler (2009) also observed high variability in pathogenicity among *N. australe* isolates causing lesions that ranged from 140 to 319 mm on one-year-old rooted canes. A study by van Niekerk (2004) also observed that one *N. australe* isolate caused lesions twice as large as the other two isolates tested. While the 2008 survey presented in Chapter 2 showed that *N. australe* incidence in nurseries was significantly less than for *N. luteum* and *N. parvum,* this species was equally widespread as *N. luteum* being present in six out of eight nurseries with botryosphaeriaceous infections.

The single isolate of *N. macrolavatum* was found to be similarly pathogenic as *N. luteum* and *N. australe* on both tissue types tested. This species was also re-isolated beyond the lesions

indicating endophytic behaviour. This species was first reported in Western Australia as a pathogen of *Eucalyptus globulus* and was found to be more pathogenic than *N. australe*, a known pathogen of *Eucalyptus* in Australia (Burgess *et al.*, 2005). The isolation of *N. macroclavatum* from an asymptomatic grafted plant in this study represented the first report of this fungus, both in New Zealand and as a pathogen of grapevines (Billones *et al.*, 2010b). Its presence on asymptomatic plant suggests endophytic or latent infection of this species. However, the single isolate was shown to be highly pathogenic indicating a need to further investigate the prevalence of this species in nurseries and vineyards and as well as other potential hosts.

The isolates of *D. mutila* were less pathogenic to green shoots than the three *Neofusicoccum* species tested and they did not differ from *B. dothidea* and *D. seriata* isolates. In one-year-old canes however, *D. mutila* followed *N. parvum* with lesion lengths that were similar to those of *N. australe* and significantly longer than those of *N. luteum*. A study in South Africa showed that *D. mutila* was equally pathogenic to *N. parvum* and *N. australe* based on *in vitro* screening of mature canes (van Niekerk *et al.*, 2004). Other studies in Portugal (Phillips, 1998), Australia (Taylor *et al.*, 2005) and California (Urbez-Torres & Gubler, 2009) have shown *D. mutila* to be weakly pathogenic to different tissue types. However, Amponsah (2011) found the three New Zealand isolates of *D. mutila* varied, with one isolate being as pathogenic on green shoots as *N. luteum, N. australe* and *N. parvum* while the other two isolates were less pathogenic. Variation in pathogenicity among the *D. mutila* isolates was also observed in this study, with mean lesion lengths ranging from 3 to 85 mm and 25 to 138 mm in green shoots and canes, respectively. In New Zealand vineyards, *D. mutila* was the second most prevalent species (Baskarathevan *et al.*, 2008) while this species was the third most prevalent in New Zealand grapevine nurseries (Chapter 2).

The *B. dothidea* isolates tested here had relatively low pathogenicity on both tissue types. In Spain, it was also found to be one of the least pathogenic among six species tested although that study only tested one *B. dothidea* isolate (Martos, 2008). In Portugal (Phillips, 1998), France (Larignon *et al.*, 2001), and Chile (Morales *et al.*, 2010) studies on *B. dothidea* showed that this species can cause cankers and dieback in grapevines, however, these studies did not describe its pathogenicity relative to the other botryosphaeriaceous species. In California, however, this species was found to be of intermediate virulence among nine species tested (Urbez-Torres & Gubler, 2009). The vineyard and nursery surveys both showed that *B. dothidea* was less common in New Zealand than the other botryosphaeriaceous species (Baskarathevan *et al.*, 2008; Billones *et al.*, 2010a), indicating that this species was of low importance in New Zealand.

Diplodia seriata has been isolated from grapevines worldwide but its pathogenicity on grapevines is unclear (Phillips, 2002). This species was the primary pathogen associated with black dead arm in France and necrotic lesions were reproduced in mature canes but pathogenicity varied so much among isolates that they were divided into four virulence groups (Larignon *et al.*, 2001). Phillips (1998) however, found this species to be a weak pathogen and suggested that it was a secondary pathogen or saprophyte. In Spain, this species was also the least pathogenic among six species tested on red and white grape varieties (Martos, 2008). In this study, differences in pathogenicity towards green shoots and canes were observed among *D. seriata* isolates. This species caused barely discernable or no lesions when inoculated on green shoots and yet on canes it caused similar lesions to *N. luteum* and *N. australe*. Amponsah *et al.* (2008b) also found *D. seriata* to be a weak pathogen in green shoots. In contrast, pathogenicity studies in California showed that *D. seriata* was weakly pathogenic on mature canes but more virulent than *N. parvum* and *N. luteum* on green shoots (Urbez-Torres & Gubler, 2009). A study in Australia also showed that this species did not produce any lesions on inoculated canes (Taylor *et al.*, 2005). The conflicting results from the different pathogenicity studies suggest that the pathogenic behaviour of this species may be affected by other factors such as virulence group, grape variety, temperature and host tissue maturity, which should be investigated in further studies using more isolates and under field conditions.

While this study has shown significant differences in pathogenicity among botryosphaeriaceous species and among isolates of the same species, information on the factors that play a direct role on the variability was not known. Recent studies, however, have linked phytotoxins to botryosphaeriaceous infections. Martos *et al.* (2008) showed that *B. dothidea*, *D. seriata*, *Do. viticola*, *N. luteum* and *N. parvum* produced hydrophilic high molecular weight compounds that were toxic to mature leaves of grapevines. Furthermore, they also found that *N. luteum* and *N. parvum* produced lipophilic low molecular weight phytotoxins that were not consistently detected from other species. Evidente *et al.* (2010) further isolated lipophilic phytotoxins from one isolate of *N. parvum* and identified them as (3R, 4R)-(-)-4-hydroxymellein, (3*R*,4*S*)-(-) 4-hydroxymellein, isosclerone and tyrosol. These metabolites were all phytotoxic to plants, but (3*R*,4*R*)-(-)-4-hydroxymellein and isosclerone were the most toxic.

Toxins were also reported to be involved in the pathogenicity of other trunk pathogens of grapevines. A phytotoxic metabolite called “eutypine” was first isolated from liquid cultures of *E. lata* and was detected in crude sap and plants infected by *E. lata* but were not present in healthy plant materials (Tey-Rulh *et al.*, 1991). Eutypine is normally synthesized by *E. lata* in the trunk and is believed to be transported to the herbaceous parts of the plants by the ascending sap from the xylem, resulting in the reduced growth of shoots and leaves and

dryness of branches and inflorescences (Tey-Rulh *et al.*, 1991; Amborabé *et al.*, 2001). Mahoney *et al.* (2003) reported that other metabolites were also produced by this fungus, including eulatachromene, eulatinol and benzofuran, suggesting that Eutypa dieback disease of grapevines may be caused by several metabolites rather than a single compound.

The fungi that cause Petri disease and are associated with the development of Esca disease of grapevines, *Ph. chlamydospora* and *Togninia minima,* were also shown to produce three metabolites *in vitro* and *in planta* (Bruno *et al.*, 2007). These exopolysacharides were found in the xylem sap of infected grapevines and caused similar symptoms to field vines affected by Esca and Petri disease. Aside from toxins, a study by Santos *et al.* (2006) further showed that the extra-cellular enzymes produced by *Phaeoacremonium* spp. and *Ph. chlamydospora* induced senescence symptoms in plant cells and inhibited callus formation in young grafted plants. They also found that the most virulent isolates of *Phaeoacremonium* spp. produced higher amounts of these enzymes than less virulent ones.

The reported ability of *N. parvum* and *N. luteum* to produce unique toxins may explain their high pathogenicity relative to the other species observed in this study. To date, no studies on toxins associated with *N. australe* has been reported. Given that this species is closely related and similarly pathogenic to *N. luteum*, it is also possible that *N. australe* can produce similar toxins as *N. luteum*. However, this hypothesis needs further investigation.

The difference in pathogenicity among *N. luteum* isolates in this study were found to be associated with nursery source, with isolates from certain nurseries being more pathogenic on green shoots but less pathogenic on canes, while other nursery isolates gave the reverse results. Some nursery isolates, on the other hand, were consistently pathogenic to both tissue types. However, some nurseries did not produce their own scion canes; rather they utilised the pruned canes from different vineyards for grafting (Verstappen, pers. comm., 2009). The sources of the scion cuttings were only provided by Nurseries 3, 5 and 9. Nursery 5 grew all of theirs, Nursery 9 grew about 50% of theirs and Nursery 3 grew very few of their scion cuttings. The outsourcing of different scion materials, therefore, may also result in the introduction of new botryosphaeriaceous species or strains in the nurseries and contribute to the variability in pathogenicity between nursery isolates. It is likely that the botryosphaeriaceous pathogens isolated were present in the cutting materials used, but this study did not establish the original suppliers of the cuttings to the nurseries.

Variability in pathogenicity among *N. parvum* isolates was also observed although it was not shown to be associated with nursery sources. The number of *N. parvum* isolates from different

nurseries was not equal, with three nurseries having only one isolate each. The fact that few or no isolates were gained from five of the seven nurseries was probably associated with their more obvious external symptoms, as discussed in Chapter 2. Since most of the isolates were from two nurseries, this indicates that quality control may have been poorer in those nurseries. However, since variability among vineyard isolates has also been reported by other researchers, it seems likely that it is also due to genetic diversity of pathogen populations. Baskarathevan (2011) found high diversity among *N. parvum* and *N. luteum* New Zealand populations. This study further observed that some isolates belonging to different genetic groups of *N. parvum* and *N. luteum* appeared to be related to virulence although the correlation was not strong.

In this study, variability in pathogenicity was apparent among isolates of most species but only *N. luteum* and *N. parvum* had sufficient numbers of isolates for the underlying factors that might affect variation to be considered. The variability in pathogenicity observed appeared to be related to nursery source and type of tissue inoculated. According to Urbez-Torres and Gubler (2009), differences in pathogenicity could be due to several factors including variability in virulence of isolates, type of inoculated tissue, different incubation periods and conditions, type of inoculum used, age of host and differences in susceptibility of varieties to the pathogen. In this study, only one type of inoculum and variety were used, therefore these factors were eliminated as probable causes of variability among isolates. Variation was observed among isolates even when lesion lengths were converted to a ratio of the positive control lesion in an attempt to correct for variability in assessment periods. However, infection processes that drive the variability in pathogenicity among isolates of the same botryosphaeriaceous species are still unknown. Since some botryosphaeriaceous species have been shown to produce phytotoxins it can be hypothesised that the variable pathogenicity of individual isolates observed in this study may be related to the amount of toxins they can produce. While individuals within a species may possess a gene or genes that encode for toxin production, these genes may not necessarily be equally expressed in all isolates. The different toxins that were found to be involved in the pathogenicity of different botryosphaeriaceous species (Martos *et al.*, 2008; Evidente *et al.*, 2010) may also have different effects on tissue types potentially causing differences in symptoms as was observed in this study. However, quantitative differences in toxin production by individual isolates of different trunk pathogens including those from botryosphaeriaceous species have not been investigated since only one isolate of each species was used for previously cited studies (Bruno *et al.*, 2007; Martos *et al.*, 2008; Evidente *et al.*, 2010; Luini *et al.*, 2010). A study on the quantitative production of toxins of different botryosphaeriaceous isolates can further confirm this hypothesis and clarify the role of toxins on the variability of pathogenicity among isolates of these pathogens.

While this study has provided no evidence on the role of toxins on the variability of pathogenicity among isolates of botryosphaeriaceous species, one study has shown that different levels of enzyme production were correlated to pathogenicity of Petri disease in grapevines. A study by Santos *et al.* (2006) has shown significant variability in the production of protease, cellulase, xylanase and polygalacturonase among *Ph. chlamydospora* isolates and that the quantity of enzymes produced had a strong correlation with the degree of virulence. Some botryosphaeriaceous species were also reported to produce laccase, a multicopper oxidase produced by several species of fungi that has been shown to be an important virulence factor in many fungal plant pathogens (Mayer & Staples, 2002). A study by Saldanha *et al.* (2007) showed that different isolates of *L. theobromae* produced different levels of laccase and that the production of this enzyme was correlated to the genetic diversity among the nine isolates tested. This study, however, did not investigate whether the different levels of laccase was correlated to pathogenicity of this species. A study by Baskarathevan (2011) also showed that different isolates of *N. parvum* produce different amounts of laccase. However, that study found no correlation between pathogenicity of isolate and production of laccase. Therefore, the role of enzymes on the pathogenicity of botryosphaeriaceous species still remains unknown and needs further investigation.

In most pathogenicity studies, re-isolations from infected tissues were done mainly to complete Koch's postulate (Phillips, 1998; 2002; Larignon *et al.*, 2001; Urbez-Torres & Gubler, 2009) and none of the studies included re-isolation distances as a measure of pathogenicity. In this study, re-isolation was done up to 100 mm beyond the edge of the lesions to determine the endophytic ability of the species. The results showed that most isolates of *N. parvum, N. luteum, N. australe* and *D. mutila,* and all *B. dothidea* isolates were able to move endophytically beyond the lesions while isolates of *D. seriata* were only re-isolated within the necrotic part of the lesion. This result suggested that the mode of *D. seriata* colonisation within tissues differed from the other botryosphaeriaceous species tested. It appeared that this species was purely necrotrophic, killing the tissue prior to colonisation. This observation agreed with Amponsah *et al.* (2011) who reported the apparent endophytic development of *N. luteum, N. australe* and *D. mutila.*

The endophytic behaviour of the botryosphaeriaceous species has not been thoroughly investigated to-date although they were recognised as endophytes in the late 1980s (Slippers & Wingfield, 2007). A high number of orchard and forestry trees were found to have high levels of endophytic colonisations by botryosphaeriaceous species (Burgess *et al.*, 2006). Several botryosphaeriaceous species were also isolated from healthy mango tissues (Johnson *et al.*, 1992), while Sakalidis *et al.* (2011) reported that 11 botryosphaeriaceous species were

endophytes of baobab trees in Australia. *Botryosphaeria dothidea* was confirmed as an endophyte in Eucalyptus and was also associated with cankers (Smith *et al.*, 1996; Perez *et al.*, 2010). Latent infection was also observed in *B. dothidea* infection on pistachio (Ahimera *et al.*, 2003) and *Sphaeropsis sapinea* infection on Austrian and Scottish pine trees (Flowers *et al.*, 2003). The high frequency of endophytic infection of botryosphaeriaceous species on various hosts suggests that most if not all members of this family might have an endophytic phase (Slippers & Wingfield, 2007). However, like other endophytes, botryosphaeriaceous species can cause diseases when their hosts are under stress; some of the stress factors that can trigger disease expressions were reported to include drought, extensive physical damage, plant competition and use of varieties unsuitable for the area (Slippers & Wingfield, 2007).

The endophytic abilities observed with the botryosphaeriaceous isolates obtained from New Zealand nurseries has great implications for the industry, since the young, symptomless but infected vines can be unwittingly sold to grape growers. Such a situation was reported for *S. sapinea,* a frequent endophyte of asymptomatic red pine seedlings, and was considered a major means of long distance dissemination of this pathogen (Stanosz *et al.*, 2005). In the 2008 survey presented in Chapter 2, 34% of the botryosphaeriaceous species isolates were recovered from Grade 1 and 2 plants and 38% from rootstock and scion cuttings that were all asymptomatic. This pathogenicity study has also provided evidence that the botryosphaeriaceous isolates from nurseries can move beyond the lesions without obvious symptoms. These results, therefore, suggest that asymptomatic cuttings used as propagation materials for grafting could have resulted in infected grafted plants. They may also produce propagules that can contaminate the propagation process and cause secondary infections. Furthermore, the infections intercepted from Grade 1 and 2 plants provided evidence that infected plants passed through the grading process undetected and so may be a common method for introduction of infections into new vineyards. Investigation into the sources of infections in the mothervine blocks and nursery system was done in Chapter 5 to further confirm this hypothesis.

3.2 ***Section 2.*** Genetic variability of *N. luteum* isolates from different pathotype groups

3.2.1 INTRODUCTION

One of the most significant characteristics of biological organisms is the diversity of traits among the individuals within a species (Agrios, 2005). The genetic diversity of pathogen populations is generally influenced by sexual reproduction which introduces variation into the progeny, potentially enabling some progeny to cope with competitors and environmental change, thereby allowing a species to evolve rapidly (Carlile *et al.*, 2001; Kück & Pöggeler, 2009). In fungi, the methods of reproduction differ with different kinds of propagules which are associated with meiotic and mitotic nuclear divisions (Anderson & Kohn, 1998). In addition, fungi have novel mechanisms of genetic exchange and recombination that are not limited to sexual reproduction (Anderson & Kohn, 1998). These can generate genetically different individuals within an asexually reproduced population, however, the degree of variability is generally lower than that observed in individuals produced by sexual reproduction (Agrios, 2005; Obanor *et al.*, 2010).

In plant pathogens, genetic diversity in a species population can provide a range of adaptions which suit different environmental factors and so improve their ability to survive and infect their hosts (Obanor, 2006). Some studies have shown that genotype is correlated to pathogenicity of some fungal pathogens. For example, Alaniz *et al.* (2009) observed a correlation between genotype and virulence among isolates of *C. macrodidymum* and *C. liriodendri*, in which the isolates from two major clades were significantly more virulent than the other isolates from the different clades. Baskarathevan (2011) also observed that some isolates belonging to the genetic groups of *N. parvum* and *N. luteum* appeared to be correlated with virulence although the relationships were relatively weak.

Variability in pathogenicity within a species has been commonly observed for botryosphaeriaceous species. For example, Larignon *et al.* (2001), found four virulence groups among *D. seriata* isolates in France. In South Africa, van Niekerk, (2004) reported that different isolates of *D. seriata* and *N. australe* showed different degrees of lesion formation in mature canes. In California, Urbez-Torres and Gubler, (2009) also observed variability in lesion lengths between isolates of the species, *L. theobromae*, *N. parvum*, *N. luteum, N. australe* and *B. dothidea,* with *N. luteum* showing the highest variability with lesions ranging from 73.1 to 313.6 mm.

The pathogenicity studies of different botryosphaeriaceous species collected from different nurseries presented in Chapter 3 Section 1 also showed high variability in pathogenicity among isolates of different botryosphaeriaceous species. This variability was particularly apparent in *N. luteum* isolates, with high variability among isolates in lesion lengths on green shoots and one-year-old canes. The mean ratios with respect to the positive control means ranged from 0.04 to 2.08 and 0.04 to 2.58, for green shoots and canes, respectively. Pathogenicity also varied significantly among *N. luteum* isolates from different nurseries, which indicated that the genotype may be influenced by origin. Therefore, there is a need to investigate the relationship between pathogenicity and genetic diversity of the predominant botryosphaeriaceous species from the different nurseries. Further understanding of the genetic diversity of the populations of plant pathogens may provide for greater understanding of pathogen biology and infection processes which are essential in developing control strategies at the nursery level.

The different PCR-based tools for investigating diversity of a fungal species were presented in Section 1.8.1. The UP-PCR is a DNA fingerprinting method that can amplify DNA from organisms and generate fingerprints without previous knowledge of the sequences was found to be highly robust and more reproduceable than RAPD method (Bulat *et al.*, 1998). This method has been found to be an effective tool for studying the genetic variability of fungal populations, including *Trichoderma* species (Lubeck *et al.,* 1999; Cumagun *et al.,* 2000) and grapevine canker pathogens like *Ph. chlamydospora* (Pottinger *et al.*, 2002), *N. parvum* and *N. luteum* (Baskarathevan, 2011) and *Cylindrocarpon* spp. (Pathrose *et al.*, 2011).

The aim of this study was to analyse the genetic variability among isolates of the *N. luteum* pathogenicity groups using UP-PCR. It further investigated the genetic diversity among the *N. luteum* isolates at an intra-plant, intra-nursery and inter-nursery level using the same molecular tool.

3.2.2 MATERIALS AND METHODS

3.2.2.1 Species and isolates selection

From the seven botryosphaeriaceous species obtained from the 2008 nursery survey (Chapter 2) and tested for pathogenicity on grapevines (Chapter 3, Section 1), *N. luteum* was selected for genetic variability analyses. The criteria for selection of this species were based on its prevalence and distribution in different nurseries and the variability displayed between isolates with respect to pathogenicity on one-year old canes based on the ratio with the positive control (results presented in Chapters 2 and 3 Section 1; Appendix C.2.10). The 67 *N. luteum* isolates were ranked from highest to lowest based on their mean lesion ratios, with respect to the positive control, in trunks of one-year-old grapevines. From these rankings, ten isolates each of

high, moderate and low pathogenicity were randomly selected and assigned into three pathotypes. The 10 isolates that gave the ratios of 0.70 or higher were placed in Pathotype Group A, nine isolates with ratios between 0.30 to 0.62 were placed under Pathotype Group B (moderate pathogenicity) while the 10 isolates that gave ratios of 0.30 or lower were placed under Pathotype Group C (Table 3.5). One isolate (826) originally placed under Group B was later confirmed as *N. australe* and was excluded from the analysis.

Of the 29 selected isolates, ten were from nine failed grafted plants (FG) two of which were obtained from the same plant (L483 and L485). Six isolates were from five apparently healthy grafted plants (HG) with one plant yielding two isolates (L228 and L229). Five isolates were from four scion cuttings (SC) with isolates L559 and L560 obtained from the same cutting. Eight isolates were from rootstock cuttings (RC) with two cuttings yielding two isolates each (L085-L086 and L105-L106). These isolates originated from different nurseries with nine from Nursery 3, 16 from Nursery 5 and four from Nursery 7.

3.2.2.2 Single spore and hyphal tip isolations

For each of the selected isolates that were stored in 20% glycerol at -80°C, two mycelium plugs were taken out and thawed, and then plated separately onto PEA (Appendix B.1) and incubated at 25°C under near UV light in a 12:12 h light:dark regime for 3 to 4 weeks until pycnidium development. Pycnidia (20-30) were picked from the bases of the plates and crushed using a sterile needle in sterile 1.7 ml tubes containing 1 ml SROW. A drop of the suspension from each tube was examined using a light microscope. If conidia were present, the suspension was withdrawn, vortexed for 30 s and conidial concentration determined using a haemocytometer. The concentration and was adjusted to 10^2 and 10^3 conidia/ml SROW and 100 µl of the adjusted spore suspensions were spread onto PDA amended with 1 ml/L Triton X-100 (Science Lab, USA) using a sterile hockey stick, with three replicate plates per isolate and concentration. Triton X was reported to slow down microbial growth allowing the differentiation of single colonies (Beuchat & Hwang, 1996). Plates were incubated at 25°C in a 12:12 h light:dark regime for 7-10 days until single colonies were visible. Single colonies of *N. luteum* isolates were excised and sub-cultured onto PDAC, incubated at 25°C for 1 week and stored at 4°C for 4 weeks until processed.

Of the 29 isolates grown in PEA, 17 isolates produced conidia from which single spore isolates were successfully obtained. For the 12 isolates that did not produce conidia in culture, hyphal tip isolation was done to produce clonal isolates. Hyphal tip isolation is a well established methodology used when spores are unavailable (Leslie & Summerell, 2006). From a PEA culture of each isolate, 5 mm mycelium plugs were transferred onto water agar plates and incubated at room temperature until colonies of 2–3 cm diameter formed. Using a stereo

microscope set up inside a laminar flow cabinet, hyphal tips (~2 mm) were excised with a sterile scalpel from the edge of each colony and sub-cultured onto fresh PDAC, followed by incubation for 4 to 5 days at 25°C in a 12:12 h light:dark regime and stored at 4°C for 3 to 4 weeks until processed.

Table 3.5 *Neofusicoccum luteum* isolates selected for genetic variability analysis with their corresponding plant and nursery sources and pathotype groups based on their pathogenicity in trunks of one-year-old grapevines.

Pathotype Group	Isolate No.	Nursery source	[b]Plant type	Infection position	Means of ratio with positive control	Pathotype
A	228	7	HG1[a]	Above graft union	2.58	High pathogenicity
	229	7	HG1[a]	Below graft union	2.41	
	244	7	HG2	Above graft union	2.18	
	248	7	HG3	Above graft union	0.79	
	070	3	FG1	Above graft union	0.89	
	106	3	RC3[a]	Middle	0.78	
	114	3	RC6	Top	0.78	
	086	3	RC1[a]	Base	0.78	
	456	5	FG2	Above graft union	0.73	
	468	5	FG5	Below graft union	0.72	
B	105	3	RC3[a]	Top	0.62	Moderate pathogenicity
	460	5	FG3	Above graft union	0.58	
	085	3	RC1[a]	middle	0.55	
	089	3	RC2	base	0.55	
	472	5	FG6	Above graft union	0.53	
	113	3	RC5	base	0.52	
	483	5	FG8[a]	Scion bud	0.35	
	523	5	HG5	Scion bud	0.33	
	110	3	RC4	Base	0.30	
C	481	5	FG7	Below graft union	0.04	Weak pathogenicity
	493	5	FG9	Below graft union	0.06	
	557	5	SC3	Top	0.07	
	552	5	SC1	Base	0.08	
	560	5	SC4[a]	Middle	0.08	
	559	5	SC4[a]	Top	0.08	
	555	5	SC2	base	0.09	
	485	5	FG8[a]	Below graft union	0.10	
	501	5	HG4	Below graft union	0.11	
	465	5	FG4	Below graft union	0.19	

[a] Plant samples with multiple infections
[b] FG – Failed grafted plants; HG- healthy grafted plants; RC – rootstock cuttings; SC – scion cuttings

3.2.2.3 Genomic DNA extraction

From hyphal tip or single spore isolates stored at 4°C, one mycelial plug was cut and plated onto a PDA and incubated at 25°C in a 12:12 h light:dark regime for 4 days. Four mycelial plugs (7 mm) were cut from the edge of each isolate and sub-cultured onto 10 ml potato dextrose broth (PDB; Difco™ New Jersey, United States) in deep Petri dishes. The PDB cultures were incubated at 25°C in a 12:12 h light:dark regime for 4 days and then the mycelia (2-3 cm diameter) were scooped out using a sterile micropipette tip and transferred onto a sterile Miracloth™ (CALBIOCHEM®, Germany) on top of 10–20 absorbent paper towels. The Miracloth™/paper towel layer was folded and pressed for a few seconds until excess PDB was absorbed. The agar plug was removed from the pressed mycelia and the remaining mycelium was wrapped in aluminium foil, labelled, snap frozen in liquid nitrogen and stored at -80°C until required for DNA extraction.

The genomic DNA was extracted using the Puregene® system (Gentra systems, Minneapolis, USA). Frozen mycelium was ground to a fine powder using a sterile mortar and pestle that had been pre-cooled with liquid nitrogen. Approximately 100 mg of ground frozen mycelium was placed into a pre-cooled 1.7 ml tube, 500 µl of Cell Lysis solution added to it, mixed by pipetting the liquid several times and incubated in a water bath at 65°C for 1 hour. The tube was removed from incubation, inverted 10 times and 1.5 µl of RNAase A (10 mg/ml, Invitrogen) was added. The sample was inverted 25 times to mix and incubated at 37°C for 15-30 min. The tube was cooled to room temperature and 167 µl of Protein Precipitation solution was added to the cell lysate and mixed by vortexing at high speed for 20 s. The mixture was centrifuged at 16,000 ×*g* for 3 min and the supernatant was transferred to a new sterile 1.7 ml tube. The DNA was precipitated by adding 500 µl of ice-cold isopropanol to the supernatant, inverting 30–50 times and centrifuging at 16,000 ×*g* for 1 min to pellet the DNA. The supernatant was decanted and the tube was drained briefly on clean absorbent paper. The DNA pellet was washed by adding 300 µl ice-cold 70% ethanol, by inverting the tube gently (~10 times) and by centrifuging for 1 min at 16,000 ×*g*. The ethanol was gently decanted and the tube containing the DNA pellet was air-dried for 15-30 min. Finally, 30 µl of sterile distilled water was added to the DNA pellet and left overnight at 4°C to rehydrate.

3.2.2.4 Check for DNA quality and quantity

To check DNA quality, 3 µl of each DNA sample was mixed with 3 µl loading dye and separated by electrophoresis on a 1% agarose gel in 1x TAE at 10 V/cm for 45 min, with 3 µl of a 1 Kb plus DNA ladder (Invitrogen Life Technologies, Carlsbad, California, USA). The gels were stained with ethidium bromide and viewed using the VersaDoc Imaging system Model 3000 as described in Section 2.2.5.1.

The DNA concentration was determined by spectrophotometry using the Nanodrop 3.0.0 spectrophotometer (Nanodrop Technologies Inc., Delaware, USA). Samples with concentrations lower than 10 ng/µl were discarded and DNA extraction was repeated. DNA samples of 20-25 ng/µl were prepared by diluting the original extracts with SNW and the stock solutions were stored at -4°C while aliquots were stored at 4°C for 8 weeks until processed by PCR.

3.2.2.5 Selection of primers for UP-PCR analyses

In a preliminary experiment, eleven UP-PCR primers were screened to select the five primers most likely to detect polymorphisms. The sequences of the UP-PCR primers and their respective annealing temperatures are shown in Table 3.6. The DNA extracted from three isolates (L105, L456, L826), which were randomly selected from three nurseries of different regions, was amplified with each of the 11 primers. Each 25 µl UP-PCR reaction volume contained 1× PCR buffer (Roche Diagnostics, Basel, Switzerland), 200 µM each of dGTP, dCTP, dATP and dTTP, 2.5 mM $MgCl_2$, 20 pmoles of the selected primer, 1.25 U of FastStart *Taq* DNA polymerase (Roche Diagnostics, Mannheim, Germany), and 20-25 ng genomic DNA.

Table 3.6 UP-PCR primers and their respective annealing temperatures

Primer Name	Primer Sequence	Annealing temperature (°C)
AA2M2	$^{5'}$CTGCGACCCAGAGCGG$^{3'}$	50
AS4	$^{5'}$TGTGGGCGCTCGACAC$^{3'}$	50
AS15	$^{5'}$GGCTAAGCGGTCGTTAC$^{3'}$	52
AS15Inv	$^{5'}$CATTGCTGGCGAATCGG$^{3'}$	52
L15	$^{5'}$GAGGGTGGCGGTTCT$^{3'}$	52
L15/AS19	$^{5'}$GAGGGTGGCGGCTAG$^{3'}$	52
L45	$^{5'}$GTAAAACGACGGCCAGT$^{3'}$	51
L21	$^{5'}$GGATCCGAGGGTGGCGGTTCT$^{3'}$	55
3-2	$^{5'}$TAAGGGCGGTGCCAGT$^{3'}$	52
Fok 1	$^{5'}$GGATGACCCACCTCCTAC$^{3'}$	52
0.3-1	$^{5'}$ÇGAGAACGACGGTTCT$^{3'}$	50

Amplification using a Bio-Rad iCycler Thermal cycler (Hercules, California, USA) was achieved by the following temperature regimes: denaturation at 94°C for 3 min, then 5 cycles of 94°C for 50 s, annealing at the appropriate temperature (Table 3.6) for 2 min and 1 min primer extension at 72°C for 1 min, followed by 34 cycles of 94°C for 50 s, annealing at the appropriate temperature (Table 3.6) for 90 s and 1 min primer extension at 72°C, with a final extension at 72°C for 10 min.

Eight microlitre aliquots of the UP-PCR amplification products were separated by electrophoresis on 1% agarose gels as described in Section 2.2.5.1, except that 250 ml of molten agarose was prepared and poured into a larger (20 cm x 21 cm) gel tray. The products were separated by electrophoresis at 6 V/cm for approximately 2 h 45 min and stained using the methods in Section 2.2.5.1.

From the eleven primers tested, five were selected for the final UP-PCR analyses. The criteria for selection of the primers were based on the total number of bands amplified, the number of polymorphic bands and the ease with which the bands could be distinguished from each other. The UP-PCR analyses were repeated for all the *N. luteum* isolates using the five selected primers.

3.2.2.6 Genetic variation analysis

Bands from each primer that were strong and reproducible were counted and scored as follows: presence of band (1) and absence of band (0). Bands that were not distinctly separated or were closely clustered were not counted. The binomial table generated using Microsoft Excel 2010 was converted to a "Nexus" file using Mesquite 2.74 program (Mesquite Software Inc., Oregon State University and University of British Columbia, USA). The converted file was analysed using PAUP software version 4.0b10 (Swofford, 1999) to generate a neighbour joining tree to determine the relationships between genotypes of 29 *N. luteum* isolates. Different branches of the neighbour joining tree were correlated to the plant source, nursery source and pathotype group.

3.2.3 RESULTS

3.2.3.1 Selection of UP-PCR primers

Of the 11 primers tested, eight primers produced multiple bands of different sizes while three did not produce bands (Table 3.7). For the three isolates used for primer selection, L826 gave a banding pattern that was consistently different from L106 and L456 with all eight primers that produced bands (Figure 3.7). When the identity of L826 was clarified from the ARDRA data (Appendix D.1), it was found to be mistakenly transcribed as *N. luteum* although the actual identity was *N. australe.* This isolate was excluded in the succeeding analysis and the selection of primers was only based on the banding patterns of L106 and L456.

Of the 11 primers tested, L15/AS19, AA2M2, AS4, AS15Inv and L21 gave the highest number of bands and polymorphic bands (Table 3.7; Figure 3.7). Primers L15, L45 and 3-2 had small numbers of bands or few polymorphic bands while primers AS15, Fok 1 and 0.3-1 did not produce any amplimers (Table 3.7; Figure 3.7).

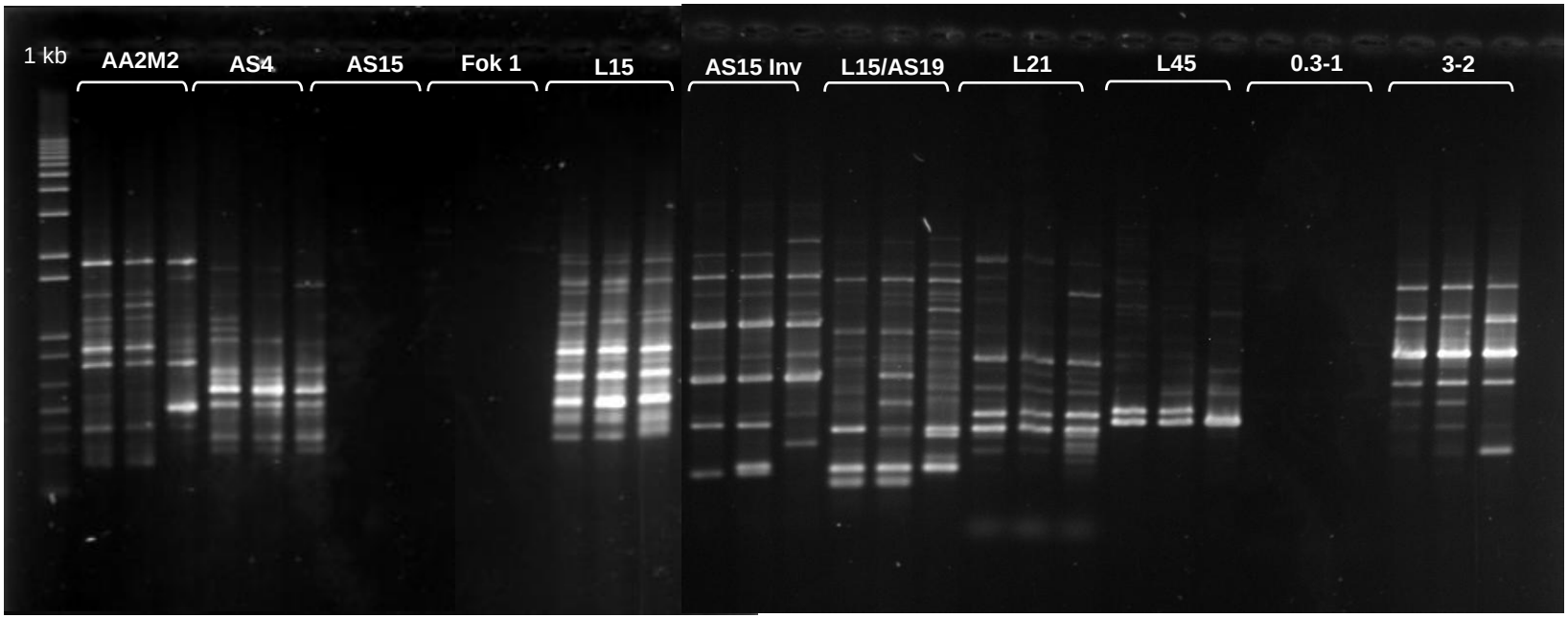

Figure 3.7 Banding patterns of the amplification products from 11 UP-PCR primers after separation by electrophoresis on a 1% agarose gel. Primer names are shown at the top of the gel. Each primer was tested on three isolates (L105, L456 and L826), from left to right in each group). Lane 1 is the 1 Kb plus DNA ladder and numbers on the far left denote molecular weights of bands in the ladder.

Table 3.7 Bands produced by 11 UP-PCR primers through amplification of DNA from two isolates of *N. luteum*.

Primers	Total Bands	Polymorphic bands	% Polymorphism	Description
AA2M2	13	3	23%	Good band separation
AS4	12	3	25%	Good band separation
AS15	0	0	0	No amplification
AS15 Inv	10	3	30%	Good band separation
Fok 1	0	0	0	No amplification
L15	12	1	8%	Low polymorphism
L15/AS19	19	7	37%	High number of bands
L21	8	2	25%	Good band separation
L45	3	2	66%	Very small number of bands
0.3-1	0	0	0	No amplification
3-2	7	2	28%	Small number of bands

3.2.3.2 UP-PCR analysis of the N. luteum isolates

The UP-PCR primers AA2M2, AS4, AS15Inv, L15/AS19 and L21 were selected for the genetic variability analysis of the 29 *N. luteum* isolates as these primers provided well distributed and polymorphic DNA fingerprint patterns. Representative gel photographs after electrophoresis with PCR products from each primer are shown in Figures 3.8.

From the five primers selected, a total of 75 scorable bands were produced, of which 48% were polymorphic. These bands were used to generate a binomial matrix for the phylogenetic analysis and construction of a neighbour joining tree (Figure 3.9). PAUP analysis showed high genetic diversity among *N. luteum* isolates, with 27 isolates having unique UP-PCR genotypes while two isolates had the same genotype (L228 and L229). The neighbour joining tree had two major branches and each was further divided into two sub-branches. These four sub-branches were defined as four major clades, with four isolates clustering in Clade I, ten in Clade II, six in Clade III and nine in Clade IV (Figure 3.9).

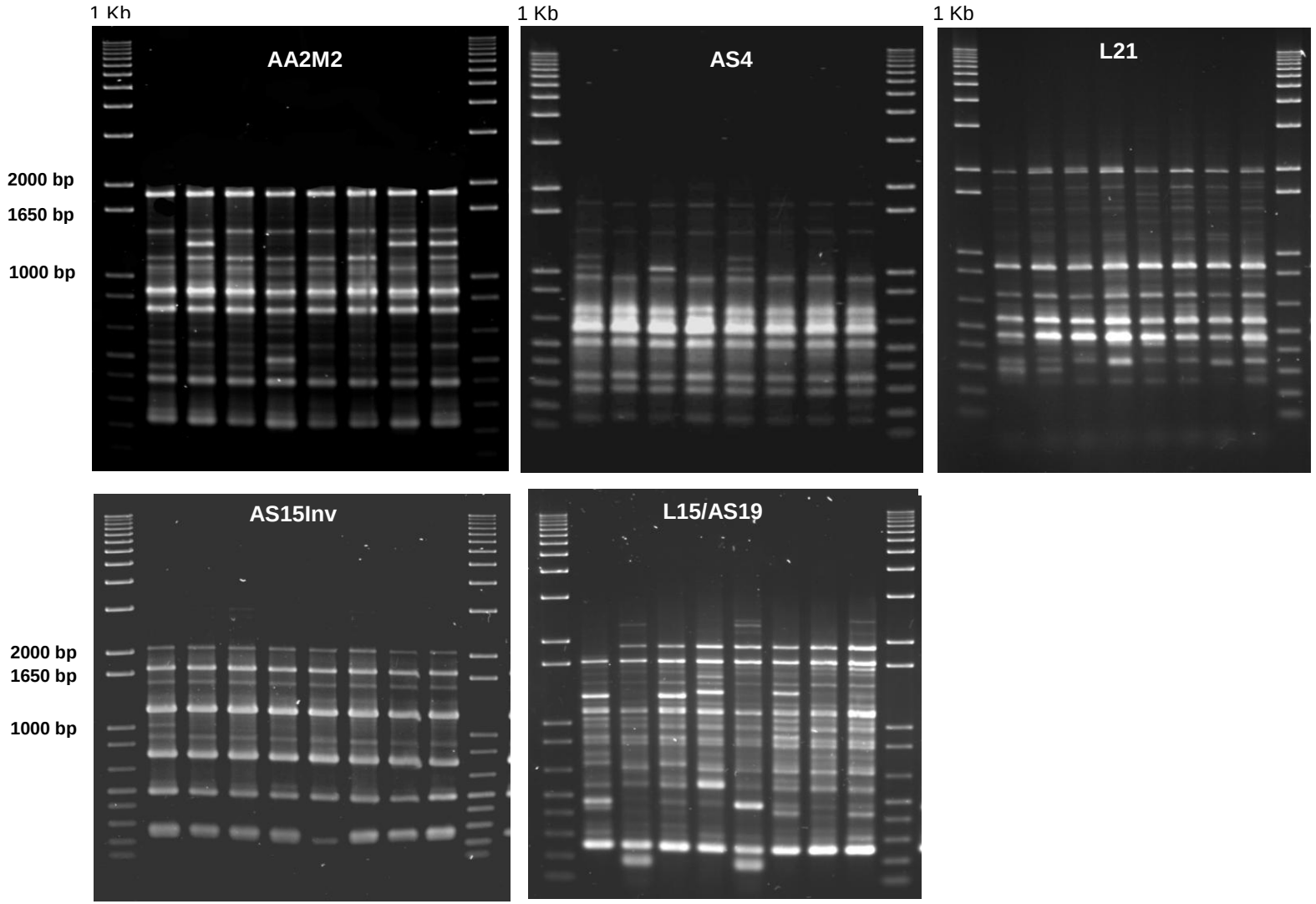

Figure 3.8 Representative photographs of UP-PCR banding profiles of *N. luteum* isolates from different pathotype groups. Primer name is shown at the top of each gel. Lane 1 for each gel is the 1 Kb plus DNA ladder and numbers on far left denote molecular weights of bands in the ladder. From left to right of each gel are banding patterns of representative isolates 070, 085, 086, 089, 105, 106, 110 and 113.

3.2.3.3 Intra-plant variability

Multiple genotypes were obtained from four out of five plant samples with two infections indicating the occurrence of intra-plant genotype variation. With the exception of the isolates L228 and L229 from HG1 that were clonal, the four isolate pairs (L85/L86; L105/L106; L483/L485 and L559/L560) recovered from the same plant samples were of different genotypes and were randomly distributed in three clades.

3.2.3.4 Inter- and intra-nursery variability

Intra- and inter-nursery comparisons showed that different genotypes were present within a nursery and similar genotypes in different nurseries. The isolates from any one nursery were highly variable, with nine isolates from Nursery 3 randomly distributed in Clades I, II and III. For Nursery 7 isolates, three clustered in Clade III, two of which were clonal (L228 and L229) while one isolate was in Clade II. For Nursery 5 isolates, seven were randomly distributed in Clades I, II and III while the remaining nine isolates clustered in Clade IV. The clustering of nine isolates from Nursery 5 in Clade IV showed high similarity among this group indicating a genotype-nursery relationship.

3.2.3.5 Genetic variability within pathogenicity groups

There was no obvious relationship between genotype and pathogenicity in canes (Figure 3.9). Although the three isolates from Pathotype A with the highest ratio values (>2.0; Table 3.5) clustered in Clade III and seven out of ten isolates belonging to Pathotype C clustered in Clade IV, these isolates of the same pathotype all originated from the same nursery, therefore, the clustering may also be due to origin. The remaining isolates from pathotype Groups A, B and C were randomly distributed among the four clades of the neighbour joining tree.

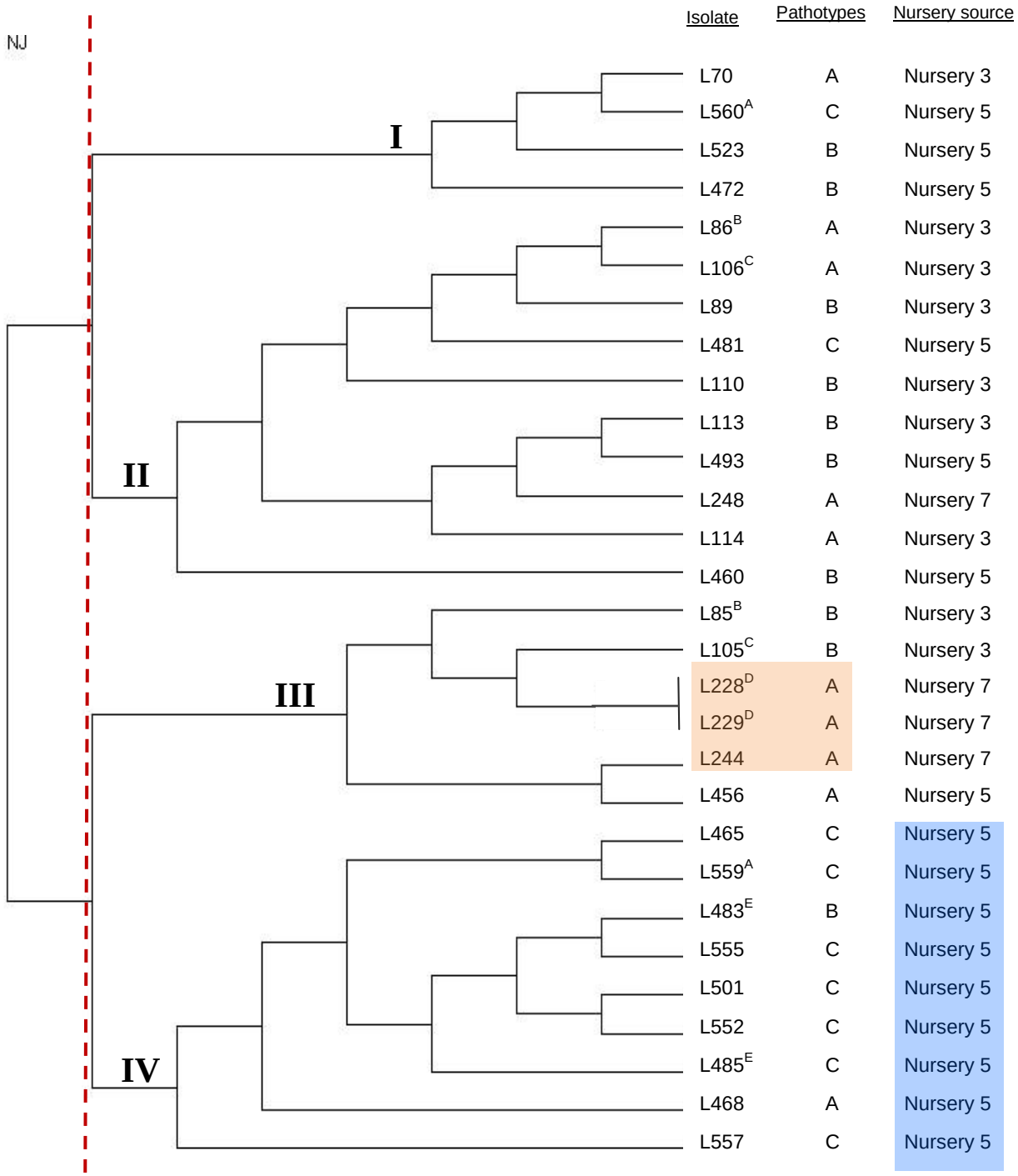

Figure 3.9 Neighbour joining dendogram depicting patterns of genetic variability among 29 *N. luteum* isolates from different nurseries based on UP-PCR analysis using five primer combinations. The red dotted line indicates the changes at which the branches were defined into four major clades (roman numerals). Where letters on the right of the isolate numbers match, this indicates that the isolates were from the same plant sample. Isolates shaded in orange indicate clustering by pathotype and blue indicate clustering by nursery origin.

3.2.4 DISCUSSION

This study used UP-PCR analysis to investigate the genetic variability among isolates of *N. luteum* collected from different nurseries that showed different degrees of pathogenicity on green shoots and canes. The analysis produced high numbers of polymorphic bands that allowed the differentiation of different *N. luteum* isolates and showed the genetic diversity of the nursery isolates of this species.

The analysis of multiple isolates from five plant samples showed the potential for pathogen movement, since the isolates recovered from above (L228) and below (L229) the graft union in one healthy grafted plant were of one genotype. A study by Gambetta *et al.* (2009) using xylem-mobile dyes and the xylem-limited bacterial pathogen *Xyllela fastidiosa* demonstrated that xylem conduits bridge the graft unions, so they could allow the passive movement of pathogens. Therefore, these open conduits may facilitate the movement of the *N. luteum* infections from either rootstock or scion and allow for development of systemic infections in young vines. However, it is also possible that multiple infections by the same genotype can occur in the same plant by splash dispersal from a single source of conidia from a nearby plant surface.

The analysis also demonstrated intra-plant genotype variation, indicating separate infection events of an individual plant sample. One grafted plant and three cuttings contained multiple isolates of different genotypes, indicating that they were infected by inoculum from different sources within the mothervine blocks or the propagation systems. Intra-vine variability was also observed among *Ph. chlamydospora* isolates suggesting that multiple introductions had occurred (Pottinger *et al.*, 2002; Mostert *et al.*, 2006). Given that multiple infections by genetically distinct isolates are occurring, investigations were done (Chapter 5) into the sources of botryosphaeriaceous species inoculum and their potential dispersal mechanisms within the nursery system as this knowledge could enhance the development of control strategies.

Inter- and intra-nursery comparisons showed that different genotypes were present within a nursery and in different nurseries in New Zealand. Except for Nursery 7, no clonal isolates were found among those obtained from Nursery 3 and Nursery 5. Furthermore, the genetically distinct isolates from different nurseries were randomly distributed among three clades suggesting they are highly diverse and that a mechanism exists for either frequent incursion of new isolates to the nursery and/or the presence of an active recombination mechanism. However, nine of the isolates from Nursery 5 were clustered in Clade IV indicating that these isolates were closely related. Baskarathevan *et al.* (2009) found a high degree of genetic variability among *N. parvum* isolates from New Zealand vineyards with

higher inter-vineyard variability than intra-vineyard. Che-Omar (2009) also found similar results with *N. parvum* isolates from six blueberry farms in New Zealand; the 30 isolates from three individual plant samples from each farm were each of unique genotype. These results are in contrast with genetic variability studies of other fungal pathogens that have predominantly asexual reproductive mechanisms. For example, Pottinger *et al.* (2002) detected low genetic variation among New Zealand populations of *Ph. chlamydospora,* for which they found only nine genotypes out of the 45 isolates analysed using UP-PCR. They also found that these NZ isolates shared high genetic similarity with the Italian isolates analysed. A study by Mostert *et al.* (2006) also showed low genetic variation among *Ph. chlamydospora* isolates from South Africa, which comprised 19 genotypes among 68 isolates analysed using amplified fragment lengths polymorphism (AFLP). Obanor *et al.* (2010) also observed low levels (13%) of genetic variation among *S. oleagina* populations in New Zealand and showed great similarity with isolates from Australia and Italy using UP-PCR analysis. A study by Alaniz *et al.* (2009) also showed low genetic variability among *C. liriodendri* and *C. macrodidymum* isolates.

Botryosphaeriaceous species were concluded to be predominantly asexual in their reproduction since their teleomorph stages were rarely encountered in the field (Jacobs & Rehner, 1998; Phillips, 2002; Slippers & Wingfield, 2007). Previous research on *D. sapinea* also supported this hypothesis since spore-trapping studies had shown that it spread exclusively via conidia (Swart *et al.*, 1991) and genetic diversity studies using simple sequence repeat (SSR) markers had provided some evidence that this species is obligately asexual (Burgess *et al.*, 2004). The high numbers of clonal isolates observed among species of *B. dothidea* (Michailides, 1991; Ma *et al.*, 2004), *L. theobromae* (Burgess *et al.*, 2005) and *N. parvum* (Slippers & Wingfield, 2007) were consistent with species that are predominantly asexual in their reproduction. The high genetic variability of *N. luteum* isolates found in this study and in the studies reported by Baskarathevan *et al.* (2011) and Che Omar (2009) were, therefore, unexpected. These suggest that despite the widespread asexual reproduction observed among botryosphaeriaceous species in New Zealand, genetic recombination is occurring in nature, resulting is highly diverse populations. According to Carlile *et al.* (2001), although 20% of all known fungi reproduce purely asexually, some genetic recombination has been observed in most of these species. Unlike other organisms, fungi have novel mechanisms of genetic exchange and recombination that are not limited to sexual reproduction, which include heterokaryosis and parasexualism (Anderson & Kohn, 1998). However, parasexual crosses occur less frequently than sexual ones and their occurrence in natural populations has not been proven conclusively (Anderson & Kohn, 1998). According to Agrios (2005), while the frequency and degree of variability between asexually produced individuals are lower, the number of individuals produced asexually is so high that the total

amount of variability produced by relatively few individuals in the asexual populations is probably greater than the variability from those sexually produced populations. Since species with abundant genetic variability are more likely to survive the challenges of a changing environment (Carlile *et al.*, 2001), the high genetic diversity among *N. luteum* isolates indicates that this species is likely to be more resilient in changing conditions and would be more difficult to control than those pathogens that are highly clonal and have low genetic variability. Therefore, this genetic diversity should be taken into consideration in developing control strategies for this pathogen.

The high genetic diversity of *N. luteum* populations within nurseries may be partially due to the use of propagation materials from different sources. Although most nurseries grow their own rootstock material, their scion materials are usually provided by different vineyards through pruning (Verstappen, pers. comm., 2009). However, some nurseries also produce the majority of scion wood propagation materials in their own mothervine blocks. The movement of plant materials from vineyards to nurseries or one nursery to another may introduce new pathogen populations, thereby increasing the genetic diversity of the pathogen within the nursery. However with *Ph. chlamydospora,* which is known to spread through infected plant materials from nurseries (Fourie & Halleen, 2004b; Whiteman *et al.*, 2007; Aroca *et al.*, 2010), high clonality and low genetic variation has been reported. This may be associated with vegetative reproduction, since infection of cuttings is sometimes due to endophytic colonisation directly from the mothervines (Edwards & Pascoe, 2004; Whiteman *et al.*, 2007), but may also be due to the fact that this species reproduces in the field predominantly through asexual processes (Pottinger *et al.*, 2002; Mostert *et al.*, 2006). Pottinger *et al.* (2002) further found that *Ph. chlamydospora* isolates from different genetic groups belonged to one mycelial compatibility group, further confirming that all isolates were genetically similar. Baskarathevan (2011) found four vegetative compatibility groups among *N. parvum* isolates from New Zealand vineyards, indicating the genetic variability of this species. Therefore, it is possible that botryosphaeriaceous species employ a different mechanism in genetic exchange and recombination than *Ph. chlamydospora*. This hypothesis can be confirmed by doing vegetative compatibility tests among *N. luteum* isolates to determine if hyphal anastomosis occurs among isolates and establish the mechanisms for genetic recombination.

This study found no correlation between genotypes and pathogenicity in canes since the highly, moderately and weakly pathogenic isolates were randomly distributed in four clades. To further confirm this result, the genetic variability results were further compared with the pathogenicity of isolates on green shoots, but similar trends were observed. The isolates that were highly pathogenic and weakly pathogenic on green shoots were randomly scattered

throughout the neighbour joining tree indicating that pathogenicity on green shoots was also not correlated with genotype. Although some other genetic variability studies found some correlation between genotype and virulence, it is important to note that this study was developed in the reverse order to the studies of Baskarathevan (2011) and Alaniz *et al.* (2009). Those studies investigated the genetic variability of a wide range of isolates and then selected groups of isolates for pathogenicity tests on detached green shoots from the branches of the neighbour joining tree they had generated and Baskarathevan (2011) further confirmed the results using rooted canes. Their pathogenicity studies also inoculated and assessed the test plants in one batch using similar tissues.

While the UP-PCR method used in this study was effective in showing the genetic diversity of different *N. luteum* isolates, this method was not able to provide any conclusive correlations between pathogenicity and genotypic variability. The lack of correlations observed may be due to the limitations of UP-PCR primers which according to Bulat *et al.* (1998), are primarily designed to target intergenic and non-coding areas of the genome. Since pathogen virulence is generally regulated by a single or multiple genes (Agrios, 2005), these specific areas of the genome were most likely not targeted by the UP-PCR primers. Therefore, the RAPD and AFLP methods that randomly target different regions of the genome (Hu *et al.*, 1995; McDonald, 1997) may be more appropriate tools in investigating relationships between pathogenicity and genotypes.

In summary, high levels of genetic variability were observed among isolates of *N. luteum* from different nurseries with only two clonal isolates found which was unexpected since previous reports have indicated that this group of fungi primarily reproduce asexually in the field and that other studies have shown some botryosphaeriaceous species to have many clonal isolates. The high genetic variability indicates that *N. luteum* can employ different mechanisms of genetic exchange. In addition, the presence of different genotypes within plants and nurseries, and between nurseries, indicated that infections may occur from different sources of inoculum. The high genetic diversity of isolates in this fungal group indicates that they might have a greater capacity to survive in changing environmental conditions, potentially making them more difficult to control, compared to those pathogens that are less diverse.

Chapter 4
Susceptiblity of common rootstock and scion varieties to botryosphaeriaceous species

4.1 INTRODUCTION

In the year ending June 2009, white varieties of grapevines dominated in the ten vineyard regions across New Zealand, of which Sauvignon blanc and Chardonnay covered 53% and 12% of the total producing area, respectively. The other popular white varieties comprised Pinot Gris (5%) and Riesling (3%). Of the red varieties, Pinot noir was dominant (15%) followed by Merlot (4%) and Cabernet Sauvignon (2%) (New Zealand Winegrowers Statistical Annual, 2010). The selection of scion varieties was driven by how well they were suited to the terroir and by the market.

Grafted vines comprised 94% of the vines in New Zealand vineyards by June 2009 (New Zealand Winegrowers Statistical Annual 2010). The most popular rootstock varieties used in New Zealand grapevine nurseries were 101-14, 3309, Riparia gloire, Schwarzmann, 5C and SO4 (Graham, pers. comm., 2008; Jaspers *et al.*, 2007). Traditionally, rootstocks were selected for their agronomical qualities such as soil type response, vigour of growth and tolerance or resistance to root parasites such as phylloxera and nematodes. In New Zealand, the choice of rootstocks is generally based on what is commonly found to be suitable for the varieties and the area (Jaspers, pers. comm., 2011). The grafted plants propagated by the nurseries are usually pre-ordered by the growers.

Studies on disease susceptibility or resistance of the varieties have generally focused on soil-borne pests and pathogens like phylloxera and nematodes for rootstocks, and for wine grapes, on foliar and fruit disease like downy mildew, powdery mildew and Botrytis bunch rot (Jackson and Schuster, 1994). The susceptibility of rootstock and wine grape varieties to some trunk pathogens including *Cylindrocarpon* spp. (Jaspers *et al.*, 2007; Alaniz *et al.*, 2010) and the esca pathogens *Phaeoacremonium* spp. and *Ph. chlamydospora* (Eskalen *et al.*, 2001; Feliciano & Gubler, 2001; Marchi, 2001) has been investigated. These studies showed that different varieties had different levels of susceptibility to the pathogens, but none were truly resistant. For botryosphaeriaceous species, a study by Amponsah (2011) showed that the detached green shoots of Sauvignon blanc, Riesling, Chardonnay, Pinot noir and Cabernet Sauvignon were all equally susceptible to four botryosphaeriaceous species. Martos (2008) on the other hand, observed differential susceptibility on four rootstock and six

scion varieties when inoculated with six botryosphaeriaceous species. Of the six species tested, *N. luteum* and *N. parvum* caused the longest necroses on rootstocks and scions, and were not significantly different except with variety Macabeo in which *N. luteum* caused significantly longer necrosis than *N. parvum*.

In this study, the 2008 nursery sampling in Chapter 2 showed that botryosphaeriaceous infections were more common in some varieties. Most of the isolates were recovered from Schwarzmann (24%) and Pinot gris (23%) and they were found less frequently in the seven other varieties sampled. However, the selection of plant samples by each of the nurseries was not random and sample types and sizes were not uniform, thus, the data must be interpreted with care. Since New Zealand wine export is dependent on very few grape varieties at present, there is a need to conduct a comprehensive study to determine the relative susceptibilities of the commonly used rootstock and scion varieties to gain a general understanding of the potential risk that botryosphaeriaceous species pose to the industry. Since there are currently no effective control measures for prevention of botryosphaeriaceous infections in both nurseries and vineyards, the identification of varieties with some resistance to these pathogens could contribute to the management of the disease.

The objective of this research chapter was to determine the susceptibility of scion and rootstock varieties commonly used in New Zealand grapevine nurseries and vineyards to the most prevalent botryosphaeriaceous species found in the nurseries.

4.2 MATERIALS AND METHODS

4.2.1 Grapevine varieties

The selected rootstock varieties used for the susceptibility test was based on the six most popular rootstock varieties used by a large commercial nursery, Corban's Viticulture Ltd. (Graham, pers. comm., 2008). The three white and three red *V. vinifera* varieties selected for the susceptibility test represented the six varieties that covered the greatest area of New Zealand vineyards as reported in the Winegrowers Statistical Annual 2010 except that Riesling replaced Pinot gris because there were insufficient canes available for testing (Table 4.1).

Table 4.1 Six commonly used rootstock and scion varieties tested for susceptibility to botryosphaeriaceous species.

Rootstock varieties	Scion Varieties
101-14 Millardet	Sauvignon blanc (white)
5C Teleki	Chardonnay (white)
3309 Courdec	Riesling (white)
Riparia gloire	Pinot noir (red)
Schwarzmann	Merlot (red)
SO4	Cabernet Sauvignon (red)

4.2.2 Fungal isolates

The 2008 botryosphaeriaceous infection and pathogenicity studies presented in Chapter 2 and Chapter 3 Section 1 identified *N. luteum, N. parvum* and *N. australe* as the most prevalent and most pathogenic botryosphaeriaceous species present in grapevine nurseries. Three isolates of each species were selected for this test. The criteria that were used for selection were whether the isolates had been identified by molecular methods and were known to have moderate to high pathogenicity. The isolates for each species are presented in Table 4.2.

Table 4.2 The three isolates of the three botryosphaeriaceous species, with their nursery sources, that were used to test the susceptibility of the different grapevine varieties.

Species	Isolate No.	Source Nursery
N. parvum	P069	Nursery 3
	P116	Nursery 3
	P178	Nursery 1
N. luteum	L086	Nursery 3
	L106	Nursery 3
	L228	Nursery 7
N. australe	A088	Nursery 3
	A240	Nursery 7
	A511	Nursery 5

4.2.3 Inoculum preparation

Routine culture and long term storage are known to reduce the pathogenicity in some pathogens (Tuite, 1969; Agrios, 2005). However, such pathogens may regain their virulence if they are returned to their hosts under proper conditions (Agrios, 2005). Since the selected isolates for this study had been sub-cultured several times and kept in cold storage for at least 4 months prior to this study, each was inoculated into detached green shoots of Sauvignon blanc and after 4 days incubation at ambient temperature were re-isolated from the induced lesions onto PDAS as described in Sections 3.1.2.3.1 and 3.1.2.3.2. The botryosphaeriaceous colonies on the four replicate tissue isolations from each shoot were sub-cultured onto PDAS plates, giving a total of 24 plates per isolate. All plates were incubated at ambient temperature for 3 days until the colonies were approximately 20 mm in diameter then stored at 4°C for a maximum of 6 weeks. Two days prior to each inoculation period, two plates for each isolate were taken out of the cold storage and placed at ambient temperature and allowed to grow further. The mycelium plugs for inoculation were cut from the edges of the actively growing colonies.

4.2.4 Susceptibility test

Apparently healthy dormant one-year-old cuttings of each selected variety were purchased in October 2008 from Corban's Viticulture and kept in a cold room at 2°C for 8 weeks until they could be rooted. All cuttings were rooted and transferred in 1.5 L pots with potting mix as described in Section 3.1.2.4.1 and allowed to grow inside the greenhouse for 2 months prior to inoculation.

Inoculations were done inside the Lincoln University greenhouse from March to April 2009 for a period of 6 weeks, two replications of each variety and species per week. All test plants were wounded at the side of the stem between the middle internodes using a flame-sterilised cork borer (4 mm) and the bark within the 4 mm circle was peeled off using a sterilised scalpel to expose the wood. Mycelium plugs (4 mm) were cut from the edges of the growing isolates, inoculated onto the wound and covered with Parafilm™. Control plants for each variety were inoculated with sterile agar. All plants were individually covered with plastic bags and sprayed with water before closing the bags for 24 h, to provide high humidity during the initial infection phase. The 12 replicates per variety-isolate combination were arranged in a randomized complete block design, but because they were assessed after 18 days growth, there were only three batches growing at any one time. During the week of the first replication, a few extra plants were inoculated with mycelium plugs of each isolate and lesions were measured 15 days after inoculation to determine the best time for the assessment during the trial.

After 18 days growth, plants were lifted out of their pots, and roots and foliage were trimmed prior to washing under running tap water. The bark surrounding the likely infection area was peeled-off and the characteristic dark necrotic lesions that developed above and below the inoculation point were measured using a digital calliper. Since the results of the pathogenicity test in Chapter 3 Section 3.1.3.3 had shown that cane lesions and re-isolation distances for *N. parvum*, *N. luteum* and *N. australe* isolates were positively correlated, the length of necrotic lesions was used to measure susceptibility. However, re-isolation of the pathogens was done by plating onto PDAS 1 cm cane sections that were cut from the last 1 cm of the lesion and 1 cm beyond it for each plant of replications 1 and 12. The plates were incubated for 72 h at room temperature and assessed for characteristic growth of the inoculated isolates.

4.2.5 Data analyses

All data from the assay were analysed using SPSS Statistics 17. Since minor necroses were observed around the inoculation sites in negative control plants, their mean lesion lengths for each block were calculated and deducted from individual lesions in the corresponding block. The adjusted data were tested for homogeneity of variance using Levene's test at $P \leq 0.05$. Effects of varieties, species, isolates and their interactions on lesion lengths were determined using two-way ANOVA, with significance at $P \leq 0.05$. Means were separated by pairwise comparison using LSD ($P \leq 0.05$ significance level).

4.3 RESULTS

Except for the wound necrosis (Figure 4.1A), no external symptoms were observed 18 days after inoculation, and all inoculated plants showed normal development that was similar to the negative control plants. When the stems were debarked, all test plants that were inoculated with botryosphaeriaceous isolates exhibited the characteristic light to dark brown lesions on debarked wood (Figure 4.1 D to F) that progressed upward and downward from the inoculation point. Minor necrotic edges were also observed around the inoculation points of negative control plants (Figure 4.1 B and C) but botryosphaeriaceous species were not isolated from any control plants. In contrast, botryosphaeriaceous colonies typical of *N. australe*, *N. luteum* or *N. parvum* were consistently re-isolated from representative samples of inoculated plants.

Figure 4.1 Test plants used for susceptibility test. A) Cane without visible external symptoms beyond the inoculation wound; B and C) debarked control canes inoculated with sterile agar; D to F) debarked canes inoculated with botryosphaeriaceous isolates showing brown lesions of different lengths. Arrows point to the inoculation wound.

4.3.1 Susceptibility among plant types

The statistical data for this chapter are summarised in Appendix C.3. While all varieties inoculated with botryosphaeriaceous isolates produced lesions, significant differences were observed between plant types ($P<0.001$; Appendix C.3.1) with rootstocks producing significantly longer ($P\leq0.05$) lesions upwards and downward of the inoculation point, (means of 11.2 and 10.1 mm, respectively) than for scions (means of 9.9 and 7.3 mm, respectively). Significant interactions between plant types and species were observed for upward lesions ($P=0.007$), downward lesions ($P<0.001$) and overall lesion lengths ($P<0.001$)

The significant interactions were associated with upward, downward and total mean lesions caused by *N. parvum* that were significantly longer in rootstocks than in scions (Figure 4.2; Appendix C.3.1) while *N. luteum* and *N. australe* caused similar mean lesion lengths on both plant types (Figure 4.2). The upward and downward lesions had similar plant type-species interaction to those observed in overall lesions (Appendix C.3.1).

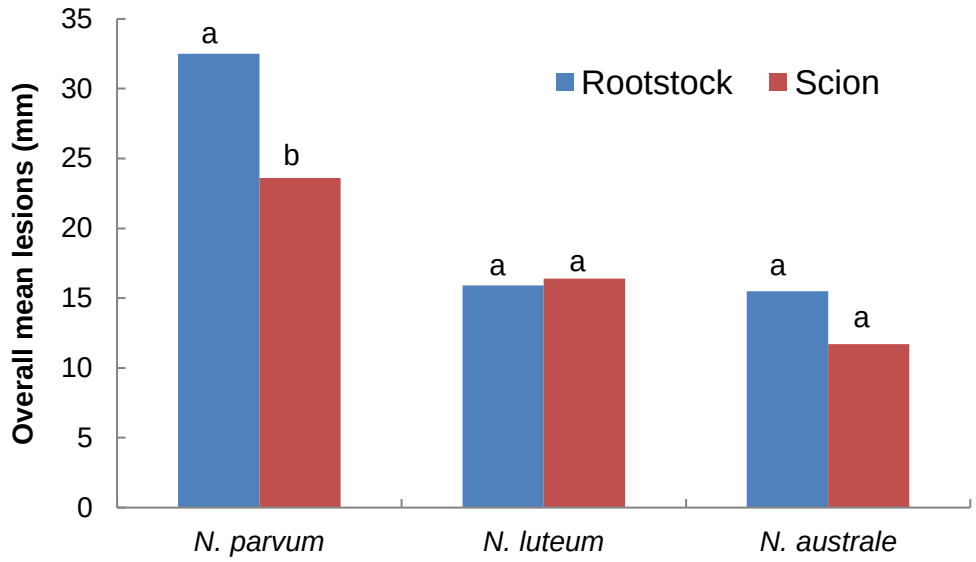

Figure 4.2 Overall mean lesion lengths on six combined rootstocks and six combined scion varieties inoculated with three botryosphaeriaceous species. Pairs of bars with different letters are significantly different at *P*≤0.05.

4.3.2 Susceptibility among rootstock varieties

The upward, downward and overall mean lesions differed significantly (*P*<0.001, *P*=0.014 and *P*<0.001, respectively) among rootstock varieties (Appendix C.3.2). Of the six varieties tested, 5C and SO4 were the most susceptible producing the longest overall lesions of 26.7 and 25.6 mm, respectively (Figure 4.3) which were significantly longer (*P*≤ 0.05) than for Schwarzmann (17.8 mm) and 101-14 (17.4 mm). The upward and downward lesions had similar variety effects to those observed on overall lesions (Appendix C.3.2). There were no significant interactions between rootstock varieties and species based on upward (*P*=0.689), downward (*P*=0.471) or overall lesions (*P*=0.465)

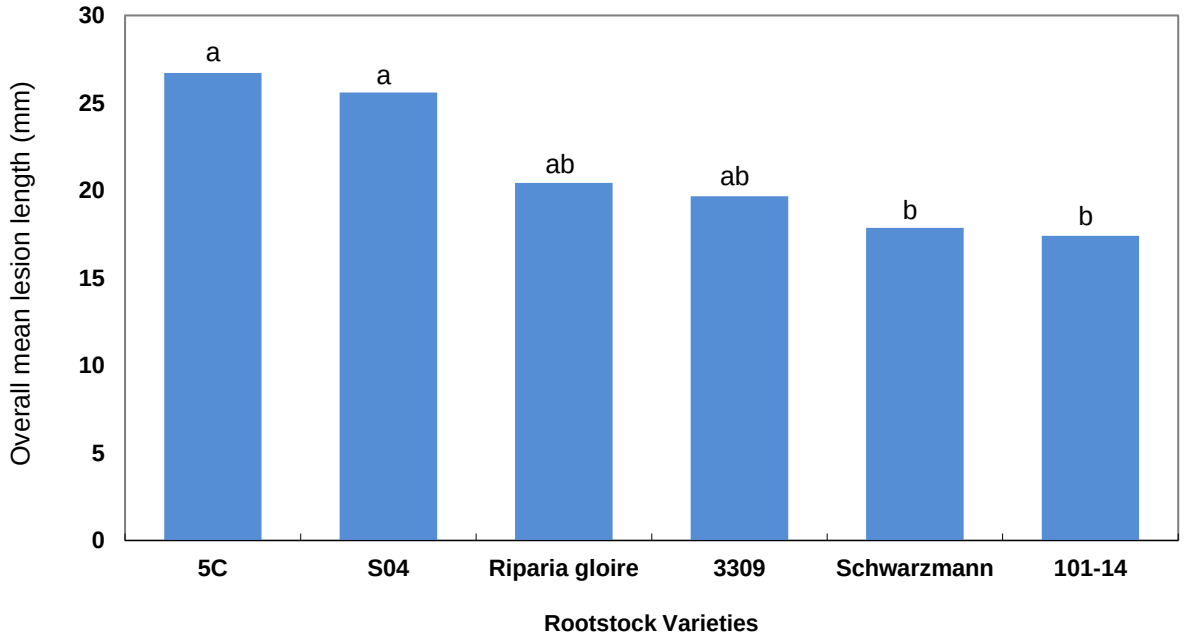

Figure 4.3 Overall mean lesion lengths on six rootstock varieties inoculated with three botryosphaeriaceous species. Bars with different letters are significantly different at $P \leq 0.05$.

4.3.3 Susceptibility among scion varieties

The upward, downward and overall mean lesions differed significantly ($P=0.002$; $P=0.002$; $P=0.001$, respectively) among scion varieties (Figure 4.4, Appendix C.3.3). Of the six varieties tested, Merlot was the most susceptible, followed by Pinot noir with overall lesion means of 20.9 and 20 mm, respectively, which were significantly longer ($P \leq 0.05$) than for Cabernet Sauvignon (14.7 mm) and Chardonnay (14.2 mm). The upward and downward lesions had similar variety effects to those observed on overall lesions (Appendix C.3.3). There were no significant interactions between scion varieties and species based on upward lesions ($P=0.894$), downward lesions ($P=0.077$) or overall lesions ($P=0.628$; Appendix C.3.3)

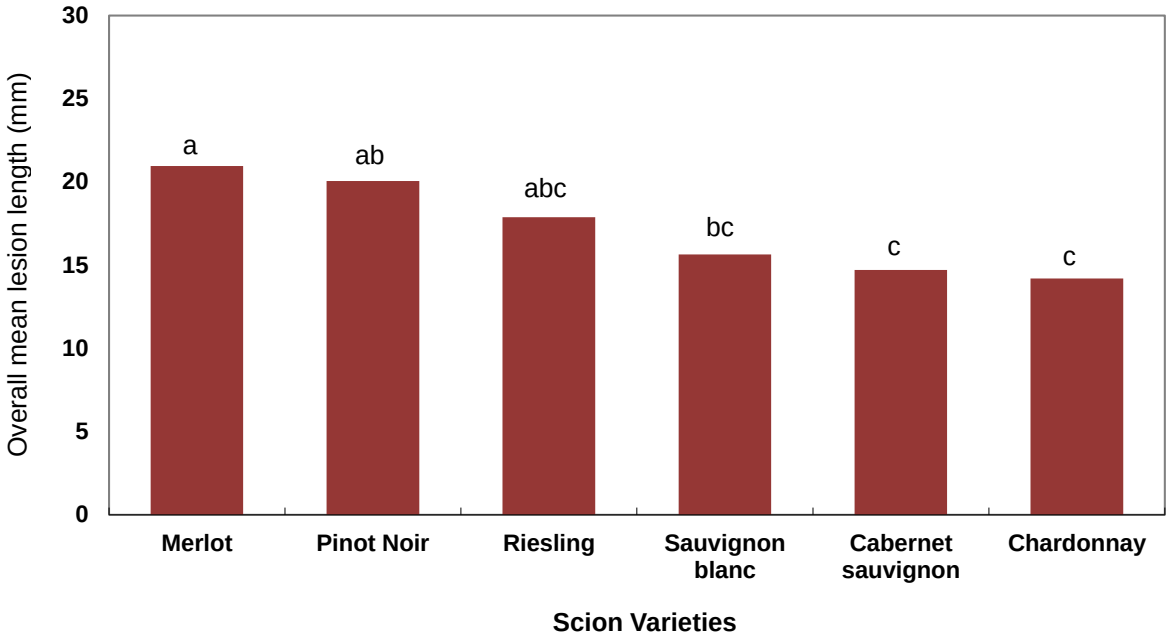

Figure 4.4 Overall mean lesion lengths on six scion varieties inoculated with three botryosphaeriaceous species. Bars with different letters are significantly different at $P<0.05$.

4.3.4 Pathogenicity of three botryosphaeriaceous species on rootstocks

All three botryosphaeriaceous species produced lesions in all rootstock varieties tested. Upward and downward lesion lengths differed significantly among the three botryosphaeriaceous species tested ($P<0.001$; Figure 4.5; Appendix C.3.2) with *N. parvum* causing the longest mean lesions at 16.6 and 15.8 mm, respectively, that were significantly longer ($P\leq0.05$) than for *N. luteum* (means of 8.2 and 7.6 mm, respectively) and for *N. australe* (means of 8.6 and 6.9 mm, respectively). Mean lesions of *N. luteum* and *N. australe* did not differ significantly from each other. The overall lesions had similar species effects to those observed on upward and downward lesions (Figure 4.5; Appendix C.3.2).

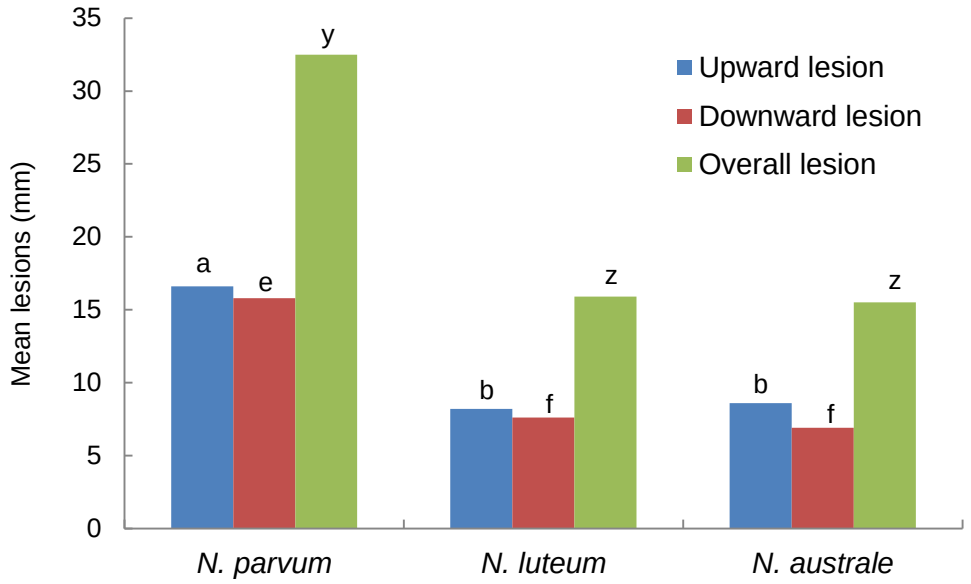

Figure 4.5 Upward, downward and overall mean lesion lengths caused by three botryosphaeriaceous species on the rootstock varieties. For each lesion type, bars with different letters are significantly different at $P \leq 0.05$.

4.3.5 Pathogenicity of three botryosphaeriaceous species on scions

All three botryosphaeriaceous species produced lesions in all scion varieties tested. Upward and downward mean lesion lengths on scion varieties differed significantly among the three botryosphaeriaceous species tested ($P<0.001$), with *N. parvum* causing the longest mean lesions at 13.5 and 10.1 mm, respectively, that were significantly longer ($P \leq 0.05$) than for the other two species (Figure 4.6; Appendix C.3.3). The upward and downward lesions produced by *N. luteum* (means of 9.5 and 6.9 mm, respectively) were also significantly longer ($P \leq 0.05$) than for *N. australe,* which produced the shortest lesions (means of 6.7 mm and 5.0 mm, respectively) among the three species tested. The overall lesions had similar species effects to those observed on upward and downward lesions (Figure 4.6; Appendix C.3.3).

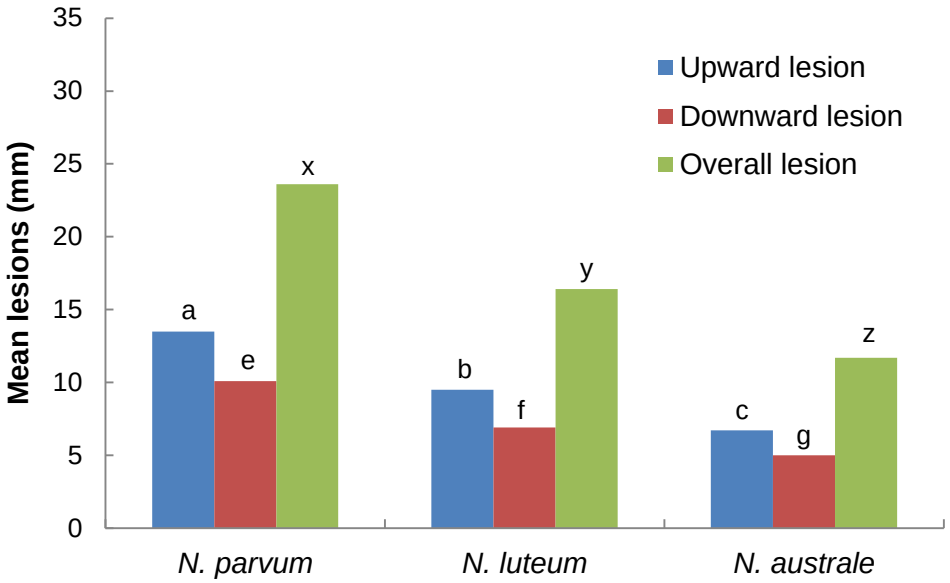

Figure 4.6 Upward, downward and overall mean lesion lengths caused by three botryosphaeriaceous species on the scion varieties. For each lesion type, bars with different letters are significantly different at $P \leq 0.05$.

4.3.6 Pathogenicity of different botryosphaeriaceous isolates on rootstocks

All isolates of each botryosphaeriaceous species produced lesions in all rootstock varieties tested but lesion lengths varied significantly among isolates ($P<0.001$). Of the nine isolates tested, *N. parvum* isolate P069 caused the longest overall lesions with a mean of 43.3 mm that differed significantly from all other isolates ($P \leq 0.05$), followed by isolate P178 with an overall lesion mean of 34.7 mm that differed significantly from the remaining isolates ($P \leq 0.05$; Figure 4.7). The third *N. parvum* isolate P116 caused similar mean overall lesion lengths to all isolates of *N. luteum* (means of 13.5 to 18.6 mm) and *N. australe* (means of 11.2 to 19.2 mm). The upward and downward lesions had similar isolate effects to those observed on overall lesions (Appendix C.3.4). There were no significant interactions between rootstock varieties and isolates based on upward ($P=0.892$), downward ($P=0.642$) or overall lesions ($P=0.690$; Appendix C.4.4).

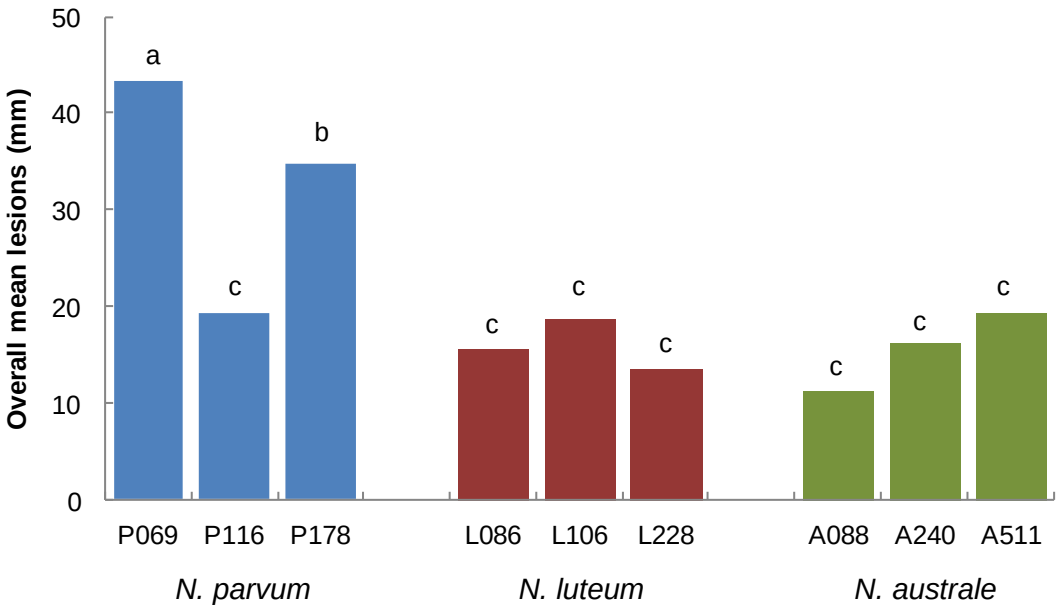

Figure 4.7 Overall mean lesion lengths of different botryopshaeriaceous isolates on rootstock varieties. Bars with different letters are significantly different at *P*≤0.05.

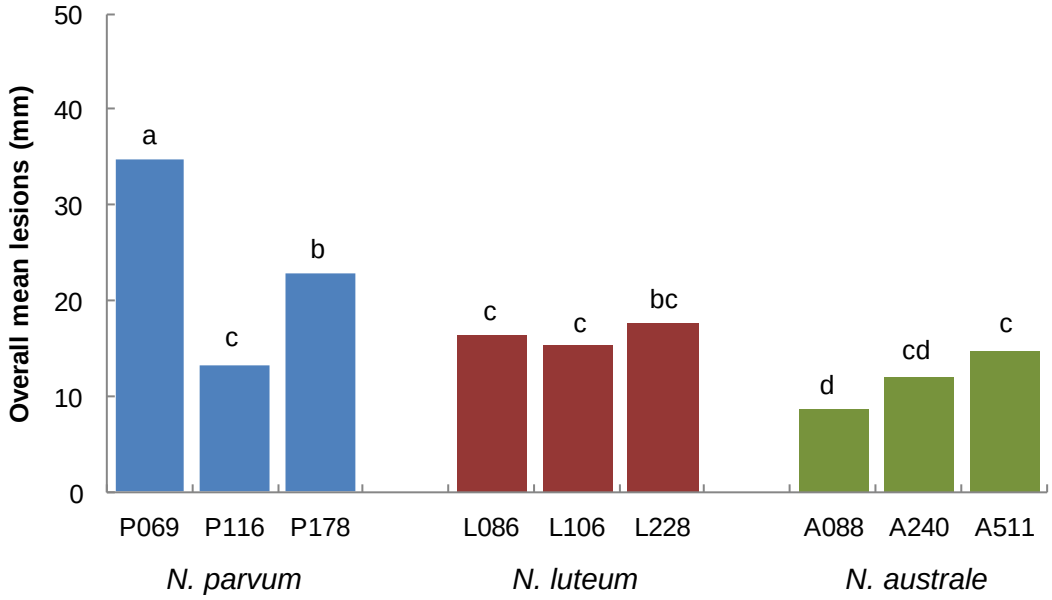

Figure 4.8 Overall mean lesion lengths of different botryosphaeriaceous isolates on scion varieties. Bars with different letters are significantly different at *P*≤0.05.

4.3.7 Pathogenicity of different botryosphaeriaceous isolates on scions

All isolates of each botryosphaeriaceous species produced lesions in all scion varieties tested but upward, downward and overall lesions varied significantly among isolates (*P*<0.001; Figure 4.8; Appendix C.3.5). The means for overall lesion lengths differed significantly (*P*≤0.05) among *N. parvum* isolates, with P069 causing the longest overall

lesions, with a mean of 34.6 mm that was significantly longer ($P \leq 0.05$) than for all other isolates. The overall mean for P178 (22.9 mm) differed significantly ($P \leq 0.05$) to the means of remaining isolates except for *N. luteum* isolate L228. The third *N. parvum* isolate P116 caused similar mean lesion length (13.1 mm) to three isolates of *N. luteum* (15.3 to 17.5 mm) and two isolates of *N. australe* (11.8 to 14.6 mm). The upward and downward lesions had similar isolate effects to those observed on overall lesions (Appendix C.3.5). There were no significant interactions between scion varieties and isolates based on upward ($P=0.862$) and overall lesions ($P=0.187$) but a significant interaction between scion variety and downward lesions was observed ($P=0.001$). The significant interaction was associated with downward lesions caused by isolate P069 that were significantly longer in Merlot (mean of 27.9 mm) than in other scion varieties (means of 9.6 to 17.4 mm; Appendix C.3.5).

DISCUSSION

This research is the first comprehensive study to assess the susceptibility of the different scion and rootstock varieties commonly used in New Zealand vineyards to the most prevalent botryosphaeriaceous species. A total of six rootstocks and six scion varieties were tested for their susceptibility to three isolates each of the most common botryosphaeriaceous species (*N. parvum*, *N. luteum* and *N. australe)* found in New Zealand nurseries. All scion and rootstock varieties showed cane lesions when inoculated with botryosphaeriaceous isolates but mean lesion lengths varied significantly among varieties suggesting that some varieties were more susceptible than others.

Previous studies showed that different rootstock varieties had different levels of susceptibility to *Cylindrocarpon* spp. (Jaspers *et al.*, 2007; Alaniz *et al.*, 2010) and Esca pathogens *Phaeoacremonium* spp. and *Ph. chlamydospora* (Eskalen *et al.*, 2001; Feliciano & Gubler, 2001; Marchi, 2001). However, these susceptibility studies each used different varieties for the reason that commonly used varieties differ in every country. In the South African study of *Ph. chlamydospora* infections, the rootstocks used were 101-14 Mgt, 99 Ritcher and 110 Richter (Fourie & Halleen, 2002, 2004b, 2006; Halleen *et al.*, 2003; Retief *et al.*, 2006). In Spain, Martos (2008) investigated the susceptibility of 41B, 140Ru, R110 and SO4 rootstocks to six botryosphaeriaceous species including *N. luteum* and *N. parvum*. For studies on Petri disease in Spanish nurseries, rootstock 110 Richter, 140 Ruggeri, 1103 Paulsen and 161-49 were the varieties used (Aroca *et al.*, 2006; Gramaje *et al.*, 2009; Aroca *et al.*, 2010) while 110 Richter was also used for *Cylindrocarpon* spp. research in Uruguay (Alaniz *et al.*, 2010; Alaniz *et al.*, 2011). SO4 was also used for studying different rootstock diseases in Italy (Zanzotto *et al.*, 2007), Spain (Aroca *et al.*, 2006) and Uruguay (Alaniz *et al.*,

2010). Therefore, most of the data from the described studies did not provide information on the relative susceptibilities of rootstock varieties commonly used in New Zealand, SO4, 5C, 101-14, 3309, Riparia gloire and Schwarzmann, which were used in this study.

The results obtained in this study showed that all rootstock varieties inoculated with the mycelia of the three botryosphaeriaceous species were infected by the pathogens to different degrees, with 5C and SO4 being the most susceptible among the six varieties tested. On rootstocks, *N. parvum* caused significantly longer lesions than on scions, which resulted in a significant interaction between plant types and species. However in the 2008 nursery survey (Section 2.3.5.3), the incidence of the species showed that Schwarzmann had the highest *N. luteum* infection incidence at 62% while 3309 and 5C were free from *N. luteum* infections. For *N. parvum*, 101-14 had the highest infection incidence at 56% while 5C was again free from infections. While the 2008 sampling represents natural infections, the selection of nurseries and plant samples were not randomised and sample types and sizes were not uniform in this study, thus, this infection incidence may be skewed towards particular varieties which had greater representation than others. In contrast, in this study the most commonly used rootstock varieties were represented as an equal number of replicates and tested for susceptibility in a randomised designed.

Susceptibility studies in Spain also showed that SO4 was highly susceptible to *N. luteum* and *N. parvum,* following 41B which was the most susceptible (Luque *et al.*, 2007; Martos, 2008). Except for SO4, the other rootstocks tested in this study were different from the ones used in Spain. However, this study and the cited study in Spain used mycelium plugs for inoculation and plants were kept under greenhouse conditions while in natural conditions conidia are generally the primary inoculum for botryosphaeriaceous species (Larignon & Dubos, 2001; Urbez-Torres *et al.*, 2010b). A study by Amponsah (2011) had shown that both conidia and mycelium can cause infections on woody trunks of young vines but mycelium infections appeared to develop faster than conidium infections for the four botryosphaeriaceous species tested. The differences in infection rates between the two types of inoculum are thought to be linked to the lag phase that may be involved in conidial attachment, germination and penetration in host tissues (Amponsah, 2011). Since the relative susceptibility of the different varieties may be affected by the type of inoculum and the environmental conditions, it is therefore important to confirm the results of this study using conidia under field conditions.

The equal susceptibility of 5C and SO4 to mycelia of botryosphaeriaceous species observed in this study may be due to their genetic relatedness since both are hybrids of the parent species *V. berlandieri* x *V. riparia* (Jackson & Schuster, 1994; Keller, 2010b). Jackson and

Schuster (1996) reported that 5C was used in cooler climates because of its ability to bring ripeness forward while SO4 is considered ideal for poor soils with high stone contents. Riparia gloire variety is a pure line of *V. riparia* while Schwarzmann, 101-14 and 3309 are hybrids of *V. riparia* and *V. rupestris* parents (Jackson & Schuster, 1994; Keller, 2010b). The susceptibility studies by Martos (2008) and Luque *et al*., (2007) had shown that the rootstock 41B was highly susceptible to *N. luteum* and *N. parvum* while rootstock 140Rugeri was the least susceptible among four varieties tested. The 41B rootstock is a hybrid of *V. vinifera* and *V. berlandieri* parents while 140Rugeri is a hybrid of *V. berlandieri* and *V. rupestris* (Pongracz, 1983). Since this study has provided some evidence that the six commonly used rootstocks in New Zealand nurseries have shown different levels of susceptibility to botryosphaeriaceous species, this indicates that the selection for varieties for propogation may need to be considered in conjunction with other control methods. Greater testing for the health status of the mothervines, replacement of infected mothervines and chemical treatments may also reduce the risk of rootstock infection. Since the rootstock section comprises 60-70% of the whole young grafted plant and provides anchorage and support in the vineyards, it is particularly important to the health of the vine.

This study also tested the six scion varieties (Sauvignon blanc, Chardonnay, Riesling, Pinot noir, Merlot and Cabernet Sauvignon) popularly used in New Zealand vineyards. The overall results obtained from this study showed that these varieties were infected by the three botryosphaeriaceous species to different degrees. The differing susceptibilities observed among the scion varieties were not observed in other research which used some of same botryosphaeriaceous species. In France, Sauvignon blanc, Cabernet Franc and Cabernet Sauvignon were the most susceptible varieties when inoculated with black dead arm pathogens *B. dothidea*, *D. seriata* and *D. mutila,* while Merlot and Semillon had very low infection incidence of these pathogens (Larignon and Dubos, 2001). A pathogenicity study by Urbez-Torres *et al.* (2008), showed that one-year-old Chardonnay were more susceptible than Thompson seedless to *L. theobromae* while the green shoots of Thompson seedless were more susceptible to *L. theobromae* than the Chardonnay shoots. Susceptibility studies in Spain, on the other hand, showed that Cabernet Sauvignon was the least susceptible compared to Macabeo, Parellada, Xarel-lo, Garnacha Tinta and Tempranillo when inoculated with *N. luteum* and *N. parvum* mycelia (Luque *et al.,* 2007; Martos, 2008). However, a recent study by Amponsah (2011) showed no statistically significant differences when detached green shoots of Cabernet Sauvignon, Chardonnay, Pinot noir, Sauvignon blanc and Riesling were inoculated with *N. luteum, N. australe*, *N. parvum* and *D. mutila,* although there were significant differences between species, with mean lesion lengths ranging from 14.0 to 27.4 mm. When this experiment was repeated on trunks of young vines of the same varieties using mycelium plugs and conidia, similar species effects were obtained but still no

statistically significant differences were observed. Except for the study by Amponsah (2011), all the above-cited researches on scion susceptibility also used mycelial plugs for inoculation, although the type of inoculum used was not specified by Larignon and Dubos (2001).

While this study showed differential susceptibility among scion varieties, the results observed differed to what had been observed in the 2008 nursery sampling (Chapter 2). In the 2008 sampling, an association between botryosphaeriaceous infections and varieties was observed, with Riesling having the highest *N. luteum* infections (38%) followed by Pinot gris (33%) and Pinot noir (21%) while Sauvignon blanc had only 8% *N. luteum* infections. For *N. parvum*, Pinot gris had the highest incidence (67%) followed by Sauvignon blanc (33%) while Pinot noir and Riesling were free of *N. parvum* infections. The few Chardonnay samples were not infected by *N. luteum* or *N. parvum*. The disparity in infection incidence was again likely to be due to the non-random collection of samples in 2008 that were not uniform within and across the nurseries.

Natural infections by botryosphaeriaceous species associated with some varieties have been reported, but other studies found no association between infections and varieties. In New South Wales, Australia, *D. seriata* was the pathogen most commonly isolated from Semillon grapevines (Castillo-Pando *et al.,* 2001) while *L. theobromae* was the major cause of cane dieback of Dawn seedless table grapes in Western Australia (Wood & Wood, 2005). In contrast, Urbes-Torrez *et al.* (2006a) showed that all wine grapes (nine varieties) and raisin-table grapes (four varieties) they surveyed had similar infection incidence by botryosphaeriaceous species. Therefore, well-designed susceptibility studies for different scion varieties, using conidia under field conditions, are needed to confirm the relative susceptibility of the different varieties under natural conditions.

The relative susceptibility of the different rootstock and scion varieties shown in this study has a great implication in the nursery system. In New Zealand, there has been an increasing popularity of red varieties like Pinot noir and Merlot in the last 6 years (New Zealand Winegrowers Statistical Annual, 2010), which has also been reflected by an increase in production of these varieties by the nurseries. Since these two varieties were found to be highly susceptible to the botryosphaeriaceous species, greater monitoring on the quality of propagation materials and sanitation practices in the nursery system is necessary to ensure that grafted plants are pathogen-free. This is particularly important for nurseries that outsource their scion cuttings from vineyards since Baskarathevan *et al.* (2011) had shown that botryosphaeriaceous infection was widespread in all wine regions of the country.

Of the three species tested in this study, the overall lesions caused by *N. parvum* were longer than those caused by *N. luteum* and *N. australe* in all varieties. These results were consistent with the pathogenicity test presented in Chapter 3, Section 1 in which *N. parvum* was generally found to be the most pathogenic among seven species, using both detached green shoots and rooted canes. The pathogenicity of *N. luteum* and *N. australe* also confirmed the results in Chapter 3 pathogenicity studies. Furthermore, symptoms caused by *N. parvum* on rootstock were more severe than on scions while symptoms caused by *N. luteum* and *N. australe* were similar for both plant types. Other pathogenicity studies also reported that *N. parvum* was one of the most pathogenic botryosphaeriaceous species on grapevines worldwide (Phillips, 2002; van Niekerk *et al.*, 2004; Luque *et al.*, 2007; Martos, 2008; Urbez-Torres & Gubler, 2009;). However, susceptibility studies in Spain showed that *N. luteum* consistently caused longer lesions on six scion varieties while *N. parvum* caused longer lesions on four rootstock varieties, although those studies used only one isolate for each species (Luque *et al.*, 2007; Martos, 2008). In contrast to the work presented here, Urbez-Torres and Gubler (2009) reported that *N. luteum* and *N. australe* were equally pathogenic to *N. parvum* when inoculated onto trunks of table, raisin and wine grapes in California. Amponsah *et al.* (2008b), on the other hand, found *N. luteum* and *N. australe* caused longer lesions on green shoots and was reisolated at longer distances from the lesion on young vine trunks than *N. parvum*. However, *N. parvum* caused longer external lesions on trunks than all other species tested. That study also used one isolate of each species. Since high variability in pathogenicity among isolates of *N. parvum*, *N. luteum* and *N. australe* was observed in the pathogenicity studies (Chapter 3) and had been consistently reported in other studies (van Niekerk *et al.*, 2004; Urbez-Torres & Gubler, 2009), therefore, the use of one isolate for a susceptibility test may not reflect the general level of pathogenicity of the tested species.

The high variability in pathogenicity among isolates of any species shown in Chapter 3, Section 1 and in this study was also reported by Urbez-Torres and Gubler (2009), who observed high variability in mean lesion lengths caused by eight isolates each of *N. parvum*, *N. luteum* and *N. australe* collected from different regions in California. In this study, significant variation was observed for only *N. parvum,* for which two isolates were significantly more pathogenic than the third isolate. Furthermore, Isolate P069 caused a significantly longer downward lesion in scion variety Merlot than the other two isolates which suggested that some isolates have different pathogenicity characteristics to others. Since these isolates came from different nurseries it suggests the possibility of inter-nursery and intra-nursery variation. The genetic diversity analysis of *N. luteum* isolates presented in Chapter 3 Section 2 indicated inter-nursery and intra-nursery variability among *N. luteum*

isolates. A similar study by Baskarathevan (2011) also showed that there were seven genetic groups among 49 *N. parvum* isolates, of which only eight were clonal isolates.

The three botryosphaeriaceous species used in this study caused longer upward lesions than downward lesions. In contrast, Urbez-Torres *et al.* (2008) reported that *L. theobromae* and *D. seriata* consistently caused longer downward lesions than upward lesions in all tissue type tested. This upward movement may only extend to the *Neofusicoccum* species as this was consistently observed in the three species tested in this study. However, this hypothesis needs further investigation since the direction of pathogen movement through canes may affect the development of infection of canes in mothervines. In the 2008 nursery survey, botryosphaeriaceous infections were more frequently found in the basal parts of the cuttings, although this trend was not highly significant. However, it was thought to indicate the potential movement from the mothervine into the canes during the growing season, as was reported by Whiteman (2004) with *Ph. chlamydospora*.

In summary, this study showed that all commonly used varieties were able to be infected by *N. parvum, N. luteum* and *N. australe* so there appears to be no potential for use of resistance as a control measure. This study had further shown that the different isolates of these three species have different levels of pathogenicity providing more evidence that these pathogens are genetically diverse. Since genetically diverse pathogens have greater capacity to survive in changing environments, development of control strategies for botryosphaeriaceous infections in the nurseries could be a challenge. The differences between isolates and species may mean that they react differently to control measures and so an integration of many methods may be necessary to combat this problem. The investigation of inoculum sources in the nursery system presented in Chapter 5 will provide a better understanding on how these pathogens survive and spread in the nursery system, and so indicate the important factors to be considered in attempting to control these pathogens.

Chapter 5
Sources of inoculum in the nurseries

5.1 INTRODUCTION

In viticulture, scion-rootstock grafting has been used for over 100 years to combine phylloxera resistance of rootstocks with the fruit qualities of *V. vinifera* wine grapes that are susceptible to this soil-borne pest (Gambetta *et al.*, 2009). The value of grafted plants was recognised in New Zealand in the early 1970s (Anonymous - Romeo Bragato Conference, 2010) and grafted vines are now occupying 94% of the country's total vineyard area (New Zealand Winegrowers Statistical Annual, 2010).

In New Zealand grapevine nurseries, the grafting season starts in winter (June-August) when one-year old rootstock and scion canes are harvested from the mothervines and cut to length. These cuttings are washed to remove dirt, soaked for several hours or overnight in hydration tanks that may also contain Chinosol® or Sporekill™ (didecyldimethylammonium Chloride-DDAC) in the solution, and stored at 2-4°C for 3-4 months. In spring (September-November), cuttings are removed from cold storage and may be rehydrated in hydration tanks overnight. After rehydration, cuttings are taken out of the tanks and the upper buds of rootstocks are removed manually using secateurs. Rootstocks and scion cuttings of similar diameter are then cut using grafting tools (omega or v-cut type) and the two sections are joined. The grafted section is sealed by dipping into molten wax or bound by a grafting tape. The grafted plants are placed upright in wooden boxes containing callusing media (perlite or vermiculite) that cover them up to the top bud and kept at 28°C at 85% RH for 3-4 weeks to promote callus formation and root development. After callusing, the resulting vines are acclimatised in a shade house for a week prior to planting in nursery fields where they grow for at least one season. During the following winter when the young plants are dormant, they are uprooted, washed, trimmed and graded, and then stored at 2-4°C until sold to vineyards. This is the standard grafting process but it may vary over time between nurseries or regions. Some nurseries incorporate hygiene and disease management practices into their nursery systems but they often do not have clear evidence on the efficacy of these practices (Jaspers, pers. comm., 2008).

While the success of grafting to prevent the devastation caused by phylloxera is recognised worldwide, the process seems to be associated with the spread of a number of diseases in vineyards. Plant pathogens that were reported to be systemic within the scion or rootstock cuttings used in grafting include *Xyllela fastidiosa* causing Pierce's disease of grapevines

(Gambetta *et al.*, 2009) and several viral diseases (Pearson & Goheen, 1998). The use of infected propagation materials in grapevine nurseries was also considered a major means of spread of wood pathogens including *Ph. chlamydospora* and *Phaeocremonium* spp. (Fourie and Halleen, 2004; Whiteman *et. al.,* 2007; Zanzotto *et al.,* 2007; Aroca *et al.,* 2010; Spagnolo *et al.,* 2011), *P. viticola* (Pearson & Goheen, 1998) and members of the *Botryosphaeriaceae* (Spagnolo *et al.*, 2011). The use of infected materials may also lead to secondary spread by contaminating the propagation process. A study on *Ph. chlamydospora* has shown that pruning and grafting tools, hydration tanks, propagation materials, nursery air and soil were contaminated by its spores. These spores were able to infect the "clean" plant materials in the nursery environment during the normal propagation processes (Whiteman *et al.*, 2007). Viable propagules of *Ph. chlamydospora* and *Phaeocremonium* spp. were also detected in different stages of grafting in Spanish nurseries (Aroca *et al.*, 2010).

The 2008 nursery survey presented in Chapter 2 showed that rootstock and scion cuttings had botryosphaeriaceous infections which led to the conclusion that use of this infected materials could be the major cause of infection of the grafted plants. Survey data also showed that infections in grafted plants were frequently found near the graft union, which suggested that the grafting process can be a contributing factor to disease development. A recent study also showed that botryosphaeriaceous species were frequently found in standing vines and propagation materials (Spagnolo *et al.*, 2011), but so far there has been no in-depth investigation into the sources of inoculum and the spread of infection during the grafting process.

Conventional methods to detect pathogens in the environment include microscopy and serial dilution plating assays (Tuite, 1969). In recent years, molecular techniques (previously described in Section 1.1.11) have been routinely used for detecting pathogens from different sources because they are fast and more sensitive than the conventional techniques.

In this study, the main objective was to investigate the sources of botryosphaeriaceous inoculum in the nursery mothervine blocks and to determine whether botryosphaeriaceous contamination was present in the different stages of the grafting process. To achieve this objective, samples from mothervine blocks and propagation systems were collected in three commercial nurseries and tested for the presence of botryosphaeriaceous propagules using conventional and molecular methods.

5.2 MATERIALS AND METHODS

Following the 2008 nursery survey in Chapter 2, three commercial nurseries were selected for this study based on the following criteria: botryosphaeriaceous infections and species present, accessibility and willingness to participate in the study (Table 5.1).

Table 5.1 Grapevine nurseries selected for the study based on the incidence of botryosphaeriaceous infection and number of species found.

Nursery	Botryosphaeriaceous infection incidence (%)	No. of species detected
Nursery 3	41	7
Nursery 5	63	3
Nursery 9	26	6

5.2.1 Sample collection from mothervine blocks

Collection of plant materials, plant debris and soil samples was conducted in the three selected nurseries (Table 5.1) in July-August 2009 prior to harvesting of vines. Trapping for water-borne conidia was conducted during the growing season in December 2009, January, February, April and July 2010 in the mothervine blocks of Nursery 3 and in April 2010 in two vineyards in Blenheim that supplied scion cuttings to Nursery 3.

5.2.1.1 Cuttings

Three varieties each of scion and rootstock were selected from the mothervine blocks of Nursery 5 and 9 based on their popularity and number of vines available for sampling (Table 5.2). Nursery 3 had only one scion variety available for sampling, so the six varieties needed included five rootstock varieties instead. For each variety, five mothervines were randomly selected within a row and labelled with plastic tags for a total of 30 vines in each nursery. From each vine, one ~30 cm segment (~3 internodes) was cut from the basal part of five different canes (Figure 5.1). Each segment was further cut into three sections (~10 cm) with each section marked as base, middle and top cuttings and these were placed together in a re-sealable plastic bag. Plants samples were transported to Lincoln University in an insulated box with ice packs and stored at 8°C, until being processed within 2 weeks.

Figure 5.1. A mothervine showing the positions (arrows) where cutting samples were collected.

Table 5.2 Rootstock and scion varieties selected for sampling from three nurseries.

Nursery	Plant type	Mothervine variety	Total vines sampled	Total cuttings collected per vine	Pooled samples for molecular analysis
Nursery 3	Rootstock	101-14	5	5	1
		Schwarzmann	5	5	1
		3309	5	5	1
		Riparia gloire	5	5	1
		Gravesac	5	5	1
	Scion	Sauvignon blanc	5	5	1
Nursery 5	Rootstock	101-14	5	5	1
		Schwarzmann	5	5	1
		SO4	5	5	1
	Scion	Pinot gris	5	5	1
		Riesling	5	5	1
		Pinot noir	5	5	1
Nursery 9	Rootstock	101-14	5	5	1
		Schwarzmann	5	5	1
		5C	5	5	1
	Scion	Sauvignon blanc	5	5	1
		Pinot gris	5	5	1
		Gewurztraminer	5	5	1

5.2.1.2 Plant debris samples

Plant debris consisting of dead grapevine woody materials were collected from the ground around each mothervine from which cutting samples had been collected (Table 5.3). Plant debris collected from the five vines of each variety were placed together in a new plastic bag and labelled accordingly. All samples were transported to Lincoln University inside an insulated box with ice packs and stored at 8°C for one week until assessed.

5.2.1.3 Soil samples

Three soil cores of 3 cm diameter and 20 cm depth were collected into new plastic bags from around each of the same three randomly selected mothervines of each variety at a distance of ~20 cm from the trunks (Table 5.3). Plant debris was removed prior to sampling and cores were collected using a soil auger. After transport to Lincoln University, each core soil sample was mixed thoroughly in a sterile plastic bag and ~50 cm^3 of each sample was placed in a sterile 50 ml centrifuge tube and stored at –80°C for approximately 10 months until assessed.

Table 5.3 Plant debris and soil samples collected from different nurseries.

Nursery	Mothervine variety	Total plant debris collected per vine	Pooled samples for molecular analysis	Total soil samples collected per vine	*Pooled samples for molecular analysis
Nursery 3	101-14	1	1	3	2
	Schwarzmann	1		3	2
	3309	1		3	-
	Riparia gloire	1		3	2
	Gravesac	1		3	-
	Sauvignon blanc	1		3	2
Nursery 5	101-14	1	1	3	-
	Schwarzmann	1		3	-
	SO4	1		3	-
	Pinot gris	1		3	-
	Riesling	1		3	-
	Pinot noir	1		3	-
Nursery 9	101-14	1	1	3	-
	Schwarzmann	1		3	-
	5C	1		3	-
	Sauvignon blanc	1		3	-
	Pinot gris	1		3	-
	Gewurztraminer	1		3	-

*Only four varieties from Nursery 3 were included for molecular analysis

(-) not analysed

5.2.1.4 Trapping of water-borne conidia

Rainwater run-off traps were made from 5 L plastic containers with screw cap lids which had been cut open on one side and the opening covered with nylon mesh to minimise plant debris contamination in the trap (Figure 5.2). Water traps were placed close to the trunks of four randomly selected 101-14 and five 3309 vines in Nursery 3 and five randomly selected vines each of Pinot noir and Sauvignon blanc in each of two vineyards in Blenheim. The plastic containers were tied to the trunks under the canopy of each vine and rainwater was collected after a significant rainfall (at least 2 mm) once each month (December 2009, January, February, April and July 2010 for Nursery 3 and April 2010 for Blenheim). The water samples were transferred to clean 250 ml plastic bottles and placed in an insulated box containing ice packs for transport to Lincoln University. All samples were passed through a 150 µm sieve to remove debris and allowed to settle overnight at 4°C (Jaspers, pers. comm., 2009). Amponsah (2011) had shown that the conidia of botryosphaeriaceous species commonly found in New Zealand had a length and width range of 19-32 µm and 7-13 µm, respectively, and could pass through the 150 µm sieve. The top 200 ml was decanted slowly and the remaining 50 ml was used for the assessment. Samples were stored at 4°C overnight until processed.

Figure 5.2 Rainwater run-off traps made of 5 L plastic container (arrow) installed underneath the canopy of a rootstock mothervine.

Table 5.4 Rainwater run-off samples collected from different locations and periods.

Sampling location	Variety	Sampling date	No. of samples collected	No. of pooled samples for molecular analysis
Nursery 3	3309	December 2009	5	1
		January 2010	5	1
		February 2010	5	1
		April 2010	5	1
		July 2010	5	1
	101-14	December 2009	4	1
		January 2010	4	1
		February 2010	4	1
		April 2010	4	1
		July 2010	4	1
Blenheim	Pinot noir	April 2010	5	1
	Sauvignon blanc	April 2010	5	1

5.2.2 Sample collection in the propagation system

Samples from the propagation process were arbitrarily collected in October-November 2009 during commercial operations of the same commercial nurseries where plant and soil samples were collected (Tables 5.2 and 5.3), except that the water samples from a wash pit and hydration tanks of Nursery 3 were collected in August 2010.

Although nurseries generally followed the standard methods described for the propagation process (Section 5.1) there were some differences amongst the three nurseries surveyed, thus, sample size and methods used in some propagation steps for each nursery were not uniform and were modified accordingly to suit the individual system. For easy understanding of the sampling process and interpretation of results, samples were categorised based on five stages in the nursery system and presented in a diagram (Figure 5.3). The first pre-harvesting stage included all the samples collected from the mothervine blocks. The second stage was pre-cold storage which included all steps after the harvesting of cuttings until cold storage. The third stage was post-cold storage which included all steps from the removal of cold-stored cuttings until they were ready for grafting. The fourth stage was grafting which included all activities during and after grafting and the last stage was callusing which included the steps before and after the grafted plants were callused. Different samples collected from the propagation steps are summarised in Table 5.5 and details of the sampling method for each propagation step are further described in Appendix F.

5.2.3 Sample collection

For sample collections from hydration and rehydration tanks, solutions were agitated for approximately 2 min and samples were placed in 50 ml sterile plastic tubes. All samples were placed in an insulated plastic box with ice packs and transported to Lincoln University for assessment.

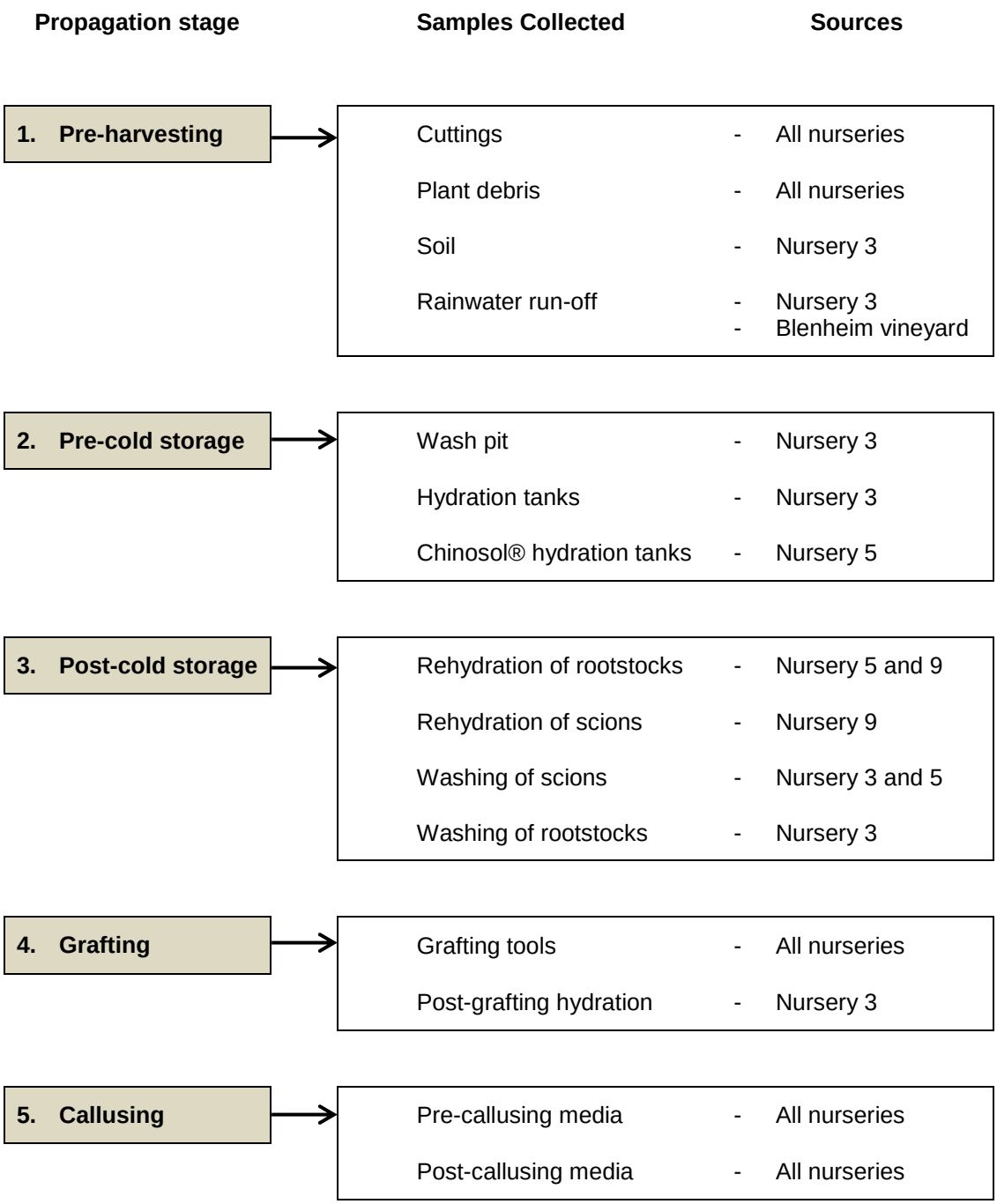

Figure 5.3 The different stages in the nursery system and the corresponding samples collected from each stage at different nurseries.

Table 5.5 Samples collected from the nursery mothervine blocks and propagation system pooled for molecular analysis.

Nursery	Propagation steps sampled	[A]Total samples collected	[B]Pooled samples for molecular analysis
Nursery 3	Wash pit	3	1
	Pre-storage hydration	4	1
	Pre-grafting wash of rootstocks	6	1
	Pre-grafting wash of scions	6	1
	Post-grafting hydration	18	3
	Grafting tool 1	3	1
	Grafting tool 2	3	1
	Grafting tool 3	3	1
	Perlite (pre-callusing, reused-unsterilised)	12	1
	Perlite (post-callusing)	4	1
Nursery 5	Chinosol pre-storage hydration	5	1
	Pre-grafting rehydration (rootstocks)	10	1
	Pre-grafting wash of scions	5	1
	Grafting tool 1	3	1
	Grafting tool 2	3	1
	Grafting tool 3	3	1
	Perlite (pre-callusing; reused-sterilised)	4	1
	Perlite (post-callusing)	4	1
Nursery 9	Pre-grafting rehydration (rootstocks)	10	1
	Pre-grafting rehydration (scions)	5	1
	Grafting tool 1	3	1
	Perlite/vermiculite (pre-callusing; new)	4	1
	Perlite/vermiculite (post-callusing)	4	1

A& B - The total samples in column A were pooled and the resulting sample is shown in column B

Figure 5.4 Different propagation stages in grapevine nurseries. A) Chinosol hydration tanks at Nursery 5; B) scion cuttings inside net bags during pre-grafting rehydration at Nursery 9; C) omega-cut grafting tool at Nursery 9; D) v-cut grafting tool at Nursery 5; E) rootstock cutting bundles partially covered with a wooden pallet during pre-grafting rehydration at Nursery 9; F) post-grafting hydration at Nursery 3 where bases of grafted plants were soaked in water overnight prior to callusing; and placement of grafted plants in G) perlite callusing medium at Nursery 3; and H) perlite-vermiculite combination at Nursery 9.

5.2.4 Sample processing

5.2.4.1 Washing for surface propagules in cuttings and plant debris

To detect botryosphaeriaceous propagules on surfaces of cuttings and pieces of woody debris previously collected from the mothervines (Sections 5.2.1.1 and 5.2.1.2), 50 ml of SROW + 0.01% Tween 80 was added to each new plastic bag containing the samples. The bags were agitated for 30 s and allowed to stand for 30 min before agitating again for 30 s. The wash water from each bag was transferred to a 50 ml sterile tube. All wash water samples were centrifuged at 3,220 x *g* for 1 h. Supernatants were transferred to new tubes and pellets were re-suspended in 0.5 ml SROW. Re-suspended pellets from the five cuttings of each mothervine were combined in one tube and mixed by shaking for 30 s. One ml of each suspension was transferred to a sterile 1.7 ml tube and stored at -80°C for molecular analysis. The remaining suspensions were stored at 4°C overnight and analysed using microscopy and serial dilution plate assays.

5.2.4.2 Internal infection of cuttings

After washing, the sections of the cuttings were air-dried and each section was placed in a new re-sealable bag for isolation. Each section was surface-sterilised using the method described in Section 2.2.3. Tissue samples (1 cm) were cut from the base of each 10 cm section of the original 30 cm segment and the three samples were placed onto a PDAC plate, marked to show the position of each tissue sample. Plates were incubated at room temperature for 5-7 days and botryosphaeriaceous-like colonies were sub-cultured onto PDAC. Isolates were allowed to grow at room temperature for 2 weeks then stored at 4°C for 4 months until ARDRA.

5.2.4.3 Wash water from propagation steps and rainwater samples

All water samples from propagation steps and rainwater traps that contained plant materials and other debris were passed through a 150 µm sieve and filtrates were centrifuged at 3,220 x *g* for 1 h. The resulting supernatants were transferred to new tubes and the pellets were re-suspended in 2 ml SROW. One ml of the re-suspended pellet was transferred to a sterile 1.7 ml tube and stored at -80°C for 8 months for molecular identification and the remaining suspensions were stored at 4°C overnight prior to microscopy and serial dilution plating.

5.2.4.4 Soil samples

To reduce the number of samples for analysis, only those soil samples which came from around mothervines with a high number of surface propagules were used in the analysis. These were from mothervine blocks of three rootstocks and one scion variety from Nursery 3 (Table 5.3).

Fungal propagules were separated from the soil following the methods described by Probst (2011). This method is based on the assumption that when soil is mixed in weak agar solution and the soil particles are allowed to settle, propagules in the soil will remain suspended in the agar solution for a certain period of time.

The selected frozen soil samples were thawed and the three samples from each variety were placed in a clean plastic bag and mixed thoroughly. From each composite sample, two 10 g soil samples were transferred to a media bottle containing 45 ml of 0.01% agar solution. Bottles were placed on a wrist action shaker (Griffin and George Ltd., London, UK) and shaken for 10 min at maximum speed and allowed to stand for 6 min. After standing, approximately 35 ml of each supernatant was transferred to a sterile 50 ml plastic tube and centrifuged at 3,220 x *g* at 4°C for 30 min. All supernatants were discarded and ~0.25 g of each pellet was immediately used for molecular analysis.

5.2.4.5 Callusing Media

Prior to analysis, the perlite and vermiculite samples from each nursery were mixed thoroughly in a plastic bag and a sub-sample of ~10 g was placed in a media bottle with 100 ml SROW and shaken for 10 min with a wrist-action shaker set at maximum speed and allowed to stand for 6 min as before. After standing, approximately 50 ml of each supernatant was transferred to a new bottle and passed through a 150 µm sieve to further remove the suspended particles. The filtrates were collected and centrifuged as previously described and the resulting pellets resuspended in 2 ml SROW, with 1 ml being stored at -80^{o}C for molecular analysis as before. The remaining samples were stored at 4^{o}C overnight and used for microscopy and serial dilution plating.

5.2.5 Detection of botryosphaeriaceous conidia by microscopy

The resuspended pellets from all replicate wash water samples were mixed thoroughly to give one pooled sample (Table 5.5). Then one drop of each suspension was placed on a haemocytometer slide and characteristic botryosphaeriaceous conidia identified and counted in five of the haemocytometer squares (each containing 10^{-4} ml). Conidial counts were conducted in two counting areas of the haemocytometer and repeated for a total of four

replicate counts. Initial attempts at examining wash solutions from callusing media and soil suspensions showed that they contained many inorganic particles that interfered with the conidial count, thus, these samples were not examined further.

5.2.6 Detection of botryosphaeriaceous conidia by serial dilution plating

Selective media for botryosphaeriaceous species was not available for this study. To obtain single colonies from samples, PDA was amended with Triton-X 100 at 1 ml/L and three antibiotics (streptomycin, penicillin and chloramphenicol) at 0.05 g/L each. Using the resuspended pellets from all wash water samples, 5-fold serial dilutions were made and 100 µl of each 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions were plated on PDA + Triton X/antibiotics with four replicate plates per sample. The plates were incubated at 25°C for 7-10 days and the characteristic botryosphaeriaceous colonies were counted and sub-cultured onto PDA plates. Isolates were allowed to grow at room temperature for 2 weeks and stored at 4°C for 4 months for molecular analysis. Soil samples collected from the different nurseries were not included in the serial dilution assay due to the very high and diverse microbial populations normally present in soils.

5.2.7 Confirmation of conidia recovery from samples by centrifugation

To confirm that the centrifugation method used was effective in the recovery of botryosphaeriaceous conidia from different liquid samples, 100 µl aliquots from the resulting supernatants of each sample were plated (three replicate plates) and incubated using the method described in Section 5.2.6 and observed for botryosphaeriaceous colonies.

5.2.8 Detection of botryosphaeriaceous inoculum from the nursery system using botryosphaeriaceous multi-species primers

5.2.8.1 Pooled samples for molecular analysis

Due to the high cost of reagents for molecular analysis, samples collected from the mothervine blocks and propagation stages were pooled to reduce the number of samples (Tables 5.2 to 5.5). The thawed samples were combined accordingly in 15 ml tubes, centrifuged 3,220 x *g* for 15 min and the resulting pellets were used for DNA extraction as described in Section 5.2.8.2. The resulting supernatants were checked for botryosphaeriaceous propagules by plating assays using the methods described in 5.2.7.

5.2.8.2 DNA extraction from samples

DNA extraction from collected samples was conducted using the PowerSoil™ DNA isolation kit (MO BIO Laboratories, CA, USA). Approximately 0.25 g of each pellet was added to the PowerBead™ tube containing the aqueous solution of acetate and salts, vortexed briefly and DNA extraction was conducted according to the manufacturer's instructions. The resulting DNA suspension (~100 µl) was stored at -20°C for 2 weeks until processed.

5.2.8.3 Detection of botryosphaeriaceous spp. using multi-species primers

Prior to PCR, all DNA samples were quantified using a Nanodrop 3.0.0 spectrophotometer as described in Section 3.2.2.4.

The multi-species primers BOT100F ($^{5'}$AAACTCCAGTCAGTRAAC$^{3'}$) and BOT472R ($^{5'}$TTTCTCAAGGMTGACCTCGGA$^{3'}$) which amplify a portion of the rRNA gene region, as described by Ridgway *et al.* (2011) were used to detect botryosphaeriaceous DNA in all samples. Each PCR reaction contained 1x PCR buffer (with 1.5 mM $MgCl_2$), 1 U Faststart Taq Polymerase (Roche), 200 µM each of dATP, dTTP, dGTP and dCTP, 5 pmole of each primer and 10-20 ng of template DNA. The amplification was performed with initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 58°C and extension for 1 min at 72°C, followed by a final extension at 72°C for 10 min. After amplification, 5 µl aliquots of the PCR products were separated by electrophoresis in 1% agarose as described in Section 2.2.5.1.

To confirm that all samples contained PCR-ready fungal DNA, PCR was repeated using the universal primers ITS1-F (5'CTTGGTCATTTAGAGGAAGTAA3') and ITS4 (5' TCCTCCGCTTATTGATATGC3') primers as described by White *et al.* (1990) but using the amplification conditions described above. To ensure that the SROW used for washing the plant samples, grafting tools and callusing media were free of botryosphaeriaceous contamination, one sample (1 ml) was collected before each washing procedure, labelled as "blank" and included in the molecular assays.

5.2.9 Identification of botryosphaeriaceous isolates from internal infections and external contaminations of cuttings using ARDRA

Twenty percent of isolates from within the cuttings from Nursery 3 and 5 and 30% of the isolates from Nursery 9 were randomly selected for molecular identification using the ARDRA described in Chapter 2 Section 2.2.5.1. However, the iterative and sequential process of restriction enzymes was modified to reduce the number of steps (Figure 5.5). The initial digestion with *Hae*III identified *N. parvum* (Group A) and *B. dothidea* (Group B) separating

them from all other species (Group C). All isolates in Group C were digested with *Sac*II which identified *N. luteum* (Group E) and *N. australe* (Group F) separating them from the remaining isolates (Group D). The Group D isolates were further digested with *Nci*I which identified *D. seriata* and *D. mutila* and separated them from other species (Group I). All isolates recovered from the serial dilution plating assays were also identified using the above method.

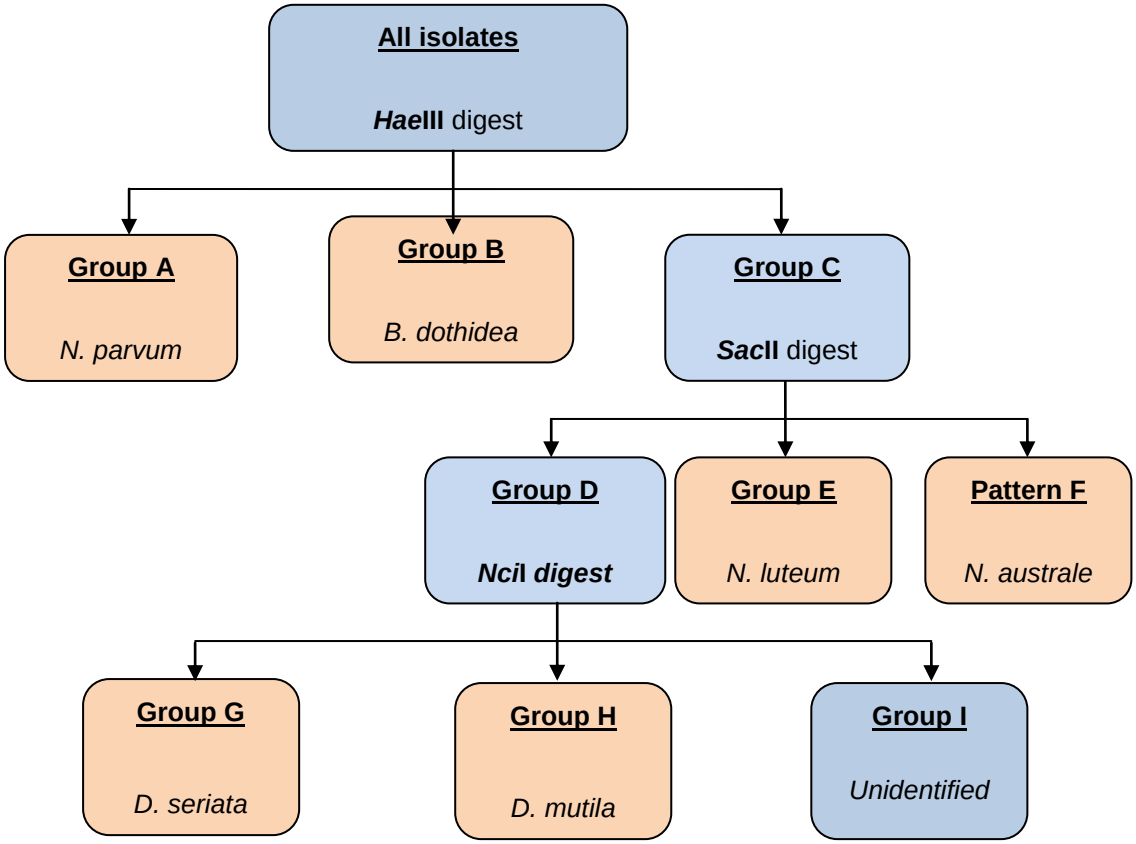

Figure 5.5 Diagram of the modified iterative restriction digestion analyses (ARDRA) used for identifying representative botryosphaeriaceous isolates from three commercial nurseries.

5.2.10 Sequence analysis of PCR products from multi-species primers

Since ARDRA is only suitable for use on pure cultures, an attempt was made to identify the botryosphaeriaceous species that were amplified using the multi-species primers by sequence analysis. The PCR products from the propagation steps that were clear and bright were selected for DNA sequence analyses, which was conducted using the Bot100F primer and followed the methods described in Section 2.2.5.2.

5.2.11 Data Analyses

Incidence of cuttings with surface botryosphaeriaceous propagules, based on microscopy, and of internal infection, from isolation, were analysed using Pearson's Chi-square test of independence at $P \leq 0.05$ using SPSS 17 with respect to the different nurseries, species and varieties.

Quantitative data from microscopy, serial dilution assays and PCR detection (presence/absence) were not statistically analysed. Due to its subjective nature and high variability of the conidial counts, these counts were categorised into three scales: low ($\leq 10^3$), moderate ($>10^3$ to 10^4) and high ($>10^4$). For serial dilution plating, there were high rates of contamination, making the colony counting difficult, with low recovery of single botryosphaeriaceous isolates. Data for this assay were categorised as positive when botryosphaeriaceous colonies were sub-cultured or negative when none were recovered. Due to the low number of pooled samples (Tables 5.2 to 5.5) used in the molecular analyses, these data were also not analysed, only being presented as positive or negative for botryosphaeriaceous isolates in the results section of this chapter.

5.3 RESULTS

5.3.1 Confirmation of conidia recovery from samples by centrifugation

Single colonies of bacteria and other fungi (*Penicillium* spp.) were observed in some of the plated supernatants but no botryosphaeriaceous colonies were observed in any of the plated samples.

5.3.2 Botryosphaeriaceous propagules on cuttings surfaces

5.3.2.1 Detection by microscopy

In all three nurseries, botryosphaeriaceous conidia that were hyaline and fusiform in shape, so characteristic of *Neofusicoccum* spp., were detected by microscopy in water after washing the cuttings from each vine (Figure 5.6A). Nursery source was found to be a significant factor for the incidence of surface propagules ($P<0.001$; Table 5.6; Appendix C.4.1) with Nursery 3 having the highest incidence at 100%, followed by Nursery 5 at 97% while only 33% of the samples from Nursery 9 were positive for surface propagules. The number of conidia from the surfaces of cuttings from these three nurseries ranged from low to high (Table 5.6).

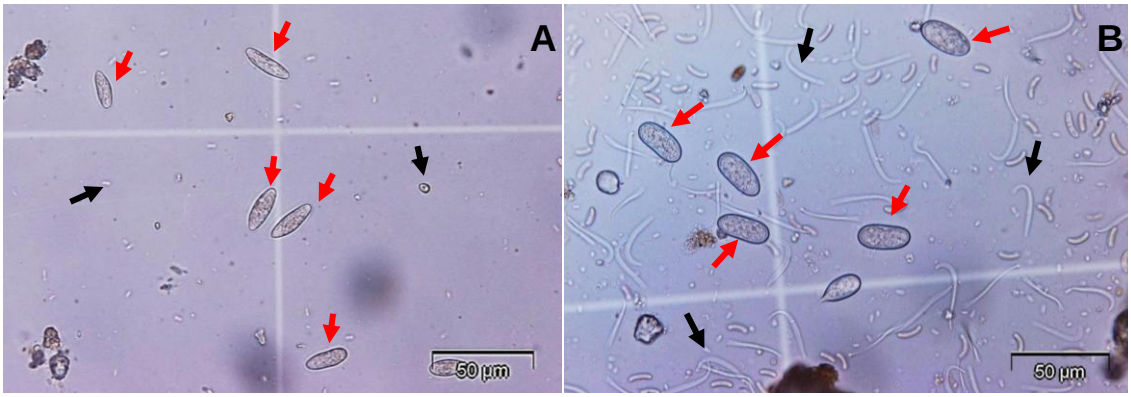

Figure 5.6 Conidia (red arrows) characteristic of A) *Neofusicoccum* spp. obtained from the surface of a Riesling cuttings from Nursery 5 and B) of *D. mutila* obtained by washing the plant debris from around 3309 mothervines from Nursery 3. Spores of other fungal groups are indicated by black arrows.

Variety was also a significant factor (P<0.001) in the proportion of cuttings with surface propagules but this appeared to be associated with nursery source (Table 5.6; Appendix C.4.1). Comparisons of surface propagule incidence for the same varieties from different nurseries reflected the differences observed between nurseries. For example, in Nurseries 3 and 5, 101-14 and Schwarzmann had 100% surface propagule incidence, but the incidences were only 40% and 20%, respectively in Nursery 9. Similarly, surface propagule incidence in Sauvignon blanc was 100% in Nursery 3 and 40% in Nursery 9. Furthermore, Pinot gris from Nursery 5 had 100% surface propagule incidence while none were detected from Nursery 9 vines.

5.3.2.2 Serial dilution plating assay

A great deal of contamination was observed on all 10^{-2} and 10^{-3} dilution plates. The over-lapping colonies made it difficult to enumerate the botryosphaeriaceous colonies although individual botryosphaeriaceous colonies were obtained from some 10^{-4} dilution plates. No fungal colonies were observed in any samples diluted to 10^{-5}.

Of the five vines tested per variety, botryosphaeriaceous colonies were recovered from very few mothervines in Nursery 3, with three Riparia gloire and one each of 101-14 and Sauvignon blanc vines yielding isolates, while none was recovered from the other three varieties (Table 5.6). For Nursery 5, botryosphaeriaceous colonies were also isolated from four out of five Pinot gris vines and from a Riesling vine, while no colonies were recovered from any vines of the four other varieties. All mothervines from Nursery 9 tested negative for botryosphaeriaceous surface propagules using the plating assays.

All botryosphaeriaceous isolates from each vine obtained from plating assays were confirmed as botryosphaeriaceous species by ARDRA (Table 5.6). For Nursery 3, the single isolate from 101-14 was identified as *N. parvum*, while three isolates from Riparia gloire were *N. parvum* (n=2) and *N. luteum* (n=1) and the single isolate from Sauvignon blanc was also identified as *N. luteum*. For Nursery 5, four representative isolates from Pinot gris and one from Riesling were identified as *N. luteum*.

Table 5.6 Detection of botryosphaeriaceous propagules on plant surfaces of different grapevine varieties from three nurseries using microscopy techniques, plating assays and PCR with botryosphaeriaceous multi-species primers (Bot100F/Bot472R).

Nursery	Variety	[A] Positive vines (N=5)	Microscopy		Plating assays (10^{-4})		PCR (pooled samples)
			[B] Conidial count/variety	Identification (morphology)	Positive vines (n=5)	Identification (ARDRA)	[C]Bot100F -Bot472R
3	101-14	5	Moderate	*Neofusicoccum* spp.	1	*N. parvum*	+
	Schwarzmann	5	High	*Neofusicoccum* spp.	0	-	+
	3309	5	Moderate	*Neofusicoccum* spp.	0	-	+
	Riparia gloire	5	High	*Neofusicoccum* spp.	3	*2 N. parvum, 1 N. luteum*	+
	Gravesac	5	Moderate	*Neofusicoccum* spp.	0	-	+
	Sauvignon blanc	5	High	*Neofusicoccum* spp.	1	*N. luteum*	+
5	101-14	5	High	*Neofusicoccum* spp.	0	-	-
	Schwarzmann	5	High	*Neofusicoccum* spp.	0	-	+
	SO4	5	Low	*Neofusicoccum* spp.	0	-	-
	Pinot gris	5	High	*Neofusicoccum* spp.	4	*N. luteum*	+
	Riesling	5	High	*Neofusicoccum* spp.	1	*N. luteum*	+
	Pinot Noir	4	Moderate	*Neofusicoccum spp.*	0	-	+
9	101-14	2	Low	*Neofusicoccum* spp.	0	-	-
	Schwarzmann	1	Low	*Neofusicoccum* spp.	0	-	-
	5C	5	Low	*Neofusicoccum* spp.	0	-	-
	Sauvignon blanc	2	Low	*Neofusicoccum* spp.	0	-	-
	Pinot gris	0	0	-	0	-	-
	Gewurztraminer	0	0	-	0	-	-

[A] Significant at $P<0.001$ using Pearson Chi-square test.
[B] low - $\leq 10^3$; moderate - $>10^3$ to 10^4; high - $>10^4$
[C] (+) positive or (-) negative to PCR using botryosphaeriaceous multi-species primers. The calculated detection level of PCR using multi-species primers is 25 conidia per 1 µl of DNA template (Ridgway *et al.,* 2011). This method, therefore, can detect 100 conidia per ml of 25 ml liquid sample that yielded 100 µl of DNA template. The higher volume of combined samples may detect as little as 17 conidia/ml for a 150 ml sample.

5.3.2.3 Molecular detection

In the molecular assay using the multi-species primers Bot100F and Bot472R, single PCR products approximately 370 bp in size were amplified from wash samples of all six mothervine varieties from Nursery 3 (Figure 5. 7A; Table 5.6). The wash samples of Pinot gris, Riesling, Pinot noir and Schwarzmann from Nursery 5 were also positive for amplimers using the multi-species primers while the wash samples for SO4 and 101-14 were negative. All samples from Nursery 9 were negative for PCR amplification. The calculated detection level of PCR using multi-species primers is 25 conidia per 1 µl of DNA template (Ridgway *et al.,* 2011). In the assay that used the ITS1-ITS4 primers, multiple PCR products of different sizes were amplified in all wash water samples indicating that DNA was present in all samples and the non-amplification with the multi-species primers was due to absence of botryosphaeriaceous species DNA (Figure 5.7). No PCR products were amplified from the blank sample and the negative control (SNW) for both the specific and universal primers.

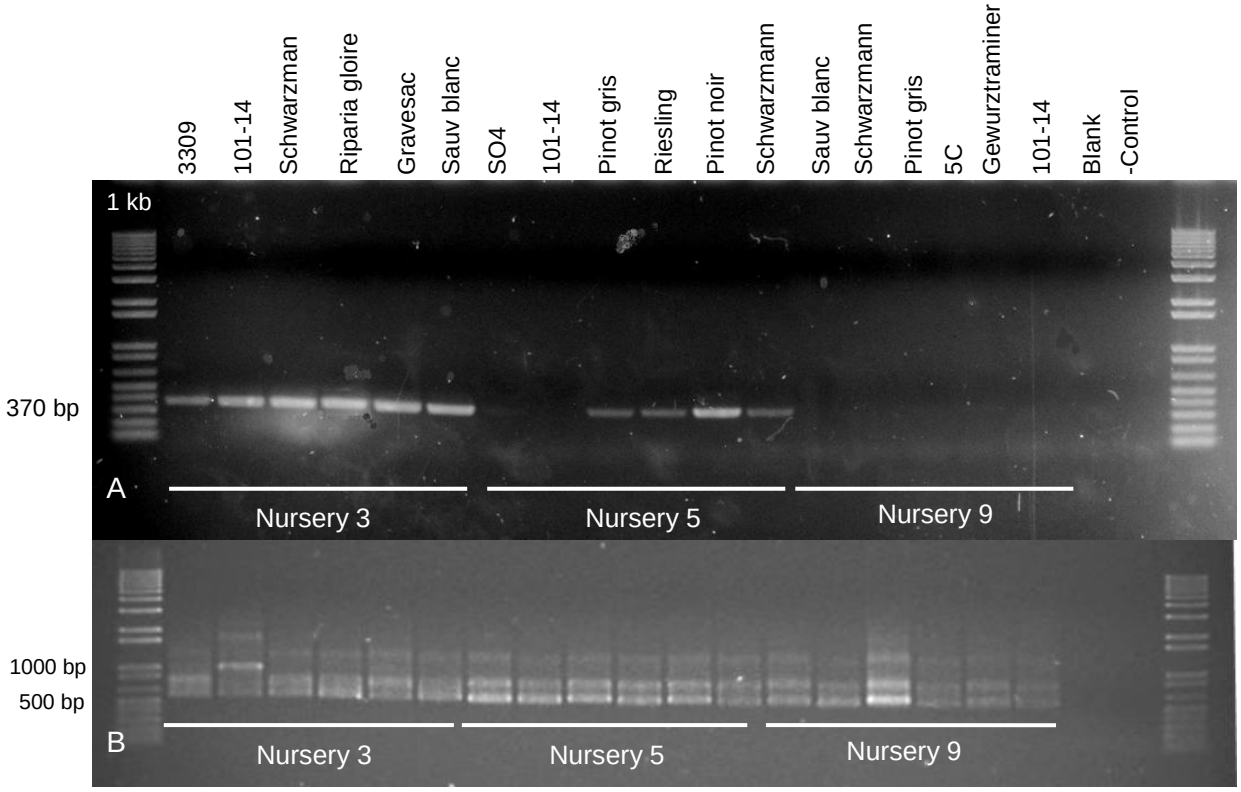

Figure 5.7. Amplification of botryosphaeriaceous DNA from wash samples of cuttings from six mothervine varieties per nursery using A) Bot100F/Bot472R multi-species primers and B) ITS1/ITS4 primers. Varieties are indicated at the top of the gel and and nursery sources at the bottom of each gel. Numbers on the far left of top gel denote molecular weight of the PCR products while numbers on the far left of the bottom gel denotes the molecular weight of DNA ladder.

5.3.3 Botryosphaeriaceous infections in cuttings

Botryosphaeriaceous infections were present in some cuttings from the mothervines of the three participating nurseries. The incidence of botryosphaeriaceous infection varied significantly between nurseries (P<0.001) with the highest incidence in Nursery 3 (68%), followed by Nursery 5 (65%) and Nursery 9 (15%; Table 5.7; Appendix C.4.2). The proportion of isolates recovered from each nursery also varied significantly (P<0.001) with highest number from Nursery 3 (n=171), followed by Nursery 5 (n=157) while only 27 isolates were recovered from Nursery 9 (Table 5.7; Appendix C.4.3).

Table 5.7 Incidence of botryosphaeriaceous infections and number of botryosphaeriaceous isolates recovered from plant materials collected in three grapevine nurseries.

Nursery	Total samples collected	*Botryosphaeriaceous infection incidence	*Botryosphaeriaceous isolates recovered
3	150	68%	171
5	150	65%	157
9	150	15%	27

*Significant at P<0.001 using Pearson Chi-square test.

Botryosphaeriaceous infection incidence was not influenced by plant type with rootstock and scion having equal infection incidence at 49% (P=0.979; Appendix C.4.2). Variety, on the other hand, was a significant factor (P<0.001) in botryosphaeriaceous infection incidence (Table 5.10; Appendix C.4.2). Riesling from Nursery 5 had the highest infection at 96% followed by Riparia gloire from Nursery 3 (84%) while both Pinot noir (Nursery 5) and Gravesac (Nursery 3) had 68% infection incidence. Gewurztraminer and 5C from Nursery 9 had the lowest infection incidence at 4% (Table 5.10). While variety was found to be a significant factor (P<0.001) for number of isolates recovered, the sample size for each variety was not equal (Table 5.10), and the recovery rate might have been skewed towards other varieties because some of them had greater number of samples than others.

Isolation position was also a significant factor (P<0.001) with the highest proportion of isolations obtained from the bases of the cuttings (41%), followed by the middle section (32%) and lowest at the top section at 27% (Appendix C.4.2).

Table 5.8 Incidence of botryosphaeriaceous infections and number of botryosphaeriaceous isolates recovered from different grapevine varieties collected in three grapevine nurseries.

Variety	Nursery	Total samples collected	*Botryosphaeriaceous infection incidence	*Botryosphaeriaceous isolates recovered
Rootstock				
101-14	3, 5 & 9	75	58%	68
Schwarzmann	3, 5 & 9	75	33%	30
Riparia gloire	3	25	84%	34
3309	3	25	56%	23
5C	9	25	4%	1
SO4	5	25	56%	68
Gravesac	3	25	68%	24
Scion				
Sauvignon blanc	3 & 9	50	46%	42
Riesling	5	25	96%	46
Pinot gris	5 & 9	50	42%	35
Pinot noir	5	25	68%	29
Gewurztraminer	9	25	4%	1

*Significant at $P<0.001$ using Pearson Chi-square test.

5.3.4 Identification of botryosphaeriaceous isolates from plant materials.

A total of six botryosphaeriaceous species were identified from the representative isolates recovered from cuttings collected at three nurseries using ARDRA (Table 5.9; Figure 5.8; Appendix D.2).

The proportion of *N. luteum* and *N. parvum* in different nurseries were significantly different ($P<0.001$; Table 5.9; Appendix C.4.4). Of the 79 representative isolates included in the identification, 47 (59%) were identified as *N. luteum* with highest incidence at Nursery 5 (n=25) and Nursery 3 (n=21) and 20% of the representative isolates were identified as *N. parvum* with the highest recovery from Nursery 3 (n=14) and only one isolate each from Nursery 5 and Nursery 9. Nursery source was also a significant factor for *N. australe* and *D. seriata* incidence at $P=0.029$ and $P\leq0.001$, respectively (Table 5.9; C.4.4). Most of the *N. australe* isolates (six of the seven found) were from Nursery 5 and all of the *D. seriata* isolates were from Nursery 9.

Table 5.9 Identification of representiative botryosphaeriaceous isolates recovered from three nurseries using ARDRA.

Species	Nursery 3	Nursery 5	Nursery 9	Total
[A]*N. luteum*	21	25	1	47
[A]*N. parvum*	14	1	1	16
[B]*N. australe*	-	6	1	7
[A]*D. seriata*	-	-	6	6
B. dothidea	1	-	1	2
D. mutila	-	1	-	1
Total	**36**	**33**	**10**	**79**

[A] Significant at $P \leq 0.001$ using Pearson Chi-square test
[B] Significant at $P = 0.029$ using Pearson Chi-square test

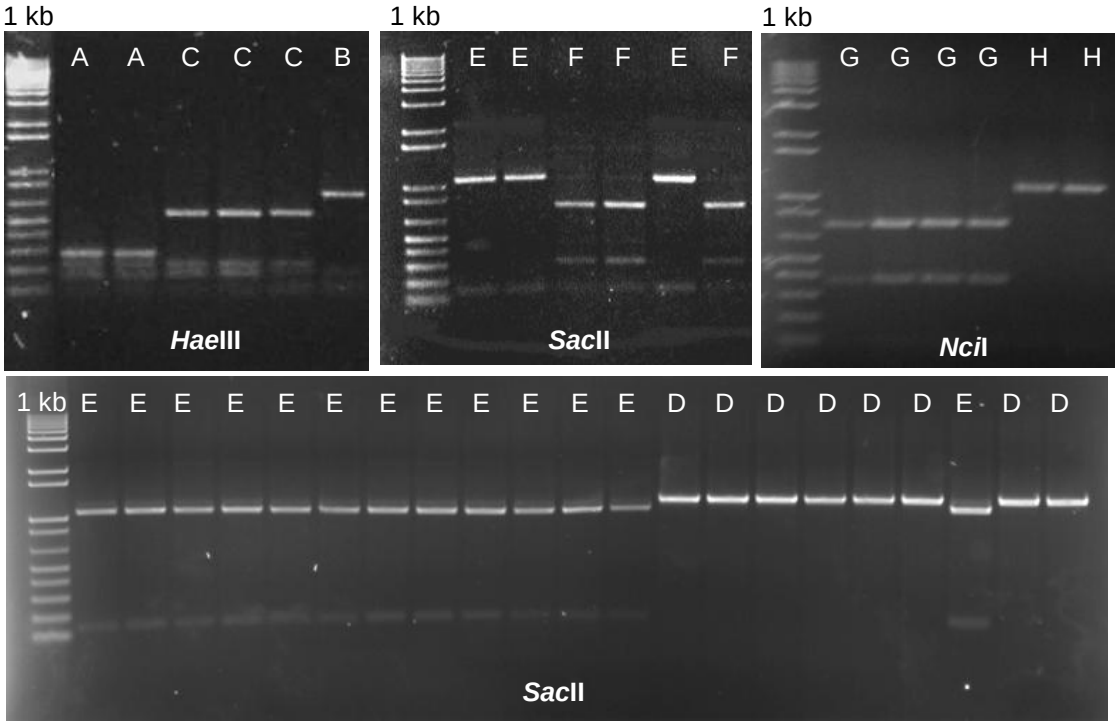

Figure 5.8 Representative gel photographs for the identification of representative botryopshaeriaceous isolates based on ARDRA using *Hae*III, *Sac*II and *Nci*I enzyme digestion. Letters represent the different restriction pattern groups: A) *N. parvum*; B) *B. dothidea*; C) unidentified isolates were further digested using *Sac*II enzyme; E) *N. luteum*; F) *N. australe*; D) unidentified isolates further digested with *Nci*I; G) *D. seriata;* and H) *D. mutila.*

Table 5.10 Detection of botryosphaeriaceous propagules on plant debris of different grapevine varieties from three nurseries using microscopy dilution plating assay and PCR with the botryosphaeriaceous multi-species primers (Bot100F/Bot472R) and universal primers (ITS1/ITS4).

Nursery	Variety	No. of samples per variety	[A] Microscopy		[B] Dilution Plating (10^{-4})		PCR (pooled samples)	
			Detection (conidial count)	Identification (Morphology)	No. of isolates recovered	Identification (ARDRA)	[C] Bot100F-Bot472R	ITS1-ITS4 primers
Nursery 3	101-14	1	low	*Neofusicoccum sp.*	**-**	-	**+**	**+**
	Schwarzmann	1	low	*D. mutila*	**-**	-		
	3309	1	High	*D. mutila*	**1**	*D. mutila*		
	Riparia gloire	1	0	-	**-**	-		
	Gravesac	1	0	-	**-**	-		
	Sauvignon blanc	1	moderate	*D. mutila, Neofusicoccum sp.*	**1**	*N. parvum*		
Nursery 5	101-14	1	0	-	**-**	**-**	**-**	**+**
	Schwarzmann	1	0	-	**-**	**-**		
	SO4	1	0	-	**-**	-		
	Pinot gris	1	0	-	**-**	-		
	Riesling	1	0	-	**-**	**-**		
	Pinot noir	1	0	-	**-**	**-**		
Nursery 9	101-14	1	0	-	**-**	**-**	**-**	**-**
	Schwarzmann	1	0	-	**-**	**-**		
	5C	1	0	-	**-**	**-**		
	Sauvignon blanc	1	0	-	**-**	**-**		
	Pinot gris	1	0	-	**-**	**-**		
	Gewurztraminer	1	0	-	**-**	**-**		

[A] low ≤10^3 mean conidia/vine; moderate >10^3 to 10^4 mean conidia/vine, high >10^4 mean conidia/vine
[B] (+) positive to botryosphaeriaceous single colonies; (-) negative to botryosphaeriaceous single colonies
[C] (+) positive or (-) negative to PCR using botryosphaeriaceous multi-species primers.
The calculated detection level of PCR using multi-species primers is 25 conidia per 1 µl of DNA template (Ridgway *et al.,* 2011). This method, therefore, can detect 100 conidia per ml of 25 ml liquid sample that yielded 100 µl of DNA template The higher volume of combined samples may detect as little as 17 conidia/ml for a 150 ml sample.

5.3.5 Botryosphaeriaceous propagules in plant debris

5.3.5.1 Detection by microscopy

High numbers of conidia (10^5/sample) typical of *D. mutila* were detected by microscopy on plant debris collected from the 3309 mothervine blocks, while a moderate number of conidia (10^4/sample) typical of *D. mutila* and *Neofusicoccum* spp. were observed from samples collected from the Sauvignon blanc blocks of Nursery 3 (Figure 5.6B; Table 5.10). From the same nursery, low numbers of conidia were also observed on samples collected from 101-14 (*Neofusicoccum* sp.) and Schwarzmann (*D. mutila*) blocks while none were observed from Riparia gloire and Gravesac blocks. Botryosphaeriaceous conidia were not detected from plant debris samples collected from Nursery 5 and Nursery 9.

5.3.5.2 Serial dilution plating assay

A wide variety of microbial colonies that over-lapped with each other were observed on 10^{-2} and 10^{-3} dilution plates and they could not be differentiated. At 10^{-4}, however, single colonies were recovered from samples collected from 3309 and Sauvignon blanc mothervine blocks. The isolate obtained from 3309 was identified by ARDRA as *D. mutila* and the isolate obtained from Sauvignon blanc as *N. parvum* (Table 5.10). No botryosphaeriaceous colonies were recovered from other plant debris samples assessed.

5.3.5.3 Molecular detection

When the combined plant debris wash samples from each nursery were analysed by PCR, botryosphaeriaceous DNA was amplified from the Nursery 3 sample while samples from Nursery 5 and Nursery 9 did not produce any PCR products (Figure 5.9). When the same samples were analysed using the ITS1/ITS4 universal primers, two PCR products were amplified from Nursery 3 and 5 samples, but none from Nursery 9. When the PCR for both primer pairs was repeated for the Nursery 9 sample using DNA diluted at 1:4 and 1:10, both samples were still negative to amplification. No PCR products were obtained from the negative control samples (SNW) using the two primer pairs.

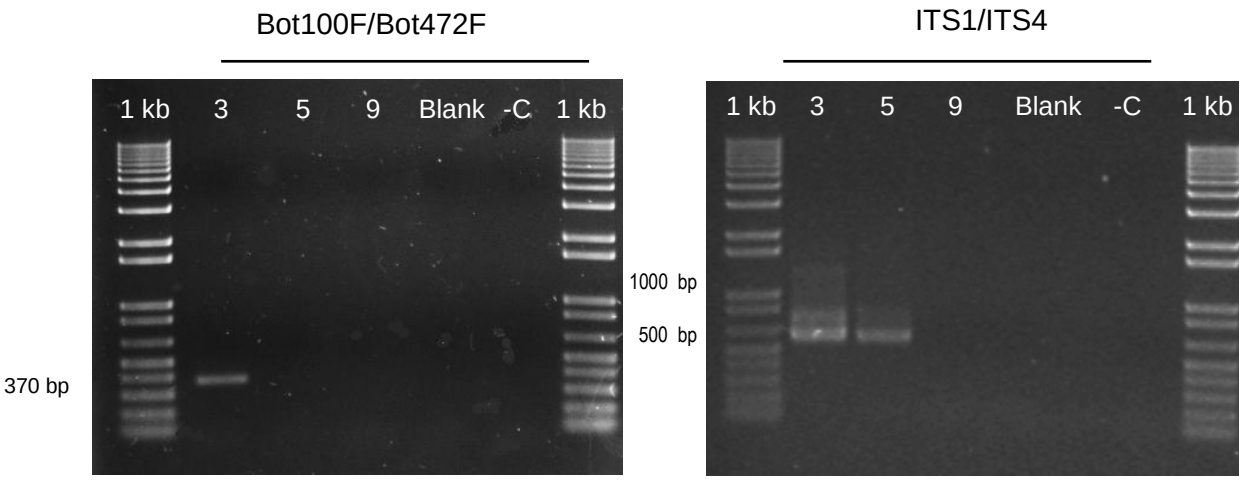

Figure 5.9. Amplification of botryosphaeriaceous DNA from plant debris washwater samples using Bot100F/Bot472R multi-species and ITS1/ITS4 universal primers. Numbers on top represent nurseries, blank and –C (SNW) are negative controls. Numbers on the far left of each gel denote molecular weight of the PCR products.

5.3.6 Botryosphaeriaceous propagules in soil

5.3.6.1 Detection using multi-species primers

Botryosphaeriaceous DNA was not amplified from the soil samples collected from Schwarzmann, Sauvignon blanc, 101-14 and Riparia gloire blocks at Nursery 3 using the botryosphaeriaceous multi-species primers (Figure 5.10). However, non-botryosphaeriaceous DNA was amplified using the ITS1/ITS4 universal primers (Figure 5.10). No PCR products were amplified from the negative controls for both the specific and universal primers.

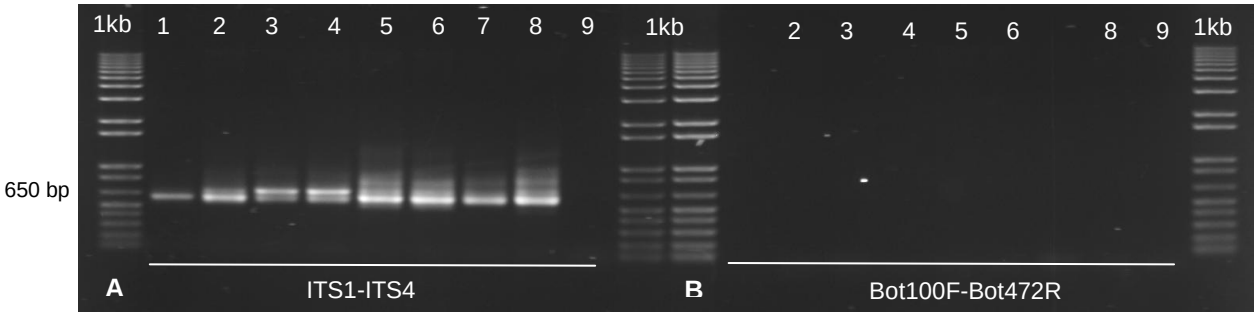

Figure 5.10 Amplification of ribosomal DNA from Nursery 3 soil samples using A) ITS1/ITS4 universal primers and B) Bot100F/Bot472R multi-species primers. Lanes 1-2, Schwarzmann; 3-4 Sauvignon blanc; 5-6, 101-14; 7-8, Riparia gloire; and 9, negative control SNW. Numbers on the far left of the gel denotes molecular weight of the bands in 1 kb plus DNA ladder.

5.3.7 Botryosphaeriaceous propagules in rainwater

5.3.7.1 Detection by microscopy and serial dilution plating assays

Microscopy analyses for rainwater samples was attempted but discontinued due to the large amount of plant debris and microbial contamination. With the serial dilution plating assay, a wide variety of overlapping microbial colonies was observed on 10^{-2} and 10^{-3} dilution plates, so the colonies could not be differentiated. Single colonies of bacterial and other fungi were observed on 10^{-4} and 10^{-5} dilution plates but no botryosphaeriaceous colonies were obtained from any of the samples.

5.3.7.2 Molecular detection

The rainwater samples collected from each month in Nursery 3 and Blenheim vineyards were combined for each variety and analysed by PCR. Rainwater samples collected from 3309 vines in December 2009 and July 2010 and from 101-14 vines in July 2010 were positive for botryosphaeriaceous DNA using the multi-species primers (Figure 5.11B). The remaining samples, including those collected from Blenheim in April 2010 were negative by PCR using the multi-species primers (Figure 5.11B). For the ITS1/ITS4 primers, single or double amplimers were obtained from all rainwater samples except for one sample collected from 3309 in February 2010 (Figure 5.11A). No PCR products were obtained from the same sample when the PCR was repeated using DNA diluted to 1:4 and 1:10. No PCR products were obtained from control sample (SNW) for either primer pair (data not shown).

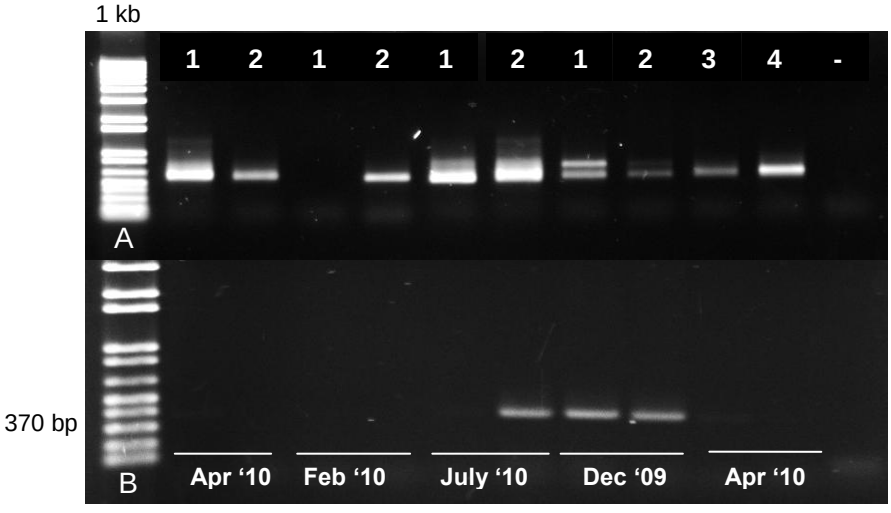

Figure 5.11 PCR products amplified from rainwater samples using A) ITS1/ITS4 universal primers and B) Bot100F/Bot472R multi-species primers. Samples were collected at different months of the growing season. Numbers on top of the gel represent the mothervine varieties where rainwater was trapped: 1, 3309; 2, 101-14; 3, Pinot noir and 4, Sauvignon blanc. Numbers on the far left of the gel denote the molecular weight of the PCR products.

5.3.8 Botryosphaeriaceous contamination in the propagation system

5.3.8.1 Detection by microscopy

All samples collected from the different propagation steps of Nursery 5 and 9 were negative for botryosphaeriaceous conidia (Table 5.11). For Nursery 3, a low number of conidia (10^3) that resembled *Neofusicoccum* spp. were detected in one of the six samples of pre-callusing medium while none were detected from all other samples.

5.3.8.2 Serial dilution plating assays

For serial dilution plating assays, various single colonies were observed on all plates inoculated with 10^{-4} dilutions but none of the colonies observed were typical of botryosphaeriaceous species, thus, no botryosphaeriaceous isolates were obtained from any of the samples analysed (Table 5.11).

5.3.8.3 Molecular detection

Botryosphaeriaceous DNA (~370 bp) was amplified from the different propagation steps using the multi-species primers although incidence differed between nurseries (Figure 5.12A; Table 5.11). In Nursery 3, all wash and liquid samples tested positive to botryosphaeriaceous DNA. In Nursery 5, only the three grafting tools tested positive to botryosphaeriaceous DNA. In Nursery 9, the single grafting tool and the post-callusing media tested positive to botryosphaeriaceous DNA. The presence of fungal DNA in samples was confirmed when single and multiple PCR products were amplified from all samples using the ITS1/ITS4 universal primers (Figure 5.12B; Table 5.11). No PCR products were obtained from the negative control samples (SNW) using the two primer pairs.

5.3.8.4 Sequence analysis of PCR products

DNA sequencing of the six samples that produced bright bands on gels did not provide conclusive proof of identity. Nursery 3 samples from the wash pit, pre-cold storage hydration tanks and pre-grafting scion wash tanks were identified as being of the *N. parvum*/*N. ribis* complex (Table 5.11). However, Nursery 3 samples from the wash solutions of pre-grafting rootstock, pre-callusing and post-callusing media could not be identified due to the presence of multiple species in individual PCR products.

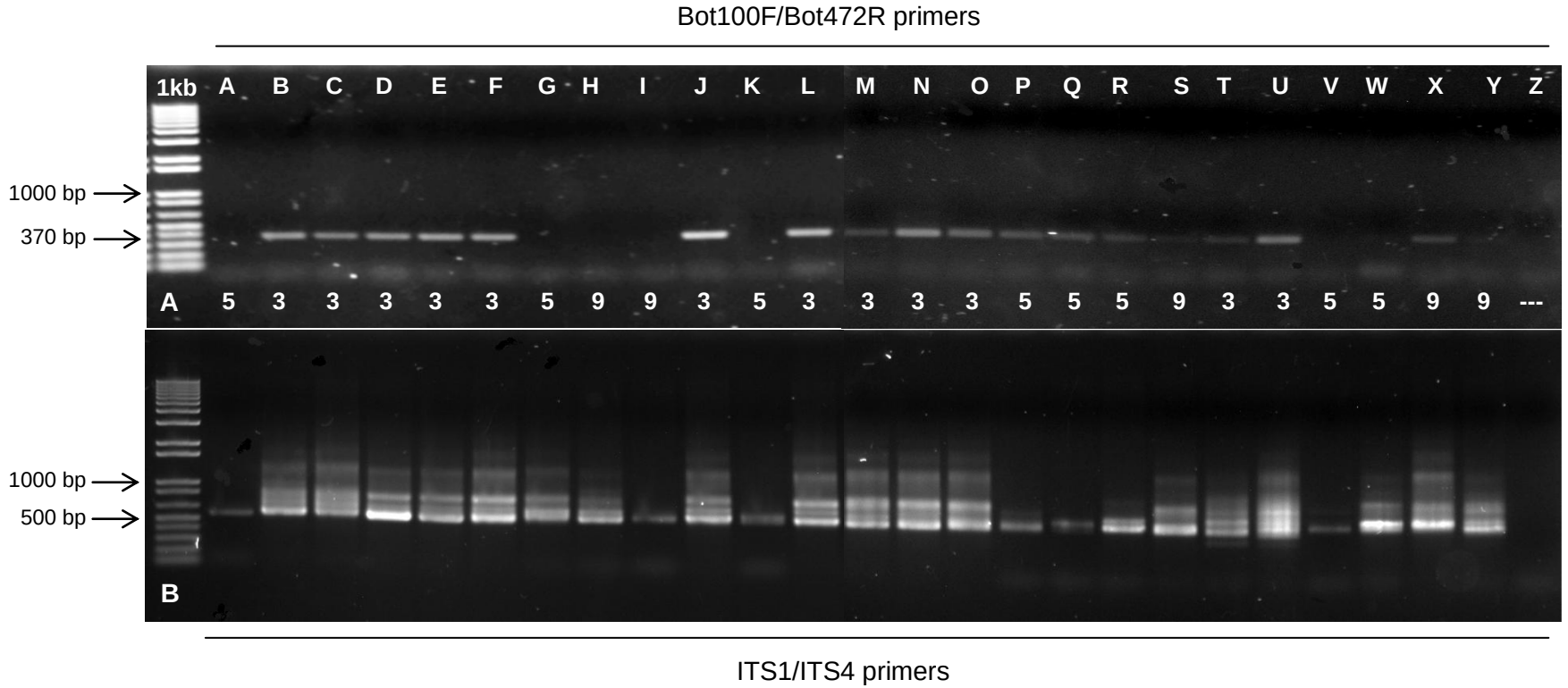

Figure 5.12 PCR products amplified from different propagation step samples using A) multi-species Bot100F/Bot472R and B) universal ITS1/ITS4 primer pairs. Letters at the top of the the gel represent the different propagation step samples and numbers represent the nurseries A) hydration/Chinosol tanks; B) pre-cold storage hydration; C) wash pit; D-F) post-grafting hydration; G-H) pre-grafting rootstock rehydration; I) pre-grafting scion rehydration; J-K) scion washing; L) rootstock washing; M-S) grafting tools; T-Y) callusing media; Z) SNW – negative control. Numbers on far left denote molecular weight of the PCR products and bands in the 1 kb DNA ladder.

Table 5.11 Detection of botryosphaeriaceous contamination in different propagation steps of three nurseries.

Nursery	Propagation steps	[A]Microscopy (mean count)	[B]Dilution Plating	[A]Molecular techniques		
				[C]PCR (Bot primers)	PCR (ITS1-ITS4)	[D]DNA sequencing
Nursery 3	Wash pit	-	-	+	+	*N. parvum/ N. ribis*
	Hydration tanks	-	-	+	+	*N. parvum/ N. ribis*
	Pre-callusing hydration 1	-	-	+	+	mixed
	Pre-callusing hydration 2	-	-	+	+	NA
	Pre-callusing hydration 3	-	-	+	+	NA
	Pre-graft rootstock washing	-	-	+	+	mixed
	Pre-graft budwood washing	-	-	+	+	*N. parvum/ N. ribis*
	Grafting tool 1	-	-	+	+	NA
	Grafting tool 2	-	-	+	+	NA
	Grafting tool 3	-	-	+	+	NA
	Perlite (pre-callusing – reused- unsterilised)	**low**	-	+	+	NA
	Perlite (post-callusing)	-	-	+	+	mixed
Nursery 5	Hydration tank/Chinosol	-	-	-	+	NA
	Rehydration Tank	-	-	-	+	NA
	Budwood washing	-	-	-	+	NA
	Grafting tool 1	-	-	+	+	NA
	Grafting tool 2	-	-	+	+	NA
	Grafting tool 3	-	-	+	+	NA
	Perlite (pre-callusing, re-used-sterilised)	-	-	-	+	NA
	Perlite (post-callusing)	-	-	-	+	NA
Nursery 9	Rehydration tank (Rootstock)	-	-	-	+	NA
	Rehydration tank (budwood)	-	-	-	+	NA
	Grafting tool	-	-	+	+	NA
	Perlite/vermiculite (pre-callusing, new)	-	-	-	+	NA
	Perlite/vermiculite (post-callusing)	-	-	+	+	NA

[A] low ≤10^3 conidia/sample; moderate >10^3 to 10^4 conidia/sample, high >10^4 conidia/sample

[B] (+) positive to botryosphaeriaceous single colonies; (-) negative to botryosphaeriaceous single colonies

[C](+) positive or (-) negative to PCR using botryosphaeriaceous multi-species primers.
The calculated detection level of PCR using multi-species primers is 25 conidia per 1 µl of DNA template (Ridgway *et al.,* 2011). This method, therefore, can detect 100 conidia per ml of 25 ml liquid sample that yielded 100 µl of DNA template. The higher volume of combined samples may detect as little as 17 conidia/ml for a 150 ml sample.

[D] DNA sequence analysis using Bot100F primer

NA - not analysed

5.3.9 DISCUSSION

This is the first comprehensive study to investigate nurseries as sources of infection for botryosphaeriaceous species using both conventional and molecular techniques. This study was able demonstrate that nursery plant materials are major sources of the botryosphaeriaceous infections which resulted in infected grafted plants (Chapter 2). Aside from internal infections, this study also showed that botryosphaeriaceous propagules were present on plant surfaces and plant debris, as well as in rainwater that can disperse in the mothervine blocks and cause secondary infections to healthy vines. It further showed that botryosphaeriaceous contaminations were present in the different stages of the propagation process. Previous work on contaminations in the nursery plant materials and propagation systems were focused mostly on Petri disease pathogens - *Ph. chlamydospora* and *Phaeocremonium* spp. (Ridgway *et al.*, 2002; Retief *et al.*, 2006; Whiteman *et al.*, 2007; Aroca *et al.*, 2010). The limited investigations previously done on sources of botryosphaeriaceous infection in nurseries have focused only on trunks and arms of standing vines, and rootstocks of dormant grafted young vines and canes that could be used for propagation materials (Spagnolo *et al.*, 2011).

This study has shown that both the rootstock and scion cuttings had high infection incidence suggesting that both plant types could potentially spread the disease. This result was consistent with the one obtained from the 2008 survey presented in Chapter 2, which showed that rootstock and scion cuttings from different nurseries were equally infected with botryosphaeriaceous species. This is in contrast with another trunk pathogen *Ph. chlamydospora* which was mainly found in rootstock cuttings (Ridgway *et al.*, 2002; Whiteman *et al.*, 2007; Zanzotto *et al.*, 2007; Aroca *et al.*, 2010) with the role of scions being unclear (Whiteman *et al.*, 2007).

The proportion of cuttings with internal infections was associated with nursery source with 68% and 65% infection incidence in Nursery 3 and Nursery 5, respectively and only 15% incidence in Nursery 9. This reflects the results of the 2008 sampling presented in Chapter 2 which showed that among eight nurseries surveyed, Nursery 5 had the highest infection incidence at 63% followed by Nursery 3 at 41% while Nursery 9 had 26% infection incidence. The most prevalent species isolated from the infected cuttings were *N. luteum* and *N. parvum.* These data are consistent with those obtained from the 2008 sampling (Chapter 2), and confirms that these two species are the most prevalent in the nursery system. While overall results showed that *N. luteum* and *N. parvum* were the most common species, species distribution differed between nurseries. *Neofusicoccum parvum* was more frequently found in Nursery 3, *N. luteum* and *N. australe* in Nursery 5, while *D. seriata* dominated in

Nursery 9. These results were similar to those presented in Chapter 2 suggesting that different species dominated in different nurseries. Differences in infection and species incidence among nurseries may be due to nursery practices, age of mothervines and environmental factors including climate and presence of alternate hosts in surrounding areas of the nurseries.

Washing the cuttings also showed that high numbers of botryosphaeriaceous conidia were present on their surfaces, by using microscopy and plating assays which further demonstrated that some conidia were viable. The proportion of the cuttings with surface propagules was associated with nurseries with 100% and 97% incidence in Nursery 3 and Nursery 5, respectively, and only 33% incidence in Nursery 9. The high percentage of samples with surface conidia suggests that these contaminated propagation materials could potentially spread inoculum within the mothervine blocks or during the propagation process. As botryosphaeriaceous species are known to be splashed-dispersed, one possible mode of secondary infection is through splashing of conidia onto healthy vines during rainy periods. Amponsah *et al.* (2009a) found botryosphaeriaceous conidia from rainwater trapped in one New Zealand vineyard throughout the year. Botryosphaeriaceous spores were also trapped during or after periods of rainfall and/or high humidity in South African vineyards (van Niekerk *et al.*, 2010). These splash-borne conidia were shown by Baskarathevan *et al.* (2010) to move up to 2 m from the focal point in a single rainfall period and were influenced by wind speed and direction. In this study, botryosphaeriaceous DNA was detected in rainwater collected from the mothervine blocks in different months, indicating that the water-borne propagules could spread in the mothervine blocks during the growing season. The sources of these water-borne propagules are most likely from infected mothervines that produce conidial ooze on their surfaces. These splashed-dispersed conidia could infect the cuts made to healthy mothervines during shoot training and harvesting of cuttings.

Botryosphaeriaceous species were reported to be saprophytic, parasitic and endophytic in a wide range of woody hosts (Phillips, 2002). In this study, viable botryosphaeriaceous conidia were obtained from surfaces of plant debris in one nursery suggesting these pathogens can survive on dead plant materials. In a South African study, van Niekerk *et al.* (2010) showed that botryosphaeriaceous species were able to colonise and produce pycnidia with viable conidia from dead plant tissues. They recovered high numbers of botryosphaeriaceous pycnidia and viable conidia from pruning debris in vineyards. A study by Amponsah *et al.* (2008a) showed that *N. australe, N. luteum* and *D. mutila* produce abundant conidia on necrotic infected shoots that has been air-dried and later incubated under moist conditions for 24 h. These air-dried necrotic stem pieces were able to produce pycnidia and viable conidia after three months of storage at room temperature. Incubating dead infected

grapevine shoots in moist conditions has been used as a routine method of producing botryosphaeriaceous conidia in this and other studies (Amponsah *et al.*, 2008a). Since trimming of vines is a standard practice in grapevine nurseries, this information can be useful in developing sanitation programs to minimise or reduce inoculum sources by removal of vine debris in mothervine blocks.

For this study, none of the soil samples collected from around four infected rootstock varieties tested positive for botryosphaeriaceous DNA suggesting that these pathogens cannot survive in soil for a long period, although it is also possible that the inoculum level could have been below the detection level of PCR using multi-species primers. Previous studies had detected *Ph. chlamydospora* DNA from soil samples collected around infected mothervines, suggesting that soil could be a potential source of inoculum for that pathogen (Retief *et al.*, 2006; Whiteman *et al.*, 2007). A *Ph. chlamydospora* isolate with a unique endogenous marker developed by Ridgway *et al.* (2005) was inoculated by Whiteman (2004) into the soil and was shown to move from soil and infect the vine.

Whitelaw-Weckert *et al.* (2006) demonstrated infection by a botryosphaeriaceous pathogen which they had added to a potting mixture consisting of river sand : loam : Canadian peat moss (2:2:1). They were able to isolate *D. mutila* at 2 cm and 20 cm from the basal end of the Pinot noir shoot six months after inoculation of the medium with pathogen mycelium, however, they did not do isolations from the roots of the inoculated plants. Based on their findings, they concluded that *D. mutila* infections could be initiated by soil-borne inoculum. Castillo-Pando *et al.* (2001) also isolated *D. seriata* from died-back roots of Semillon grapevines in one vineyard in Hunter Valley, New South Wales, but they did not carry out investigations on whether this infection was through soil-root transmissions. In contrast, Amponsah (2010) demonstrated that wounded roots were not infected by *N. luteum*, *N. australe*, *N. parvum* or *D. mutila* when conidia or mycelium were applied to wounded roots that were grown for three months prior to isolation attempts. He hypothesised that the isolations of *D. seriata* from roots (Castillo-Pando *et al.,* 2001) and *D. mutila* from trunks of root inoculated plants (Whitelaw-Weckert *et al.,* 2006) were caused by the pathogen moving from the infected shoot or stem rather than the upward movement from root infections. The development of a molecular marker for a single strain of botryosphaeriaceous species (Baskarathevan, 2011) could allow tracking of this pathogen in natural environments. This marker strain could be used for investigations on the survival of botryosphaeriaceous species in soils and provide information on the persistence of these pathogens in soils. Root inoculations using marked strains will also confirm whether these pathogens can infect roots and move upwards to the trunks and shoots.

Botryosphaeriaceous contaminations in different propagation steps were detected by PCR, with the proportion of propagation steps testing positive to contamination also being associated with nurseries. All propagation steps at Nursery 3 tested positive for botryosphaeriaceous DNA while most propagation steps at Nursery 5 and Nursery 9 were negative to botryosphaeriaceous contamination. These findings suggested that although nurseries differed in amount of surface contamination, differences in nursery practice may also account for the differences in contamination levels. Interestingly, all grafting tools from the three nurseries tested positive for botryosphaeriaceous DNA. Since rootstock and scion cuttings from Nursery 3 and Nursery 5 prior to grafting tested positive to surface contamination and cuttings from all nurseries tested positive for internal infection, this indicated that they were probably the source of contamination for grafting tools.

Pre-callusing and post-callusing media from Nursery 3 tested positive for botryosphaeriaceous DNA. Botryosphaeriaceous-like conidia were also detected in one of the pre-callusing perlite samples using microscopy, although their viability was not confirmed by agar plate assays. Since Nursery 3 re-use their perlite season to season, the presence of botryosphaeriaceous propagules from pre-callusing samples suggested the possibility that these propagules might have remained from a previous use. In addition, the newly purchased pre-callusing perlite/vermiculite from Nursery 9 tested negative while post-callusing media tested positive for the pathogen, indicating that the young grafted plants may have infested the previously clean callusing media. This may have been from pycnidia on their surfaces or from mycelium in/on infected plant fragments. Amponsah *et al.* (2008a) showed that the optimum temperature for botryosphaeriaceous pycnidia to ooze out their conidia is 24-25°C, and that it requires high relative humidity. Another study also showed that conidial germination of botryosphaeriaceous species were optimum at 25-30°C at 90-100% relative humidity (Amponsah, 2011). The 28°C and 85% relative humidity conditions used for callusing seem to be a favourable environment for conidial secretion by botryosphaeriaceous pathogens, so it is possible that sporulation and germination may occur during callusing. However, further studies are required to validate this hypothesis.

In this study, conventional and molecular techniques were also used to detect and identify the botryosphaeriaceous pathogens in order to produce comprehensive data on the presence and viability of the pathogens within the nursery system. Each method has its advantages and limitations, thus, combining the three methods was to able compensate somewhat for the lack of sensitivity or accuracy in some of the methods. Microscopy and agar plate assays are traditional methods popularly used for detecting the presence and viability of fungal pathogens. These methods, however, are time consuming and often the identification of fungi to species level based on morphological characteristics is difficult and

very subjective (Alef & Nannipieri, 1995). These conventional methods also lacked the sensitivity required to detect the pathogen due to the large variety of microorganisms and other contaminants likely to be present in the samples analysed (Whiteman, 2004). However, confirmation of their viability is important since this indicates that they have the potential to infect their hosts.

Microscopy analysis for surface propagules on different plant materials yielded higher numbers of conidia that resembled those of botryosphaeriaceous species compared to serial dilution plating assay. This method was able to quantify conidia present in the wash solutions from plant cuttings, although it was not able to detect conidia from the propagation samples. The negative results for this analysis of some propagation steps may not necessarily mean that the pathogen was absent because the method lacks the sensitivity to detect low numbers of conidia or because there were many contaminants that interfered with the count. This method used morphological characteristics to identify and enumerate the conidia, which was subject to error since other fungal spores could have had similar morphological characteristics making the conidial counts inaccurate. The use of a haemocytometer had its limitations since the standard method of counting spores in the five squares of the instrument can enumerate 2000 spores/ml of suspension (Tuite, 1969) but not lower concentrations of spores. Smith *et al.* (1988) also reported that variability of 51 to 91% was observed among haemocytometer squares. Since this method also cannot differentiate viable conidia from non-viable ones, nor identify the species of the mycelial fragments, it can over- or underestimate the infection potential of these pathogens. The use of viability stains like tetrazolium salts was successful in estimating viability of different fungal species (Pittis & Shattock, 1994; Walley & Germida, 1995) and can be used in estimating viability of botryosphaeriaceous conidia in future studies.

The use of agar plate assays was a very useful method for detecting pathogen propagules as it allows quantification of all viable propagules by counting the colony-forming units present in the samples (Alef & Nannipieri, 1995). In this study, the plating assay was able to demonstrate the viability of botryosphaeriaceous conidia from plant surfaces and debris but generally only those samples with high and moderate conidial counts were positive in the plating assays. None of the samples from the propagation process tested positive for botryosphaeriaceous species using this method. This was probably because the high levels of contamination meant that botryosphaeriaceous colonies were only successfully obtained at 10^{-4} dilutions. To calculate the sensitivity of this assay, the original suspension should contain at least 100,000 spores/ml in order to obtain one colony from the 100 µl of plated suspension diluted at 10^{-4}. Thus, spore concentrations lesser than 10^5 were not detected which equates with the moderate and high microscope counts and indicated consistency

between the two methods. If a selective or semi-selective medium had been developed, it could have improved the sensitivity of this assay. Since the use of morphological characteristics for identification was found to be difficult and unreliable for the *Neofusicoccum* spp. in Chapter 2, isolates recovered from the agar plate assay in this study were identified using ARDRA. Therefore, the agar plate assay alone was not sufficient to provide the detailed analysis required in this study.

PCR using the Bot100F and Bot472F primers (Ridgway *et al.*, 2011) used in this study was successful in detecting botryosphaeriaceous DNA from plant surfaces and plant debris confirming the results of microscopy and agar plate assays. It was also able to detect botryosphaeriaceous DNA in the propagation process that was not detected by the less sensitive conventional tools. This method is highly sensitive and was shown to detect 1 pg DNA using standard PCR, which is equivalent to 25 botryosphaeriaceous conidia (Ridgway *et al.*, 2011). Thus, the detection of 25 conidia from 1 µl of DNA template would equate to ~100 conidia/ml of a 25 ml liquid sample that yielded 100 µl of DNA template (Section 5.3.2.3). These multi-species primers successfully detected botryosphaeriaceous propagules from soil, grapevine wood and rainwater samples in other studies (Amponsah, 2011; Ridgway *et al.*, 2011). The general fungal PCR using the ITS1 and ITS4 universal primers gave positive results for all samples except for one plant debris and one rainwater sample, demonstrating that the negative results from the multi-species primers was due to its specificity to botryosphaeriaceous fungi. The non-amplification of the two samples was most likely due to the presence of inhibitory substances in the samples, rather than the lack of fungal DNA since all samples were tested for DNA concentrations prior to PCR. Since botryosphaeriaceous propagules were detected only by PCR, this result demonstrated the relative insensitivity of the microscopic examinations and serial dilution plating which were unable to detect them. It is also important to note, that while the PCR-based techniques are highly sensitive, they cannot differentiate between viable and non-viable propagules.

The multi-species primers used for this study were designed to detect six species of the 12 lineages of the *Botryosphaeriaceae* including those commonly found in New Zealand vineyards but not identify them. Therefore, the identity of the species detected from water samples in the different propagation system was not resolved at the species level. Studies done by Amponsah (2011) and Ridgway *et al.* (2011) combined PCR with single-stranded conformation polymorphism (SSCP) analysis to resolve the identities of the mixed botryosphaeriaceous species amplified by the multi-species primers. Their analyses were able to distinguish the identity of the five individual species and the *N. parvum/N. ribis* complex. The rDNA- ITS sequences that were amplified by the multi-species primers for these two species were identical and could not be differentiated by SSCP. However, the

ARDRA described by Alves *et al.* (2005) and sequence analysis of elongation factor gene (van Niekerk *et al.*, 2004) was reported to differentiate these two species. Due to time constraints and equipment being unavailable during this stage of the research, SSCP was not used. DNA sequencing was attempted for some of the representative PCR samples and it was able to identify *N. parvum*/*N. ribis* but was not able to differentiate PCR products from samples that contained two or more botryosphaeriaceous species. The use of a PCR-SSCP system is recommended for future studies as this method allows the identification of multiple species detected by the specific primers in individual environmental samples.

While this study was able to provide comprehensive information on potential sources of infections in the nursery system, quantitative data on the actual amount of inoculum in each component of the nursery system was not determined. Therefore, the infection potential of each inoculum source is difficult to estimate and, that conclusions on whether these contaminations can cause secondary infections in the propagation system cannot be made based on these data. However, Amponsah, (2011) showed that two conidia represented the minimum number of *N. luteum* conidia tested, could cause lesions on detached green shoots. While this above-cited research can be used as a possible baseline for estimating infection potentials of the nursery contaminants, future studies on the minimum level of inoculum required for cane infection is also recommended. It is also important to determine if these contaminations can cause secondary infections during the propagation process. A pilot study by Ridgway *et al.* (2011) had shown that the above-mentioned botryosphaeriaceous multi-species primers were also suitable for quantitative PCR (qPCR) that could allow the quantification of pathogen levels in different environmental samples. Therefore, qPCR using the multi-species primers could be a useful tool for future studies for quantifying the amount of inoculum present in the nursery system.

A comprehensive understanding of how the propagation process can be potentially contaminated will allow development of disease management strategies. In this study, conidia were found on the surfaces of infected plant cuttings collected from the mothervines in very high numbers, such that even the least sensitive methods of microscopy and agar plate assays were able to detect them. Their concentrations in the propagation process, on the other hand, were significantly lower and they were only detected by the more sensitive PCR method. The significant differences in inoculum levels between these two sources suggests that either the conidia had mostly germinated and infected thecuttings by the time they had reached the nursery or that the current sanitation practices of these three nurseries are effective in reducing botryosphaeriaceous contamination. Washing or disinfecting of cuttings prior to cold storage and grafting may have reduced these surface contaminants. This hypothesis was supported by the positive results of PCR detection with samples from

the wash pit and hydration tanks of Nursery 3, suggesting that botryosphaeriaceous propagules had been washed-off from the plant surfaces, although numbers were very low. Later rehydration steps prior to grafting might have reduced the inoculum further. This hypothesis was validated in Chapter 7 by investigating the persistence of botryosphaeriaceous conidia on plant cutting surfaces to determine if they could be removed during washing. It is also important to investigate if these conidia could survive and remain viable during cold storage.

During the sampling for this study, it was noted that the three nurseries disinfect their grafting tools regularly using different methods. For Nursery 3, grafting tools were sprayed with Sporekill™ before staff breaks while at Nursery 5 they washed their tools with 10% bleach solution at the beginning and end of each day's grafting. Nursery 9 staff also sprayed their grafting tools with 70% ethanol prior to morning, lunch and afternoon breaks. Since PCR-based techniques are based on the presence of pathogen genomic DNA, the positive results do not necessarily mean that the sanitation programs used by the three nurseries were not effective in killing the pathogen since dead propagules could still be detected by PCR. For example, Ridgway *et al.* (2005) were able to detect non-viable *Ph. chlamydospora* spores in soil after three weeks. It was also possible that the contamination of the grafting tools observed in this study were due to fragments of internally infected wood and not surface propagules found on the cuttings.

In summary, this research provided evidence that infected rootstock and scion cuttings are likely to be the major source of infection for grafted plants. This study also gave evidence that low levels of botryosphaeriaceous contamination were present in the propagation system which suggests that the sanitation practices of some nurseries may play a role in the reduction of these contaminations. However, the relative importance and infection potentials of each contamination have not been clarified and future investigations would be valuable for the development of control strategies in the nursery system.

Chapter 6
Infection pathways within the mothervine

6.1 INTRODUCTION

Past studies have shown that the use of infected propagation materials are a major means of spread of several trunk disease pathogens including *Ph. chlamydospora, Phaeoacremonium* spp. and botryosphaeriaceous species (Ridgway *et al.*, 2002; Whiteman *et al.*, 2007; Aroca *et al.*, 2010). The frequent isolations of *Ph. chlamydospora* and *Phaeoacremonium* spp. from rootstock mothervines and their decreasing incidence along the shoots indicated that infections can originate from the mothervine trunks and spread into the cuttings during the growing season (Pascoe & Cottral, 2000; Whiteman, 2004).

In the 2008 and 2009 sampling presented in Chapter 2 and Chapter 5, infections from cuttings were mostly found at the base and middle part of the asymptomatic cuttings which raised the question about whether the mothervines were sources of infection in the canes. However, the genetic variability studies also found multiple genotypes of *N. luteum* on the same infected cuttings (Section 3.2.3.3) indicating that infections may originate from different sources. Botryosphaeriaceous DNA was also detected in rainwater trapped at the rootstock mothervine blocks (Section 5.2.5.2) indicating that botryosphaeriaceous propagules could spread to adjacent mothervines during significant rain events. Furthermore, investigations on the sources of inoculum in the nurseries presented in Chapter 5 showed that the cuttings from mothervines contained surface propagules and internal infections of these pathogens. These findings also raised the question about the possible infection pathways of botryosphaeriaceous species in the mothervine blocks and the need for wounds which are known to be a major infection court for botryosphaeriaceous species (Urbez-Torres & Gubler, 2011).

The frequent isolation of botryosphaeriaceous species from asymptomatic grafted plants and cuttings (Chapters 2 and 5) resulted in the assumptions that the botryosphaeriaceous species can move endophytically without causing any obvious symptoms. Previous studies have also shown that botryosphaeriaceous species are endophytes and latent pathogens of woody plants (Slippers *et al.*, 2007; Sakalidis *et al.*, 2011). However, some studies have shown that some members of the botryosphaeriaceous species were more frequently present in the bark and outer tissues of the host rather than internal tissues. For example, *D. sapinea* was frequently isolated from the bark and phloem tissues of asymptomatic shoots

of pine trees and was rarely found in the xylem and pith (Flowers *et al.*, 2001). In peach trees, *B. dothidea* and *D. seriata* were also frequently isolated from the outer and inner bark and were associated with lenticels (Pusey, 1993). These studies have provided some evidence that some botryosphaeriaceous species can survive in the superficial tissues of their host for part of their life cycles but can move inwards under favourable conditions.

The anatomy and physiology of grapevine stem as described by Keller (2010b) is summarised briefly in this section. All tissues outside the vascular cambium which include the epidermis, cortex and the phloem are collectively referred to as the 'bark'. During the maturation of grapevine shoots, the secondary phloem cells form a cork cambium called phellogen, which later produces cork (phellem) and secondary cortex (phelloderm). The phellem, phellogen and phelloderm together form the outer bark or periderm which is a multilayer of dead but elastic and suberized cork cells and waxes. The periderm which includes the outermost layers of secondary phloem dies and turns brown starting at the base and moving up toward the tip as the shoot matures into a cane. This cork layer is interspersed with lenticels that allow gas exchange for the interior tissues. This layer also contains phenolic compounds called stilbenes which are phenylpropanoid derivatives that are toxic to fungi and may contribute to disease resistance as phytoalexins (Goodwin *et al.*, 2000; Chong *et al.*, 2009). Stilbene production in grapes was reported to be induced by infections of powdery mildew (Schnee *et al.*, 2008) and *B. cinerea* (Langcake & Pryce, 1976; Bezier *et al.*, 2002) as well as injuries (Langcake & Pryce, 1976). To date, no investigation has been done on the presence of these botryosphaeriaceous species on the surface tissues of grapevines shoots and the possible infection pathways involved.

To investigate the infection pathways of botryosphaeriaceous species within the mothervine, three experiments were done. The first experiment investigated whether the infections from mothervine trunks could move internally into the current season's shoots by determining the genotypes of isolates recovered from the trunks and shoots (Figure 6.1 Study 1). The second experiment investigated the spatial distribution of different botryosphaeriaceous species and genotypes in the bark and wood on an entire dormant shoot (Figure 6.1 Study 2). The third experiment investigated the genotypes obtained from the surface and internal infections of dormant canes (Figure 6.1 Study 3).

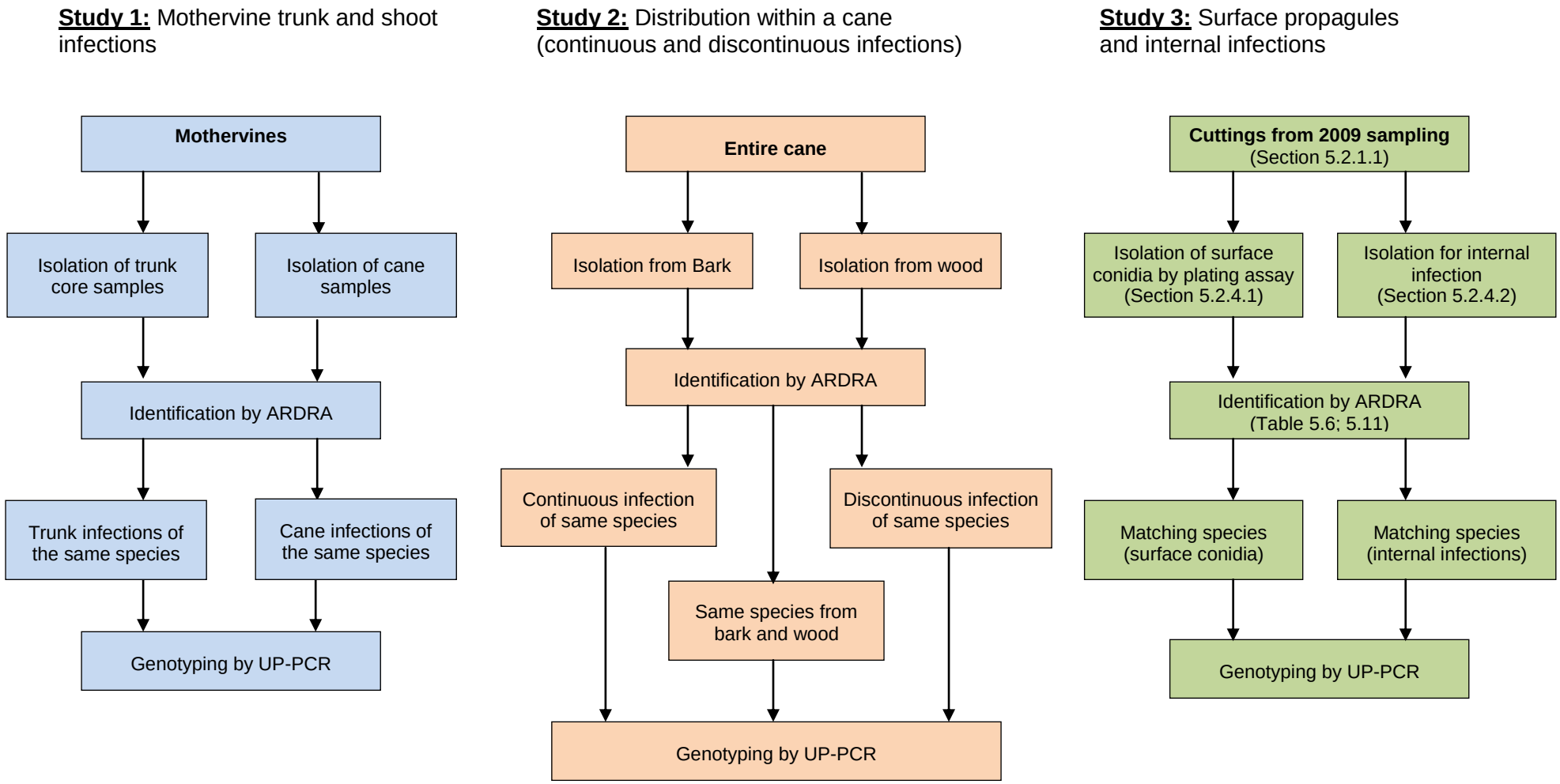

Figure 6.1 Procedural steps involved in three studies involving genotyping of botryopshaeriaceous species by UP-PCR. The three studies are indicated on top of each diagram.

6.2 MATERIALS AND METHODS

In this chapter, the term “shoots” referred to the entire mature stem cut from the mothervines while “canes” referred to the sections of the shoots that were cut at set distances. The 1 cm sections cut from canes and used for isolations were referred to as “segments”.

6.2.1 ***Study 1:*** Mothervine trunk and cane infections

To investigate if infections from the mothervine trunks could spread into the current season’s shoots, isolations were made from trunk and shoot tissues of the same mothervines. The isolates of the same species obtained from each source were genotyped to determine if single or multiple genotypes were involved in the infections (Figure 6.1 Study 1).

6.2.1.1 Collection of core samples

To identify the infected mothervines at Nursery 3, ten mothervines of variety 3309 were selected randomly in one row and tagged in January 2010. A non-destructive sampling was done to determine if trunks were internally-infected with botryosphaeriaceous species following the method of Whiteman (2004). The trunk was sprayed with 96% ethanol and bark was scraped off an area for core sampling. Two cores (20 mm depth) were made in each trunk by drilling 10 cm above the base and below the head (uppermost trunk section) of the trunk using an auger (6 mm diameter) attached to a battery operated drill (Figure 6.1A). Core samples were placed separately into sterile plastic tubes, labelled and placed into insulated boxes with ice packs during transport to Lincoln University, where they were held at 2°C for 2-3 days until isolation. The drill wounds were filled with a builder’s acrylic sealant and sprayed with fungicide (Nustar – Flusiazole rate: 1 mg a.i./L), which had been shown to be effective in preventing wound infection by botryosphaeriaceous species (Amponsah, 2011) . Core samples from ten randomly selected mothervines of 101-14 of the same row were further collected in February 2010 following the same methods.

6.2.1.2 Collection of cane samples

To determine whether the mothervine infections progressed internally through the shoots as they grew, isolations were made at set distances along entire shoots collected from the same 3309 mothervines selected for core sampling in February 2010. Three entire shoots (2-5 m) were cut above the head of each vine (n=10) and each shoot was further cut into sections containing three internodes (~30 cm), each being labelled according to its position with the lowermost labelled as Cane 1, followed by Cane 2, etc. For 101-14, only two mothervines (Vine 15 and Vine 16) whose trunks were positive for botryosphaeriaceous infections were sampled for shoot infections. At the time of the collection, the shoots were approximately 5 to 6 months old and were not fully hardened.

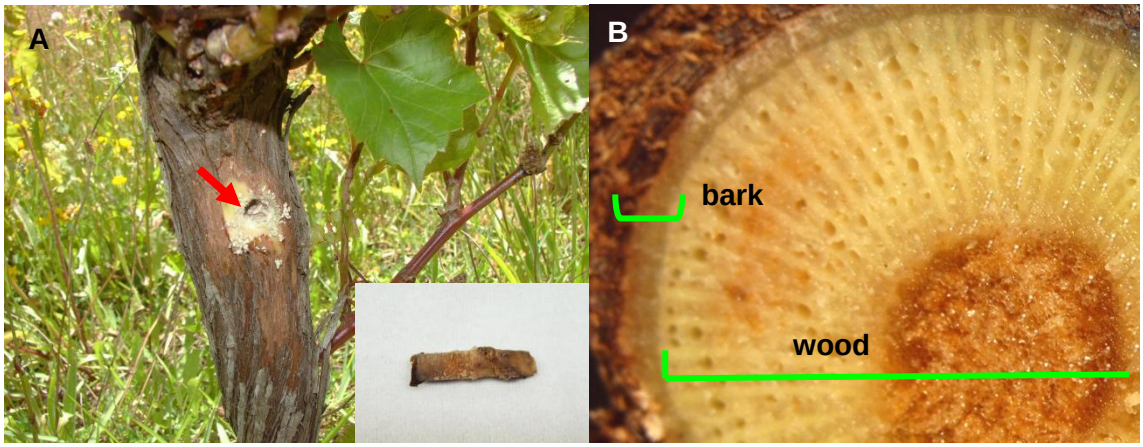

Figure 6.2 A) Mothervine with a drilled hole (red arrow) and the core sample collected (inset) and B) transverse section of a shoot indicating the bark and wood tissues used for differential isolations.

6.2.1.3 Isolations

Core samples. These were washed in 0.5% sodium hypochlorite for 30 s, rinsed twice with SROW and blot-dried on clean absorbent paper. They were then cut across into four sections, placed onto PDAC and incubated at room temperature for 4-7 days. The botryosphaeriaceous colonies that emerged were sub-cultured onto fresh PDAC for 7 days and stored at 2°C for 2-3 months. All isolates were identified using ARDRA as described in Section 5.1.10.

Cane samples. A tissue segment (1 cm) was cut from the base of each cane section and washed by squirting it with a stream of SROW for 30 s (Figure 6.3A). The bark which includes the epidermis, cortex and phloem (Keller, 2010b; Figure 6.2B) was peeled off from each woody segment, blot-dried on clean absorbent paper and plated onto PDAC. The remaining wood segments were then washed twice in SROW, blot-dried and plated onto a PDAC plate (Figure 6.3A). Each tissue sample was labelled according to the position of the section on its cane and shoot, and plates were incubated at 25°C for 4-7 days. Botryosphaeriaceous colonies were sub-cultured onto PDAC and grown for 7 days as above and stored at 2°C for 2-3 months. All isolates were identified by ARDRA as described previously in Section 5.1.10.

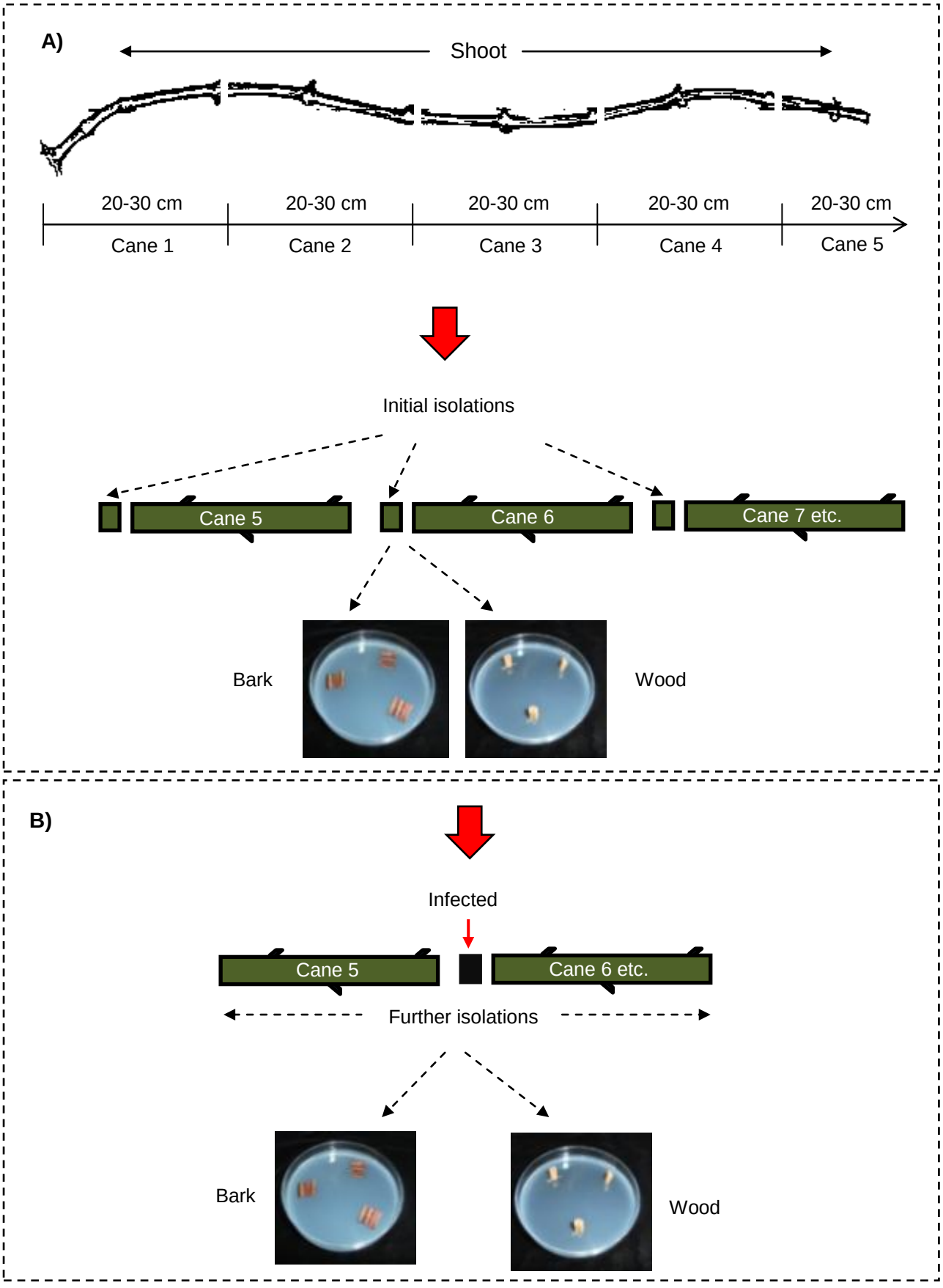

Figure 6.3 Steps for the of the isolation of botryosphaeriaceous species from entire shoots. A) Initial isolation from the base of each cane by separating bark and wood; B) succeeding sequential isolations of bark and wood from canes positive to infections.

6.2.1.4 Selection of isolates from trunks and shoots

The shoot isolates from nearest to the trunk (within 30 cm) were selected for this study. The one pair of *N. luteum* isolates from Vine 6 and Vine 9 and one pair of *N. parvum* isolates from Vine 16 were genotyped by UP-PCR (Table 6.1).

Table 6.1 Isolate pairs from mothervine trunks and their canes that were genotyped.

Species	Isolate pairs		Vine No.	Variety
	Mothervines	Canes		
N. luteum	V6C	C311	6	3309
	V9B	C321	9	3309
N. parvum	V16C	C111	16	101-14

6.2.1.5 Genotyping by UP-PCR

All selected isolates were subjected to single spore or hyphal tip isolations and mycelium growth in PDB using the methods described in Section 3.2.2.2. Genomic DNA was extracted from the 4-day old broth cultures of all hyphal tip isolates and DNA quality and quantity were further evaluated using the methods described in Sections 3.2.2.3 and 3.2.2.4.

The primers AA2M2, AS15inv and L15/AS19, which gave the highest degree of polymorphism and most countable bands (Section 3.2.3.1) were used for genotyping of *N. luteum* isolate pairs. For *N. parvum*, primers Fok 1 and 0.3-1 were used since Baskarathevan (2011) reported that they gave the highest number of polymorphic bands for this species. UP-PCR was conducted for all isolates using these primers and the methods described in Section 3.2.2.5. PCR products of each pair were loaded side by side in 1% agarose gels and run at 5V/cm for 2 h and 45 min as described in Section 3.2.2.5. The genotypes for each pair and group were compared to determine if the individuals were genetically identical.

6.2.2 ***Study 2:*** Spatial distribution of infections in bark and wood on one shoot

To further investigate the underlying causes of the apparently continuous and discontinuous distribution of botryosphaeriaceous infections within a shoot, the isolates of the same species (*N. luteum* or *N. parvum*) that occurred along the shoot were genotyped to determine the number of genetic individuals involved in the infections. The steps involved in this study are illustrated in Figure 6.1 Study 2.

6.2.2.1 Sample collection

In September 2009, an entire one-year old asymptomatic dormant shoot (~2.5 m) from a known infected 101-14 vine based on the winter 2009 sampling, was requested from Nursery 3. The cane was cut into 20 cm sections with each section labelled to determine the sequence of the cuts from base to the top (Figure 6.3). Samples were sent by courier to Lincoln University and stored at 2°C and processed within 3 weeks.

6.2.2.2 Isolation from canes

Initial steps for the isolations from the entire shoots were the same as previously described in Section 6.2.1.3 and illustrated in Figure 6.3A. The remaining samples were stored at 2°C for 2-3 weeks until further isolations. After incubation for 4-7 days at ambient temperature the botryosphaeriaceous colonies were sub-cultured onto PDAC plates, grown at ambient temperature for 7 days and stored at 2°C for 2-3 months until molecular analyses. Following the initial isolation, the sections that were positive for botryosphaeriaceous infection were subjected to further isolation by cutting the entire section as well as the section below the infected one into 1 cm segments and plating the bark and the wood separately as described above (Figure 6.3B). Plates were marked by tissue type (bark or wood) and sequential position along the shoot. All pure isolates recovered were stored at 2°C for 2-3 months and identified using ARDRA as described in Section 5.1.10.

6.2.2.3 Genotyping of isolates from continuous and discontinuous infections

For continuous infections, eight *N. luteum* isolates that were found continuously from the 99-100 and 103-106 segments of the bark and 106-107 segments of wood were selected (Figure 6.4). For discontinuous infections, two *N. luteum* isolates from segments 92 and 144 of the bark and two from segments 96 and 100 of the wood were selected. For bark to wood infections, *N. luteum* isolates from bark and wood of two segments (100 and 106) were selected.

For *N. parvum* continuous infections, seven isolates from continuous infections of bark segments 142-143, 145-147 and wood segments 146-147 were selected (Figure 6.4). For discontinuous infection, the selected isolates were from wood segments 127, 139 and 142 and for bark to wood segment isolates 142, 146 and 147 were selected. All selected isolates were genotyped by UP-PCR as described in Section 6.2.1.5.

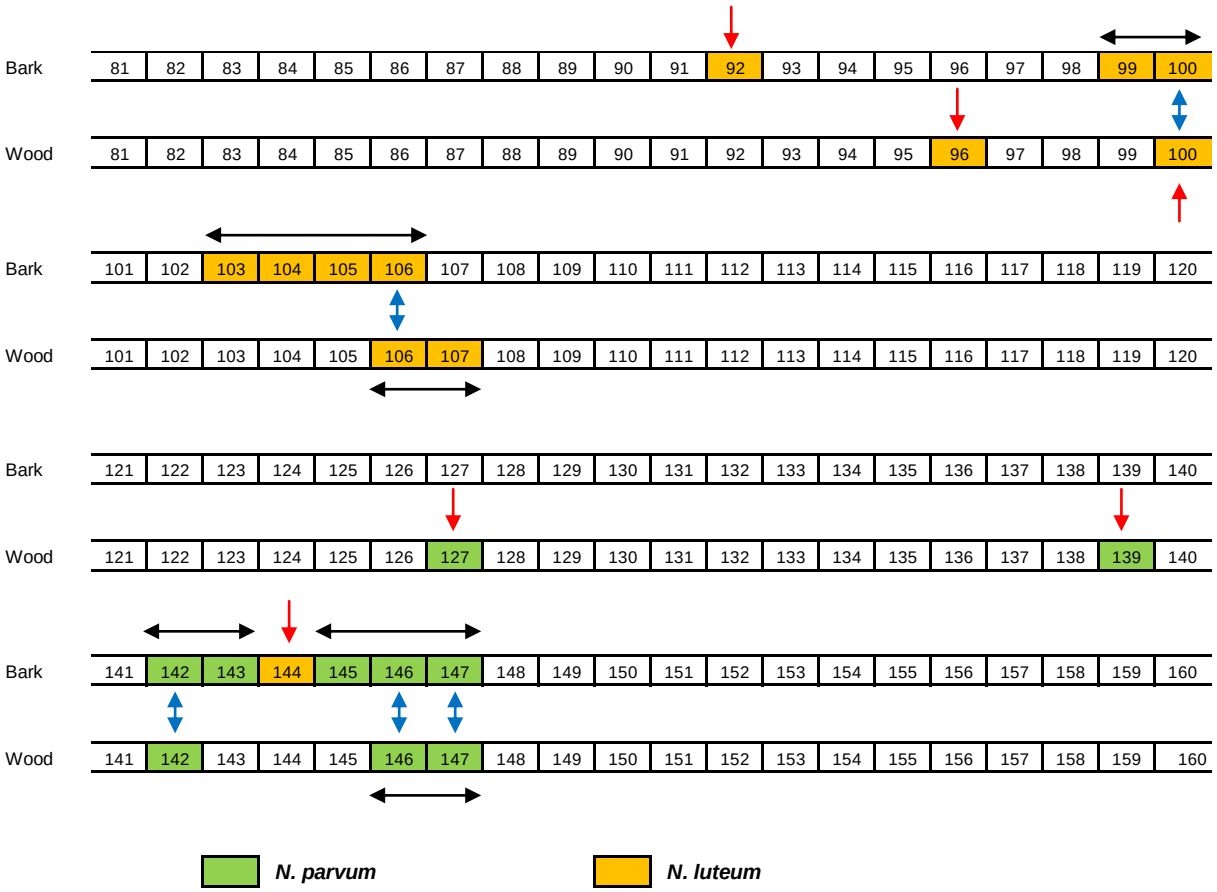

Figure 6.4 The *N. luteum* (yellow) and *N. parvum* (green) isolates obtained from continuous (black arrows) and discontinuous (red arrows) segments (1 cm) of the bark and wood used for genotyping by UP-PCR. Blue arrows indicate bark and wood infections on the same segments. The numbers indicated in each cell of diagram are the sequential position of the isolated tissue segments (bark or wood) from the entire shoot.

6.2.3 ***Study 3:*** Internal infection and surface propagules

The aim of this study was to determine if surface contaminations, as detected in Chapter 5, originated from internal infections of the cuttings or from external sources. To achieve this, botryosphaeriaceous isolates of matching species obtained from internal infections of cuttings by isolation (Section 5.3.4, Table 5.9) and from its surface contaminations by washing and plating of wash water (Section 5.3.2.2, Table 5.6) were genotyped. The procedural steps for this are illustrated in Figure 6.1 Study 3.

6.2.3.1 Selection of isolates from external contamination and internal infections and genotyping by UP-PCR

Isolates of the same species, one obtained from the surface by washing and the other from internal infection by isolation, from the same cutting were paired for genotyping. The four pairs of *N. luteum* and two pairs of *N. parvum* (Table 6.2) were genotyped by UP-PCR using the methods described in Section 6.2.1.5.

Table 6.2 Isolate pairs from external contamination and internal infections obtained from the Chapter 5 study and used for genotyping.

Species	Isolate pairs		Vine No.	Cutting variety	Nursery source
	Surface conidia	Internal infection			
N. luteum	X81	C614	N5PG1	Pinot gris	5
	X82	C621	N5PG2	Pinot gris	5
	X85	C669	N5PG5	Pinot gris	5
	X90	C750	N5RS5	Riesling	5
N. parvum	X110	C1040	N31015	101-14	3
	X117	C1149	N3RG2	Riparia gloire	3

6.3 RESULTS

6.3.1 ***Study 1:*** Mothervines trunk and shoot infections

For trunk infections, three out of ten 3309 mothervines tested positive for botryosphaeriaceous infections, with Vine 5 and Vine 9 having infections on the basal part of the trunk and Vine 6 on the crown (Table 6.3). For the 101-14 vines, two tested positive for infections, with Vine 15 having infections at the base and Vine 16 at the crown. The identities of isolates from the trunks are summarised in Table 6.3 and Appendix D.4.

For shoots, five out of ten 3309 mothervines tested positive for infections, while only one out of two 101-14 mothervines tested positive for shoot infections. The isolates were mostly recovered from bark (n=8) and only one isolate from the wood. For Vines 9 and 16, two of the three shoots had infections while only one cane each for Vines 1, 3, 4 and 6 were infected (Table 6.3). Six of the isolates were obtained from cane sections nearest to the trunk (first 30 cm) while the other two isolates were recovered from distal part of the shoot (240 to 270 cm away from the trunk; Table 6.2). Identities of the isolates from shoots are summarised in Table 6.3; Appendix D.4.

Table 6.3 Botryosphaeriaceous species isolates from mothervine trunks and canes of rootstock 3309 and 101-14 and their relative position in the vine. The isolate pairs selected for genotyping are highlighted.

Variety	Vine No.	Trunk infections		Cane infections						
		Position in the trunk	Identification by ARDRA	Shoot No.	Cane No.	Bark	Identification by ARDRA	Wood	Identification by ARDRA	Distance from the trunk (cm)
3309	1	-	-	1	10	1	*N. luteum*	-	-	270
	2		-	-		-	-	-	-	-
	3	-	-	1	2	1	*N. luteum*	-	-	30
	4	-	-	3	1	1	*N. luteum*	-	-	1
	5	Base	*D. mutila*	-		-		-	-	-
	6	Crown	*N. luteum*	3	1	1	*N. luteum*	-	-	1
	7	-	-	-	-			-	-	-
	8	-	-	-	-			-	-	-
	9	Base	*N. luteum*	1	9	1	*N. parvum*	-	-	240
			-	3	2	1	*N. luteum*	1	*N. parvum*	30
	10	-	-	ns						
101-14	11	-	-	ns						
	12	-	-	ns						
	13	-	-	ns						
	14	-	-	ns						
	15	Base	*N. parvum*	-		-	-	-	-	
	16	Crown	*N. parvum*	1	1	1	*N. parvum*	-	-	1
				3	1	1	*N. luteum*	-	-	1
	17	-	-	ns						
	18	-	-	ns						
	19	-	-	ns						
	20	-	-	ns						

(ns) not sampled
(–) negative to botryosphaeriaceous infections by isolation

6.3.1.1 Genotyping by UP-PCR

For Vine 6, the *N. luteum* isolate recovered from the trunk (V6C) was of a different genotype from the cane isolate (C311) based on the banding patterns produced by AA2M2 and L15/AS19 primers. The primer L21 gave similar banding patterns for the two isolates (Figure 6.5). For Vine 9, the isolate recovered from the trunk (V9B) was also of a different genotype from the cane isolate (C321) based on the banding pattern produced by L15/AS19 primer while primers AA2M2 and L21 were not able to differentiate the two isolates.

For Vine 16, the *N. parvum* isolates from the cane (C111) gave relatively faint bands compared to the isolate from the trunk (V16C) when amplified using primers Fok 1 and 0.3-1 making the comparison difficult. The UP-PCR for C111 was repeated for both primers using DNA diluted at 1:4 and 1:10 and both samples still gave faint bands (data not shown).

Table 6.4 Similarity in the genotypes of botryopsphaeriaceous isolate pairs from mothervine trunks and canes based on genotyping by UP-PCR.

Species	Vine No.	Isolates		Genotypes		
		Mothervine trunk	Canes	AA2M2	L21	L15/AS19
N. luteum	6	V6C	C311	Different	Similar	Different
	9	V9B	C321	Similar	Similar	Different
				Genotypes		
				*Fok 1		*0.3-1
N. parvum	16	V16C	C111	inconclusive		inconclusive

*Bands were poorly amplified.

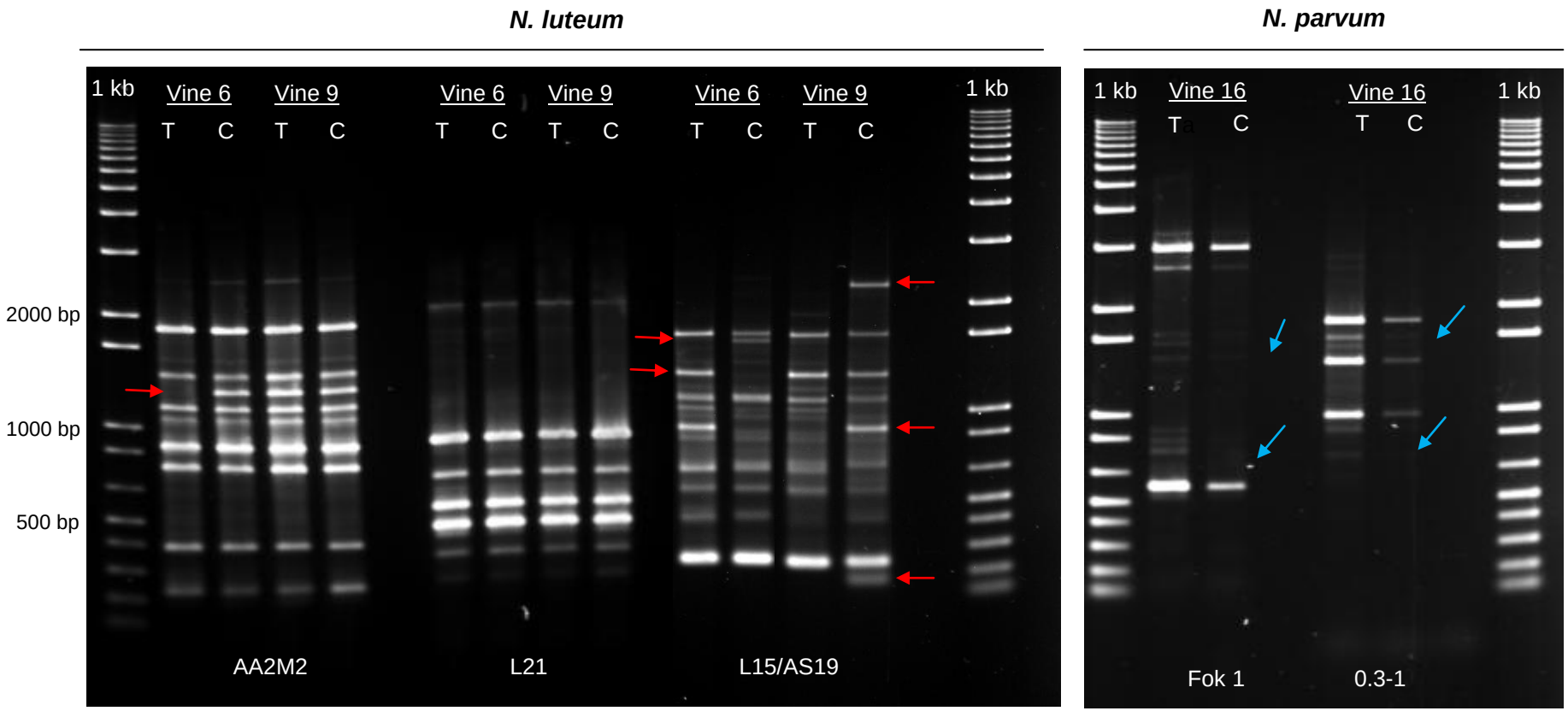

Figure 6.5 Genotyping of *N. luteum* and *N. parvum* isolates recovered from the trunks and canes of the same vines using different UP-PCR primers. Primers are indicated at the bottom of the gels. The vine numbers are indicated at the top with T representing isolate from the trunk and C representing the isolates from the canes. Red arrows indicate the differences in banding patterns between two *N. luteum* isolate pairs while blue arrows indicate very faint bands due to poor amplifications of the *N. parvum* isolate from cane (C111).

6.3.2 ***Study 2:*** **Spatial distribution of infections from one entire shoot**

For the initial isolations, canes 6, 7 and 8, were positive for infections in the bark while canes from the basal part and distal parts of the shoots were negative for infections. Further sequential isolations from all segments cut from these and the previous canes showed that botryosphaeriaceous infections were continuously found in the bark of cane sections 5 to 8 (81 to 120 cm section of the shoot) while wood infections were randomly distributed within these four sections (Figure 6.6). General observations showed that there was no relationship between the position of nodes (that were possible wound sites) and isolation from segments.

Identification of isolates by ARDRA (Appendix D.5) showed that three species were involved in the infections and were either continuously or discontinuously distributed within the infected shoot (Figure 6.6). A total of 27 *N. luteum* isolates were obtained, seven of which were from the wood, with cane sections 5 and 6 having the most *N. luteum* infections. A total of 35 *N. parvum* isolates were recovered, five of which were recovered from the wood with canes 7 and 8 having the most *N. parvum* infections. Eight *D. mutila* isolates were recovered from apparently random positions in the bark of canes 6 and 7 and none were recovered from the wood. Two botryosphaeriaceous isolates from Section 6 (110 and 112 segments) were contaminated with *Trichoderma* spp. and were not identified. None of the tissue segments had double or multiple species infections.

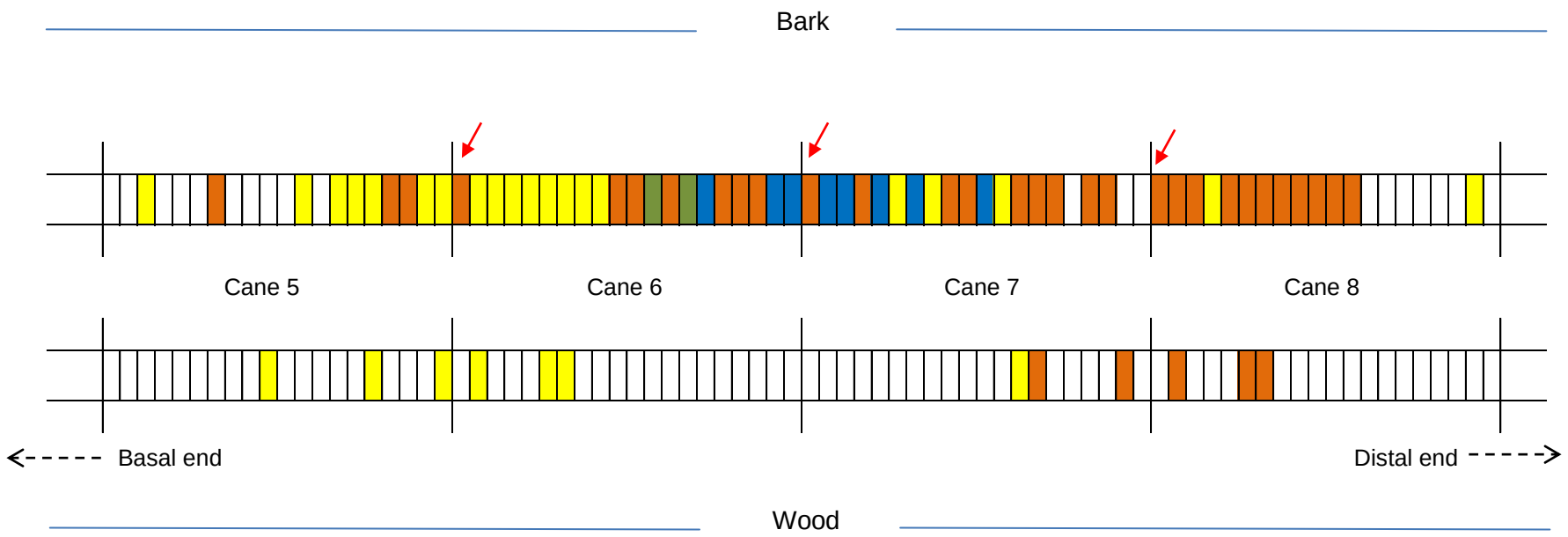

Figure 6.6 Spatial distribution of botryosphaeriaceous infections on bark and wood of a one-year old 101-14 dormant asymptomatic shoot. Lines within a cane signify the 1 cm segments for isolation, with red arrows indicating the initial isolation segments. Different colours signify species, with *N. luteum* (yellow), *N. parvum* (orange), *D. mutila* (blue), contaminated botryosphaeriaceous isolates (green) and no infection (white).

6.3.2.1 Genotyping by UP-PCR

Continuous and discontinuous infections. For the genotyping of *N. luteum* isolates, all three primers used for UP-PCR produced a high number of clear bands. The eight isolates that gave the same banding patterns using the three UP-PCR primers (Figures 6.7A-C; 6.9) were assumed to be clonal (of the same genotype). They grew continuously within bark segments 99 to 100, 103 to 106 and wood fragments 106 to 107. Conversely, four isolates that were of different genotypes were obtained discontinuously from distant parts of the canes. The isolates obtained from bark segments 92 and 144 differed by one band with primer AA2M2 (Figures 6.7A and 6.9) while isolates from wood segments 96 and 100 gave different banding patterns with primer L15/AS19 (Figure 6.7C and 6.9). Primer L21 gave the same banding patterns for all four isolates (Figure 6.7B).

For genotyping of *N. parvum*, primer 0.3-1 gave one or very few bands that were poorly amplified, so comparison for some isolates with this primer was inconclusive (Figure 6.8B). However, primer Fok 1 gave more bands and these were highly polymorphic although bands for some isolates were relatively faint (Figure 6.8A). Banding patterns from primer Fok 1 showed that *N. parvum* isolates from continuous bark and wood segments were of the same genotype (Figure 6.8A; 6.9). The same banding patterns were seen for two isolates of bark segments 142-143 and five isolates of segments 145-147 (bark) and 146-147 (wood). The *N. parvum* isolates that were from discontinuous segments were of different genotypes (Figure 6.8A; 6.9). The isolates obtained from wood segments 127 and 139 gave different banding patterns with primer Fok 1. However, the same primer gave some very faint bands for the isolate from wood segment 142 so it could not be differentiated from the other two isolates (Figure 6.8A). UP-PCR with both primers was repeated for samples with poor amplifications using DNA diluted at 1:4 and 1:10, however, the samples still gave faint bands (data not shown).

Bark and wood infections. The *N. luteum* isolate pairs from the bark and wood of the same segment were of either the same or different genotypes (Figures 6.7A&C; 6.9). Isolates from bark and wood of segment 100 were of different genotypes based on the banding patterns of primer L15/AS19, while those isolates from segment 106 were of the same genotype as they showed the same banding patterns for all three primers (Figure 6.7A to C). The *N. parvum* isolate pairs from the bark and wood of the same segment were also of either the same or different genotypes. Isolate pairs from segment 146 gave the same banding pattern while the isolates pairs from segment 147 were differentiated by primer Fok 1 (Figure 6.8A). The banding patterns for the isolate from wood segment 142 were faint and difficult to interpret (Figure 6.8B; 6.9).

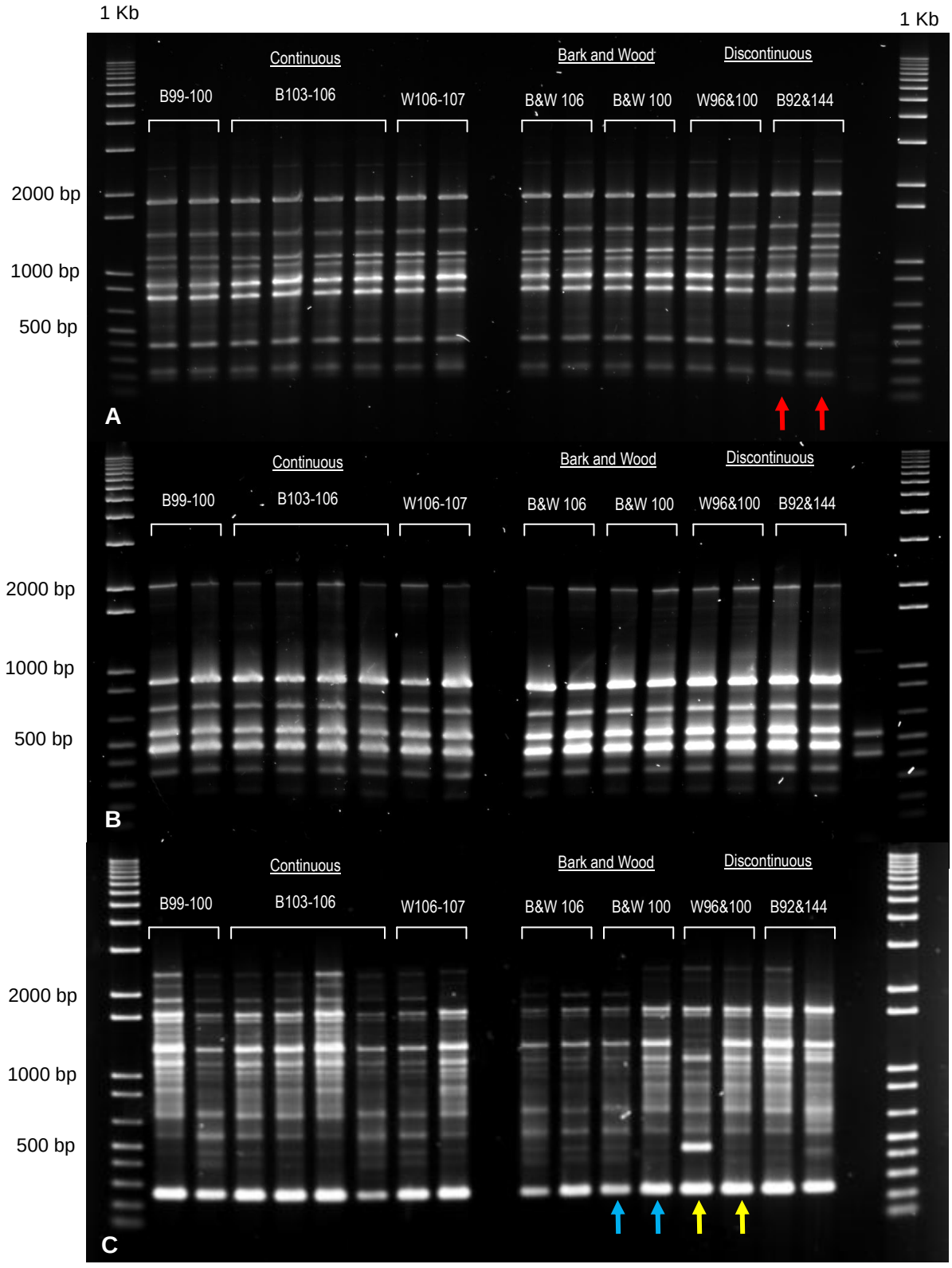

Figure 6.7 Genotyping of *N. luteum* isolates recovered from continuous and discontinuous bark and wood segments of a one-year old cane using UP-PCR primers A) AA2M2; B) L21 and C) L15/AS19. B means bark, W means wood and the numbers indicate the segment position in the cane. Arrows of the same colour are isolate pairs of different genotypes. Numbers on the left denote molecular weights of bands of the 1 Kb plus ladder.

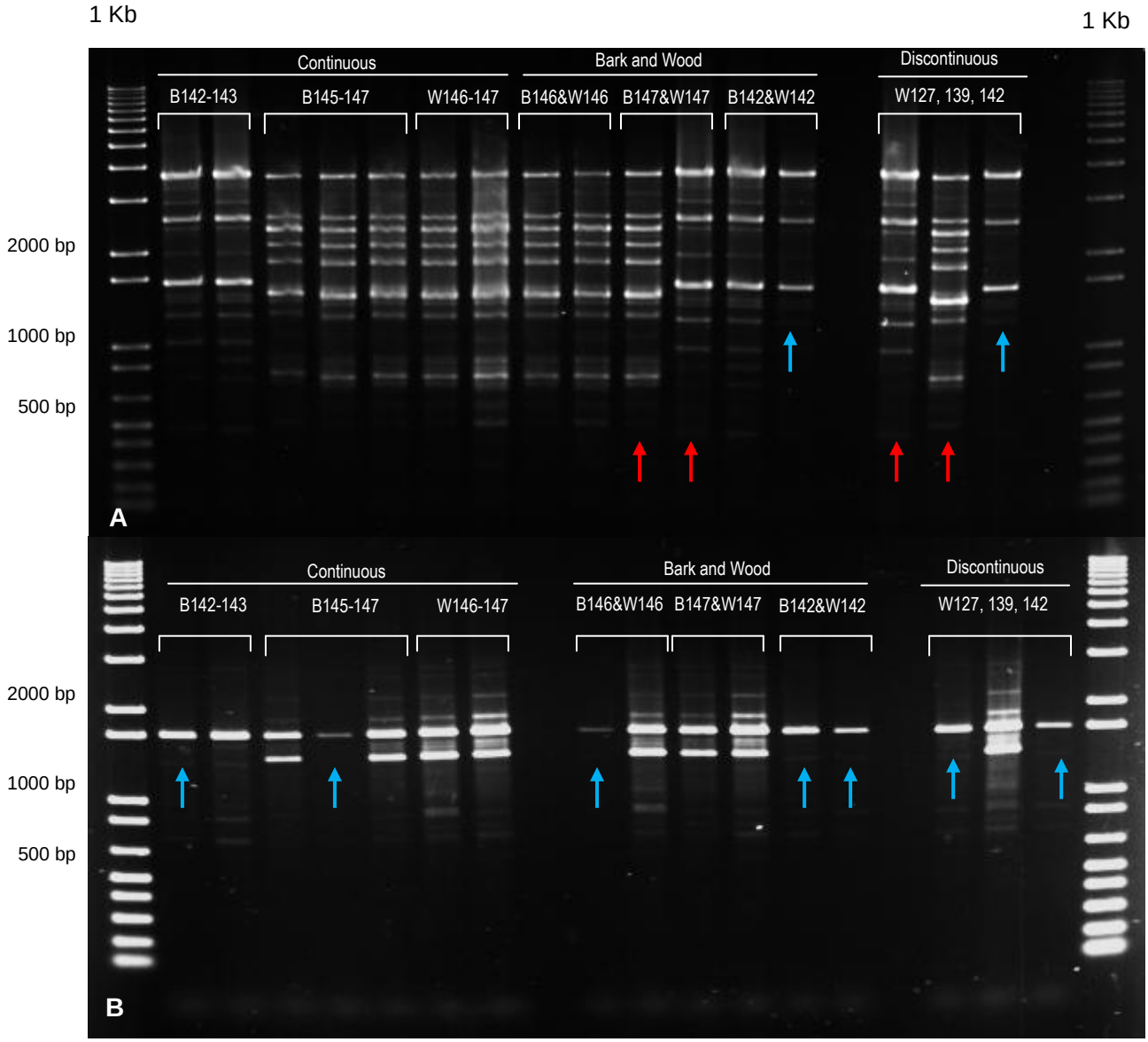

Figure 6.8 Genotyping of *N. parvum* isolates recovered in continuous and discontinuous bark and wood segments of a one-year old cane using UP-PCR primers A) Fok 1 and B) 0.3-1. B means bark, W means wood and the numbers indicate the segment position in the cane. Red arrows indicate isolate pairs of different genotypes and blue arrows indicate samples that were poorly amplified. Numbers on the far left denote molecular weights of the bands of the 1 Kb plus ladder.

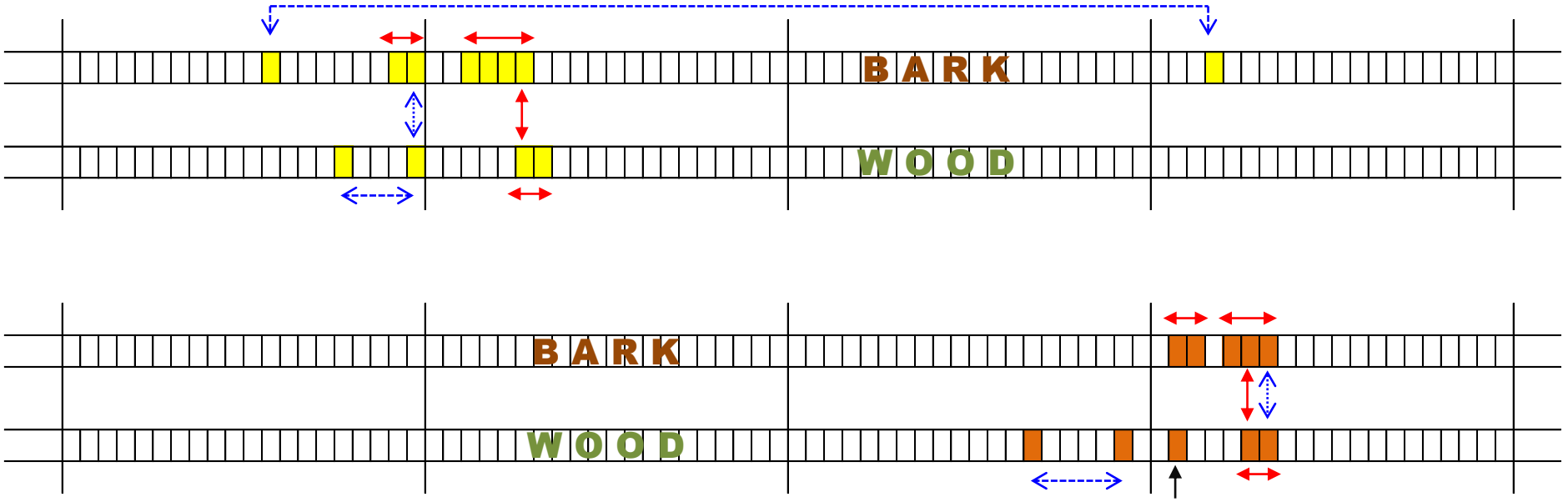

Figure 6.9 *Neofusicoccum luteum* and *N. parvum* isolates recovered in continuous and discontinuous segments from the bark and wood of a one-year-old shoot and genotyped by UP-PCR. *N. luteum* isolates are indicated in yellow and *N. parvum* in orange. Isolate pairs or groups of similar genotypes are indicated by red double-ended arrows and isolates pairs of different genotypes are indicated by dotted blue double-ended arrows. The isolate indicated by a black arrow had poor amplifications and so the result was inconclusive.

6.3.3 ***Study 3:*** **Surface propagules and internal infections.**

6.3.3.1 Genotyping by UP-PCR

All *N. luteum* isolates washed from the surface of the cuttings were of different genotypes to those isolated internally (Table 6.5; Figure 6.10). All *N. luteum* isolate pairs gave different banding patterns from each other using primers AA2M2 and L15/AS19 while no differences in banding patterns were observed using primer L21.

The *N. parvum* isolate obtained from the surface of the cutting sample 110 (X110) was of a different genotype to the internal isolate (C1040) based on the banding patterns produced using primer Fok 1. For internal isolate C1149 (Sample 117) some bands were relatively faint with primer Fok 1 and so comparison with external isolate X117 was inconclusive (Table 6.5; Figure 6.11). Primer 0.3-1 also gave a very low number of bands and some of which were very faint for all isolates tested and thus comparison between isolates was inconclusive (Table 6.5; Figure 6.11). The UP-PCR was repeated with both primers using DNA diluted at 1:4 and 1:10 and the samples still gave similar faint bands (data not shown).

Table 6.5 Genotypes of *N. luteum* and *N. parvum* isolate pairs obtained from the surface and internal infections of nursery cuttings based on UP-PCR analysis.

Species	Sample No.	Isolate pairs		Genotypes		
		Surface conidia	Internal infection	AA2M2	L21	L15/AS19
N. luteum	81	X81	C614	Different	Similar	Different
	82	X82	C621	Different	Similar	Different
	85	X85	C669	Different	Similar	Different
	90	X90	C750	Different	Similar	Different
				Genotypes		
				Fok 1		0.3-1
N. parvum	110	X110	C1040	Different		*Inconclusive
	117	X117	C1149	*Inconclusive		*Inconclusive

*Some bands were poorly amplified and comparison of patterns between isolates were inconclusive

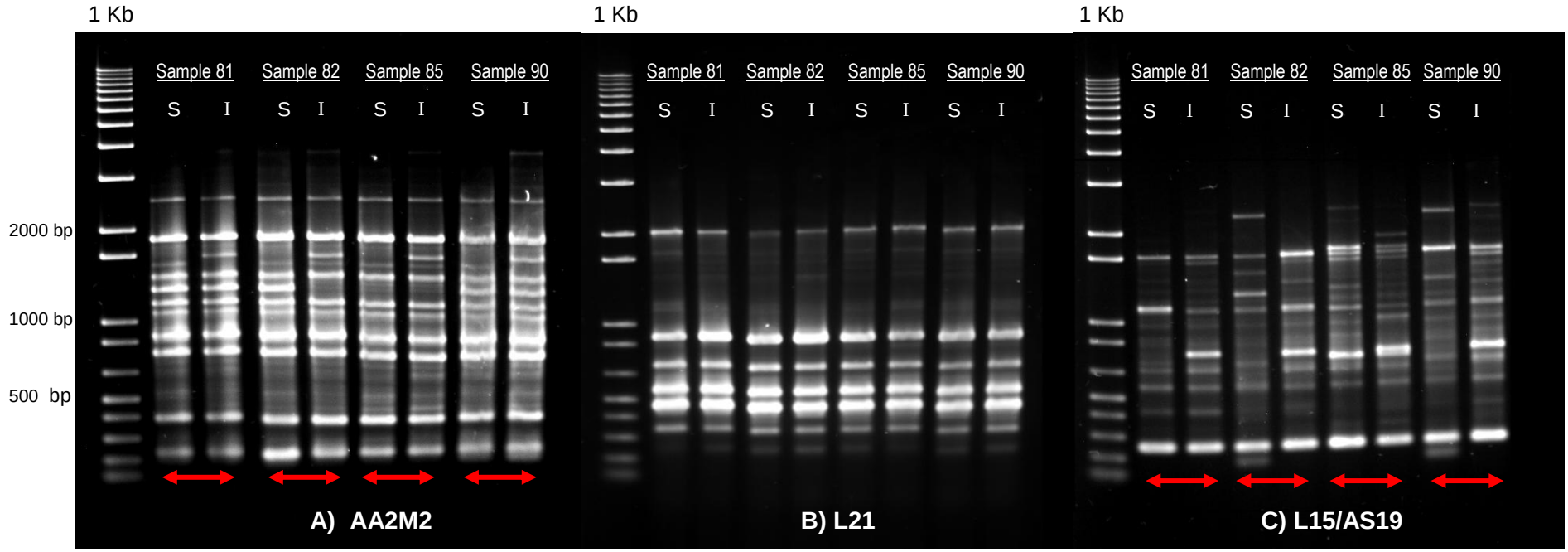

Figure 6.10 Genotyping of *N. luteum* isolates recovered from the surface and internal infections of the same cane samples by UP-PCR using primers A) AA2M2; B) L21 and C) L15/AS19. Cuttings sample numbers, and surface (S) and internal isolates (I) are indicated at the top of each gel. Red double-ended arrows indicate paired samples of different genotypes based on different banding patterns produced by primers AA2M2 and L15/AS19. Numbers on the far left denote molecular weights of the bands of the 1 Kb plus ladder.

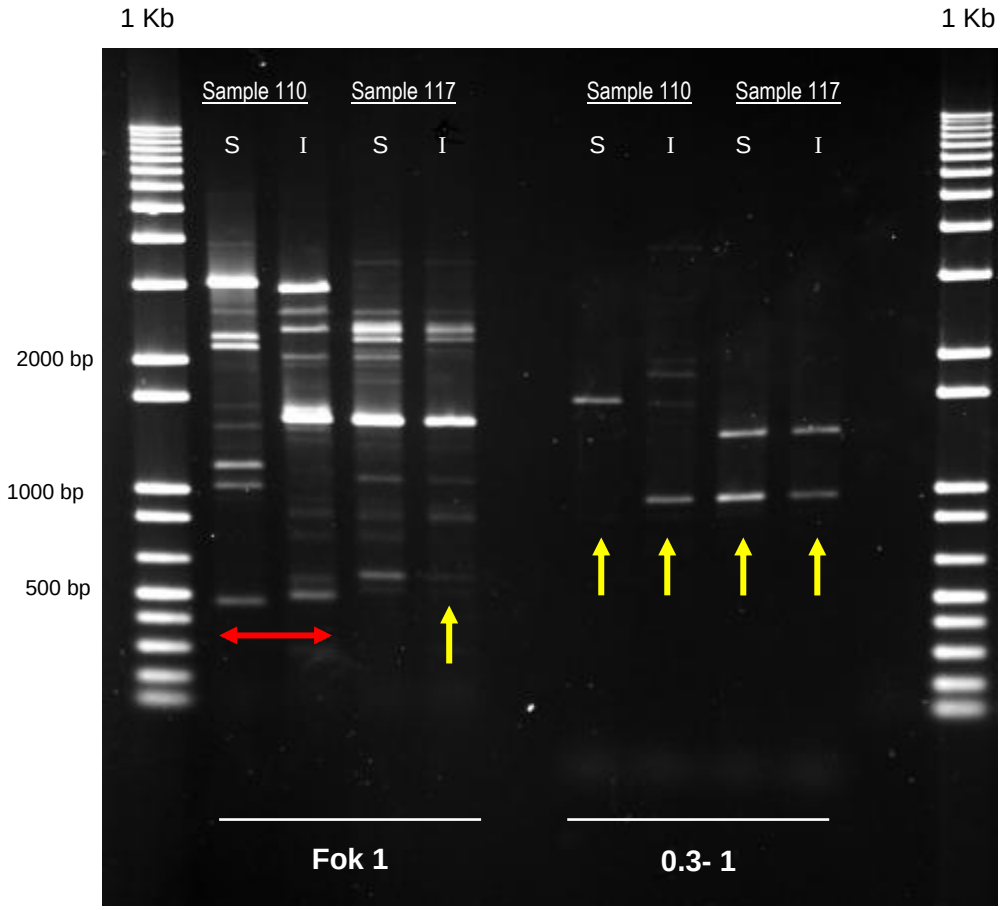

Figure 6.11 Genotyping of *N. parvum* isolates recovered from the surface and internal infections of the same cane sample by UP-PCR using primers Fok 1 and 0.3-1. Cuttings sample numbers and surface (S) and internal isolates (I) are indicated at the top of each gel. Red double-ended arrows indicate paired samples of different genotypes and yellow arrows indicate samples with poor amplifications.

6.4 DISCUSSION

This is the first comprehensive study on the infection pathways of botryosphaeriaceous species to investigate the distribution of infections within a vine and genotype the different isolates. The UP-PCR using two to three primers was useful to compare the genotypes of different botryosphaeriaceous isolates in this study. For the *N. luteum* isolates, the genotype variations were detected using two primers, but for *N. parvum* the primer 0.3-1 gave poor amplifications while the primer Fok 1 gave a high number of polymorphic bands for some isolates only. UP-PCR of >50 vineyard derived *N. parvum* isolates by Baskarathevan (2011) has shown that primer 0.3-1 can produce up to 12 bands per isolate, five of which were found to be polymorphic. The reason for the poor amplification by primer 0.3-1 and the intermittent success

of primer Fok1 in this study is unclear. It is possible that it is either due to differences in the genotype of the nursery isolates, quality of the primers or the presence of inhibitory substances like polysaccharides in the DNA. However, the results did not improve using different DNA dilutions. The quality of the genotyping in these results may be further clarified by re-extracting the isolate DNA, repeating the analyses using new stocks of primers and including a positive control from the vineyard isolates.

The results of Study 1 in this chapter have shown that *N. luteum* isolates from mothervine trunks were of different genotypes than those recovered from its shoots. Therefore, it appears that the mothervine trunk infections do not move internally into the growing shoots. However, the genotyping of *N. parvum* isolates from mothervine trunks and canes was inconclusive in this study. The limited number of valuable mothervines available for core sampling yielded only a small number of isolate pairs for genotyping, therefore this study could not conclusively demonstrate whether the systemic infections of canes could originate from mothervines. To confirm the results of this study, further genetic studies should be conducted using higher numbers of isolates of different species and more shoots from internally infected mothervines. If isolates, for which unique endogenous DNA markers (Ridgway, 2010) had been characterised, were used to infect the mothervines, the vertical transmission of the pathogens could be confirmed.

The non-systemic infections shown in Study 1 were in contrast to previous reports about *Ph. chlamydospora* infections. A study by Pascoe and Cottral (2000) had shown that *Ph. chlamydospora* was frequently isolated from rootstock mothervines leading to speculation that infections spread from the trunk of the mothervine to the shoots which were later harvested as propagation cuttings. Whiteman *et al.* (2007) also found that systemic infections of *Ph. chlamydospora* moved from the infected trunk into the shoots, by showing that cuttings collected nearest to the trunk had higher infection incidences than those collected from further away. While the above-cited studies had demonstrated the role of mothervines as primary sources of inoculum for the pathogens, none of these researchers had investigated whether the pathogens from the trunks and canes were of the same genotype which would have supported their conclusions.

The results of Study 1 also showed that shoot infections were more frequent in the bark than the wood, indicating that the sources of inoculum were most likely external rather than systemic. It seems likely that the botryosphaeriaceous infections found in the bark shoots were from splash-dispersed conidia originating from other vines or other parts of the same vine. This concurs with Slippers and Wingfield (2007), who concluded that endophytic botryosphaeriaceous infections

were predominantly introduced through horizontal transmission, i.e. via spores rather than vertical transmission (systemic) from mother plants to shoots.

The unrelated infections of mothervine trunks and shoots observed in Study 1 were unexpected since systemic infections by grapevine had been frequently observed with several trunk pathogens including botryosphaeriaceous species. Histopathology studies by Pascoe and Cottral (2000) showed that *Ph. chlamydospora* was present in the xylem parenchyma cells and that its hyphae travelled along the inside of the vessels and spread to the parenchyma, cortical and pith cells. Edwards *et al.* (2003), also showed that *Ph. chlamydospora* infections occurred via spores and fragments of hyphae carried in the sap flow from the crown of an infected trunk into the canes. Black foot disease caused by *Cylindrocarpon* species is also characterised by stunting, black discolouration of the wood and gum inclusions of the xylem vessels (Halleen *et al.*, 2007). For botryosphaeriaceous species, histopathology studies by Amponsah (2011) using SEM showed that mycelium of *N. luteum, N. parvum, N. australe* and *D. mutila* adhered to the inner walls of the xylem vessels but these pathogens were not isolated from the sap of the infected plant indicating that propagules were not translocated freely in the infected plants.

In Study 2 of this chapter, the genotyping of *N. luteum* and *N. parvum* isolates in the bark and wood of the same segment showed that some were of the same genotype while others were of different genotypes. It is important to note that, *N. luteum* was shown to be genetically-diverse using a total of five UP-PCR primers (Chapter 3 Section 2) and the use of two or three primers to identify clonal isolates may have been insufficient. The genetic variability studies of *N. luteum* in Chapter 3 Section 2 with five primers identified 75 loci of which 48% were polymorphic (a total of 36 polymorphic loci). The primers that contributed the most polymorphic bands were used to genotype the *N. luteum* in this study and were able to amplify 52 loci of which 62% were polymorphic (a total of 32 polymorphic loci), therefore, it provided a very good analysis of the clonality of the genotypes of the tested isolates that was roughly equivalent to that of Chapter 3 Section 1. Baskarathevan (2011) who used similar method to investigate the phylogeny of *N. parvum* isolates from New Zealand vineyards also found that the isolates were genetically diverse.

The observed random distribution of wood infections within the shoot in this study suggested that some infections might originate either through wounds or from bark infections. Since trimming of lateral shoots is a standard practice in mothervine blocks to keep the canes straight, (Verstappen, pers. comm., 2009), the wounds created can provide for pathogen entry. This study also showed that isolates recovered from discontinuous segments of the shoot were often of different genotypes while those from continuous infections often involved one genotype. This

result agrees with the genetic variability studies in Chapter 3 Section 2 which showed that two *N. luteum* genotypes were found on different parts of a cutting. The presence of the same genotype in continuous infections also indicates that the pathogens progressed along the shoot and bark. Furthermore, the frequent isolation of botryosphaeriaceous species in the bark of the shoot showed that these pathogens were more commonly associated with the bark and phloem vessels and not the inner tissues where xylem is located. The lack of association observed between the trimming wounds (nodes) and wood infections was in contrast to the past dogma that pruning wounds are necessary for infection by botryosphaeriaceous species (Fourie & Halleen, 2004b; Larignon & Dubos, 2001) wherein the rain-splashed conidia infect the exposed xylem of pruning wounds (Urbez-Torres & Gubler, 2011). Since most of the shoot infections observed in this study appeared to be directly from the bark, this indicates that pruning wounds may not be the only infection court for botryosphaeriaceous species.

This study represents the first observation of botryosphaeriaceous species being present in the bark of grapevines. However, the presence of botryosphaeriaceous species in bark and phloem tissues had been previously reported for other woody hosts. For example, Petrini and Fisher (1988) consistently isolated *D. sapinea* from whole stem sections of *Pinus* spp. but none were found in xylem of the plant. Smith *et al.* (1996) also reported that latent infections of *B. dothidea* were higher in leaves than xylem of Eucalyptus species. Flowers *et al.* (2001) frequently isolated *D. sapinea* from the bark and phloem of asymptomatic stems of Scots pine while infection in xylem and pith were rarely observed. Hartill and Everett (2002) also frequently isolated *N. parvum*, *N. luteum* and *B. dothidea* from intact living twigs and branches of avocado, but less frequently from the xylem and pith of the tissue. They further observed that the colonisation of the xylem was sometimes associated with wound sites. According to Kowalski and Kehr (1996) as cited by Hartill and Everett (2002), these infections should be considered as “phellophytes”, that is fungi colonising the inner bark, rather than endophytes. A study by Pusey (1993) on the differential isolation from outer and inner bark of inoculated non-wounded peach trees showed that *B. dothidea*, *D. seriata* and *L. theobromae* were more frequently found on the outer bark (35-100%) than from the inner bark (5-13%) and rarely found in the xylem tissues (0-3%). The same study further showed that in naturally infected trees, *B. dothidea* and *D. seriata* were frequently isolated from outer bark but a higher proportion of *B. dothidea* was observed in the inner bark, where it was associated with lenticels indicating their role in inwards movement of the pathogen.

The high frequency of bark infections on asymptomatic shoots observed in Study 2 further suggests that the botryosphaeriaceous isolates recovered were in their latent phase. Pathogenicity and susceptibility tests presented in Chapters 3 and 4 had shown that all

botryosphaeriaceous species isolated from nursery plant materials can cause lesions in green shoots and woody tissues providing sufficient evidence that this fungal group were pathogenic and not merely saphrophytes or endophytes of grapevines. These pathogenicity and susceptibility tests inoculated mycelia on wounded tissues under greenhouse conditions while in nature, conidia are the primary inoculum for botryosphaeriaceous infections. However, the pathogenicity studies by Amponsah (2011) has shown that both conidia and mycelia can cause infections on wounded tissues but the rate of mycelia infection is faster than conidia. Therefore, the bark infections observed in this study were most likely through conidia under natural conditions and the initial infection rates could be relatively slow. This hypothesis can be further clarified by investigating conidial infections on non-wounded dormant shoots.

While the results of Study 2 suggest that latent infections of botryosphaeriaceous species may occur in the bark, the pathways of latent infections and the succeeding steps of the disease cycle of this pathogen group in grapevines still remains unclear. Latent infections in immature fruits have been widely reported to be associated with presence of antifungal compounds, such as those in unripe avocados and mangos that inhibit infection by *C. gloeosporoides*, *Alternaria alternata* and botryosphaeriaceous species (Droby *et al.*, 1986; Prusky *et al.*, 1991; Johnson *et al.*, 1992; Hartill & Everett, 2002). The latent infection of *B. dothidea* on immature apple fruits has been well investigated by Kim *et al.* (2001; 2004). This pathogen infects immature apple fruits through natural openings such as lenticels or surface cracks and wounds but symptoms are only observed 6-8 weeks before harvest. It is believed that symptom expression in immature fruits is normally suppressed by fungitoxic compounds like benzoic acid. Histopathology studies showed that microbodies and lipid globules developed around the *B. dothidea* hyphae during its latent infection. These microbodies that were functionally defined as glyoxysomes are believed to help the pathogen survive during latent periods when nutrient conditions are limited and host defense responses are active (Kim *et al.,* 2001; 2004). The involvement of fungitoxic compounds in the latent behaviour of the above-mentioned pathogens may also apply in the disease development of botryosphaeriaceous species in canes, although this hypothesis needs further investigation.

Results of Study 3 in this chapter showed that isolates recovered from the surface of the cuttings were of different genotypes from those isolated internally from the tissues. This further supported the hypothesis of multiple sources of external inoculum, which can explain the occurrence of multiple genotype infections in mothervine trunks and canes observed in Study 1 and 2. The botryosphaeriaceous conidia are exuded in a slimy mass from the surface pycnidia on other infected vines or within a vine and splash-dispersed to host surfaces where the slime may improve adhesion (Slippers & Wingfield, 2007). The conidia can then germinate and

penetrate the cane through the lenticels, cracks and crevices in the bark but they may also colonise the secondary phloem where they remain latent for a period of time because of phenolic compounds such as stilbenes that are not only a major constituent of the lignified stem tissue of grapevines but also further produced in response to infection or injury (Langcake & Pryce, 1976). During dormancy, the phloem of the canes is inactive (Pratt, 1974) and this may limit colonisation by the pathogen. Production of microbodies on the hyphae previously described for *B. dothidea* infections may also apply to other botryosphaeriaceous species and may allow them to survive during latency; symptom expression may then occur when the host is under stress, as shown by Amponsah (2011). He observed that grapevine plants inoculated to the pith with *N. luteum* only displayed dieback symptoms during winter dormancy when the plants were inactive. He also demonstrated that symptoms of dieback were much more severe if the plants had been grown in soil maintained at 15 and 100% field water capacity the previous summer. An increase in disease severity by *B. dothidea* on peach trees was also observed when water was withheld (Pusey, 1989). The effects of stress factors in possibly triggering latent bark infections in canes to develop into active infections and their mechanisms or routes of penetration still needs further investigation.

In summary, the genotyping studies provided evidence that cane infection does not necessarily originate from mothervine trunk infections, but that multiple botryosphaeriaceous infections do originate from external sources. The frequent occurrence of botryosphaeriaceous species in the bark of canes, where they behaved as “phellophytes”, also provided evidence that they can survive as latent pathogens on cane surfaces. Moreover, the progression of the same isolates from bark to wood, without an obvious wound, suggests that pruning wounds may not be essential for penetration. However, the pathways from a latent infection to an actively pathogenic phase remain unclear. Several studies are recommended to further support these hypotheses. Sectional isolations from outer and inner bark, xylem and pith may clarify whether the pathogen grows saprophytically in the dead outer bark or is latent in the inner tissues of the cane. To determine whether and how bark infections progress into the wood, plants inoculated to induce bark infections could be planted and subjected to different stress factors and observed for disease development. Histopathological studies on the ultra-structure of one-year-old canes will elucidate the latent behaviour of botryosphaeriaceous species and clarify whether wounds are essential in the pathogenesis. Histopathological studies on botryosphaeriaceous hyphal structures as well as grapevine cane tissues may provide better understanding on the mode of survival of botryosphaeriaceous species during latent infections. This could be supported by biochemical studies that identify the different chemicals, including stilbenes that may be involved in defence mechanisms employed by the host.

Chapter 7
Botryosphaeriaceous conidia survival and infection in the nursery system

7.1 INTRODUCTION

In nature, conidia are the primary propagules of botryosphaeriaceous species; they are splash-dispersed and are believed to infect infect grapevines primarily through pruning wounds (Jacobs & Rehner, 1998; Larignon & Dubos, 2001; Urbez-Torres & Gubler, 2011). Since they can germinate between 5 and 40°C (Sutton & Arauz, 1991; Urbez-Torres *et al.*, 2010a; Amponsah, 2011), this may explain the cosmopolitan nature of this pathogen group. However, it is not known how long term cold storage affects the survival, viability and pathogenicity of these pathogens. Since botryosphaeriaceous conidia were trapped during winter months in New Zealand (Amponsah *et al.*, 2009a), Californian (Urbez-Torres *et al.*, 2010b) and French (Kuntzmann *et al.*, 2009) vineyards as well as on plant debris collected from underneath the mothervine canopy (Section 5.3.5), this indicates that these pathogens may be active during cold seasons.

The presence of botryosphaeriaceous conidia on the surfaces of dormant cuttings and the botryosphaeriaceous DNA in water samples collected from the pre-cold storage hydration tank and pre-callusing hydration bins of one nursery (Chapter 5) suggested that the propagules present on cuttings could be washed off during hydration. Hydration may therefore facilitate the dispersal of pathogens onto other cuttings and onto the ends where open wounds allow for infection. Therefore, it is important to determine if surface propagules can be effectively washed-off during hydration or can get attached and persist on cutting surfaces ultimately leading to infections.

Cold storage is considered a critical factor in the production of quality grapevine planting materials (Waite & Morton, 2007). Cold storage of cuttings and one-year rooted vines, at temperatures which ranges from 1 to 6°C (Whiteman, 2004; Fourie & Halleen, 2006; Nicholas *et al.*, 2006; Aroca *et al.*, 2010) and lasts for a few weeks to several months, allows for their preservation until they are required for processing or planting in the vineyard (Nicholas *et al.*, 2006; Waite & Morton, 2007). Since botryosphaeriaceous DNA was shown to be present in the propagation steps after cold storage (Section 5.3.8), this suggested that the pathogen was able to survive the conditions and to spread during the grafting process. Further, the presence of

botryosphaeriaceous species in or on grafted plants (Section 2.3.3.2) after cold storage demonstrated their ability to survive in cold temperatures. There is a need to investigate the effects of long cold storage on the viability and pathogenicity of these species, to provide a better understanding of their survival mechanisms and so contribute to more effective management of botryosphaeriaceous infections in the propagation process.

The main objectives of this study were to evaluate the effects of different storage temperatures and durations on germination, germ tube elongation, viability and pathogenicity of botryosphaeriaceous conidia on grapevines, and to investigate the persistence and infection of botryosphaeriaceous conidia on cutting surfaces after washing.

7.2 MATERIALS AND METHODS

7.2.1 *Experiment 1.* Effects of storage temperatures on conidial germination, viability and pathogenicity of *N. luteum* conidia

7.2.1.1 Species and isolate selection

The most prevalent species, *N. luteum,* was used in this study. The *N. luteum* isolate G51a2 was selected for this experiment as it was found to produce a unique band by UP-PCR and was being developed as a marker strain by Baskarathevan (2011) at the initial stage of this study. This isolate was also found to be highly pathogenic on green shoots and canes (Baskarathevan, 2011) and produced abundant pycnidia on green shoots. However, the development of this marker-strain was not completed at the end of this experiment and, therefore, G51a2 could not be differentiated from natural infections in this study.

7.2.1.2 Conidia production and collection

Conidia of *N. luteum* were produced using the method developed by Amponsah *et al.* (2008a). Sauvignon blanc green shoots (approximately 20 cm) were inoculated with mycelium plugs from 4-day old *N. luteum* isolate (G51a2) as described in Section 3.1.3. After 7 days incubation, necrotic lesions (6-8 cm) with visible pycnidia were cut and surface sterilised with 70% ethanol for 30 s and 0.5% sodium hypochlorite for 3 min, followed by two rinses with sterile water and air-drying for 24 h inside a laminar flow cabinet. Air dried shoots were placed in sterile Petri dishes lined with moist sterile filter paper and supported on sterile plastic straws, and placed in an incubator at 25°C overnight to induce sporulation. Conidia were harvested by placing shoots with oozing pycnidia into sterile plastic tubes containing 10 ml of pre-cooled SROW (4°C) and shaken by hand for 2 min. The conidial concentrations were determined using a haemocytometer and adjusted to $4x10^4$ per ml. All conidial suspensions were kept in an insulated box with ice packs during preparation which took approximately 1 h.

7.2.1.3 Cold storage of conidial suspensions

One ml aliquots of the conidial suspension ($4x10^4$/ml) were placed into 1.7 ml sterile tubes and covered with aluminium foil to keep them dark. Eight tubes were placed at 2±1°C and 8±1°C while six tubes were stored at ambient temperature (22±2°C). One tube of conidia stored at 2°C and 8°C was taken out of cold storage at 2, 4, 24, 48, 72 h, 1, 2 and 3 weeks for assessment. The tubes were kept in an insulated box with ice packs during the assessment period processes, which included a germination count, plating and a pathogenicity test. Conidial suspensions stored at ambient temperature were assessed after 2, 4, 24, 48 and 72 h while one additional tube was used for the germ tube measurement.

7.2.1.4 Conidial counting

The tubes were gently inverted 10 times to re-suspend the conidia and then drops of the suspension were placed with a sterile pipette onto both sides of a haemocytometer slide. The percent conidial germination and percent damaged conidia were determined by observing all conidia in five of the 10^{-4} ml squares of the haemocytometer. Conidial counts were conducted in two counting areas of the haemocytometer and repeated for a total of four replicate counts. Conidia were considered germinated if the germ tube length was at least half the width of a conidium and considered damaged when the cells were empty or when cell organelles had clumped or shrunk inside the cell. Digital images were captured using a digital camera mounted on the light microscope.

7.2.1.5 Germ tube elongation measurement

One separate tube of a conidial suspension incubated at ambient temperature was used for observing and measuring development of germ tubes. Observations were made after 0, 2, 4 and 6 h incubation by placing drops of the conidial suspension onto a haemocytometer slide as described above. Germ tubes of 10 randomly selected germinated conidia were measured at every observation period using the AnalySIS® imaging software (Soft Imaging System GmbH). Digital images were captured using a digital camera mounted on the light microscope.

7.2.1.6 Plating assay of viable conidia

From the original conidial suspensions ($4x10^4$/ml) of the treatments (Tables 7.1, 7.2 and 7.3), two-fold serial dilutions were made and 100 µl of the 10^{-2} suspension (~40 conidia) was spread onto four replicate plates of PDA containing 1 ml/L Triton-X and incubated at 25°C in a 12 h light:dark regime for 7 to 10 days. Percent conidium viability was determined by counting the colony forming units on each plate.

7.2.1.7 Pathogenicity test

One-year old cuttings of Sauvignon blanc were rooted in trays containing pumice as described in Section 3.1.2.4.1. The rooted cuttings were transferred to 350 ml plastic jars containing pumice and tap water and inoculated. The conidia stored at different storage treatments were tested for their pathogenicity by inoculating 20 µl droplets (~800 conidia/plant) onto the wounds made with a sterile scalpel on the second lowermost leaf axil of the one shoot on each plant (Figure 7.1). Control plants were inoculated with 20 µl of either freshly-harvested conidia (~800 conidia/plant) or SROW. The four replicate plants per treatment were arranged in RCBD across the shelves inside a room lit with fluorescent lights at 12:12 light:dark regime. Inoculated plants were sprayed with water daily to maintain humidity and water was added to the jars as needed. Three weeks after inoculation, infection was assessed by isolating onto PDA the 5 mm sections of the green shoot up to 25 mm above and below the inoculation point. Plates were incubated at room temperature for 5-7 days and assessed for *N. luteum* growth.

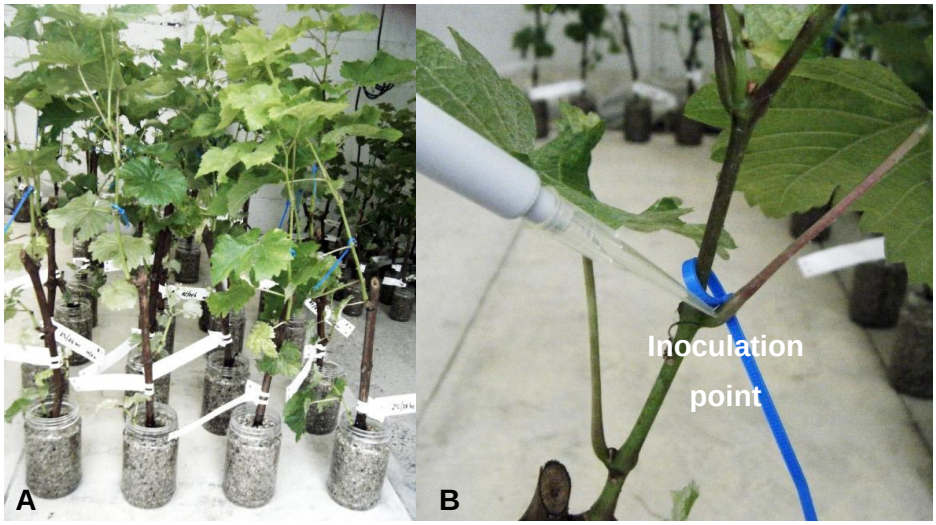

Figure 7.1 A) Rooted cuttings grown in plastic jars and used for the pathogenicity test and B) shoot inoculated with conidial suspensions at the second lower most node and marked with a cable tie.

7.2.1.8 Germinability of conidia after cold storage treatments

After assessment for germination, conidium damage, viability and pathogenicity, the remaining conidia from the 2°C and 8°C treatments were transferred to ambient temperature (22±2°C) and the subsequent conidial germinations were counted after 0, 2, 4 and 6 h using the methods described in Section 7.2.1.4. This assessment was not done for the 2 and 4 h (2 and 8°C treatments) because time did not permit and for 2 and 3 weeks (8°C treatment) because the mycelia were too well-developed.

7.2.2 *Experiment 2.* Retention and infection of *N. luteum* conidia on cuttings surfaces

7.2.2.1 Species and isolate selection

As *N. luteum* was the species most prevalent in grapevine nurseries (Table 2.3), it was selected for this test, which used the three isolates (L106, L114, L228) that gave the longest mean lesions (Appendix C.2.10) on one-year-old canes.

7.2.2.2 Production and preparation of conidial suspension

The mixed conidial suspension of three *N. luteum* isolates was produced using the green shoot method (Amponsah *et al.*, 2008a) as described in Section 7.2.1.2. The 2 x 10^4/ml conidial suspensions were kept in an insulated box with ice packs for ~30 min to prevent germination prior to inoculation. To confirm that the inoculated conidia were viable, 100 µl of the mixed conidial suspension was plated onto three replicate plates of PDA+Triton-X and incubated at 25°C for 7-10 days prior to counting colonies.

7.2.2.3 Preparation of experimental canes

Dormant one-year-old cuttings (5C variety) were purchased from a commercial nursery in July 2010 and stored at 2°C for 4 months prior to the experiment. Canes were taken out of cold storage and allowed to acclimatise for 24 h at room temperature before being hot-water-treated (HWT) in a water bath at 53°C for 30 min before being transferred to a cold 5% bleach (4.8 g/L NaOCl) solution for 15 min. A prior experiment on cuttings inoculated with *N. luteum* had shown that 53°C for 30 min was the optimum temperature that could reduce *N. luteum* infections (unpublished data). The HWT cuttings were air-dried overnight in the sterile air flow of the laminar flow hood, then cut into 10 cm sections with at least one node in each section. For each sample, three cutting sections were randomly selected and placed in a sterile plastic tube, with four sample replications per treatment.

7.2.2.4 Inoculation of cuttings

To determine the amount of conidial suspension adsorbed onto each cutting, calibration was done prior to inoculation. One sample (3 cuttings) was immersed in a graduated cylinder containing 10 ml of tap water for 10 s. The cuttings were taken out of the cylinder and the difference between the remaining water volume and the original volume showed the amount that was adsorbed on the cutting surfaces. The process was repeated three times, with fresh cuttings each time, and the average adsorption calculated as 0.5 ml per sample was used to estimate the amount of conidial suspension that could be adsorbed onto the sample surfaces.

The process for inoculation and assessment are illustrated in Figure 7.2. For inoculation, each composite sample was immersed in a sterile plastic tube containing 40 ml of the $2x10^4$/ml conidial suspension and the tube was gently inverted 10 times. At this conidial concentration, the estimated number of conidia adsorbed was 10,000 conidia per composite sample. For the negative control, cuttings were immersed in SROW. The cuttings were removed from the tubes using a sterile forceps, placed upright in a sterile rack and allowed to dry for 0, 1, 2 and 4 h in a sterile air flow inside a laminar flow hood. The 0 h treatment cuttings were washed immediately after inoculation. After each incubation period, cuttings were washed by placing them in sterile plastic tubes containing 40 ml of pre-cooled (4°C) SROW amended with Tween 80 and inverted 20 times. Washed samples were removed from the tubes using sterile forceps and placed upright in a sterile rack to air dry inside the laminar hood. No washing was done for the positive control cuttings. The resulting wash water samples were kept in an insulated box containing ice packs for approximately 30 min until centrifugation.

7.2.2.5 Conidial count by microscopy

Wash water samples from inoculated cuttings were centrifuged at 3,220 x *g* for 30 min at 4°C. The supernatant (39 ml) was gently pipetted from the tube without agitating the sample. The remaining 1 ml containing the pellet was gently shaken to re-suspend the pellet and the suspensions were used for the microscopy and plating.

Initially, conidia in the re-suspended samples were counted with a haemocytometer and a light microscope. When conidia were not detected using this method, counting was repeated by pipetting 10 µl of each suspension onto a microscope slide and counting all conidia with a light microscope at four replicate counts per sample (Figure 7.2).

7.2.2.6 Viability of conidia by plating assay

To determine the viability of the conidia washed from plant surfaces, 100 µl of the resuspended samples from each treatment was plated onto four replicate plates of PDA+Triton X. Plates were incubated at 25°C in a 12:12 light:dark regime for 7-10 days and single colonies typical of *N. luteum* were counted.

7.2.2.7 Confirmation of conidia recovery from wash samples by centrifugation

To confirm that the centrifugation method was able to recover the botryosphaeriaceous conidia from the different wash water samples, 100 µl of the resulting supernatants for each treatment were plated onto three replicate plates of PDA+Triton X following the methods described in Section 5.2.7.

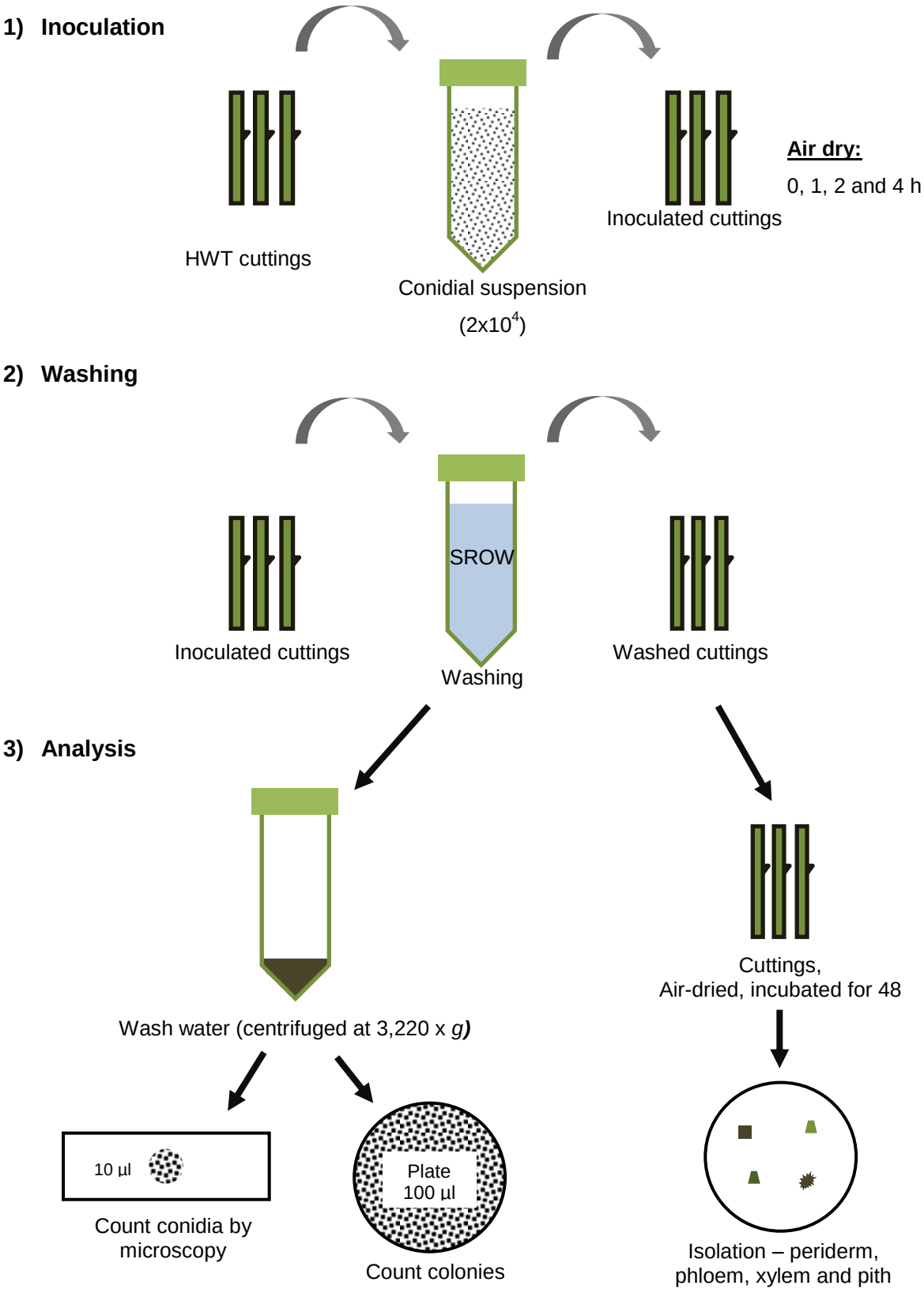

Figure 7.2 Process steps for the experiment on the retention and infection of *N. luteum* conidia on cane surfaces.

7.2.2.8 Differential isolations from inoculated cuttings

After air-drying, the washed cuttings were placed into new plastic tubes and stored at room temperature for 48 h before initial isolation. However, only one of the cuttings in each tube was used for isolation and the remaining samples were placed at 2°C for further isolation after 72 h. The 1 cm end of each section was cut off and discarded, then three segments (1 cm) from the top, middle and base of these cuttings were used for isolation. Tissue segments were washed with sterile water, blotted dry on sterile tissue and the bark (periderm and phloem) was separated from the wood and plated onto PDAC (Section 6.2.1.3). The wood tissue was further washed with SROW and blotted dry on sterile tissue and plated separately onto PDAC. Plate positions were marked based on tissue type (bark or wood) and sequential position from the cane (top, middle or base). Plates were incubated at 25°C for 5-7 days until botryosphaeriaceous colonies were observed.

The isolation was repeated for four randomly selected cuttings which had been inoculated and washed. These cuttings were selected from the 0, 4 h and negative control treatments after 72 h at 2°C. The periderm, secondary phloem, wood containing the xylem and the pith were separated from each tissue. After separating the outer tissues (periderm and phloem), the wood tissue was surface sterilised by dipping in 70% ethanol and flame-dried by passing through the Bunsen burner. The surface-sterilised wood was cut longitudinally and pith was scraped-off. The different organs of the same tissue sample were plated together on the same PDAC plate, marked to show position of each organ type, and incubated at 25°C for 5-7 days until botryosphaeriaceous colonies were observed.

7.2.2.9 Data analyses

All data from the different assays were analysed using SPSS Statistics 17. For Experiment 1, percentages of conidial germination, damaged conidia and colony forming units were calculated and one-way ANOVA was conducted for each storage temperature to determine significance of assessment periods. For the pathogenicity data (re-isolation distances), a one-way ANOVA was conducted to determine the significance of temperature treatments and control treatments for each storage time. Prior to ANOVA tests, all data were tested for homogeneity of variance using Levene's test at $P<0.05$ and when necessary arcsine transformation was done for percentage values to improve homogeneity of variance (Gomez & Gomez, 1984). Significance of differences between means was determined by making pairwise comparisons using LSD at 5% significance level. Germ tube length data were also analysed using the above tests to determine the rates of germ tube development over time.

For Experiment 2, all data from microscopy counts were converted to conidia per wash sample. The converted data for conidial count and colony counts from plating assays were tested for homogeneity and analysed by one-way ANOVA and pairwise comparisons by LSD as described above. For the isolation from inoculated washed cuttings, data were in binomial form (presence or absence of pathogen), thus, Pearson's Chi-square test of independence ($P \leq 0.05$) was used to determine if treatments were associated or independent of each other.

7.3 RESULTS

7.3.1 *Experiment 1.* Effects of temperature on conidial germination, viability and pathogenicity of *N. luteum* conidia

The statistical data for this Section are summarised in Appendix C.5. After 1 h incubation at ambient temperature, the germ tubes were observed developing, frequently at one end of the conidium without formation of appressoria (Figure 7.3A and B) but some conidia developed two germ tubes from the opposite ends of the conidia (Figure 7.3C). Germinated conidia frequently developed a single septum and occasionally two (Figure 7.3A-C). As germ tubes elongated, conidial septa and organelles disappeared (Figure 7.3D & E). Some ungerminated conidia became damaged after storage and the cells were empty (Figure 7.3F) or contents clumped .

7.3.1.1 Effects of storage at ambient temperature (22±2°C) on conidial development

Conidial germination and damage were significantly affected by storage duration at ambient temperature. For germination, 67% of conidia germinated after 2 h, peaked at 79.5% after 4 h but no further increase was observed up to 72 h ($P \leq 0.05$; Table 7.1, Appendix C.7.1).

Conidial damage at ambient temperature was also significantly affected by storage time ($P < 0.001$). Conidial structures remained normal after 2 and 4 h storage but by 24 h, 7.5% showed damage and the damaged conidia had increased to 14% after 72 h (Table 7.1). Germ tubes had developed into a network of hyphae after 72 h and so it was not possible to accurately assess germination and damaged conidia after this period (Figure 7.5).

Colonies were observed after 7 days for all incubation treatments. Viability based on colonies per plate decreased as storage times increased with 53.7% after 2 h that gradually decreased to 37.5% after 4 and 24 h (Table 7.1). However, variance between treatments was not homogenous ($P = 0.371$) and arcsine transformation did not improve variance homogeneity, thus, ANOVA was not done for this test.

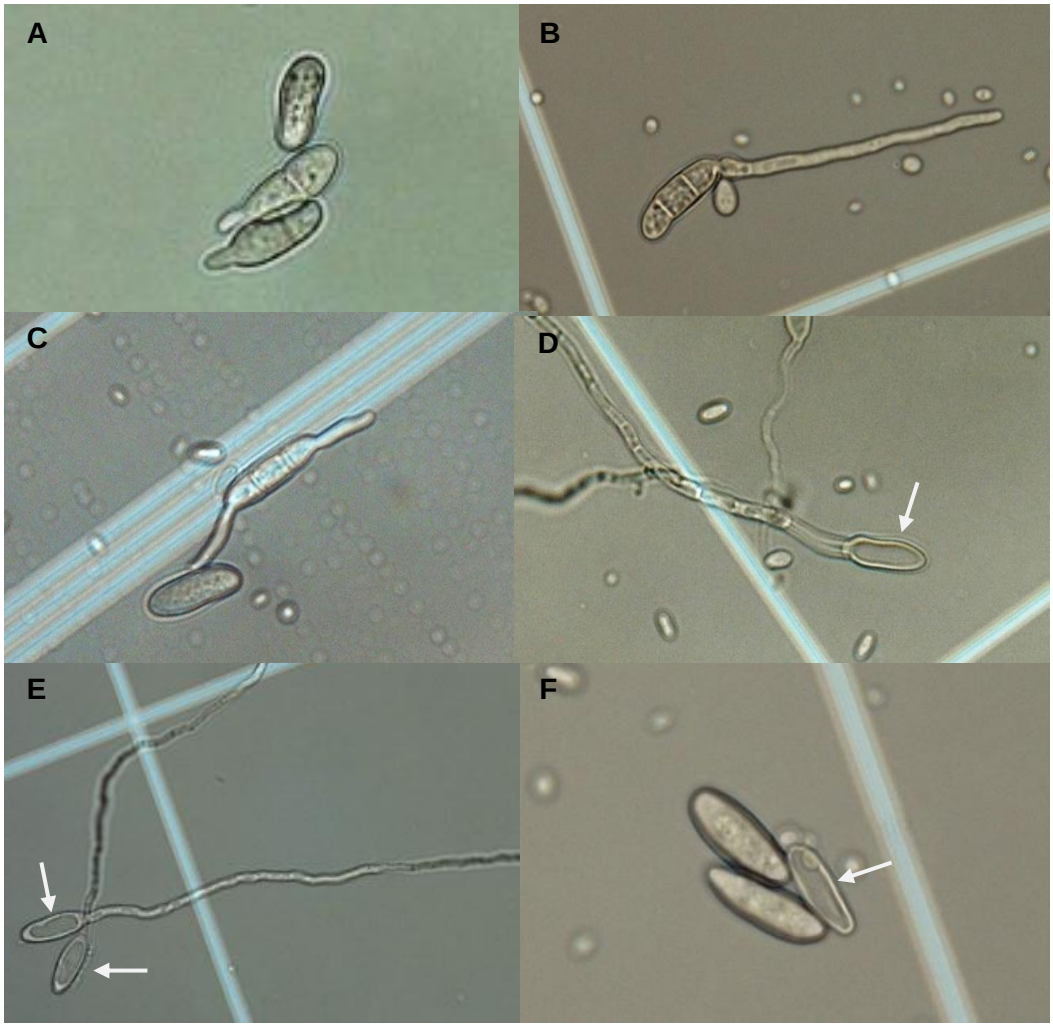

Figure 7.3 Characteristics of germinated *N. luteum* conidia (20-24 µm length). A) Conidia with one septum and germ tube; B) conidium with one germ tube and two septa; C) conidium with two germ tubes and septa; D) conidium with one germ tube and empty cell (arrow); E) conidia with single germ tubes and empty cells; D) ungerminated conidia and damaged conidium with empty cell (arrow).

Table 7.1 Germination, abnormality and viability of *N. luteum* conidia after different storage times at ambient temperature (22±2°C).

Storage time at ambient temperature	Conidium count		
	[A]Germination (%)	[A]Damaged (%)	[B]Viability (% cfu)
2 hours	67.0 a	0.0 a	53.7
4 hours	79.5 b	0.0 a	37.5
24 hours	76.2 b	7.5 b	37.5
48 hours	74.5 ab	9.0 b	nt
72 hours	73.3 ab	14.0 c	nt

[A] Means with different letters in columns are significantly different at $P \leq 0.05$ LSD.
[B] Variance of means among treatments was not homogenous ($P=0.371$).
nt – not tested

7.3.1.2 Effects of storage at ambient temperature on germ tube elongation

Average germ tube length increased significantly during 1 to 6 h incubation at ambient temperature ($P<0.001$; Appendix C.5.2). After 1 h, the mean germ tube length was 32.3 µm, which increased significantly at every assessment time with a mean germ tube length of 165 µm after 6 h (Figures 7.4 and 7.5). After 24 h, the germ tubes had developed into networks of hyphae and were no longer measured (Figure 7.5).

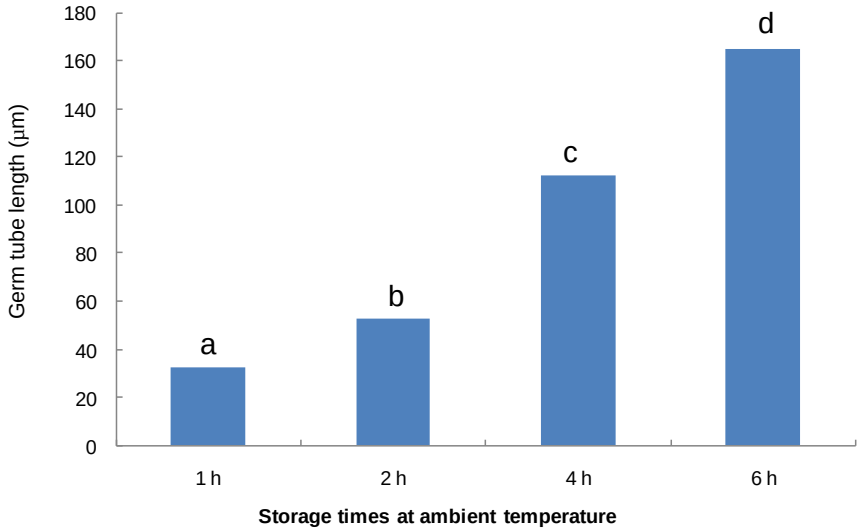

Figure 7.4 Mean germ tube lengths on *N. luteum* conidia stored for different times at ambient temperature. Bars for different parameters with different letters are significantly different at $P \leq 0.05$ LSD

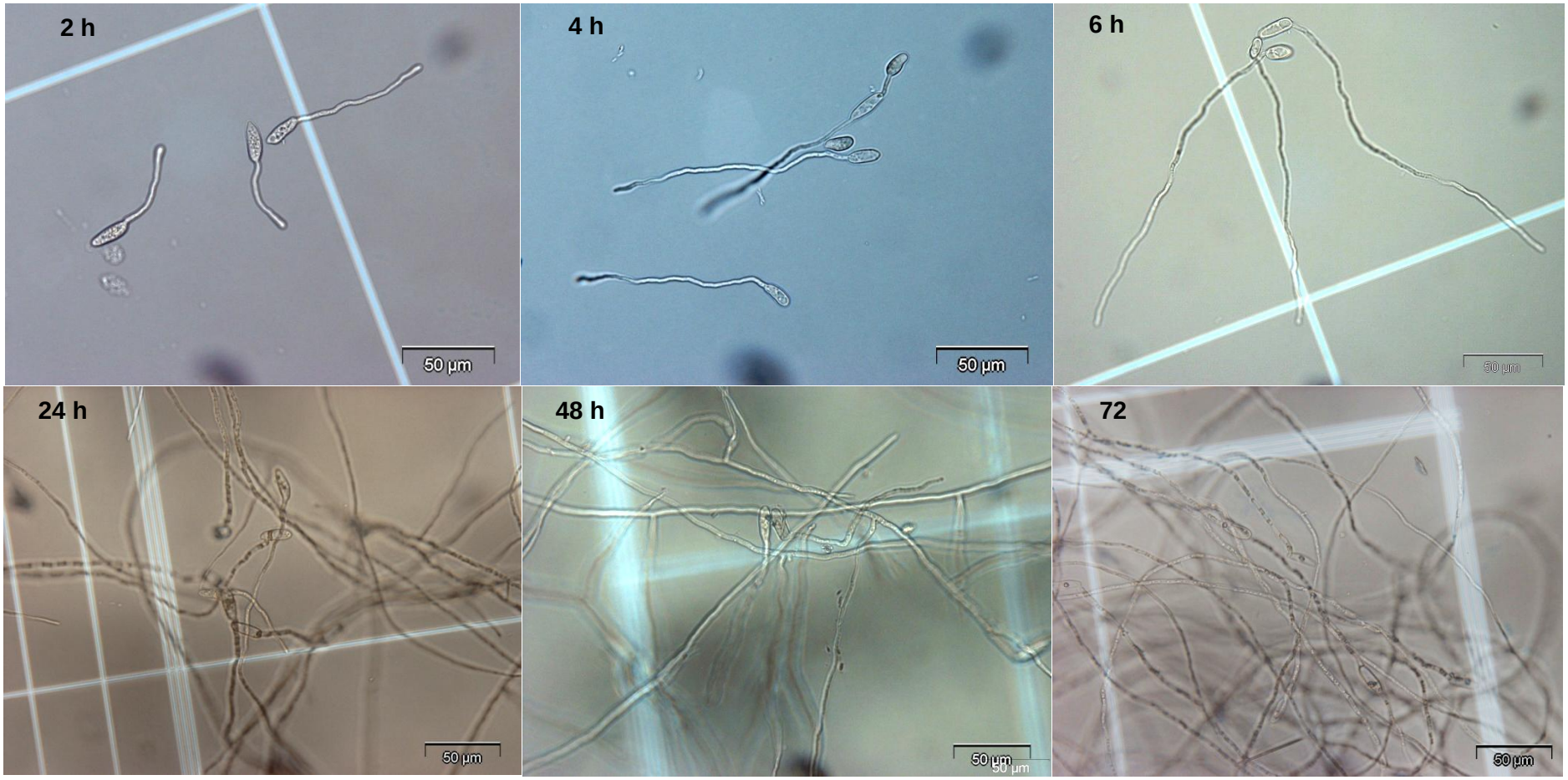

Figure 7.5 Germination and germ tube development of *N. luteum* conidia from 2 to 72 h at ambient temperature.

7.3.1.3 Effects of storage at 8°C on conidial development

Conidial germination was significantly affected by storage times at 8°C (P<0.001). No conidia had germinated after 4 h storage but 23% germinated after 24 h, and this number significantly increased (P<0.05) to 33.7% in 72 h and 55.2% after 2 weeks (Table 7.2). Conidial damage was also significantly affected (P<0.001; Appendix C.5.3) by different storage times at 8°C with 5.3% damaged after 48 h that significantly increased to 22 and 32.8% after 1 and 2 weeks, respectively (Table 7.2).

For plating assays, samples from 4, 24 and 48 h treatments were contaminated with yeasts and so colony counts were not attempted. For 72 h, 1 and 2 weeks plates, single colonies were observed after 7 days incubation for all three incubation-temperature treatment combinations. Mean conidial viability was highest at 71.3% after 72 h and lowest at 38.5% after 2 weeks (Table 7.2). However, variance for means among treatments was not homogenous (P=0.135; Appendix C.5.3) and arcsine transformation did not improve variance homogeneity, thus, ANOVA was not done for this test.

Table 7.2 Germination, abnormality and viability of *N. luteum* conidia after different storage times at 8°C.

Storage time at 8°C	Conidium count		
	[A]Germination (%)	[A]Damaged (%)	[B]Viability (% cfu)
2 hours	0.0 a	0.0 a	nt
4 hours	0.0 a	0.0 a	contaminated
24 hours	23.0 b	0.0 b	contaminated
48 hours	31.0 bc	5.3 b	contaminated
72 hours	33.7 c	9.5 b	71.3
1 week	27.3 bc	22.0 c	70.3
2 weeks	55.2 d	32.8 c	38.5

[A] Means within a column with different letters are significantly different at $P \leq 0.05$ LSD
[B] Variance of means among treatments was not homogenous (P=0.135)
nt – not tested

7.3.1.4 Effects of storage at 2°C on conidial development

Conidial germination was significantly affected by storage time at 2°C (P<0.001; Appendix C.5.4). No conidia had germinated after 24 h storage but 4.0% had germinated after 48 h and this number significantly increased (P≤0.05) to 8.8 and 17.0% after 2 and 3 weeks, respectively (Table 7.3). Conidial damage was also significantly affected by different storage time at 2°C (P<0.001; Appendix C.5.4) with 3.2% damaged after 48 h that significantly increased to 9% after 1 week and 17.8 and 18.8% after 2 and 3 weeks, respectively (Table 7.3).

For plating assays, colonies were observed after 7 days incubation for all incubation treatments. Conidial viability was significantly affected by storage times at 2°C (P<0.001; Appendix C.5.4) being significantly highest after 4 h to 72 h storage (84-86%) that significantly decreased after 2 to 3 weeks at 41% (Table 7.3).

Table 7.3 Germination, abnormality and viability of *N. luteum* conidia after different storage time at 2°C.

Storage time at 2°C	Conidia count		
	[A]Germination (%)	[A]Damaged (%)	[A]Viability (% cfu)
2 hours	0.0 a	0.0 a	nt
4 hours	0.0 a	0.0 a	86.0 a
24 hours	0.0 a	0.0 a	86.3 a
48 hours	4.0 b	3.3 a	84.0 a
72 hours	2.0 b	3.8 a	84.5 a
1 week	3.8 b	9.0 b	68.5 a
2 weeks	8.8 c	17.8 c	41.0 b
3 weeks	17.0 d	18.8 c	41.0 b

[A] Means within a column with different letters are significantly different at P≤0.05 LSD
nt – not tested

7.3.1.5 Pathogenicity test

External and internal lesions were not observed on inoculated plants although yellowing of leaves was observed after 3 weeks in all treatments, including the negative controls. However, *N. luteum* was consistently re-isolated from inoculated and positive control plants while no pathogens were isolated from any of the negative control plants.

Pathogenicity of conidia was significantly affected by storage temperatures and times. After 4 h storage, mean re-isolation distances did not differ significantly (*P*=0.273; Appendix C.5.5.) between storage treatments and temperatures (Table 7.4). However, after 24 h, there was significant effect of storage treatments (*P*=0.001) with all inoculated treatments having longer mean re-isolation distances (0.8-1.0 mm) that differed significantly from the negative control (Table 7.4). After 48 h storage, there was a significant effect of storage treatments (*P*=0.019) with mean re-isolation distances for 2°C treatment being significantly longer (12.0 mm) than all other treatments (*P*≤0.05). After 72 h storage, there was also a significant effect of storage treatments (*P*≤0.001) with the mean re-isolation distances for 2°C being significantly longer (28.3 mm) from all other treatments while the 8°C treatment was significantly longer (10.3 mm) than the control treatments. After 1 week, there was also a significant effect of storage treatments (*P*=0.029) with mean re-isolation distances for positive control (1.3 mm) being significantly longer for all other treatments while no significant differences were observed for all treatments after 2 weeks (*P*=0.108; Table 7.4).

Table 7.4 Pathogenicity of *N. luteum* conidia stored at different temperatures and times based on the mean re-isolation distances on inoculated attached green shoots.

Storage temperature	*Re-isolation distances (mm)					
	Storage times					
	4 h	24h	48h	72h	1 week	2 weeks
Ambient	0.5 a	1.0 a	1.0 b	nt	nt	nt
2°C	0.7 a	1.0 a	12.0 a	28.3 a	0.5 b	1.0 a
8°C	1.0 a	1.0 a	1.0 b	10.3 b	0.5 b	0.3 a
+ Control	1.8 a	0.8 a	2.0 b	1.0 c	1.3 a	2.3 a
- Control	0.0 a	0.0 b	0.0 b	0.0 c	0.0 b	0.0 a

* Means within a column with different letters are significantly different at *P*≤0.05 LSD
nt – not tested

7.3.1.6 Germinability of conidia after cold storage

7.3.1.6.1 Storage at 8°C followed by incubation at ambient temperature

After 24 h storage at 8°C, 23% of the conidia had germinated and this proportion increased to 54.7% after a further 6 h at ambient temperature (Figure 7.6). Variance of treatment means was not homogenous including the transformed values (*P*=0.387; Appendix C.5.7) and ANOVA was not conducted for this treatment. After 48 h at 8°C, 30% of the conidia had

germinated and their proportion significantly increased (*P*=0.009; Appendix C.5.7) to 59.5% after 6 h at ambient temperature (Figure 7.8). After 72 h at 8°C, 33.7% of the conidia had germinated and gradually increased to 38.2% after 6 h at ambient temperature (Figure 7.6). After 1 week, 25% of the conidia had germinated and this proportion gradually increased to 44.7% after 6 h at ambient temperature (Figure 7.6). However, variance of treatment means for 72 h and 1 week incubation treatments were not homogenous (*P*=0.276 and *P*=0.461, respectively; Appendix C.5.7) and ANOVA was not conducted for these treatments.

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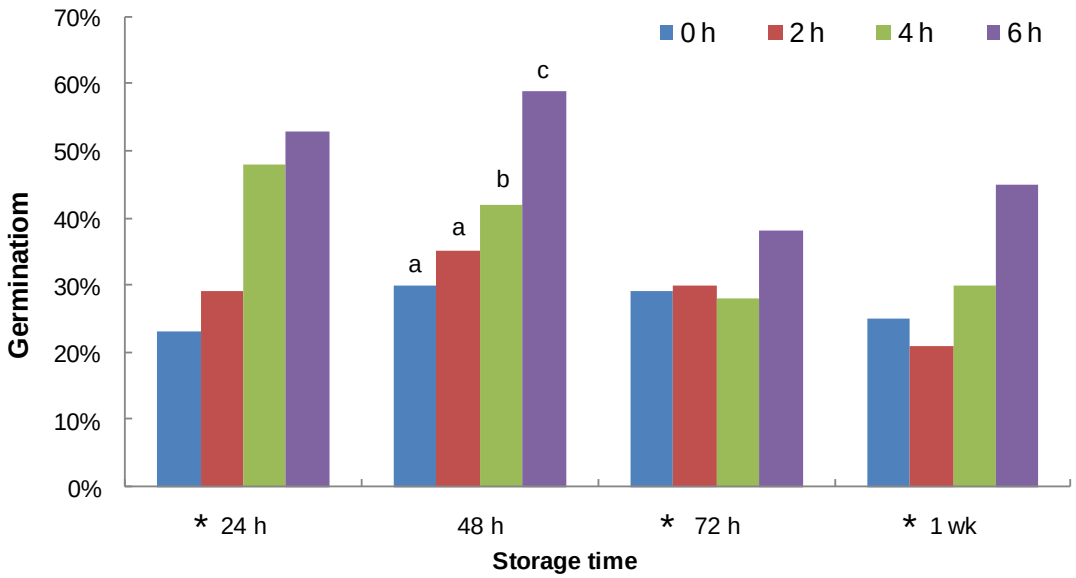

Figure 7.6 Germination of conidia stored at 8°C for different durations and transferred to ambient temperature for 0-6 h. Within different storage durations bars with different letters are significantly different at *P*≤0.05 LSD. Asterisks indicate storage durations for which mean variances were not homogenous and pairwise comparisons were not made.

7.3.1.6.2 Storage at 2°C followed by incubation at ambient temperature

After 24 h at 2°C storage, none of the conidia had germinated, but after 2 h at ambient temperature, 5.2% of the conidia had germinated and this proportion significantly increased (*P*<0.001; Appendix C.5.6) to 25.7% after a further 6 h at ambient temperature (Figure 7.7). After 48 h storage at 2°C, 4% of the conidia had germinated and this proportion significantly increased (*P*<0.001; Appendix C.5.6) after 4 h with the highest germination of 50.7% after 6 h at ambient temperature (Figure 7.7). After 72 h storage at 2°C, the sample was found to be contaminated after the initial count and was not included in the analysis. After 1 week storage at 2°C, 3.7% of the conidia had germinated and this proportion increased after each

assessment time with highest germination of 69.5% after 6 h at ambient temperature. However, variance of treatment means was not homogenous (*P*=0.109) and ANOVA was not made for this treatment. After 2 weeks storage at 2°C, 8.7% of the conidia had germinated and this proportion also significantly increased (*P*<0.001) after 4 h, with highest germination of 45.5% after 6 h at ambient temperature (Figure 7.7). After 3 weeks storage, 17% of the conidia had germinated and the proportion also increased to 66.5% after 6 h at ambient temperature but ANOVA was not conducted because the variance of treatments means was not homogenous (*P*=0.474)

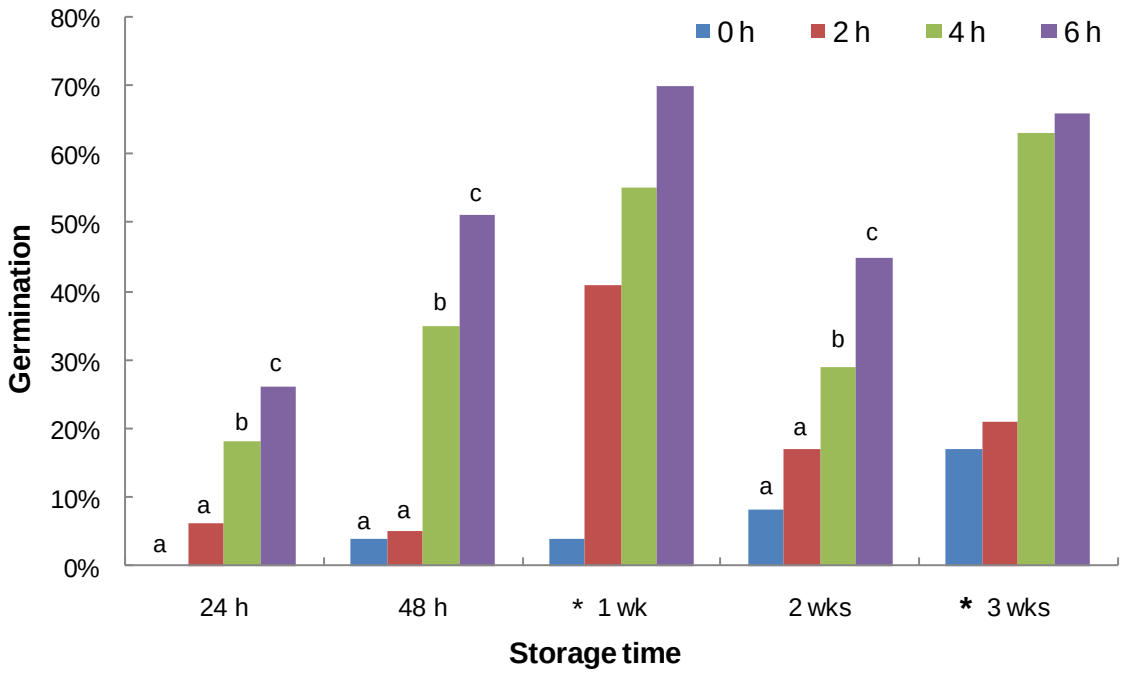

Figure 7.7 Germination of conidia stored at 2°C at different storage times and transferred to ambient temperature. Bars for different time with different letters are significantly different at *P*≤0.05 LSD. Asterisks indicate storage times which mean variances were not homogenous and pairwise comparisons were not made.

7.3.2 ***Experiment 2.*** Retention and infection by *N. luteum* conidia on cutting surfaces

7.3.2.1 Conidial count

Although 10,000 conidia were calculated as being inoculated on one composite sample, the initial count using the haemocytometer detected no conidia in wash samples. Since this method could only enumerate ≥2000 spores/ml of conidial suspension, they were counted within 10 µl of the samples pipetted onto a clean slide under a light microscope.

The number of conidia washed from the surfaces of the cuttings was significantly affected by incubation periods prior to washing (P<0.001; Appendix C.5.8). After 0 h, the mean number of conidia recovered from the composite sample was 86.2 conidia which was significantly lower (P≤0.05) than the number of conidia used for inoculation (10,000/sample) but was significantly higher than for other incubation periods and the negative control (Table 7.5). There was no significant difference in conidial recovery for cuttings washed 1, 2 and 4 h after inoculation and no conidia were found in any of the negative control samples (Table 7.5).

Table 7.5 Number of conidia washed from inoculated cuttings surfaces based on microscopy (in 500 µl) and plating assays (100 µl)

Incubation prior to washing	*Conidia/sample	*cfu/plate
Inoculum concentration	10,000 a	nc
0 h	862.5 b	28.2 a
1 h	287.5 c	12.0 b
2 h	268.7 c	4.0 c
4 h	106.2 c	1.8 cd
Negative control	0 d	0 d

* Means within a column with different letters are significantly different at *P≤0.05* LSD
nc – not counted

7.3.2.2 Viability of conidia washed from the surface of the canes

Colonies characteristic of *N. luteum* were observed from all treatment samples after 7 days incubation while no colonies were observed in any of the negative control plates. The numbers of cfu/plate were also significantly affected by incubation periods prior to washing (P<0.001; Appendix C.5.8). After 0 h, the mean number of colonies per sample was 28.2 which was significantly higher than for all other incubation periods before washing (Table 7.5; Figure 7.8B). The number of colonies significantly decreased with longer incubation times with the 4 h incubation having the lowest number of colonies at 1.8 colonies/plate (Table 7.5; Figure 7.8C to E). No colonies were observed in any of the negative control plates (Figure 7.8F). The viability of conidia in the original suspension used for inoculation was confirmed by a great number of colonies on the plates (Figure 7.8A). The colonies overlapped with each other and counting was not possible on these plates.

7.3.2.3 Confirmation of conidia recovery from wash samples by centrifugation

No single colonies typical of *N. luteum* were observed in any of the plated supernatants for all washing treatments (data not shown).

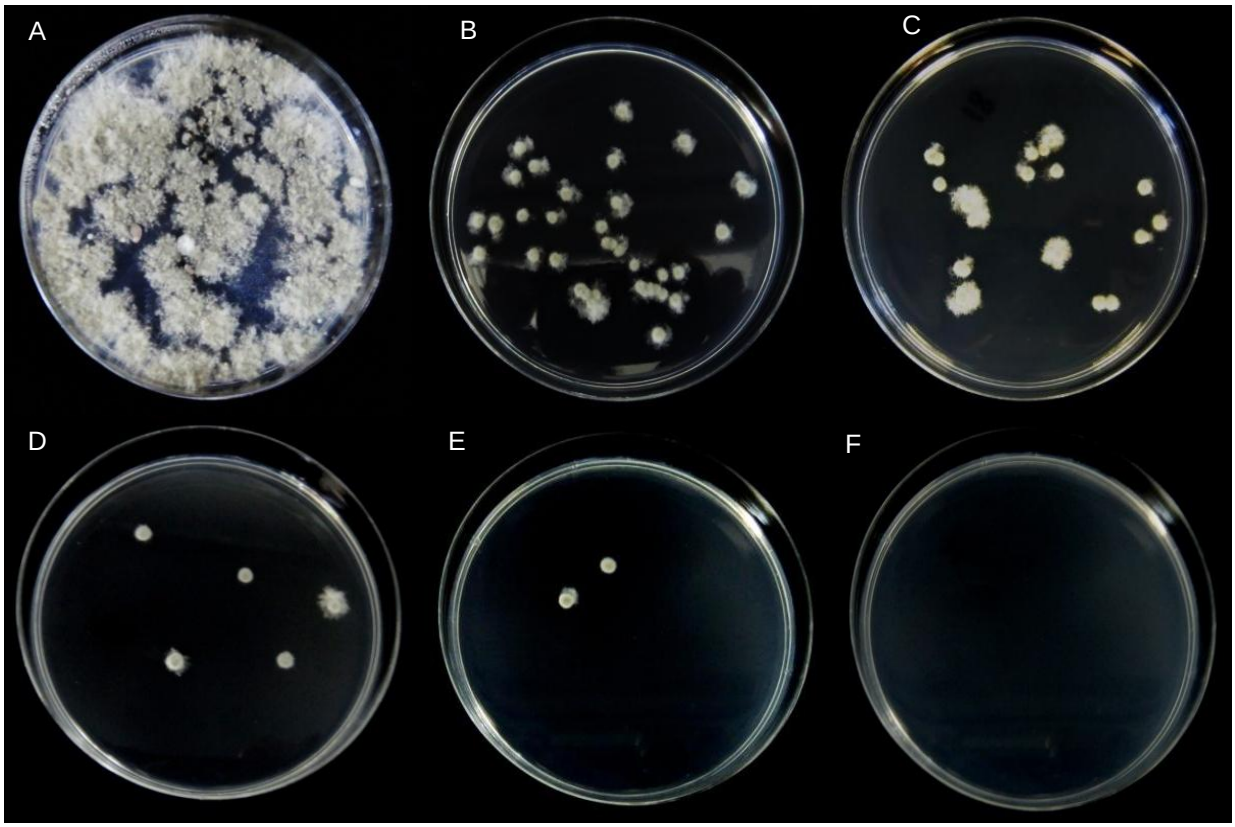

Figure 7.8 Colonies of *N. luteum* on PDA+Triton X plates which represents plating out the A) conidial suspension ($2x10^4$/ml) used for inoculation; B-E) wash liquids from washing inoculated cuttings after 0, 1, 2 and 4 h incubation and F) uninoculated cuttings.

7.3.2.4 Differential isolation from washed cuttings

For the initial isolations, colonies typical of *N. luteum* were observed growing from plated bark and wood portions taken from all parts of the cuttings from all incubation times prior to washing while no colonies were observed from the uninoculated cuttings (Figure 7.9).

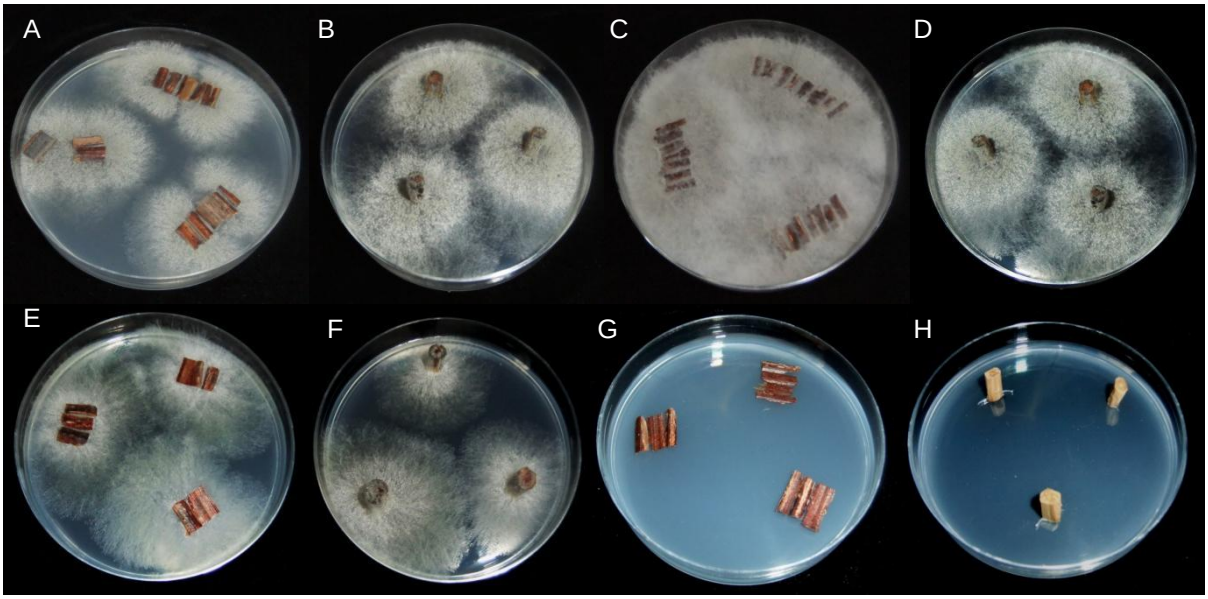

Figure 7.9 Isolation of *N. luteum* from bark and wood of inoculated cuttings after 0 and 4 h incubation prior to washing. A-B) 0 h – bark and wood; C-D) 4 h – bark and wood; E-F) positive control – bark and wood; and G-H) uninoculated control bark and wood.

For the second set of isolations from 0 and 4 h treatments (Table 7.6), *N. luteum* colonies were consistently observed in all phloem, xylem and periderm samples but appeared at different times; colonies from the phloem and xylem were observed after 3 days incubation while colonies from periderm were observed after 6 days (Figure 7.10). No *N. luteum* colonies were observed in any of the pith samples for all treatments, nor in any of the tissue samples from the negative controls (Table 7.6). A Pearson Chi-square test indicated significant association (P<0.001; Appendix C.5.9) between tissue type and *N. luteum* infections (Table 7.6).

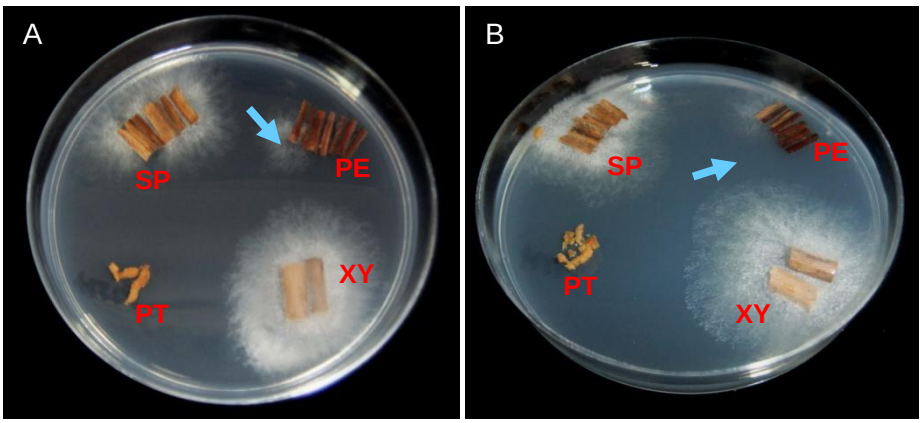

Figure 7.10 Isolation of *N. luteum* inoculated cuttings incubated and washed after A) 0 h and B) 4 h. Legends: PE - periderm, SP – secondary phloem, XY - xylem and PT – pith. Colonies from periderm (arrow) are smaller than from secondary phloem and xylem, while no growth was observed from the pith tissues.

Table 7.6 Incidence of infection in different tissue types from cuttings inoculated with *N. luteum* conidia and washed after 0 and 4 h incubation prior to isolation.

Tissue	No. of samples	*Tissues infected (n=4)		
		0 h	4 h	Negative control
Periderm	4	4	4	0
Phloem	4	4	4	0
Xylem	4	4	4	0
Pith	4	0	0	0

*Significant using Pearson Chi-square test at *P*=0.001

7.3.3 DISCUSSION

In this study, Experiment 1 represented the first comprehensive investigation on the effects of cold storage on the survival, viability and pathogenicity of *N. luteum* conidia on grapevines. Experiment 2 was also the first study to demonstrate that *N. luteum* conidia could adhere to cutting surfaces after washing and cause infections.

Conidia were able to germinate while suspended in sterile water, indicating that nutrients are not essential for germination of these botryosphaeriaceous conidia and that they can survive in relatively anaerobic conditions for a period of time. This study indicated that conidial germination was triggered by the presence of water while the rate was influenced by temperature. In contrast, Osherov and May (2001), reported that the conidia of many species need to be supplied with appropriate nutrients such as sugars, amino acids and inorganic salts in the presence of air and water; if not supplied with these compounds few or no conidia germinate and/or infect their host successfully. The results in this study were consistent with those of Arauz and Sutton (1989) and Sutton and Arauz (1991) who also found that temperature and moisture were the two most important factors that influenced conidial germination of *B. dothidea* and *D. seriata* from apples. They also reported that interruption of wetness periods by dry periods inhibited germ tube elongation and reduced the infection of apples by *D. seriata* (Arauz & Sutton, 1990).

The germination rate of *N. luteum* conidia observed at ambient temperature in this study were in a similar range (67-79% after 4 h) to those observed for other botryosphaeriaceous species by Urbez-Torres *et al.* (2010a) and Arauz and Sutton (1989). At 8°C, the germination rate of *N. luteum* conidia after 24 h observed in this study was also similar to the reported rates for *B. dothidea* and *D. seriata* (Arauz & Sutton, 1989; Sutton & Arauz, 1991). None of

the above-cited studies had investigated conidial germination at temperatures lower than 4°C. This study had showed that some conidia of *N. luteum* had germinated after 48 h at 2°C and that the number gradually increased to 17% after three weeks. The conidia remained viable for at least 3 weeks in cold storage since germination resumed when conidia were exposed to ambient temperature. Clearly the conidia of *N. luteum* follow the dictum of Isaac (1998), who concluded that the spores that can germinate rapidly often have short-term dormancy and can resume development under favourable conditions.

Furthermore, this study demonstrated the viability of the *N. luteum* conidia based on the colony forming units produced from each treatment. According to Louis and Cooke (1985), germinability and viability of conidia should be treated differently because low germinability within a spore sample does not necessarily indicate low viability, but may mean that the specified incubation period is too short for germination to occur. Therefore, this study had demonstrated that while germination was low at 2°C, even after 72 h, these conidia were able to form a high number of colonies (~80%) when subsequently incubated at ambient temperature. The viability, however, decreased with longer storage times.

Another significant finding from this study was that damaged or abnormal conidia were observed after 24 and 48 h for ambient and cold stored conidia, respectively, with the lowest damage rate at 2°C which gradually increased with longer storage times. The degradation of *N. luteum* conidia may have been due to the anaerobic conditions and lack of nutrients in the water suspensions. For most fungi, ungerminated conidia contain stored nutrients such as glycogen, lipids and phospholipids needed for ATP synthesis and maintenance of vital functions (Isaac, 1998; Gindro & Pezet, 2001). In this study, the cold temperatures may have slowed the rate of conidial metabolism but the effect of wetting which normally initiates germination and the anaerobic conditions in the water suspensions were clearly damaging. In the cold storage conditions of nurseries, the botryosphaeriaceous conidia would likely be on plant surfaces where there is some air, even in the tight packs normally used for storage. Since cold storage of cuttings and plant materials in nurseries frequently lasts for 2-3 months (Waite & Morton, 2007), it is important to investigate the survival of botryosphaeriaceous conidia on plant surfaces after such long cold storage periods.

Low temperature is widely used to extend storage life of fresh horticultural products by maintaining product quality and reducing decay due to post-harvest pathogens (Tian & Bertolini, 1996). However, many of these fungi were found to be cold-resistant. For example, Wyatt and Parish (1995), observed that conidia of *Cladosporium cladosporoides*, *Penicillium digitatum* and arthrospores of a *Geotrichum* sp. could germinate at 0°C. Tian and Bertollini (1996) also observed conidial germination of *Botrytis allii* and *P. hirsutum* below 0°C. In

grapevine nurseries, cold storage is also essential to preserve the cuttings and young grafted plants until they are used for propagation or sold to vineyards, respectively (Waite & Morton, 2007). Cold storage temperatures vary for different countries with 1-2°C in Australia (Waite & Morton, 2007), 2-3°C in Italy (Zanzotto *et al.,* 2001), 5°C in New Zealand (Whiteman, 2004) and 5-6°C in Spain (Gimenez-Jaime *et al.*, 2006; Aroca *et al.*, 2010). The survival of botryosphaeriaceous conidia in cold temperatures as shown in this study has great implications in the nursery system since these surface propagules can survive and infect plants and propagation materials while in cold storage.

In this study, no obvious symptoms were observed in any of the attached green shoots 21 days after inoculation with any of the stored *N. luteum* conidia and all plants looked similar to the negative control plants. The yellowing of leaves in all plants may have been due to the lack of nutrients available in the pumice used for growing the plants. Isolation from the shoots, however, showed that all plants inoculated with either fresh or stored conidia of *N. luteum* were infected while all negative control plants were free of the pathogen. This indicates that the pathogen was able to colonise and move internally in the tissues without causing any visible symptoms which was also reported by Amponsah (2011) when inoculating *N. luteum* on 2-year-old potted vines.

The re-isolation distances for conidia stored at 2°C for 48 and 72 h were significantly longer than for all other treatments including those for freshly-harvested conidia. Similarly, re-isolation distances for conidia stored at 8°C for 72 h were also significantly longer than for the freshly-harvested conidia. It is possible that after 48 to 72 h of cold storage, the cold-stored *N. luteum* conidia had completed some germination process (e.g. nutrient mobilisation) and so were able to colonise the tissues faster than those just harvested. To date, no information has been found on increased pathogenicity of fungal pathogens exposed to cold storage temperatures. The reduced infection rates for conidia that were stored for more than 72 h, however, may have been associated with damage of the long germ tubes of germinated conidia during inoculation. Even so, this study has shown that conidia stored at 2°C were able infect plants for at least 3 weeks and that cold storage of cuttings in nurseries may not totally protect them from botryosphaeriaceous infections. However, this study only used one isolate of *N. luteum* for all assays. Since pathogenicity and genetic variability studies presented in Chapter 3 showed that the pathogenicity of different *N. luteum* isolates was highly variable, the above hypothesis needs further confirmation using more botryosphaeriaceous isolates of two or more species. The potential for infection by conidia at low temperatures should also be investigated to determine if they can successfully infect vines during cold winter months.

Germination of some botryosphaeriaceous conidia has been well studied and was found to occur at a broad range of temperatures. Sutton and Arauz (1991) observed conidial germination of *B. dothidea* at 8 to 32°C after 24 h in free water but the optimum temperature for germination was 26.7-29.5°C while no germination was observed at 4°C after 24 h. For *D. seriata*, Arauz and Sutton (1989) observed 80% conidial germination after 4 h at 16 to 32°C, and 23 and 0% germination at 8 and 4°C, respectively, after 12 h. Urbez-Torres *et al.* (2010a) also observed 5.2 and 26.9% conidial germination at 5°C after 24 h for *B. dothidea* and *N. parvum*, respectively, although the optimum temperature at which 50% germination was reached in the shortest incubation time was 25°C for *B. dothidea* and 30°C for *N. parvum*. No conidial germination for either *Diplodia* or *Lasiodiplodia* species were observed at 5°C after 24 h, while high conidial germination was observed at 40°C. The optimum temperature for conidial germination for *D. seriata* was 30°C and pigmented *L. theobromae* conidia was 40°C. While the above-cited studies determined the optimum temperature for germination of the different botryosphaeriaceous conidia, none of these studies investigated if these conidia could survive and cause infections after longer periods at low temperatures. The study reported here has shown that at least one of the botryosphaeriaceous species can germinate slowly at 2°C, so further studies should be done with a wider range of species at 0-5°C to indicate potential for disease development at winter temperatures during pruning and harvesting of canes for nursery propagation.

Conidial germination of *N. luteum* at ambient temperature was observed after only 1 h in this study. The conidia produced one or two germ tubes at the polar ends of the cell and their lengths increased rapidly at ambient temperature, with mean germ tube length of 164 μm in 6 h. Formation of appressoria was not observed in any of the germinating conidia, an observation also made by Amponsah (2009c) during histopathology studies using SEM. However, *B. dothidea* was observed to form appressoria from the two or more germ tubes (Brown & Hendrix, 1981) when on plant surfaces, and to enter through natural openings such as lenticels and surface cracks (Kim *et al.*, 1999). Formation of appressoria by other botryosphaeriaceous species has not been reported to date. The observed germ tube elongation of *N. luteum* in this study was similar to that reported for *D. seriata,* which had a mean length of 122 μm after 4 h (Arauz & Sutton, 1989). This germination behaviour is important in understanding the mode of infection of this pathogen group. The number of germ tubes produced and the rate of elongation under favourable conditions by botryosphaeriaceous species for example may influence the rate of infections of the host. The appressorial formation also facilitates the attachment and penetration of the host by the pathogen (Agrios, 2005) and the lack of formation of this structure in *N. luteum* may explain why pruning wounds are considered the primary infection courts for botryosphaeriaceous species.

In the second experiment of this chapter, only 8% of the *N. luteum* conidia were recovered out of the 10,000 conidia inoculated when cuttings were washed immediately after inoculation. The recovery further decreased when cuttings were incubated from 1 to 4 h before washing. The low recovery of the conidia indicated that the propagules were able to adhere very quickly to cuttings surfaces. Since Experiment 1 in this chapter had also shown that more than 60% of *N. luteum* conidia were able to germinate after 2 h at ambient temperature, it further suggested that the conidia that adhered were probably able to colonise the cuttings during the incubation period.

No colonies of *N. luteum* were recovered from the supernatants for all treatments, indicating that the centrifugation method used was effective in the recovery of *N. luteum* conidia from all samples. However, it is also possible that some of the conidia were lost when the suspension ran off from the cutting surfaces during incubation in the laminar flow hood or through being attached to the walls of the tubes. This could have been determined using M2R Calcofluor white to stain the fungal cells and to visualise them with an epi-fluorescent microscope. In a study by Zelinger *et al.* (2006) Calcofluor white was used to stain and count the number of *Stagonospora nodorum* conidia that adhered onto plant surfaces. While counting of conidia from the wash water may not be accurate in quantifying the actual number of conidia that adhered in the cuttings used in this study, the differential isolations from cuttings had confirmed that these conidia had successfully colonised and caused infections. According to Zelinger *et al.* (2006), attachment and adhesion of conidia and germ tubes of fungal plant pathogens to the host surface are crucial in the establishment of disease. Spores of many fungal species are reported to be capable of rapid, non-specific attachments which often occur before germ tube emergence, preventing spores from being blown or washed from host surfaces before infection can take place (Braun & Howard, 1994). It is, therefore, possible that the rapid adhesion of *N. luteum* conidia may also be related to their production in a slimy mass which is exuded from the pycnidia, and which according to Slippers and Wingfield, (2007) play an important role in the adhesion to host surfaces. The removal of the spore matrix from 100-day-old conidia of *Colletotrichum musae* was found to depress the germination rate and reduce pathogenicity to bananas but these traits were restored when the matrix was restored in the spore suspensions (Mondal & Parbery, 1992). Therefore, the role of the slimy matrix on conidial germination, adhesion and infection of botryosphaeriaceous species could also be investigated following the methods of the above-cited study.

In this study, it was further observed that the inoculated pathogen was able to penetrate beyond the cutting surface within 48 to 72 h since all periderm, phloem and xylem tissues of inoculated cuttings were infected. Although the 10 cm end of the segments provided open wounds that may have allowed the pathogen to enter, the central segment infections were likely to have been through the bark. This infection route from bark to wood was similar to the results in the sequential isolations from the entire shoots (Section 6.3.2) where some of the bark and wood infections of the same tissue segment were caused by the same genotype. These results of the two experiments are of value to the grapevine nurseries as they demonstrate that the washing of cuttings during hydration does not effectively remove all the botryosphaeriaceous propagules present on the cutting surfaces. It also indicates that any conidia that were washed-off could infect clean cuttings in the hydration tanks. However, these hypotheses need further confirmation. A study on the surface morphology of the botryosphaeriaceous conidia and the biochemistry involved in the adhesion and colonisation similar to the study of *B. dothidea* on apple tissues (Kim *et al.*, 2001; 2004) will give a better understanding of the mode of infection on cutting surfaces. A washing experiment using commercial-length HWT one-year-old cuttings surface-inoculated with several botryosphaeriaceous species could confirm the importance of this infection mechanism. Similar studies using immature stems may also indicate whether the botryosphaeriaceous conidia can adhere to young living tissues and give better understanding on the infection process during different stages of shoot growth.

Chapter 8
Concluding discussion

The botryosphaeriaceous species that are considered serious pathogens of grapevines worldwide were also found to be widespread pathogens in New Zealand vineyards. The 2007 sampling of symptomatic vines from 43 vineyards around the country by Baskarathevan (2011) showed that nine botryosphaeriaceous species were present in vineyards with *N. parvum* being the most prevalent. The widespread occurrence of this disease in young vines within vineyards led to questions about whether the infections found in the vineyards originated from nurseries.

The overall aim of this study was to investigate the prevalence of botryosphaeriaceous infections in New Zealand grapevine nurseries, their sources of inoculum and potential infection pathways. In this chapter, the key findings of this research are summarised and used as a basis for discussion of their infection and spread methods in the nursery system. These are essential for the development of control strategies which are also proposed, in addition to future studies to confirm the findings of this research.

8.1 Prevalence of botryosphaeriaceous infection in nurseries

The 2008 nursery sampling described here was the first comprehensive study to investigate the presence of botryosphaeriaceous infections in the nurseries. Since there is little information regarding the symptoms of this disease in nursery plants, symptomatic and non-symptomatic grafted plants and propagation materials were assessed. This research was purely exploratory in order to establish the presence or absence of botryosphaeriaceous infections. However, this study provided conclusive evidence that botryosphaeriaceous infections were widespread, being present in eight out of nine nurseries with infection incidence ranging from 5 to 63%. From the 311 plants and propagation materials analysed, 23% were positive for botryosphaeriaceous infection, with failed grafted plants having the highest infection incidence of 33%, followed by Grade 1 plants (28%), rootstock cuttings (19%), scion cuttings (17%) and Grade 2 plants (7%). For grafted plants, the largest proportion of botryosphaeriaceous species (49%) were isolated from near the graft unions, which suggested that infection might occur during propagation processes. Since infections on rootstocks and scion cuttings were mostly present in the middle and basal parts, this suggested the potential for direct endophytic infection from mothervines.

From the 120 isolates recovered, a total of 115 isolates were accurately identified by morphological and molecular methods as *N. luteum* (57%), *N. parvum* (18%), *N. australe* (8%), *D. mutila* (8%), *B. dothidea* (5%), *D. seriata* (3%), and one novel isolate *N. macroclavatum*. The high proportion of Grade 1 plants with infections indicated that this disease could be carried over to new vineyards. The high proportion of rootstock and scion cuttings with infection also indicated that these infected plant materials can be a major source of infections for grafted plants. However, a true approximation of disease levels in the nurseries could only be confirmed with a complete survey that uses random sampling, with uniform sample types and sizes. This would provide a more reliable measure of the prevalence and distribution of this disease in the nurseries.

All seven botryosphaeriaceous species from the nurseries were found to be pathogenic on one-year-old Sauvignon blanc rooted canes and green shoots but pathogenicity differed significantly between species and isolates within a species, with *N. parvum*, *N. luteum* and *N. australe* being the three most pathogenic species in both assays. Except for *D. seriata,* which appeared to be necrotrophic, the six other botryosphaeriaceous species were shown to move endophytically beyond the lesions. The endophytic behaviour of these pathogens may explain the high proportion of infections found in Grade 1 plants and asymptomatic propagation materials. Susceptibility tests using three isolates each of *N. luteum*, *N. parvum* and *N. australe* against the most commonly used scion and rootstock varieties (six of each) found that all varieties were susceptible to the three species, with 5C and SO4 being the most susceptible of the rootstock varieties, and Merlot and Pinot noir being the most susceptible of the scions.

Genetic variability analysis showed that *N. luteum* isolates of different pathotypes were genetically diverse with intra- and inter-plant and nursery variability, but no association between genotype and pathogenicity was observed. The high genetic diversity of *N. luteum* populations in the nurseries may be partially due to the use of propagation materials from different sources since the scion materials of some nurseries are often collected from different vineyards during pruning. This could be determined by a phylogeny study using many isolates collected from canes in many vineyards.

Clearly, these fungi are ubiquitous in the nursery systems in New Zealand and probably throughout the grape-growing regions of the world, although there is only limited evidence of it from other regions. They were shown to be as pathogenic as the vineyard isolates on all common varieties, since they caused similar symptoms to those described in another New Zealand study by Amponsah *et al.* (2011). Therefore, management requires us to focus on the stages in the nursery disease cycle, with a view to developing methods of managing the disease problem.

8.2 Possible infection pathways in the nursery system

On the basis of the overall results achieved by this study, the likely infection pathways of botryosphaeriaceous in the different stages of the nursery system, with the supporting evidence obtained from this research, are presented below.

8.2.1 Mothervine blocks

The surfaces of cuttings from mothervines and dead grapevine materials were shown to contain abundant viable conidia and to be present in rainwater run-off, as shown by molecular identification, and so are likely to be splash-dispersed during significant rain events. These conidia can rapidly attach to the cane surface and colonise the bark and phloem but may remain in the surface tissues and not cause any symptoms until winter dormancy or after the shoots are harvested. Genotyping of the different botryosphaeriaceous isolates recovered from mothervine trunks, cutting surfaces and wood tissues showed that multiple species and isolates were involved, with more infections found in the bark than in the canes, which suggested that the bark might contain latent infections. Conidia could also infect the wood of mothervine shoots through the trimming wounds made during summer periods and through the wounds made when cuttings are harvested.

The botryosphaeriaceous conidia were shown to adhere rapidly to cutting surfaces, from where they were able to colonise the periderm, phloem and xylem. These pathogens, therefore, can colonise the vine tissues and plant trimmings and produce more conidia to be dispersed throughout the block. The *N. luteum* conidia were shown to remain viable and germinate at cold temperatures and could infect grapevines as effectively as freshly-harvested conidia indicating potential for disease development at winter temperatures during harvesting of canes for nursery propagation.

8.2.2 Harvesting and cold storage of propagation materials

Dormant canes with surface or internal infections are harvested during winter, washed, hydrated and stored at 2-5°C for 2 to 3 months until processed. Since abundant surface conidia were washed from the cuttings surface (Chapter 5), these surface propagules can contaminate hydration tanks, germinate, adhere and infect clean cuttings during hydration or storage, from where they infect through bark or open wounds. This study provided clear evidence that surface propagules were retained during washing and resulted in infections. There was also evidence to show that the conidia could remain viable and germinate at low temperatures (2 to 8°C), indicating that the pathogen could develop and survive during cold storage of cuttings or grafted plants.

8.2.3 Grafting

When infected cuttings are used for grafting, the grafting tools can become contaminated by surface propagules or infected wood fragments which may be transferred to subsequent graftlings. Despite the disinfection of grafting tools employed by the nurseries, botryosphaeriaceous DNA was detected in all grafting tools from three nurseries surveyed clearly showing that these pathogens were retained on the tools during the grafting process. However, the presence of botryosphaeriaceous DNA did not indicate that these propagules were viable, therefore, the infection potential of contamination in grafting tools still needs further investigation.

8.2.4 Callusing

Grafted plants are placed in callusing boxes and incubated at 28°C at 85% RH which is favourable for sporulation and germination of botryosphaeriaceous species. Botryosphaeriaceous DNA was detected in washings from post-callusing media in two nurseries suggesting that propagules are present and may be transferred onto the adjacent graftlings. Since the graftlings have fresh wounds, and the temperatures and moisture levels are optimum, any propagules present are likely to be able to successfully infect them.

8.2.5 Nursery planting

When the grafted plants are planted in nursery beds, they usually show no visible symptoms, but if conidia are produced, they may be dispersed in rainfall and either infect the bark or the wounds made during the trimming operations before and after harvest. Infected dormant young vines are lifted from nursery beds and those without external symptoms pass through the grading process undetected. The 2008 nursery sampling had shown that a high proportion of Grade 1 plants were infected with botryosphaeriaceous species clearly demonstrating that some of the infections were not detected during the grading process.

8.2.6 Cold storage of grafted plants

Dormant non-symptomatic young vines are placed in cold storage where surface propagules can survive, germinate and remain pathogenic. The non-symptomatic young vines that are planted into vineyards may encounter sufficient stress for them to show decline symptoms. Alternately, they may develop annual shoot dieback on which pycnidia and conidia are produced with ongoing spread of the disease through the vineyard. Previous researchers had provided evidence that water stress and winter pruning of vines can lead to

manifestation of symptoms possibly because they trigger pathogenic behaviour of latent infections.

8.3 Potential management strategies

The results presented in this thesis represent significant progress in the understanding of botryosphaeriaceous disease, its inoculum sources and potential infection pathways in grapevine nurseries. On the basis of this research, potential control strategies have been proposed to reduce or eliminate this disease in the nursery system. It is anticipated that these recommendations will advance the development of sustainable management programs that fit within the processing constraints of nursery propagation systems.

This study has clearly shown that the propagation materials from the mothervine blocks are the major source of infections in the nurseries, therefore, the control of this disease should start at the mothervine blocks. The use of resistant varieties is not a possible option at present since none of the commonly used rootstock and scion varieties are resistant to these pathogens. Since these pathogens are also genetically diverse, probably due to asexual methods of recombination, they are likely to be resilient in changing environmental conditions. Therefore, it is more likely that one control strategy will be insufficient and that an integrated disease management system will be essential for managing this problem.

To reduce the levels of inoculum, the health status of the mothervines should first be determined with non-destructive testing methods, such as the coring method used in this study to sample wood or by removing small sections of bark; it may be necessary to eradicate infected mothervines. Plant trimmings should also be removed from the mothervine blocks since the pathogens can survive and sporulate on these dead plant tissues which can therefore act as a source of secondary infections. To prevent the infection of mothervines or growing shoots, via trimming wounds, it is also recommended that trimming of vines should only be done during dry periods to prevent the splashed-dispersed conidia from infecting the wounds.

The combination of cultural methods, and biological or chemical protectants may be effective in reducing inoculum levels and infection during the growing season. According to Fourie and Halleen (2006) chemical and biological protection on wounds during all stages of propagation is essential to protect plants from decline and dieback pathogens. A study by Amponsah (2011) showed that the fungicide flusilazole was effective in reducing botryosphaeriaceous infections of pruning wounds in the vineyards, thus, this could provide protection of trimming and harvesting wounds in the mothervine blocks. A field experiment is needed to evaluate

different chemical and biological protectants in the mothervine blocks. It may also be possible to use systemic fungicides or sanitising agents for elimination of the bark infections before they penetrate into the wood.

Contaminations were detected in all steps of the propagation process but they were relatively low compared to the proportion of shoots infected within the mothervine blocks. This indicates that the propagation process may have reduced these contaminations most likely through the washing and soaking of the cuttings. Since nurseries with more stringent sanitation programs appeared to have the least contamination, it is recommended that these hygiene methods be introduced into all nurseries. This includes regular changing of hydration/rehydration solutions and amending them with recommended disinfectants. Evaluation of different sanitation products and fungicides will help determine which products are effective in eliminating or reducing contamination or incidence of latent infections. This may ensure that the cuttings are free of viable surface propagules or infections prior to cold storage, where they could remain viable and pathogenic. Regular disinfection of grafting tools is also essential as they are in direct contact with the wound and may introduce infection during grafting. Heat sterilisation or solarisation and drying of recycled callusing media can also be investigated to minimise contamination.

Since this study had demonstrated that infected asymptomatic cuttings were a major source of spread of botryosphaeriaceous infections, hot-water treatment as a potential control for botryosphaeriaceous infections should be investigated. Hot-water treatment at 50°C for 30 min was found to be effective in reducing infections of *Cylindrocarpon* spp. (Bleach *et al.*, 2008); *Ph. chlamydospora* and *Phaeoacremonium* spp. (Fourie & Halleen, 2004; Gramaje *et al.*, 2009). This study also showed that most infections were found in the outer tissues of the canes, therefore, hot-water treatment at higher temperatures but for shorter durations may eliminate these surface infections. Integration of fungicides like benomyl, sporekill, captan and *Trichoderma* formulations with hot-water treatment may further improve its efficacy in controlling dieback pathogens (Fourie & Halleen, 2004; 2006). Alternatively, fungicides mixed into the hot water may be more effective than HWT alone.

8.3.1 Future work

The future research that could be done to further improve understanding of the botryosphaeriaceous infections in grapevine nurseries has already been discussed in each chapter. However, some key research areas that are most likely to improve the understanding of the biology of these pathogens and the development of control strategies are further proposed.

1) The pathogenicity and susceptibility studies in this research used mycelium for inoculation, while in nature conidia are generally the primary inoculum for botryosphaeriaceous infections. These studies were also conducted under greenhouse conditions and may not reflect the actual situations in the field. It is therefore, important to confirm the results of these studies using conidia for inoculum under field conditions.

2) The development of an isolate-specific marker for one of the most common/pathogenic botryosphaeriaceous species for use in experiments will allow the tracking of this pathogen with PCR-based assays during infection in grapevines and the environment. This marker strain can be used for the following experiments:

    a) The survival and persistence of these botryosphaeriaceous species in soils need further investigation since previous reports provided inconsistent results on the role of soils in this pathogen transmission. Root inoculations using marked isolates will confirm whether this pathogen can infect roots and move upwards to the trunks and shoots.
    b) Histopathological studies using dormant non-wounded canes inoculated with a marker isolate would further clarify whether only pruning wounds provide infection courts for botryosphaeriaceous species. It is also important to investigate if these pathogens are capable of moving from the bark into the adjacent wood and their latency or colonisation behaviour on grapevine shoots.
    c) Washing of commercial-length one-year old cuttings as well as immature canes inoculated with a marker isolate to confirm that they provide sources of these pathogens during the different stages of propagation and storage of nursery plants.
    d) Investigate the infection potential of the nursery contaminations by introducing the marker isolate into the propagation system to confirm whether contaminations lead to new infections. The use of quantitative PCR and multi-species primers could allow the quantification of these contaminations in the nursery system and provide further information of infection potential during grafting.

3) Development of a rapid and sensitive diagnostic test, based on molecular techniques that use botryosphaeriaceous multi-species primers, could allow the detection of latent infections in cuttings and prevent the use of infected materials for propagation. This diagnostic tool could also allow screening of dormant young grafted vines for botryosphaeriaceous infections and so prevent infected asymptomatic plants from being sold to grape growers.

4) Since nursery operations can expose mothervines to many stress factors and the grafting process provides wounds for pathogen entry, there is need to investigate potential chemical and biological control agents that improve the sanitation and hygiene in the nursery system.

In summary, this research demonstrated that plants from grapevine nurseries are infected with botryosphaeriaceous species and the compromised health status of these plants may contribute to the high incidence of infections in the vineyards, threatening the long-term sustainability of the grapevine industry in New Zealand. The development of an integrated disease management strategy is needed if nurseries are to successfully manage these genetically-diverse and highly resilient pathogens. Since the indistinct symptoms of this disease in dormant propagation materials and young vines has made it difficult for nurseries to detect this problem during the grading process, the application of sensitive detection techniques as demonstrated in this research should be incorporated into any program that develops an integrated disease management strategy specific for nurseries. Similar methods could also be used for quality testing of the cuttings and grafted vines to prevent the latent infections of these pathogens being carried over to new vineyards. Once these pathogens reach the vineyards, where they can sporulate and infect the numerous wounds made during normal vineyard management, the problem becomes almost completely insoluble. Therefore, integrated management in the nursery provides the best chance of resolving the problems of Botryosphaeria dieback in vineyards for their lifetime.

Presentations and publications for thesis

Conference presentations

Billones, R. G., Jones, E. E., Ridgway, H. J., & *Jaspers*, M. V. (2009). *Prevalence and Pathogenicity of Botryopshaeria lutea isolated from grapevine nursery materials in New Zealand.* Paper presented at the The Australasian Plant Pathology Society Conference, Newcastle, NSW, Australia.

Billones, R., Jones, E. E., Ridgway, H. J., & Jaspers, M. V. (2010). *Botryosphaeriaceae infection in New Zealand nursery plant materials.* Paper presented at the 7th International Workshop on Grapevine Trunk Diseases, Santa Cruz, Chile.

Billones, R., Baskarathevan, J., Ridgway, H. J., Jones, E. E., & Jaspers, M. V. (2010). *Botryosphaeria infection in Grapevines- Where is it coming from?* Paper presented at the Romeo Bragato Conference 2010, Blenheim, New Zealand.

Journal/Magazine Publications

Billones, R., Jaspers, M., Jones E. & Ridgway H. (2009). *Botryosphaeria* sources in grapevine nurseries. *Winepress* 184, 24-25

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Appendix A

Surface sterilisation data

A.1 Evaluation of sterilisation techniques for botryosphaeriaceous species isolations.

1. Flame sterilise for 30 s.
2. 30 s in 70% ethanol and flame-dry in Bunsen burner.
3. 30 s in 70% ethanol and air dry.
4. 30 s in 70% ethanol, 5 min in 0.5% sodium hypochlorite; 30 s in 70% ethanol and air dry.

After surface sterilisation of the trunk samples, tissue samples (0.5 cm) were cut from the scion bud, 1 cm above and below the graft union and 1 cm above the stem base. The pieces from each plant were placed onto potato dextrose agar (PDA, Difco Microbiology, Detriot, USA) and incubated at room temperature for 5-7 days and colonies of botryosphaeriaceous species and other fungi and bacteria were counted. The surface sterilisation method with highest botryosphaeriaceous species recovery and least fungal and bacterial contamination was used for the succeeding isolations.

The results of the surface sterilisation techniques are shown in A.2. The technique that gave the highest botryosphaeriaceuos species recovery were the 70% ethanol dip with flame (30%) or air drying (30%). These techniques gave significantly higher recovery than the triple sterilisation (5%) and flame sterilisation (0%). Dipping the plant trunks in 70% ethanol and flame drying also led to significantly lower fungal contamination but higher bacterial contamination compared with the other three techniques (A.2).

A.2 Effects of different surface sterilisation techniques of recovery of botryosphaeriaceous species, fungal and bacterial contaminations

Surface sterilisation methods	Botryosphaeriaceous isolates recovered (%)	Contamination (%)	
		Fungi	Bacteria
1. Flame sterilise	0 (0) b	76 (95) a	6 (8) b
2. 70% ethanol dip and flame dry	6 (30) a	34 (43) b	10 (13) a
3. 70% ethanol dip and air dry	6 (30) a	56 (70) a	4 (5) b
4. Triple sterilisation (10% bleach, 3 min, 70% ethanol – 30 s, 10% bleach 3 min)	1 (5) b	50 (63) a	2 (3) b

∗ Means with different letters are significantly different at $P<0.05$ LSD.

Appendix B - Recipes

B.1 PRUNE EXTRACT/PRUNE EXTRACT AGAR

Use 25 g prunes per 500 ml flask.

1. De-stone and chop up prunes. Fill flask with distilled water to just under neck; cap flask. Place in pressure cooker (half filled with water) and boil in free steam for 30 minutes.
2. Filter product through filters – Whatman filter paper no. 3 (or two tissues) then Whatman filter paper no. 1. Add 400 ml aliquots to 500ml flasks.

Autoclave then refrigerate.

To make agar:

	To make 2L
Sucrose	10 g
Yeast extract	2 g
Agar	60 g
Prune extract	200 ml
Distilled water	1.8 L

Boil to dissolve before autoclaving.

B.2 POTTING MIX (3-4 MONTHS)

- 20% pumice
- 80% composted bark
- 2 kg/m3 Osmocote® Extract Standard 3-4 months gradual release fertiliser (16:3.5:10; N:P:K, respectively plus trace elements)
- 1 kg/m3 agricultural lime
- 500 g/m3 Hydraflo® 2 (granular wetting agent, Scott Product New Zealand, Ltd)

Appendix C STATISTICAL ANALYSES

C.1 CHAPTER 2 STATISTICAL ANALYSES

C.1.1 Botryosphaeriaceous infection incidence in grapevine nurseries

	Value	df	*P*-value
Pearson Chi-square	129.672	8	.000
Likelihood ratio	130.735	8	.000
No. of valid cases	1074		

C.1.2 Botryosphaeriaceous infection incidence in different nursery plant types

	Value	df	*P*-value
Pearson Chi-square	8.795	3	.032
Likelihood ratio	9.207	3	.027
No. of valid cases	311		

C.1.3 Multiple infection incidence in different nursery plant types

	Value	df	*P*-value
Pearson Chi-square	7.151	3	.067
Likelihood ratio	8.535	3	.036
No. of valid cases	311		

C.1.4 Pearson Chi-square test of independence on botryosphaeriaceous infection incidence on different plant and propagation materials and nursery source.

	Value	df	*P*- value
Failed grafted plants x nursery			
Pearson Chi-square	35.700	4	0.000
Likelihood ratio	39.670	4	0.000
No. of valid cases	182		
Grade 1 plants x nursery			
Pearson Chi-square	49.914	8	0.000
Likelihood ratio	53.892	8	0.000
No. of valid cases	352		
Propagation cuttings x nursery			
Pearson Chi-square	76.988	8	0.000
Likelihood ratio	72.551	8	0.000
No. of valid cases	480		

C.1.5 Pearson Chi-square test of independence on botryosphaeriaceous infection incidence on different isolation position.

	Value	df	*P*-value
Failed grafted plants x isolation position			
Pearson Chi-square	9.133	3	0.028
Likelihood ratio	10.076	3	0.018
No. of valid cases	182		
Grade 1 plants x isolation position			
Pearson Chi-square	20.533	3	0.000
Likelihood ratio	24.671	3	0.000
No. of valid cases	352		
Propagation cuttings x isolation position			
Pearson Chi-square	5.444	2	0.066
Likelihood ratio	5.995	2	0.050
No. of valid cases	480		

C.1.6 Pearson Chi-square test of independence on botryosphaeriaceous infection incidence on different grapevine varieties.

	Value	df	*P*-value
Rootstock varieties			
Pearson Chi-square	35.349	5	0.000
Likelihood ratio	44.011	5	0.000
No. of valid cases	1075		
Scion varieties			
Pearson Chi-square	139.771	5	0.000
Likelihood ratio	93.291	5	0.000
No. of valid cases	1075		

C.1.7 Pearson Chi-square test of independence on *N. luteum* incidence on different nurseries and plant materials.

	Value	df	*P*-value
Nurseries			
Pearson Chi-square	171.462	8	0.000
Likelihood ratio	128.360	8	0.000
N. of valid cases	1074		
Plant material			
Pearson Chi-square	13.798	3	0.003
Likelihood ratio	16.154	3	0.001
No. of valid cases	1074		

C.1.8 Pearson Chi-square test of independence on *N. parvum* incidence on different nurseries and plant materials.

	Value	df	*P*-value
Nurseries			
Pearson Chi-square	48.735	9	0.000
Likelihood ratio	42.875	9	0.000
N. of valid cases	1075		
Plant material			
Pearson Chi-square	10.304	3	0.016
Likelihood ratio	8.034	3	0.045
No. of valid cases	1075		

C.1.9 Pearson Chi-square test of independence between *N. luteum* infections and isolation positions from different plant materials.

	Value	df	*P*-value
Cuttings			
Pearson Chi-square	0.986	2	0.611
Likelihood ratio	1.024	2	0.599
N. of valid cases	480		
Failed grafted plants			
Pearson Chi-square	9.551	3	0.023
Likelihood ratio	11.104	3	0.11
No. of valid cases	182		
Grade 2 plants			
Pearson Chi-square			
Likelihood ratio	60		
No. of valid cases			
Grade 1 plants			
Pearson Chi-square	12.711	3	0.005
Likelihood ratio	17.026	3	0.001
No. of valid cases	352		

C.1.10 Pearson Chi-square test of independence between *N. parvum* infections and isolation positions.

	Value	df	*P*-value
Cuttings			
Pearson Chi-square	4.021	2	0.134
Likelihood ratio	5.570	2	0.062
No. of valid cases	481		
Failed grafted plants			
Pearson Chi-square	3.478	3	0.324
Likelihood ratio	3.119	3	0.374
No. of valid cases	182		
Grade 2 plants			
Pearson Chi-square	3.051	3	0.384
Likelihood ratio	2.824	3	0.420
No. of valid cases	60		
Grade 1 plants			
Pearson Chi-square	2.232	3	0.526
Likelihood ratio	3.345	3	0.341
No. of valid cases	352		

C.1.11 Pearson Chi-square test of independence between *N. luteum* infections and grapevine varieties.

	Value	df	*P*-value
Rootstock			
Pearson Chi-square	22.545	5	0.000
Likelihood ratio	28.080	5	0.000
No. of valid cases	1075		
Scion			
Pearson Chi-square	203.286	5	0.000
Likelihood ratio	90.170	5	0.000
No. of valid cases	1075		

C.1.12 Pearson Chi-square test of independence between *N. parvum* infections and grapevine varieties.

	Value	df	*P*-value
Rootstock			
Pearson Chi-square	15.560	5	0.008
Likelihood ratio	11.795	5	0.038
No. of valid cases	1075		
Scion			
Pearson Chi-square	15.058	5	0.010
Likelihood ratio	17.376	5	0.004
No. of valid cases	1075		

C.2 CHAPTER 3 STATISTICAL ANALYSES

C.2.1 Analysis of variance (ANOVA) on the mean lesion length of positive control from different batches for green shoots and cane inoculation.

Variables	Sum of squares	df	Mean square	F	*P*-value
Green shoot inoculation	40983.889	8	5111.736	5.733	0.000
Cane inoculation	62916.056	8	7864.507	4.139	0.002

C.2.2 Analysis of variance (ANOVA) on the pathogenicity of seven botryosphaeriaceous species based on means of green shoot lesions, cane lesions their respective ratios with positive control and re-isolation distances from canes.

Variables	Sum of squares	df	Mean square	F	*P*-value
Green shoot lesion	254749.761	9	28305.509	23.548	0.000
Ratio with positive control	56.217	9	6.246	22.571	0.000
Cane lesion	301281.104	9	33475.678	16.389	0.000
Cane ratio with positive control	17.840	6	2.973	5.683	0.000
Re-isolation distances	392837.091	9	43648.566	23.420	0.000

C.2.3 T-test between cane lesions and re-isolation distances seven botryosphaeriaceous species.

Variables	Standard deviation	Std error of mean	df	*P*-value
N. luteum	26.939	1.633	271	0.000
N. parvum	25.399	2.771	83	0.000
N. australe	25.392	4.488	31	0.014
D. mutila	29.208	5.163	31	0.511
B. dothidea	18.448	3.765	23	0.000
D. seriata	31.207	7.801	15	0.023

C.2.4 Analysis of variance (ANOVA) of pathogenicity among isolates of *N. luteum* from different nurseries species based on means of green shoot lesions, cane lesions, their respective ratios with positive control and re-isolation distances from canes.

Variables	Sum of squares	df	Mean square	F	*P*-value
Between nurseries					
Green shoot lesion	57953.402	5	11590.680	14.127	0.000
Green shoot ratio with positive control	2.758	5	0.552	2.054	0.072
Cane lesion	146820.064	5	29364.013	22.496	0.000
Cane lesion with positive control	44.169	5	8.364	31.617	0.000
Re-isolation distances	103314.268	5	20662.854	16.844	0.000
Between isolates					
Green shoot lesion	205069	66	2972.02	5.257	0.000
Ratio with positive control	39.201	66	0.568	2.813	0.000
Cane lesion	4629.562	66	67.095	3.214	0.000
Cane ratio with positive control	73.427	66	1.113	5.088	0.000
Re-isolation distances	2330.125	66	33.77	3.607	0.000

C.2.5 Analysis of variance (ANOVA) of pathogenicity among isolates of *N. parvum* and isolates from different nurseries species based on means of green shoot lesions, cane lesions their respective ratios with positive control and re-isolation distances of canes.

Variables	Sum of squares	df	Mean square	F	*P*-value
Between nurseries					
Green shoot lesion	24217.542	4	6054.385	2.730	0.035
Ratio with positive control	3.013	4	0.753	1.923	0.115
Cane lesion	53502.368	4	13375.592	4.563	0.002
*Cane ratio with positive control	1.743	4	0.436	1.256	0.295
*Re-isolation distances	31170.863	4	7792.716	2.730	0.035
Between isolates					
Green shoot lesion	121261.786	20	6063.089	4.886	0.000
Ratio with positive control	12.176	20	0.609	1.762	0.046
Cane lesion	173288.143	20	8664.407	4.883	0.000
Cane ratio with positive control	14.110	20	0.705	2.952	0.001
Re-isolation distances	142345.238	20	7117.262	3.922	0.000

* Variance of means are not homogenous, thus, ANOVA result is not valid

C.2.6 Analysis of variance (ANOVA) of pathogenicity among isolates of *N. australe* isolates based on means of green shoot lesions, cane lesions their respective ratios with positive control and re-isolation distances from canes.

Variables	Sum of squares	df	Mean square	F	*P*-value
Green shoot lesion	28277.219	7	4039.603	6.362	0.000
Ratio with positive control	11.248	7	1.607	11.159	0.000
*Cane lesion	23711.875	7	3387.411	4.543	0.002
*Cane ratio with control positive	5.424	7	0.775	2.265	0.064
*Re-isolation distances	12700.000	7	1814.286	2.639	0.036

* Variance of means are not homogenous, thus, ANOVA result is not valid

C.2.7 Analysis of variance (ANOVA) of pathogenicity among isolates of *D. mutila* isolates based on means of green shoot lesions, cane lesions their respective ratios with positive control and re-isolation distances from canes.

Variables	Sum of squares	df	Mean square	F	*P*-value
Green shoot lesion	18678.419	7	2668.346	23.150	0.000
Ratio with positive control	1.099	7	0.157	17.798	0.000
Cane lesion	33060.375	7	4722.911	1.764	0.141
Cane ratio with positive control	2.139	7	0.306	0.786	0.606
Re-isolation distances	13387.500	7	1912.500	0.629	0.727

C.2.8 Analysis of variance (ANOVA) of pathogenicity among isolates of *B. dothidea* isolates based on means of green shoot lesions, cane lesions their respective ratios with positive control and re-isolation distances from canes.

Variables	Sum of squares	df	Mean square	F	*P*-value
Green shoot lesion	28277.219	5	6029.142	10.364	0.000
Ratio with positive control	1.839	5	0.368	7.760	0.000
Cane lesion	3015.833	5	603.167	0.840	0.538
Cane ratio with positive control	1.040	5	0.208	2.664	0.057
Re-isolation distances	3620.833	5	724.167	1.376	0.280

C.2.9 Analysis of variance (ANOVA) of pathogenicity among isolates of *D. seriata* isolates based on means of green shoot lesions, cane lesions their respective ratios with positive control and re-isolation distances from canes.

Variables	Sum of squares	df	Mean square	F	*P*-value
Green shoot lesion	1582.750	3	527.583	3.674	0.044
Ratio with positive control	0.127	3	0.042	2.284	0.131
Cane lesion	1494.750	3	498.250	0.335	0.800
Cane ratio with positive control	0.146	3	0.049	0.436	0.731
Re-isolation distances	368.750	3	122.917	0.247	0.862

C.2.10 Analysis of variance (ANOVA) of green shoot and cane lesions, their corresponding ratios with positive control and re-isolation distances among *N. luteum* isolates.

Isolate	Green shoots		Isolate	Canes		
	Lesion length	Ratio with positive control		Lesion length	Ratio with control	Re-isolation distances
456	114.75 a	0.65 efg	106	125.0 a	0.79 cde	55.0 jk
228	106.50 ab	0.64 efg	114	122.5 a	0.78 cde	62.5 i
468	100.25 abc	0.06 h	228	116.0 b	2.58 a	52.5 k
248	98.75 abcd	1.53 abc	229	108.3 c	2.41 a	92.5 e
481	95.00 abcde	1.73 ab	244	98.3 de	2.18 a	100.0 d
483	90.25 abcdef	0.29 fgh	105	97.5 de	0.62 cde	77.5 f
465	86.25 bcdef	2.08 a	86	95.0 de	0.78 cde	60.0 ij
472	84.50 bcdefg	0.04 h	113	82.5 e	0.52 cde	100.0 d
482	80.50 bcdefgh	0.04 h	456	82.3 e	0.73 cde	150.0 a
460	79.25 bcdefghi	0.22 gh	468	81.0 ef	0.72 cde	45.0 lm
66	75.00 bcdefghij	0.11 gh	101	80.0 ef	0.65 cde	57.5 j
229	74.25 bcdefghij	0.31 fgh	517	78.5 ef	0.31 de	19.5 qr
67	73.75 bcdefghij	1.02 cde	488	75.3 f	1.15 bc	107.5 c
696	73.50 cdefghij	1.18 bcd	547	68.0 g	1.09 bc	112.5 b
864	73.25 cdefghijk	0.71 def	85	67.5 g	0.55 cde	112.5 b
492	69.75 cdefghijk	0.62 efg	89	67.5 g	0.55 cde	75.0 fg
555	69.75 cdefghijk	0.62 efg	236	66.8 g	1.48 b	102.5 d
496	69.00 cdefghijk	0.95 cde	460	65.8 gh	0.58 cde	75.0 fg
485	66.25 defghijk	0.30 fgh	248	63.8 gh	1.42 bc	63.8 h
244	64.50 efghijkl	0.87 def	543	62.5 gh	1.00 bcd	62.5 i
543	64.25 efghijkl	0.39 fgh	472	60.3 h	0.53 cde	42.5 m
559	63.25 efghijklm	0.60 efg	332	58.8 h	0.52 cde	57.5 j
487	61.50 efghijklm	0.45 efg	67	55.0 hi	1.22 bc	47.5 l
519	61.00 efghijklm	0.65 efg	457	50.0 i	0.44 cde	27.5 p
557	60.50 efghijklm	0.76 def	497	48.3 ij	0.74 cde	75.0 fg
705	60.00 efghijklm	0.64 efg	110	47.5 ij	0.30 e	57.5 j
517	59.75 efghijklm	0.96 cde	484	46.0 ij	0.71 cde	30.0 op
536	59.00 efghijklm	0.54 efg	461	43.0 ij	0.38 de	17.5 r
461	58.75 efghijklm	1.30 bcd	66	42.5 j	0.94 bcde	52.5 k
746	58.50 efghijklmn	0.69 def	70	40.0 jk	0.89 bcde	57.5 j
779	58.25 efghijklmno	0.57 efg	483	39.5 jk	0.35 de	22.5 q
775	57.75 efghijklmno	0.93 cde	509	36.5 jk	0.56 cde	37.5 n
547	57.50 efghijklmno	0.86 def	536	35.0 k	0.56 cde	67.5 h

Continuation of C.2.10

Isolate	*Green shoot		Isolate	*Cane		
	Lesion length	Ratio with positive control		Lesion length	Ratio with positive control	Re-isolation distances
778	57.25 fghijklmno	0.72 def	492	29.3 kl	0.45 cde	27.5 p
489	54.25 fghijklmno	0.76 def	496	27.0 l	0.41 de	15.0 rs
539	53.75 fghijklmno	0.98 cde	487	26.0 l	0.40 de	10.0 s
540	53.50 fghijklmno	0.43 efg	516	24.5 lm	0.38 de	20.0 qr
560	53.50 fghijklmno	0.97 cde	746	24.5 lm	0.90 bcde	35.0 no
488	51.75 ghijklmno	0.65 efg	524	24.0 lm	0.38 de	22.5 q
501	51.75 ghijklmno	0.72 def	705	23.8 lm	0.29 e	35.0 no
457	50.75 hijklmno	0.70 def	465	21.8 lm	0.19 e	17.5 r
516	50.50 hijklmno	0.71 def	539	21.0 lm	0.34 de	30.0 op
509	50.00 hijklmno	0.98 cde	523	20.8 lm	0.33 de	37.5 n
525	50.00 hijklmno	0.35 fgh	489	18.5 m	0.28 e	20.0 qr
706	49.50 hijklmno	0.56 efg	706	18.0 mn	0.40 de	32.5 o
551	49.00 hijklmno	0.80 def	525	17.8 mn	0.28 e	22.5 q
552	48.50 hijklmno	0.68 efg	779	17.8 mn	0.29 e	25.0 pq
497	46.75 ijklmnop	0.95 cde	775	13.8 mn	0.22 e	37.5 n
554	46.25 jklmnop	0.86 def	778	12.5 mn	0.20 e	25.0 pq
535	42.50 jklmnop	0.86 def	551	12.3 mn	0.20 e	72.5 g
484	40.50 klmnopq	1.03 cde	540	10.8 n	0.17 e	70.0 gh
524	34.75 klmnopq	0.92 cde	482	9.5 no	0.15 e	15.0 rs
332	33.00 lmnopq	0.41 fgh	535	9.3 no	0.15 e	12.5 s
493	31.00 mnopq	0.79 def	864	9.3 no	0.15 e	25.0 pq
101	30.75 mnopq	0.78 def	519	9.0 no	0.14 e	22.5 q
548	25.75 nopq	0.74 def	554	8.5 no	0.14 e	22.5 q
86	25.50 opq	1.12 bcd	548	8.3 no	0.13 e	27.5 p
85	22.50 opq	0.98 cde	696	7.8 no	0.13 e	27.5 p
114	22.00 opq	1.02 cde	501	7.0 no	0.11 e	22.5 q
523	22.00 opq	0.86 def	485	6.3 no	0.10 e	35.0 no
110	15.50 pq	0.97 cde	555	5.5 no	0.09 e	20.0 qr
113	7.75 q	0.94 cde	559	5.3 no	0.08 e	62.5 i
236	7.50 q	0.93 cde	560	5.0 no	0.08 e	45.0 lm
70	7.00 q	0.92 cde	552	4.8 no	0.08 e	30.0 op
89	6.25 q	0.94 cde	481	4.5 no	0.04 e	35.0 no
105	4.75 q	0.16 gh	557	4.5 no	0.07 e	45.0 lm
106	2.50 q	1.18 bcd	493	3.8 o	0.06 e	32.5 o

*Values within a column with different letters are significantly different at $P \leq 0.05$ LSD

C.2.11 Analysis of variance (ANOVA) of green shoot and cane lesions, their corresponding ratios with positive control and re-isolation distances among *N. parvum* isolates.

Isolate	*Green shoot		Isolate	*Cane			
	Lesion length (mm)	Ratio with positive control		Lesion length (mm)	Ratio with positive control	Re-isolation distances (mm)	
275	122.3 a	1.12 abcd	178	192.5 a	1.22 abcdef	180.0	a
233	119.8 a	1.14 abcd	94	160.0 ab	1.30 abcde	150.0	abc
219	118.8 a	1.66 a	116	150.0 a	0.95 cdefg	175.0	ab
69	115.0 a	1.00 abcd	225	140.0 b	0.90 defg	130.0	bcde
232	112.5 a	1.07 abcd	98	125.0 bc	1.02 bcdefg	135.0	abcd
471	112.3 a	0.85 abcd	259	85.3 c	0.88 defg	112.5	cdef
277	109.5 a	1.00 abcd	224	85.0 c	0.43 g	105.0	cdef
72	106.5 a	0.92 abcd	260	82.8 c	1.58 abcd	105.0	cdef
261	103.5 a	0.99 abcd	471	78.5 c	0.69 efg	82.5	defg
260	94.3 ab	0.90 abcd	261	77.0 c	1.21 abcdef	115.0	bcde
215	93.0 ab	1.30 ab	72	72.5 cd	1.61 abc	75.0	defg
259	88.0 abc	0.84 bcd	379	72.3 cd	1.05 bcdefg	90.0	cdefg
216	87.0 abc	1.21 abc	232	71.5 cd	1.09 bcdefg	70.0	efg
276	52.8 bcd	0.48 bc	275	68.8 cd	1.89 a	90.0	cdefg
379	42.3 cd	0.38 c	277	61.0 cd	1.71 ab	70.0	efg
224	38.0 d	0.54 bcd	233	54.8 cd	0.53 fg	65.0	fg
225	37.8 d	0.53 bcd	69	45.0 cd	1.00 cdefg	65.0	fg
178	29.3 d	0.41 cd	276	38.5 cd	1.83 a	30.0	g
116	27.0 d	0.38 d	219	35.5 cd	0.83 efg	53.5	fg
98	24.8 d	1.67 a	216	29.5 cd	0.93 cdefg	45.0	g
94	6.3 d	0.42 cd	215	14.0 d	0.59 fg	40.0	g

*Values within a column with different letters are significantly different at $P \leq 0.05$ LSD

C.2.12 Analysis of variance (ANOVA) of green shoot and cane lesions, their corresponding ratios with positive control and re-isolation distances among *N. australe* isolates.

Isolate No.	[A]Green shoot		Isolate No.	[B]Canes		
	Lesion length (mm)	Ratio with positive control		Lesion length (mm)	Ratio with positive control	Re-isolation distances (mm)
480	103.5 a	0.78 b	88	82.5	0.67	85.0
240	78.5 ab	0.75 b	240	30.3	0.67	65.0
518	60.0 b	0.96 b	355	30.0	0.46	32.5
705	60.0 b	0.96 b	480	88.7	1.36	77.5
511	58.8 bc	0.82 b	511	28.5	0.87	32.5
88	32.5 cd	2.18 a	518	54.7	1.36	67.5
355	14.8 d	0.14 c	705	23.8	0.41	35.0
826	10.0 d	0.16 c	826	8.0	0.14	45.0

[A] Values within a column with different letters are significantly different at $P \leq 0.05$ LSD

[B] Variances of means were not homogenous and ANOVA was not conducted

C.2.13 Analysis of variance (ANOVA) of green shoot and cane lesions, their corresponding ratios with positive control and re-isolation distances among *D. mutila* isolates.

Isolate No.	*Green shoot		Isolate No.	*Cane		
	Lesion length (mm)	Ratio with positive control		Lesion length (mm)	Ratio with positive control	Re-isolation distances (mm)
433	85.25 a	0.64 a	146	137.5 a	0.87 a	140.0 a
231	38.00 b	0.36 b	109	80.0 a	0.51 a	57.5 a
373	30.75 b	0.28 b	360	65.5 a	0.57 a	57.5 a
104	25.00 bc	0.21 c	433	56.3 a	0.49 a	60.0 a
103	24.75 bc	0.21 c	231	53.0 a	1.17 a	70.0 a
360	13.00 cd	0.11 cd	373	42.8 a	0.37 a	40.0 a
146	4.00 d	0.04 d	104	42.5 a	0.94 a	52.5 a
109	3.25 d	0.04 d	103	25.0 a	0.55 a	32.5 a

*Values within a column with different letters are significantly different at $P \leq 0.05$ LSD

C.2.14 Analysis of variance (ANOVA) of green shoot and cane lesions, their corresponding ratios with positive control and re-isolation distances among *B. dothidea* isolates.

Isolate No.	*Green shoot		Isolate No.	*Cane		
	Lesion length (mm)	Ratio with positive control		Lesion length (mm)	Ratio with positive control	Re-isolation distances
445	103.7 a	0.78 a	445	33.7 a	0.29 a	57.5 a
361	59.5 b	0.54 b	58	30.0 a	0.66 a	47.5 a
7	26.7 bc	0.23 bc	6	17.5 a	0.38 a	22.5 a
58	18.0 c	0.15 c	361	15.2 a	0.13 a	37.5 a
47	9.7 c	0.08 c	7	12.5 a	0.27 a	32.5 a
6	0.0 c	0.00 c	47	0.0 a	0.00 a	25.0 a

*Values within a column with different letters are significantly different at $P \leq 0.05$ LSD

C.2.15 Analysis of variance (ANOVA) of green shoot and cane lesions, their corresponding ratios with positive control and re-isolation distances among *D. seriata* isolates.

Isolate No.	*Green shoot		Isolate No.	*Cane		
	Lesion length (mm)	Ratio with positive control		Lesion length (mm)	Ratio with positive control	Re-isolation distances (mm)
344	25.5 a	0.23 a	345	54.5 a	0.48 a	25.0 a
345	8.5 ab	0.07 a	344	49.5 a	0.44 a	30.0 a
99	2.5 b	0.16 a	99	37.5 a	0.30 a	17.5 a
100	0.0 b	0.00 a	100	30.0 a	0.24 a	20.0 a

*Values within a column with different letters are significantly different at $P \leq 0.05$ LSD

C.3 CHAPTER 4 STATISTICAL ANALYSES

C.3.1 Analysis of variance (ANOVA) on the upward, downward and overall lesions of different plant types inoculated with three botryosphaeriaceous species.

Variables	S.S	df	M.S.	F	*P*-value
Upward lesions					
Plant type	502.494	1	502.494	4.732	0.030
Species	13737.927	2	6868.964	64.689	0.000
Plant type * species	1073.024	2	536.512	5.053	0.007
Error	135809.716	1279	106.184		
Downward lesions					
Plant type	2524.783	1	2524.783	35.152	0.000
Species	11977.848	2	5988.924	83.382	0.000
Plant type * species	1549.952	2	774.976	10.790	0.000
Error	91864.377	1279	71.825		
Overall lesions					
Plant type	5280.000	1	528.00	17.880	0.000
Species	51362.851	2	25681.425	86.969	0.000
Plant type * species	4803.029	2	2401.514	8.133	0.000
Error	377681.719	1279	295.295		

C.3.2 Analysis of variance (ANOVA) on the upward, downward and overall lesions of different rootstocks varieties inoculated with three botryosphaeriaceous species.

Variables	S.S.	df	M.S.	F	*P*-value
Upward lesions					
Rootstock Variety	3185.671	5	637.134	5.060	0.000
Species	9688.074	2	4844.037	38.472	0.000
Variety * species	928.351	10	92.835	0.737	0.689
Error	77938.089	619	125.910		
Downward lesions					
Rootstock Variety	1303.292	5	260.658	2.872	0.014
Species	10739.327	2	5369.663	59.167	0.000
Variety * species	877.346	10	87.735	0.967	0.471
Error	56176.631	619	90.754		
Overall lesions					
Rootstock variety	8432.353	5	1686.471	4.717	0.000
Species	40699.524	2	20349.762	56.923	0.000
Variety * species	3481.558	10	348.156	0.974	0.465
Error	221289.245	619	357.495		

C.3.3 Analysis of variance (ANOVA) on the upward, downward and overall lesions of different scion varieties inoculated with three botryosphaeriaceous species.

Variables	S.S.	df	M.S.	F	*P*-value
Upward lesions					
Scion Variety	1400.992	5	280.198	3.744	0.002
Species	5122.877	2	2561.439	34.228	0.000
Variety * species	369.856	10	36.986	0.494	0.894
Error	46322.028	619	74.834		
Downward lesions					
Scion Variety	914.218	5	182.844	3.921	0.002
Species	2788.473	2	1394.237	29.897	0.000
Variety * species	793.272	10	79.327	1.701	0.077
Error	28866.763	619	46.635		
Overall lesions					
Scion variety	4347.144	5	869.429	4.393	0.001
Species	15466.355	2	7733.177	39.071	0.000
Variety * species	1585.454	10	158.545	0.801	0.628
Error	122516.732	619	197.927		

C.3.4 Analysis of variance (ANOVA) on the upward, downward and overall lesions of different rootstocks varieties inoculated with nine botryosphaeriaceous isolates.

Variables	S.S.	df	M.S.	F	*P*-value
Upward lesions					
Rootstock Variety	3185.671	5	637.134	5.455	0.000
Isolates	17058.132	8	2132.266	18.257	0.000
Variety * isolates	3405.771	40	85.144	0.729	0.892
Error	68090.612	583	116.794		
Downward lesions					
Rootstock Variety	1303.292	5	260.658	3.117	0.009
Isolates	16014.602	8	2001.825	23.937	0.000
Variety * isolates	3023.040	40	75.576	0.904	0.642
Error	48755.662	583	83.629		
Overall lesions					
Rootstock variety	8432.353	5	1686.471	5.215	0.000
Isolates	65613.228	8	8201.654	25.362	0.000
Variety * Isolates	11325.481	40	283.137	0.876	0.690
Error	188531.617	583	323.382		

C.3.5 Analysis of variance (ANOVA) on the upward, downward and overall lesions of different scion varieties inoculated with nine botryosphaeriaceous isolates.

Variables	S.S.	df	M.S.	F	*P*-value
Upward lesions					
Scion Variety	1400.992	5	280.198	4.120	0.001
Isolates	10102.320	8	1262.790	18.566	0.000
Variety * isolates	2058.640	40	51.466	0.757	0.862
Error	39653.803	583	68.017		
Downward lesions					
Scion Variety	3185.671	5	637.134	5.455	0.000
Isolates	17058.132	8	2132.266	18.257	0.000
Variety * Isolates	3405.771	40	85.144	0.729	0.001
Error	68090.612	583	116.794		
Overall lesions					
Scion variety	4347.144	5	869.429	5.179	0.000
Isolates	33619.291	8	4202.411	25.035	0.000
Variety * isolates	8084.119	40	202.103	1.204	0.187
Error	97865.131	583	167.865		

C.4 CHAPTER 5 STATISTICAL ANALYSES

C.4.1 Frequency distribution of surface contamination incidence of plant materials by nurseries and varieties

Variable	Value	df	P-value
Pearson Chi-square			
Nurseries	47.329	2	0.000
Varieties	67.640	17	0.000

C.4.2 Frequency distribution of internal infection incidence of plant materials by nurseries plant types and varieties

Variable	Value	df	*P*-value
Pearson Chi-square			
Nurseries	107.854	2	0.000
Plant types (rootstock/scion)	0.001	1	0.979
Varieties	94.033	11	0.000
Isolation position	15.693	2	0.000

C.4.3 Frequency distribution of isolates recovered from plant materials by nurseries and varieties

Variable	Value	df	*P*-value
Pearson Chi-square			
Nurseries	144.591	2	0.000
Varieties	140.234	11	0.000

C.4.4 Frequency distribution of species recovered from plant materials by nurseries

Variable	Value	df	*P*-value
Pearson Chi-square			
N. luteum	13.807	2	0.001
N. parvum	14.452	2	0.001
N. australe	7.066	2	0.029
D. seriata	44.803	2	0.000
B. dothidea	3.126	2	0.209
D. mutila	1.412	2	0.494

C.5 CHAPTER 7 STATISTICAL ANALYSES

C.5.1 Analyses of variance (ANOVA) on the percent germination, viability and damage of *N. luteum* conidia stored at ambient temperature (20-23°C) at different time

Variables	S.S	df	M.S.	F	*P*-value
Ambient temperature					
Homogeneity of variance				3.046	0.050
% Conidial germination	340.30	4	85.075	3.013	0.052
Error	423.500	15	28.233		
Homogeneity of variance				3.320	0.039
% damage conidia	588.800	15	29.440	29.440	0.000
Error	75.000	15	5.000		
Homogeneity of variance				1.109	0.371
% viability	704.167	2	352.083	2.894	0.107
Error	1094.750	9	121.639		

C.5.2 Analyses of variance (ANOVA) on germ tube elongation of *N. luteum* conidia incubated at 0, 2, 4 and 6 h at ambient temperature

Variables	S.S	df	M.S.	F	*P*-value
Homogeneity of variance				6.828	0.001
Germ tube length	132.915	1	132.915	2358.039	0.000
Error	3.993	3	1.331	23.616	

C.5.3 Analyses of variance (ANOVA) on the percent germination, viability and damage of *N. luteum* conidia stored at 8°C at different time

Variables	S.S	df	M.S.	F	*P*-value
8°C					
Homogeneity of variance				6.314	0.001
% Conidial germination	9126.500	6	1521.083	48.123	0.000
Error	632.167	20	31.608		
Homogeneity of variance				4.302	0.006
% damage conidia	3937.357	6	656.226	23.497	0.000
Error	586.500	21	27.929		
Homogeneity of variance				2.524	0.135
% viability	2408.167	2	1204.083	15.553	0.001
Error	696.750	9	77.417		

C.5.4 Analyses of variance (ANOVA) on the percent germination, viability and damage of *N. luteum* conidia stored at 2°C at different time.

Variables	S.S	df	M.S.	F	*P*-value
2°C					
Homogeneity of variance				7.304	0.000
% Conidial germination	968.375	7	138.339	14.342	0.000
Error	231.500	24	9.646		
Homogeneity of variance				4.861	0.002
% damage conidia	1710.875	7	244.411	23.003	0.000
Error	255.000	24	10.625		
Homogeneity of variance				2.278	0.080
% viability	10029.904	6	1671.651	7.884	0.000
Error	4028.750	19	212.039		

C.5.5 Analyses of variance (ANOVA) on the pathogenicity of on re-isolation distances *N. luteum* conidia based stored at different storage temperatures and time.

Variables	S.S	df	M.S.	F	*P*-value
Pathogenicity					
4h					
Homogeneity of variance				5.803	0.006
% Conidial germination	0.067	4	0.017	1.437	0.273
Error	0.164	14	0.012		
24h					
Homogeneity of variance				6.882	0.004
% Conidial germination	0.028	4	0.007	11.118	0.001
Error	0.008	12			
48h					
Homogeneity of variance				10.448	0.001
% Conidial germination	3.069	4	0.767	4.470	0.019
Error	2.060	12			
72h					
Homogeneity of variance				10.327	0.002
% Conidial germination	17.041	3	5.680	22.966	0.000
Error	2.473	10	0.247		
1 week					
Homogeneity of variance				7.815	0.004
% Conidial germination	0.039	3	0.013	4.254	0.029
Error	0.037	12	0.003		
2 weeks					
Homogeneity of variance				7.846	0.004
% Conidial germination	0.122	3	0.041	2.513	0.108
Error	0.195	12	0.016		

C.5.6 Analyses of variance (ANOVA) on the germination rate *N. luteum* conidia at ambient temperature after storage at 2°C at different times.

Variables	S.S	df	M.S.	F	*P*-value
% Conidial germination					
Homogeneity of variance				6.187	0.009
24 h + ambient	1620.688	3	540.229	12.985	0.000
Error	499.250	12	41.604		
Homogeneity of variance				3.409	0.053
48 h + 6 h ambient	6114.688	3	2038.229	62.355	0.000
Error	392.250	12	32.688		
Homogeneity of variance				2.497	0.109
1 week + 6 h ambient	9536.250	3	3178.750	39.345	0.000
Error	969.500	12	80.792		
Homogeneity of variance				4.231	0.029
2 weeks + 6 h ambient	3018.250	3	1006.083	22.801	0.000
Error	529.500	12	44.125		
Homogeneity of variance				0.890	0.474
3 weeks + 6 h ambient	8513.250	3	2837.750	98.848	0.000
Error	344.500	12	28.708		

C.5.7 Analyses of variance (ANOVA) on the germination rate *N. luteum* conidia at ambient temperature after storage at 8°C at different times.

Variables	S.S	df	M.S.	F	*P*-value
% Conidial germination					
Homogeneity of variance				1.099	0.387
24 h + ambient	2701.188	3	900.396	4.755	0.021
Error	2272.250	12	189.354		
Homogeneity of variance				5.176	0.016
48 h + ambient	3586.000	3	1195.333	6.177	0.009
Error	2322.000	12	193.500		
Homogeneity of variance				1.456	0.276
72 h + ambient	258.750	3	86.250	1.393	0.293
Error	743.000	12	61.917		
Homogeneity of variance				0.919	0.461
1week + ambient	1302.000	3	434.000	6.634	0.007
Error	785.000	12	65.417		

C.5.8 Analyses of variance (ANOVA) on the number of conidia recovered after washing canes inoculated with *N. luteum* conidia and incubated at different periods based on microscopy and plating assay.

Variables	S.S	df	M.S.	F	*P*-value
Microscopy					
Homogeneity of variance				9.893	0.000
No. of conidia/sample	3.652	5	7.304	811.673	0.000
Error	7018750.00	78	89983.974		
Plating assay					
Homogeneity of variance				43.135	0.000
No. of colonies/plate	7054.978	4	1763.745	53.182	0.000
Error	2122.500	64	33.164		

C.5.9 Pearson Chi-square test of independence on isolation of *N. luteum* after washing of inoculated cuttings.

Wash treatments	Value	df	*P*-value
0 h	16.00	3	0.001
4 h	16.00	3	0.001
Negative control	16.00		

Appendix D

Identification of botryosphaeriaceous isolates by ARDRA

D.1 Identification of botryosphaeriaceous isolates from nursery plant materials collected during the 2008 nursery sampling (Chapter 2)

Isolate No.	Nursery	Species/Group based on the restriction digest of rDNA			
		***Taq*I**	***Sac*II**	***Hae*III**	***Nci*I**
0006	N03	Group C		*B. dothidea*	
0007	N03	Group C		*B. dothidea*	
0047	N03	Group C		*B. dothidea*	
0058	N03	Group C		*B. dothidea*	
0066	N03	Group A	*N. luteum*		
0067	N03	Group A	*N. luteum*		
0069	N03	Group B		*N. parvum*	
0070	N03	Group A	*N. luteum*		
0072	N03	Group B		*N. parvum*	
0083a	N03	Group B		Group F	*N. ribis*
0083b	N03	Group B		Group F	*N. ribis*
0085	N03	Group A	*N. luteum*		
0086	N03	Group A	*N. luteum*		
0088	N03	Group A	*N. australe*		
0089	N03	Group A	*N. luteum*		
0094	N03	Group B		*N. parvum*	
0098	N03	Group B		*N. parvum*	
0099	N03	Group C		Group F	*D. seriata*
0100	N03	Group C		Group F	*D. seriata*
0101	N03	Group A	*N. luteum*		
0103	N03	Group B		Group F	*D. mutila*
0104	N03	Group B		Group F	*D. mutila*
0105	N03	Group A	*N. luteum*		
0106	N03	Group A	*N. luteum*		
0109	N03	Group B		Group F	*D. mutila*
0110	N03	Group A	*N. luteum*		
0113	N03	Group A	*N. luteum*		
0114	N03	Group A	*N. luteum*		
0116	N03	Group B		*N. parvum*	
0146	N01	Group B		Group F	*D. mutila*
0178	N01	Group C		*N. parvum*	
0215	N03	Group B		*N. parvum*	
0216	N03	Group B		*N. parvum*	

Continuation of D.1

0219	N03	Group B		*N. parvum*	
0224	N09	Group B		*N. parvum*	
0225	N09	Group B		*N. parvum*	
0228	N09	Group A	*N. luteum*		
0231	N09	Group B		Group F	*D. mutila*
0232	N09	Group B		*N. parvum*	
0233	N09	Group B		*N. parvum*	
0240	N09	Group A	*N. australe*		
0244	N09	Group A	*N. luteum*		
0248	N09	Group A	*N. luteum*		
0259	N09	Group C		*N. parvum*	
0260	N09	Group C		*N. parvum*	
0261	N09	Group C		*N. parvum*	
0275	N09	Group B		*N. parvum*	
0276	N09	Group B		*N. parvum*	
0277	N09	Group B		*N. parvum*	
0332	N09	Group A	*N. luteum*		
0344	N11	Group C		Group F	*D. seriata*
0345	N11	Group C		Group F	*D. seriata*
0353	N11	Group C		Group F	*unknown*
0355	N11	Group A	*N. australe*		
0360	N11	Group B		Group F	*D. mutila*
0361	N11	Group C		*B. dothidea*	
0373	N11	Group B		Group F	*D. mutila*
0379	N11	Group C		*N. parvum*	
0433	N11	Group B		Group F	*D. mutila*
0445	N11	Group C		*B. dothidea*	
0456	N05	Group A	*N. luteum*		
0457	N05	Group A	*N. luteum*		
0460	N05	Group A	*N. luteum*		
0461	N05	Group A	*N. luteum*		
0465	N05	Group A	*N. luteum*		
0468	N05	Group A	*N. luteum*		
0471	N05	Group B		*N. parvum*	
0472	N05	Group A	*N. luteum*		
0480	N05	Group A	*N. australe*		
0481	N05	Group A	*N. luteum*		
0482	N05	Group A	*N. luteum*		
0483	N05	Group A	*N. luteum*		
0484	N05	Group A	*N. luteum*		
0485	N05	Group A	*N. luteum*		
0487	N05	Group A	*N. luteum*		
0488	N05	Group A	*N. luteum*		
0492	N05	Group A	*N. luteum*		

Continuation of D.1

0493	N05	Group A	*N. luteum*
0496	N05	Group A	*N. luteum*
0497	N05	Group A	*N. luteum*
0501	N05	Group A	*N. luteum*
0509	N05	Group A	*N. luteum*
0511	N05	Group A	*N. australe*
0516	N05	Group A	*N. luteum*
0517	N05	Group A	*N. luteum*
0518	N05	Group A	*N. australe*
0519	N05	Group A	*N. luteum*
0523	N05	Group A	*N. luteum*
0524	N05	Group A	*N. luteum*
0525	N05	Group A	*N. luteum*
0535	N05	Group A	*N. luteum*
0536	N05	Group A	*N. luteum*
0539	N05	Group A	*N. luteum*
0540	N05	Group A	*N. luteum*
0543	N05	Group A	*N. luteum*
0547	N05	Group A	*N. luteum*
0548	N05	Group A	*N. luteum*
0551	N05	Group A	*N. luteum*
0552	N05	Group A	*N. luteum*
0554	N05	Group A	*N. luteum*
0555	N05	Group A	*N. luteum*
0557	N05	Group A	*N. luteum*
0559	N05	Group A	*N. luteum*
0560	N05	Group A	*N. luteum*
0562	N05	Group A	*N. luteum*
0696	N07	Group A	*N. luteum*
0705	N07	Group A	*N. australe*
0706	N07	Group A	*N. luteum*
0746	N10	Group A	*N. luteum*
0774	N10	Group A	*N. luteum*
0775	N10	Group A	*N. luteum*
0778	N10	Group A	*N. luteum*
0779	N10	Group A	*N. luteum*
0826	N02	Group A	*N. australe*
0864	N02	Group A	*N. luteum*

D.2 Identification of representative isolates from internal infection of cuttings collected during 2009 nursery sampling (Chapter 5)

Nursery	Isolate No.	Species/Group based on restriction digest of rDNA (ARDRA)		
		***Hae*III**	***Sac*II**	***Nci*I**
Nursery 11	83	c	*N. luteum*	
Nursery 11	91	c	d	*D. seriata*
Nursery 11	92	c	d	*D. seriata*
Nursery 11	95	c	d	*D. seriata*
Nursery 11	131	*N. parvum*		
Nursery 11	152	c	*N. australe*	
Nursery 11	252	c	d	*D. seriata*
Nursery 11	407	c	d	*D. seriata*
Nursery 11	434	c	d	*D. seriata*
Nursery 11	447	*B. dothidea*		
Nursery 05	477	c	*N. australe*	
Nursery 05	479	c	*N. luteum*	
Nursery 05	482	c	*N. luteum*	
Nursery 05	492	c	*N. luteum*	
Nursery 05	495	c	*N. luteum*	
Nursery 05	505	c	*N. luteum*	
Nursery 05	524	c	*N. australe*	
Nursery 05	563	c	*N. luteum*	
Nursery 05	586	c	*N. luteum*	
Nursery 05	614	c	*N. luteum*	
Nursery 05	621	c	*N. luteum*	
Nursery 05	622	c	*N. luteum*	
Nursery 05	642	c	*N. luteum*	
Nursery 05	643	c	*N. australe*	
Nursery 05	683	c	*N. australe*	
Nursery 05	689	c	*N. luteum*	
Nursery 05	692	c	*N. luteum*	
Nursery 05	715	c	*N. luteum*	
Nursery 05	726	c	*N. luteum*	
Nursery 05	734	c	*N. luteum*	
Nursery 05	735	c	*N. luteum*	
Nursery 05	738	c	*N. luteum*	
Nursery 05	767	c	*N. luteum*	
Nursery 05	768	c	*N. luteum*	
Nursery 05	770	c	*N. luteum*	
Nursery 05	787	c	*N. luteum*	
Nursery 05	790	c	*N. luteum*	
Nursery 05	817	c	*N. luteum*	
Nursery 05	824	c	*N. luteum*	

Continuation of D.2

Nursery	Isolate No.	Species/Group based on restriction digest of rDNA (ARDRA)		
		***Hae*III**	***Sac*II**	***Nci*I**
Nursery 05	847	c	*N. australe*	
Nursery 05	893	c	d	*D. mutila*
Nursery 03	930	*N. parvum*		
Nursery 03	935	*N. parvum*		
Nursery 03	936	*N. parvum*		
Nursery 03	963	c	*N. luteum*	
Nursery 03	985	c	*N. luteum*	
Nursery 03	988	c	*N. luteum*	
Nursery 03	1006	*N. parvum*		
Nursery 03	1014	*N. parvum*		
Nursery 03	1040	*N. parvum*		
Nursery 03	1073	*N. parvum*		
Nursery 03	1074	*N. parvum*		
Nursery 03	1082	c	*N. luteum*	
Nursery 03	1129	c	*N. luteum*	
Nursery 03	1131	c	*N. luteum*	
Nursery 03	1147	c	*N. luteum*	
Nursery 03	1149	*N. parvum*		
Nursery 03	1152	*N. parvum*		
Nursery 03	1169	c	*N. luteum*	
Nursery 03	1171	c	*N. luteum*	
Nursery 03	1174	c	*N. luteum*	
Nursery 03	1176	c	*N. luteum*	
Nursery 03	1180	c	*N. luteum*	
Nursery 03	1184	*N. parvum*		
Nursery 03	1190	c	*N. luteum*	
Nursery 03	1208	*N. parvum*		
Nursery 03	1236	*N. parvum*		
Nursery 03	1269	c	*N. luteum*	
Nursery 03	1277	*N. parvum*		
Nursery 03	1278	c	*N. luteum*	
Nursery 03	1283	c	*N. luteum*	
Nursery 03	1285	c	*N. luteum*	
Nursery 03	1301	c	*N. luteum*	
Nursery 03	1311	c	*N. luteum*	
Nursery 03	1325	c	*N. luteum*	
Nursery 03	1329	*N. parvum*		
Nursery 03	1346	c	*N. luteum*	

D.3 Identification of isolates from washing of plant surfaces, plant debris and isolation of cuttings for internal infections during the 2009 nursery sampling (Chapter 5)

Source	Nursery	Variety	Vine No.	Isolate No.	Species/Group based on restriction digest of rDNA (ARDRA)		
					*Hae*III	*Sac*II	*Nci*I
Surface propagules	5	Pinot gris	1	X81	Group C	*N. luteum*	
by plating	5	Pinot gris	2	X82	Group C	*N. luteum*	
assay	5	Pinot gris	4	X84	Group C	*N. luteum*	
	5	Pinot gris	5	X85	Group C	*N. luteum*	
	5	Riesling	5	X90	Group C	*N. luteum*	
	3	101-14	5	X110	*N. parvum*		
	3	Riparia gloire	2	X117	*N. parvum*		
	3			X118	*N. parvum*		
	3			X120	Group C	*N. luteum*	
	3			X127	Group C	*N. luteum*	
Plant Debris	3	3309		PD17	Group C	Group D	*D. mutila*
plating assay	3	Sauvignon blanc		PD22	*N. parvum*		
Internal infection				C623	Group C	*N. luteum*	
				C651	Group C	*N. australe*	
				C665	Group C	*N. australe*	
				C750	Group C	*N. luteum*	
				C1170	Group C	*N. luteum*	

D.4 Identification of isolates from different parts of the mothervine (Chapter 6)

Plant source	Variety	Vine No.	Shoot No.	Cane No.	Tissue type	Species/Group based on restriction digest of rDNA (ARDRA)		
						*Hae*III	*Sac*II	*Nci*I
Mothervine	3309	5			core	Group C	Group D	*D. mutila*
trunks	3309	6			core	Group C	*N. luteum*	
	3309	9			core	Group C	*N. luteum*	
	101-14	15			core	*N. parvum*		
	101-14	16			core	*N. parvum*		
Shoots	3309	1	1	10	bark	Group C	*N. luteum*	
	3309	3	1	2	bark	Group C	*N. luteum*	
	3309	4	3	1	bark	Group C	*N. luteum*	
	3309	6	3	1	bark	Group C	*N. luteum*	
	3309	9	3	2	bark	*N. parvum*		
	3309	9	3	2	wood	Group C	*N. luteum*	
	101-14	16	1	1	bark	Group C	*N. luteum*	
	101-14	16	1	1	bark	*N. parvum*		

D.5 Identification of isolates from sequential isolations from an entire shoot (Chapter 6)

Tissue type	Segment No.	Species/Group based on restriction digest of rDNA (ARDRA)		
		***Hae*III**	***Sac*II**	***Nci*I**
Bark	83	Group C	*N. luteum*	
Bark	87	*N. parvum*	Group D	*D. mutila*
Bark	92	Group C	*N. luteum*	
Bark	94	Group C	*N. luteum*	
Bark	95	Group C	*N. luteum*	
Bark	96	Group C	*N. luteum*	
Bark	97	*N. parvum*		
Bark	98	*N. parvum*		
Bark	99	Group C	*N. luteum*	
Bark	100	Group C	*N. luteum*	
Bark	101	*N. parvum*		
Bark	102	Group C	*N. luteum*	
Bark	103	Group C	*N. luteum*	
Bark	104	Group C	*N. luteum*	
Bark	105	Group C	*N. luteum*	
Bark	106	Group C	*N. luteum*	
Bark	107	Group C	*N. luteum*	
Bark	108	Group C	*N. luteum*	
Bark	109	Group C	*N. luteum*	
Bark	110	*N. parvum*		
Bark	111	*N. parvum*		
Bark	113	*N. parvum*		
Bark	115	Group C	Group D	*D. mutila*
Bark	116	*N. parvum*		
Bark	117	*N. parvum*		
Bark	118	*N. parvum*		
Bark	119	Group C	Group D	*D. mutila*
Bark	120	Group C	Group D	*D. mutila*
Bark	121	*N. parvum*		
Bark	122	Group C	Group D	*D. mutila*
Bark	123	Group C	Group D	*D. mutila*
Bark	124	*N. parvum*		
Bark	125	Group C	Group D	*D. mutila*
Bark	126	Group C	*N. luteum*	
Bark	127	Group C	Group D	*D. mutila*
Bark	128	Group C	*N. luteum*	
Bark	129	*N. parvum*		
Bark	130	*N. parvum*		
Bark	131	Group C	Group D	*D. mutila*
Bark	132	Group C	*N. luteum*	
Bark	133	*N. parvum*		

Continuation of D.5

Tissue type	Segment No.	Species/Group based on restriction digest of rDNA (ARDRA)		
		***Hae*III**	***Sac*II**	***Nci*I**
Bark	135	*N. parvum*		
Bark	137	*N. parvum*		
Bark	138	*N. parvum*		
Bark	141	*N. parvum*		
Bark	142	*N. parvum*		
Bark	143	*N. parvum*		
Bark	144	Group C	*N. luteum*	
Bark	145	*N. parvum*		
Bark	146	*N. parvum*		
Bark	147	*N. parvum*		
Bark	148	*N. parvum*		
Bark	149	*N. parvum*		
Bark	150	*N. parvum*		
Bark	151	*N. parvum*		
Bark	152	*N. parvum*		
Bark	159	Group C	*N. luteum*	
Wood	90	Group C	*N. luteum*	
Wood	96	Group C	*N. luteum*	
Wood	100	Group C	*N. luteum*	
Wood	101	*N. parvum*		
Wood	106	Group C	*N. luteum*	
Wood	107	Group C	*N. luteum*	
Wood	133	Group C	*N. luteum*	
Wood	134	*N. parvum*		
Wood	139	*N. parvum*		
Wood	142	*N. parvum*		
Wood	146	*N. parvum*		
Wood	147	*N. parvum*		

Appendix E
Sequence analyses

E.1 Nucleotide sequence of ITS region obtained from molecular identification using ITS1 primer of representative botryosphaeriaceous species isolates from the 2008 nursery sampling

E.1.1 *Neofusicoccum luteum* isolates

Isolate L067

GTCAACCTTGAGAAAAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGTTTTCTACTACGCTTGAG
GCAAGACGCCACCGCCGAGGTCTTCGAGGCGCGTCCACAGAGGACGGAGCCCAATACCAAGCAGAGCTTGAGGGTTGAAATGA
CGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTC
ACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAGTTTATTAACTT
GTTTCTCAGACTGCGACGTTTACTGACTGGAGTTTTGTGGTCCTCTGGCGGGCGCTGGCCGGCCCCCCGAACGGGGGTCGGTG
CGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTAACACATGGGGTGGGAGAGTCGAGCCGGAGCTCGAATCAACTCGGT
AATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACATTTTTACTTCCTCTA

Isolate L106

TACCTGATCCGAGGTCAACCTTGAGAAAAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGTTTTC
TACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTCGAGGCGCGTCCACAGAGGACGGAGCCCAATACCAAGCAGAGCTTG
AGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTG
AATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTT
AGTTTATTAACTTGTTTCTCAGACTGCGACGTTTACTGACTGGAGTTTTGTGGTCCTCTGGCGGGCGCTGGCCGGCCCCCCGA
ACGGGGGTCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATGGGGTGGGAGAGTCGAGCCGGAGCTCG
AATCAACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTACTTCCT

Isolate L228

CAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGTTTTCTACTACGCTTGAGGCAAGACGCCACCGCCGAG
GTCTTCGAGGCGCGTCCACAGAGGACGGAGCCCAATACCAAGCAGAGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCC
CTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTC
GCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAGTTTATTAACTTGTTTCTCAGACTGCGACGT
TTACTGACTGGAGTTTTGTGGTCCTCTGGCGGGCGCTGGCCGGCCCCCCGAACGGGGGTCGGTGCGGAGGACCGCGGCCCGCC
AAAGCAACAGAGGTAGGTACACATGGGGG

Isolate L864

TACCTGATCCGAGGTCAACCTTGAGAAAAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGTTTTC
TACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTCGAGGCGCGTCCACAGAGGACGGAGCCCAATACCAAGCAGAGCTTG
AGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTG
AATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTT
AGTTTATTAACTTGTTTCTCAGACTGCGACGTTTACTGACTGGAGTTTTGTGGTCCTCTGGCGGGCGCTGGCCGGCCCCCCGA
ACGGGGGTCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTAACACATGGGGTGGGAGAGTCGAGCCGGAGCTC
GAATCAACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTAC

E.1.2 *Neofusicoccum parvum* isolates

Isolate P069

TACCTGATCCGAGGTCAACCTTGAGAAATAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGTTTT
CTACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTTAAGGCGCGTCCGTGGAGGACGGGGCCCAATACCAAGCAGAGCTT
GAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACT
GAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTT
TAGTTTATTAACTTGTTTTTCAGACTGCGAAGTTCACTGACTGGAGTTTTATGGTCCTCTGGCGGGCGCTGGCCAGCCCCCCG
AAGGGCGCCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATTGGGTGGGAGAGTCGAGCCGGAGCTCG
AATCAACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGAC

Isolate P072

TACCTGATCCGAGGTCAACCTTGAGAAATAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGTTTT
CTACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTTAAGGCGCGTCCGTGGAGGACGGGGCCCAATACCAAGCAGAGCTT
GAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACT
GAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTT
TAGTTTATTAACTTGTTTTTCAGACTGCGAAGTTCACTGACTGGAGTTTTATGGTCCTCTGGCGGGCGCTGGCCAGCCCCCCG
AAGGGCGCCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATAGGGTGGGAGAGTCGAGCCGGAGCTCG
AATCAACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTACTTCCTCT

Isolate P116

TACCTGATCCGAGGTCAACCTTGAGAAATAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGTTTT
CTACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTTAAGGCGCGTCCGTGGAGGACGGGGCCCAATACCAAGCAGAGCTT
GAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACT
GAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTT
TAGTTTATTAACTTGTTTTTCAGACTGCGAAGTTCACTGACTGGAGTTTTATGGTCCTCTGGCGGGCGCTGGCCAGCCCCCCG
AAGGGCGCCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATTGGGTGGGAGAGTCGAGCCGGAGCTCG
AATCAACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTACTTCCTCTATT

Isolate P471

TACCTGATCCGAGGTCAACCTTGAGAAATAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGTTTT
CTACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTTAAGGCGCGTCCGTGGAGGACGGGGCCCAATACCAAGCAGAGCTT
GAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACT
GAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTT
TAGTTTATTAACTTGTTTTTCAGACTGCGAAGTTCACTGACTGGAGTTTTATGGTCCTCTGGCGGGCGCTGGCCAGCCCCCCG
AAGGGCGCCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATAGGGTGGGAGAGTCGAGCCGGAGCTCG
AATCAACTCGGTAATGATCCTTCCGCAGGTCACCTACGGAAACCTTGTTACGACTTTTACTTCCTC

E.1.3 *Neofusicoccum australe* isolate

Isolate A088

GTCAACCTTGAGAAAAATTCAAAGGTTCGTCCGGCGGGCGACGCCATGCGCTCCAAAGCGAGGTGTTTTCTACTACGCTTGAG
GCAAGACGCCACCACCGAGGTCTTCGAGGCGCGTCCGCGGAGGACGGAGCCCAATACCAAGCAGAGCTTGAGGGTTGAAATGA
CGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTC
ACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAGTTTATTAACTT
GTTTCTCAGACTGCGACGTTTACTGACTGGAGTTTTGTGGTCCTCTGGCGGGCGCTGGCCGGCCCCCGAACGGGGGTCGGTGC
GGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATGGGGTGGGAGAGTCGAGCCGGAGCTCGAATCAACTCGGTAA
TGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTACTTCCTCTA

E.1.4 *Botryosphaeria dothidea* isolate

Isolate B007

TACCTGATCCGAGGTCAACCTTGAGAAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCCCTGCGCTCCGAAGCGAGATGTATG
TTCTACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTTGAGGCGCGCCCGCAAAGGACGGTGCCCAATACCAAGCAGAGC
TTGAGGGTTGTAATGACGCTCGAACAGGCATGCCCTTCGGAATACCAAAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCA
CTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGT
TTTAGTTTATTAAATGTTTTTCAGACTGCATCGTTTACTGACTGGAGTTTGATGGTCCTCTGGCGGGCGCTGGCCACCCCCCC
GGGGAGGGGGGCGGCCGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACAAAGGGTGGGAGGATCGGGCCGGAG
CCCGAATCAACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTACTTCCTCTA

E.1.5 *Diplodia mutila* isolate

Isolate S103

TCAACCTTGAGAAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCCAACCGCTCCAAAGCGAGGTGTATTCTACTACGCTTGAG
GGCTGAACAGCCACCGCCGAGGTCTTTGAGGCGCGTCCGCAGAGAGGACGGCGCCCAATACCAAGCAGAGCTTGAGGGTTGTA
ATGACGCTCGAACAGGCATGCCCCTCGGAATGCCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCA
ATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAGTTTATTA
ACTTGTTTTTCAGACGTCGACGTTTACTGACTGGAGTTTGAAGGTCCTCTGGCGGAAGCGCGC

E.1.6 *Diplodia seriata* isolate

Isolate O099

GTTCAGAAGGTTCGTCCGGCGGGCGACGCCAACCGCTCCAAAGCGAGGTGTATTCTACTACGCTTGAGGGCTGAACAGCCACC
GCCGAGGTCTTTAAGGCGCGTCCGCAGAGAGGACGGCGCCCAATACCAAGCAGAGCTTGAGGGTTGTAATGACGCTCGAACAG
GCATGCCCCCCGGAATGCCAGGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTAT
CGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAGTTTATTAACTTGTTTATCAGAC
GTCGACGTTTACTGACTGGAGTTTGAAGGTCCTCTGGCAGAAAGCGCGCGGGGGGGCCCGCGAGGGCCTCCTCGCGGCAAAGA
GCCGCCAAAGCAACAGAGGTATGTTCACAAAGGGTGGGAGATTCGAGCCGAAGCCCGAGAACTCGGTAATGATCCTTCCGCAG
GTTCACCTACGGAAACCTTGTTACGACTTTTACTTCCTCT

E.1.7 *Neofusicoccum ribis* isolates

Isolate R083A

CGATCCTACCTGATCGAGGTCACCTTGAGAATAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGT
TTTCTACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTTAAGGCGCGTCCGTGGAGGACGGAGCCCAATACCAAGCAGAG
CTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTC
ACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAG
TTTTAGTTTATTAACTTGTTTTTCAGACTGCGAAGTTCACTGACTGGAGTTTTATGGTCCTCTGGCGGGCGCTGGCCAGCCCC
CCGAAGGGCGCCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTGGGTACACATTGGGTGGGAGAGTCGAGCCGGAGC
TCGAATCAACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTACTTCCTCTAATGTGACCAAG
GA

Isolate R083B

GGATCGTACCTGATCGAGGTCACCTTGAGAATAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGT
TTTCTACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTTAAGGCGCGTCCGTGGAGGACGGAGCCCAATACCAAGCAGAG
CTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTC
ACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAG
TTTTAGTTTATTAACTTGTTTTTCAGACTGCGAAGTTCACTGACTGGAGTTTTATGGTCCTCTGGCGGGCGCTGGCCAGCCCC
CCGAAGGGCGCCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTGGGTACACATTGGGTGGGAGAGTCGAGCCGGAGC
TCGAATCAACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTACTTCCTCTAAATTGGACCAA
AGA

E.2 Nucleotide sequence of β-tubulin gene of isolates (R083A and R083B) initially identified as *N. ribis* by ARDRA.

Isolate R083A

TGTTTGCAAACTCCGCTCCGCGCCCCGCTGACGCGAATCGACACCACAGGCAGACCATTTCCGGCGAGCATGGCCTGGACGGC
TCTGGCGTGTAAGTCTGCGCCGTTTCCCGCGCGAATGGCAATGGCTGACCCGCAGCAGCTACAATGGCACCTCCGACCTGCAG
CTCGAGCGCATGAACGTCTACTTCAACGAGGTACTCTCTCACTAATTGCACAAACACATAAAGTATGGCAATCTTCTGAACGC
GCAGCAGGCGTCCAACAACAAGTACGTTCCTCGTGCCGTCCTCGTCGACCTCGAGCCCGGCACCATGGATGCCGTCCGCGCCG
GCCCCTTCGGCCAGCTCTTCCGCCCCGACAACTTCGTCTTCGGCCAGTCTGGCGCCGGTAACAACTGGGCCAAGGGTCACTAC
ACTGAGGTTA

Isolate R083B

TGTTGCAAACCTCCGCTCCGCGCCCCGCTGACGCGAATCGACACCACAGGCAGACCATTTCCGGCGAGCATGGCCTGGACGGC
TCTGGCGTGTAAGTCTGCGCCGTTTCCCGCGCGAATGGCAATGGCTGACCCGCAGCAGCTACAATGGCACCTCCGACCTGCAG
CTCGAGCGCATGAACGTCTACTTCAACGAGGTACTCTCTCACTAATTGCACAAACACATAAAGTATGGCAATCTTCTGAACGC
GCAGCAGGCGTCCAACAACAAGTACGTTCCTCGTGCCGTCCTCGTCGACCTCGAGCCCGGCACCATGGATGCCGTCCGCGCCG
GCCCCTTCGGCCAGCTCTTCCGCCCCGACAACTTCGTCTTCGGCCAGTCTGGCGCCGGTAACAACTGGGCCAAGGGTCACTAC
ACTGAGGT

E.3 Nucleotide sequence of ITS region, β-tubulin, elongation factor α-1 gene obtained from molecular identification of novel species *N. macroclavatum*.

E.3.1 rDNA sequence

Isolate M353

CGATCCTACCTGATCGAGGTCACCTTGAGAATAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGT
TTTCTACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTTAAGGCGCGTCCGTGGAGGACGGAGCCCAATACCAAGCAGAG
CTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTC
ACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAG
TTTTAGTTTATTAACTTGTTTTTCAGACTGCGAAGTTCACTGACTGGAGTTTTATGGTCCTCTGGCGGGCGCTGGCCAGCCCC
CCGAAGGGCGCCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTGGGTACACATTGGGTGGGAGAGTCGAGCCGGAGC
TCGAATCAACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTACTTCCTCTAATGTGACCAAG
GA

E.3.2 β-tubulin sequence

Isolate M353

GTTGCAAACACTGCCGCTCCGCGCCCGCTGACGCGAATCGACACCACAGGCAGACCATTTCCGGCGAGCACGGCCTGGACGGC
TCTGGCGTGTGAGTCTGCGCCGTTTCCCGCGCGAATGACAATGGCTGACCCGCAGCAGCTACAATGGCACCTCCGACCTGCAG
CTCGAGCGCATGAACGTCTACTTCAACGAGGTACTCTCTCACTAATTGCACAAACACGTAAAGTATGGCAATCTTCTGAACGC
GCAGCAGGCGTCCAACAACAAGTACGTTCCTCGTGCCGTCCTCGTCGACCTCGAGCCCGGCACCATGGATGCCGTCCGCGCCG
GCCCCTTCGGCCAGCTCTTCCGCCCCGACAACTTTGTCTTCGGCCAGTCTGGCGCCGGTAACAACTGGGCCAAGGGTCACTAC
ACCTGAGGTA

E.3.3 Elongation factor α-1 gene sequence

Isolate M353

CTGCACGCGCTGGGTGCTGGGTTCCCGCACTCAATTTGCCTTATCGCTTCGGTGAGGGGCATTTTGGTGGTGGGGTCGGCCCG
CGCTAAGCCTCGTTTGGGCTCGGCAAAATCTCCGCATCTGGTTTTTTTGCGACCGGCGTGCGACCGAAGCGCGCCCCTCGCCA
GACACGCCACGCATGTGCGACCAGACGCTAACGGCCGTCCCAGGAAGCCGCCGAGCTCGGTAAGGGTTCCTTCAAGTA

E.4 Nucleotide sequence of ITS region obtained from molecular identification of representative botryosphaeriaceous species from the propagation system using ITS1 primers.

E.4.1 Nursery 3 callusing medium

GCTACTGGGCTGGTAACTAACTTTCACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAAT
GTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTTCGAG
CGTCATTTCAACCCTCAAGCTCTGCTTGGTATTGGGCTCCGTCCTCTGTGGACGCGCCTTGAAGACCTCGGGGGGGGGGTCTT
GCCTCAAGCGAAGAAAAAAACACCTCGCTTTGGAGGGAACGGGGGGCCCCGCCGGACGAACCTTTGAATTTTTTTTAAGGGTA
GCCCGCGAAAGGA

E.4.2 Nursery 3 grafting tool 2

GGGTTTTCGCTATAACTAAACTTTCACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAAT
GTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTTCGAG
CGTCATTTCAACCCTCAAGCTCTGCTTGGGTATTGGGCCCCGTCCTCCACGGACGCGCCTTAAAGACCTCGGCGGTGGCGTCT
TGCCTCAAGCGTAGTAGAAAACACCTCGCTTTGGAGCGCACGGCGTCGCCCGCCGGACGAACCTTTGAATTATTTCTAAAGGT
TGAACCTCGGAA

E.4.3 Nursery 3 hydration tank

GGCGGTGGGCTAGTAACTAAACTTTCACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA
TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTTCGA
GCGTCATTTCAACCCTCAAGCTCTGCTTGGGTATTGGGCCCCGTCCTCCACGGACGCGCCTTAAAGACCTCGGCGGTGGCGTC
TTGCCTCAAGCGTAGTAGAAAACACCTCGCTTTGGAGCGCACGGCGTCGCCCGCCGGACGAACCTTTGAATTATTTCTCAAGG
TGAACCTCGGAA

E.4.4 Nursery 3 post-grafting hydration

GGGTGGGCAGGGCCGCGGTAGCTAACTTTCACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAA
GTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGT
TCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTATTGGGCCCCGTCCTCCACGGACGCGCCTTGAAGACCTCGGCGGTGGC
GTCTTGCCTCAAGCGTAGTAGAAAACACCTCGCTTTGGAGCGCACGGCGTCGCCCGCCGGACGAACCTTTGAATTATTTCTCA
AGGTTGACCTCGGAAGGAA

E.4.5 Nursery 3 post-grafting hydration

TAGCTGCGGCTGCCCGCGGTAACTAACTTTCACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATA
AGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTG
TTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTATTGGGCTCCGTCCTCTGTGGACGCGCCTTGAAGACCTCGGCGGTGG
CGTCTTGCCTCAAGCGTAGTAGAAAACACCTCGCTTTGGAGCGCACGGCGTCGCCCGCCGGACGAACCTTTGAATTATTCTTA
AGGGTGGACTCCGAAAGGA

E.4.6 Nursery 3 post-cold storage rootstock washing

TAGCTGCGGCTGCCCGCGGTAACTAACTTTCACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATA
AGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTG
TTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTATTGGGCTCCGTCCTCTGTGGACGCGCCTTGAAGACCTCGGCGGTGG
CGTCTTGCCTCAAGCGTAGTAGAAAACACCTCGCTTTGGAGCGCACGGCGTCGCCCGCCGGACGAACCTTTGAATTATTCTTA
AGGGTGGACTCCGAAAGGA

E.4.7 Nursery 3 post-cold storage scionwood washing

GGGGCGTCGCATAGCTAGACTTTCACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATG
TGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTTCGAGC
GTCATTTCAACCCTCAAGCTCTGCTTGGGTATTGGGCCCCGTCCTCCACGGACGCGCCTTAAAGACCTCGGCGGTGGCGTCTT
GCCTCAAGCGTAGTAGAAAACACCTCGCTTTGGAGCGCACGGCGTCGCCCGCCGGACGAACCTTTGAATTATTTCTCAAGGTG
AACCTCGGAA

E.4.8 Nursery 3 wash pit

CGGGTCGCTATAAACTAACTTTCACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGT
GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTTCGAGCG
TCATTTCAACCCTCAAGCTCTGCTTGGGTATTGGGCCCCGTCCTCCACGGACGCGCCTTAAAGACCTCGGCGGTGGCGTCTTG
CCTCAAGCGTAGTAGAAAACACCTCGCTTTGGAGCGCACGGCGTCGCCCGCCGGACGAACCTTTGAATTATTTCTCAAGGTTG
AACCTCGGAAAGA

Appendix F
Sampling steps for nursery propagation system

F.1 Pre-cold storage hydration

Water samples were requested from Nursery 3 in the 2010 season and were sent to Lincoln University by courier. In Nursery 3, three 50 ml water samples were collected from the wash pit and four 50 ml samples from the post-collection hydration tanks. Water samples from the wash pit were collected from the catch basin of the wash pit where cuttings are hosed down prior to post-collection hydration. After washing, cuttings were placed in post-collection hydration tanks for overnight soaking prior to cold storage. The water samples were collected by agitating the catch basin and the tank and samples were collected in 50 ml plastic tubes and sent by courier in an insulated bag containing ice packs during transport to Lincoln University.

For Nursery 5, five samples (50 ml) of Chinosol solution (670 g/kg 8-hydroxyquinoline sulphate) in the form of a wettable powder that was used for post-collection hydration in June and July 2009 were collected in October 2009 (Figure 5.3C). This Chinosol solution had been reused several times for hydration through-out the 2009 harvest season. The solution was agitated for approximately 2 min prior to collection and placed in 50 ml plastic tubes and placed in a cooler and transported to Lincoln University for analysis.

No pre-cold storage hydration samples were collected in Nursery 9.

F.2 Pre-grafting rehydration

Nursery 3 did not rehydrate their plant materials prior to grafting. To determine if cuttings were contaminated prior to grafting, six rootstock cuttings were randomly picked from the grafting table and washed by placing one cutting in a new plastic bag containing 50 ml SROW + Tween 80 and agitated for 30 s. The wash water for each sample was transferred to a sterile 50 ml centrifuge tube. For scions, five pieces of scion cuttings were randomly picked from the grafting table and washed in plastic tubes containing 50 ml of sterile water as above and the wood pieces removed from the tube. Washing was repeated six times for a total of six samples.

In Nursery 5, rootstock cuttings were rehydrated prior to grafting but scion cuttings were normally not rehydrated. To rehydrated rootstocks, cuttings that were packed in perforated plastic bags (~500 pcs/pack) were placed in metal bins filled with tap water and left overnight

for soaking (Figure 5.2). After soaking, cuttings were lifted out from the tanks and allowed the water to drain before processing. The two rehydration tanks at Nursery 5 had been drained the day prior to sampling but some water (2-3 cm) were still available for sampling at the bottom of the tanks. From each tank, the water was agitated thoroughly and five samples were collected in 50 ml sterile plastic tubes for a total of 10 samples. For budwood, five samples were randomly picked from the grafting table and washed using the same method described previously for Nursery 3. The process was repeated five times for a total of five samples.

For Nursery 9, both the rootstock and scion cuttings were rehydrated prior to grafting but in separate rehydration containers. Scion cuttings of different varieties were placed in separate net bags and soaked in tap water inside a plastic barrel for 4-5 h or until the bags were observed to sink to the bottom of the barrel (Figure 5.3D). For rootstocks, rehydration was done by placing the 8 to 10 bundles of cuttings (~200 pieces per bundle) into a large plastic bin with wooden pallets on top to keep the cuttings underwater and soaked for approximately 6 h (Figure 5.3E). The cuttings were lifted out of the tank and allowed to drain prior to grafting. Five samples from each hydration tanks were collected by agitating the tanks thoroughly and 50 ml water were placed in sterile plastic tubes. All samples were placed in a cooler with ice packs and transported to Lincoln University.

For Nursery 3, instead of pre-grafting hydration, hydration was done after grafting. This step involved soaking of the basal part of the grafted plants overnight in plastic trays containing 3-4 cm of tap water prior to callusing (Figure 5.3F). Two water samples were collected in 50 ml tubes from each of the nine trays used for pre-callusing hydration. Each tray was agitated prior to sampling. All samples were placed in a chilly bin containing ice packs and transported to Lincoln University for analysis.

F.3 Grafting Tools

Nursery 3 and Nursery 9 were using omega-cut grafting tools (Figure 5.3A) while Nursery 5 used the V-cut design (Figure 5.3B). Three grafting tools in Nursery 3 and 5 and one at Nursery 9 that were used during commercial operation were washed during morning (10:30 am), lunch (12:30 pm) and afternoon (3:00 pm) breaks of the nursery staff. Washing was done by squirting ~50 ml of SROW + Tween 80 (0.01%) on the blades and platform of the grafting tools using a wash bottle, and collecting the water by a sterile funnel that directed the water into a new plastic bag. Wash water samples were transferred to sterile plastic 50 ml tubes.

F.4 Callusing Media

For Nursery 3, perlite was used as a standard callusing medium for their grafted plants (Figure 5.4a). The perlite was normally reused several times during the grafting season. At the end of each season, the medium was air-dried and saved for the following season. For sample collection, ~200 g of previous-season perlite was collected in new plastic bags from each of the six wooden bins prior to the 2010 callusing. From each bin, one sample was collected at the top 15 cm and another from about ~50 cm deep giving a total of 12 samples. Six perlite samples were further collected after callusing from three callusing boxes of the 2010 season.

For Nursery 5, perlite was also the standard medium that was reused throughout the season (Figure 5.4a). The medium was normally saved for the next year but steam-sterilised at 70-80^{o}C for 3 h and allowed to cool for at least two days prior to use. For sampling, four samples each (~200 g) of sterilised perlite were collected from the top 15 cm and ~50 cm of the bin. Additional two samples of recently used perlite were sent by courier four weeks after the first sampling.

For Nursery 9, newly purchased perlite and vermiculite were normally used for callusing plants. Perlite was used at the bottom layer of the callusing box while vermiculite was used at the upper layer covering the graft union and the budwood (Figure 5.4b). This callusing media were re-used several times during the season and discarded at the end of each season. Two samples (~200 g) each of perlite and vermiculite that has not been used for callusing were collected. Additional two samples each of recently used perlite and vermiculite were requested from the Nursery and sent to Lincoln University for analysis.