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Botryosphaeria disease in New Zealand blueberry gardens: identification of pathogens, inoculum sources and factors affecting disease development

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A thesis

submitted in partial fulfilment

of the requirements for the Degree of

Doctor of Philosophy (Plant Pathology)

at

Lincoln University

by

K.M Shanika Tennakoon

Lincoln University

2016

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

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by

Shanika Tennakoon

Botryosphaeriaceae species cause dieback and death of blueberry plants but the species involved and factors affecting infection and spread are not well understood. Sampling at blueberry farms found these pathogens in five of seven farms sampled; overall incidence was 41.4%, with *N. australe* (79.0%), *N. luteum* (8.0%), *N. ribis* (8.0%) and *N. parvum* (5.0%). Sampling of nursery plants found infections in all four nurseries with 45% incidence in mainly asymptomatic plants, which were infected with *N. australe* (66.0%), *N. parvum* (31.5%) and *N. ribis* (2.5%). Asymptomatic propagation cuttings from one nursery were found to have external contamination of Botryosphaeriaceae DNA (90.0%) and internal infection (65.0%) by the main four species found in the blueberry farms and nurseries. Propagation media received from four nurseries contained Botryosphaeriaceae DNA (44.0% incidence), with species identified using SSCP as *N. australe*, *N. luteum*, *N. parvum*/ *N. ribis* and *D. mutila*. However, when conidia of *N. australe* and *N. ribis* were added to the propagation media of potted blueberry plants to investigate the potential for root infection, genotyping by UP-PCR showed that the isolates recovered from the roots and stems of plants were not the isolates added to the soil. Thus, inocula in propagation mixtures is unlikely to cause infection of nursery plants.

Isolates of the four main species recovered from farms and nurseries were pathogenic on blueberry stems but pathogenicity differed significantly between species and isolates within a species, with *N. ribis* being the most pathogenic, then *N. parvum*, *N. luteum* and *N. australe*. Conidia of these species caused necrotic lesions on wounded crowns and trunks, and infection progressed down into roots and up into shoots of the plants. These conidia also caused infection of hard and soft shoots, flower and leaf buds, as well as fruits, incidence and progression usually being greater in wounded than non-wounded tissues. No lesions developed in non-wounded soft and hard shoots. Wound age (0-28 days) in soft and

hard shoots and trunks showed decreasing pathogen progression by *N. ribis* over time, with progression of 16.7, 33.3 and 35.0 mm respectively, by 28 days. However, lesions did not develop from wounds inoculated after 7, 4 and 4 days, respectively. Herbicide damage of hard green shoots allowed for infection and lesion development by *N. ribis,* levels being similar to the wounded control. The seven blueberry cultivars inoculated with *N. parvum* and *N. ribis* conidia differed in disease development with Centra blue, Maru and Ocean blue showing lowest susceptibility.

Conidia of the main three *Neofusicoccum* species were released from pycnidia at 15-30°C and under relative humidities (RHs) of 80-100%, with greatest numbers usually released by *N. parvum*. Overall, the greatest numbers of oozing pycnidia and conidia released were observed at higher temperatures (25-30°C) and RHs (92-100%). Infection of wounded and non-wounded shoots of potted blueberry plants by *N. ribis* progressed through the tissues irrespective of the early incubation conditions, 20 or 25°C and 90 or 100% RH. Further, a field study with potted plants showed that *N. ribis* infection of hard shoots and trunks could take place in summer, autumn and winter, but was least in winter.

In vitro fungicide experiments with four isolates each of *N. australe*, *N. luteum*, *N. parvum* and *N. ribis* showed differences in mycelial growth, conidial germination and germ tube growth between isolates and species, and between products. *In vivo* evaluation carried out with fungicides on wounded and non-wounded plant tissues on potted and field blueberry plants showed that carbendazim and tebuconazole were the most effective for protecting blueberry plants from infection by *Neofusicoccum* species. The biological products Serenade™ and Vinevax™ provided some control if applied 7-14 days before inoculation.

Overall, this study has shown that *Neofusicoccum* species are widespread in New Zealand farms and nurseries and can infect all aerial tissues of the plants, with production of conidia from all tissues. The potential infection of non-wounded tissues has indicated that fungicides need to be applied overall, not just on wounds as previously believed, and that inoculum sources should be eliminated with all possible control methods.

Key words: *Neofusicoccum* species, widespread infection, pathogenicity, wounding, humidity, temperature, fungicides, biocontrol

Acknowledgements

I am forever indebted to my academic supervisors…

I am very much thankful to Associate Prof. Marlene Jaspers for her guidance, expertice, commitment, for helping me with sample collection and have gone beyond the duties to fight away my worries, concerns and anxieties. I express my deepest gratitude to Dr. Eirian Jones for her encouragement, patience, and shown faith in my work and directing me to persuade a career in Science. I also extend my heartfelt thanks to Associate Prof. Hayley Ridgway for guiding all the molecular work through out my research, precious advice on how to improve scientific writing and encouraging me to finalize the thesis.

I also like to express my sincere gratitude to Mr. Geoff Langford (Plant and Food Research Lincoln) for his valuable guidance.

I extend my sincere gratitude…

To blueberry growers of New Zealand and the Lincoln University for the research funds and scholarships that supported my work. I am grateful to ICCP, New Zealand plant protection society and Lincoln University ELLS summer scholarships for the travel grants to attend conferences and workshops in and out of New Zealand.To owners and staff of blueberry farms and nursery, for plant materials, space to conduct trials, their expertise and time.

To the University academic, technical and administrative staff who were always there to support; Professor Nick Dickinson, for his trust, support and encouragement and for being such an inspiring person; Candice Barclay, for her incredible patience and technical support.

To my very special plant pathology- molecular team, my family for the last three years. Thank you all for being there for me and making my life (apart from studies) more enjoyable. A big thank you to all my friends overseas, who gave me motivation when I was having a bad day.

I would like to dedicate this dissertation to my family. To my dad Ajantha and my mom Anula who trusted and supported and encouraged me to go away from my home country and see the world and persuade my dreams. To my loving brother Eranda who encouraged me throughout my life.

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Chapter 1

Introduction

1.1 Blueberry industry in New Zealand

Blueberries are grown commercially in 27 countries including Canada, South America, Australia, New Zealand, and northern and eastern Europe (Blueberry Council New Zealand, 2003). Two species of blueberry [*Vaccinium corymbosum* (highbush) and *V. ashei* (rabbiteye)] were introduced to New Zealand from North America in 1950 and development of the industry has been rapid since 1975 (Poll and Wood, 1985). By June 2007 there were 560 ha managed by 96 growers, yielding 2940 tonnes with crop values of NZ$14.5 m in the export market and NZ$25 m in the domestic market (Fresh Facts, 2007). The New Zealand industry has been an active participant in developing blueberries as a global fruit following their domestication in the early 20th century, and 10 new cultivars have been developed which are suitable for New Zealand conditions. Overall, 75-100 named cultivars are believed to exist currently, and new cultivars are being produced annually (Future Horticulture, 2013).

1.2 Blueberry diseases

Blueberry growers experience crop losses due to various diseases and pests. Several viral, bacterial and fungal diseases have been described on blueberries worldwide (Caruso and Ramsdell, 1995). Common fungal diseases include phytophthora root rot (caused by *Phytophthora cinnamomi*), botrytis blight/grey mould (caused by *Botrytis cinerea)*, mummy berry (caused by *Monilinia vaccinii-corymbosi*), anthracnose (caused by *Colletrotrichum gloeosporioides* and *Colletrotrichum acutatum)*, early rot (caused by *Guignardia vacinii*) and blueberry leaf rust (caused by *Naohidemyces vacinii*) (Langford, 2010). Wood diseases caused by several fungi including *Phomopsis vaccinii*, *Fusicoccum putrefaciens*, *Botryosphaeria cortices* and *B. dothidea* have been reported to produce canker symptoms which can result in the die back of twigs, branches or entire stems (Hartman *et al.*, 2008). *Botryosphaeria cortices* and *B. dothidea* have also been reported to cause leaf spots, and fruit rots on blueberry and have been responsible for devastating disease out breaks in the

United States (Milholland, 1995). Among these disease symptoms, dieback, stem cankers and stem blight are the most important resulting in yield reduction and plant death. In New Zealand this disease is an emerging problem; dieback and crown rot were estimated to affect about 18% of blueberry plants in the main production areas, costing about $500,000 annually due to yield losses and replanting costs (Sammonds *et al.,* 2009). Therefore, identification of the Botryosphaeriaceae species associated with dieback and crown rot of blueberry plants, and knowledge of the infection processes and the factors that increase infection risk are essential for the development of control strategies and thereby reduction of yield losses in New Zealand.

1.3 Botryosphaeria dieback in blueberry

Botryosphaeriaceae species have been associated with canker and dieback symptoms in a broad range of different perennial fruit crops worldwide, and their importance as canker-causing and fruit rot agents has been extensively recognized (Espinoza *et al.,* 2009). Florida blueberry growers have identified stem blight and dieback symptoms as economically the most important disease problems they face (Wright and Harmon, 2010). Stem blight caused by *B. cortices* and *B. dothidea* (anamorph *Fusicoccum aesculi*) was reported to be a widespread and destructive disease in blueberries in southeastern United State (Milholland, 1971a; Creswell and Milholland, 1988; Smith, 2009). However, in Florida two dominant species, *Lasiodiplodia theobromae* and *Neofusicoccum ribis* were identified as causative agents of stem blight in southern highbush blueberries (Wright and Harmon, 2010). A study conducted in Chile to identify the canker-causing pathogen species in blueberries, revealed three species of Botryosphaeriaceae namely *Neofusicoccum arbuti*, *N. australe*, and *N. parvum* (Espinoza *et al.,* 2009). Choi (2011) reported a bark dieback in blueberries caused by *F. aesculi* in Korea. A recent study conducted by Yu *et al.* (2012) reported that *F. aesculi* caused dieback and stem blight in blueberries in southwest China. These reports have indicated that Botryosphaeriaceae species are probably ubiquitous in blueberries.

Botryosphaeriaceae species occurrence in blueberries in New Zealand was investigated in a preliminary study which isolated pathogens from dieback symptoms on 70 blueberry specimens from three central North Island regions (Sammonds *et al*., 2009). The study found Botryosphaeriaceae species associated with 86% of the lesions in these plants. During

the study, the conidia of 40 representative cultures were examined morphologically, resulting in identification of *N. parvum*, *N. luteum/australe* and *Diplodia seriata* with one unidentified Botryosphaeriaceae species, apparently of the *Neofusicoccum* type. Molecular identification of 14 isolates selected to be representative of the *Neofusicoccum* isolates from these 40 isolates confirmed the presence of *N. luteum, N. parvum* and *N. australe*. Another identification study with the remaining 99 isolates from the sampling study used a combination of morphological and molecular techniques for identification. Identified species were *D. seriata, N. parvum*, *N. luteum*, *N. australe* and *N. ribis* (Che Omar, 2009). Species identified from dieback in New Zealand blueberries to-date were the same as those identified from grapevine dieback in New Zealand. All these blueberry studies were conducted using the isolates collected from the North Island of New Zealand; therefore a second study obtained samples from diseased South Island blueberry farms and some additional North Island farms to identify the species and their prevalence. The 18 isolates collected were identified as *N. australe*, *D. seriata* and *N. ribis* but not *N. parvum* which had been previously found in North Island blueberry farms and is commonly found in other hosts such as grapevine (Antunovich, 2010). Moreover, in the South Island *N. australe* was the dominant species among the isolates taken from the Timaru region, whereas isolates taken from the Canterbury region were identified as *N. australe*, *D. seriata* and *N. ribis*. However from this data it is difficult to come to a conclusion regarding the distribution of Botryosphaeriaceae species in blueberries as only a few growers were surveyed and many isolates were not identified with molecular methods. Therefore more samples need to be taken that represent more growers, to develop an understanding of the species distribution in New Zealand. At the start of this research programme, there were approximately 200 isolates from blueberry in the Plant Microbiology culture collection, many of which had not identified to species level. To determine the most pathogenic and common species in New Zealand, these isolates need to be identified and respective isolates of each species need to be inoculated into blueberry tissues.

1.3.1 Botryosphaeriaceae species identification based on morphology

The genus *Botryosphaeria,* which belongs to the Ascomycotina, is a species rich genus. Due to the complex taxonomy of this family, teleomorphic and anamorphic names are being used at the same time when referring to these species (Crous *et al*., 2006). Identification of these fungi to the species level is complicated due to several factors. They rarely form teleomorphs in nature, therefore identification of species is usually based mainly on morphological characters of the anamorph, which is the most common form found in nature (Jacobs and Rehner, 1998). Most of the recent research papers have used the anamorphic names for the *Neofusicoccum* groups and the teleomorphic names for other species only when the teleomorphic state has been found frequently in nature. The same format will be used in this thesis. The main morphological features used for identification include conidial shape, size, colour, septation, wall thickness and texture of conidia, and details of conidiogenesis. However, these characters require careful interpretation, as there is substantial overlap between many species. Thus, conidial size represents a continuous character that may change with age or on different substrates and hosts, and it is also variable between isolates (Pennycook and Samuels, 1985; Slippers *et al.,* 2007). The diversity of anamorph states of Botryosphaeriaceae species have added to the taxonomic confusion (van Niekerk *et al.,* 2004). Moreover, as species may vary in minor morphological features identification can be difficult for those not familiar with these fungi (Shoemaker, 1964). Colony morphology, chromogenicity and temperature effects, and mycelial growth rate have also been used for species recognition (Amponsah *et al.,* 2011).

To date, taxonomic studies have depended on morphological characters and analyses of nucleotide sequences of multiple genes, which have allowed the identification of at least 21 different species in the Botryosphaeriaceae occurring in grapevines worldwide (Urbez-Torres, 2011). Botryosphaeriaceae species occurring on grapevines in different countries have been shown to differ in pathogenicity; this has led to confusion and conflicting reports about which species of Botryosphaeriaceae are important pathogens of grapevines (Phillips, 2002). In New Zealand some work has been done regarding the identification of Botryosphaeriaceae species in blueberries, with the studies of Sammonds *et al.* (2009), Che Omar (2009) and Antunovich (2010). It is essential to identify the remaining species from the study of Antunovich (2010) using accurate and reproducible identification methods.

This would help to indicate the most important Botryosphaeriaceae pathogens of blueberry in New Zealand and allow for investigation of the factors affecting their distribution.

1.3.2 Botryosphaeriaceae species identification in blueberries based on molecular techniques

In recent years, molecular techniques have increasingly been used for the identification of animals, plants and micro-organisms. For the accurate identification of Botryosphaeriaceae species it is necessary to use molecular methods along with morphological identification (Phillips *et al*., 2002). In prior research, different multigene molecular methods have been developed for PCR-based detection of some Botryosphaeriaceae species (Zhou and Stanosz, 2001; Phillips *et al*., 2002; Alves *et al*., 2004; Slippers *et al*., 2007). However amplification of ITS regions or restriction fragment length polymorphism alone cannot clearly discriminate between the species, *N. ribis* and *N. parvum.* Partial sequences of the elongation factor 1-α (EF1-α) was needed in combination with other gene analyses for accurate identification of these two species (Slippers *et al*., 2007). Alves *et al.* (2005) developed an amplified ribosomal DNA restriction analysis (ARDRA) procedure for the identification of Botryosphaeriaceae species, in which combinations of two restriction enzymes made it possible to discriminate 10 Botryosphaeriaceae spp, but not between the closely related species such as *N. luteum* and *N. australe,* or *N. parvum* and *N. ribis*. Therefore the *SacII* restriction enzyme developed by Baskarathevan (2009) was used to differentiate *N. luteum* and *N. australe* and the *Nci*I restriction enzyme developed by Che Omar (2009) allowed her to differentiate *N. parvum* and *N. ribis.* Che Omar (2009) identified 93 isolates of Botryosphaeriaceae species from northern New Zealand blueberry farms.

1.3.3 Disease Symptoms

The susceptibility of blueberry to infection by Botryosphaeriaceae species and the development of disease symptoms vary with the species, age of the host, cultivar susceptibility and environmental factors such as rainfall, water logging, high temperatures and other of stress factors which may affect symptom development (Michailides and Morgan, 1992; Ahimera *et al.,* 2003). These symptoms can develop slowly and lead to a gradual decline in vigour and yield (Phillips, 1998). Specific symptoms also vary, depending on the cultivar type, inoculum concentration and isolate virulence (Che Omar, 2009).

Neufusicoccum corticis and *F. aesculi* have been reported to cause twig and stem dieback, leaf spots, and fruit rots on blueberries and are responsible for devastating disease outbreaks in the USA (Milholland, 1995). In Florida, stem blight is caused by *L. theobromae* and *N. ribis* in highbush blueberries, where symptoms are reported to be rapid wilting (Figure 1.1.A) and reddening of leaves on affected branches (Figure 1.1. B). In severe cases, infection progresses into the crown of the plant and results in systemic branch dieback (Figure 1.1.C) over a period of weeks or months, eventually killing the plant (Wright and Harmon, 2010). Pecan-brown discolouration which frequently on one side of an affected trunk (Milholland, 1971b) can be seen in the woody tissues (Figure 1.1.D) of the infected branches.

Figure 1.1: Stem blight disease caused by the botryosphaericaceae species. (A) Rapid wilting of the infected plant, (B) reddening of leaves on affected branches in close proximity to healthy stems of the same plant, (C) infection progresses into the crown of the plant and results in systemic dieback, and (D) wood staining in the trunk/crown of the blueberry bush.

1.4 Disease cycle of the Botryosphaeriaceae species in New Zealand blueberry gardens

Very little is currently known about the disease cycle of Botryosphaeria blueberry dieback in New Zealand blueberries. The large and increasing numbers of Botryosphaeriaceae species found have complicated the epidemiological studies of this disease (Baskarathevan *et al.,* 2012). However, the research on Botryosphaeria diseases within other crop species and observations of disease incidence in blueberries has provided some relevant information for the epidemiological studies.

1.4.1 Over winter survival of the pathogens

Prunings and dead tissues on living bushes can be infected. Diseased woody parts as well as infected wood debris left in the field commonly have pycnidia of Botryosphaeriaceae species embedded in them. The blueberry canker survey conducted by Carroll (2007) in New York revealed that many of the samples collected from blueberry bushes after being incubated in moist chambers developed fruiting bodies in the bark, which, were used for pathogen identification. Sammonds *et a.l* (2009) isolated several Botryosphaeriaceae species from symptomatic blueberry tissues (necrosis in branches, roots and from crowns) in New Zealand. Van Niekerk *et al.* (2004) isolated Botryosphaeriaceae species from pruning debris in vineyards, further confirming the importance of debris as a source of inoculum. A recent study by Elena and Luque (2015) showed that canes of grapevines which had been removed by pruning 2 years earlier and were naturally infected with *D. seriata* were able to produce viable conidia for at least a further 42 months when held under vineyard conditions. In pistachio in California, *Fusicoccum aesculi*, the causal organism of panicle and shoot blight disease, was reported to over-winter in nut mummies, which contained pycnidia (Michailides, 1991).

1.4.1.1 Primary spread

Young blueberry plants may become infected during the propagation process and thereby introduce the disease into a blueberry field. For infection of mature bushes, the sources of primary inoculum are likely to be the pycnidia that had formed on current-season's infected rachides, fruit, blighted shoots, petioles and leaf lesions, as reported for pistachio by Michailides (1991). In pistachio, they constitute the main sources of primary inoculum and

the same pycnidia can provide viable conidia for summer and autumn infections for up to 6 years (Michailides and Morgan, 2004). Under humid conditions, pycnidia of Botryosphaeriaceae species produce pycnidiospores (conidia) that are exuded in gelatinous matrices forming cirrhi, which are ribbon-like masses of spores (Phillips, 2002). The conidia in cirrhi are released by water splash, which is provided by rain or irrigation. Dispersal by splash is normally effective for only a few meters, although the dispersing droplets may be further dispersed by wind. In the USA, a blueberry growers' article by Cline (2013) reported that *F. aesculi* overwintered as fruiting bodies in dead and infected stems of blueberry, and then spores were carried by wind and rain from infected stems to wounds on healthy plants. Infection is generally believed to be through wounds, which could be caused by pruning and trimming, mechanical injury (such as caused by harvesters or wind-blown grit), freezing injury and herbicide injury.

1.4.1.2 Secondary spread

Many host tissues killed by Botryosphaeriaceae species have been reported to produce pycnidia that oozed conidia in moist conditions and then to be rain-splashed, as shown by spore trapping studies conducted for crops such as apple, peach and grapevine. The study conducted by Amponsah *et al.* (2009) in a Canterbury vineyard showed that Botryosphaeriaceae spores were present in rainwater run-off throughout the entire year, with the hghest number of conidia being detected during December, January and February, when summer temperatures were high. In North Carolina, USA, spore trapping studies were carried out by Creswell and Milholland (1988) which showed that in two blueberry farm sites, *F. aesculi* conidia were present in rainwater traps throughout the growing season, although they were barely detectable from December to February (winter) when temperature and rain fall were low. They further stated that large numbers of spores may be released during heavy rains, but that light rain may be more conductive to spore deposition and infection. In South Africa, van Niekerk *et al*. (2010) reported that spore release events and the number of spores released were dependent on both high relative humidity (RH) and rainfall occurring before and during the period of spore release. In this study, Botryosphaeriaceae spores were not captured in the absence of rainfall. High RH

could trigger spore release from the pycnidia, but rainfall played the important role in spore dispersal of Botryosphaeriaceae species.

In apple orchards, Sutton (1981) demonstrated that average and maximum temperature variables relating to rainfall amount and duration were positively correlated to the numbers of conidia of *F. aesculi* caught in rainwater traps. Due to the high variation in climatic conditions among countries and even within regions in the same country, further studies should be carried out in New Zealand blueberry gardens to determine the variation in abundance of Botryosphaeriaceae conidia with the weather events, which would improve understanding of the favorable conditions for spore dispersal, and therefore likely infections.

1.4.2 Inoculum from non-blueberry hosts

The Botryosphaeriaceae fungi are considered to be opportunistic pathogens that may behave as saprophytes, parasites and/or endophytes, causing die-back and canker diseases (Smith *et al*., 1996) on a wide range of hosts. Therefore shelter species and other plant species around the blueberry fields may host Botryosphaeriaceae species and act as a potential inoculum sources. The wide host range of this family could potentially provide important sources of primary inoculum for blueberry farms. In New Zealand, *Eucalyptus* species are commonly used as shelter belts for orchards and other agricultural systems. *Neofusicoccum macroclavatum*, a recognized pathogen of *Eucalyptus globulus* in Western Australia was also recovered from a grapevine by Billones-Baaijens *et al.* (2012) who subsequently found that this New Zealand isolate of *N. macroclavatum* was highly pathogenic on grapevine and *E. globulus* seedlings. Che Omar (2009) also revealed that a *N. parvum* grapevine isolate was equally pathogenic on blueberries as the *N. parvum* isolates recovered from blueberries. Due to the plurivorous nature of the Botryosphaeriaceae species there is some evidence to support the hypothesis of cross infection between other hosts and blueberries. Therefore it is important for growers to understand whether the surrounding neighboring crops and shelter belts are equally as susceptible to Botryosphaeriaceae species as blueberry plants.

1.5 Pathogenicity studies

Very little work has been done to examine the pathogenicity of Botryosphaeriaceae species to blueberry in New Zealand. With grapevine, most of the earlier international pathogenic studies had been conducted using mycelial discs, however Amponsah *et al.* (2011) investigated conidial and mycelial infections using the more common Botryosphaeriaceae species found infecting grapevines in New Zealand; they showed that mycelial infections appeared to develop faster than conidial infections in woody grapevine tissues. They further explained that this was probably because conidia needed to attach, develop germ tubes, penetrate and develop within the internal tissues. Moreover, Mercure *et al.* (1994) stated that the ability of fungal spores to germinate and to produce infection structures was associated with the ability of a spore to attach to the tissue surface prior to infection. Creswell and Milholland (1987) showed that responses of three blueberry cultivars to *F. aesculi* were similar for mycelial inoculation and inoculation with high concentrations of conidia. Espinoza *et al.* (2009) also showed that conidia and mycelia were equally pathogenic on 2-year old blueberry cultivar 'O'Neal' stems when inoculated with *N. parvum.* They further stated that necrotic discolouration was considerably larger from conidial than mycelial inoculation. Therefore more assessments need to be done with different species, multiple isolates with a number of cultivars, and with mycelia and conidial suspensions for inoculation.

Since most Botryosphaeriaceae species do not produce abundant pycnidia and conidia in culture, some researchers have produced conidia from pine needles embedded in agar (van Niekerk *et al.,* 2004). However, Amponsah *et al.* (2008) developed a more rapid method for producing large numbers of conidia of Botryosphaeriaceae species from grapevine tissues. They used detached green shoots of grapevines which were inoculated with five Botryosphaeriaceae species (*N. luteum, N. australe, D. seriata, N. parvum* and *D. mutila).* The lesions were excised and the abundant pycnidia oozed conidia within 24-36 hours if incubated under high humidity, with *N. luteum, N. australe and D. mutila* producing the greatest numbers of conidia. It is important to have conidia for pathogenicity studies in blueberries, therefore this method could be developed using woody tissues and green shoots of blueberries with different Botryosphaeriaceae species pathogenic for blueberries.

A method should be developed using detached blueberry stems for pathogenicity studies. Detached stem assays have been used for pathogenicity studies with Botryosphaeriaceae species, as they were relatively simple and fast (Espinoza *et al.,* 2009; Amponsah *et al.,* 2011). Most of these experiments were conducted in chambers maintained at high humidity under optimum temperatures for fungal growth, which were however completely different from field conditions. Amponsah *et al.* (2011) showed that on detached soft grapevine shoots held in a humid chamber, *N. luteum* was able to germinate and infect much more quickly than on attached soft shoots held in a shadehouse. Milholland (1971b) also conducted a pathogenicity study using two isolates of *F. aesculi* on ten blueberry cultivars, using an attached stem assay in a greenhouse. He used the whole plants of Wolcott and Bluecrop sprayed with conidia and then measured the lengths of the lesions from point of infection, and also used a rating scale from 0 to 5 for five types of disease reaction. Similar studies should be conducted with New Zealand isolates to ensure relevance to field conditions.

1.6 Factors that may affect disease development

1.6.1 Effect of humidity/free water on conidium germination

Amponsah *et al*. (2010) studied the effect of relative humidity on viability and germination of conidia of three Botryosphaeriaceae species namely, *N. luteum*, *N. australe* and *D. mutila,* which are important grapevine pathogens. Their study showed that RH of 100% resulted in the quickest and highest rate of conidial germination of 91.8% within 3 h, whereas in 97% RH it took 6 h for germination to reach 67.2%, which was close to the maximum of 69.9% after 48 h. At 93% RH no conidia had germinated after 6 h but germination reached 43.9% after 24 h, and 48.9% after 48 h. In the same study the germinating conidia had developed mycelium networks when exposed to 100 and 97% humidity for 6 hours whereas network development was absent in 93% RH even after 48 h exposure. Arause and Sutton (1990) stated that requirements for ascospore germination were similar to those for conidia. They found that maximum germination of conidia of *D. seriata* occurred in free water and declined as relative humidity (RH) was reduced from 100 to 92%; no germination was observed at 88.5% RH. Studies conducted in Chile showed that three *Neofusicoccum* species (*N. arbuti*, *N. australe* and *N. parvum*) obtained from blueberry stem cankers, grew in media

with water availability (a_w) between 0.900 and 0.996 (Latorre *et al.,* 2012). However, more research work needs to be carried out on factors affecting blueberry infection by Botryosphaeriaceae species, especially conidium germination, infection processes and variation between species under different humidity and free water conditions.

1.6.2 The effect of tissue type and wound age on susceptibility

Species in the Botryosphaeriaceae have been reported to be wound parasites (Taylor, 1958; Hildebrand and Weber, 1994; *Smith*, 2009). However, Milholland (1971b) showed that *F. aesculi* spore penetration of non-wounded stems could also take place through open stomata. Cell layers beneath the epidermis then constituted a thickened layer of periderm which restricted the fungus to the outer portion of the lesion. On pistachio fruit, the studies of Michailides (1991) using light and scanning electron microscopy showed that germ tubes of conidia of *F. aesculi* entered through the stomata of the upper or lower surfaces of leaves, rachises, and shoots, and also through the lenticels. His experiment revealed that the mode of penetration and stage of plant development influenced both the type and extent of disease development. Further, he stated that penetration of stems by the fungus through open stomata did not result in necrosis or dieback; instead, small raised lesions developed but failed to enlarge. However further studies are needed to investigate the mode of infection and the histological effect of New Zealand Botryosphaeriaceae isolates on different tissue types of blueberries.

The age of the wound may also play a major role in disease susceptibility. On wounds made in succulent stems (3-4 months old) of Bluechip blueberries, the susceptibility to *F. aesculi* conidial suspensions (4×10^4 conidia/mL) decreased with wound age (Creswell and Milholland, 1987). In that study, the percent isolation of *F. aesculi* from attached succulent stems inoculated at 0, 1, 7, 14 and 28 days after wounding was 93, 86, 84, 71, and 86%, respectively. In the same study, succulent stems and woody stems (1-year old) were inoculated with the same concentration of spores and the same pattern of susceptibility was observed, with decreasing incidence with increasing wound age, and similar lesion lengths on succulent stems and woody stems (Creswell and Milholland, 1987). In grapevine, van Niekerk *et al.* (2011a) studied the susceptibility of pruning wounds, at various wound ages and different pruning times during the pruning season, to all trunk disease pathogens

in grapevines in South Africa. Individual pruning wounds were spray-inoculated with 1 mL of a suspension of 1×10^4 spores /mL of either *Eutypa lata*, *Phaeomoniella chlamydospora*, *N. australe* or *Phomopsis viticola* directly after pruning, and at 1, 2, 3, 7, 10, 14, 17 and 21 days after pruning. Results supported the previous studies, as pruning wound susceptibility declined with increasing wound age irrespective of the time of year pruning took place. In blueberry, these same investigations should be conducted as the improved understanding of the mode of pathogen penetration and timing of wound susceptibility may help in development of control measures.

1.6.3 Effect of wounding at different times of year on susceptibility

Several studies have indicated that conidia of Botryosphaeriaceae species are present in the field for long periods of time during an annual crop cycle (Creswell and Milholand, 1988; Amponsah *et al.,* 2009), and so infection may take place whenever favourable climatic conditions for the pathogen coincide with susceptible wounds. Amponsah *et al.* (2009) also showed that conidia of all isolates of *N. luteum*, *N. australe*, *N. parvum and D. mutila* tested were able to infect 100% of the inoculated wounded green shoots and trunks of grapevines indicating the potential for infection several times in a year, when shoot trimming and winter pruning take place. Creswell and Milholland (1988) took samples of blueberry bushes in Dublin County in North Carolina in 1985 and 1986, and determined by tracking symptoms from the infection points, that natural infection sites were available in blueberry fields. Pruning wounds made between the 1984 and 1985 growing seasons accounted for 61 and 68% of apparent infection sites in 1985 and 1986, respectively. The other infection sites (growth cracks or injuries, below-ground buds, and undetermined) accounted for 15, 11, and 13% of infection sites, respectively, during 1985, and for 27, 0 and 5% of infection sites, respectively, in 1986. Therefore improving understanding of the susceptible sites is very important for the development of successful control measures.

A study conducted by Serra *et al.* (2008) in Italy during three consecutive dormant seasons investigated grapevine pruning wound susceptibility to conidia of *Pa chlamydospora*, *Phaeoacremonium aleophilum* and *D. seriata*, which was assessed each week for 4 months after pruning in January, February and March. Results revealed that for all tested pathogens, wound susceptibility remained high for up to 4 months after pruning and wounds made in

late winter were less susceptible to infection than wounds made in early winter. These studies were also conducted in different locations, in which there were varied abiotic and biotic factors, therefore comparison of results was difficult. Even though studies have been conducted with other berry crops, with regard to the wound susceptibility with age and the time of the year, similar studies should be conducted with blueberries as the difference in tissue characteristics may affect the infection capabilities of the Botryosphaeriaceae pathogens.

1.6.4 Effect of herbicide injuries on host for disease susceptibility

Subtoxic levels of herbicides can increase or decrease resistance to plant diseases via indirect effects on the crop (Duke *et al*., 2007). In Mississippi during the late 1980s, the incidence of stem blight in blueberries coincided with injury to the base of young canes as a result of damage caused by contact herbicides such as paraquat; however the incidence of stem blight then declined as growers avoided injuring blueberry canes with paraquat (Smith, 1997). Cline (1997) stated that in fields of 2-3 year old 'Premier' rabbiteye blueberries, 25-95% of plant death was due to herbicide injury from improperly used Gramoxone. In the same study, *F. aesculi* was isolated from 50% of Gramoxone injured stems. Cankers caused by *F. aesculi* in apples were also reported to be increased by glyphosate weed control programs (Johal and Huber, 2009). Glyphosate, dicamba, and 2, 4-D are examples of commonly used herbicides that cause chemical injury to desirable plants when used incorrectly. Excessive concentrations of these chemicals cause twisting and curling of stems, stem swelling, weakened cell walls, rapid cell growth, as well as cellular and vascular damage and death (Small and Whiting, 2014). These damage sites may be ideal ports for pathogen penetration and so should be investigated in New Zealand blueberry farms. This will help growers to understand the importance of correctly applying herbicides at the correct concentrations.

1.6.5 Threshold numbers of conidia required for infections of wounds

Sufficient inoculum is known to be required before an infection can take place, even if biotic and abiotic factors are favorable. Amponsah *et al.* (2014) studied the effect of different *N. luteum* conidium concentrations ($10^{2,}$ 10^3, 10^4, 10^5 and 10^6 conidia/mL) used to inoculate wounds on detached grapevine shoots. Results revealed that incidence of lesion

development in inoculated plants was 100% irrespective of conidial concentrations, although greatest concentrations caused longest lesions. Creswell and Milholland (1987) assessed the effect of *F. aesculi* inoculum concentration and type on 2-year old blueberry cultivars (‘Bluechip’, ‘Powderblue’ and ‘Murphy’). After piercing the stem, one isolate was tested at four concentrations (1, 5, 10, and 50×10^3 conidia/ per inoculation site) and another isolate at three concentrations (1, 5, and 10×10^3 conidia/mL). These were compared with mycelial inoculation (a 2 cm mycelial disc per wound). They showed that conidial inoculum applied at a rate of 5× 10^3 conidia per wound or higher caused similar lesion lengths as mycelial discs but lower concentrations caused shorter lesions. Studies are needed to assess the effects of Botryosphaeriaceae conidial numbers when used to infect New Zealand blueberry cultivars.

1.6.6 Effect of water stress of the host on disease susceptibility

Schoeneweiss (1986) concluded that *Botryosphaeria* species have a wide host range, but they are capable of attacking some hosts only when the host is weakened or has experience stressful conditions. Several researchers have shown that water stress in various host plants, either as a predisposing factor or during the incubation period, affects the colonization of canker causing pathogens (Pusey, 1989; Ma *et al.,* 2001). The effect of water stress on Botryosphaeria die back in pistachio caused by *F. aesculi* was studied by Ma *et al.* (2001). In laboratory tests, spore germination, germ tube elongation, and mycelial growth of *F. aesculi* increased as water potential (ψ) decreased from 0 to –2.0 MPa and declined as ψ decreased below –2.0 MPa. In greenhouse studies, drought stressed 2-year-old pistachio trees (leaf ψ being < –0.635 MPa) developed more severe Botryosphaeria blight disease than the non-stressed trees (ψ > –0.485MPa). Van Niekerk *et al.* (2011b) conducted an experiment consisting of four irrigation regimes, with field capacities (FC) of soils maintained at 100, 70, 50 or 20%. The one year old grapevines growing in these soils were pruned and inoculated with spore suspensions of four Botryosphaeriaceae species (*N. australe*, *N. parvum*, *L. theobromae* or *D. seriata*). Measurements of stomatal conductance, photosynthetic rate and leaf spectrometry were made to monitor physiological stress. Results revealed that plants which had been subjected to the lowest irrigation regime, which showed higher levels of physiological stress, had longest mean lesions which developed from inoculated stem wounds. In contrast, Amponsah *et al.* (2014) found that grapevine plants inoculated with

spore suspension of *N. luteum* (10^4/mL) showed that mean lesion length was in proportion to the moisture supplied, with lesion length being shortest in plants held at 25% FC and highest at 100% FC. A second experiment conducted using three FC levels 100%, 25% and 15%, showed that longest lesions developed at high FC of 100% and low FC of 15%. Water stress effects on Botryosphaeriaceae infection of blueberries has not been studied as rigorously as on grapevines. Therefore knowledge on water stress on blueberry may help with recommendations for appropriate irrigation management in the fields.

1.7 Control strategies

1.7.1 Cultivar susceptibility

The potential for controlling this disease by use of resistant cultivars needs to be investigated. In the southeastern United States Smith (2004) reported that stem canker and stem blight had re-emerged in the previous decade due to the rapid introduction of southern highbush blueberry cultivars, which had not been tested for susceptibility to botryosphaeriaceaous species prior to mass planting. The commercial blueberry industry in the southeastern USA had previously been based largely on rabbiteye cultivars. Highbush blueberry is one of the most commercially significant berry crops, mainly cultivated in the United States and Canada, but also in Europe, Australia, Chile and New Zealand (Prodorutti *et al.,* 2007). Screening for tolerant cultivars and their subsequent use is essential to overcome this disease. Critical factors in these screening protocols include stem age, isolate virulence, inoculation environment, and timing of evaluation (Smith, 2009). To date, none of the cultivars tested in the southeastern United States were found completely resistant to the disease, but there were great differences in their susceptibility (Smith, 2004).

In New Zealand breeding programs have mainly focused on improvement of yield and fruit quality (Sammonds *et al*., 2009). Little attention has been paid to disease resistance, which may have allowed for increased the incidence of Botryosphaeria die back in the blueberry industry in New Zealand. There is no information on the relative susceptibility to Botryosphaeriaceae species in the wide range of cultivars used in New Zealand. However, anecdotal reports from blueberry growers have indicated that some of the new cultivars bred by HortResearch in the late 1990s for New Zealand conditions are particularly seusceptible to Botryosphaeriaceae disease. In the USA, the susceptibility of many blueberry

cultivars was determined by inoculating them with *F. aesculi* (Milholland, 1971b; Creswell and Milholland, 1987; Smith, 2009). Polashock and Kramer (2006) also studied the relative resistance of 50 blueberry cultivars to Botryosphaeria dieback (caused by *F. aesculi*) and twig blight (caused by *Phomopsis vaccinii*). They found that half-high cultivars and low bush cultivars were the more resistant to *F. aesculi,* and that individual cultivars resistant to one pathogen were not necessarily resistant to the other.

The first New Zealand survey (Sammonds *et al*., 2009) recovered many isolates of different Botryosphaeriaceae species from symptomatic tissue of the blueberry cultivars 'Marimba' and 'Elliot' both of which were confirmed in separate studies in the USA, to be resistant to *F. aesculi*. Clearly the cultivars found to be resistant to *F. aesculi* do not also express resistance to New Zealand isolates of other Botryosphaeriaceae species. A pilot study was conducted in New Zealand using detached green shoots to evaluate the pathogenicity of *N. parvum* isolates on cultivars 'Maru' and 'Centurion' (Che Omar, 2009). Results showed that *N. parvum* isolates were pathogenic to both cultivars tested. However, many more cultivars need to be tested for their resistance to several Botryosphaeriaceae species, with sufficient replicates to provide statistically valid conclusions.

1.7.2 Chemical and biological control methods and field sanitation

Other control measures for Botryosphaeria dieback in crops could include chemical and biological control spraying programs, field sanitation and cultural practices. Control of stem blight with fungicides has been very difficult to achieve (Milholland, 1995). Root dip treatments for controlling blueberry stem blight caused by *F. aesculi* in container-grown nursery plants were studied by Cline and Milholland (1992). Results revealed that dipping highbush blueberry roots into a benomyl-kaolin clay slurry at benomyl concentrations of 2000-3000 µg/mL did provide protection; however the effect lasted only for few months. In an experiment conducted by Smith (2009) each blueberry plant was treated every 10 days with one of nine fungicides. Succulent, partially hardened-off stems were collected from each plant after the fourth or fifth fungicide application. The stems were wounded and inoculated with mycelial blocks of *F. aesculi* and then incubated at 25°C in 100% RH for 15 days. Results showed that stems treated with cyprodinil + fludioxonil or pyraclostrobin had shorter lesions than stems receiving no fungicide treatment, while stems treated with

captan, Ziram, and tebuconazole had lesions as long as those receiving no fungicide. Chemicals that protect pruning wounds from infection by these diseases need to be evaluated in field experiments, with respect to the most common Botryosphaeriaceae species in New Zealand.

The main challenges for recommending a protectant are that (1) pruning wounds are being colonized with a broad range of unrelated species, and (2) the chemicals have to be retained in or on the wood, throughout the susceptible period of the wound, without been washed off. Rolshausen *et al.* (2010) evaluated four fungicides (1% thiophanate-methyl, 5% boric acid in a wound-sealing paste, 1% pyraclostrobin, and a paste containing cyproconazole and iodocarb) against *F. aesculi, D. seriata, Dothiorella viticola* and *L. theobromae* on pruning wounds of grapevine. They concluded that these products were unable to control the entire spectrum of pathogens efficiently, but thiophanate-methyl was overall the most efficacious product. Amponsah *et al.* (2012a) tested fungicides on pruning wounds of grapevine inoculated with *N. luteum* and found that the most effective fungicides were flusilazole, carbendazim, tebuconazole, thiophanate-methyl and mancozeb. It is important to develop a fungicide treatment method, for protecting pruning wounds of blueberries. As well as reducing the pruning wound infections, these treatments may also play an important role in reducing numbers of spores released and so controlling secondary spread of the disease when conditions are favorable. Smith and Hendrix (1984) showed that application of one long lasting fungicide (captafol) during winter reduced primary infection of apple buds by *D. seriata* which takes place during the winter in Georgia, USA, and therefore was effective in controlling the secondary infections; moreover it helped to reduce the fungicide cost. In New Zealand, captan, thiram, copper and mancozeb sprays are recommended, for protecting wounds in blueberry plants, especially after damage-causing events like hail storms (Langford, 2010). However, the research that proves the efficacy of these products against New Zealand blueberry canker causing agents still needs to be done. Since secondary infection might occur through tissues other than pruning wounds it might also be useful to test seasonal fungicide applications.

Biological products may also be effective as wound protectants, although they have not been investigated against Botryosphaeria diseases of blueberry. Halleen *et al.* (2010) conducted two field trials to evaluate benomyl, flusilazole and commercially available

Trichoderma harzianum-containing products (Isolate T77, Vinevax and Trichoseal spray) as grapevine wound protectants to prevent infection by *Eutypa lata*. They reported that the fungicides benomyl and flusilazole were the most effective treatments, and the *Trichoderma* treatments T77 and Trichoseal spray caused significant reductions in the incidence of *E. lata.* In addition, the studies by Yin *et al.* (2011) in China revealed that *Bacillus amyloliquefaciens* was an effective biological control agent for poplar canker (*Poplus* species) disease caused by *F. aesculi.*

The success of any fungicide program has been shown to be greatly enhanced if combined with sanitation and cultural practices designed to reduce the amount of inoculum in the field (Urbez-Torres, 2011). In New Zealand, Langford (2010) recommended removing disease inoculum by pruning out dead tissue and burning the prunings in order to maintain field sanitation in blueberry farms. However, the real risk imposed by these tissues has not been investigated for blueberry and so experiments should be conducted to determine whether dead, infected tissues can release conidia, for how long and from which types of tissues.

1.8 Aim and research objectives

Although some Botryosphaeriaceae species have been identified as important blueberry pathogens in several countries, more species seem to be causing blueberry dieback in New Zealand than in other countries. Therefore, research needs to be done on the infection processes and the factors that increase infection risk, knowledge that is essential to the development of control strategies. This research programme will improve understanding of pathogens involved with Botryosphaeria dieback in blueberries and their epidemiology, and to develop effective control methods. The objectives of this research are as follows:

Objective 1: Investigate which species cause infection of blueberries in farms and nurseries, as well as the tissues affected and sources of inoculum commonly available in blueberry farms and nurseries.

Objective 2: Investigate the pathogenicity of key Botryosphaeriaceae species on a range of blueberry tissues, wounded and non-wounded, and the relative resistance of commonly used and experimental cultivars.

Objective 3: Identification of the cultural and environmental factors that affect disease development and spread. This will provide information on how management and environmental factors affect disease development, which can be used to ensure that cultural practices and control products are used to the best effect.

Objective 4: Investigate potential control strategies to protect infection courts. This experiment will help to identify ways to manage infection effectively with a range of biological products and fungicides.

Chapter 2

Botryosphaeriaceae species associated with dieback in blueberry farms and nurseries in New Zealand

2.1 Introduction

In New Zealand some early reports have identified Botryosphaeriaceae species as pathogens of kiwi fruit, apple and poplar (Pennycook and Samuels, 1985). Amponsah *et al.* (2011) also showed that Botryosphaeriaceae species were able to infect shelter, fruits and ornamental plants. In that study 11 non-grapevine isolates including a *Neofusicoccum luteum* isolate from blueberry were tested for their pathogenicity on wounded detached green grapevine shoots. All the isolates were able to cause lesions which produced pycnidia and conidia, indicating that many host plants could act as potential inoculum sources for Botryosphaeriaceae infections of berry crops. Botryosphaeriaceae species are considered to be important pathogens of grapevines and are relatively common causes of dieback of grapevines in New Zealand (Amponsah *et al.,* 2011; Billones-Baaijens *et al.,* 2011). Nine Botryosphaeriaceae species (*N. parvum*, *N. luteum*, *N. australe*, *N. ribis*, *Diplodia mutila*, *D. seriata*, *Fusicoccum aesculi (B. dothidea)*, *Dothiorella iberica* and *Do. samentorum*) were reported to infect grapevines in New Zealand (Baskaratheven *et al.,* 2012).

In New Zealand, blight of blueberry stems and plant crowns is increasingly becoming a problem in new production areas (Sammonds *et al*.*,* 2009). Five Botryosphaeriaceae species (*D. seriata, N. parvum*, *N. luteum*, *N. australe* and *N. ribis*) were recovered from the initial surveys which isolated and identified the species from symptomatic blueberry tissues sent in by six New Zealand growers in the central North Island (Che Omar, 2009; Sammonds *et al.,* 2009). However in a later sampling of dieback pathogens in the North and South Islands, some of the isolated fungi (Antunovich, 2010) were not identified to species level.

Botryosphaeriaceae species were also shown by Billones-Baaijens *et al.* (2011) to be prevalent in grapevine propagation materials and grafted plants in New Zealand grapevine propagation nurseries, with about 5-63% of samples collected being infected. It therefore seemed likely that infected materials are present in blueberry propagation nurseries. This study therefore aimed to i) complete the identification of the unnamed fungal isolates from

New Zealand blueberry farms survey in 2009/2010, ii) to complete sampling in blueberry farms across non-sampled areas of the North and South Islands, and iii) to investigate sources of Botryosphaeriaceae primary inoculum in blueberry propagation nurseries.

2.2 Materials and Methods

2.2.1 Blueberry gardens and nursery sampling

Blueberry tissue samples were collected from seven blueberry farms which included four in Canterbury, one in Marlborough, one in the Waikato and one in the Hawke's Bay, from September 2013 to January 2014. Ten samples which exhibited symptoms of rapid wilting, reddening of leaves, stunted growth and dieback were collected from bushes on each farm. Sampled tissues included stems, buds, leaves, fruit mummies and stem prunings collected from the ground. Samples were collected from farms in Christchurch, Timaru and Blenheim, while staff from other farms sent their samples to Lincoln University. Most of these samples showed stem necrosis externally and internally. Samples were sent to the Lincoln University Plant Pathology laboratory and stored at 9°C for a maximum of one week until they were assessed.

Blueberry nursery owners from different regions of New Zealand were contacted and asked to send 10-20 samples each of propagated plants. Samples were sent by courier to the Lincoln University Plant Pathology laboratory from June to August 2013. Of the four nurseries contacted, three nurseries sent newly propagated plants (10-12 cm) and one nursery sent potted plants of 60-80 cm. Samples were examined and isolations were carried out.

Nonidentified isolates (45) remaining from the surveys of blueberry farms conducted in 2009 and 2010 in other regions of New Zealand were also retrieved from the Plant Pathology Culture Collection, grown on PDA and identified using morphological characters and molecular techniques, as described in Sections 2.2.3 and 2.2.4. Each nursery and blueberry farm was assigned a number for identification and their locations or names were not specified for confidentiality reasons.

2.2.2 Isolation of Botryosphaeriaceae species from plant samples

For each of the large field plants, 0.5 cm sections were cut from 1 cm beyond the lesion edges as well as 1 cm within the lesion. For each young nursery plant, 0.5 cm sections were cut from 1 cm above the base, in the middle and near the top of the main stem. These plant sections were surface sterilized by soaking in 70% ethanol for 30 s, and air dried under sterile air in a laminar flow hood. Each plant piece was further cut into four pieces that were placed onto PDA (DIFCO™, New Jersey, USA) amended with chloramphenicol (CP, 0.5 g/L). The plates were incubated at 25°C in a diurnal light regime (12 h dark and 12 h white light) for three to five days. Colonies growing from the plant samples that showed typical growth of Botryosphaeriaceae species were sub-cultured onto fresh PDA. In order to produce pure cultures, colonies that were morphologically identified as Botryosphaeriaceae species on the PDA plates were subsequently sub-cultured onto 2% water agar (WA). Plates were incubated at 25°C in diurnal dark: light for two days. Mycelial tips were cut aseptically when viewing the culture with a stereo microscope and sub-cultured onto individual PDA plates for selection of Botryosphaeriaceae like isolates. Isolates were stored at 4°C until they were re-cultured for morphological identification.

2.2.3. Identification of Botryosphaeriaceae isolates based on morphological characters

All isolates were sub cultured onto PDA and incubated in 12 h dark and 12 h light at 25°C. After 3, 7 and 30 days incubation, the cultures were observed morphologically and allocated into groups based on colony growth rate and colony colour change with time as described by Amponsah *et al.* (2011). Ten representative isolates were taken from each colony morphology group for conidial characterisation. Isolates were sub-cultured onto prune extract agar (PEA; Appendix A) and incubated under 12 h light and 12 h dark at 25°C for 30 days to induce sporulation. Pycnidia were scraped from the bottom of the agar in the PEA plates, placed on sterile glass slides with a drop of sterile water and crushed using a sterile scalpel blade in order to release conidia. Conidial characteristics were compared with the descriptions of Amponsah *et al* (2011) and Billones-Baaijens (2011). Conidial morphology was used to identify the isolates to genus level, but was not able to identify the isolates to species level. Any isolates which did not produce pycnidia were identified using molecular techniques. Isolates morphologically identified as Botryosphaeriaceae spp. were stored in 20% glycerol at -80°C as mycelial plugs.

2.2.4 Identification of Botryosphaeriaceae isolates using molecular techniques

2.2.4.1 Amplified ribosomal DNA restriction analysis (ARDRA)

The Botryosphaeriaceae isolates recovered from the nurseries and blueberry farms were identified using amplified ribosomal DNA restriction analysis (ARDRA) as described by Alves *et al.* (2005) and Sammonds *et al.* (2009). The isolates were grown on PDA at 25°C in 12 h light and 12 h dark for three days. DNA was extracted using the REDExtract-N-Amp™ Plant PCR Kit (Sigma Aldrich, Missouri, USA). Aerial mycelium (approximately 2 mm) on the edge of each culture was lifted off using a sterile pipette tip and the mycelium was added to 100 µL of Extraction Solution in a 1.7 mL tube. The mixture was vortexed briefly and incubated at 95°C in a heat block for 10 min. Then 100 µL of Dilution Solution was added to each tube and it was briefly centrifuged for 2 min at 3,220×*g*. Samples were stored at -20°C until used for PCR.

The amplification of the ribosomal RNA gene was conducted using the REDExtract-N-Amp™ Plant PCR ready mix, with primers ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') primers (White *et al.,* 1990; O' Donnell, 1993; Alves *et al.,* 2005). For each sample, the reaction mixture contained 10 µL of the REDExtract-N-Amp™ Plant PCR Ready Mix, 1 µL of each primer (5 µM), 4 µL of sterile nanopure water (SNW) and 4 µL of DNA extract. Negative controls contained nanopure water instead of the DNA extract. Samples were briefly vortexed and centrifuged for 10 s at 3,220×*g*. The thermal cycle was conducted using a BioRad iCycler Thermal cycler (California, USA). The thermal cycle consisted of initial denaturation at 94°C for 3 min, followed by 40 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. Amplified PCR products were separated by gel electrophoresis. A 1% agarose gel [1 g agarose (Bioline, London, UK) in 100 mL 1× Tris-Acetate EDTA buffer (TAE; 40 mM Tris acetate, 2 mM Na_2 EDTA, pH 8.5)] was prepared and immersed in 1× TAE buffer. The 1kb plus DNA Ladder™ (0.1 ng/µL; Invitrogen) molecular weight marker was loaded to the first or last well of each gel. For each amplified PCR product or 1kb plus DNA Ladder™ 5 µL was combined with 3 µL of loading dye [40% (w/v) sucrose; 0.25% bromophenol blue; 0.25% xylene cyanol] before being loaded into each well. The samples were separated using electrophoresis at 10 V/cm for 45 min in 1× TAE buffer.

The gel was stained by immersing in an ethidium bromide solution (0.5 µg/mL) for 15 min, then de stained by immersing in water for another 15 min. The stained gel was photographed under UV light using a FireReader^{TM} Gel Doc imaging system (UVITEC Cambridge, TLS Total Lab Systems Ltd).

After amplification, each PCR product (~1200 bp) was digested using one or more restriction enzymes in an iterative process (Figure 2.1). Initial digestion was done using the *Taq*I enzyme with the resulting banding pattern determining which enzyme was used next. The *Taq*I digestion reaction contained 2 U of enzyme, 2.5 µL of 10× Buffer 4 [50 mM potassium acetate, 20 mM Tris- acetate, 10 mM magnesium acetate, 1 mM dithiothreitol (DTT)], 0.3 µL of bovine serum albumin (BSA), 17 µL of SNW and 5 µL of the PCR product. Samples were incubated at 65°C for 2 h and inactivated at 80°C for 30 min. Digested DNA was separated by electrophoresis in a 1.5% agarose gel immersed in 1× TAE buffer as previously described, except that the digested products (20 µL) were mixed with the loading dye (4 µL). The gel was run at 10 V/cm for 1 h followed by staining, destaining and visualization using UV as described previously. According to the banding pattern each sample was assigned into groups as described by Alves *et al.* (2005) and Sammonds *et al.* (2009). Based on the resultant digest pattern (Figure 2.1) the samples were then digested with one or more other restriction enzymes, *Hae*III, *Sac*II and *Nci*I, according to manufacturer's recommendations. For each reaction, the final volume of 20 µL was prepared by adding 2 U of enzyme, 2 µL of 10× recommended buffer, 12.8 µL of SNW and 5 µL of PCR product. For digestion with *Hae*III and *Sac*II, samples were incubated at 37˚C for 16 h and inactivated by heating to 80˚C and 65°C, respectively, for 25 min. The *Nci*I digest was incubated at 37°C for 14 h. The DNA fragments were separated by electrophoresis in a 1.5% agarose gel immersed in 1× TAE buffer as previously described, except that the digested products (20 µL) were mixed with the loading dye (4 µL). The stained gels were photographed under UV light using a FireReader^{TM} Gel Doc imaging system (UVITEC Cambridge, TLS Total Lab Systems Ltd).

Initial digestion with *Taq*I allowed placement of the isolates into one of three groups. Group A isolates with four bands (364, 292,189 and 53-92 bp) were either *N. australe* or *N. luteum*. Group B isolates with three bands (427-428, 291-292 and 53-117 bp) were either *N. parvum* or *N. ribis*. Group C isolates with four bands (426-432, 291-292, 173-189 and 51-63 bp) were either *N. parvum*, *D. seriata*, *Do. samentorum* or *Do. Iberica*.

In order to distinguish the Group A isolates, *N. australe*/*N. luteum*, initial PCR products of those isolates were digested with the *Sac*II enzyme. This resulted in two bands (1071 and 102 bp) for *N. luteum* and three bands (766, 304 and 102 bp) for *N. australe*. To identify the isolates in Groups B and C, the initial PCR products of those isolates were digested with the *Hae*III enzyme. Digestion identified *N. parvum* which gave five bands (258, 254, 203, 157 and 58-83 bp) and *B. dothidea* with three bands (716, 157 and 58-836 bp). The remaining isolates were identified by digesting the initial PCR product with the *Nci*I enzyme. *Neofusiccocum ribis* isolates remained uncut with the enzyme (1173 bp). For *D. seriata* digestion resulted in three bands (747, 334 and 93 bp) and for *D. mutila* resulted in two bands (1079 and 93 bp). Those isolates that were not differentiated using the restriction enzymes were identified using DNA sequencing.

2.2.4.2 Sequencing of isolates

Isolates' identity was confirmed by sequencing of randomly selected isolates (≥15%). Twelve isolates of *N. australe*, four isolates of *N. parvum,* three isolates of *N. luteum* and three isolates of *N. ribis* were randomly selected for sequencing. An initial PCR product of each was amplified using NL4 and ITS1 primers as described in Section 2.2.4.1. Then the PCR products were sequenced using primer ITS4 at the Lincoln University Sequencing facility using an ABI PRISM® 310 Genetic Analyser (Applied Biosystems, Foster City, California). Identities were further confirmed by amplifying and sequencing the β-tubulin gene using primers: Bt2a (5'GGTAACCAAATCGGTGCTGCTTTC3') and Bt2b (5'ACCCTCAGTGTAGTGACCCCTTGGC3') as described in Section 2.2.4.1. Phylogenetic analyses were conducted using MEGA version 6 (Tamura *et al.,* 2011) and DNAMAN 5. Three or four published ribosomal RNA gene sequences, which included the ITS region and were at least 500 bp, were retrieved from the NCBI database (www.ncbi.nlm.nih.gov) and aligned with the sequences of the isolates from each species in this study using MEGA 6.

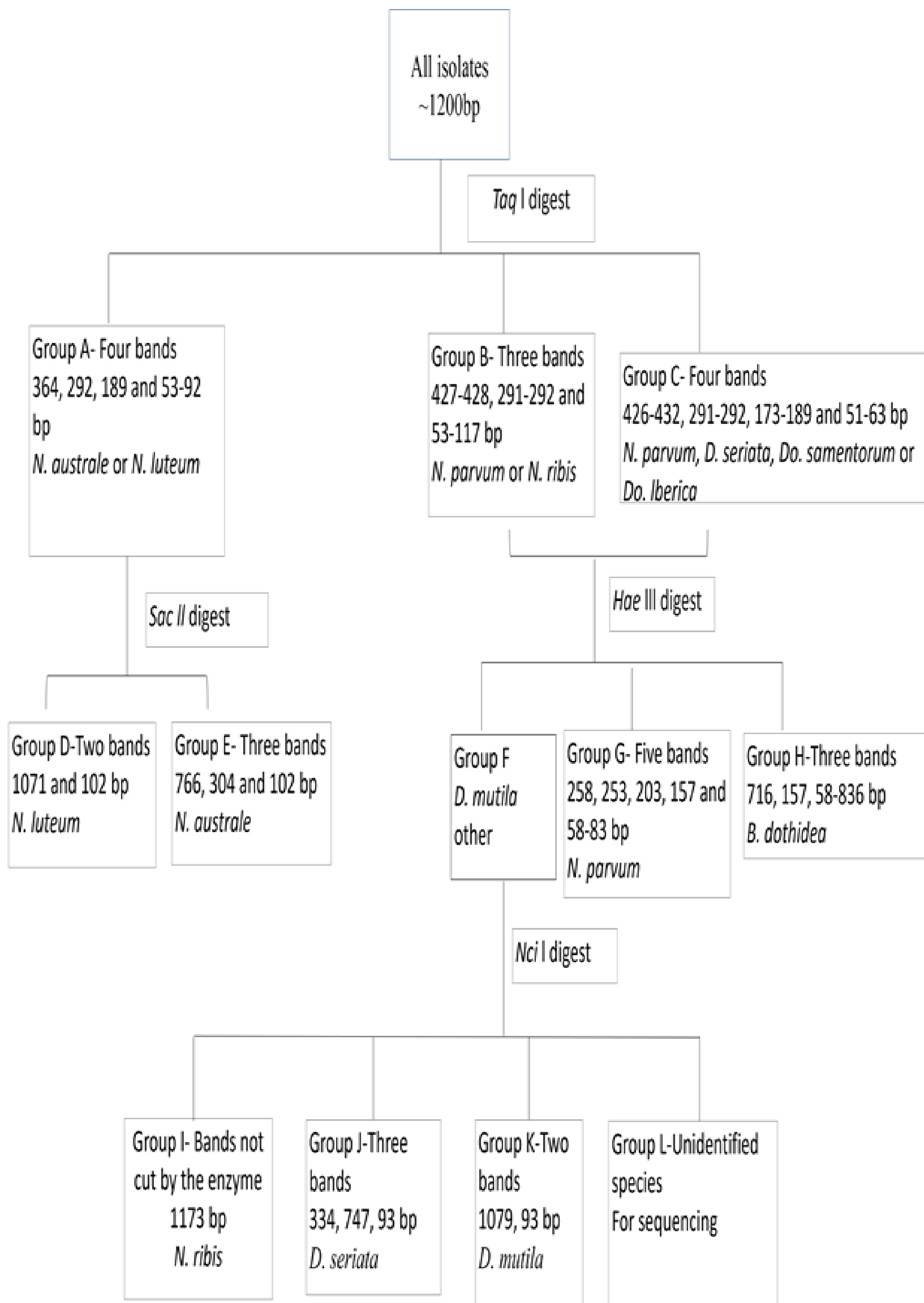


Figure 2.1: Diagram of the restriction enzymes used in an iterative amplified ribosomal DNA restriction analysis (ARDRA) for identification of the Botryosphaeriaceae to species level.

For the out group, the rRNA sequence that included the ITS region of *Guignardia philoprina*, was obtained from the NCBI database. A phylogenetic tree was generated using New Zealand isolates and the ITS sequences of representative Botryosphaeriaceae species (Appendix B.11-13) retrieved from GenBank.

2.2.5 Assessment of Botryosphaeriaceae species in nursery propagation cuttings

To investigate Botryosphaeriaceae species infestation in nursery propagation cuttings, a blueberry nursery in the North Island was visited on November 2014 for sampling. From the source block, 20 plants were randomly selected and five 15-20 cm long soft green shoots (suitable for propagation) taken from each plant. Samples consisted of different cultivars ('Dolce Blue', Rahe, Brite blue, Blue down, Vacc Velluto, Vacc Ocean blue, Tasty, 'Powderblue', Centra blue, Skyblue, Dixie, Oneal, Reka, Pettie blue, Misty and Marimba). The five shoots from each plant were placed in a new plastic bag and stored in an insulated container with ice during travel to Lincoln University, where they were stored at 9°C for up to 2 days until processed.

2.2.5.1 Surface propagules in cuttings

2.2.5.1.1 Washing to remove propagaules

To detect the presence of Botryosphaeriaceae species propagules on the surfaces of the propagation cuttings, 100 mL of sterile water+ 0.01% Tween 80 was added to each plastic bag containing samples. The bags were shaken thoroughly by hand in order to wash off the propagules and then filtered through a 50 µm sieve and allowed to settle overnight at 4˚C. The supernatant was removed carefully and the rest (~50 ml) was frozen at -80˚C until further processing.

The thawed samples (under 4°C in the refrigerator) were centrifuged at 10°C for 15 min at 10,000 × g. Pellets were re-suspended in 400 µL of sterile water and the DNA was extracted from a 300 µL aliquot using the Power Soil^{TM} DNA isolation kit (MO BIO laboratories, USA) according to manufacturer's instructions. DNA concentrations were determined using a Nanodrop 3.0.0 spectrophotometer (Nanodrop Technologies, USA), and then samples of 20-25 ng/µL were prepared and stored at -20°C.

2.2.5.1.2 Detection of Botryosphaeriaceae species in wash water using multi species primers

In order to determine whether the samples contained fungal DNA, universal primers ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to conduct PCR. The PCR mixture contained 10× PCR buffer (with 1.5 mM $MgCl_2$), 200 µM each of dATP, dTTP, dGTP, dCTP, 1 µL of each primer (5 µM; Invitrogen), 1U of FastStart Taq polymerase (Roche) and 1 µL (~20 ng) of template DNA. The thermal cycle included an initial denaturation of 5 min at 95°C, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C and 1 min at 72°C and final extension at 72°C for 10 min, as described by Ridgway *et al.* (2011). After the amplification 5 µL aliquots from each PCR product were mixed with 3 µL of loading dye and separated by electrophoresis in a 1% agarose gel as described in Section 2.2.4.1. The second PCR was conducted using multispecies primers specific to Botryosphaeriaceae species, BOT 100F (5'-AAACTCCAGTCAGTRAAC-3') and BOT 472R (5'-TCCGAGGTCAMCCTTGAG-3'). The reaction mix and the thermal cycling protocol was as described for the universal primers. After amplification, 5 µL aliquots from each amplified PCR product were each mixed with 3 µL of loading dye and separated by electrophoresis in a 1% agarose gel as described in Section 2.2.4.1. The multi- species primers amplified a portion of the rRNA gene region (371-372 bp) as described by Ridgway *et al.* (2011) and presence of a band confirmed the presence of Botryosphaeriaceae DNA in the samples.

2.2.5.1.3 Detection of Botryosphaeriaceae species in wash water using nested PCR

To increase the PCR sensitivity, a nested PCR protocol was followed using the PCR products initially amplified with the universal primers. Each PCR product was diluted 1:200 in sterile nanopure water (SNW) and 1 µL was used as the template for the secondary amplification with multispecies specific primers BOT 100F (5'-AAACTCCAGTCAGTRAAC-3') and BOT 472R (5'-TCCGAGGTCAMCCTTGAG-3') following the thermal cycling protocol described for the universal primers (Section 2.2.5.1.2). After amplification, 5 µL aliquots from each amplified PCR product was mixed with 3 µL of loading dye and separated by electrophoresis in a 1% agarose gel as described in Section 2.2.4.1. Presence of a band helped to confirm the presence of Botryosphaeriaceae DNA in the samples.

2.2.5.1.4 Single stranded conformational polymorphism (SSCP) identification of Botryosphaeriaceae species from wash water

To identify different Botryosphaeriaceae species present in wash samples single stranded conformational polymorphism (SSCP) analysis was used. The nested PCR products obtained from the above experiment were used. For each nested PCR product, 2 µL was mixed with 20 µL of loading dye (95% formamide, 20 mM EDTA, 10 mM NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol, 10 mM NaOH, 20 mM EDTA pH 8) and heat denatured for 7 min at 99°C. The reaction was immediately placed on wet ice for 5 min. Then 22 µL of each sample was loaded into a well on a non-denaturing polyacrylamide gel (0.625×MDE® Gel Solution; Lonza Rockland Inc.) in 1× TAE buffer. The samples were separated using electrophoresis for 17 h at 200 V using a cooling system that maintained the temperature in the gel tank at 22°C (Ridgway *et al.,* 2011).

Gels were silver stained using the method of Bassam and Gresshoff (2007). The gel was fixed in an aqueous fixative solution of 10% (v/v) ethanol and 0.5% (v/v) acetic acid for 3 min and then stained in another fixative solution containing 0.002% (w/v) silver nitrate for 5 min. The gel was then quickly rinsed with reverse osmosis (RO) water and washed for 2 min in RO water, and developed using a solution of 3% (w/v) NaOH and 0.001% formaldehyde at 20°C for 10-45 min (until clear bands were observed) followed by a quick rinse in RO water. The wet gel was scanned with a photocopier and then placed on a dryer (Biorad Model 583 gel dryer) set at 64°C for 3 h for permanent storage. Identification of the wash samples were made on the basis of similarities between band positions with the standard samples which had previously been identified by Baskarathevan (2011) and Billones-Baaijens (2011) [*N. luteum*(isolate L70), *N. australe* (isolate B3192), *D. mutila* (isolate M232), *D. seriata* (N520) and *N. parvum* (P223)].

2.2.5.2 Internal infections of propagation cuttings

After washing the plant cuttings, all shoots were surface sterilised using the method described in Section 2.2.2. Tissue samples (1 cm) were taken from the base, mid and tip of the plants. These three segments were placed onto one PDA plate amended with chloramphenicol at 0.5 g/L (PDAC). Three leaves from each plant which showed discoloration or spots were also selected, surface sterilised and three discoloured segments

of each were plated onto PDAC. The plates were incubated at 25˚C in a diurnal light regime (12 h dark and 12 h white light) for three to five days. Colonies growing from the plant samples that showed typical growth of Botryosphaeriaceae species were sub-cultured onto fresh PDAC. The cultures were subcultured onto WA and purified using the mycelial tip cutting technique described in Section 2.2.2 prior to subculture onto PDA. Morphological identification and molecular identification using ARDRA was done as described in Sections 2.2.3 and 2.2.4.

2.2.6 Analysis of nursery propagation media for presence of Botryosphaeriaceae species

2.2.6.1 Collection of nursery media samples

Blueberry nurseries were contacted and asked to send samples of their used propagation mixes. Four nurseries sent samples, which included propagation mix (for rooting of cuttings), potting mix (for growing the small plants) and potting saw dust (for growing on the large plants prior to sale), depending on their use in the specific nursery. Ten samples were sent from each nursery for each type. Samples were stored at -80°C until used for identification.

2.2.6.2 Identification of Botryosphaeriaceae species DNA from samples

Samples of soil media were removed from the -80°C freezer and DNA extraction was conducted using the PowerSoil™ DNA isolation kit (MO BIO Laboratories, CA, USA). Approximately 0.25 g of each sample was added into a Power Bead™ tube containing an aqueous solution of acetate and salts. The tubes were then vortexed briefly. DNA was extracted by following the manufacturer's recommendations and the final DNA extraction solution (~100 µL) was stored at -20°C until used. A nested PCR was carried out as described in Section 2.2.5.1.2 and 2.2.5.1.3 and identification was carried out using the SSCP method as described in Section 2.2.5.1.4.

2.2.7 Botryosphaeriaceae infection of blueberry propagation cuttings by soil root transfer

The experiment was conducted in November 2014 in a nursery in the North Island within their nursery propagation facilities. Two different types of propagation cuttings were used for this experiment: soft green cuttings 10-12 cm long and 3 months old hard rooted

cuttings (10-15 cm). The soft green cuttings were transferred into 5×5 cm cells in potting trays (Figure 2.16) and the rooted hard cuttings were transferred into 9.5×9.5 cm pots, which both contained propagation media (tested and found free of Botryosphaeriaceae infestation as described in Section 2.2.6). The nursery provided 36 soft cuttings from one batch of cultivar 'Blue Bayou'. For rooted hard cuttings they provided 12 hard rooted cuttings from a batch of cultivar 'Blue Bayou', which were used as controls, another 12 hard rooted cuttings from a batch of cultivar 'Dolce Blue' which were used for *N. ribis* inoculation, and another 12 hard rooted cuttings from a batch of cultivar 'Powderblue'which were used for *N. australe* inoculation. The soil media was allowed to dry out for one day prior to inoculation.

2.2.7.1 Soil infestation and isolation of pathogens from plants

The conidial suspensions (10^4/mL) used for inoculation were made from two isolates each of *N. australe* (S1-131 and S1-174) and *N. ribis* (S1-158 and S1-175), which were prepared as described in Section 3.2.4. To give the inoculum good contact with the plants, 250 mL of inoculum was poured into a hole (3 cm in depth) made in the soil at 2 cm from each potted hard rooted cutting. For the soft cuttings, 50 mL of inoculum was poured into each 5×5 cm cell in potting trays. The 12 plants for each treatment, including controls, were arranged in a completely randomized design (CRD). All plants were maintained in the nursery environment to develop normally. The nursery manager was asked to monitor their condition and to send photos of any discolouration or dieback symptoms displayed by the plants. After six months when some plants started to display discolouration, the plants were all sent to the Lincoln University plant pathology laboratory. Plants were left under lights (12 h light: 12 h dark) at room temperature, with occasional hand-watering, for one to two weeks until they were processed.

For each rooted hard cutting, the stems were removed from the roots and surface sterilized as described in Section 2.2.2. From the main stem, ten 0.5 cm sections were cut from the base, one section from the middle and one from the tip of the main stem. The sections were separated into bark and wood prior to isolation. From the three side-shoots that grew closest to the base, the first 3 cm was removed and surface sterilised with 70% ethanol prior to cutting into 0.5 cm segments for which bark and wood were separated for isolation.

Three tissue segments taken from each of two leaves close to the tip of each plant and three leaf segments from each of three leaves that showed lesions were also plated.

The root systems of ready-rooted hard cuttings were washed with tap water to remove soil and other debris. Then the roots randomly selected for isolation were surface sterilised by dipping in 20% bleach (sodium hypochlorite 53 g/ L) for 3 min, washing with sterile water for 1 min and air drying for 30 min under sterile air in a laminar flow hood. For each plant, the three selected hard roots were cut into 1 cm pieces for up to 10 cm (depending on length) from the plant base. The three selected soft roots were cut into 1 cm pieces for up to 5 cm from the plant base (depending on length).

For the rooted soft cuttings, the ~10 cm stems were separated from the root system and surface sterilised with 70% ethanol, washed and dried. Stems were then cut into 0.5 cm segments which were plated onto PDAC. Five soft roots were removed from the root system and washed with tap water and then surface sterilised by dipping in 20% bleach for 3 min, followed by washing with sterile water for 1 min and air drying for 30 min under sterile air in a laminar flow hood. Each root was cut into 1 cm pieces, up to 5 cm from the plant base for isolation.

All the plant tissues were plated onto PDAC. The plates were incubated at 25°C in a diurnal light regime (12 h dark and 12 h white light) for three to five days. Colonies growing from the plant samples that showed typical growth of Botryosphaeriaceae species were sub-cultured onto fresh PDA. These cultures were subcultured onto WA and purified using the mycelial tip cutting technique described in Section 2.2.2 and subcultures made on PDA. Morphological identification and molecular identification using ARDRA was done as described in Sections 2.2.3 and 2.2.4. Mycelial plugs of isolates identified as *N. australe* and *N. ribis* were stored in -80°C until extraction of genomic DNA was performed for further genetic analysis, which was used to differentiate the inoculating isolates from naturally occurring isolates.

2.2.7.2 Genomic DNA extraction

The purified mycelial plugs of isolates from the plant survey, which had been identified (Appendix B), and all the isolates from Section 2.2.7.1 which had been stored in -80°C were used for this assay. One plug from each isolate was plated onto PDA and incubated at 25°C in a diurnal light regime (12 h dark and 12 h white light) for three days. Three mycelial plugs (7 mm) were cut from the edge of each growing mycelium and sub cultured in 15 mL potato dextrose broth (PDB; Difco™ New Jersey, United States) in deep Petri dishes. The cultures were incubated at 25°C in a diurnal light regime (12 h dark and 12 h white light) for three days. Each whole growing mycelium was scooped out using a sterile micropipette and transferred onto sterile Miracloth™ (CALBIOCHEM® Germany) on top of 10-20 sterile paper towels. The Miracloth™ with paper towels was folded and pressed until excess PDB was removed. Then the mycelial plug was scraped off using a sterile scalpel leaving the fungal hyphae, which was wrapped in aluminium foil. Then the aluminium packets were labelled, snap frozen in liquid nitrogen and stored at -80°C until required for DNA extraction.

Genomic DNA of each isolate was extracted from the frozen mycelium using the plant tissue DNA isolation protocol of the PUREGENE® genomic extraction kit (Gentra systems, Minneapolis, USA). Frozen mycelium was ground using a sterile mortar and pestle that had been precooled with liquid nitrogen. Approximately 100 mg of ground frozen mycelium was transferred into a precooled 1.7 mL tube and then used for DNA extraction following the manufacturer's instructions. Finally, 30 µL of sterile distilled water was added to the DNA pellet to rehydrate it.

To check the DNA quality, 3 µL of each DNA sample was mixed with 3 µL of loading dye and separated by gel electrophoresis on a 1% agarose gel in 1×TAE at 10 V/cm for 50 min. The gels were stained with ethidium bromide and photographed under UV light using a FireReader™ Gel Doc imaging system (UVITEC Cambridge, TLS Total Lab Systems Ltd) as described in Section 2.2.4. DNA concentrations were measured using a NanoDrop spectrophotometer (Nanodrop Technologies Inc., Delaware, U SA). All the genomic DNA samples were diluted to working concentrations of 20-25 ng/µL and stored at -20°C until processed by PCR.

2.2.7.3 Selection of primers for UP-PCR analyses for *N. ribis* isolates

A total of 11 primers were screened to select the three primers most likely to detect polymorphism. The sequences of the UP-PCR primers and their respective annealing temperatures are listed in Table 2.1. The DNA extracted (as described in Section 2.2.7.2) from eight isolates (S1-83, S1-88, S1-92, S1-96, S1-106, S1-141, S1-150 and S1-159), which were randomly selected from different farms (Appendix B.14), as well as the inoculating isolates (S1-158 and S1-175). These DNA samples were then amplified with each of the first six primers listed in the Table 2.1. For each primer, a UP-PCR was conducted in a 25 µL reaction volume containing 1× PCR buffer (Roche Diagnostics, Basel, Switzerland), 200 µM each of dGTP, dCTP, dATP, dTTP, 20 pmol of the UP-PCR primer, 2.5 mM $MgCl_2$, 1.25 U FastStart *Taq* polymerase (Roche Diagnostics, Mannheim, Germany) and 20-25 ng genomic DNA.

Table 2.1: UP-PCR primers, sequences and their annealing temperatures

Primer Name	Primer Sequence	Annealing temperature (°C)
AA2M2	$^{5'}$CTGCGACCCAGAGCGG$^{3'}$	50
AS4	$^{5'}$TGTGGGCGCTCGACAC$^{3'}$	55
AS15	$^{5'}$GGCTAAGCGGTCGTTAC$^{3'}$	55
AS15Inv	$^{5'}$CATTGCTGGCGAATCGG$^{3'}$	52
L15/AS19	$^{5'}$GAGGGTGGCGGCTAG$^{3'}$	52
L21	$^{5'}$GGATCCGAGGGTGGCGGTTCT$^{3'}$	58
L45	$^{5'}$GTAAAACGACGGCCAGT$^{3'}$	51
L15	$^{5'}$GAGGGTGGCGGTTCT$^{3'}$	52
3-2	$^{5'}$TAAGGGCGGTGCCAGT$^{3'}$	52
Fok 1	$^{5'}$GGATGACCCACCTCCTAC$^{3'}$	52
0.3-1	$^{5'}$CGAGAACGACGGTTCT$^{3'}$	50

PCR amplifications were performed in a Veriti Thermal Cycler-200 as follows: denaturation at 94°C for 3 min, then 5 cycles of 94°C for 50 s, annealing at the appropriate temperature (Table 2.1) for 2 min and primer extension at 72°C for 1 min, followed by 34 cycles of 94°C for 50 s, annealing at the appropriate temperature (Table 2.1) for 90 s and 1 min primer

extension at 72°C, with a final extension at 72°C for 10 min. Then the UP-PCR was repeated with the remaining five primers listed in the Table 2.1 with four selected isolates (S1-83, S1-88, S1-92, S1-96 and S1-106) which showed least polymorphism for the previous UP PCR with the first six primers.

All UP-PCR amplification products were separated on 1% agarose gels by loading 12 µL of PCR product combined with 4 µL of loading dye [40% (w/v) sucrose; 0.25% bromophenol blue: 0.25% xylene cyanol] into wells. Each gel also had 10 µL of 1kb plus DNA ladder (0.1 µg/ µL; Invitrogen) combined with 4 µL loading dye as a molecular weight marker. Electrophoresis of the gel was at 10 V/cm for 50 min in 1 × TAE buffer then stained and photographed as described in Section 2.2.4. Three primers were selected for the final UP PCR analyses. The criteria for the selection of primers were based on the total number of bands amplified, the number of polymorphic bands and the ease with which bands helped to distinguish standard isolates (S1-158 and S1-175) which had been inoculated into soil. The UP- PCR analyses were repeated with selected primers for all the *N. ribis* isolates recovered from the propagation cuttings in Section 2.2.7 with two standard isolates (S1-158 and S1-175) which had been inoculated into soil media. The banding patterns of standards were compared with all the *N. ribis* isolates to determine if the individuals were genetically identical.

2.2.7.4 Selection of primers for RAPD analyses for *N. ribis* isolates

A total of four primers were screened to select the primer most likely to detect polymorphism. The sequences of the RAPD primers are listed in Table 2.2. The DNA extracted from nine isolates listed in Section 2.2.7.3 (except isolate S1-141), which were randomly selected from different farms (Appendix B.14), was amplified with each of the RAPD primers (Singh and Hughes 2006) listed in the Table 2.2.

Table 2.2: RAPD primers, sequences and their annealing temperatures

Primer Name	Primer Sequence
UBC 517	$^{5'}$GGTCGCAGCT$^{3'}$
UBC 598	$^{5'}$ACGGGCGCTC$^{3'}$
UBC 600	$^{5'}$GAAGAACCGC$^{3'}$
Operon H-19	$^{5'}$CTGACCAGCC$^{3'}$

PCR was conducted in a 25 µL reaction volume prepared as described in Section 2.2.7.2 except that the 20 pmol of UP-PCR primer was replaced with 10 pmol of a RAPD primer. PCR amplifications were performed in a Veriti Thermal Cycler-200 as follows: initial denaturation at 94°C for 6 min, followed by 45 cycles of denaturation at 92°C for 1 min, an annealing temperature of 36°C for 1 min and extension at 72˚C for 1 min, with a final extension at 72°C for 6 min. All the PCR products were separated by electrophoresis on a 1.3% agarose gel (1.3 g agarose in 100 mL 1× Tris-Acetate EDTA buffer). PCR products and a 1 kb plus ladder were mixed with the loading dye as described in Section 2.2.7.2. Electrophoresis of the gel was at 100 volts for 50 min in a 1× TAE buffer. The agarose gels were stained and photographed as described in Section 2.2.4.

From the four primers, one primer was selected for the final RAPD analyses. The criteria for selection were similar to the criteria described in Section 2.2.7.3. The RAPD analysis was repeated with the selected primer for all the *N. ribis* isolates recovered from the propagation cuttings in Section 2.2.7. The banding patterns of standards (S1-158 and S1-175) were compared with all the *N. ribis* isolates to determine if the individuals were genetically identical.

2.2.8 Data analyses

All the data collected from the sampling survey were categorical and in binomial form (presence or absence of pathogen). Statistical analyses were conducted using GenStat version 16. Data of incidence of the different Botryosphaeriaceae species in nurseries and farms were analysed with Pearson chi square tests of independence at $P \leq 0.05$.

2.3 Results

2.3.1 Nursery and blueberry farm survey

Most of the samples collected showed symptoms externally and internally. Nursery 1 submitted one-year-old blueberry plants which were 60-80 cm in height and of which some plants showed symptoms such as lesions on the shoots. Nursery 2, Nursery 3 and Nursery 4 submitted tube plants which were 10-12 cm tall. Plants from Nursery 3 did not show any external or internal symptoms, while some of the plants from Nursery 2 and Nursery 4 showed lesions on the main stems.

2.3.2 Identification of nursery Botryosphaeriaceae isolates

2.3.2.1 Morphological characterization of Botryosphaeriaceae isolates

Morphological characterization included 38 isolates from nurseries, 75 isolates from blueberry farms and 45 isolates remaining to be identified from the surveys conducted in 2009 and 2010. Isolates were placed in two groups based on their colony colour (Figure 2.2).

The colonies of the Group A isolates were light to bright yellow after growing in PDA for three days. The colour became light green/grey to dark green after seven days growth. In addition, the colony edges were fluffy after three days. Colony colour changed to green to dark grey or black after 30 days incubation. Isolates grown in PEA to encourage conidium production, black to grey coloured pycnidia form at the bottom of the agar in contact with the bottom of the plate. Conidia extracted from them were aseptate, hyaline and fusiform. This morphology of isolates matched the descriptions for *N. luteum* and *N. australe (*Amponsah *et al*., 2011*)*.

The mycelia of the Group B isolates were light green with white margins after three days of incubation. After seven days, colony colour changed to dark green/grey and the aerial mycelia became greyish to dark green. The aerial mycelia and the whole colony became black after 30 days. Pycnidia were produced at the bottom of the PEA plates after four to six weeks of incubation. Some isolates produced many pycnidia, some few and others produced no pycnidia. Conidia were also fusiform, aseptate and hyaline, although they were more round in shape than those in Group One. Isolates in this group were morphologically identified as *N. parvum* and *N. ribis.*

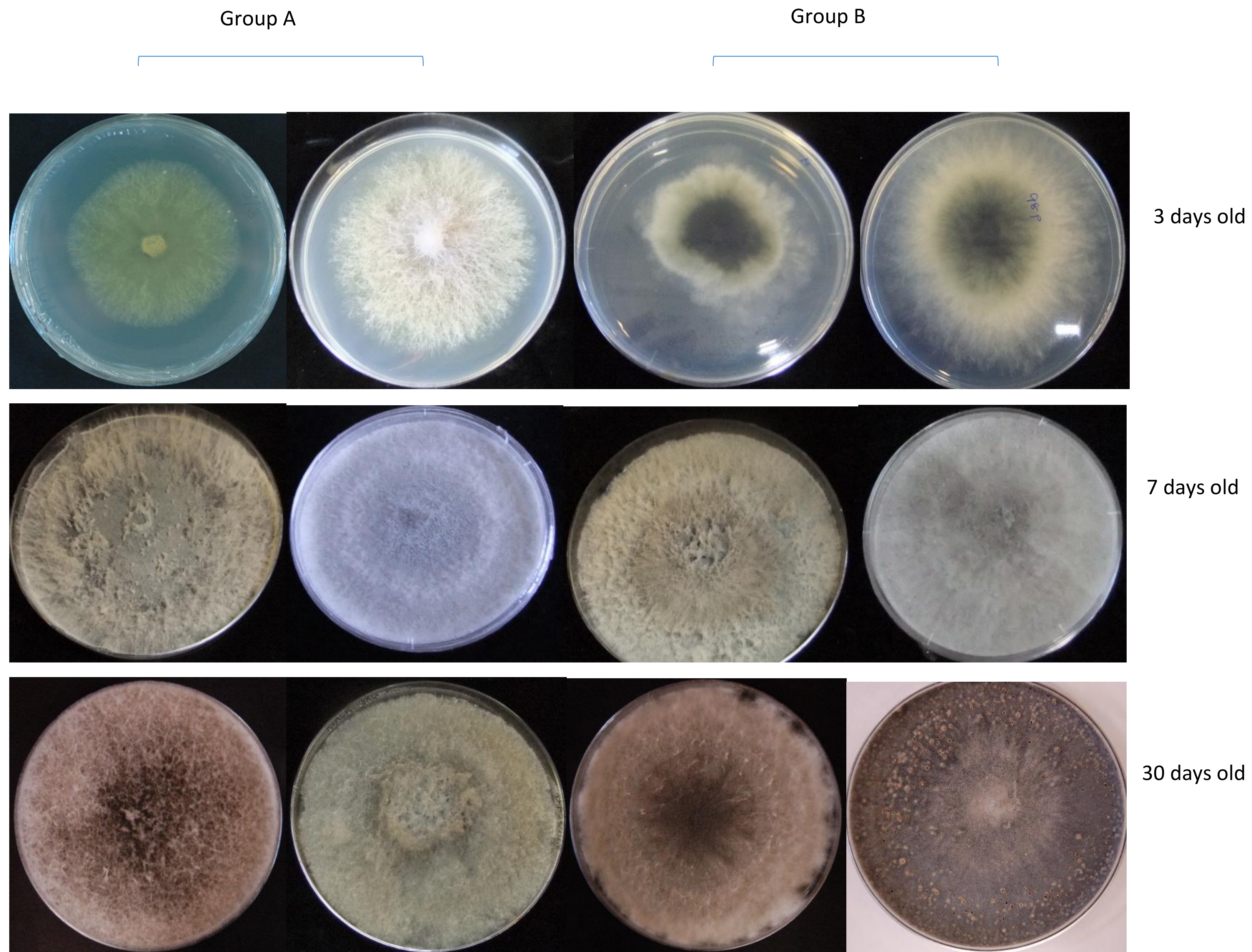


Figure 2.2: Culture morphology of Botryosphaeriaceae species cultured on PDA and incubated in 12 h dark and 12 h light at $25^{o}C$. Group A and B represent the two different morphologies observed based on colony colour and growth characteristics, with Group A species being *N. australe* and *N. luteum* and Group B species being *N. ribis* and *N. parvum*, from left to right

2.3.2.2 Identification of Botryosphaeriaceae isolates by ARDRA

The morphological characters of colonies and conidia were not sufficient to identify the isolates accurately to species level. Therefore, all the isolates recovered from the samples were identified using ARDRA.

The initial PCR with ITS1 and NL4 primers produced a product of ~1200 bp for all of the 158 isolates to be identified (Figure 2.3). When the DNA of all the isolates was initially digested with *Taq*I the resulting banding patterns (Figure 2.4) placed 119 isolates into Group A (*N. luteum* and *N. australe*) and 39 isolates into Group B (*N. parvum* and *N. ribis)*. The DNA of the 119 Group A isolates was further digested using *Sac*II and the resulting banding patterns (Figure 2.5) identified 105 isolates as *N. australe* and the remaining 14 isolates as *N. luteum*. Further digestion of Group B isolates with *Hae*III resulted in two different types of banding patterns (Figure 2.6) with 35 isolates giving the banding pattern of *N. parvum*. The remaining isolates were digested with *Nci*I and identified as *N. ribis* (Figure 2.7).

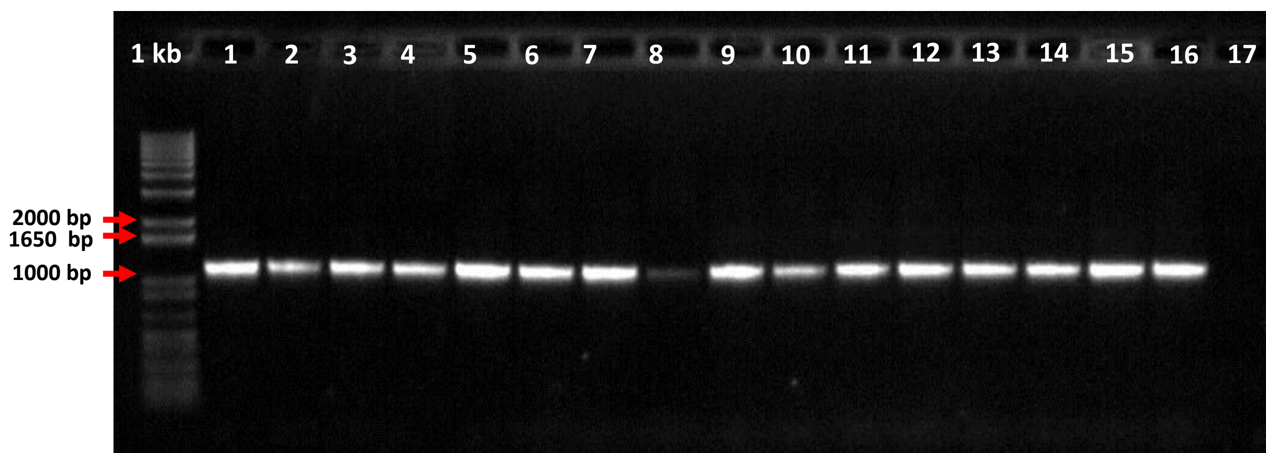


Figure 2.3: Gel of amplified 1200 bp fragments of the ribosomal RNA gene of different Botryosphaeriaceae isolates using the ITS1/NL4 primers. Numbers in the far left denote the molecular weight of the 1 kb plus DNA ladder. Lanes 1-16 contain representative Botryosphaeriaceae isolates; lane 17, negative control.

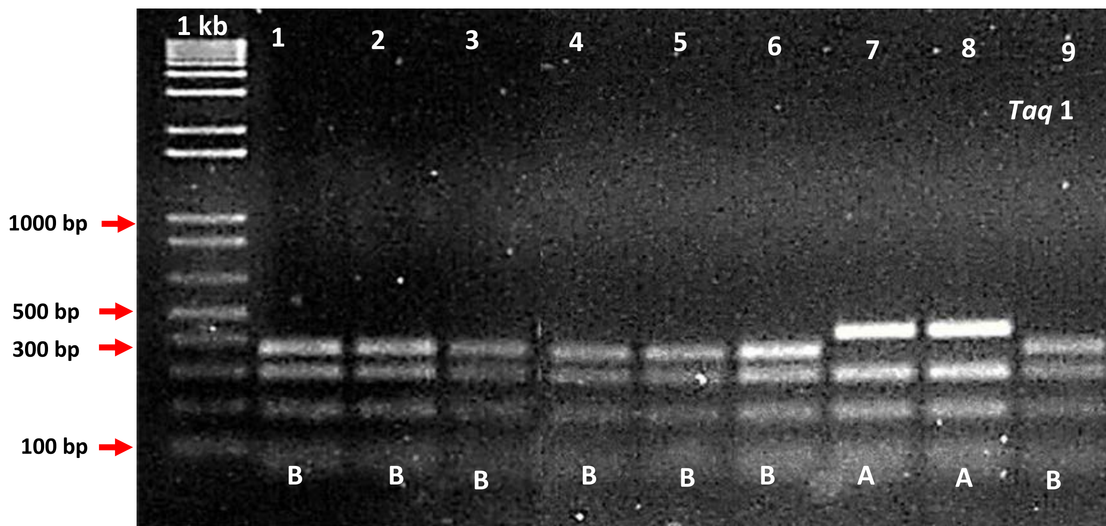


Figure 2.4: PCR product of ITS1/NL4 primers digested with *Taq*I. Lanes 1-6 and 9 show the banding pattern of Group B isolates; lanes 7 and 8 show the banding pattern of Group A isolates. The numbers on the far left denote the molecular weights of the 1 kb plus DNA ladder.

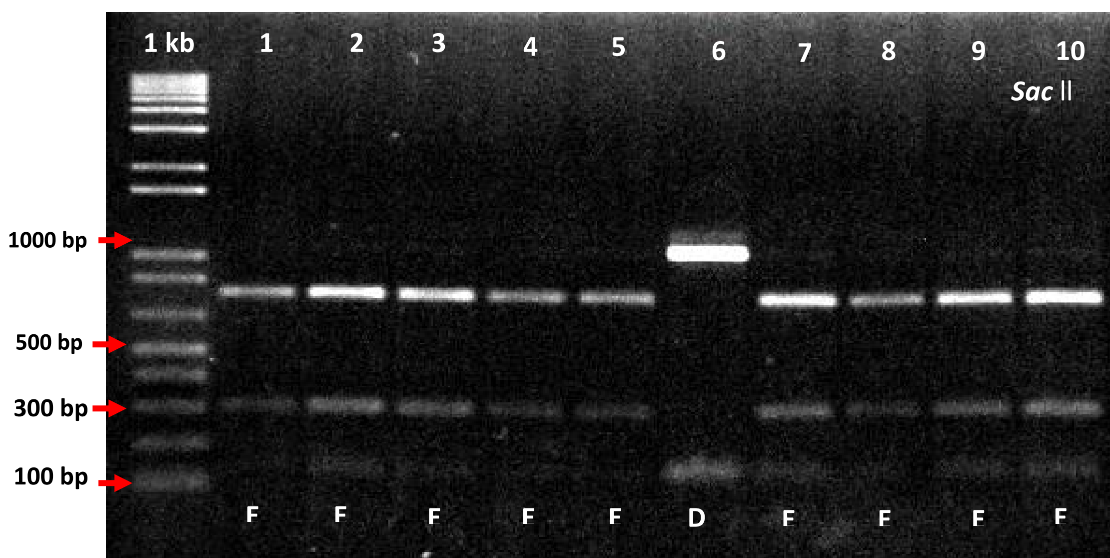


Figure 2.5: PCR product of ITS1/NL4 primers digested with *Sac*II. Lane 6 shows the banding pattern of Group D (*N. luteum*). Lanes 1- 5 and 7-10 show the banding pattern of Group E (*N. australe*). The numbers on the far left denote the molecular weights of the 1 kb plus DNA ladder.

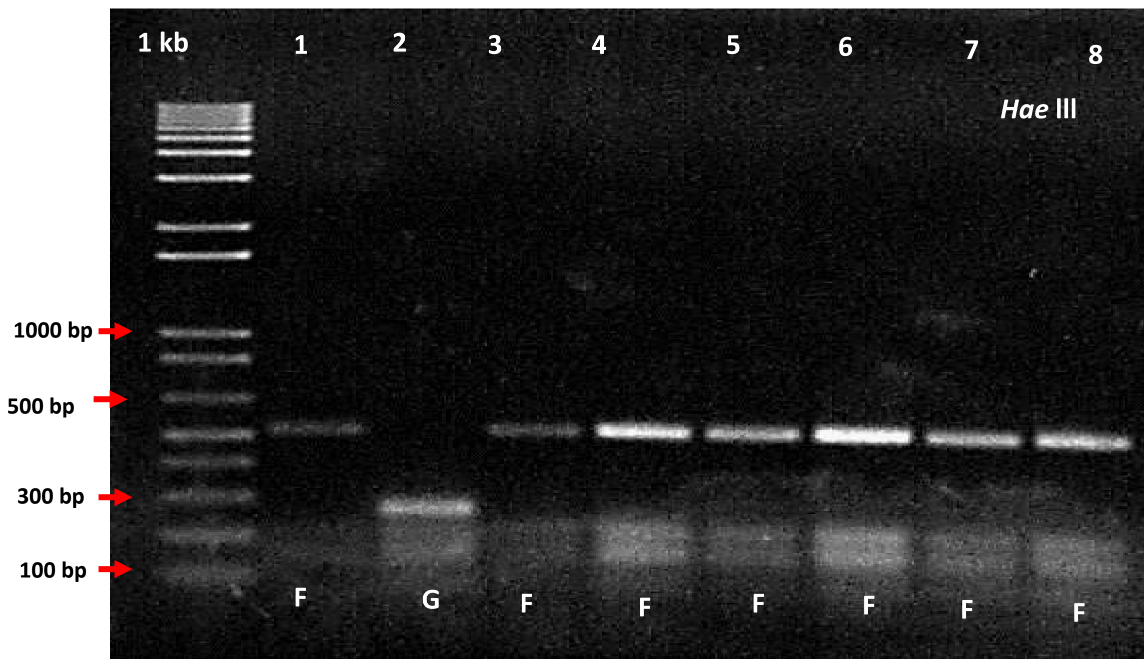


Figure 2.6: PCR product of ITS1/NL4 primers digested with *Hae*lll. Lanes 1, 3-8 are Group F (*Diplodia seriata/mutila*; *N. ribis*); Lane 2- Group G (*N. parvum*). The numbers on the far left denote the molecular weights of the 1 kb plus DNA ladder.

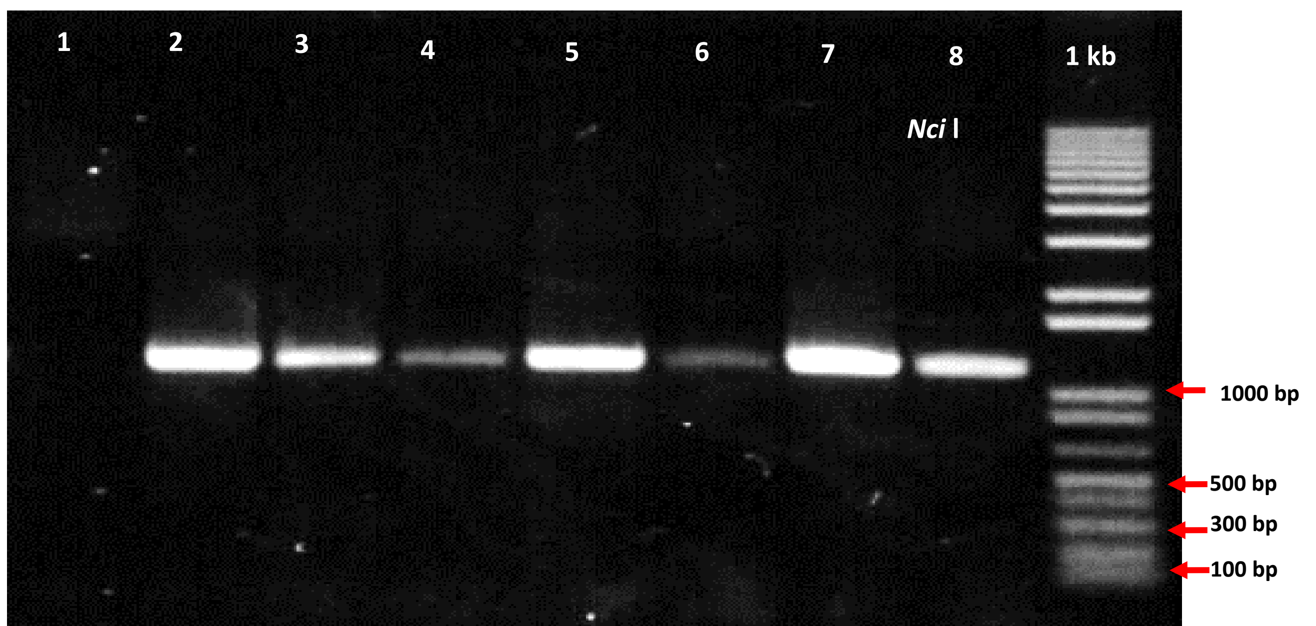


Figure 2.7: PCR product of ITS1/NL4 primers digested with *Nci*I show *N. ribis* isolates which remained uncut with the enzyme. The numbers on the far right denote the molecular weights of the 1 kb plus DNA ladder.

Sequence analysis of isolates

Sequences of the 22 representative *Neofusicoccum* species isolates are given in Appendix F. The neighbour joining tree generated with representative sequences is presented in Figure 2.8, and is composed of two main branches. The first branch is divided into two sub branches, with *N. australe* occupying the first sub branch and *N. luteum* occupying the second sub branch. The second branch is also divided into two sub branches with *N. parvum* occupying the first branch and *N. ribis* occupying the second branch. All the representative isolates of *N. australe*, *N. luteum*, *N. parvum* and *N. ribis* were clustered with their matches from the NCBI database confirming their initial identification using ARDRA. The tree is rooted to the out group *Guignardia philoprina*.

2.3.3 Botryosphaeriaceae infection incidence

2.3.3.1 Overall infection incidence in blueberry nurseries and farms

The numbers and proportion of the different Botryosphaeriaceae species isolated from the nurseries and the blueberry farms in the 2013/2014 or the 2009/2010 surveys are presented in Tables 2.3, 2.4 and 2.5, respectively.

Out of the nursery samples received (n=40), 18 (45%) samples were positive for Botryosphaeriaceae species infection, with a total of 38 isolates recovered. Pearson chi square tests indicated that there was no significant association between the overall incidence of Botryosphaeriaceae infection and nursery source (P= 0.131; Appendix B.1). Nursery 4 had the highest infection incidence (80.0%) followed by Nursery 1 and 2 (40.0%). The lowest infection incidence was observed in Nursery 3 (20.0%) (Figure 2.9). *Neofusicoccum australe* (66.0%) was the dominant species in the nurseries, being isolated from all four nurseries sampled. This was followed by *N. parvum* (31.5%) which was isolated from three nurseries and *N. ribis* (2.5%) which was isolated from only one nursery. No *N. luteum* isolates were recovered from any of the nurseries sampled. The isolation position was not a significant factor in the overall infection incidence (P=0.830; Appendix B.2). Of the 40 nursery plants, 25% of the stem samples were infected in the middles and bases and 20% in the top sections.

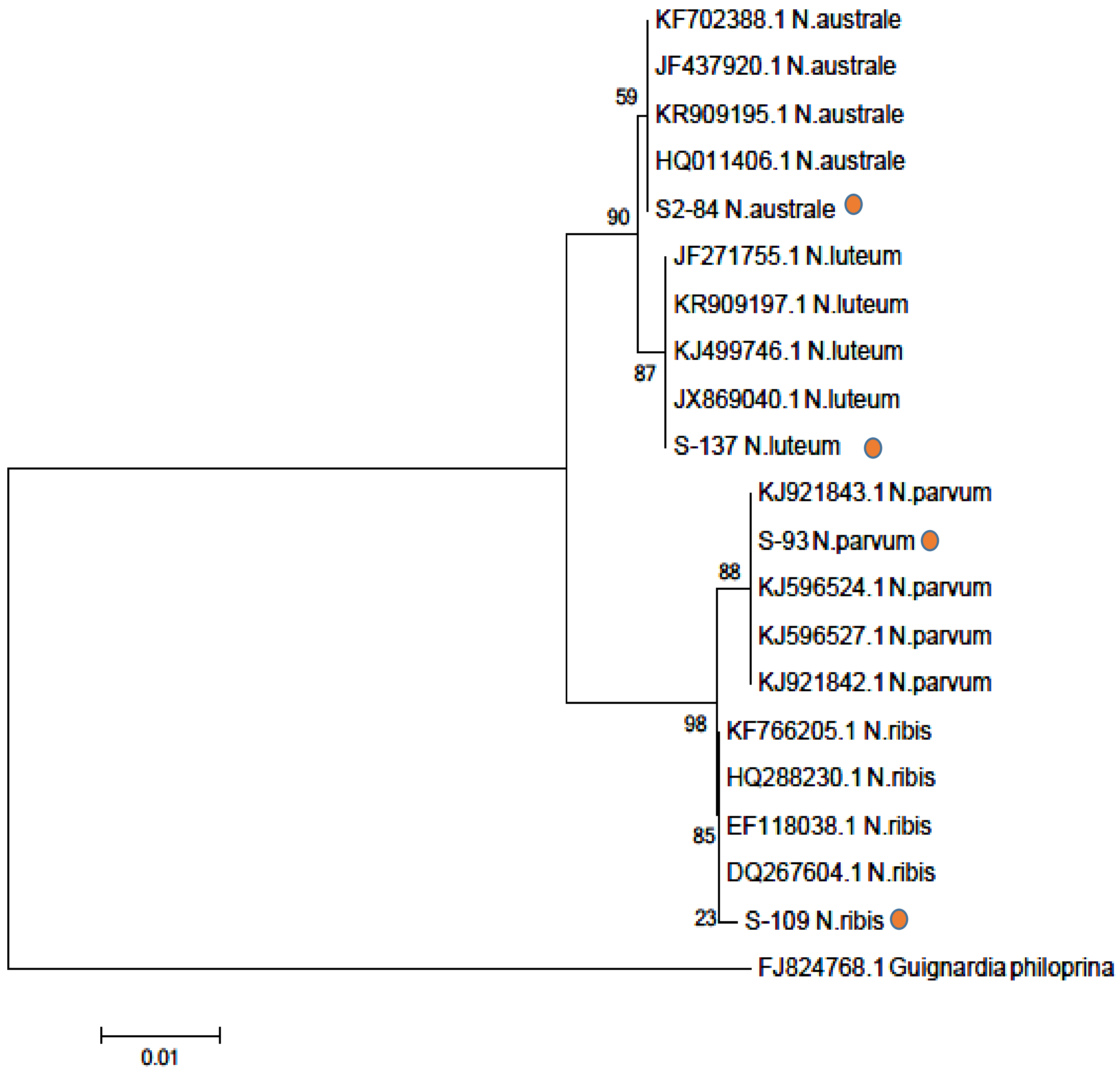


Figure 2.8: Neighbour joining tree of representative isolates of Botryosphaeriaceae species from New Zealand nurseries, farms and representative isolates of Botryosphaeriaceae species from NCBI data base. Isolates marked with an orange circle were isolated in this study from blueberry tissues collected from New Zealand blueberry farms and nurseries.

Table 2.3: Incidence and distribution of Botryosphaeriaceae species in samples from the four nurseries.

Species	No. of isolates	Percentage (%)	No. of nurseries infected
N. australe	25	66.0	4
N. luteum	0	0.0	0
N. parvum	12	31.5	3
N. ribis	1	2.5	1

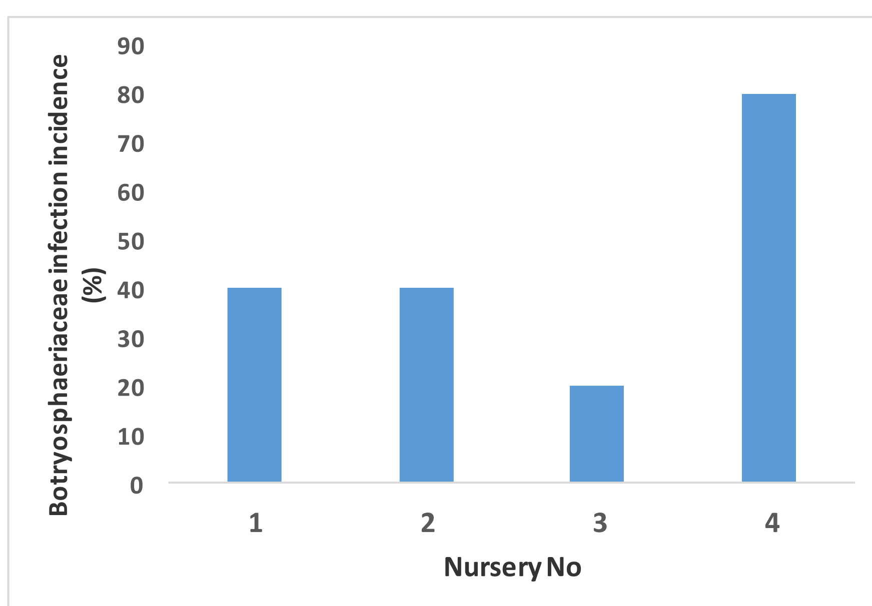


Figure 2.9: Infection incidence of Botryosphaeriaceae species in samples from New Zealand blueberry nurseries.

Pearson Chi-square tests were conducted to determine the relationships between nursery source and isolation positions on the incidence of *N. australe* and *N. parvum* infections (the most predominant species) indicated that nursery source was not a significant factor for *N. australe* or *N. parvum* (*P*=0.058 and *P*=1.00; Appendix B.3 and B.5, respectively). Nursery 4 had the highest incidence of recovery of *N. australe* (70%), followed by Nursery 1 (30%) and Nursery 2 and Nursery 3 (20%). For *N. parvum*, Nursery 4 had the highest (40%) incidence, followed by Nursery 3 (30%) and Nursery (10%). In Nursery 3 none of the plants were infected with *N. parvum*.

For *N. australe* infection incidence, isolation position was not a significant factor (*P*=0.822; Appendix B.4). Of the plants assessed, incidence in samples from the middle of the plants was 17.5%, from the top of the plants was 15.0% and from the base was 12.5%. For *N. parvum* incidence, isolation position was not a significant factor (*P*=0.897; Appendix B.6). Of the plants assessed, the incidence of infection in the stem base was 40% but only 7.5% in the top and middle of the stems.

Seventy samples were received from the blueberry farms, of which 29 (41.43%) were positive for Botryosphaeriaceae species, with a total of 75 isolates recovered (Table 2.4). The Pearson Chi square-test indicated that infection incidence was significantly affected (*P*<0.001; Appendix B.7) by farm source, with the greatest incidence in Farm 1 (100.0% of samples) and least in Farms 2 and 4 (30.0% of samples) (Figure 2.10). No Botryosphaeriaceae species isolates were recovered from Farms 6 and 7. The most dominant species was *N. australe* (59 isolates; 79.0%) which was isolated from five farms. The Pearson Chi square-test conducted to determine the relationships between blueberry farms and incidence of *N. australe* infections showed a significant effect (*P*<0.001; Appendix B.9). In Farm 1, all the samples were infected with *N. australe* (14.3% of all samples), followed by Farm 3 (8.6%), Farm 2 (4.2%), Farms 4 and 5 (2.8%). The next most dominant species was *N. ribis* (8.0%) which was isolated from three farms and *N. luteum* (8.0%) which was isolated from one farm. The least dominant species isolated was *N. parvum* (5.0%) which was isolated from two farms. From the samples obtained from the blueberry farms, there was no significant association between isolation position with respect to lesions, and overall infection incidence (*P*= 0.545; Appendix B.8). In symptomatic shoots, infection incidence was 22.9% at 1 cm above the lesion, 21.4% at 1 cm below the lesion point and 15.7% within the lesion. Isolation position was not a significant factor (*P*= 0.129; Appendix B.10) for *N. australe* infection incidence, with 22.9% at 1 cm below, 12.9% above and 11.4% within the lesions. No other analysis were carried out as incidence of other Botryosphaeriaceae species was low.

For the isolates from the 2009/2010 survey, the most dominant species was *N. australe* (47.0%) and this was followed by *N. parvum* (20.0%), *N. luteum* (18.0%) and *N. ribis* (15.0%), respectively (Table 2.5). All the isolates identified from each survey was listed in Appendix B.10-B.12.

Table 2.4: Incidence and distribution of Botryosphaeriaceae species in samples from seven farms (2013 survey).

Species	No. of isolates	Percentage (%)	No. of farms infected
N. australe	59	79	5
N. luteum	6	8	1
N. parvum	4	5	2
N. ribis	6	8	3

Table 2.5: Incidence and distribution of Botryosphaeriaceae species in samples from five farms (2009/2010 surveys).

Species	No. of isolates	Percentage (%)	No. of farms infected
N. australe	21	47	5
N. luteum	8	18	5
N. parvum	9	20	5
N. ribis	7	15	5

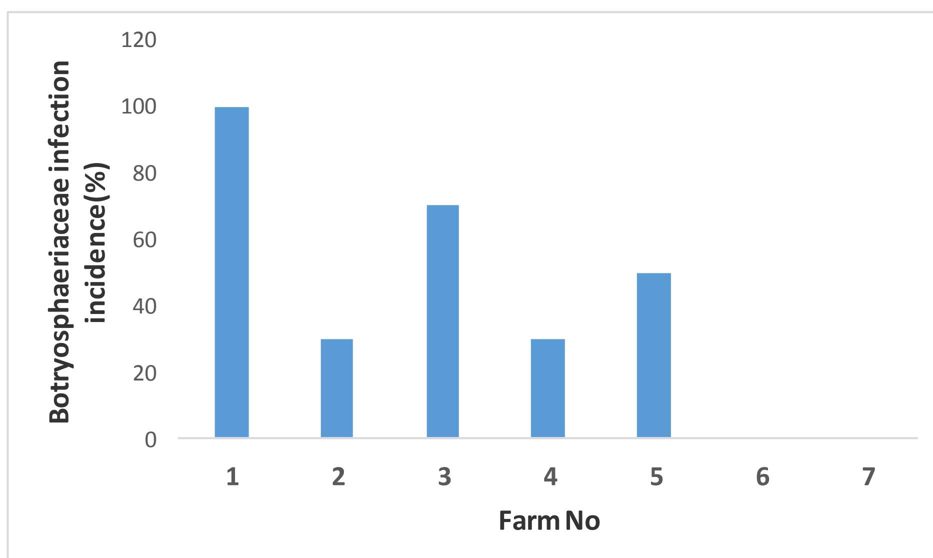


Figure 2.10: Infection incidence of Botryosphaeriaceae species in samples from New Zealand blueberry farms (2013 survey).

In addition, *Pestalotiopsis* species were found alone or coexisting with Botryosphaeriaceae species in some samples received, and also in a farm from which no Botryosphaeriaceae species were isolated. The details of the species morphology and pathogenicity are given in the Appendix B.15.

2.3.4 Identification of surface propagules on cuttings

The initial PCR using the universal fungal primers confirmed that all 20 wash water samples contained fungal DNA (Figure 2.11). When PCR was conducted using the BOT primers only, none of the samples gave a positive band for the presence of Botryosphaeriaceae DNA. When the sensitivity of the PCR was increased with nested PCR 18 samples out of 20 were positive for the presence of the Botryosphaeriaceae DNA (Figure 2.12). When the nested PCR products were analysed using the SSCP method (Figure 2.13), results indicated that *N. australe*, *N. luteum*, *N. parvum*/ *N. ribis*, *D. mutila* and *D. seriata* were present in the samples (Table 2.7). The species most commonly detected was *N. australe* (9 samples), this was followed by *D. mutila* (4 samples) and *N. luteum* (3 samples). One sample each indicated presence of *N. parvum*/*ribis* and *D. seriata*. Botryosphaeriaceae species obtained from each washed sample are listed in the Table 2.6.

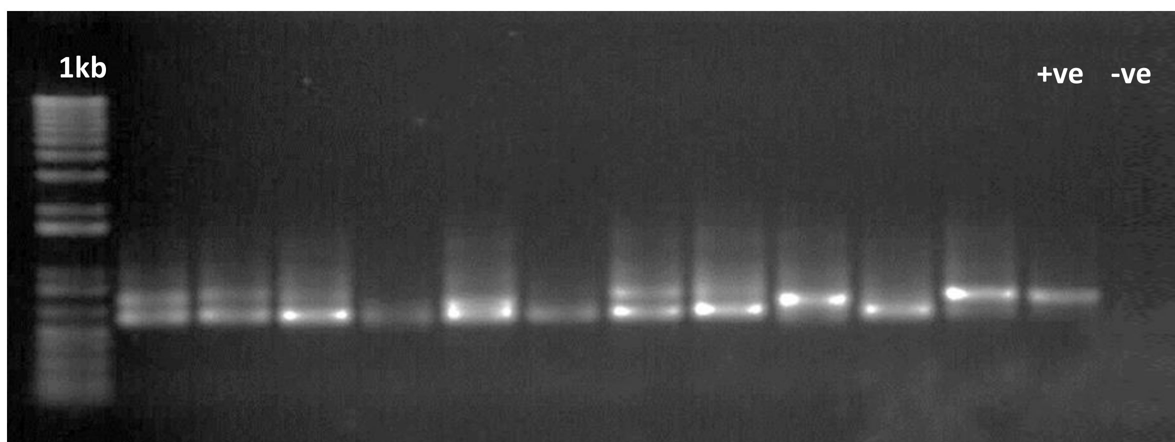


Figure 2.11: Gel showing PCR products produced with the primers ITS1/NL4 from wash water samples. The far right lane shows the negative control (water) and *Neofusicoccum luteum* (isolate L70) DNA as a positive control (+ ve)The far left lane has the 1 kb plus DNA ladder.

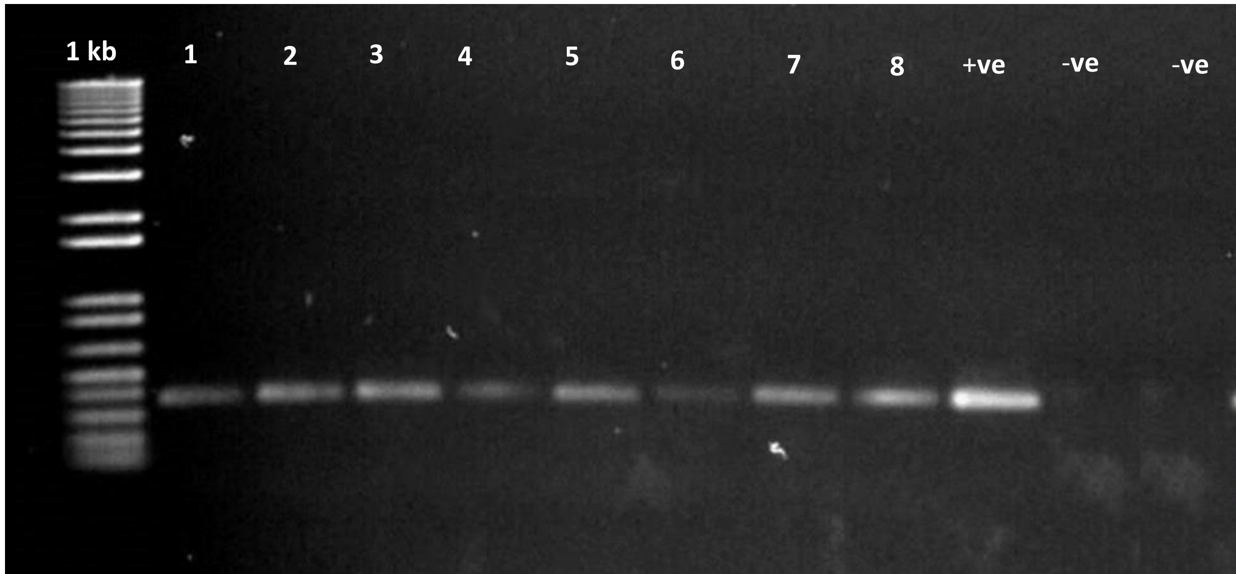


Figure 2.12: Gel from the nested PCR using the diluted (1:200) product of the ITS1/ITS4 PCR. *Neofusicoccum luteum* (isolate L70) DNA included as a positive (+ve) control , two negative controls from initial PCR and diluted PCR, respectively shown in the far right lanes. The far left lane has the 1 kb plus DNA ladder.

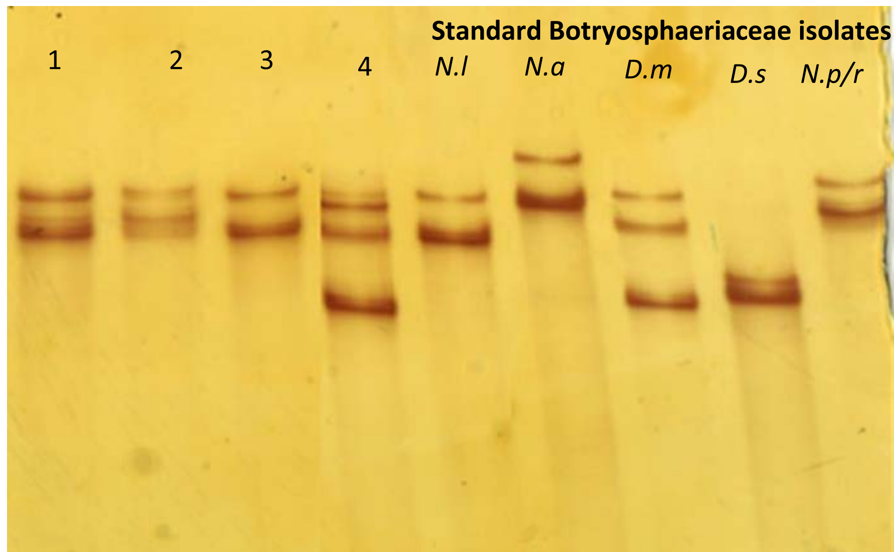


Figure 2.13: SSCP gel showing separation of amplicons produced using BOT primers. Lanes 1-4 represent the wash water from nursery cuttings. The right lanes show the standards: *N.l- N. luteum, N.a- N. australe, D.m- D. mutila, D.s- D. seriata* and *N.p/r- N. parvum/N. ribis.* Note: The bands on the gel have a slight curvature which was taken into account during the identification process.

Table 2.6: Botryosphaeriaceae isolates recovered from washing samples of propagation cuttings and isolating from tip, middle and base of the same cuttings.

Sample No.	Species recovered from washed samples	Species recovered from propagation cuttings		
		Tip	Mid	Base
1	*D. mutila* (1)[a]	0	0	*N. parvum* (1)
2	*N. luteum* (1)	0	0	*N. ribis* (1)
3	*N. australe* (1)	0	0	0
4	*D. seriata* (1)	*N. parvum* (1)	*N. luteum* (1)	0
5	*N. australe* (1)	0	0	0
6	*N. australe* (1)	0	*N. parvum* (1)	0
7	*N. australe* (1)	0	0	0
8	*N. australe* (1)	0	0	*N. parvum* (1) *N. ribis* (1) *D. seriata* (1)
9	*D. seriata* (1) *N. parvum/N. ribis* (1)	0	0	*N. parvum* (1)
10	0	0	0	*N. ribis* (1)
11	*N. luteum* (1) *D. mutila* (1)	0	0	*N. parvum* (2) *D. mutila* (1)
12	*N. luteum* (1) *D. mutila* (1)	0	*N. parvum* (2)	*N. australe* (1)
13	*N. parvum* (1)	0	0	0
14	*N. australe* (1)	*N. parvum* (2)	*N. parvum* (1)	*N. parvum* (1)
15	*N. australe* (1) *D. seriata* (1)	0	*N. parvum* (1)	0
16	*D. mutila* (1)	0	0	0
17	*N australe* (1)	0	0	0
18	*N. australe* (1)	0	*N luteum* (1)	*N. luteum* (1)
19	*N. australe* (1)	0	0	0
20	0	0	*N. ribis* (1)	0

[a] No. of isolates recovered given in the brackets

2.3.5 Internal infections of propagation cuttings

Botryosphaeriaceae infections were present in the some of the cuttings from the source plants of the nursery. Out of 20 plants sampled 13 plants (65.0%) were infected with Botryosphaeriaceae species (Table 2.6). No isolates were recovered from the leaf samples from the cuttings. The results indicated that nursery source plants were infected with *N. australe*, *N. luteum*, *N. parvum*, *N. ribis*, *D. mutila* and *D. seriata.* The most common species detected was *N. parvum* (16 isolates), followed by *N. ribis* (3 isolates) and *N. luteum* (3 isolates). One isolate was obtained for each of *N. australe*, *D. mutila* and *D. seriata*. Multiple isolates were recovered from some plants. Overall, bases of the cuttings showed highest infection incidence (45.0%) and this was followed by the mid (35.0%) and tips (10.0%) of the cuttings. However all the propagation cuttings were either contaminated externally or internally with Botryosphaeriaceae species.

2.3.6 Nursery soil sample analysis

Of the seven nurseries contacted four nurseries, responded to the survey by sending samples of propagation media for analysis. Three nurseries had already sent their plant samples involved with this study and one new nursery (Nursery 5) was from the Waikato region (Table 2.7). Most nurseries sent propagation medium, potting medium and potting sawdust, which had been held from the previous year. Nurseries 2 and 4 also sent one and three samples, respectively, of newly prepared potting mix and sawdust.

Table 2.7: Nursery propagation material sample type sent by different New Zealand blueberry nurseries.

Nursery	Sample type		
	Propagation medium	Potting medium	Potting sawdust
2	11*	11*	10
3	NP	10	NP
4	13*	13	20
5	NP	6	4
Total	24	40	34

NP = not provided
*For Nursery 2, one sample each of both propagation medium and potting medium were newly prepared, and for Nursery 4, three samples each of both propagation medium and sawdust were newly prepared.

2.3.6.1 Detection of Botryosphaeriaceae DNA using PCR

The initial PCR using the universal fungal primers confirmed that all the samples contained fungal DNA. When the PCR was repeated using the BOT primers, only two samples (blueberry propagation mix from Nursery 2) gave a positive band for Botryosphaeriaceae DNA (Figure 2.14). When the sensitivity of the PCR was increased with nested PCR, more samples were positive for the presence of Botryosphaeriaceae DNA (Figure 2.15). Out of the 98 samples received, 43 samples were positive for the presence of Botryosphaeriaceae DNA (44.0%). No isolates were recovered from the newly prepared samples sent from Nurseries 2 and 4. Results obtained from the nested PCR are summarised in Table 2.8.

Table 2.8: Propagation material samples obtained from blueberry nurseries which gave a positive reaction for the presence of Botryosphaeriaceae DNA using nested PCR.

Nursery	Sample type	Number of samples	Number of samples positive for Botryosphaeriaceae DNA
2	Propagation medium	11	7
	Potting medium	11	8
	Potting sawdust	10	3
3	Potting medium	10	6
4	Propagation medium	13	3
	Potting medium	13	6
	Potting sawdust	20	5
5	Potting medium	6	4
	Potting sawdust	4	1

2.3.6.2 Single stranded conformational polymorphism (SSCP) detection of Botryosphaeriaceae fungi

Results from the SSCP indicated that *N. australe*, *N. luteum*, *N. parvum*/ *N. ribis* and *D. mutila* were present in the samples received (Table 2.6). The most common species detected was *N. australe,* which occurred in Nurseries 2, 4 and 5, followed by *N. luteum* which was detected in Nurseries 2 and 5. Nurseries 3 and 4 were positive for the presence of *N. ribis*/ *N.parvum* although *N. ribis* and *N. parvum* could not be discriminated using SSCP. Only two samples, both from Nursery 2, were positive for the presence of *D. mutila*.

Overall, all the nurseries were positive for the presence of at least one Botryosphaeriaceae species in the propagation medium sent. The results obtained from the SSCP are summarised in Table 2.9.

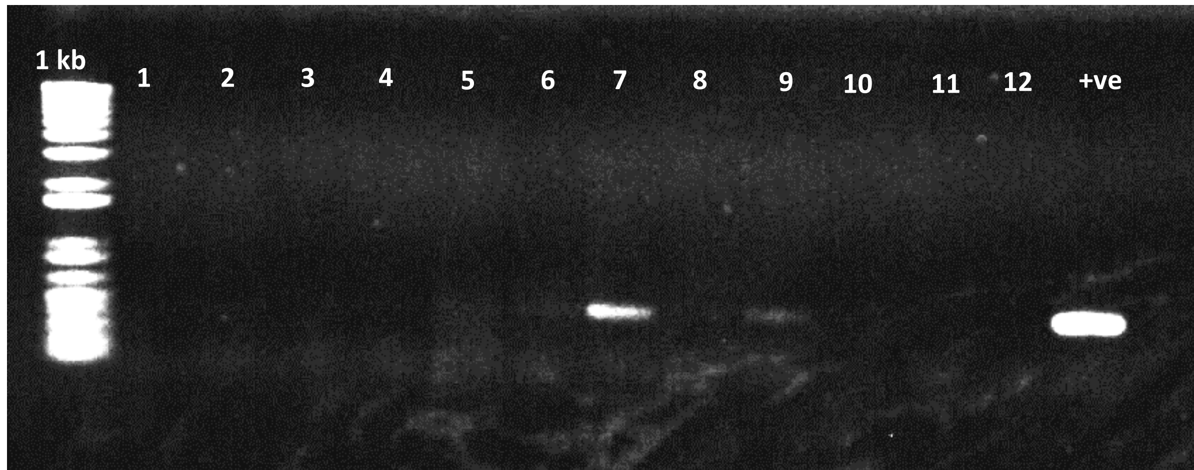


Figure 2.14: PCR product generated by BOT 100F/BOT 472R primers using genomic DNA of the soil samples (propagation medium) supplied by Nursery 2. The samples in lanes 7 and 9 showed a positive band using the BOT primers. *Neofusicoccum luteum* (isolate L70) DNA was included as a positive (+ve) control in the far right lane. The far left lane has the 1 kb plus DNA ladder.

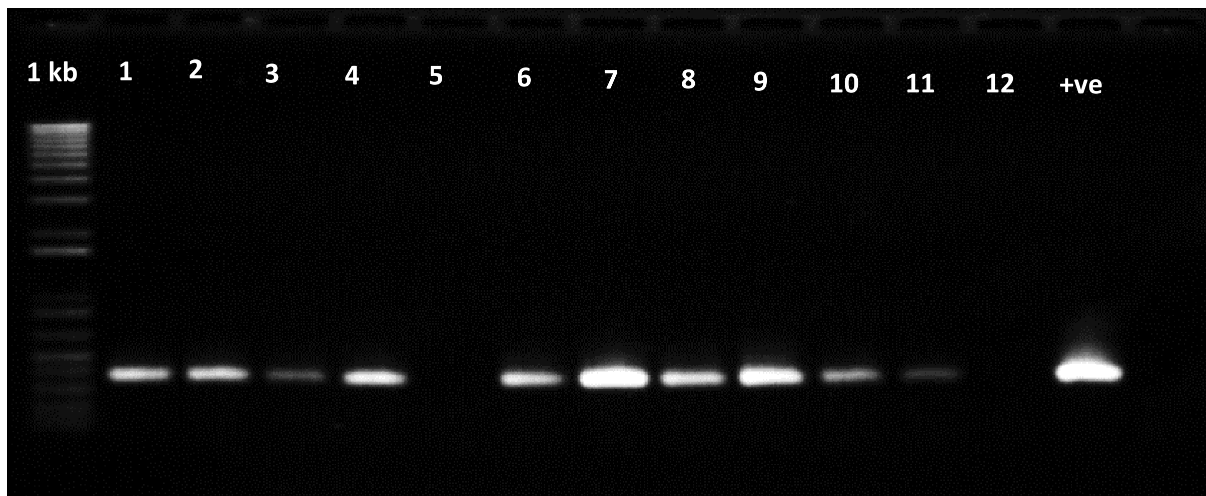


Figure 2.15: Gel of nested PCR products produced using the diluted (1:200) product of the ITS1/ITS4 PCR. *Neofusicoccum luteum* (isolate L70) DNA included as a positive (+ve) control in the far right lane. The far left lane has the 1 kb plus DNA ladder.

Table 2.9: Presence of Botryosphaeriaceae species in nursery propagation and potting mixtures.

Nursery	Sample type	Number of samples positive for Botryosphaeriaceae species				
		N. australe	*N. luteum*	*N. parvum /N.ribis*	*D. mutiila*	*D. seriata*
2	Propagation medium	4	3	0	0	0
	Potting medium	4	4	0	0	0
	Potting sawdust	3	1	0	2	0
3	Potting medium	0	0	3	0	0
4	Propagation medium	3	0	0	0	0
	Potting medium	6	0	0	0	0
	Potting sawdust	3	0	2	0	0
5	Potting medium	2	2	0	0	0
	Potting sawdust	1	0	0	0	0

0 = no samples positive for Botryosphaeriaceae species.

2.3.7 Botryosphaeriaceae infection of blueberry propagation cuttings by soil root transfer

When the plants were assessed 6 months after inoculation of potting medium, some plants started to show discolouration in the leaves but no lesions or dieback were observed in potted soft rooted cuttings or rooted hard cuttings (Figure 2.16). When the isolations were carried out from different plant tissues, isolates were recovered from some plant tissues of soft rooted cuttings and rooted hard cuttings which were identified by ARDRA as *N. parvum* and *N. ribis*. Out of 12 soft rooted cuttings and 12 rooted hard cuttings for which soil was infested with *N. australe*, no isolates identified as *N. australe* were recovered. For these plants two *N. parvum* isolates were recovered from the bark (5th and 6th positions from the base of the cutting) of one rooted hard cutting and two *N. ribis* isolates from the 7th and 8th positions of the stem of a soft rooted cutting.

Figure 2.16: Potted soft and rooted hard cuttings showing symptoms 6 months after soil infestation with *N. ribis* A) soft cuttings B) rooted hard cuttings.

For rooted hard cuttings for which soil was infested with *N. ribis,* isolates of *N. ribis* were mostly recovered from the hard roots, soft roots and the base of the plants where they were restricted to the bark. Out of 12 rooted hard cuttings eight plants (66.7%) were positive for the presence of *N. ribis*, with a total of 15 isolates recovered (Table 2.10). For the soft rooted cuttings *N. ribis* isolates were recovered from some soft roots (no hard roots were developed) and the soft stems of the plants. Out of the 12 rooted soft cuttings six were infected (50.0%) with *N. ribis,* with a total of 12 isolates recovered (Table 2.11). In rooted hard cuttings *N. parvum* (4 isolates) were recovered from the bark of the base of a one plant, being found in segments up to 4 cm continuously in plant no. 4. In soft rooted cuttings *N parvum* isolates were recovered from the stems of two plants (5th position in one plant and 8th position in another plant). For the controls of hard rooted cuttings, one plant

was infected with *N. parvum*, with one isolate being recovered from the bark at the base of the plant.

Table 2.10: Isolation position and number of *N. ribis* isolates recovered from different tissues of rooted hard cuttings.

	Soft roots		Hard roots		Base of plant	
Sample no	**No. of isolates**	**[a] Position recovered**	**No. of isolates**	**[a] Position recovered**	**No. of isolates**	**[c] Position recovered**
1	2	1 & 3	1	2	0	
2	0		0		0	
3	1	6	0		0	
4	0		0		1	8
5	0		0		0	
6	0		0		0	
7	0		0		1	7
8	0		0		0	
9	0		3	4 & 5 [b] 1	0	
10	1	1	0		0	
11	0		0		1	7
12	1	1	2	3 & 4	1	8

[a] numbers given in ascending order from base downwards for roots
[b] different roots
[c] numbers given in ascending order from base

Table 2.11: Number of *N. ribis* isolates recovered from different tissues of soft cuttings.

	Soft roots		Stem of the plant	
Sample no	**Number of isolates**	**[a]Position recovered**	**Number of isolates**	**[b]Position recovered**
1	0		0	
2	1	3	2	3 & 4
3	0		2	4 & 5
4	0		2	2 & 4
5	0		2	7 & 8
6	0		0	
7	0		0	
8	0		0	
9	2	2 & 3	0	
10	0		1	5
11	0		0	
12	0		0	

[a] numbers given in ascending order from base downwards for roots
[b] numbers given in ascending order from base

2.3.8 Selection of primers for UP-PCR analyses for *N. ribis* isolates

Of the 11 primers tested, 10 primers produced multiple bands of different sizes while primer Fok1 did not produce bands. From the 10 standard isolates, isolate S1-141 was excluded from the analysis as it was found to have been mistakenly identified as *N. ribis.* The initial UP-PCR was conducted with six primers (AA2M2, AS4, AS15, AS15Inv, L15/AS19, L21 and L45), for which the clearest bands were produced by primers AS4, L21 and AA2M2 (Figure 2.17). Results showed that the inoculating isolate S1-175 produced unique bands that enabled it to be discriminated from the other isolates but isolate S1-158 did not (Figure 2.17). Further UP-PCR conducted with the remaining primers (L15, 3-2, Fok1 and 0.3-1) also did not produce any polymorphic bands among the four isolates tested including for isolate S1-158. Therefore the presence of isolate S1-175 was determined by UP-PCR genotyping using three primers (AS4, L21 and AA2M2) with all the isolates recovered from the plants grown in soil infested with *N. ribis* isolates (S1-158 and S1-175).

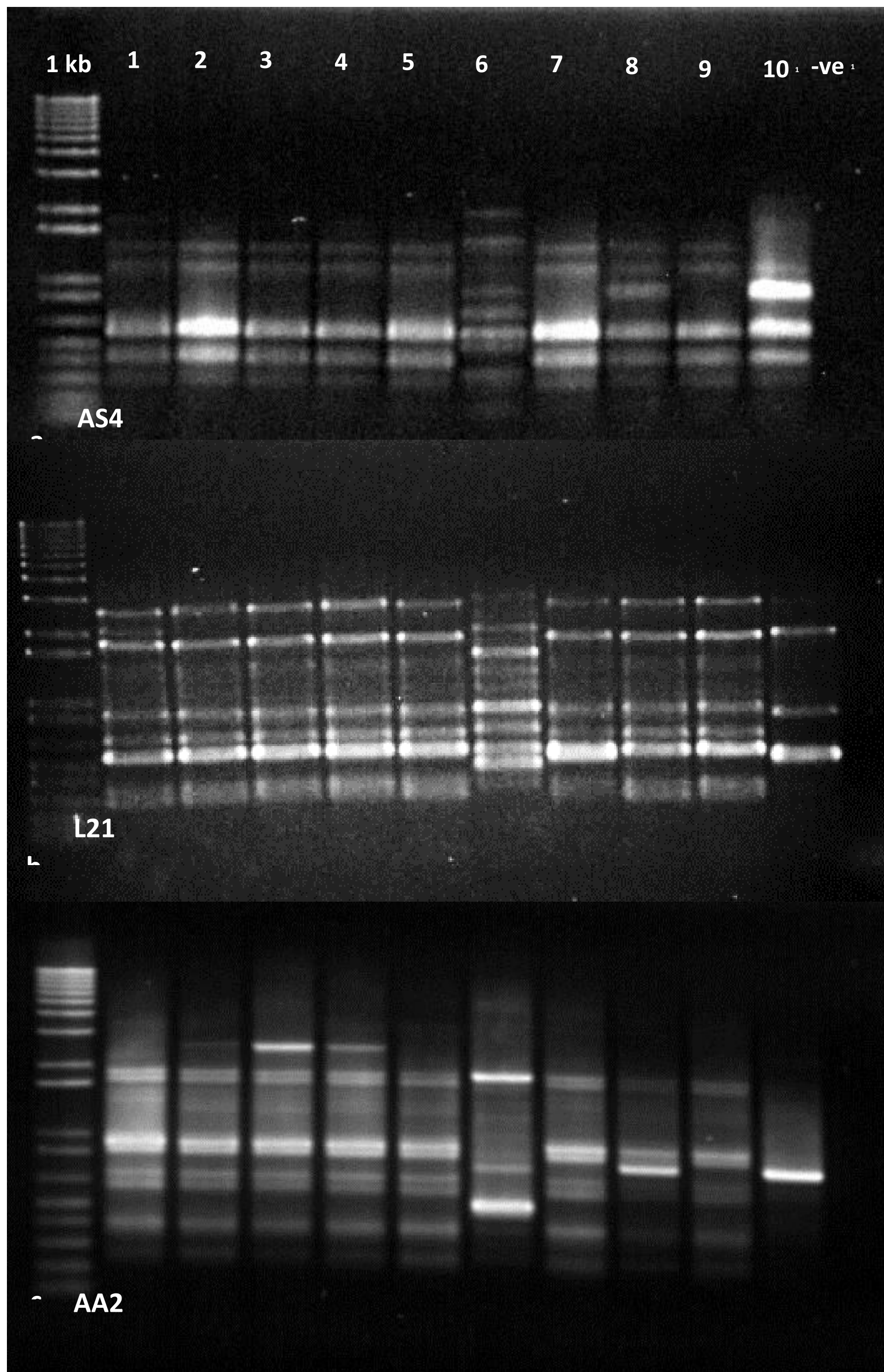


Figure 2.17: Gels of UP-PCR fingerprints from genomic DNA of *N. ribis* isolates. Primers were: a) AS4, b) L21 and c) AA2M2. From left to right isolates are S1-83, S1-88, S1-92, S1-96, S1-106, S1-141 (non-*N. ribis* isolate), S1-150 and S1-159, as well as inoculating isolates S1-158 and S1-175. The far left lanes have the 1 kb plus DNA ladder.

For the genotyping of *N. ribis* isolates from the soil infestation experiment, all the three primers used for UP-PCR produced a high number of clear bands. However no isolate from plants grown in the infested soil showed the unique band pattern of isolate S1-175 with the three primers (Figure 2.18). As isolate S1-158 did not have a unique UP-PCR fingerprint its presence or absence could not be confirmed with this method.

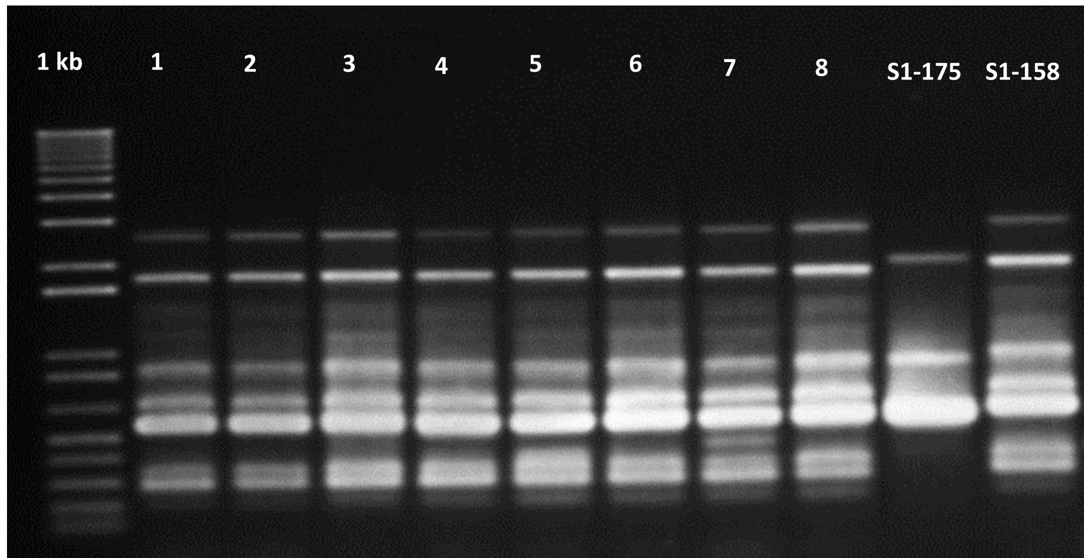


Figure 2.18: Gel of UP-PCR amplification with primer L21 of genomic DNA from *N. ribis* isolates recovered from plants grown in soil infested with conidia of *N. ribis* S1-158 and S1-175. From left to right, lanes 1-8 are isolates recovered from rooted cuttings grown in the infested soil. The far left lane has the 1 kb plus DNA ladder.

2.3.9 Selection of primers for RAPD analyses of *N. ribis* isolate

Of the five primers tested on the representative isolates, all the primers produced multiple bands. Both *N. ribis* isolates (isolate S1-175 and S1-158), produced unique fingerprints with primer Operon H-19 (Figure 2.19).

Further analysis conducted with primer Operon H-19 using the isolates recovered from the plants which had been grown in soil inoculated with two *N. ribis* isolates (S1-175 and S1-158), showed that no isolate had the unique fingerprints of either isolate S1-175 or S1-158.

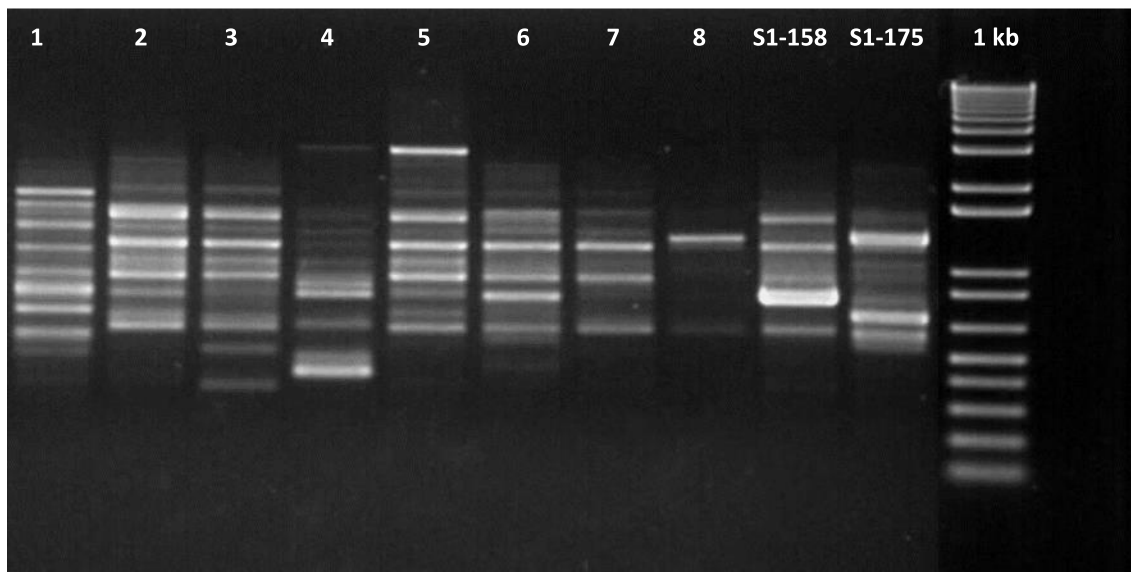


Figure 2.19: Gel of RAPD amplification with primer Operon H-19 of genomic DNA from *N. ribis* isolates recovered from plants grown in soil infested with conidia of *N. ribis* S1-158 and S1-175. From left to right, lanes 1-8 isolates recovered from rooted cuttings. The far right lane has the 1 kb plus DNA ladder.

2.4 Discussion

This research expanded the initial surveys in blueberry farms from throughout New Zealand which sampled tissues showing disease symptoms identified as being due to Botryosphaeriaceae species. The blueberry farm surveys conducted in 2009 and 2010 included a few blueberry farms in the North and South Islands of New Zealand and the current study assessed more blueberry farms in both islands for presence of Botryosphariaceae species. Botrosphaeriaceae infections were found to be present in five out of the seven farms sampled, with 41.43% overall incidence.

In nurseries, Botrosphaeriaceae infections were widespread, being present in all the four nurseries sampled, with 45% of nursery plants and 100% of cuttings being infected. Assessment of the wash water from the propagation cuttings was positive for the presence of the Botryosphaeriaceae DNA in 18 out of 20 samples and isolation of cutting segments showed that 13 of these 20 cuttings were internally infected. These investigations showed that all the propagated cuttings were either contaminated externally or internally with Botryosphaeriaceae species. The Botryosphaeriaceae species washed from plant surfaces may have been splashed-dispersed as propagules onto plant surfaces (Amponsah *et al.*,

2009; van Niekerk *et al*., 2010) from where they could be dispersed onto other healthy plant materials in the nursery by washing and by watering of the propagated plants. Infection may then occur when wounds are made during propagation processes. Billones-Baaijens *et al.* (2013) also recovered Botryosphaeriaceae propagules from the surfaces of scion and rootstock cuttings from grapevine nurseries, as well as internal infections by these pathogens. The current study showed that *N. australe*, *N. luteum*, *N. parvum*, *N. ribis*, *D. mutila* and *D. seriata* cuttings were present in wash water and internal infections of the nursery plants which reflected the infection status of the plants in source blocks used by the nurseries.

This research has shown that infections by Botryosphaeriaceae species are widespread in blueberry farms and nurseries in New Zealand. However, only a limited number of farms and nurseries participated since managers of some blueberry nurseries and farms believed that it might affect the marketability of their products. Further, samples were selected based on their obvious symptoms or being from weak plants for which the managers wanted the problems to be identified. A more extensive survey of nurseries, with random sampling of propagation materials, would help to confirm the prevalence of Botrosphaeriaceae species.

The method developed by Ridgway *et al.* (2011) using the BOT 100F and BOT 472F primers used in this study was successful in detecting the presence of Botryosphaeriaceae species in the wash samples. The sensitivity of these BOT 100F/ BOT 472F was shown to be 1 pg in a standard PCR and 0.1 pg in a nested PCR. The detection of 0.1 pg was approximately equivalent to the DNA from 2.5 conidia, or 250 conidia per 10 mL sample (Ridgway *et al*., 2011). The method has been successfully used in the detection of Botryosphaeriaceae propagules from soil, grapevine wood and rainwater samples (Ridgway *et al*., 2011). However this method cannot differentiate between the viable and non-viable spores, and therefore cannot estimate the presence of viable conidia in the samples. The SSCP method was also used for identification of the species present in the each sample, but was not able to distinguished *N. parvum* from *N. ribis*. The sequences of the two species were identical within the 372 bp amplicon produced by the Bot 100F/Bot 472F primers (Ridgway *et al*., 2011).

Species identification by morphological and molecular methods found that the main four species infecting plants in blueberry farms in New Zealand were *N. australe,* which was the most common species, and *N. parvum*, *N. luteum* and *N. ribis*, with a few incidences of *D. mutila* and *D. seriata*. Sammonds *et al.* (2009) who conducted a limited survey of blueberry farms, in Hamilton, Ohaupo and Hastings in the North Island of New Zealand, from which they recovered 93 isolates, identified a selected number of isolates by ARDRA as being *N. luteum, N. parvum*, *N. luteum/australe and D. seriata,* with a fifth species being of the *Neofusicoccum* type which could not be positively identified morphologically. However, Che Omar (2009) identified all the blueberry Botryosphaeriaceae isolates from that survey using ARDRA. The remaining 61 isolates from different symptomatic plants had a higher percentage of *N. australe* (34%) followed by *N. luteum* (23), *N. parvum* (21%) and *N. ribis* (21%). The pathogenicity study in the Chapter 3, showed that *N. parvum* and *N. ribis* caused longer lesions than *N. australe*, in which the pathogen could be found well beyond the lesion. It therefore seems likely that staff in nurseries and farms might not completely eliminate the infection from tissues if they pruned out only the necrosis. Also growers might tend to remove the most damaged plants while keeping the less infected /symptomatic plants which could be infected with *N. australe*. This may be the reason for high prevalence of *N. australe* in the nurseries and farms in New Zealand.

Several studies had been done overseas regarding identification of Botryosphaeriaceae species associated with dieback in blueberries. Milholland (1995) stated that stem blight caused by *F. aesculi* was a widespread disease of highbush and rabbiteye blueberries in the southern United States. However, in the current study no *F. aesculi* (Teleomorph: *Botryosphaeria dothidea*) was isolated and only one *F. aesculi* isolate was isolated from a North Island farm in the 2009/2010 survey. Blueberries were introduced to New Zealand from the United States so this is unexpected. However, in the earlier studies conducted in the United States, the Botryosphaeriaceae species were identified by their morphological features, not by molecular methods. More recently in Florida USA, Wright and Harmon (2010), who conducted a study identifying isolates of Botryosphaeriaceae species from blueberry with molecular methods, stated that *N. ribis* and 23 other named fungal species had earlier been considered synonyms of *B. dothidea.* Espinoza *et al.* (2009) sampled 1-2 years old stems from eight blueberry commercial plantations in Chile, including northern

high bush and southern high bush blueberry cultivars. They identified their isolates by sequencing the ITS ribosomal DNA and found that the three main species were *N. arbuti*, *N. australe* and *N. parvum*, although they did not mention the percentages of each species. Xu *et al.* (2015) conducted field surveys of 20 blueberry plantations in eight provinces across China and recovered 69 Botryosphaeriaceae isolates from twig dieback and stem blight in blueberries. They identified three species by DNA sequencing, *F. aesculi*, *N. parvum* and *L. theobromae,* of which the former species were the most prevalent and widely distributed species in all blueberry growing regions, while *L. theobromae* was only found in southern regions.

In New Zealand, Botryosphaeriaceae species have mainly been reported as pathogens of grapevines. Baskarathevan *et al.* (2012) sampled 43 vineyards in New Zealand, with recovery of 336 isolates, which were identified by ARDRA and DNA sequencing as nine Botryosphaeriaceae species (*N. parvum*, *N. luteum*, *N. australe*, *N. ribis*, *D. mutila*, *D. seriata*, *F. aesculi*, *Dothoriella sarmentorum* and *Do. iberica*). *Neofusicoccum parvum* was the predominant species (34%) in that study and similar incidence levels were reported for *D. mutila* (18%), *D. seriata* (16%), *N. luteum* (14%) and *N. australe* (11%). Amponsah *et al.* (2011) also sampled necrotic tissues in 20 New Zealand vineyards, but largely within the South Island, and recovered 77 Botryosphaeriaceae isolates which were identified as belonging to five species, of which *N. parvum* was the predominant species followed by *N. luteum, D. mutila*, *N. australe*, and *D. seriata*. In contrast, when Billones-Baaijens *et al.* (2013) studied the prevalence of Botryosphaeriaceae species in New Zealand grapevine nurseries, results showed that *N. luteum* (57%) was the most prevalent followed by *N. parvum* (18%), *N. australe* (8%), *D. mutila* (8%), *F. aesculi* (5%) and *D. seriata* (3%). They suggested that the predominance of *N. luteum* might be due to the less obvious symptoms caused, since the plants with clear necrotic symptoms, such as those caused by *N. parvum,* were likely to be removed during the grading processes in nurseries.

Baskarathevan *et al.* (2012) examined the distribution patterns of the New Zealand Botryosphaeriaceae species and concluded that temperature was likely to be the factor that determined species distribution. They found that *N. australe* and *N. luteum* isolates were distributed in both the North and South Islands but the majority of *N. australe* isolates were found in the South Island (which had cooler temperatures) and the majority of *N. luteum*

isolates were found in the North Island (warmer temperatures). They isolated *D. seriata*, *Do. sarmentorum*, *Do. iberica* and *F. aesculi* only from cooler South Island vineyards. In contrast, the current survey isolated *D. seriata* and *D. mutila* from propagation cuttings taken from one North Island nursery. In the current study of blueberry farms and nurseries, there was a similar pattern of species distribution for *N. australe,* of which more were recovered from the South Island (62.7%) than North Island (37.3%). Che Omar (2009) also showed that the recovery of *N. australe* (34%) in the North Island was higher than to *N. luteum* (23%). In the current study no *N. luteum* isolates were recovered from the South Island farms. Due to the low numbers of farms and nurseries and their predominance in the North Island, no conclusions can be drawn about the regional distribution of species in the current study. However, the results showed that there seemed to be higher numbers of infections by *N. australe* in farms and nurseries and in the vineyards. This may be due to the host effect rather than the environmental conditions in New Zealand. The identification of the main Botryosphaeriaceae species which infect blueberries throughout New Zealand growing regions will ensure that the most prevalent species are used in further experiments for this research programme.

Genotyping studies by Billones-Baaijens *et al.* (2015) showed that the isolates recovered from the trunk and the shoots of the same grapevine were mostly of different genotypes. Also, the isolates recovered from within the bark of the cuttings were mostly of the different genotypes from the isolates recovered from the adjacent internal tissues. These findings suggested the probability of multiple infections from different external sources. Further, this experiment showed that most isolates were within the bark not the wood suggesting that they might be latent in the surface tissues. Botryosphaeriaceae species are generally believed to be wound pathogens wherein grapevine pruning wounds get infected with conidia (Urbez-Torres and Gubler, 2009; Halleen *et al*., 2010). However, Phillips (1998) showed that non-wounded, green, non-succulent attached shoots could be infected with *F. aesculi*. Amponsah *et al.* (2014) also showed that infected non-symptomatic grapevine plants begin to show symptoms when they undergo stress conditions or become winter dormant. All these results with grapevines indicated that propagation cuttings become infected internally or externally and the resulting plants are also likely to be infected, although they may be non-symptomatic when sold and only develop symptoms later on

when established in vineyards. The same scenario is likely to be relevant to blueberry farms. However, only one nursery willingly participated in this experiment, therefore it was difficult to comment about the infection incidence of the propagation cuttings within blueberry nurseries in New Zealand. It is clearly important that managers of nurseries understand the disease cycle of Botryosphaeriaceae species within their propagation systems. However, this experiment showed the importance of developing a method to control the nursery contamination by Botryosphaeriaceae species, perhaps by disinfecting the propagation cuttings.

An experiment was conducted to determine whether the propagation media used in nurseries could be infested with Botryosphaeriaceae species. Results indicated low numbers of propagules since the initial PCR using BOT primers resulted in only two sample positive for the presence of Botryosphaeriaceae DNA. However when the PCR sensitivity was increased by nested PCR, 43 out of the 98 samples received were positive for the presence of Botryosphaeriaceae DNA (44.0%). The high incidence of Botryosphaeriaceae DNA might have been due to the the fact that most of the samples sent by nurseries were previous year's propagation mixtures, which are commonly reused. In contrast, no bands representing Botryosphaeriaceae DNA were observed in the newly prepared samples. In nurseries they usually left the piles of mixtures outside to use later. These mixtures could become contaminated during rain by splash borne spores. Also these mixtures contain plant materials, which may be already infected with Botryosphaeriaceae species. However, there was no evidence about the viability of any spores in these mixtures nor whether Botryosphaeriaceae conidia in propagation mixtures could infect the plants. Billones-Baaijens (2011) demonstrated that necrotic trimmed vine shoots on the ground were contaminated with conidia of Botryosphaeriaceae species and so collected soil samples from around four infected grapevine rootstocks mother plants and tested them for the presence of Botryosphaeriaceae DNA using multispecies primers. Her negative results suggested that these pathogens could not survive in soil for a long period of time but she also suggested that inoculum levels may have been below the level of detection by PCR using multi- species primers. However, in the current study the presence of Botryosphaeriaceae DNA in propagation mixtures indicated the potential ability of soil-borne inoculum for infection of nursery cuttings during the propagation process.

An experiment conducted using conidia of *N. australe* and *N. ribis* to contaminate the soil-free propagation mixture showed that 50% of soft cuttings were infected with *N. ribis* at three months after inoculation but none were infected with *N. australe.* Most of the isolates were recovered from the shoot tissues, including one *N. parvum* isolate which was probably due to earlier contamination of the shoot used. In hard rooted cuttings, no infection occurred with *N. australe* but 66.7% of cuttings were positive for the presence of *N. ribis* isolates. Most of the isolates were recovered from the soft roots, hard roots and the bases of the plants. Some isolates were recovered from the 7th and 8th positions of the plants, which suggested splash dispersal from the soil during plant watering or prior infection as these plants did not show the expected continuous isolation from the base. Since no *N. ribis* isolates were recovered from the non-inoculated controls, it seemed likely that infection had occurred from the infested potting medium.

A similar experiment was conducted by Whitelaw-Weckert *et al.* (2006) using mycelia and conidia of *D. mutila*. The inoculum was applied into soil to infect the roots of 3 year old potted Pinot noir vines. After six months, isolations were carried out and *D. mutila* was recovered at 2 cm and 20 cm from the basal end of the stem of a one plant and at 20 cm from the basal end of the stem of another plant. The isolates they recovered could have been from splash-dispersed inoculum from the soil during watering of the plants as their isolations did not show continuous infection from the roots. They concluded that *D. mutila* infections could be initiated by the soil borne inoculum, as did Castilo-Pando *et al.* (2001) who isolated *D. seriata* from grapevine roots. However, Amponsah *et al.* (2012b) demonstrated that wounded roots of grapevines were not infected by mycelial or conidial inoculum of *N. luteum*, *N. australe*, *N. parvum* or *D. mutila* when isolations were made after 3 months. This variation with the current study may be due to the use of a different host type. Whiteman (2004) used a natural *Ph. chlamydospora* isolate with a unique endogenous marker, for which the identification method was developed by Ridgway *et al.* (2005), and showed that this inoculum was able to move from infected field soil into the young grapevines. If a marker isolate was used for the current experiment it would be simpler to prove that the isolated used for inoculation were the same as those recovered from the plants.

Another way to show that the inoculating isolates were the same as the recovered isolates is to attempt molecular fingerprinting, as done in the current study, to determine if the genotype of the recovered isolate is the same as that of the deliberately inoculated isolate. Initial screening using isolates from a *N. ribis* population, which included the two isolates inoculated into soil, showed bands clearly distinguishing one inoculating isolate of *N. ribis* (S1-175) but not the other isolate (S1-158). However, none of the recovered isolates showed the S1-175 banding pattern. Further work using RAPD PCR was performed on isolates S1-175 and S1-158 with primer Operon H-19. This produced a unique fingerprint for both isolates. However, no isolates recovered from the plants had the fingerprints of isolates S1-175 and S1-158. This showed that the isolates recovered from the plants were not the isolates added to the soil which concurred with the results of Amponsah *et al.* (2012c). Thus, inocula in propagation mixtures are not the sources of infection and it is unclear how *N. ribis* infected the roots from which it was isolated. Amponsah *et al.* (2012c) suggested that in the study by Castilo-Pando *et al.* (2001) stem infection might have progressed downwards into the roots, as might have been the case in the current study. This was investigated in Chapter 3.

Chapter 3

Pathogenicity and cultivar susceptibility of *Neofusicoccum* species and isolates on blueberries

3.1 Introduction

Most pathogenicity studies with blueberries have described the pathogenicity of only one or a few Botryosphaeriaceae species (Milholland, 1971b; Creswell and Milholland, 1987; Espinoza *et al.,* 2009). However, Creswell and Milholland (1987) investigated the pathogenicity of 11 isolates of *Fusicoccum aesculi* on 12 blueberry cultivars using mycelial plugs inoculated onto wounded succulent stems of 2 year old plants. Four weeks after inoculation, mean lesion lengths differed on the different cultivars, varying between 33 and 13 mm, with the largest lesions of 33 and 29 mm on 'Bluechip' and 'Blueray', respectively, and the shortest on Centurion, *V. darrowi* and Premier, with 16, 14 and 13 mm, respectively. A second experiment was conducted using three cultivars ('Bluechip', 'Murphy' and 'Powderblue') and the same 11 isolates. Results revealed highly significant isolate and cultivar differences.

Espinoza *et al.* (2009) also performed a pathogenicity test on 2 year old potted blueberry cultivars 'Brigitta' and 'O'Neal' using mycelial plugs of three isolates of *N. parvum*, which were inoculated onto wounded stems. After 25 days, all the isolates were shown to be pathogenic, causing reddish brown cankers from 13.3 to 95.5 and 11.3 to 70.7 mm in length on blueberry cultivars 'Brigitta' and 'O' Neal', respectively. Also in a cultivar susceptibility experiment they assessed seven blueberry cultivars 'Elliot', 'Brigitta', 'O' Neal', 'Brightwell', 'Duke', 'Bluecrop' and 'Misty'. Mycelial inoculation was done on wounded shoots of two year old potted blueberry plants using two isolates of *N. parvum* and one isolate of *N. australe*. Results showed that one isolate of *N. parvum* was able to produce significantly longer cankers than the other two isolates tested. Canker length was also significantly different between cultivars, with mean lesion lengths after 15 days of 59.4, 30.3, 29.2, 24.2, 21.2, 20.8, and 18.4 mm, respectively. This experiment was repeated with the conidia of one isolate of *N. parvum* with all the seven cultivars and a similar trend was observed with lesion lengths after 25 days of 37.5 to 9.7 mm.

With grapevines, van Niekerk *et al.* (2004) conducted a pathogenicity assay on excised green shoots using 21 isolates from eight Botryosphaeriaceae species isolated from grapevines. Results showed that *N. australe* and *N. parvum* were the most virulent, and the least virulent species were *Diplodia* sp*.* and *D. porosum*. In contrast, assays conducted on excised mature canes using 16 isolates from eight Botryosphaeriaceae species showed that *N. ribis* and *N. australe* were the most virulent species, and *N. luteum* and *L. theobromae* the least virulent. They also conducted an assay on attached canes and mature wood of a grapevine cultivar Periquita. The species that caused most severe lesions were *N. parvum* (15.0 mm long)*, D. porosum.* (13.2 mm), *N. australe* (12.9 mm) and *N. rhodina* (11.7 mm). *Diplodia seriata,* the *Fusicoccum* species and a *Diplodia* sp. caused significantly smaller lesions (8.1–7.2 mm). Most of these studies in grapevines used mycelial plugs as inoculum, largely because Botryosphaeriaceae species do not produce conidia readily in agar. In blueberries, pathogenicity studies were also mostly done using mycelial plugs. However, Amponsah *et al.* (2008) developed a method to produce conidia on grapevine green shoots and they were therefore able to conduct more realistic pathogenicity assays. When conidia of multiple isolates of each species were inoculated onto excised or attached green shoots and trunks of five grapevine cultivars, all species except *D. seriata* caused significant lesions, irrespective of the host plant source, with *N. luteum* causing longest and *D. mutila* shortest lesions, and *N. australe* and *N. parvum* causing intermediate lesions.

The relative pathogenicity of different Botryosphaeriaceae species on different hosts has been widely debated due to some conflicting results (Urbez-Torres, 2011). These conflicting results may be due to the type of inoculum used, type of inoculated tissue, age and susceptibility of cultivar used, as well as different assessment periods and parameters (Billones- Baaijens *et al.,* 2012. In addition, isolates from the same species were recently shown to vary significantly in pathogenicity on detached grapevine shoots, lesions from some isolates differing by up to a factor of 10 (Amna Shafi, pers. comm. 2015). In New Zealand there are no reported pathogenicity studies, assessing the pathogenicity of different Botryosphaeriaceae species on different blueberry tissues and on the susceptibility of the cultivars commonly used in New Zealand.

The aims of this study were to: i) develop a method to produce conidia using blueberry shoots of different maturity stages, ii) to determine the pathogenicity of multiple isolates

from the Botryosphaeriaceae species most commonly associated with blueberries on different tissue types of blueberries, and iii) to determine the susceptibility of different tissues of different cultivars.

3.2 Materials and Methods

3.2.1 Management of potted blueberry plants

Initial isolations which were conducted with plant materials sent by blueberry nurseries in New Zealand indicated that Nursery 3 had low incidence of Botryosphaeriaceae infections (Chapter 2). Therefore, the blueberry plants required for most of this PhD research programme were purchased from this nursery. The plants were of the cultivar 'Tiff blue' and each consisted of one 3-5 cm shoot, with 1-5 tiny (5-10 mm long) new leaves. Plants were repotted into the recommended mix and placed into a greenhouse (temperature 15-20°C), however all the plants died. This may have been due to the size of the plants or to the transplantation shock they faced. Therefore another batch of stock plants were purchased from Nursery 2 which showed the next lowest number of Botryosphaeriaceae species infections. The plants were of the cultivar 'Dolce Blue', were ~30 cm in height and showed no obvious symptoms of disease when delivered. The plants were repotted into 2 L pots of potting mix which contained 40% peat, 40% bark (chunky ~2-4 mm) and 20% vermiculite with enough dolomite to bring the pH to the required level of 4.5-5.0. To ensure adequate nutrients, the potting mix was amended with a 12-14 month Osmocote mix (N:P:K at a ratio of 16:11:10). Plants were grown in a shade house at the Lincoln University Nursery from early December 2013. They were hand-watered as needed and weeds were controlled by hand removal every month. Every 6 months, a side dressing of the same Osmocote mix was added to the plants. These plants were used for all the experiments in the current research programme, unless otherwise stated.

3.2.2 Statistical analysis

In this and the following experiments, data of pycnidial and conidial numbers, lesion lengths, pathogen isolation distances and isolation incidences were analysed by general analysis of variance (ANOVA) using Genstat 16 to determine treatment effects. Comparisons between means of individual treatments used Fisher's protected LSDs at $P \leq 0.05$.

3.2.3 Pathogenicity of *Neofusicoccum* isolates on detached hard and soft green blueberry shoots

In this thesis, the soft green shoots were ~5 mm in diameter, light green in colour and usually 1-2 months old. The hard shoots were 6-8 mm in diameter, dark green and 2-4 months old. These excised shoots (20-25 cm long) were immediately placed in Universal bottles containing sterile water and the bottle tops wrapped with Parafilm™ (Pechiney Plastic Packaging Co., Chicago, IL) to support the shoots. The isolates previously recovered from symptomatic blueberry plants were used in these experiments (details given in Appendix C). The shoots were each inoculated with one of the four pathogenic isolates each of *N. australe* [S1-131, S1-174, S1-111, S1-90], *N. luteum* [S1-142, S1-130, S1-167, S-118], *N. ribis* [S1-110, S1-175, S1- 158, S1-150] and *N. parvum* [S1-58, S1-173, S1- 98, S1-138]. Each soft green or hard shoot was surface sterilised by swabbing with 70% ethanol, superficially wounded with a sterile scalpel (~1-2 mm deep and 4-6 mm in diameter) and inoculated with a 3 mm diameter mycelial plug (Figure 3.1 A) cut from the edge of a 3-day-old PDA culture of one of the isolates or sterile agar for the controls. The ten replicates per treatment were placed in an enclosed completely transparent humid chamber in a randomised block design (RBD) held at room temperature (20-25°C) with 12 h light and 12 h dark, with frequent misting for the first three days. Soft green shoots were removed after seven days and hard green shoots after 10 days. Then the lesion lengths (Figure 3.1B) were measured using a digital caliper (Mitutoyo, U.K Ltd). The infected tissues were surface sterilised by dipping for 30 s in 70% ethanol and air dried in a laminar flow cabinet for 10 min. Isolations were made onto PDA using 1 cm stem pieces cut from above and below the inoculation point. After 4 days incubation at room temperature (20-25°C) with 12 h light and 12 h dark, colonies were identified by their morphological resemblance to the inoculating isolates. These colonies were used to inoculate three fresh wounds per treatment using green blueberry shoots from the same source to demonstrate the similarities of the symptoms and to prove the Koch's postulates. Isolations were again conducted from the 7 day old lesions as described previously.

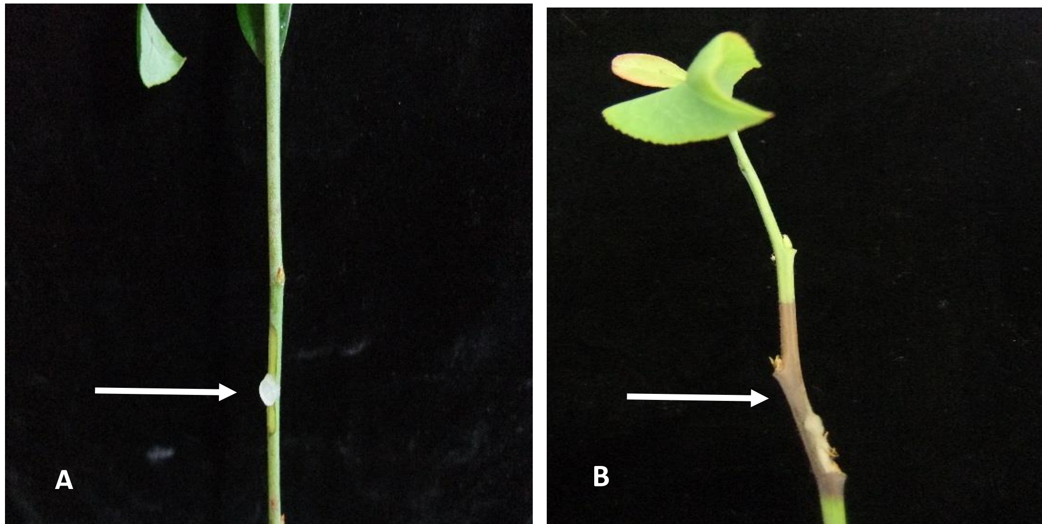


Figure 3.1: Soft green blueberry shoots (A) inoculated with a mycelial agar plug (arrow) and (B) lesion which *N. ribis* caused seven days after inoculation (arrow).

3.2.4 Induction of sporulation on detached green shoots of blueberry

Soft green shoots (20-25 cm cut from the tip) were detached from the potted plants (Section 3.2.1), the base of each shoot being inserted into a Universal bottle filled with water and the top of the bottle wrapped with Parafilm. The six replicate shoots per treatment were allocated to different blocks and the shoots within each block were wounded and inoculated at the same time before beginning with the next block, the whole process being completed in 1 day. Wounding and inoculation with mycelium agar discs used the same isolates and methods described in Section 3.2.3, following the methods of Amponsah *et al.* (2008)**.** All remaining assessment activities followed the same block order to ensure that treatment times remained the same for all shoots. The shoots were placed in an enclosed transparent humid chamber in a randomised block design (RBD) and held at room temperature (20-25°C) with 12 h light and 12 h dark, with frequent misting for three days. After 8 days incubation when pycnidia had developed, the lesions were excised, surface sterilised by dipping in 70% ethanol for 30 s and air dried overnight in a laminar flow cabinet. Sections (1.5 cm) cut from central areas of the dried stem lesions were placed on sterilised moist paper towels in Petri dishes and incubated for 12 h at 25°C to allow pycnidia to ooze cirrhi of conidia. Shoot sections were held with tweezers to allow counting of oozing pycnidia under a stereo microscope (x 10 magnification) and each section was then placed into a 1.5 mL Eppendorf tube containing 1 mL of sterile water with 0.01% Tween 80 (BDH

Chemicals LTD, UK). The tubes were vortexed for 2 min to disperse the conidia into the water and the concentrations of conidia in the suspensions were determined using a haemocytometer.

3.2.5 Conidial infection of *N. ribis* in bark and wood of wounded and non-wounded hard green shoot prunings

Hard green shoots of cultivar 'Dolce Blue', which were leafless and had just been pruned off in a blueberry farm were collected in June 2013. To mimic natural infection of these prunings they were inoculated and maintained in a moist environment similar to what might be expected in a heap of prunings. The shoots were surface sterilised by swabbing with 70% ethanol for 30 seconds and air dried in a laminar flow cabinet prior to wounding (as described in Section 3.2.3) or not wounding. A conidial suspension was made for each isolate of *N. ribis* [S1-175, S1- 158, S1-150], which was enumerated as described in Section 3.2.4 and then the individual suspensions were mixed, with the volumes used being sufficient to give equivalent numbers of conidia for each isolate, and diluted to 10^4/mL. Conidia were inoculated onto wounds or similar intact, marked positions with 50 µL of the mixed isolate conidial suspension. To investigate infection progression over 10 days, five shoots were inoculated for each assessment time (1 day, 2 days, 3 days, 7 days and 10 days). Each treatment also comprised a wounded and non-wounded water-inoculated control of five shoots each. The shoots for each day's assessment were placed on a plastic tray covered with moist paper towels, inoculated shoots being well separated from non-inoculated shoots, and enclosed within a new polythene bag. The trays were incubated at room temperature (15-20°C). To assess shoots for pathogen progression at each assessment time, the shoot were surface sterilised by dipping in 70% ethanol and air dried as described in Section 3.2.3. Isolation onto PDAC was conducted as described in Section 3.2.3 using tissue pieces cut at 1 cm intervals, from 5 cm above to 5 cm below the inoculation point, except for 1 cm around the inoculation point. The bark and wood of each piece were plated separately. The plates were incubated in 12 h light and 12 h dark conditions at 25°C for five days and *N. ribis* isolates identified by colony appearance. Distance data were analysed using ANOVA for each isolation day.

3.2.6 Pathogenicity of conidia of three *Neofusicoccum* species on attached wounded soft green shoots and hard green shoots

A mixed isolate conidial suspension (10^6/mL) was made from three isolates each for *N. australe* [S1- 131, S1-174, S1-111], *N. ribis* [S1-175, S1- 158, S1-150] and *N. parvum* [S1-58, S1-173, S1- 98] as described in Section 3.2.4 and 3.2.5. The 'Dolce Blue' blueberry plants to be inoculated were 2 y old and approximately 900 mm in height when used for inoculation in January 2014. On each plant, two soft and two hard shoots were wounded and immediately inoculated with the same species. Different plants were used for different species and the non-inoculated controls (sterile water).

3.2.6.1 Inoculation technique

The following technique was used to inoculate plants for all experiments throughout this thesis. The shoots were wounded at 30 cm from their bases using a sterile scalpel to make an angled incision which created a flange of tissue (Figure 3.2) that was 1-2 mm deep and 5-8 mm long and remained attached to the shoot. This flange was created below the wound to hold the inoculum in place. Further, Parafilm was wrapped around the stem below the flange, in such a way as to create a continuous lip, which also helped to prevent the droplet of inoculum from running off. Each wound was inoculated immediately with a 50 µL drop of the conidial suspension and control plants were inoculated with 50 µL of sterile water. Each plant was covered with a transparent new plastic bag lightly sprayed inside with water and left in place for 48 h to provide sufficient humidity for infection.

In this experiment, ten replicate plants were used for each treatment and plants were arranged in a completely randomised design (CRD) in an open gravel area similar to field conditions. Hard green shoots were assessed for presence of lesions after 10, 20, 25, 30, 50 and 60 days. At 60 days after inoculation lesions were measured and pathogen isolations were carried out from lesion edges as before. Soft green shoots were assessed for presence of lesions after 1, 2, 3, 4, 6, 7, 10 and 14 days, and after 14 days lesions were measured and isolations were carried out from lesion edges as previously described.

Figure 3.2: Soft green blueberry shoot wounded to create a flange of tissue (arrow).

3.2.6.2 Pathogenicity of conidia of two *Neofusicoccum* species on wounded and non-wounded attached soft green shoots and hard green shoots

A mixed isolate conidial suspension (10^6/mL) was made from three isolates each for *N. australe* [S1- 131, S1-174, S1-111] and *N. ribis* [S1-175, S1- 158, S1-150] as described in Section 3.2.4 and 3.2.5. Three year year old plants of cultivar 'Dolce Blue' were used and the experiment was carried out in February 2016. Soft green shoots and hard green shoots were wounded using a sterile scalpel as described in Section 3.2.6.1 or non-wounded, the inoculation site being marked with a permanent marker pen and wrapped with Parafilm to form a lip as described in Section 3.2.6.1. The sites were immediately inoculated with 50 µL drops of a mixed conidial suspension. Control plants were inoculated with sterile water. Plants were arranged in a CRD in a shadehouse. Six plants were used for each treatment combination and two shoots were inoculated on each plant. Non-wounded shoots and wounded shoots were observed weekly for disease progression. At 14 days after inoculation for soft green shoots and at 60 days after inoculation for hard green shoots, the lesion lengths were measured with a digital caliper as described in Section 3.2.3 and isolations were carried out as described in Section 3.2.5.

3.2.7 Susceptibility and disease progression in different blueberry tissues inoculated by three *Neofusiccocum* species

The susceptibility to infection of fruits, buds, fruit stems, trunks and crowns (Figure 3.3) was determined with three isolates each for *N. australe* [S1-131, S1-174, S1-111], *N. ribis* [S1-175, S1-158, S1-150] and *N. parvum* [S1-58, S1-173, S1-98]. Mixed isolate conidium suspensions (10^4/ mL for flower buds, fruits and fruit stems and 10^6/mL for trunks and crowns) for inoculation were made as described previously in Section 3.2.5. The experiments were begun in February 2014.

The different tissue types were located on different 2 year old plants and the ten replicate plants for each treatment combination were placed in a shadehouse area at the Lincoln University Nursery, in a CRD layout. The inoculation techniques and incubation times differed between the tissue types and so are described separately below.

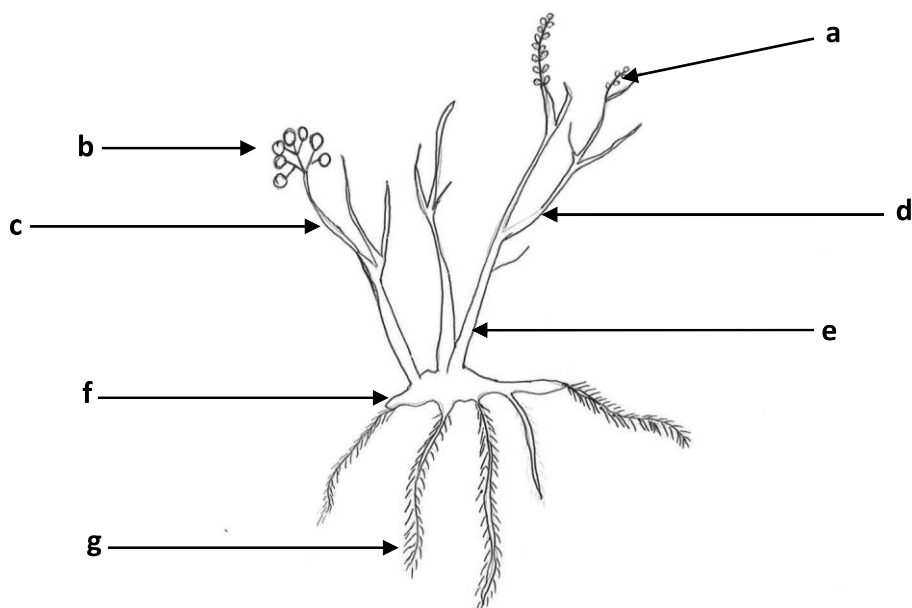


Figure 3.3: Diagram of blueberry plant showing the different parts used for inoculation and isolation (a) buds, (b) fruits, (c) fruit stem, (d) green shoots attached to the inoculated trunk, (e) trunk, (f) crown and (g) roots.

3.2.7.1 Flower buds, fruits and fruit stems

For fruit and bud infection, 20-40 fruit/bud bunches selected on each plant (growth stages shown in Figure 3.9 in Results Section 3.3.7.2) were surface sterilised by swabbing with 70% ethanol and allowed to air dry for ~30 s. Of these, 10-20 replicate bunches were wounded and 10-20 replicate bunches were non-wounded. Fruits were wounded by pricking (~1 mm deep) 10 times each with a sterile needle. The buds were individually wounded by scratching lightly with a needle. The needle was resterilised between tissue types and plants by dipping in 70% ethanol and allowed to air dry for ~30 s. The selected fruits and buds were each drop inoculated with 20 µL of the mixed conidial suspension or sterile water (control plants). To prevent the droplet from running off the tissues, the pipette tip was used to spread the droplet to cover the wounded and non- wounded areas. The stems of two additional non-treated fruit bunches on each plant were also wounded to create a 1-2 mm flange at 2 cm below the fruit bunch and inoculated with 20 µL of the mixed conidial suspension of a species, or sterile water for the control, and surrounded with a Parafilm lip. Each inoculated tissue on a plant was immediately covered with a separate plastic bag, misted inside with water and left for 48 h to provide high humidity. The buds were examined after 1, 2, 3, 4, 6, 7, 9 and 10 days for any discolouration. These buds were then removed from the inoculated plants and non-inoculated controls, surface sterilised and used for isolation, as described previously (Section 3.2.5). Fruits were examined after 3, 6, 9, 12 and 14 days, and 10 apparently infected fruits and fruits from non-inoculated controls per plant were removed after 14 days for isolation as described previously. Another 10 apparently infected fruit from each plant were surface sterilised and placed onto a moist sterilised paper towel within a Petri dish. They were incubated in 12 h light and 12 h dark at 25°C and observed for pycnidial formation after 1, 2, 3 and 4 days, and the number of fruit with pycnidia was recorded. Fruit stems were observed for development of lesions along stems and into the fruits after 10, 15 and 20 days, and were harvested on the 20th day to be used for isolation as previously described.

3.2.7.2 Crowns and trunks

Trunks were wounded and inoculated with 50 µL of the mixed isolate conidial suspensions (10^6/mL) (or controls with sterile water) as described for shoots (Section 3.2.6.1). For crown inoculation, the area at the base of each plant was exposed by removing the upper soil layers, superficially washed with tap water and wounded to create a ~6 mm diameter flange as described previously. Each wound was inoculated with 50 µL of a mixed conidial suspension, and controls with sterile water, and covered with Parafilm. Plants were covered with moistened plastic bags for 48 h.

Extra plants were set up to allow for exploratory dissections that would indicate the most appropriate time for assessment. Three plants were inoculated for each treatment (species and wounding) and for assessment every month.

Trunks and crowns were observed monthly for four months for lesion development. Then the plants were harvested and lesions were measured using a digital caliper. For trunks, lesion development was difficult to observe due to the loose bark which was a common feature in most plants. Therefore bark was removed from the inoculated areas and lesions measured. The potential disease progression into the shoots was examined by making isolations from the two closest shoots to the point of inoculation on the trunk. Isolations were carried out with 1 cm pieces cut up to 10 cm from the attachment point of the shoot to the trunk. Isolations and assessments were carried out as described previously. For crowns it was difficult to measure the lesion length therefore isolation was carried out with 1 cm pieces cut along a horizontal line up to 4 cm on each side of the inoculation point. To investigate potential progression of the disease through the inoculated crown to the roots, three woody roots were randomly taken from each plant. They were washed in tap water, surface sterilized and 1 cm pieces, taken up to 10 cm from the point of attachment of the root to the crown, were used for isolation.

3.2.8 Susceptibility of blueberry cultivars to *N. parvum* and *N. ribis*

The susceptibility of seven blueberry cultivars ('Blue Bayou', 'Centra Blue', 'Dolce Blue', 'Maru', 'Ocean Blue', 'Powderblue' and 'Rahi') to infection by Botryosphaeriaceae species was studied using 2-year-old potted plants from December to February 2014. A mixed isolate conidial suspension (10^4/ mL for fruits, flower buds and leaf buds and 10^6/mL for

soft green shoots and hard green shoos) for inoculation was made from two isolates each for *N. ribis* [S1-175, S1- 158] and *N. parvum* [S1-173, S1-98] as described in Section 3.2.3 and 3.2.5. The different tissue types were located on different 2 year old plants and the six replicate plants for each treatment combination (six plants for each species and cultivar) were placed in a shadehouse area at the Lincoln University Nursery, in a CRD layout. The inoculation techniques and incubation times differed between the tissue types and so are described separately below.

3.2.8.1 Soft green shoots and hard green shoots

Soft green shoots and hard green shoots were wounded and each wound was inoculated with 50 µL of a conidial suspension of a species as described in Section 3.2.6.1. Controls were inoculated with sterile water. Plants were misted with water and covered with plastic bags for 48 h. In this experiment, for each species and cultivar, three stems (soft green or hard green) were inoculated per plant. Plant inoculation sites were observed frequently for lesion development, at 1, 2, 3, 4, 6, 8, 10 and 14 days for soft green shoots and 10, 20, 25, 30, 50 and 60 days for hard green shoots. The soft green shoots were removed 14 days after inoculation and hard green shoots at 60 days, when the lesions were measured using a digital caliper. The infected tissues were surface sterilised by dipping in 70% ethanol for 30 s and air dried in a laminar flow cabinet for 10 min. Isolations were made onto PDAC using 1 cm stem pieces cut from above and below the inoculation point. After 4 days incubation at room temperature (20-25°C) with 12 h light and 12 h dark, colonies were identified by their resemblance to the inoculating isolates.

3.2.8.2 Fruits, flower buds and leaf buds

The fruits were about 50% of their mature size, hard and green. The 20-30 fruit and flower bud bunches selected on each plant (growth stage shown in Figure 3.9 in Results Section 3.3.7.2) were surface sterilised by swabbing with 70% ethanol and allowed to air dry for ~30s. Of these, 10-20 replicate bunches (depending on availability) were wounded and 10-20 replicate bunches were non-wounded. Fruits were wounded by pricking (~1 mm deep) 10 times each with a sterile needle. The buds were individually wounded by scratching lightly with a needle. The needle was resterilised between tissue types and plants by dipping in 70% ethanol and allowed to air dry for ~30 s. The selected fruits and flower buds were

each drop inoculated, with 20 µL of the mixed conidial suspension or sterile water (control plants). To prevent the droplet from running off the tissues, the pipette tip was used to spread the droplet to cover the wounded and non-wounded area. After inoculation each plant was immediately covered with a separate plastic bag, misted inside with water and left for 48 h to provide high humidity. For leaf bud infection, 5-10 leaf buds were selected on two shoots each for wounding and non-wounding. The buds were surface sterilised as described previously and buds were wounded by light scratching with a sterile needle. Each wounded and non-wounded bud was drop inoculated with 20 µL of the mixed conidial suspension or sterile water (control plants), the droplet being spread with the pipette tip as before. Each inoculated shoot was covered with a separate plastic bag, misted with water and left for 48 h.

The flower buds were examined after 1, 2, 3, 4, 6, 7, 9 and 10 days for any discolouration and then removed and used for surface sterilisation and isolation, as described previously. Fruits were examined after 3, 6, 9, 12 and 14 days, and then 10 apparently infected fruits per plant were removed for isolation as described previously. The leaf buds were examined after 1, 4, 6, 8 and 10 days for discolouration and then 10 wounded and non-wounded inoculated leaf buds were removed from each plant for isolation as described previously.

3.3 Results

3.3.1 Pathogenicity of *Neofusicoccum* isolates on detached soft green and hard blueberry shoots

On wounded, inoculated soft green shoots and hard green shoots, lesion development began after two and three days of incubation, respectively. No lesions developed for any control treatments and these were not included in the statistical analysis. In soft green shoots, lesion lengths were significantly affected by the *Neofusicoccum* isolates ($P<0.001$; Appendix C.1.A) (Table 3.1) and species ($P<0.001$; Appendix C.1.B) (Table 3.2). Overall, *N. ribis* and *N. parvum* were the most pathogenic species, although they caused similar mean lesion lengths ($P>0.05$), being 77.7 mm and 75.1 mm, respectively. The isolates of *N. luteum* had shorter lesions ($P<0.05$), with a mean lesion length of 60.0 mm which was significantly greater ($P<0.05$) than the mean lesion length for *N. australe* (39.8 mm).

Table 3.1: Mean lesion lengths caused by isolates of four *Neofusicoccum* species 7 days after inoculation for soft green shoots and 10 days after inoculation for hard green shoots of blueberry cultivar 'Dolce Blue'.

Species	Isolate	Mean lesion length (mm)	
		Soft green shoots	**Hard green shoots**
Neofusicoccum australe	S1- 90	20.1 a[1]	20.4 abcd
N. australe	S1-111	29.3 a	23.7 abcd
N. australe	S1-174	51.7 b	17.6 a
N. australe	S1-131	58.1 bcd	19.4 ab
Neofusicoccum luteum	S1-130	55.8 bc	30.4 cdef
N. luteum	S1-142	58.1 bcd	20.1 abc
N. luteum	S1-118	59.7 bcd	25.6 abcde
N. luteum	S1-167	66.4 bcde	27.1 abcdef
Neofusicoccum parvum	S1-58	63.3 bcde	34.5 ef
N. parvum	S1-138	75.5 defg	37.5 fg
N. parvum	S1-173	76.3 defg	28.2 abcdef
N. parvum	S1-98	85.4 fg	24.4 abcdef
Neofusicoccum ribis	S1-150	67.8 bcdef	34.6 ef
N. ribis	S1-158	73.2 cdef	47.6 g
N. ribis	S1-175	78.3 efg	31.0 def
N. ribis	S1-110	91.4 g	29.4 bcdef
LSD		**18.27**	**10.64**

[1]Values within columns followed by the same letter are not significantly different according to Fisher's protected LSD at P=0.05. Isolates mean effect in soft green shoots (a-g) was significant (P< 0.001; LSD =18.27). Isolates mean effect in hard green shoots (a-f) was significant (P< 0.001; LSD =10.64)

In hard green shoots, lesion lengths were significantly affected by the *Neofusicoccum* isolates (Table 3.1) and species (Table 3.2) (P<0.001 for both; Appendices C.1.C and C.1.D). *Neofusicoccum ribis* and *N. parvum* were the most pathogenic (P<0.05), with similar (P>0.05) mean lesion lengths of 35.6 mm and 31.2 mm, respectively. The smallest lesions (P<0.05), were produced by *N. australe* with a mean lesion length of 20.3 mm. Results showed a trend of *Neofusicoccum* species producing longer lesions in soft green shoots than in hard green shoots.

Table 3.2: Mean lesion lengths caused by four *Neofusicoccum* species 7 days after inoculation with mycelial agar plugs onto detached soft green shoots and 10 days after inoculate onto detached hard green shoots of blueberry cultivar 'Dolce Blue'.

	Mean lesion length (mm)	
Species	**Soft green**	**Hard green shoots**
N. australe	39.8 a[1]	20.3 a
N. luteum	60.0 b	25.8 ab
N. parvum	75.1 c	31.2 bc
N. ribis	77.7 c	35.6 c
LSD	**9.13**	**5.75**

[1]Values within columns followed by the same letter are not significantly different according to Fisher's protected LSD at P=0.05. Species mean effect in soft green shoots (a-c) was significant (P< 0.001; LSD =9.13). Species mean effect in hard green shoots (a-c) was significant (P< 0.001; LSD =5.75)

Fungal colonies characteristic of the inoculated isolates were recovered from the lesion edges of all the inoculated detached shoots. No isolates were recovered from the control non-inoculated shoots.

3.3.2 Induction of sporulation on detached green shoots of blueberry

Oozing pycnidia were observed on the lesions for all isolates after 12 h of incubation (Figure 3.4). Numbers of oozing pycnidia were significantly affected by isolate (P<0.001; Appendix C.2.A). The highest mean number of pycnidia was for *N. parvum* isolate S1-173 (272.5 pycnidia /15 mm shoot length), and *N. ribis* isolates S1-175 and S1-150 (228.3 and 215.9 pycnidia / 15 mm shoot length, respectively) which were significantly higher (P<0.05) than for *N australe* S1-90 (51.0 pycnidia/15 mm shoot length) and *N. luteum* isolates S1-130 and S1-118 (82.0 and 96.2 pycnidia /15 mm shoot length, respectively). There was significant variation in numbers of pycnidia between isolates in all species except *N. ribis* and considerable overlap between species (Table 3.3). When the isolate data were combined to determine the species effect, a second analysis showed that there was a significant effect of species (P=0.004; Appendix C.2.B). Pycnidial production was highest (P<0.05) for *N. ribis* and *N. parvum*, which were higher (P<0.05) than *N. luteum* and *N. australe,* as shown in Table 3.4.

Figure 3.4: Blueberry shoot (infected with *N. ribis*) with oozing pycnidia (arrow).

Numbers of conidia produced were significantly affected by isolate (P=0.004; Appendix C.3.C) and species (P< 0.003; Appendix C.3.D), being higher (P< 0.05) for *N. ribis* and *N. parvum,* which had similar numbers (P>0.05), than for *N. australe* and *N. luteum* which also had similar numbers (P> 0.05; Tables 3.3 and 3.4). This method was therefore considered suitable for the production of pycnidia and conidia: the isolates which produced the greatest numbers of conidia were used for further studies as indicated in Table 3.3.

Table 3.3: Mean number of oozing pycnidia and conidia (no. /mL× 10^5) produced on 15 mm lesion lengths caused by *Neofusicoccum* species isolates on detached green blueberry shoots (cultivar 'Dolce Blue').

Species	Isolate	Oozing pycnidia	Conidia (x 10^5/mL)
Neofusicoccum australe	S1- 90	51.0 a[1]	1.1 ab
N. australe	S1-111*	161.2 bcde	1.6 abcd
N. australe	S1-174*	133.2 abcd	0.9 ab
N. australe	S1-131*	129.5 abcd	1.30 abc
Neofusicoccum luteum	S1-130*	82.0 ab	0.7 a
N. luteum	S1-142*	135.0 abcd	1.0 ab
N. luteum	S1-118	96.2 ab	0.7 a
N. luteum	S1-167*	208.3 cdef	1.3 abc
Neofusicoccum parvum	S1-58*	123.2 abc	1.3 abc
N. parvum	S1-138	127.9 abcd	1.3 abc
N. parvum	S1-173*	272.5 f	2.0 bcde
N. parvum	S1-98*	138.3 abcde	2.9 e
Neofusicoccum ribis	S1-150*	215.9 def	1.5 abcd
N. ribis	S1-158*	171.7 bcde	2.6 de
N. ribis	S1-175*	228.3 ef	2.3 cde
N. ribis	S1-110	170.8 bcde	1.0 ab
LSD		**90.23**	**1.16**

[1]Values within columns followed by the same letter are not significantly different according to Fisher's protected LSD at P=0.05. Numbers of oozing pycnidia were significantly affected by isolate (a-f) (P< 0.001; LSD =90.23). Numbers of conidia oozed were significantly affected by isolates (a-e) (P= 0.004; LSD =1.16)

* Isolates selected for use in further studies

Table 3.4: Mean numbers of oozing pycnidia and conidia (no. /mL × 10^5) produced on 15 mm lesion lengths caused by *Neofusicoccum* species on detached green blueberry shoots (cultivar 'Dolce Blue').

Species	Pycnidia	Conidia (x 10^5/mL)
N. australe	118.7 a[1]	1.2 a[1]
N. luteum	130.4 ab	0.9a
N. parvum	165.5 bc	1.9 b
N. ribis	196.5 c	1.8 b
LSD	**45.12**	**0.58**

[1]Values within columns followed by the same letter are not significantly different according to Fisher's protected LSD at *P*=0.05. Numbers of oozing pycnidia were significantly affected by species (a-c) (*P*= 0.004; LSD =45.12). Numbers of oozing conidia were significantly affected by species (a-b) (*P*= 0.003; LSD =0.58).

3.3.3 Conidial infection of *N. ribis* in bark and wood of wounded and non-wounded hard green shoot prunings

Overall the infection incidence was 100% for inoculated wounded shoots. For non-wounded shoots infection incidence was 100% for all the treatments except for Day 1 which showed 90% infection incidence. No isolates were recovered from the non-inoculated control shoots. Pathogen progression showed significant effects of wounding on Day 1 (*P*=0.002; Appendix C.3.A) and on Day 2 (*P*<0.001; Appendix C.3.B), with mean distances being significantly greater (*P*<0.05) for wounded shoots than non-wounded shoots (25.0 and 6.0 mm; 28.0 and 19.0 mm, respectively). There were no significant effects of wounding in Days 3, 7 and 10 (*P*=0.069, *P*=0.114 and *P*=0.198, respectively). There were no significant effects of tissue type (bark vs wood) for Days 1, 3, 7 and 10 (*P*=0.850, *P*=0.213, *P*=0.814 and *P*=0.661, respectively; Appendix C.3). However, for Day 2 mean pathogen progression was significantly greater (*P*=0.04) for bark (26.0 mm) than for wood (21.0 mm). The interactions between tissue type (bark and wood) and wounding were not significant for Days 1, 3 and 7 (*P*=0.350, *P*=0.213 and *P*=0.484, respectively), but were significant for Days 2 and 10 (*P*=0.04 for both), which appeared to be associated with large differences in progression distances between bark and wood (Table 3.5).

Table 3.5: Mean pathogen progression (mm) in the non-wounded and wounded shoots on different days after inoculating hard green shoot prunings of blueberry 'Dolce Blue' cultivar with *Neofusicoccum ribis*.

Day	Non-wounded shoots			Wounded shoots		
	Bark	Wood	Mean progression	Bark	Wood	Mean progression
1	4.0	8.0	6.0	28.0	22.0	25.0
2	24.0	14.0	19.0	28.0	28.0	28.0
3	32.0	32.0	32.0	34.0	42.0	38.0
7	54.0	50.0	52.0	58.0	60.0	59.0
10	68.0	76.0	72.0	84.0	72.0	78.0

There were significant effects of wounding for Days 1 and 2 (P=0.002 and P<0.001, respectively) but not for Days 3, 7 and 10 (P=0.069, P=0.114 and P=0.198, respectively). There was a significant effect of type (bark vs wood) on Day 2 (P=0.040) but not for Days 1, 3, 7 and 10 (P=0.850, P=0.213, P=0.814 and P=0.661, respectively). There were significant interactions between wounding and tissue types for Days 2 and 10 (P=0.040 for both) but not for Days 1, 3 and 7 (P=0.350, P=0.213 and P=0.484 *P*, respectively).

3.3.4 Pathogenicity of conidia of three *Neofusicoccum* species on attached wounded soft green shoots and hard green shoots

The conidia of all the *Neofusicoccum* species tested caused brown to black lesions along the shoots (Figure 3.5). The lesions started to develop within three to four days in both soft green shoots and hard green shoots. In the non-inoculated control, no lesions developed apart from some minor discolouration at the wound site. The lesion lengths were significantly affected by species, both in soft green shoots after 14 days and hard green shoots after 60 days (P<0.001 for both; Appendix C.4). In both types of shoots, there were no significant differences (P>0.05) in the sizes of the lesions caused by *N. ribis* and *N. parvum,* which were both significantly (P<0.05) longer than for *N. australe* (Table 3.6). Fungal colonies characteristic of the inoculating isolates were obtained from the lesion edges of all the inoculated shoots and no colonies were recovered from the control shoots.

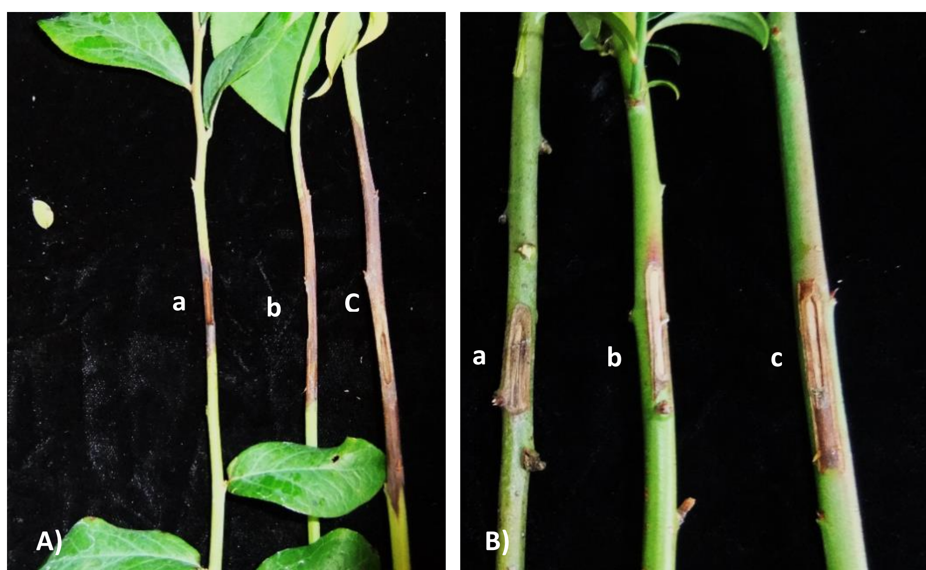


Figure 3.5: Lesion development in attached A) soft green shoots and B) hard green shoots inoculated with conidia of (a) *N. australe*, (b) *N. parvum* and (c) *N. ribis*.

Table 3.6: Mean lesion lengths caused by conidia of three *Neofusicoccum* species inoculated onto attached, wounded soft green shoots after 14 days and hard green shoots after 60 days in blueberry cultivar 'Dolce Blue'.

	Lesion lengths (mm)	
Species	**Soft green shoots**	**Hard green shoots**
N. australe	24.4 a[1]	18.8 a
N. parvum	69.4 b	34. 5 b
N. ribis	90.1 b	41.3 b
LSD	**20.95**	**8.59**

[1]Values within columns followed by the same letter are not significantly different according to Fisher's protected LSD at *P*=0.05. Species mean effect in soft green shoots (a-b) was significant (*P*< 0.001; LSD =20.95). Species mean effect in hard green shoots (a-b) was significant (*P*< 0.001; LSD =8.59).

3.3.5 Pathogenicity of conidia of two *Neofusicoccum* species on attached wounded and non-wounded soft green shoots and hard green shoots

The conidia of two *Neofusicoccum* species tested caused brown to black lesions on the wounded soft green shoots and hard green shoots which started to develop within three to four days in both shoot types. The lesion lengths were significantly affected by species, both in soft green shoots after 14 days (*P*<0.001; Appendix C.5.A) and hard green shoots after 60 days (*P*= 0.004; Appendix C.5.B), with mean lesion lengths being significantly greater for *N.*

ribis (58.8 and 46.8 mm for soft and hard green shoots, respectively) than for *N. australe* (29.8 and 25.8 mm for soft and hard green shoots, respectively). In non-wounded tissues, *N. australe* caused no lesions but sites inoculated with *N. ribis* had some brown dots in some soft green shoots and hard green shoots (Figure 3.6) which did not develop into lesions. In the non-inoculated controls, no lesions developed apart from some minor discolouration at the wound sites.

In soft green shoots, pathogen progression (Table 3.7) showed a significant effect of species (P<0.001; Appendix C.5.C), with mean progression being significantly greater (P<0.05) for *N. ribis* (47.1 mm) than *N. australe* (30.4 mm). There was a significant effect of wounding (P<0.001; Appendix C.5.D), with mean progression being significantly greater for wounded shoots (47.1 mm) than non-wounded shoots (30.4 mm). There was a significant effect of tissue type on pathogen progression (P=0.007), with means of 44.2 and 33.3 mm respectively, for bark and wood, respectively. There were no significant interactions between species and wounding (P=0.057), species and tissue type (P=0.665), wounding and tissue type (P=0.388) and species, wounding and tissue type (P=0.828) (Table 3.7). In hard green shoots, there was a significant effect of species (P=0.014; Appendix C.5.E), with mean pathogen progression being significantly greater for *N. ribis* (39.2 mm) than *N. australe* (28.8 mm). There was a significant effect of wounding (P=0.003), with mean progression being significantly greater for wounded shoots (40.4 mm) than non-wounded shoots (27.5 mm). There was no significant effect of tissue type on pathogen progression (P=0.362). There were no significant interactions between species and wounding (P=0.191), species and tissue type (P=0.132), wounding and tissue type (P=0.919) and species, wounding and tissue type (P=0.478). No Botryosphariaceae isolates were recovered from control shoots and incidence was 100% in inoculated shoots.

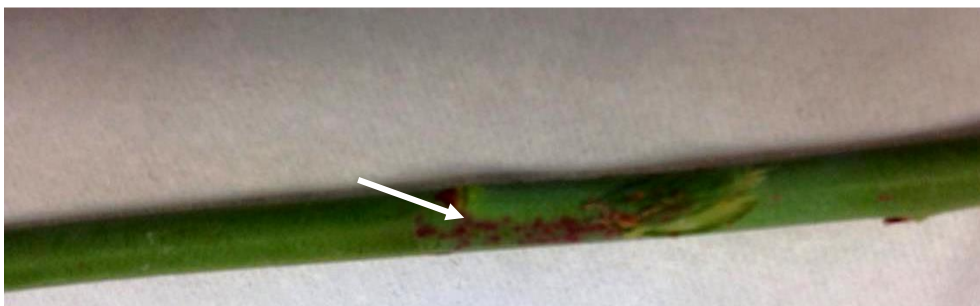


Figure 3.6: Lesions (arrow) which developed in the non-wounded hard green shoots inoculated with *N. ribis.*

Table 3.7: Mean pathogen progression caused by conidia of two *Neofusicoccum* species inoculated onto wounded and non-wounded soft green shoots after 14 days and hard green shoots after 60 days in blueberry cultivar ‘Dolce Blue’.

	Soft green shoots						Hard green shoots					
	Non-wounded			Wounded			Non- wounded			Wounded		
Species	**Bark**	**Wood**	**Mean**	**Bark**	**Wood**	**Mean**	**Bark**	**Wood**	**Mean**	**Bark**	**Wood**	**Mean**
N. australe	31.7	20.0	25.8	38.3	31.7	35.0	25.0	25.0	25.0	30.0	35.0	32.5
N. ribis	43.3	26.7	35.0	63.3	55.0	59.2	33.3	26.7	30.0	55.0	41.7	48.3
Mean effect	37.5	23.3		50.8	43.3		29.2	25.8		42.5	38.3	

In soft green shoots pathogen progression was significantly affected by species (P<0.001), wounding (P<0.001) and tissue type (P=0.007). There were no significant interactions for species × wounding (P=0.057), species × tissue type (P=0.665), wounding × tissue type (P=0.388) and species× wounding × tissue type (P=0.828). In hard green shoots there was a significant effect of species (P=0.014) and wounding (P=0.003) but not tissue type on pathogen progression (P=0.362). There were no significant interactions for species × wounding (P=0.191), species × tissue type (P=0.132), wounding × tissue type (P=0.919) and species × wounding × tissue type (P=0.478).

3.3.6 Susceptibility and disease progression in different blueberry tissues inoculated by three *Neofusicoccum* species

3.3.6.1 Flower buds, fruits and fruit stems

On fruits, inoculation by all three *Neofusicoccum* species caused necrotic lesions on wounded berries within 3-6 days and non-wounded berries within 9-12 days followed by mummification of the berries after a further 14-20 days in wounded and non-wounded fruits (Figure 3.7A). Infection incidence was significantly affected by species (P<0.001; Appendix C.6.A) and by wounding (P<0.001) but not by an interaction between species and wounding (P=0.897). Highest incidence was caused by *N. ribis* (P<0.05) with mean percent infection incidence of 76.8% (67.0 and 86.5% for non-wounded and wounded fruits, respectively) (Table 3.8). This was followed by *N. parvum* (P<0.05), with mean percent infection incidence of 65.8% (57.0 and 74.5% for non-wounded and wounded fruits, respectively). The lowest (P<0.05) mean percent infection incidence of 54.0% was shown by *N. australe* (46.0 and 62.0% incidence for non-wounded and wounded fruits, respectively). Mean incidence was 74.3% for wounded fruits, being significantly higher than for non-wounded fruits (56.7%).

For buds, inoculation by all three *Neofusicoccum* species caused necrotic light brown overall discolouration on wounded buds within 2-3 days and non-wounded buds within 3-5 days (Figure 3.7C). The species effect on infection incidence was significant (P=0.009; Appendix C.6.B) as was the wounding effect (P<0.001) but not the interaction between species and wounding (P=0.965). Highest mean incidence was caused by *N. ribis* (P<0.05), with 74.5% (66.0 and 83.0% for non-wounded and wounded flower buds, respectively), followed (P<0.05) by *N. parvum,* with 67.5% (60.0 and 75.0% for non-wounded and wounded buds, respectively) (Table 3.8). The lowest mean percent infection incidence was caused by *N. australe* (P<0.05) with 57.8% (49.5 and 66.0 for non-wounded and wounded buds, respectively). Mean incidence was 74.7% for wounded buds being significantly higher (P<0.05) than for non-wounded buds (58.5%).

Figure 3.7: Symptoms on different blueberry tissues caused by *Neofusicoccum* species A) arrow (a) shows non-infected fruits and arrow b infected fruits, B) dead fruits with oozing pycnidia shown by arrow c, C) necrotic buds infected by *N. ribis*, D) fruit stem infected by *N. ribis* with infection spreading into fruits, E) elliptical lesion on trunk infected with *N. ribis*.

Table 3.8 Infection incidence of wounded and non-wounded fruits and buds inoculated with *Neofusicoccum* species

Species	Fruits			Buds		
	No wound	Wound	Mean	No wound	Wound	Mean
N. australe	46.0	62.0	54.0 a[1]	49.5	60.0	57.8 a
N. parvum	57.0	74.5	65.8 b	60.0	75.0	67.5 ab
N. ribis	67.0	86.5	76.8 c	66.0	83.0	74.5 b
LSD			**7.83**			**9.03**

[1] Values within columns followed by the same letter are not significantly different according to Fisher's protected LSD at P=0.05. Species effect on infection incidence of fruits (a-c) was significant (P< 0.00;: LSD =7.83) as was the effect of wounding (P < 0.001) but not species × wounding (P= 0.897). Species effect on infection incidence of buds (a-b) was significant (P= 0.009; LSD =9.03) as was the effect of wounding (P < 0.001) but not species × wounding (P= 0.965).

For pycnidial development in fruit (Figure 3.7B), there was a significant effect of species (*P*=0.001; Appendix C.6.C). The highest mean percent of fruits with pycnidia was produced by *N. ribis* (73.5%) which was not significantly greater (*P*>0.05) than for *N. parvum* (66.0%) with both having significantly higher (*P*<0.05) mean numbers than for *N. australe* (50.5%).

When the wounded fruit stems were inoculated with different species, discolouration of the fruit stem started to develop in both directions. The lesions caused by *N. ribis* developed faster than those caused by *N. parvum* and *N. australe*. When the lesions reached the fruits, they became light brown colour and shrivelled (Figure 3.7D). Pycnidial formation on the fruit stem was also observed. The numbers of infected fruit that developed on the inoculated fruit stems were significantly affected by species (*P*=0.003; Appendix C.6.D). The highest mean infection incidence was caused by *N. ribis* followed by *N. parvum* with 81.0% and 68.0%, respectively, which were not significantly different (*P*>0.05). The minimum mean infection incidence was caused by *N. australe* with 39.3% which was significantly (*P*<0.05) less than for the other species.

Fungal colonies characteristic of the inoculated isolates were recovered from the lesion edges of all the above inoculated tissues. No *Neofusicoccum* isolates were recovered from control tissues on which no necrotic lesions developed.

3.3.6.2 Crowns and trunks

All the species caused light brown discolouration in the wood of the trunk, which could be measured only after removal of the loose bark (Figure 3.7 E). There was a significant effect (*P*=0.002; Appendix C.6.E) of species on overall lesion development, with *N. ribis* causing the longest mean lesion (61.5 mm) which was significantly longer (*P*<0.05) than means for both *N. parvum* and *N. australe* which were not significantly different to each other (39.0 and 44.0 mm, respectively). Direction of lesion development (upward versus downward) did not differ (*P*=0.076) nor was there a significant interaction between species and direction (*P*=0.139). The pathogen progression assessed by recovery of *Neofusicoccum* isolates from the wood tissue pieces was significantly affected by species (*P*<0.001; Appendix C.6.G). The mean pathogen progressions was highest for *N. ribis* and *N. parvum,* with 77.0 and 67.0 mm, respectively, which did not differ significantly (*P*>0.05) from each other (Table 3.10). These were significantly higher (*P*<0.05) than for *N. australe* (51.0 mm). The infection

progression through the trunk to shoots showed that there was a significant species effect (*P*=0.034; Appendix C.6.I), with longest mean distance for *N. ribis* (57.0 mm) which was significantly different (*P*<0.05) from *N. australe* (27.5 mm). However the mean distance for *N. parvum* (40.8 mm) was similar (*P*>0.05) to that of *N. ribis* and *N. australe*.

Table 3.9: The mean lesion development (mm) upward and downward from the inoculation points in trunks of blueberry cultivar 'Dolce Blue' inoculated by three *Neofusicoccum* species.

	Direction lesion development (mm)		Overall totals for species
Species	Upward	Downward	
N. parvum	19.50	19.30	38.8 a[1]
N. australe	22.60	21.70	44.3 a
N. ribis	34.0	27.40	61.5 b
			LSD 6.05
Direction means	25.4	22.8	

[1] Means followed by the same letter do not differ significantly at *P*= 0.05 according to Fisher's protected LSD. Significant effect of species (*P*=0.002; LSD= 6.05) for overall direction effect, no significant effect on interaction between direction and species (*P*=0.076).

In the crowns, all species caused light brown discolouration which was similar to the trunk symptoms. The pathogen progression in the crown was significantly affected by species (*P*<0.001; Appendix C.6.F). The longest mean distance colonised was by *N. ribis* (76.0 mm) which was significantly longer (*P*<.05), than for *N. parvum* (63.0 mm) which was also significantly (*P*<0.05) longer than for *N. australe* (39.0 mm) (Table 3.10). When infection progression from the crown to the root was assessed, there was no significant species effect (*P*=0.108; Appendix C.6.H), although the species trends were similar to effects in other tissues, with mean of pathogen progression being 20.5, 7.5 and 6.5 mm for *N. ribis, N. parvum* and *N. australe,* respectively (Table 3.10). However, no necrotic discolouration was observed in the roots.

Fungal colonies characteristic of the inoculated isolates were recovered from the lesion edges of all the above inoculated tissues. No *Neofusicoccum* isolates were recovered from control tissues on which no necrotic lesions developed.

Table 3.10: Mean pathogen progression (mm) by three *Neofusicoccum* species in trunk and shoot from trunk inoculation, and in crown and root from crown inoculation, of the blueberry plants

Species	Trunk	Shoot	Crown	Root
N. australe	51.0 a[1]	27.5 a	39.3 a	6.5
N. parvum	67.0 b	40.8 ab	63.0 b	7.5
N. ribis	77.0 b	57.0 b	76.0 c	20.5
LSD	**12.62**	**21.82**	**12.18**	

[1] Values within columns followed by the same letter are not significantly different according to Fisher's protected LSD at P=0.05. Species mean effect for pathogen progression in trunks (a-b) was significant (P< 0.001; LSD =12.62). Species mean effect for pathogen progression in shoots (a-b) was significant (P=0.034; LSD =21.82). Species mean effect for pathogen progression in crown (a-c) was significant (P< 0.001; LSD =12.18).

3.3.7 Susceptibility of blueberry cultivars to *N. parvum* and *N. ribis*

3.3.7.1 Soft green shoots and hard green shoots

Inoculated soft green shoots started to develop light brown to reddish lesions (Figure 3.8) along the stems of all the cultivars 3-4 days after inoculation by *N. parvum* and *N. ribis*. There was a significant species effect (P<0.001; Appendix C.7.A) over all the cultivers, with longer lesions for *N. ribis* (94.8 mm) than for *N. parvum* (19.6 mm). There was also a significant cultivar effect (P<0.001; Appendix C.7.A; Table 3.11) and a significant interaction between cultivars and species (P<0.001; Appendix C.7.A). The greatest species difference was observed on 'Centra Blue' for which the mean lesion length was 11.1 mm for *N. parvum* and 149 mm for *N. ribis,* which were significantly different (P<0.05) (Table 3.11). Overall, cultivar 'Maru' produced the smallest mean lesions of 26.6 mm for *N. parvum* and 36.6 mm for *N. ribis*.

Table 3.11: Lesion lengths (mm) developed in soft green shoots of different cultivars of blueberries 14 days after inoculation with *Neofusicoccum* species

Species	Cultivar							Species mean
	Blue Bayou	Centra Blue	Dolce Blue	Maru	Ocean Blue	Powder blue	Rahi	
N. parvum	17.3 ab	11.1 a[1]	24.2 ab	26.6 ab	17.3 ab	19.4 ab	21.5 ab	19.4
N. ribis	78.1 c	149.0 e	118.3 d	36.6 b	79.2 c	87.3 c	115.4 d	94.8
Cultivar mean	47.7 ab	80.1 d	71.3 cd	31.6 a	48.2 ab	53.4 bc	68.5 cd	

[1]Values within the rows and columns followed by the same letter are not significantly different according to Fisher's protected LSD at P=0.05. There was a significant species effect (P<0.001) and cultivar effect (P<0.001; LSD= 17.97). There was a significant cultivar and species effect (P<0.001; LSD = 25.41).

In hard green shoots, lesions (Table 3.12) started to develop in all the cultivars 10-12 days after inoculation by *N. parvum* and *N. ribis*. There was a significant species effect (*P*<0.001; Appendix C.7.B) and a significant cultivar effect (*P*<0.001) on lesion development, as well as a significant cultivar and species interaction (*P*<0.001). Overall, the lesions in hard green shoots inoculated with *N. parvum* were smaller than for *N. ribis,* with mean lesion lengths of 14.5 and 40.9 mm, respectively. The mean lesion lengths from *N. parvum* inoculation were not significantly different between the cultivars (*P*>0.05) however, they were from *N. ribis* inoculation. The greatest species and cultivar interaction was observed for 'Dolce Blue' and 'Powderblue', with mean lesion lengths of 63.9 mm and 55.9, mm respectively, for *N. ribis,* while for *N. parvum* they were 16.3 mm and 17.6, respectively, which were not significantly different (*P*>0.05). Overall, 'Centra Blue', 'Maru' and 'Ocean Blue' were best able to resist lesion development in shoots when inoculated with *N. ribis* and *N. parvum* (Table 3.12).
Fungal colonies characteristic of the inoculated isolates were recovered from the lesion edges of all the above inoculated tissues. No *Neofusicoccum* isolates were recovered from control tissues on which no necrotic lesions developed.

Table 3.12: Lesion lengths (mm) developed in hard green shoots of different cultivars of blueberries 60 days after inoculation with *Neofusicoccum* species

Species	Cultivar							Species mean
	Blue Bayou	Centra Blue	Dolce Blue	Maru	Ocean Blue	Powderblue	Rahi	
N. parvum	9.8 a[1]	13.0 a	16.3 a	18.7 a	13.5 a	17.6 a	12.8 a	14.5
N. ribis	39.5 c	29.2 b	63.9 d	30.4 bc	29.2 b	55.9 d	38.4 bc	40.9
Cultivar mean	24.8 a	21.1 a	40.1 b	24.5 a	21.3 a	36.8 b	25.6 a	

[1]Values within the rows and columns followed by the same letter are not significantly different according to Fisher's protected LSD at P=0.05. There was a significant species effect (P<0.001) and cultivar effect (P<0.001; LSD= 6.56). There was a significant cultivar and species effect (P<0.001; LSD = 9.28).

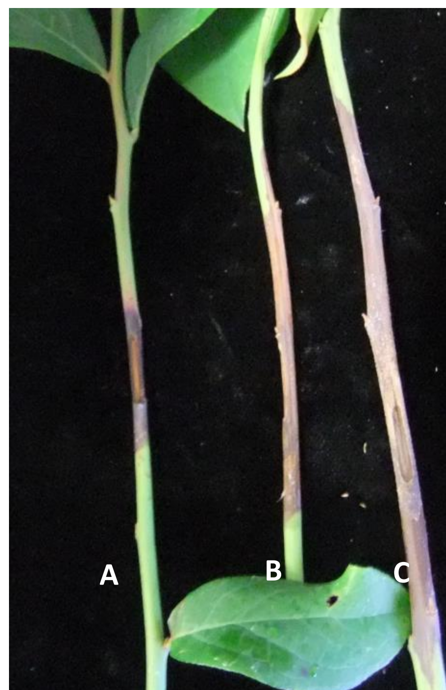


Figure 3.8: Lesion development in attached soft green shoots of cultivars A) 'Ocean Blue' B) 'Maru' C) 'Dolce Blue' after inoculation with conidia of *N. parvum*.

3.3.7.2 Fruits, flower buds and leaf buds

On fruits, both *Neofusicoccum* species caused necrotic lesions on wounded berries within 4-8 days and non-wounded berries within 10-12 days (Figure 3.9) followed by mummification of the berries after a further 14-25 days. Infection incidence was not significantly affected by cultivars (*P*=0.081; Appendix C.8.A), although there was a trend that 'Blue Bayou' (16.7%) and Ocean blue (17.1%) had lowest infection incidence and 'Dolce Blue' (38.7%) had highest infection incidence (Table 3.13). Infection incidence was not significantly affected by species (*P*=0.271). The overall infection incidence for *N. parvum* was 21.7% and for *N. ribis* 26.2%. There was a significant effect of wounding (*P*=0.005; Appendix C.8.A), with overall infection incidence for non-wounded fruits and wounded fruits being 18.1 and 29.8% respectively. There was no significant interaction between cultivar, species and wounding (*P*= 0.669; Appendix C.8.A). For flower buds, both *Neofusicoccum* species caused necrotic light brown lesions (Figure 3.9) on wounded buds within 3-4 days and non-wounded buds within 3-6 days. Infection incidence was significantly affected by cultivars (*P*=0.009; Appendix C.8.B), with cultivar Blue bayou (21.2%) showing lowest infection incidence (*P*<0.05; Table 3.14), which was significantly different (*P*<0.05) from other cultivars. The highest infection incidence was showed by cultivar 'Dolce Blue' (45.0%) which was significantly different from other cultivars (*P*<0.05). Infection incidence was significantly affected by species (*P*< 0.001), with overall infection incidence for *N. parvum* being 24.4% and for *N. ribis* 41.3%. There was a significant effect of wounding (*P*<0.001; Appendix C.8.B), with overall infection incidence for non-wounded flower buds and wounded flower buds being 28.0 and 41.3%, respectively. There was no significant interaction between cultivar, species and wounding (*P*= 0.189; Appendix C.8.B).

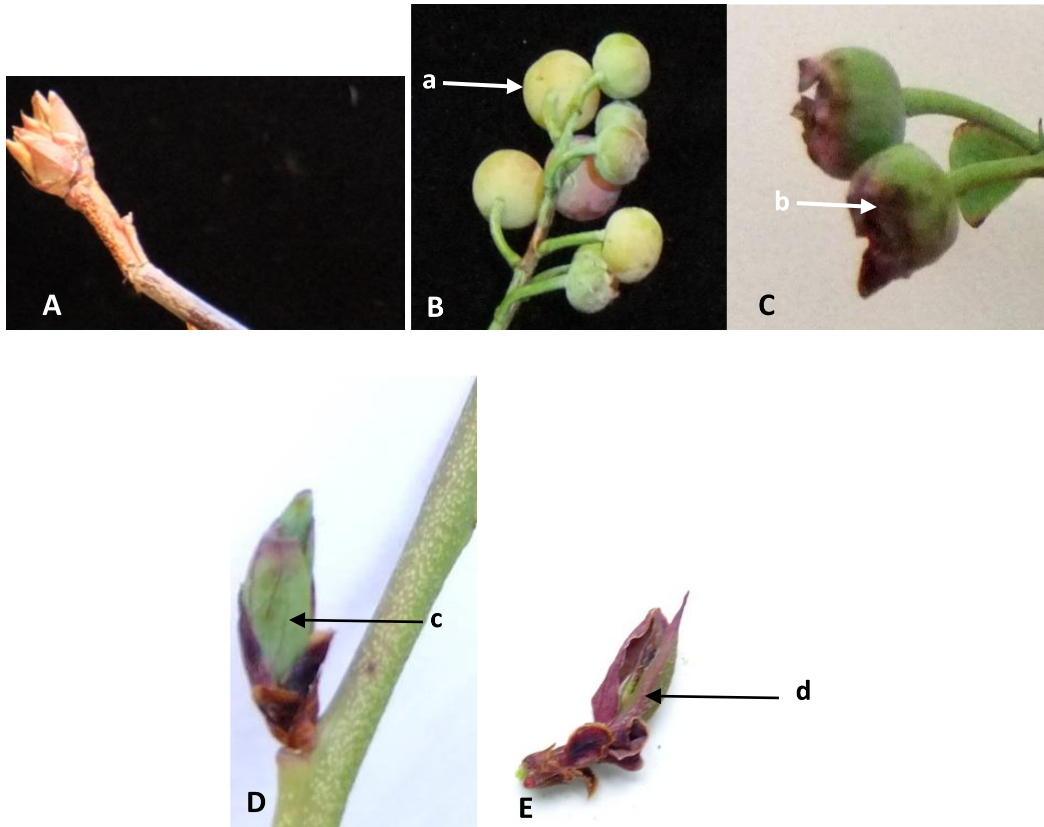


Figure 3.9: Symptoms on different blueberry tissues caused by *Neofusicoccum ribis*. infection A) necrotic flower buds infected by *N. ribis*, B) non-infected fruits denoted by arrow (a), C) infected fruits denoted by arrow (b), D)non-infected leaf bud denoted by arrow (c) and E) infected leaf bud denoted by arrow (d)

Table 3.13: Mean percent infection incidence in non-wounded and wounded fruits of different cultivars inoculated with two *Neofusicoccum* species

Species	*N. parvum*		*N. ribis*		Cultivar mean infection incidence
	Non-wounding	Wounding	Non-wounding	Wounding	
Blue Bayou	21.7	15.0	6.7	23.3	16.7
Centra blue	11.7	36.7	31.7	30.0	27.5
Dolce Blue	21.7	55.0	16.7	61.7	38.7
Maru	15.0	20.0	16.7	38.3	22.5
Ocean Blue	15.0	16.7	16.7	20.0	17.1
Powderblue	16.7	16.7	31.7	25.0	22.5
Rahi	20.0	21.7	11.7	36.7	22.5
Species mean	17.4	26.0	18.8	33.6	

There was no significant species effect (*P*=0.271) and cultivar effect (*P*=0.081). There was a significant wounding efect (*P*=0.005) and no significant interaction of cultivar × species × wounding (*P*=0.669)

Table 3.14: Mean percent infection incidence in non-wounded and wounded flower buds of different cultivars inoculated with two *Neofusicoccum* species

Species	*N. parvum*		*N. ribis*		Cultivar mean infection incidence
	Non-wounding	Wounding	Non-wounding	Wounding	
Blue Bayou	18.3	25.0	16.7	25.0	21.3 a[1]
Centra Blue	10.0	20.0	51.7	65.0	36.7 bc
Dolce Blue	26.7	45.0	40.0	68.3	45.0 c
Maru	15.0	38.3	30.0	60.0	35.8 bc
Ocean Blue	20.0	23.3	35.0	48.3	31.7 ab
Powderblue	21.7	26.7	41.7	63.3	38.3 bc
Rahi	20.0	31.7	45.0	38.3	33.8 bc
Species mean	18.8	30.0	37.2	52.6	

[1]Values within the columns followed by the same letter are not significantly different according to Fisher's protected LSD at *P*=0.05. There was a significant species effect (*P*<0.001) and cultivar effect (*P*=0.009; LSD= 11.72). There was a significant wounding efect (*P*<0.001) and no significant interaction of cultivar × species × wounding (*P*=0.189; LSD= 23.45)

On leaf buds, both *Neofusicoccum* species caused necrotic lesions (Figure 3.9) on wounded buds within 3-4 days and on non-wounded buds within 5-6 days. Infection incidence was significantly affected by cultivars (P<0.001; Appendix C.8.C), with the cultivar Blue bayou (9.2%) showing lowest infection incidence and 'Dolce Blue' (38.0%) showing highest infection incidence (Table 3.15). The infection incidence was significantly affected by species (P<0.001), with overall infection incidence for *N. parvum* being 15.4% and for *N. ribis* 32.4%. There was a significant effect of wounding (P=0.005; Appendix C.8.C), with overall infection incidence for non-wounded leaf buds and wounded leaf buds being 19.9 and 27.9%, respectively. There was no significant interaction between cultivar, species and wounding (P= 0.051; Appendix C.8.C). Fungal colonies characteristic of the inoculated isolates were recovered from the lesion edges of all the above inoculated tissues. No *Neofusicoccum* isolates were recovered from control tissues on which no necrotic lesions developed.

Table 3.15: Mean percent infection incidence in non- wounded and wounded leaf buds of different cultivars inoculated with two *Neofusicoccum* species

Species	*N. parvum*		*N. ribis*		Cultivar mean infection incidence
	Non-wounding	Wounding	Non-wounding	Wounding	
Blue bayou	8.3	3.3	11.7	13.3	9.2 a[1]
Centra blue	8.3	16.7	38.3	56.7	30.0 cd
Dolce Blue	13.3	38.3	31.7	51.7	33.8 d
Maru	11.7	31.7	23.3	46.7	28.3 bcd
Ocean Blue	15.0	11.7	25.0	23.3	18.8 ab
Powderblue	18.3	6.7	18.3	45.0	22.1 bc
Rahi	15.0	16.7	40.0	28.3	25.0 bcd
Species mean	12.8	17.9	26.9	37.9	

[1]Values within the columns followed by the same letter are not significantly different according to Fisher's protected LSD at P=0.05. There was a significant species effect (P<0.001) and cultivar effect (P<0.001; LSD= 10.41). There was a significant wounding efect (P=0.005) and also significant interaction of cultivar × species × wounding (P=0.051; LSD = 20.83).

3.4 Discussion

In the pathogenicity assay using mycelia on detached soft green and semi hard green shoots, isolates of *N. ribis* and *N. parvum* were found to be most pathogenic (longest lesions) followed by *N. luteum* and *N. australe*. Similar results were reported by Espinoza *et al.* (2009) who studied the pathogenicity of *N. arbuti*, *N. australe* and *N. parvum* isolates on two year old detached blueberry shoots of cultivars 'Brigitta' and 'O'Neal' using mycelial agar plugs. Their results showed that *N. parvum* was the most virulent species followed by *N. australe,* and the least virulent was *N. arbuti*. In contrast Amponsah *et al.* (2011) reported that in grapevines *N. luteum* was the most pathogenic species on detached and attached shoots and trunks, followed by *N. australe* and *N. parvum*. Studies by van Niekerk *et al.* (2004) using mycelium inoculum to infect detached green shoots of 'Perquita' grapevines showed that *N. australe* and *N. parvum* were the most pathogenic species, however, for detached mature canes of 'Chardonnay' and 'Cabernet Sauvignon' *N. ribis* was the most pathogenic followed by *N. australe*, and least pathogenic were *N. luteum* and *Lasiodiplodia theobromae.* This variation might be due to a number of experimental factors, including cultivar or isolate differences, or the maturaty stage of the host tissue.

In the current study, there was some variation in pathogenicity between isolates of the same species. Variation in pathogenicity of isolates of a Botryosphaeriaceae species was also reported by Cresswell and Millholand (1987) who investigated the susceptibility of three blueberry cultivars to 11 isolates of *Fusicoccum aesculi* which they subsequently placed into two virulence groups. In a pathogenicity study conducted by Milholland (1971b), using 18 blueberry cultivars and six isolates of *F. aesculi,* all the highbush cultivars were found to be highly susceptible to all six isolates, but the rabbiteye cultivars were susceptible to only five of the six isolates tested. However in that paper they did not mention the identification procedure used for these isolates, and as molecular identification had not been developed at that time the identity of the isolates may not have been confirmed. Espinoza *et al.* (2009) studied the pathogenicity of three isolates of *N. parvum* as mycelial inoculum on detached stems of blueberry cultivar 'O'Neal' and showed that the pathogenicity of two isolates was similar with regards to lesion lengths produced, whereas one isolate produced longer lesions. In the same study, the pathogenicity of three blueberry isolates each of *N. arbuti* and *N. parvum* as mycelial inoculum were tested on apple and

kiwifruits. In apple fruits, isolates of *N. arbuti* produced similar and smaller lesions than isolates of *N. parvum* which generally produced larger lesions which differed in length between isolates. In kiwifruit, isolates of both species caused lesions of different lengths.

Variation in pathogenicity among isolates of Botryosphaeriaceae species on other crops has also been reported. In New Zealand, Amponsah *et al.* (2011) found that the species effects were not clear-cut as the variation among isolates of the three most pathogenic species placed them in some statistically similar groups. The greatest isolate variation was observed for *D. mutila,* with one isolate producing long lesions similar to those of *N. parvum* and the other two isolates producing very short lesions. A susceptibility study conducted by Billiones-Baaijens *et al.* (2013), using 114 Botryosphaeriaceae grapevine isolates which represented seven species, showed that for *N. luteum* and *N. parvum* the lesion lengths produced on excised green shoots were statistically different. In subtropical regions of eastern Australia, Savocchia *et al.* (2007) studied the pathogenicity of two isolates of *N. luteum* and six isolates of *D. seriata* isolated from declining grapevines using excised grapevine shoots. Results showed that all the isolates were pathogenic on green shoots. However, one isolate of *N. luteum* was highly pathogenic and produced longer lesions compared to the other *N. luteum* isolate. In *D. seriata* inoculated shoots one isolate produced much longer lesions compared to the other five isolates which also varied in their pathogenicity.

The pathogenicity and virulence of the different isolates may be related to the host-pathogen responses since plants invaded by pathogenic or non-pathogenic organisms tend to accumulate phytoalexins that inhibit the growth of microorganisms (Albersheim and Valent, 1978). Production of phytoalexins in the plants is known to be triggered by the elicitors of the microorganisms, however Albersheim and Valent (1978) stated that plants were unlikely to recognize elicitors of every strain of a bacterial or fungal species and virus, and so the defensive responses varied. Martos *et al.* (2008) studied the phytotoxic metabolites of grapevine isolates of *F. aesculi*, *D. seriata*, *D. viticola*, *N. luteum* and *N. parvum in vitro* and showed that although all the fungi tested were capable of producing phytotoxic metabolites, they had variable levels of activity. Moreover, *N. parvum* and *N. luteum* produced high levels of phytotoxic metabolites, which they concluded could be involved with virulence in grapevines. However no work has been published about the

phytotoxic metabolites produced by Botryosphaeriaceae species associated with dieback of blueberries, which needs to be studied to improve understanding of the isolate variation in virulence.

Other metabolites may also affect the pathogenicity of these pathogens. Dekker *et al.* (2001) studied the synthesis of enzymes involved in the degradation of starch and lignocellulose in a Botryosphaeriaceae sp. [later identified as *B. rhodina,* which is the teleomorph of *L. theobromae* (Giese *et al.* 2005)]. This species was grown in starch, pectin, cellulose or xylan in which it produced amylase, pectinase, cellulase, xylanase and lacase. McClendon *et al.* (1960) reported the production of pectinases and cellulases by *N. ribis* and Saldanha *et al.* (2007) the production of laccase and pectinase from one isolate of *N. ribis* and eight isolates of *L. theobromae,* which belonged to three RAPD genetic groups. For the *L. theobromae* isolates, they showed that production of laccase varied significantly between isolates, which demonstrated a strong relationship with genetic groups but such a relationship was not found with pectinase production of the different isolates. They also showed that the one isolate of *N. ribis* produced the highest level of pectinase compared to the eight *L. theobromae* isolates. Baskarathevan (2011) did a preliminary study on laccase production by three *N. parvum* isolates and showed that *N. parvum* produced both types of laccase (PPO-I and PPO-II) and that the amount produced varied between the isolates. They also reported that there was no relationship between the virulence of the isolates and the production of laccase. These studies have shown that *Botryosphaeriacea* species and isolates produced different levels of cell wall degrading enzymes that are associated with invasion of different hosts. However the roles of the different enzymes in infection of blueberries needs further studies with different Botryosphaeriaceae species and isolates. Since more than one enzyme may be involved with the virulence of the isolates, multiple isolates and enzymes need to be studied to provide explanations for isolate variations in virulence.

All the isolates tested in the current study were more pathogenic on soft green shoots than on hard green shoots for both attached and detached shoots. Similar results were reported by Milholland (1971b), who showed that attached succulent stems of blueberry cultivar Berkley inoculated with *F. aesculi* conidia produced longer lesions 14 days after wounding and inoculation than in 1 year old stems. Cresswell and Milholland (1987) also reported that

three isolates of *F. aesculi* produced longer lesions in attached succulent stems than in woody stems of blueberry cultivars 'Harrisons', 'Powderblue' and 'Murphy' 5 weeks after inoculation. Similarly in grapevines *N. luteum* and *D. seriata* produced longer lesions on one year old canes than on lignified shoots and trunks of the grapevine cultivar 'Chardonnay' (Savocchia *et al*., 2007).

Most of the studies on the pathogenicity of Botryosphaeriaceae in blueberries have been conducted using mycelial agar plugs. Since conidia are believed to cause much of the natural infection, it was considered important to develop a method to produce conidia in order to assess their pathogenicity. The method developed by Amponsah *et al* (2008) using grapevine green shoots was successfully modified using blueberry tissue with the four most common *Neofusicoccum* species isolated from blueberries. In the current study, higher numbers of pycnidia and conidia were produced by *N. ribis* followed by *N. parvum*, *N. luteum* and *N. australe*. This was not consistent with the results of Amponsah *et al.* (2008) in grapevines, which tested three isolates each of *N. australe*, *N. luteum*, *N. parvum*, *D. seriata* and *D. mutila*. In that study, no conidia were produced by *N. parvum* isolates and the highest numbers of conidia were produced by *N. luteum* followed by *N. australe* isolates. However, recent New Zealand research (pers. comm. Amna Shafi, 2015) has shown that there was great variation between isolates of *N. parvum* in their ability to produce conidia on grapevine stems, with most of the isolates tested producing very low numbers of conidia while some produced similar numbers to the *N. luteum* isolates.

The results from the current study showed that the most pathogenic isolates (which caused longer lesions) in blueberry stems produced more pycnidia and conidia than the isolates which produced shorter lesions. However this may have been associated with the fact that the experimental tissue from the most pathogenic isolates represented older lesions which were more ready to sporulate. Although this relationship has not been reported for Botryosphaeriaceae species on the many hosts affected, studies with other diseases have shown such effects. Studies done on wheat (*Triticum aestivum* L.) cultivars infected by *Septoria tritici* (Eyal, 1971; Gough and Smith, 1976; Gough 1978) showed that the leaf necrosis area was correlated with the number of pycnidia produced. Also Shaner *et al.* (1975) stated that the number of pycnidia was correlated with the cultivar susceptibility since fewer pycnidia were produced in resistant cultivars than in susceptible cultivars.

Further, Gough (1978) showed that pycnidia of *S. tritici* in wheat leaves of the resistant cultivar ('Oasis') produced significantly lower numbers of spores than the moderately resistant cultivar ('TAM W-101') and susceptible cultivars ('Triumph 64' and 'Improved Triumph'). In the current study, the sporulation experiment showed that the numbers of conidia were not always as large as could have been expected for the large numbers of pycnidia formed on the blueberry tissues. This was in contrast to the study by Amponsah *et al.* (2008) in which a similar experiment with grapevine shoots resulted in a more consistent relationship between numbers of pycnidia produced and conidia released. However Gough (1978) stated that number of spores released from a pycnidium from a host plant may vary widely with respect to the environmental conditions. In the current study, all oozing pycnidia were counted, although the amount of ooze varied greatly, as did the size and colour of the pycnidia. These pycnidia clearly did not release the same number of conidia although some may have released more conidia during the vortexing of shoots with water. Eyal (1971) showed that with *Septoria tritici* infection of wheat there was no correlation between the number of spores within a pycnidium and the volume of the pycnidium. However, this was in contrast to the studies by Gough (1978) who stated that there was a correlation between the number of spores released from a pycnidium and the pycnidial volume. In the current study it was considered possible that not all pycnidia were sufficiently mature for optimum sporulation which could have been achieved with longer incubation. However, the successful production of pycnidia and conidia on blueberry tissues using *Neofusicoccum* species was sufficient for further pathogenicity studies.

In the experiment set up to assess the infection progression in bark and wood of pruned hard shoots, after inoculation with *N. ribis* conidia onto wounded and non-wounded hard green shoot prunings, there was an overall trend of higher infection progression in wounded shoots than in non-wounded shoots. It also showed that pathogen progression increased with incubation period. This experiment therefore showed that prunings could act as sources of inoculum in blueberry farms. Also, since some farms use prunings to propagate new plants these could act as sources of inoculum to introduce the disease to nurseries and farms. Several studies in grapevines have shown that pycnidia of Botryosphariaceae species were associated with canker and dieback found in old pruning wounds, pruning debris and trunks of infected grapevines (van Niekerk *et al*., 2010; Urbez-Toress, 2011). However in the

current study the bark had crinkled with time, even though the conditions were maintained at high relative humidity, therefore it was difficult to observe any lesion development or pycnidia in the bark. This may have been due to the relatively short incubation period; further pathogenicity studies on attached blueberry shoots using the same spore concentration (10^4/mL) showed that lesions took a longer time to develop (nearly 2 months), and that pycnidia were also observed in these lesion areas. Elena and Luque (2015) studied the pruning debris of grapevines as a potential inoculum source of *Diplodia seriata*, casual agent of Botryosphaeria dieback. In that study, pruned canes naturally colonized by *D. seriata* were collected 2 years after pruning and kept outside in a vineyard under natural conditions. Conidial production was monitored at 6, 12, 18 and 42 months. Overall results showed decreasing conidial production per pycnidium and also decreasing viability of conidia throughout the study period. However by 42 months after pruning, viable conidia were still dectected. All these studies showed that pruning debris could act as an important inoculum source in the field.

The pathogenicity assay using conidia on attached soft green and hard green shoots showed that the time taken for the symptom development was greater for the attached than detached shoots, means being 63.1 mm 7 days after inoculation in detached soft green shoots and 61.3 mm after 14 days in attached soft green shoots. For detached hard green shoots, lesions of 20.3 mm length developed after 10 days, whereas in attached hard green shoots lesions of 31.5 mm developed after 60 days. In the current study, this variation could have been affected by the inoculum type used since mycelium agar plugs were used in the detached shoots and conidia in the attached shoots. This could also have been due to plant defence mechanisms being more active in attached shoots than detached shoots. Amponsah *et al.* (2012b) also reported that Botryosphaeria lesions took 10 days to develop on detached grapevine shoots and 60 days on attached shoots. Espinoza *et al.* (2009) reported similar effects on blueberries; two isolates of *N. parvum* inoculated as mycelial agar plugs produced shorter lesions in attached shoots of O'Neal cultivar (13.5 and 20.3 mm lesions within 25 days) and produced longer lesions in detached shoots of the same cultivar (63.9 and 70.7 mm lesions within 25 days). Further, these authors also showed that longer lesions were produced by conidia of the same isolates on attached shoots of the same cultivar (75.0 and 55.0 mm lesions within 25 days). In contrast, Creswell and Milholand

(1987) reported that there was no significant difference between mycelium vs conidial inoculum type in lesion development by a *F. aesculi* isolate tested on three different cultivars ('Bluechip', 'Powderblue', 'Murphy'). The differences in the results reported from the various studies could be due to experimental differences such as conidial numbers, the species used, isolate virulence, cultivar susceptibility and the growth stage of the tissues.

In the pathogenicity assay using conidia of two *Neofusicoccum* species on attached wounded and non-wounded soft and hard green shoots, overall pathogenicity was higher in wounded than non-wounded tissues and higher for *N. ribis* than *N. australe,* which was also shown for the previously discussed experiments. However lesion development differed between the experiments; mean lesion lengths caused by *N. ribis* in soft green shoots in the above experiment was 90.1 mm and 58.8 mm in this experiment. These experiments were carried out in two different seasons, and it is possible that the pathogens were sensitive to the environmental conditions. In non-wounded shoots of both types, *N. australe* produced no lesions of any kind although *N. ribis* produced brown dots at the inoculation point which did not develop into clear lesions. Milholland (1971b) also reported that when attached wounded and non-wounded blueberry succulent stems were inoculated with *F. aesculi* conidia, small slightly raised lesions which failed to develop were observed in non-wounded shoots, whereas wounded shoots produced long lesions. Milholland (1971b) studied the spore germination, penetration, and establishment of *F. aesculi* using non-wounded succulent stems removed from cultivar Berkley plants 2, 6, 24, and 48 hr after inoculation. Results showed that the fungus penetrated only through open stomata on non-wounded stems and although penetration was observed 2 h after inoculation most penetration occurred after 48 h. Further, they stated that after penetration through stomata cells division proliferated only in the layers of cells beneath the epidermis, thereby restricting the fungal growth to the outer portion of the lesion. In contrast, when the fungus had invaded through wounded tissues mycelium grew rapidly through vascular tissue and down the stem. In the current experiment, isolations showed that the pathogens were able to progress endophytically in both tissues, although there was a trend for the pathogen progression to be higher in wounded tissues than in non-wounded tissues. This appears to conflict with the findings of Milholland (1971b) who reported that after infection through stomata the pathogen did not progress beyond the "small raised lesions". However,

different pathogens were used in the two studies. Although the current study showed that the pathogen could infect and develop in both wounded and non-wounded shoots, further studies should be conducted to investigate whether symptoms developed later from the endophytic growth, and the conditions under which this happens.

In the pathogenicity assay using flower buds, fruits, fruit stems, crowns and trunks, all the tissues were susceptible to infection by *N. australe*, *N. parvum* and *N. ribis*. Both wounded and non-wounded fruits and flower buds were infected, with disease symptoms developing for all the species used. Higher infection incidences were observed for wounded fruits and flower buds than non-wounded tissues. Espinoza *et al.* (2009) reported a significant effect of inoculation method (wounded and non-wounded) on size of rot lesions which developed on inoculated fruits of apple and kiwifruit, although no data were shown for the effects of wounding. In the current study, as with previous experiments, *N. ribis* showed highest infection incidence for wounded and non-wounded tissues, followed by *N. parvum* and *N. australe*. The same trends were observed for pycnidial formation when fruits were incubated. However, in the current study pycnidial formation in the fruits was conducted with randomly selected fruits, which were taken without considering whether they were wounded or non-wounded. Therefore no comparison was made between numbers of pycnidia in wounded and non-wounded fruits.

There are no reported studies related to infection of blueberry reproductive structures by Botryosphaeriaceae species. In grapevines, Wunderlich *et al.* (2011) isolated Botryosphaeriaceae pathogens from dormant buds, flowers and berries of grapevines. They also reported that symptomatic berries were soft, oozing juice, covered in mycelial growth and formation of black pycnidia. Steel *et al.* (2007) also recorded isolation of Botryosphaeriaceae species, *Pestalotia* species and *Phomopsis viticola* from grape flowers and berries throughout the growing season, although Botryosphaeriaceae species were isolated in low frequency. When Amponsah *et al.* (2012c) inoculated leaf buds of grapevines with *N. luteum* conidia most of the infected buds failed to burst although some buds developed into asymptomatic shoots, but with internal discolouration which originated from the inoculated point. Further, they demonstrated that inoculation of wounded fruit resulted in rotting of the fruit followed by formation of pycnidia and pathogen progression into the supporting shoots. When the wounded and non-wounded fruits and flower buds of

blueberries were inoculated in the current studies most of the buds and fruits developed discolouration and lesions. Further, fruit infection progressed downwards into the supporting shoots. Also, infection of the wounded stems that supported the fruit led to lesions on the stems followed by fruit rot which resembled the trend observed in grapevines.

When wounded trunks were inoculated with *Neofusicoccum* species, internal lesions developed similarly in both directions. Castillo-Pando *et al.* (2001) also stated that *F. aesculi* could spread in both directions within the xylem of grapevine trunks. However in grapevine, Amponsah *et al.* (2012c) showed that upward movement in the grapevine trunks by *N. luteum* was greater than the downward movement. These diferences in results may be attributed to the different hosts and species used. In the current studies, disease development assessed by isolation in blueberry crowns also showed that *N. ribis* progressed most quickly, followed by *N. parvum* and *N. australe,* with all species being able to move from the crown to the hard woody roots. The survey of New Zealand blueberry farms by Sammonds *et al* (2009) reported that *Botryosphaeria* - like isolates were recovered from crowns (70% of incidence) and from the roots of the symptomatic tissues (data was not presented). However, Amponsah *et al.* (2014) found that when grapevine fine roots were inoculated with conidia and mycelium of *N. luteum*, *N. australe*, *N. parvum* and *D. mutila* they were not able to recover any of the isolates from the inoculated roots after 3 months. It therefore seemed likely that the root infection observed in the study, by Sammonds *et al.* (2009) was the result of pathogen progression from the stem, not by direct infection of the roots.

In the susceptibility studies with blueberry plants of seven different cultivars, *N. parvum* and *N. ribis* species were pathogenic to some extent in all the tissue types of all the cultivars and there was 100% infection incidence in all inoculated tissues. However, in hard green shoots, soft green shoots, flower buds and leaf buds, there were significant differences in disease development between cultivars and species. In general, shortest lesions were caused on soft green shoots with cultivar 'Maru', while cultivars 'Centra Blue', 'Maru' and 'Ocean Blue' had shortest lesions in hard green shoots. In leaf buds and flower buds infection incidence was lowest in 'Blue Bayou', followed by 'Ocean Blue', and in fruit all cultivars were similarly

infected. In these tissues, the non-wounded ones were also infected and developed symptoms after inoculation with *N. parvum* and *N. ribis*.

In the current study, six rabbiteye (*Vaccinium ashei*) cultivars and one highbush (*V. corymbosum*) cultivar ('Blue Bayou') were used for pathogenicity studies. They were selected because most blueberry fields in New Zealand were planted with the rabbiteye cultivars, and these were the cultivars available in the nursery that supplied the plants for this study. The availability of data from other studies for comparison of the data was limited. Studies by Che Omar (2009) used mycelial plugs of three *N. parvum* isolates to infect rabbiteye cultivars, using detached soft green shoots of 'Maru' and hard and soft green shoots of 'Centurion'. Results after 7 days showed that lesion lengths on soft green shoots were longer in 'Centurion' (49.0 mm) than in 'Maru' (38.0 mm). On 'Centurion' 7 days after inoculation, lesion lengths in soft green shoots (49.0 mm) were longer than in hard green shoots (34.0 mm) which was similar to the trend found in the current study. However the mean lesion length reported by Che Omar (2009) for *N. parvum* after 7 days was much longer than for the cultivars tested in the current study, in which incubation times were longer, being 60 days for hard and 14 days for soft green shoots. This could be due to the differences in the cultivars used or isolate virulence. However, it is also likely that the differences were due to the fact that her studies were conducted on detached shoots. In the current study attached shoots were used, which had been shown to have slower pathogen progression than detached shoots. The use of detached shoots in pathology studies is very risky as they are physiologically very different from attached shoots consequently the results are frequently very different also.

In the USA, Polashock and Kramer (2006) evaluated disease progression caused by *F. aesculi* on 50 blueberry cultivars, which included half-high, low bush, highbush, southern highbush and rabbiteye cultivars. The stem tips of the second flush of the blueberry plants were inoculated with mycelial plugs of *F. aesculi* and results revealed that half high bush cultivars had shortest lesions followed by lowbush and highbush cultivars. The southern highbush and rabbiteye cultivars had longest lesions. Overall, the mean lesion length observed for rabbiteye cultivars after 4 weeks was 36.5 mm which differed from the current study, the means on soft shoots of rabbiteye cultivars, being 19.6 mm with *N. parvum* and 94.8 mm for *N. ribis* after 14 days. This variation was likely to have been associated with differences in the pathogen species and isolates used. Milholland (1971b) evaluated susceptibility of ten

commercial high bush cultivars and eight rabbiteye cultivars against *F. aesculi*; attached green shoots were inoculated with mycelial plugs of six different *F. aesculi* isolates and lesions were measured 7 and 14 days after inoculation. Results showed significant differences in disease development between cultivars and between isolates, with highbush cultivars generally being highly susceptible. In another study, Creswell and Milholland (1987) evaluated succulent stems of six blueberry cultivars with mycelial plugs of *F. aesculi* isolates and results again showed that high bush cultivars were more susceptible than rabbiteye cultivars. They also inoculated succulent and woody wounded stems of cultivars 'Harrison', 'Murphy' (highbush) and 'Powderblue' (rabbiteye), using conidia (concentration not specified) of three isolates of *F. aesculi.* They found that lesion lengths were greater for succulent stems of cultivars 'Harrison' and 'Powderblue' than 'Murphy', with no difference between cultivars for woody stems. They also reported that succulent stems were more susceptible than woody stems, with mean lesion lengths of 48.0 mm and 21.0 mm, respectively, and concluded that succulent stems, about 3-4 months after new growth occurred, more accurately reflected the susceptibility of the cultivars than older or very young stems. Recently, Espinoza *et al.* (2009) evaluated the susceptibility of seven blueberry cultivars, which included six high bush cultivars 'Brigitta', 'Bluecrop', 'Duke', 'Elliot', 'Misty' and 'O' Neal' and one rabbiteye cultivar 'Brightwell'. The wounded lignified stems were inoculated with mycelial plugs of *N. australe* and *N. parvum* and the lesion length evaluated after 15 days. Results showed that lesion lengths were shortest in 'Misty', 'Blue Crop' and 'Duke' and longest in 'Eliot', and that *N. parvum* was most pathogenic. This experiment was repeated using a conidial suspension (10^6/ mL) of *N. parvum* and results showed a similar trend, except that 'Brigitta' also had shortest lesions.

This pathogenicity study with potted plants has shown that all above-ground parts of blueberries except leaves were susceptible to Botryosphaeriaceae infections. It also demonstrated that *N. ribis* was generally most pathogenic followed by *N. parvum* and then *N. australe,* and the same trend was observed for numbers of conidia produced. Further, inoculation studies with *N. ribis* indicated that wounding was not necessary for infection of shoots, as was also confirmed by bark infection. Since *N. ribis* and *N. parvum* were also able to infect non-wounded flowers, fruits and buds, from which infection may spread into supporting stems, it is clear that in blueberries these Botryosphaeriaceae species are not just wound pathogens as has been shown for other crops. Further, these tissues develop pycnidia and release conidia, therefore providing inoculum in the field and also leading to dieback which is the main form of damage acknowledged by growers.

Chapter 4

Factors that affect disease development and spread

4.1 Introduction

Initial sampling of necrotic blueberry stems from New Zealand farms (Chapter 2) resulted in the isolation of *N. australe*, *N. luteum*, *N. parvum* and *N. ribis,* which indicated a role in the die-back disease that is frequently reported in blueberry farms. Since some Botryosphaeriaceae species can also act as endophytes, becoming saprobic or pathogenic when conditions are favourable (Smith *et al.,* 1996), this indicates the potential for infected plants to act as new inoculum sources when used to establish blueberry farms. In nurseries, Botryosphaeriaceae infections of propagating material may occur both in the field, and during propagation processes as shown in Chapter 2, in which Botryosphaeriaceae species were isolated from asymptomatic nursery plants and propagation material. Van Niekerk *et al.* (2006) also stated that early infections which take place during the propagation of grapevines may stay latent until the plants undergo abiotic or biotic stress. However, the factors that increase the infection and development of Botryosphaeriaceae species in blueberries have not been well studied. Information on host and environmental factors that affect disease development, are essential to ensure the appropriate timing of cultural practices and application of control products to provide effective control.

The objectives of this research were to determine the factors that affected *Neofusiccocum* species infection and disease development, namely i) effect of temperature and relative humidity on conidial (inoculum) production, ii) Effects of conidial numbers on wound infection, iii) effect of wound age on susceptibility of different tissue types, iv) effect of wounding and environmental factors on disease development, v) effect of wounding at different times of the year on susceptibility, and vi) effect of herbicide injuries on infection and disease development[1].

[1]Results from this chapter have been published in a modified form as:-Tenakoon K.M.S. Jaspers, M.V. Ridgway H.J. and Jones E. E. (2015). Herbicide injuries on blueberry provide suitable infection sites for *Neofusicoccum ribis*. New Zealand Plant Protection 68, 411-414.

4.2 Materials and Methods

4.2.1 Effect of temperature and relative humidity on sporulation from lesions

The effect of environmental conditions on inoculum production from stem lesions was determined for *N. australe, N. ribis* and *N. parvum* in December 2014. Soft green shoots of blueberry cultivar 'Dolce Blue' were detached, wounded and inoculated with mycelium plugs of two pathogenic isolates each of *N. australe* [isolates S1-131, S1-174], *N. ribis* [isolates S1-175, S1-158] and *N. parvum* [isolates S1-173, S1-98] and allowed to develop lesions and pycnidia as described in Section 3.2.4. To determine the effect of different relative humidities (RH) and temperatures on numbers of oozing pycnidia and conidia, different saturated salt solutions and water were used to achieve RHs of 80-81% [$(NH_4)_2SO_4$], 92-96% (KNO_3) and 100% (water) (Greenspan, 1977). The saturated salt solutions were prepared and 10 mL poured into separate 25 mL tubes. A filter paper strip was placed into the solution of each tube to act as a wick, to maintain uniform vapour inside the tube. Shoot sections (1 cm), same infection period, with lesions and pycnidia were suspended in each tube on a string (Figure 4.1) and tubes were incubated at 15°C, 20°C, 25°C or 30°C for 24 h. The shoot sections were then evaluated for presence of oozing pycnidia and numbers of conidia as described in Section 3.2.4. There were four replicates for each isolate and RH treatments which were arranged in a CRD for each temperature supplied by individual incubators which had all been set to provide 12 h light and 12 h dark. Data were analysed for each temperature as this factor could not be randomised within the layout.

4.2.2 Effects of conidial numbers on wound infection

The effect of different conidial concentrations of *N. ribis* [isolates S1-175, S1- 158, S1-150] and *N. parvu*m [isolates S1-58, S1-173, S1- 98] on wound infection was determined using two year old potted blueberry plants cultivar 'Dolce Blue' in February 2014. For each of the isolates a 10^7/mL conidial suspension was prepared as described in Section 3.2.5 and these were then mixed in equal volumes to obtain a mixed isolate conidial suspension for each species. A dilution series was then made to obtain suspensions containing $5×10^4$, $1×10^5$, $5×10^5$ and $5×10^6$ conidia/mL.

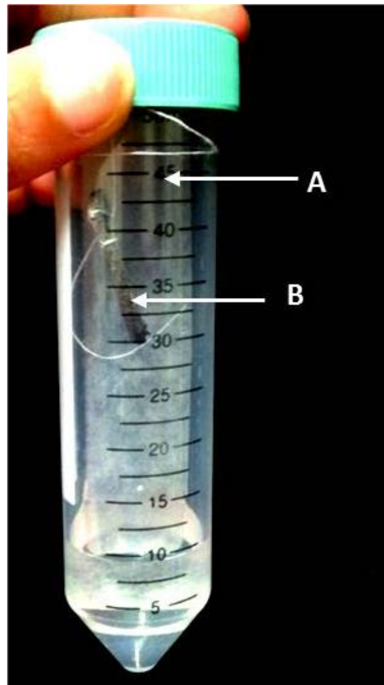


Figure 4.1: Tube setup used to test the effect of relative humidity (RH) and temperature on conidial production from stem lesions. Tube shows saturated salt solutions or water and a filter paper wick (arrow A) which provided a specific RH in the air. A stem piece (arrow B) with a *N. ribis* lesion and pycnidia was tied with a string to allow the stem to be suspended inside the tube.

Hard green shoots and soft green shoots were selected for the inoculation. The hard green shoots and soft green shoots emerging from the main stem were wounded at 10 cm from their bases using a sterile scalpel as described in Section 3.2.6.1. Wounds were immediately inoculated with 50 µL drops of the conidial suspensions and control plants were inoculated with sterile water. Each inoculation point was covered with Parafilm by wrapping it to form a lip which held the solution in place, as described in Section 3.2.6.1. Each plant was then covered with a transparent plastic bag which was sprayed inside with a fine mist of water and left for 48 h to provide humid conditions conducive to spore germination and infection. Ten replicate plants were used for each treatment and two shoots of each type were inoculated on each plant. The plants were arranged in a completely randomised design (CRD) in an open area similar to field conditions at the Lincoln University nursery and the soil watered by hand as required. Lesion lengths were measured with a digital caliper, after 14 days for soft green shoots and after 30 days on the bark of the hard green shoots, and then the bark was removed and the lesions that developed in the wood were measured. Lesion lengths were measured using a digital caliper and the infected tissues were surface

sterilised by dipping for 30 s in 70% ethanol and air dried in a laminar flow cabinet for 10 min. Isolations were made onto PDA using 1 cm stem pieces cut from above and below the inoculation point as described in Section 3.2.3. The plates were incubated in 12 h light and 12 h dark conditions at 25°C for five days and *N. ribis* and *N. parvum* isolates identified by colony appearance.

4.2.3 Effect of wound age on susceptibility of different tissue types

This experiment was conducted during July 2014. Plant tissues of three different phenological development stages were used for the experiment. The soft green shoots, hard green shoots (those emerging from the main stem) and the woody trunk (main stem) of 2 year old blueberry plants cultivar 'Dolce Blue' were wounded at 10 cm from their bases using a sterile scalpel as described in Section 3.2.6.1. To prevent natural infection, plants were kept in a greenhouse where wounds were made and then inoculated after 0, 1, 4, 7, 10, 14 and 28 days. The wounds were inoculated with 50 µL drops of a mixed conidial suspension (10^6/mL) of *N. ribis* [isolates S1-175, S1- 158, S1-150] which was prepared as described in Section 3.2.5. Six plants were used for each treatment and tissue type and two shoots were inoculated on each plant. The controls were inoculated 1 and 28 days after wounding with sterile water. Plants were immediately covered with separate new plastic bags sprayed inside with a fine mist of water and left in place for 48 h. Plants were arranged in a CRD and maintained as described in Section 4.2.2. Lesion lengths were measured using a digital caliper after 14 days on soft green shoots, and after 30 days on hard green shoots and woody trunks. Lengths of stem extending from 5cm below to 5cm above the inoculation point were cut and surface sterilised by dipping in 70% ethanol and air dried, and Isolation onto PDA was conducted as described in Section 3.2.3 using tissue pieces cut at 1 cm intervals, but excluding 1 cm around the inoculation point. The plates were incubated in 12 h light and 12 h dark conditions at 25°C for five days and *N. ribis* isolates identified by colony appearance.

4.2.4 Effect of wounding and environmental factors on infection

The two growth chambers (Conviron PGV36; Controlled Environments Limited), used for this experiment were maintained at 20°C and 25°C. Each chamber was used to provide two relative humidities, 90% and 100%. Although the chambers were able to provide 90%

relative humidity, automatically they could not provide 100%. To achieve that a misting unit was installed which sprayed the chamber walls with fine water droplets for 30 s every hour, providing RH of 99–100%. The relative humidity and temperature were measured using Tinytag® relative humidity (0-95%) and temperature (-40 – +75ºC) data loggers (Gemini Data Loggers, UK). Two year old potted blueberry plants cultivar 'Dolce Blue' were used for this experiment which was conducted in March 2014. Hard green shoots were wounded using a sterile scalpel as described in Section 3.2.6.1 or non-wounded, the inoculation site being marked with a permanent marker pen and wrapped with Parafilm to form a lip as described in Section 3.2.6.1. The sites were immediately inoculated with 50 µL drops of a mixed conidial suspension (10^6/mL) of *N. ribis* [isolates S1-175, S1- 158, S1-150] which was prepared as described in Sections 3.2.4 and 3.2.5 Control plants were inoculated with sterile water. Plants were arranged in a CRD in each growth chamber, for each temperature and RH treatment, and incubated for 48 h. Plants were then transferred into a shade house where they were exposed to natural conditions and the soil watered by hand as required. Six plants were used for each treatment and two shoots were inoculated on each plant. Non-wounded shoots and wounded shoots were observed weekly for disease progression. At 30 days after inoculation, the lesion lengths were measured with a digital caliper as described in Section 3.2.3 and isolations were carried out as described in Section 3.2.5.

4.2.5 Effect of wounding at different times of the year on susceptibility

Potted 2 year old blueberry plants cultivar 'Dolce Blue' were used for this experiment which was conducted from August 2014 to March 2015. Hard green shoots and trunks of the plants were wounded using a sterile scalpel and wrapped with Parafilm to form a lip, then immediately inoculated as described in Section 3.2.6.1 with 50 µL drops of a mixed conidial suspension (10^6/mL) of *N. ribis* [isolates S1-175, S1- 158] prepared as described in Sections 3.2.4 and 3.2.5. The inoculated plants were covered with separate new polythene bags misted inside with water to provide high humidity and left in place for 48 h. Control plants were inoculated with water. Six replicate plants were used for each treatment and two shoots were inoculated on each plant. Treatments were set up at different times of the year, being winter (August), summer (December) and autumn (March). Plants for each season were placed separately in a CRD in a shade house and examined for disease development weekly until 30 days. The external lesion lengths were then measured with a

digital caliper and sequential 1 cm segments cut from 5 cm above to 5 cm below the inoculation point were used for isolations, from the bark and wood separately, as described in Section 3.2.5.

4.2.6 Effect of herbicide injuries on disease development

Blueberry plants, cultivar 'Centurion blue' (10 years old), established in the field at Lincoln University were used for this experiment, which was conducted in November 2014. Three widely used herbicides (Geoff Langford, Pers Comm 2014), glufosinate-ammonium, paraquat+diquat and carfentrazone-ethyl, were applied at the recommended field rates (Table 4.1). The 1-year-old hard green shoots on the outer sides of the bushes, which arose directly from the trunks, were selected for treatment, two shoots per plant. The unbranched lower sections of these shoots were carefully sprayed with a hand-sprayer until covered with fine droplets for a length of 50 cm, beginning 10 cm from the trunk. The treated areas were observed for signs of discolouration 1, 2, 3, 4, 6, 7 and 10 days after spraying. The areas for inoculation (≈1 cm^2) were then marked centrally within the discoloured sections and a Parafilm lip was wrapped around at each site. The marked areas were inoculated as described in Section 3.2.6.1 with 50 µL of a mixed isolate conidial suspension (10^6 conidia/mL) of *N. ribis* (isolates S1-175 and S1- 158) prepared as described in Sections 3.2.3 and 3.2.5. The non-herbicide treated control plants were wounded and inoculated with sterile water as described in Section 3.2.6.1. There were also two plants treated with each herbicide but not inoculated, which provided a check that any developing lesions were caused by the applied pathogen and that the plants were initially free of this pathogen. Each inoculated shoot was covered with a new polythene bag, misted inside with water to provide high humidity and left in place for 48 h. Two shoots were treated on each plant, with six replicate plants for each treatment. Plants were selected for each treatment according to a CRD. For each herbicide, two shoots on one extra plant were inoculated as before to allow for exploratory dissections that would indicate the most appropriate time for assessments. Plants were assessed 30 days after inoculation. The central 21 cm sections of the shoots (from 10 cm below to 10 cm above the inoculation points) were harvested. They were surface sterilised by soaking in 70% ethanol for 30 s and air dried in a laminar flow cabinet for 10 min. The bark was then removed from each section in a single sheet to expose the light brown lesions, which were measured using a digital caliper. Tissue pieces

were cut at 1 cm intervals for isolation. Bark and wood tissues were plated separately onto PDA, with tissue placement on the agar plates indicating their positions on individual shoots as described in Section 3.2.5.

Table 4.1: Details of the herbicides used to create injury to blueberry plants prior to inoculation with *Neofusicoccum ribis* conidia

Active ingredient	Trade name	Chemical class	Manufacturer	Use rate (mL/100 litres)
glufosinate-ammonium	Buster®	Ammonium phosphate	Bayer Crop Science	750
carfentrazone-ethyl	Shark®	Triazoline	FMC	300
paraquat+ diquat	Preeglone®	Bipyridyl	Syngenta	550

4.2.7 Statistical analysis

Data were shown to be normally distributed with Genstat and so raw data were analysed. Data of lesion lengths and pathogen isolation distances, pycnidial and conidial numbers, were analysed by general analysis of variance (ANOVA) using Genstat 16 to determine treatment effects. Comparisons between means of individual treatments used Fisher's protected LSDs at $P \leq 0.05$. Data which included binomial data were analysed by generalized linear mixed model (GLM) using Genstat 16 and SEDs provided to show significance of differences between means.

4.3 Results

4.3.1 Effect of temperature and relative humidity on sporulation from lesions

The effect of relative humidity on the number of oozing pycnidia and released conidia that were produced from stem lesions of three *Neofusicoccum* species was determined separately for each temperature.

15°C

The mean number of oozing pycnidia at 15°C was 74.3. There was no significant effect of relative humidity (RH) on the number of oozing pycnidia (*P*=0.316; Table 4.2.), but the effect of isolate was significant (*P*<0.001; Appendix D.1.A). The highest mean number of oozing pycnidia was from *N. parvum* isolate S1-173 (97.8), followed by *N. australe* isolate S1-131 (95.3), *N. parvum,* S1-98 (93.8) and *N. ribis* S1-175 (83.8) which were not significantly different from each other (*P*>0.05; Table 4.2). The lowest mean number of oozing pycnidia was from *N. ribis* isolate S1-158 (17.3), which was significantly different from all other isolates (*P*<0.05). The interaction between RH and isolates was not significant (*P*= 0.278; Appendix D.1. A1). When the isolates were analysed as replicates of a species, the effect of species was significant (*P*=0.002) with the highest mean number of oozing pycnidia from *N. parvum* (95.8) followed by *N. australe* (76.5) which were not significantly different from each other (*P*>0.05) but were significantly greater (*P*<0.05) than for *N. ribis* (50.5; Table 4.2). The interaction between RH and species was not significant (*P*= 0.642; Appendix D.1. A2). There was no significant effect of relative humidity (RH) on the number of oozing pycnidia (*P*=0.316; Appendix D.1.A2).

The mean number of conidia released at 15°C was 1.4×10^5/mL. The effect of RH on conidial production was not significant (*P*=0.293; Appendix D.1.B) nor was the interaction between RH and isolates (*P*=0.742; Appendix D.1.B). The effect of isolates on number of conidia was significant (*P*=0.015; Appendix D.1.B), with the highest mean number being from *N. parvum* isolate S1-173 (2.5×10^5/mL) followed by *N. parvum* S1-98 (2.4×10^5/mL) and *N. australe* isolate S1-174 (1.8×10^5/mL), which were similar (*P*<0.05) (Table 4.3). The lowest mean number of conidia was from *N. ribis* isolate S1-158 (1.0×10^4 /mL).

Table 4.2: The effect of relative humidities (RH) at 15°C on numbers of oozing pycnidia from 15 mm lengths of shoots infected with different Botryosphaeriaceae species.

RH (%)	*N. australe*			*N. parvum*			*N. ribis*			
Isolates	**S1-131**	**S1-174**	**Means**	**S1-98**	**S1-173**	**Means**	**S1-158**	**S1-175**	**Means**	**Means for RH**
80-81	98.0	74.2	86.1	109.0	128.5	118.8	31.2	64.5	47.9	84.2
92-96	125.0	29.2	77.1	79.0	70.2	74.6	11.0	82.0	46.5	66.1
100	63.0	70.0	66.5	93.2	94.8	94.0	9.5	105.0	57.2	72.6
Means of isolates	95.3 c[1]	57.8 b		93.8 c	97.8 c		17.3 a	83.8 bc		
Means of species	76.6 b			95.8 b			50.5 a			

[1]Values within columns followed by the same letter are not significantly different according to Fisher's protected LSD at *P*=0.05.There was no significant effect of relative humidity (RH) on the number of oozing pycnidia for isolates and species (*P*=0.316 for both). The effects of isolate and species on the number of oozing pycnidia were significant (*P*<0.001; LSD= 34.03 and *P*= 0.002; LSD= 24.06, respectively). The interactions between RH and isolates or species were not significant (*P*= 0.278 and *P*= 0.642, respectively).

Table 4.3: The effect of relative humidities (RH) at 15°C on numbers of conidia (x10^5/mL) released from 15 mm lengths of shoots infected with different Botryosphaeriaceae species.

RH (%)	*N. australe*			*N. parvum*			*N. ribis*			
Isolates	**S1-131**	**S1-174**	**Means**	**S1-98**	**S1-173**	**Means**	**S1-158**	**S1-175**	**Means**	**Means for RH**
80-81	0.6	1.0	0.8	1.8	1.5	1.7	0.1	0.6	0.4	1.0
92-96	0.9	0.7	0.8	2.1	3.5	2.8	0.1	0.4	0.2	1.3
100	0.2	3.6	1.9	3.3	2.4	2.9	0.1	1.5	0.8	1.9
Means of isolates	0.6 ab[1]	1.8 bcd		2.4 cd	2.5 d		0.1 a	0.9 abc		
Means of species	1.2 a			2.5 b			0.5 a			

[1]Values within columns followed by the same letter are not significantly different according to Fisher's protected LSD at *P*=0.05.There was no significant effect of relative humidity (RH) on the number of released conidia for isolates and species (*P*=0.293 for both). The effects of isolate and species on the number of released conidia were significant (*P*= 0.015; LSD= 1.61 and *P*= 0.004; LSD= 1.14, respectively). The interactions between RH and isolates or species were not significant (*P*= 0.742 and *P*= 0.363, respectively).

When the isolates were analysed as replicates of a species, there was no significant effect of RH on conidial production (*P*=0.293; Appendix D.1.B) nor a significant interaction between RH and species (*P*=0.363; Appendix D.1.B). There was a significant effect of species on conidial production (*P*=0.004; Appendix D.1.B). Overall the greatest mean number of conidia was from *N. parvum* (2.5×10^5/ mL) which was significantly different (*P*<0.05; Table 4.3) from *N. australe* (1.2×10^5/ mL) and *N. ribis* (5.0×10^4/ mL), which were not significantly different from each other (*P*>0.05; Table 4.3).

20°C

The mean number of oozing pycnidia at 20°C was 84.2. There was no significant effect of relative humidity (RH) on the number of oozing pycnidia (*P*= 0.673; Appendix D.1.C). The effect of isolate on the number of oozing pycnidia was significant (*P*<0.001; Appendix D.1.C). The highest mean number of oozing pycnidia were from *N. parvum* isolates S1-98 (122.0) and S1-173 (115.4) which was significantly higher (*P*<0.05; Table 4.4) than from all the other isolates except *N. ribis* isolate S1-175 (86.8) which was not different (*P*>0.05) from *N. parvum* S1-173. The lowest mean number of oozing pycnidia was from *N. ribis* isolate S1-158 (40.8) which was not significantly different (*P*>0.05) from *N. australe* S1-131 but was significantly different (*P*<0.05; Appendix D.1.C) from all the other isolates. The interaction between RH and isolates was significant (*P*= 0.006; Appendix D.1.C). The highest mean number of oozing pycnidia was from *N. parvum* isolate S1-173 at 92-96% RH (166.0 pycnidia) which was significantly different (*P*<0.05) from all other isolates at the different RHs. The lowest mean numbers of pycnidia were produced by *N. ribis* isolates S1-158 (22.5) and *N. australe* S1-174 (27.5) at 100 and 92-96% RH, respectively, which were not significantly different from each other but significantly different from all the other isolates (*P*<0.05). When the isolates were analysed as replicates of a species, there was no significant effect of relative humidity (RH) on the number of oozing pycnidia (*P*=0.673; Appendix D.1.C). The effect of species was significant (*P*<0.001; Appendix D.4.C). The highest mean number of oozing pycnidia was from *N. parvum* (118.7) which was significantly different (*P*<0.05; Table 4.4) from both *N. ribis* (63.8) and *N. australe* (70.0), which were not significantly different (*P*>0.05) from each other. There was a significant interaction (*P*<0.001) between RH and species. The highest number of oozing pycnidia was from *N. parvum* at 92-96% RH (154.9), which was significantly different from other treatments. This was followed by *N. australe* at 100% RH (111.1) which was significantly different from other treatments, whereas *N. australe* also had the lowest number of oozing pycnidia (35.3) at 92-96% RH.

The mean number of conidia released at 20°C was 7.0×10^4/mL. The effect of RH on conidial production was not significant (*P*=0.129; Appendix D.1.D) nor was there a significant interaction between RH and isolates (*P*=0.348; Appendix D.1.D). The effect of isolates on number of conidia was significant (*P*< 0.001; Appendix D.1.D). The highest mean numbers of conidia were from *N. parvum* isolates S1-98 and S1-173 (1.7 and 1.6×10^5 /mL, respectively)

which were not significantly different (*P*>0.05) from each other (Table 4.5) but which were significantly different (*P*<0.05) from all other isolates.

Table 4.4: The effect of relative humidities (RH) at 20°C on numbers of oozing pycnidia from 15 mm lengths of shoots infected with different Botryosphaeriaceae species.

RH (%)	*N. australe*			*N. parvum*			*N. ribis*			Means for RH
Isolates	S1-131	S1-174	Mean	S1-98	S1-173	Mean	S1-158	S1-175	Mean	
80-81	38.0	89.5	63.8	120.2	86.8	103.5	49.0	89.8	69.4	78.9
92-96	43.0	27.5	35.2	143.8	166.0	154.9	50.8	74.0	62.4	84.2
100	108.0	114.3	111.1	102.0	93.5	97.8	22.5	96.5	59.5	89.5
Means of isolates	63.0 ab[1]	77.1 b		122.0 d	115.4 cd		40.8 a	86.8 bc		
Means of species	70.0 a			118.7 b			63.8 a			

[1]Values within columns followed by the same letter are not significantly different according to Fisher's protected LSD at *P*=0.05.There was no significant effect of relative humidity (RH) on the number of oozing pycnidia for isolates and species (*P*=0.679 for both). The effect of isolate and species on the number of oozing pycnidia was significant (*P*< 0.001; LSD= 33.63 and *P*<0.001; LSD= 23.78, respectively). The interaction between RH and isolates or species was significant (*P*= 0.006; LSD= 58.25 and *P*< 0.001; LSD= 41.19, respectively).

Table 4.5: The effect of relative humidities (RH) at 20°C on numbers of conidia (10^5/mL) released from 15 mm lengths of shoots infected with different Botryosphaeriaceae species.

RH (%)	*N. australe*			*N. parvum*			*N. ribis*			Means for RH
Isolates	S1-131	S1-174	Means	S1-98	S1-173	Means	S1-158	S1-175	Means	
80-81	0.1	0.5	0.3	1.8	2.3	2.0	0.1	0.8	0.4	0.9
92-96	0.1	0.2	0.1	1.6	2.2	1.9	0.1	0.7	0.4	0.8
100	0.1	0.2	0.2	1.8	0.4	1.1	0.1	0.2	0.1	0.5
Means of isolates	0.1 a[1]	0.3 a		1.7 b	1.6 b		0.1 a	0.6 a		
Means of species	0.2 a			1.7 b			0.3 a			

[1]Values within columns followed by the same letter are not significantly different according to Fisher's protected LSD at *P*=0.05.There was no significant effect of relative humidity (RH) on the number of released conidia for isolates and species (*P*=0.129 for both). The effect of isolate and species on the number of released conidia was significant (*P*< 0.001 with LSDs= 0.67 and 0.82, respectively). The interaction between RH and isolates or species was not significant (*P*= 0.348 and *P*= 0.552, respectively).

When the isolates were analysed as replicates of a species, there was no significant effect of RH on conidial production (*P*=0.129; Appendix D.1.D) nor was there a significant interaction between RH and species (*P*=0.552; Appendix D.1.D). The effect of species on number of conidia was significant (*P*< 0.001), with highest mean number from *N. parvum* (1.7 ×

10^5/mL) which was significantly different (*P*<0.05) from both *N. australe* (2.0×10^4/mL) and *N. ribis* (3.0×10^4/mL), which were not significantly different from each other (*P*>0.05).

25°C

The mean number of oozing pycnidia at 25°C was 93.6. There was a significant effect (*P*<0.001; Appendix D.1.E) of RH on the number of oozing pycnidia, with the highest mean number (133.8) at 100% RH, which was significantly higher (*P*<0.05) than that at 92-96% and 80-81% RH (67.8 and 79.3, respectively), which did not differ significantly (*P*>0.05; Table 4.6) from each other. The effect of isolates on the number of oozing pycnidia was also significant (*P*<0.001; Appendix D.1.E). The highest mean numbers of oozing pycnidia were from *N. parvum* isolates S1-175 (148.2) and S1-158 (146.4) which were not significantly different (*P*>0.05) from each other. The lowest number of oozing pycnidia was from *N. australe* isolate S1-131 (46.3) which was not significantly different (*P*>0.05) from *N. australe* S1-74 (57.2) or *N. ribis* S1-173 (71.2). The interaction between RH and isolates was significant (*P*=0.018; Appendix D.1.E), which appeared to be associated with the greater effects of RH on *N. parvum* isolates than on *N. australe* and *N. ribis* isolates. The highest mean number of oozing pycnidia was produced by *N. ribis* isolate S1-175 at 100% RH (187.5) which was significantly different (*P*<0.05) from all the other isolates. The lowest mean number of oozing pycnidia was produced by *N. australe* isolate S1-131 at 80-81% RH (23.2) followed by *N. ribis* isolate S1-173 at 80-81% (23.5) RH and *N. australe* isolate S1-174 at 92-96% RH (24.2) which were not significantly different (*P*>0.05) from each other. When the isolates were analysed as replicates of a species, the effect of RH on the number of oozing pycnidia was significant (*P*<0.001; Table 4.6) as was the effect of species (*P*<0.001). The highest number of oozing pycnidia was from *N. parvum* (147.3) which was followed by *N. ribis* (81.9) which were significantly different (*P*<0.05). The lowest was produced by *N. australe* (51.8) which was significantly different (*P*<0.05) from the other species. There was a significant interaction (*P*<0.001) between RH and species, which appeared to be associated with the different effects of RH on *N. parvum* isolates compared to *N. australe* and *N. ribis* isolates (Table 4.6). The highest mean number of oozing pycnidia was produced by *N. parvum* (171.8), at 80-81% RH which was significantly different from other treatments (*P*<0.05; Table 4.6). The lowest mean number of oozing pycnidia was produced by *N. ribis* (30.0) at

80-81% RH followed by *N. australe* at 92-96% RH (33.4) and at 80-81% RH (36.0), which were not significantly different (P>0.05) from each other.

Table 4.6: The effect of relative humidities (RH) at 25°C on numbers of oozing pycnidia from 15 mm lengths of shoots infected with different Botryosphaeriaceae species.

RH (%)	*N. australe*			*N. parvum*			*N. ribis*			Means for RH
Isolates	S1-131	S1-174	Means	S1-98	S1-173	Means	S1-158	S1-175	Means	
80-81	23.2	48.8	36.0	171.5	172.0	171.8	23.5	36.5	30.0	79.3 a[1]
92-96	42.5	24.2	33.4	124.0	117.5	120.8	45.0	53.8	49.4	67.8 a
100	73.0	98.8	85.9	143.8	155.0	149.4	145.0	187.5	166.2	133.8 b
Means of isolates	46.2 a	57.2 ab		146.4 c	148.2 c		71.2 ab	92.6 b		
Means of species	51.7 a			147.3 c			81.9 b			

[1]Values within columns followed by the same letter are not significantly different according to Fisher's protected LSD at P=0.05.There was a significant effect of relative humidity (RH) on the number of oozing pycnidia for isolates and species (P<0.001for both with LSDs= 27.93 for both). The effect of isolate and species on the number of oozing pycnidia was significant (P< 0.001 for both with LSDs= 39.50 and 27.93, respectively). The interaction between RH and isolates or species was significant (P= 0.018; LSD= 68.42 and P<0.001; LSD= 48.38, respectively).

The mean number of conidia released at 25°C was 1.5×10^5/mL. The effect of relative humidity on conidial production was significant (P=0.005; Appendix D.1.F). The highest mean number of conidia was at 100% RH (2.1×10^5 /mL) which was significantly higher (P<0.05; Table 4.7) than at 92-96% (1.0×10^5 /mL) and 80-81% RH (1.4×10^5 /mL), which did not differ significantly (P>0.05) from each other. The effect of isolates was significant (P<0.001), the highest mean number of conidia being produced by *N. parvum* isolate S1-98 (4.0×10^5 /mL) which was not significantly different (P>0.05) from other isolates (Table 4.7). The lowest mean number of conidia was produced by *N. australe* isolate S1- 131 (3.0×10^4 /mL) which was significantly different (P<0.05) from *N. australe* isolate S1-174 (1.0×10^5 /mL) and *N. ribis* isolate S1-158 (9.0×10^4/mL). There was no significant interaction between RH and isolate (P=0.064). When the isolates were analysed as replicates of a species, the effect of species was significant (P=0.005; Appendix D.1.F), with the highest mean number being produced by *N. parvum* (2.8×10^5/mL) which was significantly higher (P<0.05) than for *N. ribis* (1.1×10^5/mL) and *N. australe* (7.0×10^4/mL), which were not significantly different from each other (P>0.05; Table 4.7). There was a significant effect of relative humidity on the number of released conidia (P= 0.005). There was a significant interaction between RH and species (P=0.017). Overall, the highest mean number of conidia was produced by *N. parvum* (3.5×10^5/mL*)* at 80-81% RH which was significantly different from the other

treatments (*P*<0.05; Table 4.7). The lowest mean number of conidia was produced by *N. australe* (2.0 × 10^4/mL) at 92-96% RH followed by *N. ribis* at 80-81% RH (4.0 × 10^4/mL) and 92-96% RH (5.0 × 10^4/mL), and *N. australe*, (4.0 × 10^4/mL) at 80-81% RH ,which were not significantly different (*P*>0.05) from each other.

Table 4.7: The effect of relative humidities (RH) at 25°C on numbers of conidia (10^5/mL) released from 15 mm lengths of shoots infected with different Botryosphaeriaceae species.

RH (%)	***N. australe***			***N. parvum***			***N. ribis***			
Isolates	**S1-131**	**S1-174**	**Mean**	**S1-98**	**S1-173**	**Mean**	**S1-158**	**S1-175**	**Mean**	**Means for RH**
80-81	0.2	0.6	0.4	4.7	2.3	3.5	0.2	0.5	0.4	1.4 a[1]
92-96	0.3	0.2	0.2	3.9	0.8	2.3	0.5	0.5	0.5	1.0 a
100	0.6	2.1	1.3	3.3	2.0	2.7	1.8	2.9	2.4	2.1 b
Means of isolates	0.3 a	1.0 ab		4.0 c	1.7 b		0.9 ab	1.3 b		
Means of species	0.7 a			2.8 b			1.1 a			

[1]Values within columns followed by the same letter are not significantly different according to Fisher's protected LSD at *P*=0.05.There was a significant effect of relative humidity (RH) on the number of released conidia for isolates and species (*P*= 0.005 for both with LSDs= 0.65 for both). The effect of isolate and species on the number of released conidia was significant (*P*< 0.001 for both with LSDs= 0.92 and 0.65, respectively). The interaction between RH and isolates or species was significant (*P*= 0.064; LSD= 1.59 and *P*=0.017; LSD= 1.12, respectively).

30°C

The mean number of oozing pycnidia at 30°C was 104.6. There was a significant effect (*P*<0.001; Appendix D.1.G) of RH on the number of oozing pycnidia. The highest mean number of oozing pycnidia was produced at 100% RH (128.0) which was significantly higher (*P*<0.05; Table 4.8) than at 92-96% RH (92.9) and 80-81% RH (93.0) which were not significantly different (*P*>0.05) from each other. The effect of isolates on the number of oozing pycnidia was also significant (*P*<0.001). The highest mean numbers of oozing pycnidia were from *N. parvum* isolates S1-98 (158.2) and S1- 173 (141.6) which were not significantly different from each other (*P*>0.05). The lowest mean number of oozing pycnidia was from *N. ribis* isolate S1-158 (36.2) which was significantly different (*P*>0.05) from all other isolates. The interaction between RH and isolates was significant (*P*<0.001; Appendix D.1.G). The highest mean numbers of oozing pycnidia were produced by *N. parvum* isolates S1-173 and S1-98 at 100% RH which were not significantly different (*P*>0.05) from each other. The lowest mean number of oozing pycnidia was produced by *N. ribis* isolate S1-158 (16.5) at

100% RH which was significantly different (P<0.05) from all other isolates. When the isolates were analysed as replicates of a species (Appendix D.1.G), there was a significant effect (P<0.001) of RH on the number of oozing pycnidia (Table 4.8). The effect of species on the number of oozing pycnidia was significant (P<0.001). The highest mean number of oozing pycnidia was from *N. parvum* (149.9) which was followed by *N. australe* (108.8) which were significantly different (P<0.05). The lowest mean number was produced by *N. ribis* (55.3) which was significantly different (P<0.05) from the other species. The interaction between RH and species was significant (P<0.001). For *N. parvum,* RH of 100% resulted in the highest mean number of oozing pycnidia (218.5) which was significantly different from all other treatments. The lowest number of oozing pycnidia was from *N. ribis* at 92-96% RH (38.9) which was significantly different from all the other treatments (P<0.05).

Table 4.8: The effect of relative humidities (RH) at 30°C on numbers of oozing pycnidia from 15 mm lengths of shoots infected with different Botryosphaeriaceae species.

RH (%)	*N. australe*			*N. parvum*			*N. ribis*			
Isolates	**S1-131**	**S1-174**	**Mean**	**S1-98**	**S1-173**	**Mean**	**S1-158**	**S1-175**	**Mean**	**Means for RH**
80-81	95.5	99.5	97.5	128.0	72.7	100.4	72.8	89.8	81.2	93.0 a[1]
92-96	112.2	105.7	109.0	138.2	123.5	130.9	19.2	58.5	38.9	92.9 a
100	125.0	114.5	119.7	208.5	228.5	218.5	16.5	74.8	45.6	128.0 b
Means of isolates	110.9 c	106.6 c		158.2 d	141.6 d		36.2 a	74.3 b		
Means of species	108.8 b			149.9 c			55.2 a			

[1]Values within columns followed by the same letter are not significantly different according to Fisher's protected LSD at P=0.05.There was a significant effect of relative humidity (RH) on the number of oozing pycnidia for isolates and species (P<0.001for both with LSDs= 16.86 for both). The effect of isolate and species on the number of oozing pycnidia was significant (P< 0.001 for both with LSDs= 23.85 and 16.86, respectively). The interaction between RH and isolates or species was significant (P< 0.001 for both with LSDs= 41.31 and 29.21, respectively).

The mean number of conidia released at 30°C was 2.5×10^5/mL. The effect of RH on conidial production was not significant (P= 0.443; Appendix D.1.H), but there was a significant effect of isolates (P<0.001) on numbers of conidia. The highest mean number of conidia was produced by *N. parvum,* isolate S1-98 (4.1×10^5/mL) which was significantly different (P<0.05; Table 4.9) from all other isolates. The lowest numbers of conidia were produced by *N. ribis* isolates S1-158 (1.4×10^5/mL) and S1-98 (1.4×10^5/mL), and *N. australe* isolate S1-174 (1.9×10^5/mL) which were not significantly different (P>0.05) from each other. There was no significant interaction between RH and isolates (P=0.132). When the isolates were analysed as replicates of a species (Appendix D.1.H), there was no significant effect of RH on

conidial production (*P*=0.443; Appendix D.1.H) or a significant interaction between RH and species (*P*=0.246; Appendix D.1.H). There was a significant effect of species (*P*<0.001) on conidial production. Overall the highest mean number of conidia was from *N. parvum* (3.8 × 10^5/mL) which was significantly different (*P*<0.05) from both *N. australe* (2.2× 10^5/mL) and *N. ribis* (1.4 × 10^5/mL), which were not significantly different (*P*>0.05) from each other.

Table 4.9: The effect of relative humidities (RH) at 30°C on numbers of conidia (10^5/mL) released from 15 mm lengths of shoots infected with different Botryosphaeriaceae species.

RH (%)	*N. australe*			*N. parvum*			*N. ribis*			
Isolates	**S1-131**	**S1-174**	**Mean**	**S1-98**	**S1-173**	**Mean**	**S1-158**	**S1-175**	**Mean**	**Means for RH**
80-81	1.4	2.0	1.7	5.4	2.9	4.1	1.1	2.7	1.9	2.6
92-96	3.4	2.1	2.7	3.8	5.0	4.4	1.2	0.6	0.9	2.7
100	2.5	1.8	2.1	3.0	2.9	2.9	1.9	0.8	1.4	2.1
Means of isolates	2.4 ab	1.9 a		4.1 c	3.6 bc		1.4 a	1.4 a		
Means of species	2.2 a			3.8 b			1.4 a			

[1]Values within columns followed by the same letter are not significantly different according to Fisher's protected LSD at *P*=0.05.There was no significant effect of relative humidity (RH) on the number of released conidia for isolates and species (*P*= 0.443 for both with LSDs= 0.92 for both). The effect of isolate and species on the number of released conidia was significant (*P*< 0.001 for both with LSDs= 1.30 and 0.92, respectively). The interaction between RH and isolates or species was not significant (*P*= 0.132; LSD= 2.25 and *P*=0.246; LSD= 1.59, respectively).

4.3.2 Effects of conidial numbers on wound infection

All the plants produced light brown to black lesions in the inoculated shoots for all conidial concentrations tested. No lesions were produced in the non-inoculated control shoots and these were not included in the statistical analysis. On soft green shoots, there was a significant effect of species (*P*=0.002; Appendix D.2.A) on lesion lengths, with mean lesions produced by *N. ribis* (61.8 mm) being significantly longer (Table 4.10) than those produced by *N. parvum* (44.7 mm). There was a significant effect of conidial concentrations (*P*<0.001; Appendix D.2.A) on lesion length. The longest mean lesion (91.2 mm) was produced on shoots inoculated with the highest concentrations of 5 × 10^6 conidia/mL, which differed between *N. parvum* and *N. ribis,* being 69.2 and 113.1 mm (Table 4.10), respectively. Inoculation with the lowest concentrations of 5 × 10^4 conidia/mL resulted in the shortest mean lesion of 23.0 mm for *N. parvum,* while for *N. ribis* lesions were similar for 5 × 10^4 conidia/mL and 1 × 10^5 conidia/mL, resulting in mean lesion lengths of 39.7 and 37.9 mm, respectively.

Table 4.10: Effect of conidial concentrations (conidia per mL) of *N. parvum* and *N. ribis* on mean lesion lengths (mm) which were visible on soft green shoots and the hard green shoots (outer bark and underlying wood) when attached to the blueberry plants.

	Soft green shoots			Hard green shoots					
				Bark			Wood		
Conidial conc.	***N. parvum***	***N. ribis***	**Mean lengths (mm)**	***N. parvum***	***N. ribis***	**Mean lengths (mm)**	***N. parvum***	***N. ribis***	**Mean lengths (mm)**
5×10^4	23.0	39.7	31.3 a[1]	19.6	26.7	23.1 a	22.5	40.1	31.3 a
1×10^5	39.8	37.9	38.9 ab	29.2	34.8	32.0 b	32.2	48.1	40.1 b
5×10^5	46.7	56.5	51.6 b	34.2	43.2	38.7 c	32.7	47.2	40.0 b
5×10^6	69.2	113.1	91.1 c	34.3	46.2	40.3 c	35.4	50.9	43.2 b
Species mean	44.7	61.8		29.3	37.7		30.7	46.6	

[1]Values within columns followed by the same letter are not significantly different according to Fisher's protected LSD at P=0.05.There was a significant species effect for all the treatments (P=0.002 for soft shoots; P<0.001 for both bark and wood of hard shoots) and a conidial concentration effect (P<0.001; LSD=14.59 for soft shoots, P<0.001; LSD=4.90 for bark and P=0.003; LSD=6.17 for wood of hard shoots). There was a significant interaction between species and conidial concentration for soft shoots (P=0.021; LSD = 20.63) but not for bark (P=0.605; LSD =6.93) and wood (P=0.968; LSD =8.73) of hard shoots.

The significant interaction (*P*=0.02; Appendix D.1.A) between species and conidial concentration was associated with greater increase in lesion length by *N. ribis* than *N. parvum* with increasing conidial concentration (Table 4.10). For the pathogen inoculated plants infection incidence was 100% and was 0% for controls.

On hard green shoots there was a significant effect of species (*P*<0.001; Appendix D.2.B) on lesion development, with mean lesion length produced by *N. ribis* (37.7 mm) being significantly longer than for *N. parvum* (29.3 mm). There was a significant effect of conidial concentrations (*P*<0.001; Appendix D.2.B) on lesion length. The highest concentration of 5×10^6 conidia/mL produced the longest mean lesion (40.3 mm) with means for *N. parvum* and *N. ribis,* being 34.3 and 46.2 mm*,* respectively, (Table 4.10). Inoculation with the lowest concentrations of 5×10^4 conidia/mL resulted in the shortest mean lesion (23.2 mm), with mean lesions of 19.6 and 26.7 mm for *N. parvum* and *N. ribis,* respectively. The interaction between species and conidial concentration was not significant (*P*=0.605; Appendix D.2.B).

When the bark was removed from the hard green shoots light brown discolouration was observed in the wood due to the infection (Figure 4.2 E and F). There was a significant effect of species (*P*<0.001; Appendix D.2 .C) on lesion length, with mean lesion length produced by *N. ribis* (46.6 mm) being significantly longer than for *N. parvum* (30.7 mm). There was a significant effect of conidial concentrations (*P*=0.003; Appendix D.2.C) on lesion length. The longest mean lesion (41.0 mm) was produced on shoots inoculated with the highest concentrations of 5×10^6 conidia/mL, means being 35.4 and 46.6 mm for *N. parvum* and *N. ribis,* (Table 4.10), respectively. Inoculation with 5×10^4 conidia/mL resulted in the shortest mean lesion, means being 31.3 mm overall, and 22.5 and 40.1 mm for *N. parvum* and *N. ribis,* respectively. The interaction between species and conidial concentration was not significant (*P*=0.968; Appendix D.2.C). For the pathogen inoculated plants infection incidence was 100% and was 0% for controls, for bark and wood.

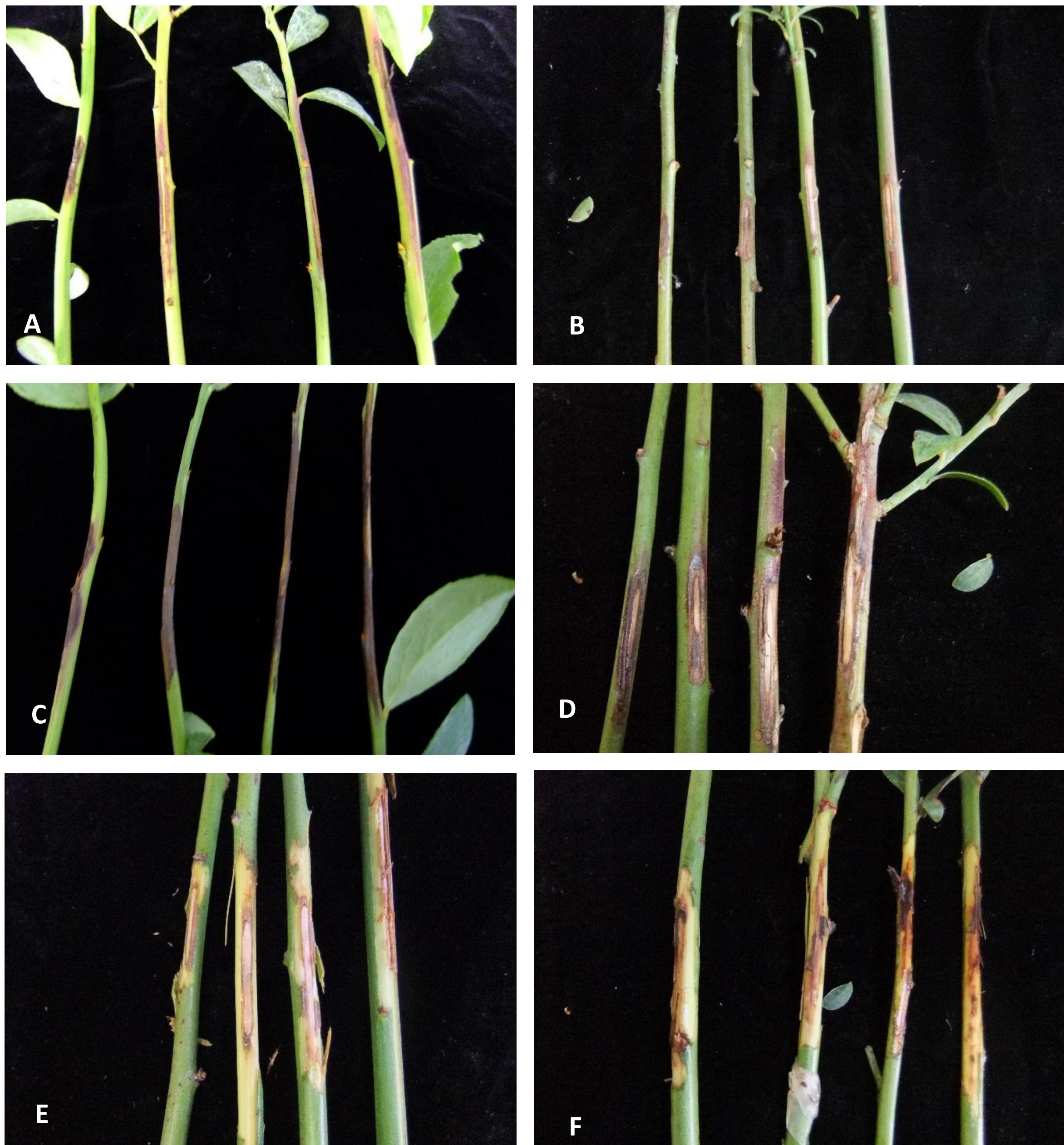


Figure 4.2: Lesions developed in different tissues after inoculation with different concentrations of conidia of *Neofusicoccum* species. Shoots were placed according to ascending order of conidial concentrations (5×10^4, 1×10^5, 5×10^5 and 5×10^6 conidia/mL). A) soft green shoots inoculated with *N. parvum,* B) hard green shoots inoculated with *N. parvum,* C) soft green shoots inoculated with *N. ribis,* D) hard green shoots inoculated with *N. ribis,* E) wood lesions caused by *N. parvum,* and F) wood lesions caused by *N. ribis*.

4.3.3 Effect of wound age on susceptibility of different tissue types

On soft green shoots, lesions started to develop in fresh wounds and one day old wounds within 3-4 days after inoculation with *N. ribis.* However, inoculated 4 and 7 day old wounds took 10-14 days for lesions to be clearly visible and no lesions appeared on older wounds. No lesions developed on the non-inoculated wounded control treatments. There was a significant effect of wound age (for 0, 1, 4 and 7 day old wounds) on lesion length ($P< 0.001$; Appendix D.3.A), with mean lesion lengths for fresh wounds (103.3 mm) being similar to those produced from 1 day old wounds (88.3 mm) and significantly longer ($P<0.05$; Table 4.11) than 7 day old wounds (24.3 mm), which were significantly different from 4 day old wounds (57.4 mm) ($P<0.05$). On soft green shoots there was a significant effect of wound age on pathogen movement ($P<0.001$; Appendix D.4.A). The mean pathogen movement in the fresh wounds and 1 day old wounds (96.7 mm for both) were significantly greater ($P<0.05$; Table 4.11) than for all the other wound ages (16.7-61.7 mm). The lowest mean pathogen movement (16.7 mm) observed in 28 day old wounds was significantly different from all other times except 7 and 14 day old wounds ($P<0.05$; Table 4.11). For the pathogen inoculated plants there was 100% infection incidence and 0% infection incidence for controls.

In hard green shoots and woody trunks, light brown to reddish brown lesions started to develop within 7 to 10 days on inoculated fresh and 1 day-old wounds, and at 14-16 days for 4 day old wounds, but no lesions were observed for wounds greater than 4 days old. On hard green shoots, at 30 days after inoculation, there was a significant effect of wound age (for 0, 1 and 4 day old wounds) on lesion lengths ($P=0.002$; Appendix D.3.B) with the mean lesion length produced from fresh wounds (28.2 mm) being significantly longer ($P<0.05$; Table 4.11) than those produced from wounds of 1 and 4 days old (20.5 and 15.4 mm, respectively), which were not significantly different from each other. There was also a significant effect of wound age on pathogen movement ($P<0.001$; Appendix D.4.B). Mean pathogen movement was not significantly different between the fresh wounds and 1 day old wounds (90.0 and 85.0 mm, respectively), which were significantly greater ($P<0.05$; Table 4.11) than for all the other wound ages (33.3-63.3 mm). The lowest mean pathogen movement (33.3 mm) was observed in 28 day old wounds, which was significantly different from other treatments ($P<0.05$; Table 4.11). For the pathogen inoculated plants there was 100% infection incidence and 0% infection incidence for controls.

Table 4.11: Pathogen movement (mm) and length of lesions (mm) which developed in the soft green and hard green shoots, as well as woody trunks of blueberry plants inoculated with *N. ribis* conidia onto wounds of different ages.

Wound age (days)	Soft green shoots		Hard green shoots		Woody trunk	
	Pathogen progression	Lesion length (mm)	Pathogen progression	Lesion length (mm)	Pathogen progression	Lesion length (mm)
28	16.7 a[1]	NA[2]	33.3 a	NA	35.0 a	NA
14	23.3 ab	NA	46.7 b	NA	40.0 a	NA
10	35.0 b	NA	50.0 b	NA	46.7 a	NA
7	30.0 ab	24.3 a	63.3 c	NA	60.0 b	NA
4	61.7 c	57.4 b	63.3 c	15.4 a	63.3 bc	11.6 a
1	96.7 d	88.3 bc	85.0 d	20.5 a	73.3 cd	16.9 ab
0	96.7 d	103.3 c	90.0 d	28.3 b	81.7 d	22.0 b
LSD	14.51	31.00	12.35	6.18	12.27	5.34

[1]Values within columns followed by the same letter are not significantly different according to Fisher's protected LSD at *P*=0.05. The lesion lengths were significantly affected by wound age for all the treatments (*P*<0.001; LSD=31.00 for soft shoots, *P*=0.002; LSD=6.18 for hard shoots and *P*=0.003; LSD=5.34 for trunks) and the pathogen movement (P<0.001 for all treatments, with LSD s=14.51, 12.35 and 12.27 for soft shoots, hard shoots and trunks, respectively).

[2] NA- No lesions developed to be assessed

On woody trunks, 30 days after inoculation, there was a significant effect of wound age (for 0, 1 and 4 days old wounds) on lesion length (*P*=0.003; Appendix D.3.C). Mean lesion length from fresh wounds (22.0 mm) was significantly longer (*P*<0.05; Table 4.11) than for wounds that were 1 and 4 days old (16.9 and 11.6 mm, respectively), which were significantly different from each other (*P*<0.05; Table 4.11). There was also a significant effect of wound age on pathogen movement (*P*<0.001; Appendix D.4.C). Mean pathogen movement in fresh wounds (81.7 mm) was similar to that in 1 day old wounds (73.3 mm) and significantly greater (*P*<0.05; Table 4.11) than for all the other wound ages (35.0-63.3 mm). The lowest mean pathogen movement was observed in trunks with wounds that were 10, 14 and 28 days old (46.7, 40.0 and 35.0 mm, respectively) which were not significantly different (*P*>0.05) from each other, but significantly lower (*P*>0.05) than for all other treatments. For the pathogen inoculated plants there was 100% infection incidence and 0% infection incidence for controls.

4.3.4 Effect of wounding and environmental factors on infection

In wounded inoculated shoots dark brown lesions developed irrespective of the environmental factors used to incubate the plants after inoculation (Figure 4.3). In the non-wounded tissues brown spot-like lesions appeared which did not develop into clear lesions irrespective of the environmental factors (Figure 4.4).

Figure 4.3: Lesions which developed in hard green shoots on 2 year old blueberry plants incubated under different environmental conditions A) after incubation at 20°C under 90% RH (arrow), B) after incubation at 25°C under 90% RH, C) after incubating at 20°C under 100%, and D) after incubation at 25°C under 100%.

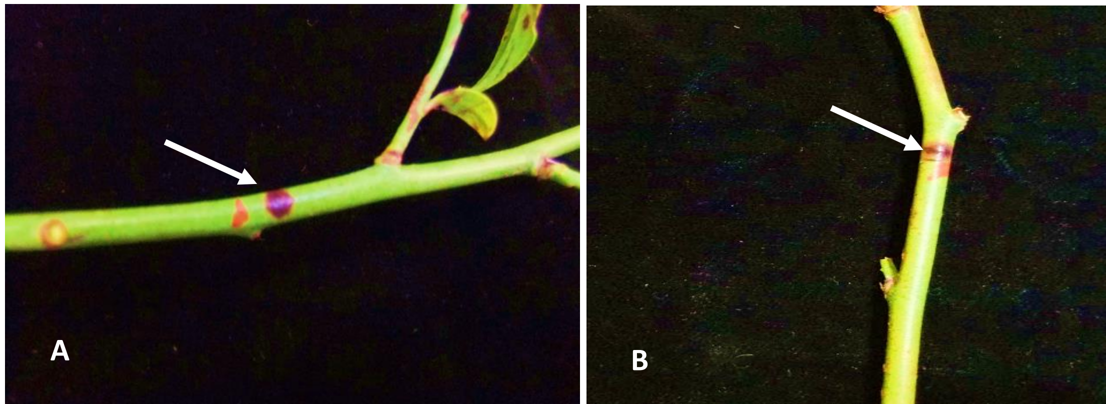


Figure 4.4: Lesions (arrows) which developed in the non-wounded shoots inoculated with *N. ribis* A) after incubation at 20°C under 90% RH, B) after incubation at 25°C under 90% RH.

Therefore the lesion lengths were measured and analysed for only the wounded shoots (Table 4.12). At 20°C there was a significant effect of RH (P<0.001; Appendix D.5.A) on lesion lengths produced, with significantly longer mean lesions at 90% RH (51.5 mm) than that at 100% RH (26.7 mm). At 25°C there was no significant effect of RH (P=0.379; Appendix D.5.B) on lesion lengths produced, with mean lesions of 32.0 and 25.7 mm at 90 and 100% RH, respectively.

For plants incubated at 20°C, sequential isolations showed a significant effect of wounding (P<0.001; Appendix D.5.C) on pathogen movement. Mean pathogen movement was significantly greater for wounded shoots (86.7 mm) than for non-wounded shoots (69.2 mm; Table 4.13). There was a significant effect of RH on pathogen movement (P<0.001; Appendix D.5.C), with significantly greater pathogen movement at 90% RH (91.7 mm) than at 100% RH (64.2 mm). There was also a significant interaction (P=0.002; Appendix D.5.C) between RH and wounding on the pathogen movement, which was associated with variation between wounding effects for the two RHs. Mean pathogen movement at 90% RH was significantly longer for wounded shoots (95.0 mm) than for non-wounded shoots (88.3 mm), but not at 100% RH, with means of 78.0 and 50.0 mm, respectively.

For plants incubated at 25°C, there was a significant effect of wounding (P=0.002; Appendix D.5.D) on pathogen movement, with mean pathogen movement being significantly greater for wounded shoots (70.0 mm) than for non-wounded shoots (52.5 mm; Table 4.13). There was a significant effect of RH on pathogen movement (P<0.001; Appendix D.5.D), with significantly greater pathogen movement at 90% RH (70.8 mm) compared with 100% RH

(51.7 mm). There was no significant interaction (P=0.865) between wounding and RH on the pathogen movement. The infection incidences were 100% for all the pathogen inoculated treatments and 0% for controls. No Botryosphaeriaceae species were recovered from the controls.

Table 4.12: The length of lesions (mm) which developed on wounded blueberry hard green shoots inoculated with *N. ribis* and then incubated at 20°C or 25°C and 90% or 100% relative humidities.

Temperature	Relative humidity		Means for Temperatures
	90%	100%	
20°C	51.5	26.7	39.1
25°C	32.0	25.7	28.9
Means for RHs	41.8	26.2	

At 20°C there was a significant effect of RH on lesion development (P<0.001) but not at 25°C (P=0.3795). There was a significant effect of wounding treatment for the pathogen movement (P<0.001) and also a significant interaction (P=0.002) between RH and wounding on the pathogen movement.

Table 4.13: Pathogen movement in the wounded and non-wounded blueberry hard green shoots inoculated with *N. ribis* and then incubated at 20°C or 25°C and 90% or 100% relative humidities.

Treatment	Pathogen movement (mm)					
	20°C			25°C		
	90% RH	100% RH	Means for wounding	90% RH	100% RH	Means for wounding
Wounded	95.0	78.0	86.5	80.0	60.0	70.0
Non-wounded	88.3	50.0	69.2	61.7	43.3	52.5
Mean for RHs	91.7	64.0	77.9	70.9	51.7	

For plants incubated at 20°C there was a significant effect of wounding treatment for the pathogen movement (P<0.001) and also a significant effect of RH (P<0.001). There was a significant interaction (P=0.002) between wounding and RH on the pathogen movement. For plants incubated at 25°C there was a significant effect of wounding treatment for the pathogen movement (P=0.002) and also a significant effect of RH (P<0.001). There was no significant interaction (P=0.865) between wounding and RH on the pathogen movement.

4.3.5 Effect of wounding at different times of the year on susceptibility

Inoculation of wounded shoots during summer resulted in the development of visible external lesions in hard green shoots and trunks, with means of 50.0 and 70.8 mm, respectively, whilst no external lesions were observed after inoculation in autumn and winter. No lesions were observed and no isolates of Botryosphaeriaceae species were recovered from any of the non-inoculated control shoots or trunks. In hard green shoots, there were no significant effects of tissue type (bark or wood) on infection incidence in all seasons (*P*=0.08 for both summer and autumn and *P*=0.07 for winter; Table 4.14). There was a significant effect of tissue type on pathogen movement in summer (*P*<0.011) and winter (*P*=0.003) but not in autumn (*P*=0.195), with consistently greater pathogen progression in bark than wood (Table 4.15). In trunks there was no significant effect of tissue type on infection incidence (*P*=0.33 for both summer and autumn and *P*=1.00 for winter) or on pathogen movement (*P*=1.00, *P*=0.823 and *P*=0.467 for summer, autumn and winter, respectively). No Botryosphaeriaceae like isolates were recovered from the non-inoculated controls.

4.3.6. Effect of herbicide injuries on disease development

The shoot areas that were sprayed with each herbicide developed dark brown to black discolouration on the bark and the leaves of those shoots became necrotic within 10 days (Figure 4.5). For inoculated shoots, pale brown discolouration was visible under the bark within 14-30 days, but it was not evident in the non-inoculated herbicide controls, even after 30 days. There was no significant effect of treatment (*P*=0.331; Appendix D.7.A) on the mean lesion lengths for the inoculated, herbicide-treated shoots and the wounded, inoculated controls (Table 4.16). All inoculated shoots, whether wounded or treated with one of the herbicides, developed lesions under the bark (100% incidence) but none developed in the non-inoculated shoots whether wounded or treated with herbicide (0% incidence). Isolations made from bark and wood showed continuous colonisation from the inoculation point. There was no treatment effect on the pathogen progression in the bark (*P*=0.200; Appendix D.7.B) and wood (*P*=0.194; Appendix D.7.C) (Table 4.16).

Table 4.14: Mean pathogen incidence in the bark and wood of wounded hard green shoots and trunks inoculated with *Neofusicoccum ribis* conidia in different seasons. Data are logit transformed and back transformed.

	Summer				Autumn				Winter			
	shoots		trunk		Shoots		trunk		shoots		trunk	
	bark	wood	bark	wood	bark	wood	bark	wood	bark	wood	bark	wood
Logit values	0.00	-1.10	0.00	-0.34	0.00	-1.10	0.00	-0.34	0.00	-2.06	-0.34	-0.34
Back transformed Values (%)	100.0	50.0	100.0	83.3	100.0	50.0	100.0	83.3	83.3	17.0	83.3	83.3

For hard green shoots, the infection incidences were not significant in all seasons (*P*=0.08for both summer and autumn and *P*=0.07for winter). In trunks the effect of tissue type on infection incidences were not significant (*P*=0.33; SED=0.33 for both summer and autumn and *P*=1.00; SED=0.49 for winter).

Table 4.15: Mean pathogen movement (mm) in the bark and wood of wounded hard green shoots and trunks inoculated with *Neofusicoccum ribis* conidia in different seasons.

	Summer				Autumn				Winter			
	shoots		trunk		shoots		trunk		shoots		trunk	
	bark	wood	bark	wood	Bark	wood	bark	wood	bark	wood	bark	wood
Pathogen movement (mm)	46.7	15.0	61.7	61.7	51.7	30.0	60.0	56.7	18.3	1.7	30.0	23.3

For hard green shoots, the effect of tissue type on pathogen movement was significant in summer (*P*<0.011) and winter (*P*=0.003) but not in autumn (*P*=0.195). In trunks the effects of tissue type on pathogen movement were not significant (*P*=1.00, *P*=0.829 and *P*=0.467, respectively) for summer, autumn and winter.

Table 4.16: The mean lengths of lesions below the bark (mm) and mean pathogen progression (mm) in bark or wood from 20 cm sections of 1-year-old hard green blueberry shoots treated with different herbicides or a wounded control, at 30 days after inoculation with *Neofusicoccum ribis* conidia.

Herbicide	Lesion (mm)	Bark (mm)	Wood (mm)
glufosinate-ammonium	11.7	18.3	19.0
paraquat+ diquat	8.8	17.3	18.0
carfentrazone-ethyl	7.3	16.0	15.0
Control (wounded, inoculated)	16. 5	14.3	15.0

Treatment effects on lesion length and pathogen progression on the bark and wood were not significant (*P*=0.331 lesion length, and pathogen progression for bark *P*=0.200 and wood *P*=0.194).

Figure 4.5: Discolouration observed in shoots of blueberry plants treated with herbicide A) discoloured branch (arrow) treated with herbicide Glufosinate-ammonium, B) shoot (arrow a) showing discolouration of the bark after treating with herbicide, and lesion which developed on the wood (arrow b) infected with *N. ribis*.

4.4 Discussion

The current study is the first in blueberries to show the effect of humidity and temperature on the release of Botryosphaeriaceae conidia from pycnidia under laboratory conditions, and therefore the influence of environmental factors on abundance of conidia in the environment. The results showed that all the species were capable of producing oozing conidia but that there were some differences between species. Overall, *N. parvum* produced the greatest numbers of oozing pycnidia and conidia under the relative humidities and temperatures used, followed by *N. australe* and *N. ribis*. There was variation in number of oozing pycnidia and conidia among the isolates in each species, which was also described in Chapter 3. Overall, the greatest numbers of oozing pycnidia and conidia were observed at higher temperatures (25-30°C) and RHs (92-100% RH). There was no increase in the numbers of conidia associated with increasing numbers of oozing pycnidia as expected for 100% RH at 30°C for *N. parvum* isolates. This anomaly was also found in Chapter 3 where it was considered likely that insufficient numbers of mature conidia may have been the cause.

The trend for greater conidial release with higher temperatures and RH observed in the current study has also been reported for Botryosphariaceae species in blueberries and other crops. Creswell and Milholland (1988) also reported that *F. aesculi* conidia were present throughout the year in the blueberry fields in North Carolina. The numbers of conidia trapped in rain water positively correlated to rain fall amount, temperature and relative humidity, and the abundance of conidia was low when the temperature and rainfall were low during winter. Amponsah *et al.* (2009) studied the abundance of Botryosphariaceae spores using rainwater run-off traps in Canterbury vineyards in New Zealand and showed that conidia were abundant throughout the year and most abundant during the summer when the temperatures were high. Kuntzman *et al.* (2009) also reported that in a French vineyard, *D. mutila* and *D. seriata* conidia were released throughout the year with peak release occuring during spring, summer and autumn. Further, conidial release could occur in mild and wet spells in winter, with *D. seriata* conidia being trapped at low temperatures, even as low as to 9°C. However, in a New Hampshire (USA) apple orchard, Holmes and Rich (1970) who studied the factors affecting release and dissemination of conidia of *Physalospora obtusa*, later renamed *Diplodia seriata* by Phillips *et al.* (2007), found that most spores were released over the

temperature range 6°C to 16°C when relative humidity was high. Gough (1978), showed that for *Septoria tritici* on wheat twice as many spores were released from pycnidia produced in a growth chamber as were released from pycnidia produced in a greenhouse. Gough (1978) concluded that this difference could be due to one or more environmental factors such as light quality, humidity, leaf water potential and temperature. Therefore experiments which aim to determine the factors affecting sporulation should incorporate these other factors. Also, field experiments which monitor temperature and relative humidity within blueberry canopies and assess the production of pycnidia and oozing conidia should also be conducted to allow for a comparison of field and laboratory results. However, the factors that are favourable for pycnidial oozing of conidia may not be the same as for the infection processes of the pathogen.

Most of the published research studies on Botryosphaeriaceae disease in blueberry have evaluated pathogenicity, cultivar susceptibility and control methods using artificial inoculation using mycelial plugs (Milholland 1972; Creswell and Milholland, 1987; Espinoza *et al*., 2009; Smith, 2009 and Lattorre *et al*., 2013). However, because the current studies were intended to investigate the factors affecting natural infection conidia were used. An experiment was conducted to investigate effects of conidial concentrations on pathogenicity, and to ensure that an optimal dose was used in future experiments. All concentrations caused 100% incidence but lesion lengths increased with increasing concentrations. Also lesion lengths were longer in soft green shoots than in the hard green shoots, and there was a trend for longer lesions in the wood than in the bark of the hard green shoots. Similar effects on lesion length were observed by Cresswell and Milholland (1987) who inoculated 2 year old blueberry plants of three cultivars (‘Bluechip’, ‘Powderblue’ and ‘Murphy’) with conidia of *F. aesculi*, using from 1×10^3 to 5×10^4 conidia per inoculation site which caused lesions of increasing length. Amponsah *et al.* (2014) also demonstrated a similar effect on detached soft green shoots of grapevines which were incubated for 10 days under laboratory conditions. All the conidial loads of *N. luteum* (2 to 2×10^4 per wound site) tested caused 100% infection incidence, but increasing conidial concentrations caused significantly longer lesions, which ranged from 8 to 42 mm. However, when Elena *et al.* (2005) assessed the effect of the inoculum dose of *D. seriata* on cane pruning wounds of potted grapevines maintained outdoors, with doses ranging from 10 to 4000 conidia

per wound, isolations showed increasing incidence of 10-100% for the increasing conidial concentrations. Overall they concluded that in order to obtained 50-70% recovery of the inoculated pathogen, dose rates of 100-1000 conidia of *D. seriata* per wound were required, which concurs with the current study since as few as 250 conidia per wound site caused 100% incidence. Espinoza *et al.* (2009) used 1.5×10^4 conidia of *N. parvum* per wound site for their pathogenicity studies on detached 2 year old blueberry stems, which was similar to the higher concentrations tested in the current study. They reported consistent isolation of the pathogen and lesions of 44-96 mm after 25 days which was approximately 1.3 to 3.0 times the lesion lengths found in the current study using attached hard green shoots after 30 days. However, the differences might have been due to the lack of defence response in detached shoots compared to those in attached shoots, physiological differences between attached and detached shoots and other environmental factors etc. Further, their stems were incubated in a humid chamber at 20°C, rather than the outdoor environment used in the current study, so this may have increased pathogen development within the tissues. Several studies on grapevines have showed that the establishment of the pathogen in a wound depends on several factors, including environment, cultivar, age of the plant, virulence of the isolates and experimental conditions (van Niekerk *et al.,* 2004; Sosnowski *et al.,* 2007 and Serra *et al.,* 2008).

In the experiment conducted to evaluate the effect of wound age on infection processes, external lesions developed in soft green shoots only when wounds were inoculated at up to 7 days old. In hard green shoots and trunks, external lesions developed only in wounds up to 4 days old. Also there was a decrease in the pathogen movement with increasing wound age. A similar effect was also reported by Creswell and Milholland (1987) from their studies on blueberries when they inoculated 0, 1, 7, 14 and 28 day old of wounds with 4×10^4 conidia of *F. aesculi* per wounding site and assessed them 4 weeks after inoculation. There was a decrease in the infected proportion in the shoots with increasing wound age. Sakamoto and Gordon (2006) studied the effect of wound age (0, 7, 14, 21 and 28 days) on infection of pruned branches of Monterey pines (*Pinus radiata*) by *Fusarium circinatum*. Infection frequency and recovery of the pathogen was assessed 10-12 weeks after inoculation. The results suggested that the susceptibility of pruning wounds to infection, decreased

with increasing wound age since freshly cut and 7 day old wounds were the most frequently infected.

Biggs (1986) conducted an investigation into the effects of wounding on infection of peach bark by *Cytospora leucostoma* and concluded that decrease in infection with wound age was related to wound healing processes; lignification and formation of lignosuberized tissues decreased the rate of colonization rather than prevented colonization, which was achieved by a periderm at least three cells thick. In the current study, an improved understanding of the susceptibility period of blueberry wounds will help to identify the most effective application timing of fungicides. Based on the results of the current study, it seems that fungicides should be applied soon after the pruning and trimming of blueberry shoots and would need to be reapplied at least once to protect wounds from Botryosphaeriaceae infection.

An experiment was conducted to evaluate the effect of environmental factors namely temperature and humidity and wounding on infection. Results showed that wounded shoots started to develop lesions irrespective of the environmental factors used, when incubations conditions were 20 or 25°C and 90 or 100% RH during the early infection processes. In non-wounded shoots only spot-like lesions were observed, also irrespective of environmental factors. Similar results were reported by Milholland (1971b) in which wounded leaf axils, shoot tips, succulent stems, in 1 year old stems and non-wounded succulent stems of blueberry cultivar 'Berkley' were inoculated with a conidial suspension (10^5/mL) of *F. aesculi* and placed in a moist chamber at 25-30°C for 48 h, before being transferred to a greenhouse under natural light at 25-30°C for 12 days. Results showed that stem blight developed on all the wounded stems, but in non-wounded stems only small slightly raised lesions developed, similar to those in the current study. The 20 mm lesions which developed in the wounded shoots were smaller than in the current study, however these differences may be due to the species, cultivar, temperature or incubation conditions used in the current study. For an examplein the study of Milholland (1971b) there was no mention of the relative humidity used, whereas in the current study the relative humidity was controlled to investigate its effects on the infection process.

Amponsah *et al.* (2014) further studied the effect of tissue wetness duration for *N. luteum* infection progression in grapevines. Results showed similar infection incidences

on wounded shoots provided with up to 24 h further surface wetness and those held for 0–24 h in 95 % RH. However in that study, the incubation conditions during the following 3 months of incubation appeared to affect levels of germination and pathogen progression since the maximum distances of pathogen isolation were longer for plants given longer wetness periods, but only for those at 95 % relative humidity and not those placed in 78 % RH. This showed the holding humidity after establishment will affect the pathogen spread internally.

The experiment conducted to observe the effect of wounding and inoculation with *N. ribis* in different seasons indicated that plants could be infected in all the seasons tested, with an overall mean incidence of 77.8%. However, external lesions were observed only in summer and no lesions were observed in the shoots that were wounded and inoculated in the autumn and winter even though isolates were recovered. Overall pathogen progression was also lower in winter-inoculated plants compared to other seasons. Similar observations were reported by Creswell and Milholland (1988) who showed that the majority of 'Bluechip' plants inoculated with *F. aesculi* became symptomatic when inoculated during March to April (spring) when conditions were warm. They also stated that some naturally infected plants were symptomless, possibly due to the infections having occurred earlier in the year or at the time of pruning in late fall or winter. Ferreira (1999) stated that carbohydrate and nitrogen concentrations were greatest in South African grapevines in the winter period of June to August, and higher growth was shown by *E. lata* in the extract obtained from the shoots in August than in June. Although such experiments have not been conducted in blueberry to evaluate the factors such as nutrient availability which may affect pathogen penetration in the plant, similar effects may have occurred in the current study because Botryosphaeriace progression was higher in summer and autumn-pruned shoots than in winter pruned shoots. In the current study, infection incidence and disease progression were also higher in the bark compared to wood of the hard shoots throughout the seasons investigated. This indicated the potential for conidial infection or saprophytic existence in the plant bark at any time of the year, with penetration of the wood later when conditions were favourable. This hypothesis was supported by the studies of Billones-Baaijens *et al.* (2015), who isolated bark and wood separately when they conducted sequential isolations along an entire grapevine cane of several metres. They found that after surface sterilisation few isolates of the

Botryosphaeriaceae were sited within the wood and most in the bark, which suggested that they were latent in surface tissues. Further, their genotyping studies showed that multiple species and genotypes were distributed along the cane bark, with only a few adjacent wood and bark infections being caused by the same genotypes, indicating that wood infection may have originated from the bark. In the current study, inoculation of shoots resulted in lower infection incidence and pathogen progression (means of 66.7% and 27.2 mm, respectively) than in trunks (means of 88.9% and 48.9 mm, respectively). However, inoculation of hard green shoots had caused 100% effective in earlier studies, which indicated low infection success in these studies, but only of the wood, since mean infection incidence in wood was 39.0% and in bark was 94.4%. Since the experiment was conducted over three seasons, it is unlikely that infection of the wood was restricted by lignin compounds as suggested by Munkvold and Marois (1995).

This is the first comprehensive study to investigate whether herbicide injuries in blueberries could provide sites of infection for Botryosphaeriaceae species. In this study, herbicide applied to plant bark caused necrosis of bark. Necrosis in the wood under the bark was caused by only the pathogen and was not visibly in plants treated only with herbicide (non-inoculated). Results showed that the herbicide damaged bark allowed the pathogen to penetrate the tissue, results in a degree of infection was seen in the inoculated controls (mechanical wounds). This phenomenon is highlighted by an incident reported in the Mississippi during the late 1980s (Smith, 1997) when incidence of stem blight in blueberries coincided with injury to the base of young canes caused by contact herbicides such as paraquat. The incidence of stem blight has declined as growers avoided injuring blueberry canes with paraquat. In the current study, the lesion lengths of inoculated plants for both herbicide treated and wounded controls were not significantly different although the results indicated that the damaged bark slowed down the pathogen penetration compared to mechanically damaged control plants. This may be due to the toxic effect of the herbicides on fungi. In the current study, there was an overall trend of pathogen progression being higher with the glufosinate-ammonium treatment in than with paraquat+diquat and carfentrazone-ethyl which were contact herbicides. Buster® (glufosinate-ammonium) is a broad spectrum non-selective contact herbicide, with a limited systemic movement. The results from this experiment indicated that it is important to apply herbicides with

shielded sprayers to prevent damage which may lead to infection by Botryosphaeriaceae species.

This study has provided information on the risk periods for wound susceptibility to Botryosphaeriace infection which will be used to target control strategies such as fungicide and biocontrol products in the research of the next chapter.

Chapter 5

Potential control strategies to protect infection courts

5.1 Introduction

Wounds, especially pruning wounds, are regarded as primary infection sites for Botryosphaeriaceae species which cause cankers and shoot dieback. Eventually infection can progress into the crown killing the entire bush (Milholland, 1995; Polashock and Kramer, 2006). One method of preventing infection is to protect the freshly made wounds, which are the main infection courts. Since Botryosphaeriaceae species also cause canker and dieback on grapevines, and many studies have investigated control measures on this host in different parts of the world. In the Barossa Valley Australia, Pitt *et al.* (2010) applied fungicides to grapevine wounds prior to inoculation with Botryosphaeriaceae species and showed that carbendazim, flusilazole, tebuconazole and fluazinam provided 30-55% protection. The relatively low degree of protection was probably related to the short-term efficacy of the fungicides on wounds, which were later infected by natural inoculum. Kotze *et al.* (2011) showed that grapevine pruning wounds stay susceptible to Botryosphaeriaceae species and to *Phomopsis viticola* infection for at least 3 weeks, which indicates that they need protection for at least that long.

Bester *et al.* (2007) studied ten fungicides *in vitro* for their efficacy on mycelial inhibition of the four most common or pathogenic species of Botryosphaeriaceae in South African grapevines (*D. seriata*, *N. australe*, *N. parvum* and *Lasiodiplodia theobromae*). Results showed that benomyl, tebuconazole, prochloraz manganese chloride and flusilazole were the most effective fungicides. Amponsah *et al.* (2102) studied 14 fungicides *in vitro* for their efficacy on mycelial inhibition and conidial germination inhibition of three pathogenic species of Botryosphaeriaceae in grapevines in New Zealand (*N. australe*, *N. luteum* and *D. mutila*). Results revealed that flusilazole, carbendazim, tebuconazole, prochloraz, procymidone, iprodione and fenarimol were the most effective for mycelial inhibition. For conidial germination inhibition

procymidone, carbendazim, and iprodione, mancozeb and flusilazole were the most effective.

In blueberries, Latorre *et al.* (2013) studied the effectiveness of fungicides and biological control agents as pruning wound-protectants against *N. parvum*. The results obtained from *in vitro* studies showed that mycelium of *N. parvum* was highly sensitive to benomyl, tebuconazole, and iprodione. Pastes (wound protectants) formulated with 0.1% benomyl, 0.5% tebuconazole, and 0.06% iprodione were also evaluated for protection of pruning wounds against *N. parvum* infection on the stems of Duke blueberries under field conditions. The pruning wounds were treated with the pastes and inoculated with mycelial plugs of *N. parvum* after 24 h. They were evaluated for the discolouration developing in the wood from the inoculated point after 18 days as well as re-isolation from the margins of lesions. Benomyl, tebuconazole and iprodione were, in order of efficacy, the most effective fungicide treatments. These fungicides completely prevented the re-isolation of *N. parvum* and provided greater than 90% reduction in development of necrotic lesions. However, the biocontrol products Serenade® MAX (*Bacillus subtilis*) and Vinevax™ (*Trichoderma* species) were not effective, resulting in 100 and 50% re isolation of *N. parvum*, respectively.

Chemical methods of control may have undesirable environmental effects (Bodle *et al.,* 1994; Rayachchetry *et al.,* 1996) whereas biological control methods hold promise of ecological compatibility and economic viability (Balciunas and Center, 1991). Kotze *et al.* (2011) studied the biological protection of grapevine pruning wounds against grapevine trunk disease pathogens *(N. australe*, *N. parvum*, *D. seriata*, *L. theobromae*, *Eutypa lata*, *Phaeomoniella chlamydospora* and *Phomopsis viticola).* Results revealed that the efficacy of the biocontrol agents (*Trichoderma* species products and *Bacillus subtilis*) was in most cases similar or superior to that observed for benomyl. John *et al.* (2004) also showed that *Trichoderma* based treatments protected pruning wounds against *E. lata* with a 67% reduction of pathogen incidence in pruning wounds. Ferreira *et al.* (1991) reported that *Bacillus subtilis* inhibited mycelial growth of *E. lata* by 88% on culture medium and completely prevented infection by the pathogen in grapevine pruning wounds.

In New Zealand, no biocontrol products or fungicides have been evaluated against the Botryosphaeriaceae species which cause infections in blueberries. Fungicides which

had potential to control these fungi, were evaluated for their ability to reduce (i) *in vitro* mycelial growth of the most common, pathogenic *Neofusicoccum* species [*N. australe, N. luteum, N. ribis* and *N. parvum*], and (ii) *in vitro* conidial germination and germ tube elongation of the same species. Bio-control agents were also evaluated for their potential to inhibit *in vitro* mycelial growth of the same *Neofusicoccum* species. Finally field evaluation of the most effective products was carried out.

5.2 Materials and Methods

5.2.1 Effect of fungicides on *in vitro* mycelial growth

Nine fungicides belonging to different chemical groups (Table 5.1) were tested *in vitro* to determine their efficacy for mycelial growth inhibition of three pathogenic blueberry isolates each of *N. australe* [S1-131, S1-174, S1-111], *N. luteum* [S1-142, S1-130, S1-167], *N. ribis* [S1-110, S1-175, S1- 158] and *N. parvum* [S1-58, S1-173, S1- 98].

Each of the fungicides tested was suspended in sterile water and the appropriate amounts added to 50°C PDA which was immediately poured into 90 mm Petri dishes, using a technique which ensured similar amounts (18-19 mL) per dish. The test range of the active ingredient (a.i.) concentrations ranged from 0.001 to 250 mg a.i. /L (Table 5.1) based on the reported activity range for each product. Each fungicide was tested at six different concentrations and control plates of unamended PDA. Each plate was inoculated centrally with a 7 mm diameter mycelial disc cut from the edge of a 4-6-day old culture grown on PDA at 25°C. The three replicate plates for each combination of fungicide concentration and isolate were laid out in a complete randomized design (CRD) and incubated at 25°C in continuous darkness. After 48 h, the mycelial growths were measured with a digital caliper across two perpendicular diameters of each colony and the mean mycelial growth inhibition was calculated as a percentage of the mean diameter of control plates. The percent inhibition was calculated for each isolate and fungicide concentration and its EC_{50} determined as described in Section 5.2.2.

Table 5.1: Fungicides used for *in vitro* testing against mycelial growth, conidial germination and germ tube growth of *N. luteum*, *N. australe*, *N. ribis* and *N. parvum.*

Active ingredient	Trade name	Chemical class	Conc. range for mycelia (mg a.i/L)	Conc. range for conidia (mg a.i/L)	Manufacturer
captan	Captan 800 WG	Cyclic imide	10-250	1-6	Arysta Lifesciences Corporation USA
carbendazim	Protek®	Benzimidazole	0.005-0.3	0.01-0.1	Tapuae partnership, NZ
fludioxonil	Maxim®	Phenylpyrrole	0.001-0.03	1-30	Syngenta Group Company
flusilazole	Nustar®	Triazole	0.1-0.6	0.1-2	DuPont (New Zealand) Ltd
iprodione	Rovral® FLO	Dicarboximide	0.1-0.6	0.01-0.1	Etec Crop Solutions Ltd
mancozeb	Dithane	Dithiocarbamates	2-8.5	0.2-2.2	Dow Agro Sciences NZ Ltd
pyraclostrobin	Cabrio® WG	Strobilurin	0.7-1.5	0.001-0.06	BASF New Zealand Ltd
tebuconazole	Hornet™ 430SC	Triazole	0.05-2	0.2-10	Nufarm Ltd
thiram	Thiram 40F	Disulphide	10-20	0.1-1	Nufarm Ltd

5.2.2 Effect of fungicides on *in vitro* conidial germination and germ tube growth

This experiment used the same nine fungicides as outlined in Section 5.2.1 (Table 5.1). Each fungicide was suspended in water and adjusted to six different concentrations based on the reported activity range for each product. The same species and isolates as used in Section 5.2.1 were used. Conidial suspensions were made as described in Sections 3.2.3 and 3.2.5 and concentrations were adjusted to 10^4 conidia/ mL based on haemocytometer counts. For each species, the conidial concentrations of the three isolates were adjusted equally and mixed together. Then 150 µL of the mixed conidial suspension was mixed with 150 µL of each fungicide concentration. For the control, the

conidial suspension was mixed with an equal amount of sterile water. Three separate drops, each containing 50 µL of each fungicide/conidial suspension or water/conidial suspension were placed separately onto three replicate glass slides. Each slide was then placed on a wet filter paper within a square plastic box which had been held in an incubator at 25°C for two days to ensure high relative humidity in the boxes and covered with its lid to maintain high relative humidity. The slides in the plastic boxes were arranged in a CRD and were then incubated at 25°C for 24 h in continuous darkness. After incubation, a drop of lactophenol cotton blue was placed on each slide, to prevent further germination and growth and to improve observation, and covered with a coverslip. A light microscope was used at ×200 magnification to determine germination of 100 conidia in each droplet. Germination was considered to have occurred if the germ tube of a conidium was at least the width of the conidium. From the mean percent germination, relative to the no fungicide controls, the percent inhibition of germination was calculated for each fungicide/conidial suspension. During the assessment of each slide, germ tubes of ten randomly selected germinated conidia were photographed using the digital camera mounted on the light microscope, and the lengths of the germ tubes were measured using the AnalySIS® imagine software (Soft Imagine System GmbH).

Probit analysis in Gen Stat 16 software was used to calculate the EC_{50} values for mycelial growth, conidial germination and germ tube elongation. For mycelial growth the EC_{50} values obtained for individual isolates and species, per replicate, were analysed with ANOVA in Gen Stat software in order to determine the fungicides and species effect. The means of isolates were used to determine the mean species effect and means were separated using Fisher's protected LSD at $P \leq 0.05$. For conidial germination and germ tube growth, mixed isolates were used for each species therefore EC_{50} values were obtained from mixed isolates.

5.2.3 Effect of potential biocontrol agents on *in vitro* mycelial growth

Two potential biocontrol agents, *Bacillus subtilis* (Trade name: Serenade® MAX Manufacturer: AgraQuest Inc) and *Trichoderma atroviride* (Trade name: Vinevax™ Manufacture: Agrimm Technologies Ltd) were tested against *Neofusicoccum* species

A suspension was made of the *T. atroviride* strains from the Vinevax™ product using 1 g/mL which the product description said was equivalent to 5 x 10^8 CFU/g. A five-fold serial dilution was made and 100 µL of the 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions were plated onto PDA in incubated at 25°C in 12 h light and 12 h dark 7-9 days. From these, a single colony of *T. atroviride* isolated from the commercial product was subcultured onto PDA and incubated at 25°C in 12 h light and 12 h dark for 7 days, and then used for all further studies. A suspension was made of the *Bacillus subtilis* cells from the Serenade® product using 1 g/mL which the product description said was equivalent to 7.3 x 10^9 CFU/g. A loop of suspension was streaking out onto a NA (Nutrient agar; Oxoid, Thermo Fisher Scientific Inc.) plate and incubated at 25°C in 12 h light and 12 h dark for three days. A pure colony was taken, subcultured onto NA and sequenced to confirm the identity (procedure in Appendix A) and used for subsequent *in vitro* studies.

In dual plate assays for *T. atroviride* and *Neofusicoccum* species, the 7 mm mycelial plugs taken from a 5 day-old culture of the *T. atroviride* and a 3 day-old culture of a *Neofusicoccum* isolate (same species and isolates as used in Section 2.5.1) were placed opposing one another on the outer edges of the PDA in 90 mm Petri dishes. To prepare the dual plates for *Bacillus subtilis* and *Neofusicoccum* species, a single colony was used to streak one side of a PDA plate and the mycelial plug of the pathogen was placed 2 cm from the bacterial streak. Control plates used mycelial plugs of the pathogen and sterile agar plugs (instead of *T. atroviride*) or water (instead of *B. subtilis*). The three replicates of dual plates for each isolate/ treatment were incubated in 12 h light and 12 h dark at 25°C for 7 days before visual assessment of the interactions of the cultures which were photographed. After this the mycelial growths were measured with a digital caliper across two perpendicular diameters of each colony and the mean mycelial growth inhibition was calculated as a percentage of the mean diameter of mycelia on control plates. The percent inhibition was calculated for each isolate and biocontrol agent.

Another experiment was conducted to determine whether *Bacillus subtilis* could inhibit germination and early growth of the same *Neofusicoccum* isolates as used for the previous experiment. A conidial suspension of 10^4/mL was prepared from each isolate and 0.1 mL was spread over a PDA plate. The plates were then allowed to dry in the

laminar flow hood for few minutes. A suspension was made of the *B. subtilis* cells and loop of suspension was inoculated onto the PDA plates in four equidistant positions approximately 1 cm from the edge of each Petri plate. Plates were incubated in 12 h light and 12 h dark at 25°C for 5 days and the diameters of the inhibition zones were measured.

For better observation of the microscopic interactions, the same pathogens and the biocontrol agents were plated onto synthetic nutrient agar (SNA; Appendix A) medium, using the same technique as above, and incubated in 12 h light and 12 h dark at 25°C for 10 days. Agar plugs (~2×2 mm) were cut from the interaction zone between the pathogen and the biocontrol agent and mounted on a glass slide with lactophenol cotton blue for microscopic observation (x600 magnification). Photographs were taken using a digital camera mounted on the light microscope.

5.2.4 Effect of cyprodinil+ fludioxonil, copper hydroxide and phosphonate (phosphorous acid) on *in vitro* mycelial growth, conidial germination and germ tube growth

Due to a request from the blueberry growers' organisation (Blueberry New Zealand; BBNZ), cyprodinil+ fludioxonil, copper hydroxide and phosphorous acid were(Table 5.2) also evaluated *in vitro* for the inhibition of mycelial growth, conidial germination and germ tube growth as described in Sections 5.2.2 and 5.2.3.

Table 5.2: Details of cyprodinil+ fludioxonil, copper hydroxide and phosphonate (phosphorous acid) used for *in vitro* testing against mycelial growth, conidial germination and germ tube growth of *N. luteum*, *N. australe*, *N. ribis* and *N. parvum.*

Active ingredient	Trade name	Chemical class	Conc. range for mycelia (mg a.i/L)	Conc. range for conidia (mg a.i/L)	Manufacturer
copper hydroxide	DuPont™ Kocide® Opti™	Inorganics	200-700	1-6	DuPont (New Zealand) Ltd
cyprodinil + Fludioxonil	Switch®	Anilinopyrimidine	0.01-0.06	0.01-0.11	Syngenta Group Company
mono+ di potassium salts (potassium phosphite)	Foschek ™	Phosphorus acid	1000-3500	250-1500	Tapuae partnership, NZ

5.2.5 Effect of fungicides, biocontrol agents and paints on *Neofusicoccum* species infection of potted blueberry plants

Two control treatments were used for each of the fungicide/ Acrylic paint evaluation experiments. The positive control plants (no fungicide treatment) were sprayed with water before inoculating with the *Neofusicoccum* fungal isolate. Negative control plants (no fungicide and no *Neofusicoccum* species inoculation) were inoculated with sterile water only. For all the treatments two shoots per plant and six replicate plants were used.

5.2.5.1 Evaluation of fungicides and paints when applied to plants before inoculation with *Neofusicoccum ribis*

Subsequent to the *in vitro* studies, in Sections 5.2.1 and 5.2.2, three fungicides (carbendazim, tebuconazole and flusilazole) and cyprodinil+ fludioxonil (in response to a request from blueberry growers) were selected for use in greenhouse studies. Acrylic paint (Resene Ceiling White) was also evaluated, being painted over wounds made on soft green and hard green shoots prior to inoculation as described below. Two year old potted plants of blueberry cultivar 'Dolce Blue' were used for the experiment.

For protection of wounded and non-wounded soft green shoots and hard green shoots, plants were sprayed with fungicides in June 2014 according to manufacturer's

recommended field rates. Plants were wounded as described in Section 3.2.6.1 and fungicides were sprayed onto each whole shoot until the surfaces were covered in fungicide droplets using an Aqua systems 2 L garden hand pressure sprayer. Acrylic paint was applied with only a paint brush onto wounds of soft green shoots and hard green shoots. After 24 h, wounded sites and selected marked sites on the non-wounded shoots were inoculated with 50 µL of the mixed conidial suspension (10^6/mL) of *N. ribis* (isolates S1-175 and S1-158) as described in Section 3.2.6.1. Positive and negative controls were set up as described in Section 5.2.5. All the treated plants were covered with polythene bags misted inside with water and left for 48 h to provide high humidity. The six replicate plants used for each treatment were arranged in a completely randomised design in an open area similar to field conditions for hard green shoots, and in a greenhouse for soft green shoots. Soft green shoots were examined after 4, 7, 10 and 14 days and hard green shoots after 7, 14, 21, 30, 45 and 60 days for discolouration of the inoculated stems. Lesions were measured in soft green shoots 14 days after inoculation and in hard green shoots 60 days after inoculation using a digital caliper. From each shoot, a 110 mm piece, which comprised 50 mm from above and 50 mm from below the ~1cm inoculation point was removed. After surface sterilisation as described in Section 3.2.3 each piece was cut into 10 mm segments (not including the inoculation site) for sequential isolation onto PDA plates. The plates were incubated in 12 h light and 12 h dark conditions at 25°C and the *N. ribis* isolates identified by colony appearance.

In June 2014, fungicide treatment of leaf buds was investigated for protection against *N. ribis* infection. Five buds were selected for each hard green shoot and two shoots selected for wounding and non-wounding of buds on each plant, with 10 buds being wounded and 10 buds non-wounded on each plant. Buds were wounded by pricking five times with a sterile needle which was resterilised by dipping in 70% ethanol between shoots. The selected buds were immediately spray treated with the same four fungicides as previously described according to manufacturer's recommended field rates (Appendix E21). After 24 h, selected buds were drop inoculated with 20 µL of the mixed conidial suspension (10^4/mL) of *N. ribis* (S1-175 and S1-158). Positive and negative controls were included as mentioned in Section 5.2.5. All the treated plants were covered with polythene bags misted inside with water and left for 48 h to provide high humidity. Six replicate plants were used for each treatment and plants were

arranged in CRD in an open area similar to field conditions. Plants were examined for discolouration in buds after 2, 3, 6, 9 and 14 days. The ten inoculated wounded and non-wounded buds were removed from each plant 14 days after inoculation, were surface sterilised in 70% ethanol for 30 s, rinsed with sterile tap water for 30 s and air dried in a the laminar flow cabinet for 10 min, then plated onto PDA. Plates were incubated in 12 h light and 12 h dark at 25°C for three days and the number of buds recorded that gave rise to colonies characteristic of *N. ribis* isolates.

5.2.5.2 Evaluation of fungicides applied after inoculation with *Neofusicoccum ribis*

The three most effective fungicides (carbendazim, tebuconazole and cyprodinil+ fludioxonil) from the experiment in Section 5.2.5.1 were assessed using 2 year old potted plants of cultivar 'Dolce Blue' in August 2014. Soft green shoots were wounded as described in Section 3.2.6.1 and wounds were immediately inoculated with 50 µL of the mixed conidial suspension (10^6/mL) of *N. ribis* (S1-175 and S1-158). All the treated plants were covered with new polythene bags misted with water and left for 24 h to provide high humidity. Positive and negative controls were set up as described in Section 5.2.5. Plants were treated with fungicides as described in Section 5.2.5.1**.** The six replicate plants used for each treatment were arranged in a CRD in a greenhouse. Plants were examined for lesion development and lesions were measured using a digital caliper after 14 days. From each shoot, a 110 mm piece, which comprised 50 mm from above and 50 mm from below the inoculation point was removed, surface sterilised and the 10 mm segments isolated sequentially onto PDA as described in Section 3.2.3. The plates were incubated in 12 h light and 12 h dark conditions at 25°C and the colonies characteristic of *N. ribis* isolates recorded.

5.2.5.3 Evaluation of fungicides under natural infection conditions

The same fungicides used in Section 5.2.5.2 were used in this experiment which was conducted in October 2014. The hard green shoots of two year old potted blueberry plants (cultivar 'Dolce Blue') were wounded or non-wounded on two shoots of each plant as described in Section 3.2.3 and the entire plants were spray treated with a fungicide or water (control) as described in Section 5.2.5.1. The six replicate plants per treatment were randomly allocated to places within the rows of a vineyard in the Lincoln University Grape Cultivar Collection, where Botryosphaeriaceae inoculum had previously been found, within 1-2 m of the vines. Plants were examined for dieback

symptoms after 12 weeks and infections assessed by isolations. These were made from the trunk (from the base up to 100 mm) and from two wounded and two non-wounded shoots (up to 100 mm from the attached point to the trunk) from each plant. The shoots were surface sterilised as described in Section 3.2.3 and divided into 10 mm pieces which were sequentially plated, bark and wood separately, onto PDA. Ten fruits per plant were also surface sterilised as described in Section 3.2.3 and plated onto PDA. Three leaves from each plant which showed discoloration or spots were also selected, surface sterilised and three segments of each were plated onto PDA. The plates were incubated in 12 h light and 12 h dark conditions at 25°C and the Botryosphaeriaceae isolates growing from the tissue identified by colony appearance and molecular identification as described in Section 2.2.3 and 2.2.4.

5.2.5.3.1 Trapping of water borne conidia and identification of Botryosphaeriaceae species

Rain water run-off traps were placed in the vineyard in the Lincoln University Grape Cultivar Collection from October 2014 to January 2015 to confirm the presence of Botryosphaeriaceae inoculum for the previous experiment. The rain water run-off traps consisted of 210 × 297 mm transparent acetate sheets folded and stapled at the lower end to create a funnel. The opposite wide end was stapled onto the arms of each vine and also tied on with a thread (Figure 5.1). Each funnel base was placed into a 250 mL container fixed to the trunk at 80 cm above the ground. There were 10 traps attached to 10 selected grapevines (third and ninth in each row).

Figure 5.1: Rain water trap set up in the vineyard.

After every significant rainfall event (>2 mm) the rain water was collected, filtered through a 50 µm sieve and allowed to settle overnight at 4°C. The supernatant was removed carefully and the rest (~50 mL) was frozen at -80°C until further processing. The thawed samples were centrifuged at 10°C for 15 min at 10,000 × *g* and the resulting pellets for each rain fall event were pooled. Pellets were re-suspended in 400 µL of sterile water and the DNA was extracted from a 300 µL aliquot using the Power Soil^{TM} DNA isolation kit (MO BIO laboratories, USA) according to the manufacturer's instructions. A nested PCR was carried out as described in Section 2.2.5.1.3 and identification was carried out using the SSCP method as described in Section 2.2.5.1.4.

5.2.5.4 Evaluation of copper hydroxide, phosphanate (phosphorous acid) and biocontrol agents

The blueberry growers' organisation (BBNZ) requested that a trial was carried out with these products. The experiment was set up in January 2015. Commonly used sprays comprising *Bacillus subtilis* (Serenade®), *Trichoderma atroviride* (Vinevax™), copper

hydroxide and phosphorous acid (Table 5.2). Soft green shoots of the potted blueberry plants (cultivar 'Dolce Blue') were wounded as described in Section 3.2.6.1 and spray treated as described in Section 5.2.5.1 with water (control), the chemicals and biocontrol agents using manufacturers' field application rates (Appendix E21). Plants treated with biocontrol agents were covered with polythene bags misted inside with water and left for 48 h to provide high humidity so that the biocontrol agents could colonize the wound, but the plants sprayed with water, copper hydroxide and phosphorous acid products were not covered. After seven days wounded tissues were inoculated with 50 µL of the mixed conidial suspension (10^6/mL) of *N. ribis* (S1-175 and S1-158). All the inoculated plants were covered with polythene bags misted inside with water and left for 48 h. Two shoots were inoculated on each plant and six plants were used for each treatment. Controls were inoculated only with 50 µL of the mixed *N. ribs* conidial suspension. All the treated plants were arranged in CRD in a greenhouse where the temperature was maintained at 20-25°C and the pots' soil watered as required. Plants were examined for lesion development and lesions were measured using a digital caliper 14 days after inoculation. Isolations were carried out as described in Section 3.2.3.

5.2.6 Data analysis

The distances of pathogen movement and lesion lengths were analysed with ANOVA and means were separated with Fisher's protected LSD at $P \leq 0.05$. Incidence data which comprised binomial data were analysed by generalized linear mixed model (GLM) and means were compared using SED values. Pearson chi square tests of independence at $P \leq 0.05$ were used to determine the association between Botryosphaeriaceae species infection incidence overall and treatments for the experiment described in Section 5.2.5.3. Statistical analyses were conducted using GenStat version 16.

5.3 Results

5.3.1 Effect of fungicides on *in vitro* mycelial growth

Mycelial growth of Botryosphaeriaceae species was inhibited by most of the fungicides at some concentrations (Figure 5.2). Fludioxonil, carbendazim, flusilazole and tebuconazole were the most effective among the fungicides tested, with EC_{50} values less than 0.1 mg a.i. /L (Tables 5.3 and 5.4). However, when the EC_{50} values of some fungicides for some isolates and species were much higher than the concentrations used in this experiment; in fact some values were much higher than could be used in the *in vitro* experiment or in the field. These were presented in the Tables as 'MC'. For some fungicides EC_{50} values were much lower than the concentrations used, these were presented as 'mC'.The analysis of EC_{50} values showed that there was a significant effect of fungicides and species, and interaction between them ($P< 0.001$ for all; Appendix E.1). The least effective fungicide was captan which was significantly different from other treatments ($P<0.05$). For the species effect, *N. australe* was the most sensitive with the lowest EC_{50} values, and being significantly ($P<0.05$) different from all other species. This was followed by *N. ribis* which was significantly ($P<0.05$) different from all other species. The least sensitive species were *N. luteum* and *N. parvum* which were not significantly different ($P>0.05$) from each other. The significant interaction between the species and the fungicides appeared to be mostly associated with the higher efficacy of thiram and captan (according to the EC_{50} values) on *N. ribis* than on the other species.

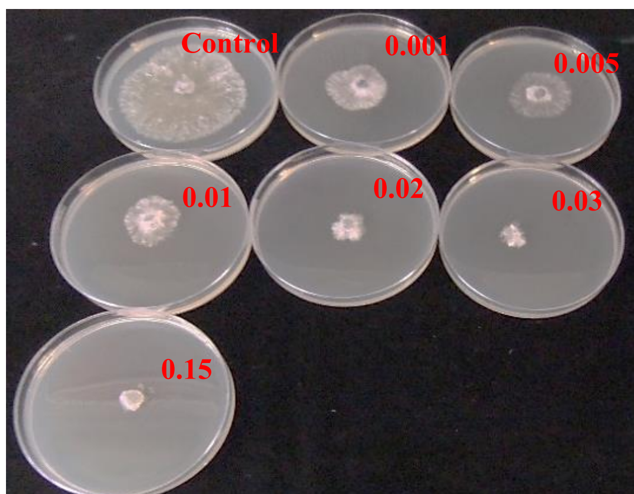


Figure 5.2: Effect of fludioxonil on mycelial growth of *Neofusicoccum luteum* isolate S1-130; plates were arranged according to the agar concentrations in mg a.i. /L.

Table 5.3: The mean EC_{50} values in active ingredient concentrations (mg a.i./L) obtained for fungicides tested *in vitro* for mycelial growth of 12 *Neofusicoccum* species isolates relative to the no fungicide control.

Fungicides	Isolates											
	N. australe			***N. luteum***			***N. parvum***			***N. ribis***		
	S1-131	**S1-174**	**S1-111**	**S1-142**	**S1-130**	**S1-167**	**S1-58**	**S1-173**	**S1-98**	**S1-110**	**S1-175**	**S1-158**
carbendazim	0.00	0.00	0.00	0.08	0.12	0.05	0.04	0.05	0.03	0.00	0.03	0.03
	-2.95[a]	**-4.08**	**-3.52**	**-1.1**	**-0.92**	**-1.26**	**-1.36**	**-1.34**	**-1.54**	**-4.23**	**-1.57**	**-1.48**
iprodione	0.36	0.19	0.16	0.71	0.73	0.85	0.89	0.70	0.69	0.78	0.70	0.86
	-0.44	**-0.72**	**-0.79**	**-0.15**	**-0.14**	**-0.07**	**-0.05**	**-0.16**	**-0.16**	**-0.11**	**-0.16**	**-0.07**
captan	62.67	36.80	45.71	38.90	30.13	18.07	32.21	32.51	17.50	6.34	18.84	64.57
	1.80	**1.57**	**1.66**	**1.59**	**1.48**	**1.26**	**1.51**	**1.51**	**1.24**	**0.80**	**1.28**	**1.81**
pyroclostrobin	0.31	0.95	4.35	1.79	1.59	1.23	1.11	1.79	0.24	1.05	1.43	1.53
	-0.50	**-0.02**	**0.64**	**0.26**	**0.20**	**0.09**	**0.04**	**0.25**	**-0.62**	**0.02**	**0.16**	**0.19**
mancozeb	15.06	26.30	110.4	7.28	14.92	21.04	9.12	10.16	9.80	4.78	20.50	9.80
	1.18	**1.42**	**2.04**	**0.86**	**1.17**	**1.32**	**0.96**	**1.01**	**0.99**	**0.68**	**1.31**	**0.99**
thiram	69.02	100.46	82.41	13.55	15.14	9.10	17.06	14.26	14.55	0.03	14.69	17.46
	1.84	**2.00**	**1.92**	**1.13**	**1.18**	**0.96**	**1.23**	**1.15**	**1.16**	**-1.54**	**14.69**	**1.24**
flusilazole	0.09	0.02	0.00	0.03	0.11	0.13	0.03	0.01	0.03	0.30	0.12	0.09
	-1.02	**-1.76**	**-3.19**	**-1.51**	**-0.98**	**-0.89**	**-1.48**	**-1.97**	**-1.54**	**-0.53**	**-0.92**	**-1.06**
tebuconazole	0.03	0.01	0.00	0.08	0.15	0.10	0.15	0.14	0.10	0.03	0.11	0.27
	-1.54	**-2.06**	**-3.02**	**-1.08**	**-0.82**	**-1.01**	**-0.83**	**-0.86**	**-0.99**	**-1.47**	**-0.97**	**-0.56**
fludioxonil	0.00	0.00	0.00	0.00	0.01	0.01	1.0	0.02	0.02	0.00	0.01	0.01
	-4.24	**-5.73**	**-7.35**	**-2.67**	**-2.11**	**-2.34**	**0.00**	**-1.62**	**-1.75**	**-5.27**	**-2.25**	**-2.23**

a- Log values used for analysis

Table 5.4: The mean EC_{50} (mg a.i. /L) values for different fungicides for *in vitro* effect on mycelial growth of four *Neofusicoccum* species relative to the no fungicide control.

Fungicide	***Neofusicoccum* species**								**Fungicides mean EC_{50}**	
	N. australe		*N. luteum*		*N. parvum*		*N. ribis*			
	Log value	Transformed data	Log value	Transformed data	Log value	Transformed data	Log value	Transformed data	Log value	Transformed data
carbendazim	-3.52	0.00	-1.09	0.08	-1.41	0.04	-2.43	0.00	-2.12	0.00 b[1]
iprodione	-0.65	0.22	-0.12	0.76	-0.12	0.75	-0.11	0.78	-0.25	0.56 d
captan	1.67	47.20	1.44	27.67	1.42	26.36	1.30	1.30	1.46	28.71 g
pyroclostrobin	0.04	1.09	0.18	1.52	-0.11	0.78	0.12	1.32	0.06	1.15 e
mancozeb	1.55	MC[a]	1.12	MC	0.99	MC	0.99	MC	1.16	14.52 fg
thiram	1.92	MC	1.09	12.30	1.18	15.24	0.29	1.95	1.12	13.21 f
flusilazole	-1.99	0.01	-1.13	0.08	-1.66	0.02	-0.84	0.15	-1.40	0.04 c
tebuconazole	-2.21	0.01	-0.97	0.11	-0.89	0.13	-1.00	0.10	-1.27	0.05 c
fludioxonil	-5.77	0.00	-2.37	0.00	-1.12	0.08	-3.25	0.00	-3.13	0.00 a
Species mean effect	-1.00	0.10 x	-0.2	0.62 z	-0.19	0.64 z	-0.55	0.28 y		

[1]Values within the rows and columns followed by the same letter are not significantly different according to Fisher's protected LSD at P=0.05. Fungicides mean effect (a-g) was significant (P< 0.001; LSD = 0.298). The mean effect of species (x-z) was significant (P < 0.001; LSD = 0.198) and species × fungicides effect was also significant (P< 0.001; LSD = 0.595).

[a]MC- Maximum concentration.

5.3.2 Effect of fungicides on *in vitro* conidial germination and germ tube growth

There was a significant effect of fungicide on EC_{50} values ($P< 0.001$; Appendix E.2) for all four *Neofusicoccum* species in reduction of conidial germination. Thiram was the most effective fungicide which was significantly different from all the other fungicides ($P<0.05$; Table 5.5). This was followed by pyroclostrobin which was significantly different from other treatments ($P<0.05$). The next most effective fungicides were carbendazim and iprodione which were not significantly different from each other ($P>0.05$). The least effective fungicide was tebuconazole. There was also a significant effect of species ($P<0.001$), with *N. luteum* being the most sensitive with the lowest EC_{50} value ($P<0.05$), followed by *N. australe*, *N. ribis* and *N. parvum* which was least effective. The significant interaction between the species and the fungicides appeared to be mostly associated with the lower efficacy of flusilazole, tebuconazole, fludioxonil and mancozeb (according to the EC_{50} values) on *N. parvum* (Table 5.5).

There was a significant effect of fungicides ($P< 0.001$; Appendix E.3) on germ tube growth (Table 5.6). Pyroclostrobin was the most effective fungicide which was significantly different from other treatments ($P< 0.05$). This was followed by carbendazim and thiram which did not differ significantly ($P> 0.05$), and then by iprodione (Figure 5.3). The least effective fungicides was tebuconazole. There was a significant effect of species, with *N. australe* being the most sensitive followed by *N. luteum, N. parvum* and *N. ribis* which was the least sensitive. The significant interaction between fungicides and species ($P<0.001$) was associated with some fungicides having high EC_{50} values for some species (Table 5.6).

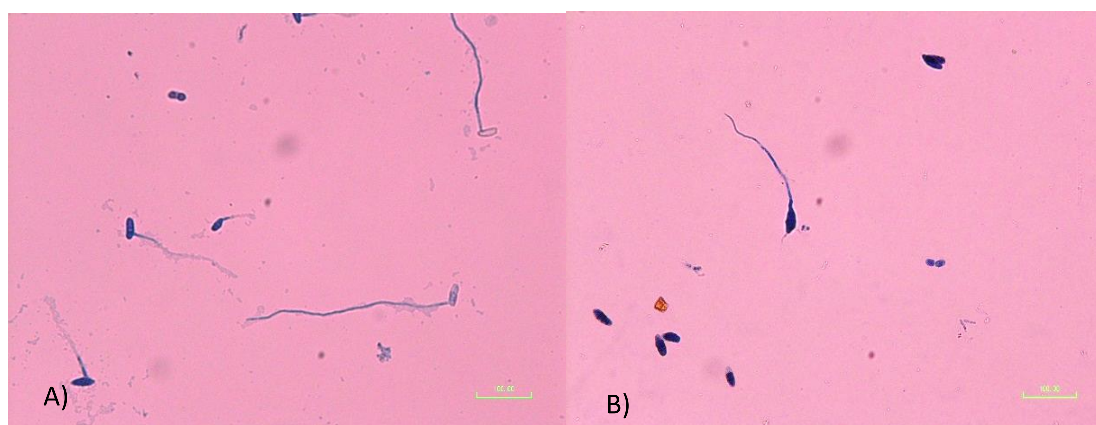


Figure 5.3: Effect of the fungicide iprodione on germination and germ tube growth of *Neofusicoccm parvum* after 24 h at 25˚C. A). Germ tube growth at 0.01 mg a.i. /L and B) germ tube growth at 0.1 mg a.i. / L. (×400)

Table 5.5: The mean EC $_{50}$ (mg a.i. /L) values for different fungicides for *in vitro* inhibition of conidial germination of four *Neofusicoccum* species.

Fungicide	*Neofusicoccum* species								Fungicides mean EC_{50}	
	N. australe		***N. luteum***		***N. parvum***		***N. ribis***			
	Log value	**Transformed data**	**Log value**	**Transformed data**	**Log value**	**Transformed data**	**Log value**	**Transformed data**	**Log value**	**Transformed data**
carbendazim	-2.58	0.00	-1.3	0.05	-1.07	0.09	-1.82	0.02	-1.69	0.02 c[1]
iprodione	-1.71	0.02	-1.36	0.04	-1.03	0.09	-2.78	0.00	-1.72	0.02 c
captan	0.16	1.44	-0.542	0.29	-0.19	0.65	0.33	2.14	-0.06	0.87 e
Pyroclostrobin	-3.70	mC[a]	-5.10	mC	-1.41	0.04	-1.21	0.06	-2.85	0.00 b
mancozeb	-0.31	0.50	-1.30	0.05	0.79	6.18	0.49	3.07	-0.09	0.82 e
thiram	-1.15	0.07	-15.14	mC	-0.05	0.90	1.0	MC[b]	-3.84	0.00 a
flusilazole	-1.25	0.06	-2.77	0.00	1.42	27.04	0.27	1.88	-0.58	0.26 d
tebuconazole	-1.02	0.10	0.98	9.46	1.23	16.87	0.79	6.21	0.49	3.12 f
fludioxonil	-0.94	0.11	-0.65	0.23	1.57	36.81	-1.80	0.02	-0.46	0.35 d
Species mean effect	-1.39	0.04 x	-3.02	0.00 w	0.14	1.39z	-0.53	0.30 y		

[1]Values within the rows and columns followed by the same letter are not significantly different according to Fisher's protected LSD at *P*=0.05. Fungicides mean effect (a-f) was significant (*P*< 0.001; LSD = 0.366). The mean effect of species (w-z) was significant (*P* < 0.001; LSD = 0.243) and species × fungicides effect was also significant (*P*< 0.001; LSD = 0.731).

[a]mc- Minimum concentration [b]MC- Maximum concentration

Table 5.6: The mean EC $_{50}$ (mg a.i. /L) values for different fungicides for *in vitro* inhibition of germ tube growth of four *Neofusicoccum* species.

Fungicide	*Neofusicoccum* species								Fungicides mean EC_{50}	
	N. australe		*N. luteum*		*N. parvum*		*N. ribis*			
	Log value	Transformed data	Log value	Transformed data	Log value	Transformed data	Log value	Transformed data	Log value	Transformed data
carbendazim	-3.63	mC[a]	-1.46	0.04	-1.21	0.06	-1.24	0.06	-1.88	0.01 b[1]
iprodione	-0.87	0.13	-0.98	0.10	-1.03	0.09	-1.22	0.06	-1.03	0.09 c
captan	0.43	2.69	0.55	3.52	-1.21	0.06	0.45	2.79	0.05	1.13 e
pyroclostrobin	-3.67	mC	-1.54	0.03	-4.53	mC	-2.12	MC	-2.97	0.00 a
mancozeb	-0.98	0.11	-0.71	0.20	0.45	2.81	-0.31	0.49	-0.39	0.41 d
thiram	-5.09	mC	-2.23	mC	-0.37	0.43	-0.63	0.23	-2.08	0.01 b
flusilazole	-1.76	0.02	-1.31	0.05	0.32	2.08	0.69	4.94	-0.51	0.31 d
tebuconazole	-0.88	0.13	1.6	MC[b]	0.90	7.87	1.06	11.45	MC	4.67 f
fludioxonil	-2.79	mC	-1.47	0.03	1.63	42.56	0.58	3.79	-0.51	0.31 d
Species mean effect	-2.14	0.01 w	-0.84	0.15 x	-0.56	0.27 y	-0.31	0.50 z		

[1]Values within the rows and columns followed by the same letter are not significantly different according to Fisher's protected LSD at P= 0.05. Fungicides mean effect (a-f) was significant (P< 0.001; LSD = 0.224). The mean effect of species (w-z) was significant (P < 0.001; LSD = 0.150) and species × fungicides effect was also significant (P< 0.001; LSD = 0.449).

[a]mc- Minimum concentration [b]MC- Maximum concentration

5.3.3 Effect of biocontrol agents on *in vitro* mycelial growth

Macroscopic observations of the interactions between biocontrol agents and *Neofusicoccum* species mainly included the formation of inhibition zones (Figure 5.4) or the biocontrol agent grew over and sporulated on the mycelium of the pathogen as described by Koetze *et al.* (2011). In dual cultures with *Bacillus subtiliis* all the isolates of each *Neofusicoccum* species were inhibited with little mycelial growth near the bacterial zone (Figure 5.4A). In dual cultures with *Trichoderma atroviride*, the biocontrol agent was observed to inhibit growth of all *Neofusicoccum* species isolates by overgrowth (Figure 5.4B), after which it sporulated.

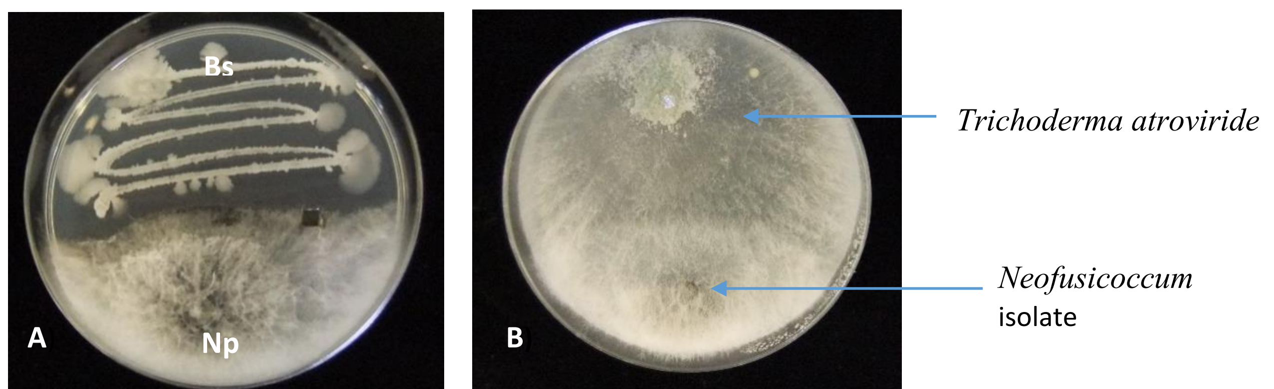


Figure 5.4: Macroscopic interactions observed for dual plate assays between *Neofusicoccum parvum* and biocontrol agents A) inhibition zone (*Bacillus subtiliis*/Bs and *N. parvum*/Np) and B) overgrowth (*Trichoderma atroviride* and *N. parvum*).

There was a significant effect of biocontrol agents (P<0.001; Appendix E.4.A) on mycelial growth of twelve different isolates of *Neofusicoccum* species (Table 5.7). The mean percent inhibition of mycelial growth was significantly greater for *B. subtilis* (52.82%) than *T. atroviride* (46.79%). There was a significant effect of isolates (P=0.021; Appendix E.4.A), with *N. parvum* isolate S1-98 being least sensitive to the biocontrol agents and *N. ribis* isolate S1-110 most sensitive, which were significantly different from each other (P<0.05; Table 5.7). There was a significant interaction between the biocontrol agents and isolates (P<0.001) which was associated with some isolates being much more inhibited by *B. subtilis* than *T. atroviride*. When data were analysed as species (Table 5.8) there was a significant effect of biocontrol agents (P<0.001; Appendix E.4.B) but no significant effect of species (P=0.102; Appendix E.4.B) and no significant interaction between the fungicides and species (P=0.895).

Table 5.7: The mean percent inhibition (%) of mycelial growth caused by *Bacillus subtilis* and *Trichoderma atroviride* on twelve different isolates of *Neofusicoccum* species.

Species	Isolate	Biocontrol agent		Isolate mean
		B. subtilis	***T. atroviride***	
N. australe	S1-131	48.57	54.29	51.43 bcd[1]
	S1-111	52.86	50.00	51.43 bcd
	S1-174	61.43	35.71	48.57 abc
N. luteum	S1-130	48.57	48.10	48.33 abc
	S1-142	54.29	43.33	48.81 abc
	S1-167	55.71	48.57	52.14 cd
N. parvum	S1-98	49.05	43.81	46.43 a
	S1-173	50.00	44.29	47.14 ab
	S1-58	50.48	45.71	48.10 abc
N. ribis	S1-158	54.76	43.33	49.05 abc
	S1-110	56.67	53.81	55.24 d
	S1-175	51.43	50.48	50.95 abcd
Mean effect of biocontrol agents		52.82	46.79	

[1]Values within the columns followed by the same letter are not significantly different according to Fisher's protected LSD at P= 0.05. Biocontrol agents mean effect was significant (P< 0.001; LSD =1.886). The mean effect of isolates (a-d) was significant (P = 0.021; LSD =4.621) and isolates × biocontrol agents effect was also significant (P< 0.001; LSD =6.535).

Table 5.8: The mean percent inhibition of mycelial growth caused by *Bacillus subtilis* and *Trichoderma atroviride* on four *Neofusicoccum* species.

Species	Biocontrol agent		Species mean
	B. subtilis	***T. atroviride***	
N. australe	54.29	46.67	50.48
N. luteum	52.86	46.67	49.76
N. parvum	49.84	44.60	47.22
N. ribis	54.29	49.21	51.75
Mean effect of biocontrol agents	52.82	46.79	

Biocontrol agents' mean effect was significant (P<0.001). The mean effect of species was not significant (P=0.102) and species × biocontrol agents effect was not significant (P=0.895).

As *T. atroviride* overgrowth in the dual plates assay made it difficult to quantify the inhibition for this biocontrol agent, it was not used in a repeat experiment which was conducted using only *Bacillus subtilis.* The results showed a small inhibition zone between *B. subtilis* and *N. parvum* isolate S1-173 (Figure 5.5A), but no inhibition zone between the other isolates and *B. subtilis* (Figure 5.5B).

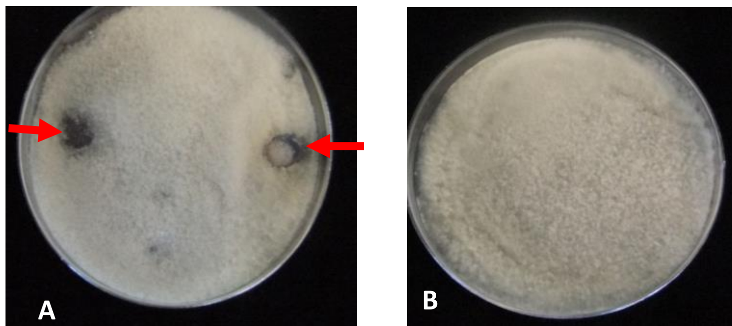


Figure 5.5: Dual plate assay results for *Bacillus subtilis* A) Inhibition zones observed between *B. subtilis* (arrows) and *Neofusicoccum parvum* isolate S1-173, B) no effect due to over growth of the biocontrol agent, *B. subtilis,* by *N. ribis* isolate S1-158.

Microscopic interactions between *T. atroviride* and *Neofusicoccum* isolates were difficult to distinguish from controls. The effect of *B. subtilis* on *Neofusicoccum* isolates was visible as swelling of hyphae as shown in Figure 5.6B.

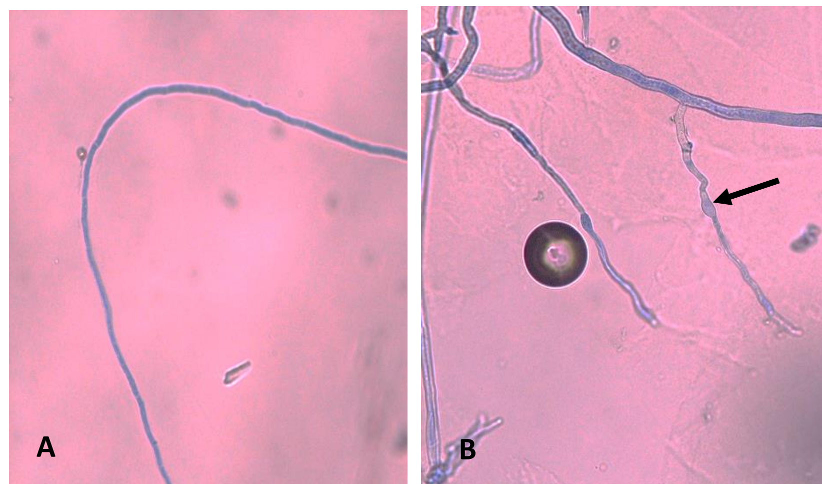


Figure 5.6: Microscopic observations of the interactions between biocontrol agents and the *Neofusicoccum australe* isolate S1-131. A) Hyphae of *N. australe* isolate S1-131 without biocontrol agent (×600). B) Hyphal swelling observed (arrow) in the hyphae of *N. australe* isolate S1-131 in the interaction zone with *Bacillus subtilis* (×600).

5.3.4 Effect of copper hydroxide, phosphonate (phosphorous acid) and cyprodinil + fludioxonil on *in vitro* mycelial growth, conidial germination and germ tube growth

For mycelial growth inhibition caused by three fungicides on twelve different isolates of *Neofusicoccum* species, there were significant effects of fungicides and isolates, and a significant interaction between the fungicides and isolates (all *P*<0.001; Table 5.9; Appendix E.5). When isolate EC_{50} values were reanalysed according to species there was no significant effect of fungicides (*P*=0.868; Appendix E.6), but a significant effect of species (*P*=0.002) and a significant interaction between the fungicides and the species (*P*<0.001) The most effective fungicide was copper hydroxide and the least effective fungicide cyprodinil+ fludioxonil (Table 5.10). In the species responce to the fungicides, *N. australe* and *N. ribis* were the most sensitive and not significantly different (P>0.05), with *N. luteum* and *N. parvum* being the least sensitive and not significantly different (P>0.05; Table 5.10). The significant interaction between species and fungicides was mostly associated with the lower efficacy of cyprodinil+ fludioxonil on *N. luteum* and *N. parvum,* and copper hydroxide on *N. australe* and *N. ribis* (according to EC_{50} values).

Table 5.9: EC_{50} values of active ingredient concentrations (mg a.i. /L) obtained for copper hydroxide, phosphonate (phosphorous acid) and cyprodinil + fludioxonil tested *in vitro* for mycelial growth of 12 *Neofusicoccum* species isolates of four species relative to the no fungicide control.

		Fungicides					
		copper hydroxide		phosphonate		cyprodinil+ fludioxonil	
Species	Isolate	Log value	Transformed data	Log value	Transformed data	Log value	Transformed data
N. australe	S1-131	2.89	767.36	2.80	628.06	-7.36	mC[2]
	S1-111	3.54	MC[1]	4.67	MC	-0.15	0.71
	S1-174	2.57	372.39	5.47	MC	-0.14	0.72
N. luteum	S1-130	3.07	1174.90	2.43	268.54	17.27	MC
	S1-142	2.86	721.11	2.77	588.84	4.87	MC
	S1-167	2.83	668.34	2.77	592.93	5.50	MC
N. parvum	S1-98	2.71	509.33	2.34	216.28	-0.73	0.19
	S1-173	2.66	461.31	2.85	701.55	27.51	MC
	S1-58	2.95	887.16	1.45	28.12	1.21	16.22
N. ribis	S1-158	3.37	MC	2.35	225.42	-2.15	0.01
	S1-110	2.87	732.82	3.18	1503.14	-2.57	0.00
	S1-175	2.79	610.94	2.43	271.02	-2.15	0.01

MC[1]- Maximum concentration mC[2]- Minimum concentration

Table 5.10: The mean EC $_{50}$ (mg a.i. / L) values for copper hydroxide, phosphonate (phosphorous acid) and cyprodinil + fludioxonil for *in vitro* effects on mycelial growth of four *Neofusicoccum* species relative to the no fungicide control.

Species	Fungicides						Species mean effect	
	copper hydroxide		phosphonate		cyprodinil+ fludioxonil			
	Log values	Transformed data	Log values	Transformed data	Log values	Transformed data	Log values	Transformed data
N. australe	3.00	MC[a]	4.31	MC	-2.55	0.00	1.59	38.55 a[1]
N. luteum	2.92	831.76	2.66	457.09	9.21	MC	4.93	MC b
N. parvum	2.77	588.84	2.21	162.18	9.33	MC	4.77	MC b
N. ribis	3.01	MC	2.65	446.68	-2.29	0.01	1.12	13.19 a
Fungicides mean EC$_{50}$	2.92	831.76	2.96	912.01	3.43	2691.53		

[1]Values within the columns followed by the same letter are not significantly different according to Fisher's protected LSD at P=0.05. Fungicides mean effect was not significant (P= 0.856). The mean effect of species (a-b) was significant (P < 0.001; LSD= 2.096) and species × fungicides effect was also significant (P< 0.001; LSD = 4.192).

[a]MC- Maximum concentration

For conidial germination the EC_{50} values showed significant effects of fungicides, species and an interaction between fungicides and species tested (*P*<0.001 for all: Table 5.11: Appendix E.7). Cyprodinil+fludioxonil was the most effective (*P*<0.05; Table 5.11) followed by copper hydroxide and phosphonate which all differed significantly (*P*<0.05). The most sensitive species was *N. luteum* followed by *N. australe*, *N. ribis* and *N. parvum* which all differed significantly (*P*<0.05).

For reduction of germ tube growth the EC_{50} values showed significant effects of fungicides, species and an interaction between fungicides and species tested (*P*<0.001 for all; Table 5.12: Appendix E.8). Phosphonate was the most effective and was significantly different from other treatments (*P*<0.05), and this was followed by copper hydroxide and cyprodinil + fludioxonil which were significantly different from each other (*P*<0.05). *Neofusicoccum australe* was the most sensitive followed by *N. ribis*, *N. parvum, with N. luteum* being the least sensitive, all being significantly different (*P*<0.05). The significant interaction between the species and the fungicides was mostly associated with the lower efficacy of cyprodinil+ fludioxonil on *N. rlbis*.

5.3.5 Effect of fungicides on bud infection when applied before inoculation with *Neofusicoccum ribis*

The effect of fungicides on bud infection by *N. ribis* was significant (*P*< 0.001: Appendix E.9) but there was no significant effect of wounding (*P*=0.197; Appendix E.9), with incidences for wounded and non-wounded buds being 35.3 and 38.3%, respectively), or a significant interaction between fungicide and wounding (*P*= 0.657; Appendix E.9; Table 5.13). Carbendazim and tebuconazole significantly reduced (*P*<0.05) the percent incidence of *N. ribis* (5.0% for both) compared with all other treatments that were not significantly different (*P*>0.05) from each other (Table 5.13). Flusilazole (46.7%) and cyprodinil+ fludioxonil (47.5%) significantly (*P*<0.05) reduced the percent incidence of *N. ribis* compared with the inoculated control (80.0%). No *N. ribis* was recovered from non-inoculated control buds, and these were not included in the analysis.

Table 5.11: The mean EC_{50} (mg a.i. /L) values for copper hydroxide, phosphonate(phosphorous acid) and cyprodinil + fludioxonil for *in vitro* inhibition of conidial germination of four *Neofusicoccum* species.

Species	Fungicides						Species mean effect	
	copper hydroxide		phosphonate		cyprodinil+ fludioxonil			
	Log value	Transformed data	Log value	Transformed data	Log value	Transformed data	Log value	Transformed data
N. australe	0.51	3.22	2.51	326.60	-1.80	0.02	0.41	2.56 b[1]
N. luteum	-5.58	≤ mC[a]	2.86	722.80	-1.73	0.02	-1.48	0.03 a
N. parvum	2.09	121.90	2.66	458.14	-0.46	0.35	1.43	26.90 d
N. ribis	1.24	17.50	3.10	1250.26	-1.51	0.03	0.94	8.80 c
Fungicides mean EC_{50}	-0.44	0.37 x	2.78	606.77 y	-1.38	0.04 w		

[1]Values within the rows and columns followed by the same letter are not significantly different according to Fisher's protected LSD at P=0.05. Fungicides mean effect (w-y) was significant (P< 0.001; LSD =0.3389). The mean effect of species (a-d) was significant (P < 0.001; LSD= 0.29) and species × fungicides effect was also significant (P< 0.001; LSD = 0.587).

[a]mC - minimum concentration

Table 5.12: The mean EC $_{50}$ (mg a.i. /L) values for copper hydroxide, phosphonate (phosphorous acid) and cyprodinil + fludioxonil for *in vitro* inhibition of germ tube growth of four *Neofusicoccum* species.

Species	Fungicides						Species mean effect	
	copper hydroxide		phosphonate		cyprodinil+ fludioxonil			
	Log value	Transformed data	Log value	Transformed data	Log value	Transformed data	Log value	Transformed data
N. australe	-0.87	0.13	-3.92	0.00	0.10	1.26	-1.56	0.03 a[1]
N. luteum	0.10	1.26	0.10	1.26	0.10	1.26	0.10	1.26 d
N. parvum	-1.08	0.08	-1.31	0.05	0.86	7.31	-0.51	0.31 c
N. ribis	-0.37	0.43	-4.50	0.00	1.77	59.30	-1.03	0.09 b
Fungicides mean EC_{50}	-0.56	0.28 x	-2.41	0.00 w	0.71	5.12 y		

[1]Values within the rows and columns followed by the same letter are not significantly different according to Fisher's protected LSD at *P*=0.05. Fungicides mean effect (w-y) was significant (*P*< 0.001; LSD =0.089). The mean effect of species (a-d) was significant (*P* < 0.001; LSD= 0.103) and species × fungicides effect was also significant (*P*< 0.001; LSD = 0.178).

Table 5.13: Effect of fungicides on *Neofusicoccum ribis* incidence in non-wounded and wounded leaf buds 14 days after inoculation. Data presented as angular transformed data (AVG%) and back transformed incidence data.

Fungicides	*N. ribis* incidence					
	Non-wounded buds		Wounded buds		Means	
	AVG[1] %	Incidence[2] (%)	AVG %	Incidence (%)	AVG %	Incidence (%)
carbendazim	7.6	5.0	9.2	5.0	8.4	5.0 a[3]
tebuconazole	3.1	1.7	13.6	8.3	8.4	5.0 a
flusilazole	43.5	51.7	40.0	41.3	41.9	46.7 b
cyprodinil+ fludioxonil	41.7	45.0	45.0	50.0	43.3	47.5 b
control(+)	59.8	73.3	70.6	86.7	65.2	80.0 c
LSD					12.90	

[1] Angular transformed data used for statistical analysis
[2] Back transformed data
[3] Values within the columns followed by the same letter are not significantly different according to Fisher's protected LSD at P=0.05. Fungicides mean effect (a-c) was significant (P< 0.001; LSD =12.90). The mean effect of wounding was not significant (P = 0.197) and fungicides × wounding effect was also not significant (P= 0.657).

5.3.6 Effect of fungicides and paints on infection of soft and hard green shoots when applied before inoculation with *Neofusicoccum ribis*

5.3.6.1 Acrylic paint as a treatment to protect pruning wounds

The paint treatment, which was applied to wounded shoots only, but as a treatment within a wider experiment which examined the effects of fungicide treatments on wounded and non-wounded soft green shoots and hard green shoots, was relatively ineffective. The analysis of wounded only shoots (Appendices E.10, E.11, E.14 and E.15) provided a significant effect of the treatments due largely to the effects of the fungicides. The paint results are described briefly here, but the remaining effects of wounding and fungicides were examined in a second series of analyses and are described below (Sections 5.3.6.2 and 5.3.6.3).

On soft green shoots, the paint treatment was ineffective, with 100% *N. ribis* incidence and 83.3 mm mean *N. ribis* progression compared with the inoculated control, for which the incidence of *N. ribis* was 100% and the mean *N. ribis* progression 93.3 mm. On hard green shoots, the results from the paint treatment were 100% *N. ribis* incidence and 36.67 mm *N. ribis* progression compared with the control, for which incidence was 100% and progression 86.67 mm. Closer examination of the applied paint using a hand lens, showed some cracks had developed in the paint after drying in soft green shoots and hard green shoots. In addition brown discolouration was observed in inoculated paint treated soft green sheets. However no discolouration was observed in controls (only paint treated) soft green shoots, confirming there were no phototoxic effects caused by the paint on soft green shoots.

5.3.6.2 Effect of fungicides on infection of soft green shoots when applied before inoculation with *Neofusicoccum ribis*

Infection incidence was significantly affected by fungicide treatments (P=0.001; Appendix E.12). Overall mean infection incidence was low in carbendazim treated shoots (16.7%) (P>0.05) which was significantly lower than for all other treatments. There was a significant effect of wounding (P= 0.024; Appendix E.12), with the mean infection incidence for non-wounded and wounded shoots being 56.6 and 83.3%, respectively. There was no significant interaction between the fungicides and wounding (P= 0.277; Appendix E.12). The effect of fungicides (excluding paint) on distance progression by the pathogen on soft green shoots was significant (P< 0.001; Appendix E.13) (Table 5.14). Overall, carbendazim was the most effective fungicide for which disease progression was significantly less than (P<0.05) from the other treatments (Table 5.14). This was followed by tebuconazole, cyprodinil+ fludioxonil and flusilazole which were not significantly different (P>0.05) from each other, but were significantly lower than the inoculated control (Table 5.14). There was a significant effect of wounding (P<0.001; Appendix E.13), with the mean distance travelled for non-wounded shoots and wounded shoots being 17.02 and 31.66 mm, respectively. There was a significant interaction between the fungicide and wounding (P= 0.002; Appendix E.13) which was probably associated with the lesser distances travelled in non-wounded versus wounded shoots, for all fungicides except carbendazim. No *N. ribis* was recovered from any of the non-inoculated control plants and this treatment was not included in the analysis.

Table 5.14: Effect of Fungicides on *Neofusicoccum ribis* progression (mm) and infection incidence (%) in non-wounded and wounded soft green shoots 14 days after inoculation.

	Non-wounded		Wounded		Means	
Fungicides	**Pathogen progression**	**Infection incidence**	**Pathogen progression**	**Infection incidence**	**Pathogen progression**	**Infection incidence**
carbendazim	1.7	16.6(-2.4)[2]	1.7	16.7(-2.4)	1.7 a[1]	16.6 (-2.4) w
tebuconazole	6.7	50.0(-1.1)	16.7	100.0(0.0)	11.7 b	73.2(-0.6) x
flusilazole	11.7	83.0(-0.3)	23.3	100.0(0.0)	17.5 b	91.7(-0.2) x
cyprodinil+ fludioxonil	5.0	33.3(-1.6)	23.3	100.0(0.0)	14.2 b	61.8 (-0.8) x
control(+)	60.0	100.0(0.0)	93.3	100.0(0.0)	76.7 c	100.0(0.0) x

[1] Values within the columns followed by the same letter are not significantly different according to Fisher's protected LSD at *P*=0.05. Fungicides effect for pathogen progression (a-c) was significant (*P*< 0.001; LSD =11.20). The effects of wounding (*P* <0.001) and fungicides × wounding were significant (*P*=0.002) for pathogen progression. For infection incidence wounding effect was significant (*P* =0.024; SED=0.30), as was fungicides effect (w-x) (*P*=0.001; SED=0.46). The fungicides × wounding effect was not significant (*P*= 0.277; SED=0.27).

[2] Logit data used for statistical analysis

5.3.6.3 Effect of fungicides on infection of hard green shoots when applied before inoculation with *Neofusicoccum ribis*

For the second analysis, the effect of fungicides was significant on infection incidence (*P*=0.013; Appendix E.16) (Table 5.15). The lowest mean incidence was for carbendazim (42.0%) which was significantly different (*P*<0.05) from cyprodinil + fludioxonil and tebuconazole (50.0% for both). There was no significant effect of wounding (*P*=0.587; Appendix E.16) nor a significant interaction between the fungicides and wounding (*P* =0.973; Appendix E.16). The effect of fungicides on pathogen progression was significant (*P*< 0.001: Appendix E.17). Carbendazim and tebuconazole were most (*P*<0.05) effective, with mean distances of 5.0 and 6.7 mm, respectively, which were less (*P*<0.05) than for flusilazole (15.0 mm), but similar (*P*>0.05) to cyprodinil + fludioxonil (10.0mm). There was a significant effect on wounding (*P*=0.015; Appendix E.17) on pathogen progression, with means for non-wounded and wounded shoots, being 20.0 and 26.0 mm, respectively. There was no significant interaction between the fungicides and wounding (*P* = 0.137; Appendix E.17).

Table 5.15: Effect of Fungicides on *Neofusicoccum ribis* progression (mm) and infection incidence (%) in non-wounded and wounded hard green shoots 60 days after inoculation.

Fungicides	Non-wounded		Wounded		Means	
	Pathogen progression	Infection incidence	Pathogen progression	Infection incidence	Pathogen progression	Infection incidence
carbendazim	3.3	33.0(-1.6)[2]	6.7	50.0(-1.1)	5.0 a[1]	42.0(-1.4)w
tebuconazole	8.3	50.0(-1.1)	5.0	50.0(-1.1)	6.7 a	50.0(-1.1)x
flusilazole	11.7	83.0(-0.3)	18.3	100.0(0.0)	15.0 b	92.0(-0.2)y
cyprodinil+ fludioxonil	6.7	50.0(-1.1)	13.3	50.0(-1.1)	10.0 ab	50.0(-1.1)x
control(+)	70.0	100.0(0.0)	86.7	100.0(0.0)	78.3 c	100.0(0.0)y

[1] Values within the columns followed by the same letter are not significantly different according to Fisher's protected LSD at P=0.05. For pathogen progression fungicides mean effect (a-c) was significant (P< 0.001; LSD =10.71). The mean effect of wounding was significant (P =0.015) and fungicides × wounding effect was not significant (P= 0.137). For infection incidence fungicide mean effect (w-y) was significant (P=0.013; SED=0.22). The mean effect of wounding was not significant (P =0.587; SED=0.30) and fungicides × wounding effect was not significant (P= 0.973; SED=0.44).

[2] Logit data used for statistical analysis

5.3.7 Effect of fungicides when applied after inoculation with *Neofusicoccum ribis* species

The effect of fungicides on the pathogen progression was significant (P< 0.001: Appendix E.18). The pathogen progression was low for carbendazim and tebuconazole, with mean distances of 25.0 and 26.7 mm, respectively, which were not significantly different (P>0.05) from each other. This was followed by cyprodinil+ fludioxonil with a mean progression of 48.3 mm, which was significantly different (P<0.05) from other treatments. The inoculated controls had the greatest progression, with a mean of 70.0 mm. External lesions were observed on only the inoculated controls. Infection incidence was 100% for all the fungicide treatments and the inoculated controls, and 0% for the non-inoculated controls.

5.3.8 Evaluation of fungicides under natural infection conditions

Of the plants placed in the field (n=24), six (25%) were positive for Botryosphaeriaceae infection (Figure 5.7) with a total of 18 isolates recovered. Botryosphaeriaceae infections were present in some plant materials (Figure 5.8) for every treatment except for tebuconazole treated plants, and were similarly frequent in bark and wood of shoots. The Pearson Chi square tests showed no significant association between overall Botryosphaeriaceae infection incidence and treatments (P= 0.187; Appendix E.19). The

highest infection incidence (50%) was found in the cyprodinil + fludioxonil treatment followed by carbendazim (16.7%), controls (16.7) and tebuconazole (0%) (Figure 5.7).

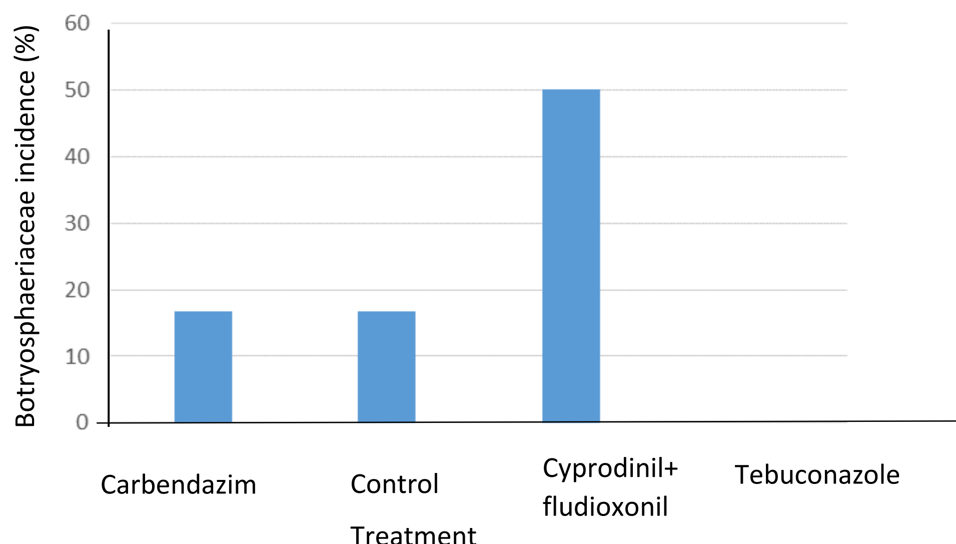


Figure 5.7: Botryosphaeriaceae incidence in blueberry plants treated with different fungicides

Under these natural infection conditions, low numbers of isolations were positive for Botryosphaeriaceae species. Number of isolates and species recovered from each treatment are shown in Tables 5.16 and 5.17. Light brown discolouration was observed in the leaves of one plant treated with cyprodinil+ fludioxonil, from which one isolate each of *N. ribis* and *N. australe* were recovered. Fruit infection was also found on the same plant, from which *N. australe* was recovered. Tip dieback, from which *N. parvum* was recovered, was observed in another plant which had been treated with cyprodinil+ fludioxonil. Of all the isolates recovered, *N. parvum* comprised 77.8%, followed by *N. australe* (11.11%), then *N. ribis* and *N. luteum* with 5.56% each.

Table 5.16: Number of Botryosphaeriaceae species isolates recovered from different plant parts treated with different fungicides.

	Hard green shoots				**Trunk**			
Fungicide	**WB**	**WW**	**NWB**	**NWW**	**Bark**	**Wood**	**Leaves**	**Fruit**
cyprodinil+fludioxonil	2	3	1	0	3	0	2	1
carbendazim	0	0	0	0	3	0	0	0
tebuconazole	0	0	0	0	0	0	0	0
control	1	1	0	0	1	0	0	0

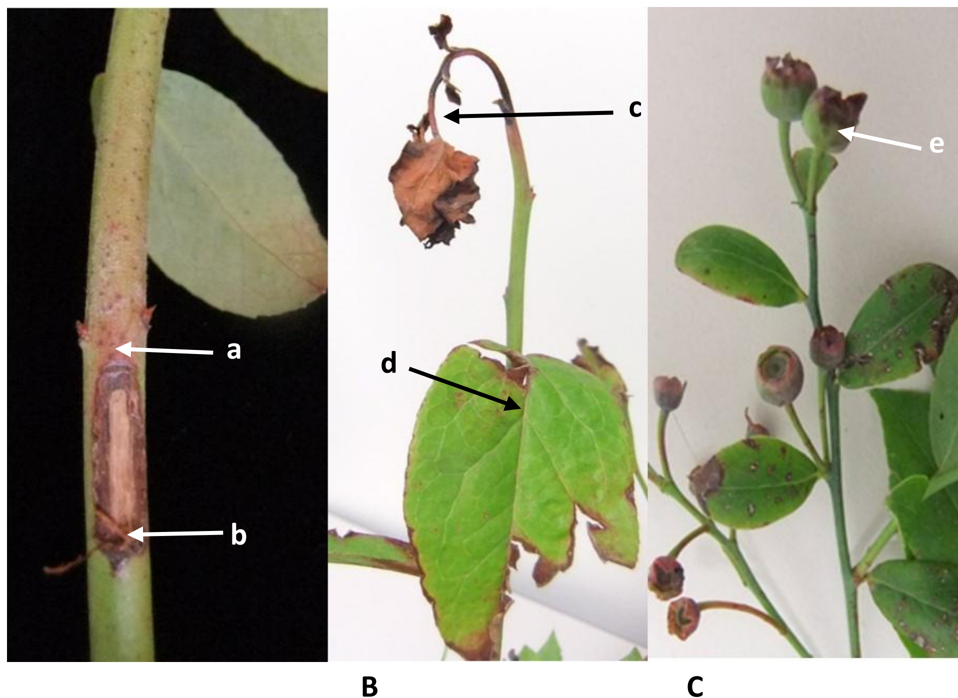
WB- wounded bark WW- wounded wood NWB- non-wounded bark, NWW non-wounded wood

Table 5.17: Number of isolates of each *Neofusicoccum* species recovered from the plant parts treated with different fungicides and controls.

	Botryosphaeriaceae species			
	N. australe	***N. luteum***	***N. parvum***	***N. ribis***
Treatment				
cyprodinil+ fludioxonil	2	1	8	1
carbendazim	0	0	3	0
tebuconazole	0	0	0	0
control	0	0	3	0
Percentage (%)	11.11	5.56	77.77	5.56

Trapping of water borne conidia and identification of Botryosphaeriaceae species

The pooled rainwater samples for each of the 15 rainfall events, provided 15 samples for nested PCR. Five samples were positive for the presence of Botryosphaeriaceae DNA, which were further identified using the SSCP method. Of the five samples. All were positive for the presence of at least one Botryosphaeriaceae species, three samples for the presence of *N. luteum*, *N. australe* and *D. seriata,* and two samples for the presence of *N. parvum*/*N. ribis*.



B **C**

Figure 5.8: Susceptibility of different blueberry tissues after being exposed to natural Botryosphaeriaceae infection conditions. A) Discolouration in the bark (denoted by arrow a) close to a wounded area in the green shoot (denoted by b). B) Tip dieback (denoted by arrow c) and discolouration observed on a leaf (denoted by arrow d). C) Infected fruit (denoted by arrow e).

5.3.9 Effect of copper hydroxide, phosphonate (phosphorous acid) and biocontrol products

Infection incidence in bark and wood was 100% for all the treatments. External lesions were visible on only the inoculated controls, in which the mean lesion length was 55.0 mm. For isolation distance, the effect of treatments was significant (*P*<0.001; Appendix E.20). There was no significant effect of tissue type (bark and wood) on pathogen progression (*P*= 0.711) and there was no significant interaction between the fungicides and tissue type on pathogen progression (*P*=0.854; Appendix E20). All of the treatments (copper hydroxide, phosphonate, *Bacillus subtilis* and *Trichoderma atroviride*) significantly reduced (*P*<0.05) *N. ribis* progression compared with the inoculated control, with there being no significant difference (*P*>0.05) between treatments (Table 5.18).

Table 5.18: Mean fungicide and biocontrol agents' effect on mean distance (mm) travelled by *Neofusicoccum ribis* on wounded soft green shoots.

Treatment	**Pathogen progression (mm)**		**Mean**
	Bark	**wood**	
phosphonate	31.7	25.0	28.3 a[1]
Trichoderma atroviride	35.0	30.0	32.5 a
Bacillus subtilis	35.0	30.0	35.0 a
copper hydroxide	33.3	38.3	35.8 a
control(+)	65.0	65.0	65.0 b
LSD			**11.36**

[1] Values within columns followed by the same letter are not significantly different according to Fisher's protected LSD at *P*=0.05. Fungicides mean effect for pathogen progression (a-b) was significant (*P*< 0.001; LSD =11.36).

5.4 Discussion

This is the first study to report fungicidal effects on inhibition of mycelial growth, conidial germination and germ tube growth of the four *Neofusicoccum* species commonly associated with the dieback of blueberries in New Zealand. The fungicides tested represented different chemical classes, which included systemic and contact fungicides, and were evaluated for effects on mycelial growth, conidial germination and germ tube growth of different isolates of four *Neofusicoccum* species. From the initial set of 9 fungicides tested fludioxonil, carbendazim, flusilazole and tebuconazole were effective in reducing mycelial growth of the four species, with EC_{50} values of less than 0.1 mg a.i. /L. For mycelial growth, *N. australe* was most sensitive and *N. luteum and N. parvum* were the least sensitive to the range of nine fungicides tested.

Recent research on fungicide efficacy against blueberry isolates of the *N. parvum* was conducted in Chile. Latorre *et al.* (2013) studied the efficacy of tebuconazole, iprodione and pyroclostrobin for the reduction of mycelial growth of two blueberry isolates of *N. parvum* and reported median effective concentrations (EC_{50}) obtained were 0.15-0.25, 0.26-0.33, and 0.52-0.68 $\mu g\text{-}mL^{-1}$. In the current study the mean EC_{50} values for *N. parvum* were 0.13, 0.75 and 0.78 $\mu g \cdot mL^{-1}$, respectively. Espinoza *et al.* (2009) also reported that mycelial growth of three isolates of *N. parvum* was affected by two of the six fungicides tested; EC_{50} values were cited only for fludioxonil and iprodione as 0.019 - 0.022 and 0.021 - 0.2 $\mu g \cdot mL^{-1}$, respectively. In the current study *N. parvum* was less sensitive to the iprodione. Bester *et al.* (2007) investigated the effect of 10 fungicides on mycelial growth of grapevine isolates of four Botryosphaeriaceae species. They found that the most effective fungicides overall were benomyl, tebuconazole and flusilazole, with EC_{50} concentration ranges of 0.36-0.55, 0.07-0.17 and 0.04-0.36 mg a.i./L. Pitt *et al* (2012) evaluated 17 fungicides for *in vitro* sensitivity against four Botryosphaeriaceae species (*N. parvum*, *F. aesculi*, *D. seriata* and *Lasiodiplodia theobromae*) from grapevines. The mycelial growth was significantly inhibited by 10 of the 17 fungicides tested. Of these, fludioxonil was the most effective followed by carbendazim, fluazinam, tebuconazole and flusilazole with mean EC_{50} values of <0.1 $\mu g/\cdot mL$, which is similar to the results of the present study. The differences in efficacy reported in the

literature and in the results of current study may be associated with formulations of the fungicides, experimental conditions and differences between isolates and species.

In the current study, there was variation between different isolates with a species in their response to the different active ingredients tested. For an example thiram had higher EC_{50} values for two isolates of *N. luteum* and *N. ribis* than the remaining isolates. Similar differences in the mycelial growth of different isolates were also observed by Amponsah *et al* (2012) who reported that metalaxyl had higher EC_{50} values for two of three isolates of *D. mutila* and *N. luteum* than the third isolate. Amponsah *et al* (2012) reported that flusilazole, carbendazim, tebuconazole and iprodione were most effective against mycelial growth of *N. australe*, *N. luteum* and *D. mutila*, with mean EC_{50} values of 0.002, 0.037, 0.055, and 0.328 mg a.i/L, respectively. The same fungicides were effective in the current study.

For conidial germination and germ tube growth, carbendazim, iprodione, pyroclostrobin and thiram were the most effective in the current study, with mean EC_{50} values of less than 0.1 mg a.i. /L. Tebuconazole was the least effective, with high EC_{50} values for germination and for germ tube elongation (3.12 and 4.67 mg a.i. /L, respectively). Amponsah *et al.* (2012a) reported that of the fungicides tested procymidone, carbendazim, iprodione, mancozeb and flusilazole were the most effective in conidial inhibition of Botryosphaeriaceae species of grapevines. However in the current study mancozeb and flusilazole were only moderately effective in inhibiting conidial germination and germ tube elongation. For conidial germination and germ tube growth, *N. luteum* and *N. australe* were the most sensitive to the fungicides tested, whereas *N. parvum* and *N. ribis* were the least sensitive. In contrast, mycelial growth studies showed that *N. australe* was the most sensitive to the fungicides tested and *N. parvum* and *N. luteum* were the least sensitive.

In this study, the fungicides that performed well in reducing mycelial growth generally did not perform well in inhibiting conidial germination and germ tube growth. The effectiveness of sterol demethylation inhibitors (DMI) against Botryosphaeriacea species varied among the DMI compounds tested (e.g. flusilazole and tebuconazole) and have been reported from other studies to be, in general, less effective in reducing conidial germination than mycelia growth *in vitro* (Torres *et al.,* 2013). This response is probably related to the generic mode of action of the triazoles which is to inhibit demethylation (DMIs), a key step on formation of

the cell membranes. So the effect will be greatest on mycelial extension as the fungus attempts to grow rather than germination (effectively they cause leaky membranes). Other researchers have also reported similar results for other grapevine trunk pathogens; in most cases, the active ingredients that effectively reduced mycelial growth did not perform well in inhibiting conidial germination and those that reduced conidial germination well did not performed well in mycelial growth reduction (Rego *et al.,* 2006; Alaniz *et al.,* 2011; Bleach, 2012). In the current study, there was a trend for lower EC_{50} values for inhibition of conidial germination and germ tube growth, which was much lower than their field rates (Appendix E21). This was also observed by Jaspers (2001) who reported fungicide sensitivity studies against *P. chlamydospora* and further stated that efficacy of these compounds in inhibiting conidial germination was an indication that they might be effective as wound protectants. Since conidia are likely to be the main type of inoculum in blueberry orchards the effectiveness of the fungicides to inhibit conidial germination is likely to be a better indication of their effectiveness to protect against infection in the orchard. Overall, in this study carbendazim was seen to be the most effective in inhibiting mycelial growth, conidial germination and germ tube growth of the four *Neofusicoccum* species.

An additional three fungicides (cyprodinil+ fludioxonil, copper hydroxide and phosphonate) were evaluated *in vitro* for inhibition of mycelial growth, conidial germination and germ tube growth at the request of Blueberry New Zealand (BBNZ). Results showed that copper hydroxide was effective in inhibiting mycelial growth, conidial germination and germ tube growth of the four species tested and that phosphorous acid was only effective in reducing the germ tube growth. Cyprodinil + fludioxonil was effective for reducing mycelial growth of some isolates but not others, being effective for three *N. ribis* isolates, one *N. parvum* isolate and one *N. australe* isolates. It was also highly effective against germination of all isolates but less effective in inhibition of germ tube elongation. The EC_{50} values for copper hydroxide found in this study (831.8 and 0.37 mg a.i/L) for mycelial growth and conidial germination, respectively) conflict with some other studies. Amponsah *et al.* (2012c) reported EC_{50} values of 69-107 mg a.i/L for copper hydroxide against mycelial growth and 4.2-5.7 mg a.i/L for conidial germination of *N. australe*, *N. luteum* and *D. mutila*. Everett *et al.* (2005) reported EC_{50} values for effects of copper hydroxide on mycelial growth and conidial germination of 587.0 and 3.6 mg a.i. /L, respectively, for *N. parvum* isolated from

avocado fruit, which was also different to the values obtained in the current study. These differences could be due to the variation among isolates and differences in experimental conditions.

There are no published reports available related to *in vitro* evaluation of phosphorous acid against Botryosphaeriaceae species which could be used for comparison. Fenn and Coffey (1984) demonstrated that it was effective on *Phytophthora* species, but much less inhibitory *in vitro* on *Pythium* species and had low activity against a selection of non-oomycetous fungi (*Alternaria alternate*, *Rhizoctonia solani*, *Thielaviopsis viticola*, *Neurospora tetrasperma* and *Colletotrichum phonioides*). Jaspers (2001) reported that phosphorous acid was ineffective at reducing mycelial growth and conidial germination of *P. chlamydospora*, with EC_{50} values similar to those in the current study. However, phosphorous acid is believed to induce resistance against a number of pathogens in the host plant after being absorbed (Fenn and Coffey 1984). Therefore these types of chemicals are better tested on plants inoculated with the pathogen rather than laboratory evaluation in artificial media.

Trichoderma atroviride (Vinevax™) has been recommended for diseases like Eutypa dieback, black dead arm and Petri disease in crops like grapes, orchards and other ornamental plants. *Bacillus subtilis* (Serenade® MAX) has been recommended for some diseases like anthracnose, fireblight, botrytis and downy mildew in crops including grapes, citrus and avocado (New Zealand Novachem Agrichemical Manual 2015). However these products are not registered for Botryosphaeria dieback in blueberries. In dual plate assays with *T. atroviride* and isolates of *Neofusicoccum* species, *T. atroviride* cultures grew over the pathogens and appeared to inhibit them but there were no clear zones between the pathogens and *T. atroviride* colonies. This result may have been affected by the experimental method which used one isolated strain from the *Trichoderma* product, which was described as containing five strains that might have had different levels of activity. John *et al.* (2004) and Kucuk and Kivanc (2004) stated that a clear zone of inhibition between the pathogen and a *Trichoderma* spiecies were most likely due to the volatile and non-volatile substances released by the *Trichoderma* species They also stated that the ability of *Trichoderma* species to overgrow the pathogen could help to reduce its growth in the dual plate assays, and is a good indication of competition. When percent inhibition was

measured results showed that it inhibited the *Neofusicoccum* species to some extent, with mean inhibition of 46.8%. When microscopic studies were carried out, coiling of *Trichoderma* sp. hyphae around those of the pathogen was expected. However it was difficult to identify the coiling compared to controls. In contrast, Kotze *et al*. (2011) who used the same procedure as used in the current study, reported that adhesion or coiling of antagonist hyphae or disintegration of pathogen hyphae were observed when three commercial strains of *T. atroviride* were tested against *N. australe, D. seriata*, *N. parvum* and *L. theobromae*. These mycoparasitic reactions (coiling and adhesion to the pathogen hyphae) facilitate the activities of the cell wall degrading enzymes of *Trichoderma* species which enable them to extract nutrients from their target fungi (Almeida *et al*., 2007).

When dual plate assays were conducted with a *B. subtilis* strain isolated from the Serenade™ commercial product and *Neofusicoccum* isolates, growth of all isolates of *N. australe*, *N. luteum, N. ribis* and *N. parvum* was reduced in comparison with the controls (mean of 52.8%). Further, small inhibition zones were formed between the colonies of the *B. subtilis* and one *N. parvum* isolate, with swelling of hyphae observed microscopically. Similar results were observed by Kotze *et al.* (2011), who tested *B. subtilis* (strain EE 1/10) against *N. australe*, *D. seriata*, *N. parvum* and *L. theobromae*. Stein (2005) stated that *B. amyloliquefaciens* and *B. subtilis* produced a range of antimicrobial dipeptides or cyclic lipopeptides while Baker and Cook (1982) stated that the antibiotics secreted from *B. subtilis* were capable of penetrating fungal hyphae and causing malformations. The formation of inhibition zones and reduction of mycelial growth in the current study was an indication of an inhibitory compound secreted by *B. subtilis*. When another experiment was conducted by spreading the pathogen conidia over PDA plates and then inoculating specific sites with *B. subtilis*, zones of inhibitions were observed for only one isolate of *N. parvum* (S1-173). However, the inhibitory compounds secreted by *B subtilis* are mainly active against mycelial growth and not conidial germination (Stein 2005) which this may account for the lack of inhibition zones. However, Alfonzo *et al.* (2009) used an agar diffusion technique to test a crude extract of media in which *Bacillus subtilis* (strain AG1) had grown. They placed the media extract into wells made in agar seeded with spores of vine wood pathogens (*Botryosphaeria rhodina*, *Verticillium dahlia*, *Phaeomoniella chlamydospora,* and *Phaeoacremonium aleophilum*). In contrast the current results, their results revealed that

the extract was effective in preventing germination of conidia of all the pathogens tested and was most effective on *Botryosphaeria rhodina*. However, the “active fractions” within the growth media were extracted into 96% ethanol before use but they did not have an ethanol only control. A similar method to that used in the current study was conducted on PDA and Kings B agar by Cazorla *et al.* (2007) who demonstrated that on both agars *B. subtilis* strains PCL1608 and PCL1612 inhibited the growth of all the target fungi, *F. oxysporum* f. sp. *radicis-lycopersici, Phytophthora cinnamomi, Pythium ultimum, Rosellinia necatrix, Rhizoctonia solani* and *Sclerotium rolfsi,* while two other *B. subtilis* strains (PCL1605 and PCL1610) inhibited growth of only some target fungi. Further, Fiddaman and Rossall (1994) demonstrated that antifungal activity against *Rhizoctonia solani* by a *B. subtilis* strain on nutrient agar was enhanced by the addition of potato starch, as well as D-glucose, complex carbohydrates and peptones. In the current study, the nutrient rich media should have been adequate and the strain was isolated directly from the commercial product, therefore the reasons for the poor efficacy in the *in vitro* experiments are not immediately clear. To get a better understanding of whether fungicides and biocontrol agents are effective in disease management, it was considered important to carry out *in vivo* experiments because biocontrol agents may act differently in the field than under laboratory conditions. Also need to be retested under different seasonal and climatic conditions to ensure consistency before recommended

In the potted plant experiments, efficacy of the fungicides, paints and biological agents was tested against only *N. ribis* because it was found to be the most pathogenic species in blueberries, causing rapid lesion development and death of the plant. The fungicides tested were the most effective in the current *in vitro* studies or had been reported to be effective on other hosts (Ma *et al.,* 2001 Bester *et al.,* 2007). In these experiments the ability of the different chemicals to inhibit *N. ribis* infection of buds, soft green shoots and hard green shoots were evaluated. Results showed that in buds wounding had no effect on *N. ribis* incidence. However some fungicides were effective; carbendazim and tebuconazole were most promising for reducing *N. ribis* infection incidence. There are no data relating to fungicides evaluation against Botryosphaeriaceae bud infections in blueberries for comparison.

With soft green shoots visible lesions, developed only in wounded controls (no fungicide treatment) and wounded paint-treated ones. In hard green shoots, there were no visible lesions observed for any of the treatments including wounded controls (no fungicide treatment) and paint treated ones. The hard green shoot experiment was set up during winter and in an open area which may have been too cold for development of lesions while inoculated soft green shoots were incubated in a glasshouse where temperatures were maintained at 20-23°C. Latorre *et al.* (2013) conducted three fungicide trials on 1 year old blueberry stems (cultivar Duke and O'Neal), one in autumn and two during spring. They showed that lengths of lesions were considerably affected by the date of inoculation and longest lesions were obtained from spring inoculations.

Pathogen incidence in soft green shoots was much greater in wounded shoots than in non-wounded shoots (83.3 and 56.6%, respectively), as was pathogen progression, being 31.6 and 17.0 mm, respectively. In hard green shoots pathogen incidence was similar in wounded and non-wounded shoots, being 70.0 and 63.0%, respectively, as was pathogen progression, being 26.0 and 20.0 mm, respectively. The lower incidence in non-wounded than wounded soft green shoots may have been due to the relatively short incubation time, since pathogen entry through the cuticle may have taken some time. *Neofusicoccum ribis* development and infection incidence have repeatedly been found to be higher in soft green shoots than in hard green shoots, indicating that soft shoots are more susceptible to infection than hard green shoots even after fungicide treatments.

Although none of the fungicides were able to completely inhibit infection for soft green shoots or hard green shoots, all the fungicides tested were clearly capable of reducing the pathogen development within the tissues. Carbendazim was the most effective at reducing *N. ribis* development, followed by tebuconazole. Infection incidence was greatly reduced by carbendazim which performed well in both soft green shoots and hard green shoots. The efficacy of carbendazim was expected because it was very effective in reducing *in vitro* mycelial growth and conidial germination. Tebuconazole on the other hand which was not highly effective in reducing conidial germination was effective in the *in vivo* study. Similar reduction in infection from the conidial inoculum by fludioxonil + cyprodinil was expected due to its effectiveness at reducing conidial germination *in vitro*, but it was not highly

effective in the *in vivo* study. This illustrates the importance of testing the fungicides *in planta* since fungicides can perform differently on living plants than under laboratory conditions. Tebuconazole was also reported to be effective in field trials by Latorre *et al.* (2013) who used benomyl, iprodione, pyraclostrobin and tebuconazole to protect wounds on 1 year old blueberry stems (cultivar Duke and O' Neal), which were inoculated with mycelial plugs of *N. parvum* 24 h after the fungicide treatments. Plants were assessed for lesion development 30 days after inoculation and results revealed that benomyl, tebuconazole and iprodione were, in order of efficacy, the most effective fungicides against *N. parvum*. Amponsah *et al.* (2012c) evaluated 12 fungicides on cane pruning wounds of 2 year old potted grapevine plants (cultivar Pinot noir), which were inoculated with conidia of *N. luteum* after 24 h. Results after 3 months incubation revealed 0% incidence in plants treated with carbendazim, iprodione, mancozeb and flusilazole, and all the other fungicides (including tebuconazole) were relatively ineffective with 20-70% incidence. However in a second experiment, with treatment and inoculation on trunk wounds of 1 year old Pinot noir grapevines, which were assessed after 6 months, the most effective fungicides were flusilazole, carbendazim, tebuconazole and thiophanate methyl, with 0, 9, 16 and 20% incidence. The results of Amponsah *et al.* (2012c) differ from those of Bester *et al.* (2007) whose glasshouse experiment used 1 year old grapevine cuttings (cultivar Chenin Blanc) placed into tubes with sufficient water to allow budding. Cuttings were then wounded, treated and inoculated with conidial suspensions of *D. seriata*, *N. australe*, *N. parvum* or *L. theobromae*. Results after 3 months incubation revealed low infection incidence in shoots treated with benomyl, tebuconazole and prochloraz which were more effective as wound protectants than flusilazole and copper ammonium acetate. They reported significant differences in lengths of lesions caused by the different species but no species x treatment interaction, which indicated that all species were similarly affected. The difference between these two cited studies may be due to the Botryosphaeriaceae species used as Amponsah *et al.* (2012c) also showed differences between species but not isolates in *in vitro* experiments with three isolates of each of *N. australe, N. luteum* and *D. mutila*. In another study (Twizeyimana *et al.,* 2013) evaluated five fungicides (azoxystrobin + propiconazole, metconazole, pyroclostrobin, fludioxonil + cyprodinil, myclobutanil) for abilities to reduce sizes of lesions that developed after inoculating wounds with mycelial fragments of *D.*

iberica, *N. australe*, *N. luteum*, *N. parvum* and *Phomopsis* sp. in avocado in California. Plants were assessed after 6 months for lesion development and results revealed that the most effective fungicides were azoxystrobin + propiconazole and metconazole, while fludioxonil + cyprodinil showed some level of reduction in lesion development compared with the controls. However the differences in performance of the fludioxonil + cyprodinil between that study and the current study could be due to the inoculum types, species used or the host tissues used.

The acrylic paint treatment in the current study was ineffective. However closer examination of the painted surfaces showed that cracks had developed in the paint which may have facilitated pathogen penetration into the shoot. These cracks were more evident in soft green shoots than hard green shoots which may have partly accounted for the greater distance of pathogen progression in soft green shoots than in hard green shoots. Pitt *et al.* (2012) tested acrylic paint on pruning wounds of grapevines against conidia of *D. mutila*, and results revealed that it was capable of providing 46% disease control in some trials. Further, the paint products which contained fungicide compounds also provided control, with 42-65% incidence for Garrison (containing cyproconazole and iodocarb) and 38% for Bacseal Super (containing tebuconazole). These pruning wound protectants could be evaluated on blueberries but they are time consuming to apply and so only suitable for large wounds.

In the experiment conducted to evaluate the activity of carbendazim, tebuconazole and cyprodinil + fludioxonil when applied to soft green shoots after inoculation with *N. ribis*, no lesions were observed in any of the treatments apart from the inoculated controls. Pathogen movement in the tissues treated by all the fungicides was less than in the controls, however, the infection incidence was 100%, which indicated that *N. ribis* was only inhibited not eliminated. Overall, disease progression was lower for tebuconazole and carbendazim, which are systemic fungicides, than for cyprodinil + fludioxonil which contains both systemic (cyprodinil) and contact (fludioxonil) active ingredients. However, these fungicides need to be applied immediately after wounding as application 24 h after inoculation provided poor protection from infection. Twizeyimana *et al.* (2013) evaluated the effect of fungicides applied to pruning wounds in branches of avocado to inhibit

infection when mycelial fragments of *D. iberica*, *N. australe*, *N. luteum*, *N. parvum* and *Phomopsis* sp. inoculated a day or a week after treatment. Results revealed that there was no significant difference between inoculation times and they concluded that a week might not be enough to observe the fungicide residual activity. This is directly applicable to the current study as the incubation period of 24 h before the fungicide treatments might be sufficient (results of Chapter 3) for the pathogen to penetrate and progress into the tissues resulting in a reduction in the effectiveness of the fungicides when the plants were already infected with the pathogen. Some studies have shown the impact of dose or the concentration of the fungicide on infected tissues. Kelley and Jones (1981) revealed that bitertanol, a Triazole product applied at 150 µg a.i. /mL was less effective than at 300 µg a.i. /mL for suppressing apple scab lesion development caused by *Venturia inaequalis* when applied 5 days after inoculation when leaf lesions had already been initiated. Twizeyimana *et al.* (2013) stated that fungicides lost their ability to control diseases due to the dilution of systemic fungicides inside the plant tissues as the plants grew or washing off of protectants by rain. However, to fully understand the efficacy of fungicides in the infected plants further work is required to evaluate the effect of different fungicide concentrations, inoculum dose and infection durations.

When three fungicides (carbendazim, tebuconazole and cyprodinil + fludioxonil) were evaluated under natural Botryosphaeriaceae species inoculum conditions, results showed that tebuconazole could protect the plants from infection for up to 12 weeks. The controls and other treatments were infected with *N. parvum* confirming the presence of *N. parvum* spores in the field. The high levels of infection in the plants treated with carbendazim and cyprodinil + fludioxonil may have been due to the uneven distribution of natural inoculum present in the field, which could have been released during the 12 weeks between treatment and assessment, causing further infections after the expected period of efficacy. The infection incidence of *N. parvum* in the plants was reflected by the presence of the pathogen in rain water samples. However the SSCP method was not able to distinguish *N. parvum* and *N. ribis* in the samples. The rain water samples showed that inoculum distribution were uneven in the field; out of 15 samples, only five were positive for the presence of Botryosphaeriaceae DNA. However, since detection by the nested PCR and SSCP method was reported be approximately 0.1 pg, which was approximately equivalent to the

DNA in 2.5 conidia (Ridgway *et al.,* 2011), and as little as two conidia of *N. luteum* provided for 100% incidence of inoculated wounds in wounded soft green shoots of grapevines, the inoculum in each trap would seem to be sufficient for infection to occur. Luque *et al.* (2008) studied efficacy of benzimidazoles (benomyl or carbendazim), cyprodinil + fludioxonil, thiabendazole and thiophanate- methyl sprayed onto cork oak trees after annual cork removal and assessed canker development by natural inoculum of Botryosphaeriaceae species after 8 months. Incidence of cankers was not greatly reduced but canker area was, with reductions for thiophanate methyl (58%), carbendazim (62%) and cyprodinil + fludioxonil (36%). The efficacy may be due to the method of treatment since they removed the cork and applied the fungicide using a portable atomizer which might have assisted the impregnation of the plant tissue by the fungicide. Pitt *et al.* (2012) evaluated nine fungicides in four commercial vineyards in Australia, painted onto wounds to prevent infection by conidia of *D. seriata* and *D. mutila.* A year later, mean pathogen incidences in treated tissues revealed that carbendazim (45%), fluazinam (57%) and tebuconazole (64%) were the most effective. They also reported that tebuconazole and cyprodinil + fludioxonil were more effective when applied at 10× the recommended rates, with means of 59 and 66% incidence, respectively. This investigation had very high rates of infection which indicated high levels of natural inoculum, due to frequent periods of rainfall after the fungicide protection had worn off (Pitt *et al.,* 2012). Also they assessed infection after 1 year providing greater chance of infection, than in the three months used in the current study. Further, the different species prevalent in study by Pitt *et al.* (2012) might have accounted for differences in efficacy compared with the current study.

Sprays of copper hydroxide and phosphonate, and biocontrol agents were unable to prevent infection, but all treatments reduced pathogen movement in the plants compared with controls and prevented development of external lesions. Blueberry growers in New Zealand have reported that treating the plants with phosphonate helped to reduce infection by Botryosphaeriaceae species, probably because external lesions were no longer visible, as shown here. Elliott and Edmonds (2008) also evaluated the effect of phosphorous acids along with other fungicides (propiconazole, thiabendazim, paclobutrazole, carbendazim, copper and tebuconazole) in minimizing the canker growth in madrones inoculated with *Fusicoccum arbuti.* Fungicides were injected into the trees and assessed one and two years

after application. In both years phosphorous acid performed well in reducing canker development compared with other fungicides. However in the current study, pathogen isolation was carried out after 14 days. If plants had been left for longer before isolation, they might have shown even less pathogen progression due to the longer term effects of the phosphorous acid. In addition, stem injection, as was carried out in the study of Elliot and Edmonds (2008), might have increased the absorption of the chemical by the plants and induced greater systemic resistance in the plants.

Limited effects of copper hydroxide were also reported by Amponsah *et al.* (2012c) in potted grapevines, with 20% incidence and 42 mm dieback compared to 100% incidence and 114 mm lesions for the inoculated control canes. The higher efficacy they reported may have been due to the different methods, since they inoculated wounds with conidia of *N. luteum* 24 h after spraying with fungicides and results were evaluated after 3 months. Fungicide activity might have been higher in the wound area compared with the current study in which plants were left for a week before inoculating. Also in the current study effectiveness may have been improved by increasing the number of applications.

Even though strains isolated from the biological products Vinevax® (*Trichoderma atroviride*) and Serenade® (*Bacillus subtilis)* did not perform that well in the *in vitro* studies, results showed some level of control on plants. Latorre *et al.* (2013) also reported that the two commercial biological products, Serenade WP and Trichonativa *(Trichoderma* species), were ineffective in controlling blueberry die-back caused by *N. parvum*. However, in that study the plants were inoculated 24 h after treatment and this may not been sufficient time to allow the biocontrol organisms to colonize the plant tissue prior to pathogen inoculation. Kotze *et al.* (2011) who used similar methods to the current study demonstrated that *Trichoderma* products (Biotricho®, Vinevax® and ECO 77®), two *T. atroviride* isolates and *B. subtilis* were capable of reducing disease incidence by *N. australe*, *N. parvum*, *D. seriata, L. theobromae, Ph. viticola*, *E. lata and Pa. chlamydospora.*

Pruning wounds have been shown to be a primary route of entry for Botryosphaeriaceae species into grapevine stems. However in the present study on blueberries, isolates were recovered in similar numbers from the both wounded and non-wounded tissues. Further, the consistent isolation of Botryosphaeriaceae from bark in plants exposed to natural

Botryosphaeriaceae inoculum indicated that Botryosphaeriaceae were able to infect without wounds. However, the more prominent lesion development was observed in the wounded tissues indicating that the Botryosphaeriaceae were able to colonise the wood more rapidly if provided with wounds. Although total protection was not achieved by any of fungicides in this experiment, results revealed that a single application of tebuconazole and carbendazim reduced disease incidence during the three months in the field, where only natural inoculum was present. However, more than one application might have provided better control of the disease. Further, the inoculum levels present in the field are likely to be much lower than those used with artificial inoculation in these experiments, so these products are likely to provide better control in commercial berry fields than in the inoculated experiments. Further experiments with these products should be conducted in commercial properties, with multiple applications being based on likely risk periods for inoculum, not on pruning times as recommended for grapevines.

Chapter 6

Concluding Discussion

This study investigated the prevalence of Botryosphaeriaceae infections in New Zealand blueberry farms, nurseries, their source of inoculum, factors affecting infection and disease expression and also the effectiveness of some control options such as fungicides and biocontrol agents for preventing infection.

Prevalence of Botryosphaeriaceae infections in New Zealand blueberry farms and nurseries

Sampling of necrotic tissues in blueberry farms in 2013-2014 showed that Botryosphaeriaceae species were associated with rapid wilting, reddening of leaves, stunted growth and dieback of blueberry plants. These pathogens were present in five out of the seven farms sampled, with 41% overall incidence. The identities of the 75 *Neofusicoccum* isolates recovered were confirmed through a combination of morphological and molecular techniques as *N. australe* (79%), *N. luteum* (8%), *N. ribis* (8%) and *N. parvum* (5%). Identification of the remaining 45 isolates from the 2009/2010 blueberry farm sampling of North and South Island farms also showed similar species dominance, with *N. australe* (47%), *N. parvum* (20%), *N. luteum* (18%) and *N. ribis* (15%). The main species identified and their frequencies were consistent with the results of Sammonds *et al.* (2009) and Che Omar (2009) who identified isolates from blueberry farms mainly from the North Island. Overall, this study have assessed samples from farms throughout New Zealand, although did not conform to a random sampling pattern the samples received represented a reasonable cross section of blueberry farms.

Blueberry nursery sampling was conducted in a similar way and represented a first attempt to conduct such an investigation in blueberry nurseries. Assessment of the samples sent by four nurseries showed that Botrosphaeriaceae infections were present in 45% of nursery plants from all nurseries. The 38 isolates recovered from these nursery samples were identified as *N. australe* (66.0%), *N. parvum* (31.5%) and *N. ribis* (2.5%). Further random sampling from within a source block of one nursery showed external contamination by these pathogens in 18, internal infections in 13 of the 20 samples, 100% infection incidence

overall, and with multiple infections in some cuttings. These isolates were identified as *N. parvum/ribis* (20), *N. australe* (10), *N. luteum* (6) *D. mutila* (5) and *D. seriata* (2).

Propagation mixtures were also infested with Botryosphaeriaceae species, with 43 of the 98 samples received (44.0%) being positive for presence of Botryosphaeriaceae DNA. Species identified using SSCP were *N. australe*, *N. luteum*, *N. parvum/ribis* and *D. mutila*. Studies by Castilo-Pando *et al.* (2001) and Whitelaw-Weckert *et al.* (2006) had concluded that Botryosphaeriaceae species infections of grapevines could be initiated by soil borne inoculum, so experiments were conducted to determine whether presence of soil-borne inoculum of *N. australe* and *N. ribis* could lead to plant infection. However results were negative. Although these plants were shown to be infected, including the roots, these were with isolates other than those used as inoculum (as demonstrated by the genotyping studies). However, when crowns of plants were inoculated with three *Neofusicoccum* species in a later experiment, the pathogens were shown to move into the roots, which may explain the results of Castilo-Pando *et al.* (2001) and Whitelaw-Weckert *et al.* (2006).

Overall, the high rate of infection in nursery plants indicated that nurseries can be a major source of infection for blueberry farms. Since the pathogen was isolated from asymptomatic plants and cuttings, it demonstrated that some infections were unlikely to be detected during plant grading. This research has shown that infections by Botryosphaeriaceae species are widespread in blueberry farms and nurseries in New Zealand. Overall results from the study indicated that three species which dominated the blueberry farms also dominated in the nurseries, being *N. australe*, *N. parvum* and *N. ribis*. A more extensive survey of nurseries, with random sampling of propagation materials, would help to confirm the prevalence of Botrosphaeriaceae species.

The sources of inoculum for nursery and farm infection are likely to be from within the individual properties but may also be from other host plants on adjoining properties. Espinoza *et al.* (2009) showed three *Neofusicoccum* isolates (*N. arbuti*, *N. australe* and *N. parvum*) recovered from symptomatic blueberry plants in Chile were pathogenic to apple fruits and kiwifruits. In New Zealand, Amponsah *et al.* (2011) showed that *Neofusicoccum* isolates recovered from non-grapevine woody hosts were pathogenic and reproduced on grapevines. *Neofusiccocum macroclavatum,* a recognised pathogen of *Eucalyptus globulus* in

Western Australia, was also isolated from grapevine propagation material, which was collected by Billones-Baaijens *et al.* (2012) from a New Zealand nursery which had a eucalyptus hedge. In New Zealand, blueberries are commonly grown close to vineyards, orchards, shelter belts and other woody plants. Therefore, these plants may act as alternative hosts, providing inoculum for blueberry plants. Since non-blueberry plants near the blueberry farms were not sampled, further sampling should include other crops in New Zealand and this will improve understanding of Botyrosphaeriaceae species inoculum sources.

Since propagating cuttings are the likely sources of infection for nurseries, these should be targeted in the control strategies. If disease-free source blocks could be developed, this would result in production of disease-free cuttings. If source blocks were frequently sprayed with fungicides, this would also reduce the likelihood of infection. Another option is the disinfection of the shoots commonly used for propagation. Since soft shoot tips are usually used for propagation, they cannot be disinfected by hot water treatment. However, disinfection of dormant grapevine canes by soaking for 30 min in benomyl was shown to be highly effective in eliminating these pathogens from the propagating material (Billones-Baaijens *et al.*, 2015). Therefore, experiments should be conducted to determine whether similar methods could be used with blueberry shoots.

Botryosphaeriaceae infection and disease expression

Most of the reported pathogenicity studies of Botryosphaeriaceae species on blueberries have been conducted using mycelial plugs which do not reflect the natural infection conditions. The development of a fast and reliable method for production of conidia of the main *Neofusiccocum* isolates allowed more realistic pathogenicity studies to be conducted. All the four main species recovered from the blueberry farms and nurseries were found to be pathogenic on soft green shoots and hard green shoots, although pathogenicity differed significantly between species and isolates within a species. Overall, *N. ribis* was the most pathogenic followed by *N. parvum, N. luteum* and *N. australe,* and all species produced pycnidia and conidia. This results was consistent for detached and attached wounded soft and hard green shoots. Further, a pathogenicity study on attached wounded and non-wounded shoots showed that *N. ribis* and *N. australe* conidia could infect the non-wounded

soft and hard green shoots, but without causing immediate lesions. Many researchers have measured lesion lengths and used these as proof of pathogenicity, but the studies reported here have shown that lack of lesion development does not equate to absence of infection or pathogen progression within shoots. Further, isolation studies from lesions demonstrated that the pathogen was endophytic, being able to move beyond the lesion edges. Therefore, growers should remove at least an additional 10 cm of shoot tissues beyond the lesion when pruning, as recommended for grapevines when removing lesions caused by *Eutypa lata* (Sosnowski *et al.,* 2007). Infection of wounded and non-wounded hard green prunings also showed that if prunings were left lying within blueberry farms, they could become infected, possibly even releasing conidia during favourable conditions to infect the pruning wounds of healthy plants. Therefore, it is important for growers to remove or bury their pruned shoots soon after winter pruning and summer trimming.

Pathogenicity studies conducted with *Neofusiccocum* species on buds and fruits also showed that these non-wounded tissues could become infected, with rotting of the tissues and infection spreading into the stems. These symptoms of disease have not been widely recognised by growers. Further, fruit rot is commonly blamed on *Botrytis cinerea* which was found to be associated with rotting fruit and stem-end scars (Johnson and McKenzie 1982)*;* since this is a coloniser of necrotic tissues, it is possible that it is a secondary pathogen of the fruits. Pycnidia and conidia of *Neofusiccocum* species were also observed on infected buds and fruit so they may also act as sources of inoculum to infect stems and so cause further dieback of the plants, especially when shoot trimming is done in the farms.

Overall, the most pathogenic species were considered to be *N. parvum* and *N. ribis* as they were detected most frequently on all tissues and formed the largest lesions on stems. For that reason the cultivar susceptibility tests were carried out using these two species. The results showed that all the cultivars tested were susceptible, with some differences in incidence and lesion lengths, which varied between tissues and between cultivars. Overall, Maru and Ocean blue seemed to be the most resistant. This was the first study to test blueberry cultivar susceptibility for Botryosphaeriaceae species in New Zealand. However, only seven cultivars were available to purchase from the nurseries. Since many more cultivars are commonly grown in New Zealand, these cultivars should be incorporated into a

further study, which should investigate effects of different conidial concentrations and a range of tissues.

All these pathogenic studies were carried out using high conidial concentrations (10^6/mL for shoots, trunks and crowns and 10^4/mL for bud infection studies). These were equivalent to 500-50,000 conidia on the inoculation sites, which is likely to be more than is usually present on tissues in the field. In preliminary shadehouse studies using soft green shoots lower concentrations (10^4/mL) took a much longer time (2-3 months) to express symptoms than the 10-14 days found with higher concentration. Therefore, higher concentrations were used during these studies to expedite the experiments.

Since *N. ribis* progressed rapidly through blueberry tissues and caused longer lesions than other species it was used for further inoculation studies of blueberry shoots and trunks. When wounds of different ages were inoculated, results showed that infection progression and lesion development was greater for fresh wounds than old wounds, and on the harder tissues lesions were observed only after inoculation of fresh wounds. However, in these tissues, the pathogen had still progressed to 33-35 mm from the old wounds. An investigation into the effect of season on infection success indicated that infection could take place throughout the year, although pathogen progression was relatively slow following winter inoculation. Higher infection incidence and pathogen progression was found in the bark than the wood which suggested that the bark might contain latent infections, which may be able to grow towards and infect wounds when trimming is done during any season. This showed the importance of applying wound protectants as soon as plants are pruned.

Dieback of plants has been anecdotally reported to occur after application of herbicides to the understory areas in the farms and so the susceptibility of herbicide damaged tissues to Botryosphaeriaceae species was investigated. This study showed that wood and bark areas damaged by three herbicides were invaded by *N. ribis* and became equally as infected as those areas with mechanical damage. Further, the herbicides caused death of affected shoots. This information will increase the awareness of the farmers for proper application methods, such as reducing potential herbicide drift problems by using shielded nozzles, lower pressures or sprayers with large orifice nozzles that increase the average droplet size.

Effects of environmental factors on sporulation, infection and disease expression

The current study is the first to show the effect of humidity and temperature on the release of Botryosphaeriaceae conidia from pycnidia in blueberries under laboratory conditions, and so the influence of environmental factors on abundance of conidia in the field. The results showed that all four species tested were capable of oozing conidial cirrhi at all the temperatures and humidities tested but that the greatest numbers of oozing pycnidia and conidia released were observed at higher temperatures (25-30°C) and RHs (92-100% RH). Under field conditions, these environmental conditions are affected by factors such as aspect and landscape of the farm as well as the climate and plant density, which may also affect conidial survival. To confirm these findings, a field experiment should be set up, with data loggers to monitor these conditions and show how they affect development of pycnidia and conidia.

The effects of environmental conditions on infection and disease development in the plants were also investigated. Earlier studies had shown that lesion development and pathogen progression was generally slower on plants in a shadehouse than in a glasshouse, indicating that the pathogen was sensitive to temperature. The experiment carried out using wounded and non-wounded shoots showed that *N. ribis* infected equally well and progressed through the tissues at similar rates when incubations conditions were 20 or 25°C and 90 or 100% RH during the early infection processes. However, this work should be further investigated using a wider range of temperatures and relative humidities. Further, the effects of other stress conditions such as water availability should also be investigated. Amponsah *et al.* (2014) demonstrated that grapevine plants with asymptomatic infections by *N. luteum* expressed symptoms only when plants were stressed by over- or under-supply of soil water or after onset of winter dormancy. Since some of the inoculated plants in the current work were asymptomatic, even though the pathogen had progressed quite long distances through the stems, the triggers which caused expression of symptoms should be investigated.

Effects of fungicides and biocontrol agents as wound protectants

The fungicide sensitivity studies conducted in the laboratory showed that the four most common species were affected by the same fungicides, although by different concentrations. These fungicides were also able to protect wounded and non-wounded plant tissues, but generally acted to reduce pathogen progression rather than preventing infection by *N. ribis* conidia. The different experiments showed variation in the fungicide responses to *N. ribis* infections, although carbendazim and tebuconazole gave good control overall. The high pathogen incidence and infection progression found with even the most effective fungicides may be associated with the high inoculum levels used. Further experiments should be conducted with lower inoculum concentrations which may better reflect natural inoculum levels. Similar experiments should be conducted in commercial properties, with multiple applications. Since the pathogenicity studies and cultivar susceptibility tests showed that most plant tissues, wounded or non-wounded, can become infected by *Neofusiccoccum* species this also shows the importance of overall spray cover of the plants, possibly whenever conidia are likely to have been dispersed.

As many growers were interested in the efficacy of biological control products this study also evaluated Vinevax® (*Trichoderma atroviride*) and Serenade® (*Bacillus subtilis)*. Overall, these products were more effective when used to prevent disease development in potted plants than *in vitro* under laboratory conditions. Vinevax® was relatively effective when allowed at least 7 days to colonise the plant tissues before inoculation. These experiments should also be repeated with lower pathogen inoculum concentrations and under field conditions to provide for control recommendations to farmers.

In summary, this study has provided information on the prevalence of disease and possible sources of inoculum of the main Botryosphaeriaceae species in New Zealand blueberry farms. It has also shown how environmental conditions affect sporulation and infection and the range of tissues which may become infected and subsequently provide further inoculum. This knowledge on the potential infection pathways could help growers to understand how to use cultural control methods integrated with fungicides and biocontrol products to reduce pathogen spread in blueberry nurseries and farms.

Presentations and publications from this thesis

Conference Presentation

Tennakoon, K.M., Jones, E.E., Ridgway, H.J., and Jaspers, M.V. (2014). *In vitro* evaluation of fungicides efficacy against *Neofusicoccum* species that cause dieback of blueberries, Poster presentation at New Zealand Plant Protection Society Conference 2014, Rotorua, New Zealand.

Tennakoon, K.M., Jaspers, M.V., Ridgway, H.J., and Jones, E.E. (2015). Herbicide injuries on blueberry provide suitable infection sites for *Neofusicoccum ribis*, Paper presented at New Zealand Plant Protection Society Conference 2015, Christchurch, New Zealand.

Tennakoon, K.M., Jaspers, M.V., Ridgway, H.J., and Jones, E.E. (2015). Are wound necessary for Botryosphaeria infections in Blueberries?, Poster presentation at The Australasian Plant Pathology Society Conference, Perth, Australia.

Journal Publication

Tennakoon, K.M., Jaspers, M.V., Ridgway, H.J., and Jones, E.E. (2015). Herbicide injuries on blueberry provide suitable infection sites for *Neofusicoccum ribis*, *New Zealand Plant protection*, 68, 411-414.

Oral presentation at blueberry growers meeting

Botryosphaeria Dieback in Blueberry: Pathogen identity, disease development and control, annual blueberry growers council meeting (2014), Hamilton, New Zealand.

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Personal communications

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Appendix A

A.1 Prune extract/ prune extract agar

1. De-stone and chop up prunes (25 g). Fill flask with distilled water to just under neck (500 mL); 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 500 mL flasks. Autoclave and refrigerate.

To make agar: 2 L

Sucrose	10g
Yeast extract	2 g
Agar	60 g
Prune extract	200 mL
Distilled water	1.8 mL

Boil to dissolve before autoclaving

A.2 Synthetic Nutrient Agar (SNA)

KH2PO4	1 g
KNO3	1 g
MgSO4.7H2O	0.5 g
KCL	0.5 g
Glucose	0.2 g
Sucrose	0.2 g
1N NaOH	0.6 mL
Agar	22 g
pH	6.5

Autoclave for 20 minutes and add the antibiotics Streptomycin sulphate (0.05 g/L), Penicillin (0.05g/L)

A.3 DNA extraction procedure for *Bacillus subtilis*

The DNA from each strain was extracted using the rapid REDExtract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich; Sigma-Aldrich Co. LLC) according to manufacturer's instructions. Approximately 1,500 bp of the 16S rRNA gene was amplified using the primers F27 (5'-AGA GTT TGA TCM TGG CTC AG-3') and R1494 (5'- CTA CGG YTA CCT TGT TAC GAC- 3'). PCR was performed in a thermal cycler (Applied Bio system Veriti, Life Technologies Ltd, New Zealand) as described by Weisberg *et al* (1991) in a total volume of 20 µL containing 10 µL REDExtract-N-Amp PCR Reaction Mix, 4 µL ultra pure water (Life technologies; Thermo Fisher Scientific Inc., USA), 0.4 µM of each forward and reverse primer (IDT, Integrated DNA Technologies Inc., Australia) and 4 µL DNA template.

Appendix B

B.1 Botryosphaeriaceous infection incidence in blueberry nurseries

	Value	df	*P*-value
Pearson Chi-square	8.50	5	0.131

B.2 Pearson Chi-square test of independence on botryosphaeriaceous infection incidence in different isolation position

	Value	df	*P*-value
Pearson Chi-square	0.37	2	0.830

B.3 Pearson Chi-square test of independence on *N. australe* incidence on different nurseries

	Value	df	*P*-value
Pearson Chi-square	7.47	3	0.058

B.4 Pearson Chi-square test of independence between *N. australe* infections and isolation positions

	Value	df	*P*-value
Pearson Chi-square	0.39	2	0.822

B.5 Pearson Chi-square test of independence on *N. parvum* incidence on different nurseries

	Value	df	*P*-value
Pearson Chi-square	6.25	3	0.100

B.6 Pearson Chi-square test of independence between *N. parvum* infections and isolation positions

	Value	df	*P*-value
Pearson Chi-square	0.22	2	0.897

B.7 Botryosphaeriaceous infection incidence in blueberry farms

	Value	df	*P*-value
Pearson Chi-square	33.33	6	$P < 0.001$

B.8 Pearson Chi-square test of independence on botryosphaeriaceous infection incidence in blueberry farms and different isolation position

	Value	**df**	***P*-value**
Pearson Chi-square	1.21	2	0.545

B.9 Pearson Chi-square test of independence on *N. australe* incidence on different farms

	Value	**df**	***P*-value**
Pearson Chi-square	35.10	6	$P < 0.001$

B.10 Pearson Chi-square test of independence between *N. australe* infections and isolation positions in farms

	Value	**df**	***P*-value**
Pearson Chi-square	4.10	2	0.129

B.11 Identification of Botryosphaeriaceae isolates from nursery plant materials collected during 2013 nursery sampling

Isolate Nursery No	Sample No	Species/Group based on the restriction digest of rDNA			
		Taq l	Sacll	Hae lll	Ncil
1	N151	Group A	*N. australe*		
1	N154	Group A	*N. australe*		
1	N155	Group A	*N. australe*		
1	N113	Group A	*N. australe*		
1	N115	Group A	*N. australe*		
1	N116	Group A	*N. australe*		
1	N136	Group A	*N. australe*		
1	N1103	Group B		*N. parvum*	
1	N1106	Group B		*N. parvum*	
2	N235	Group B		*N. parvum*	
2	N236	Group B		*N. parvum*	
2	N271	Group B		*N. parvum*	
2	N272	Group B		*N. parvum*	
2	N264	Group A	*N. australe*		
2	N288	Group A	*N. australe*		
3	N342	Group A	*N. australe*		
3	N345	Group A	*N. australe*		
3	N374	Group A	*N. australe*		
3	N375	Group A	*N. australe*		
3	N377	Group A	*N. australe*		
3	N378	Group A	*N. australe*		
4	N471	Group A	*N. australe*		
4	N472	Group A	*N. australe*		
4	N474	Group A	*N. australe*		
4	N475	Group B			*N. ribis*
4	N435	Group A	*N. australe*		
4	N414	Group B		*N. parvum*	
4	N416	Group B		*N. parvum*	
4	N417	Group B		*N. parvum*	
4	N464	Group B		*N. parvum*	
4	N465	Group A	*N. australe*		
4	N4103	Group A	*N. australe*		
4	N4106	Group A	*N. australe*		
4	N496	Group A	*N. australe*		
4	N451	Group B		*N. parvum*	
4	N454	Group B		*N. parvum*	
4	N455	Group A	*N. australe*		
4	N484	Group A	*N. australe*		

B.12 Identification of Botryosphaeriaceae isolates from farm plant materials collected during 2013/2014 farm sampling

Isolate farm No	Sample No	Species/Group based on the restriction digest of rDNA			
		Taq l	Sacll	Hae lll	Ncil
1	532	Group A	*N. australe*		
1	535	Group A	*N. australe*		
1	544	Group A	*N. australe*		
1	583	Group A	*N. australe*		
1	586	Group A	*N. australe*		
1	592	Group A	*N. australe*		
1	595	Group A	*N. australe*		
1	596	Group A	*N. australe*		
1	597	Group A	*N. australe*		
1	598	Group A	*N. australe*		
1	5113	Group A	*N. australe*		
1	5114	Group A	*N. australe*		
1	5121	Group A	*N. australe*		
1	5122	Group A	*N. australe*		
1	5123	Group A	*N. australe*		
1	5141	Group A	*N. australe*		
1	5142	Group A	*N. australe*		
1	5154	Group A	*N. australe*		
1	5162	Group A	*N. australe*		
1	5193	Group A	*N. australe*		
1	5194	Group A	*N. australe*		
1	523	Group A	*N. australe*		
1	5153	Group B			*N. ribis*
1	5163	Group B			*N. ribis*
2	611	Group A	*N. australe*		
2	613	Group A	*N. australe*		

Continuation of B.12

Isolate farm No	Sample No	Species/Group based on the restriction digest of rDNA			
		Taq l	Sacll	Hae lll	Ncil
2	614	Group A	*N. australe*		
2	616	Group A	*N. australe*		
2	623	Group A	*N. australe*		
2	626	Group A	*N. australe*		
2	631	Group A	*N. australe*		
2	634	Group A	*N. australe*		
2	615	Group B			*N. ribis*
2	622	Group B			*N. ribis*
3	7112	Group A	*N. australe*		
3	7113	Group A	*N. australe*		
3	7114	Group A	*N. australe*		
3	7115	Group A	*N. australe*		
3	7116	Group A	*N. australe*		
3	7117	Group A	*N. australe*		
3	7121	Group A	*N. australe*		
3	7122	Group A	*N. australe*		
3	7123	Group A	*N. australe*		
3	7132	Group A	*N. australe*		
3	7161	Group A	*N. australe*		
3	7163	Group A	*N. australe*		
3	7172	Group A	*N. australe*		
3	7173	Group A	*N. australe*		
3	7182	Group A	*N. australe*		
3	7193	Group A	*N. australe*		

Continuation of B.12

Isolate farm No	Sample No	Species/Group based on the restriction digest of rDNA			
		Taq I	**SacII**	**Hae III**	**NciI**
3	7194	Group A	*N. australe*		
3	7195	Group A	*N. australe*		
3	7151	Group A	*N. luteum*		
3	7152	Group A	*N. luteum*		
3	7153	Group A	*N. luteum*		
3	7154	Group A	*N. luteum*		
3	7155	Group A	*N. luteum*		
3	7156	Group A	*N. luteum*		
4	813	Group A	*N. australe*		
4	814	Group A	*N. australe*		
4	816	Group A	*N. australe*		
4	823	Group A	*N. australe*		
4	826	Group A	*N. australe*		
4	834	Group B		*N. parvum*	
4	838	Group B		*N. parvum*	
4	838	Group B		*N. parvum*	
4	836	Group B			*N. ribis*
4	837	Group B			*N. ribis*
5	913	Group A	*N. australe*		
5	914	Group A	*N. australe*		
5	916	Group A	*N. australe*		
5	923	Group A	*N. australe*		
5	926	Group A	*N. australe*		
5	934	Group B		*N. parvum*	

B.13 Identification of Botryosphaeriaceae isolates from farm plant materials collected during 2009/2010 farm sampling

Freezer no	Additional isolate no	Species/Group based on the restriction digest of rDNA			
		Taq I	SacII	Hae III	*NciI*
38	S1-38	Group B		*N. parvum*	
42	S1-42	Group B		*N. parvum*	
61	S1-61	Group B		*N. parvum*	
75	S1-75	Group B		*N. parvum*	
83	S1-83	Group B			*N. ribis*
85	S1-85	Group A	*N. australe*		
89	S1-89	Group A	*N. luteum*		
91	S1-91	Group B			*N. ribis*
93	S1-93	Group B		*N. parvum*	
101	S1-101	Group A		*N. parvum*	
109	S1-109	Group B			*N. ribis*
114	S1-114	Group A	*N. australe*		
120	S1-120	Group A	*N. australe*		
122	S1-122	Group B		*N. parvum*	
125	S1-125	Group B		*N. parvum*	
137	S1-137	Group A	*N. luteum*		
140	S1-140	Group A	*N. australe*		
141	S1-141	Group A	*N. luteum*		
146	S1-146	Group A	*N. australe*		
148	S1-148	Group A	*N. australe*		
151	S1-151	Group A	*N. australe*		
152	S1-152	Group A	*N. australe*		
153	S1-153	Group B			*N. ribis*
154	S1-154	Group B		*N. parvum*	
162	S1-162	Group A	*N. australe*		
165	S1-165	Group B			*N. ribis*

Continuation of B.13

Freezer no	Additional isolate no	Species/Group based on the restriction digest of rDNA			
		Taq I	**SacII**	**Hae III**	***NciI***
166	S1-166	Group A	*N. australe*		
167	S1-167	Group A	*N. luteum*		
168	S1-168	Group A	*N. luteum*		
169	S1-169	Group A	*N. luteum*		
177	S1-177	Group B			*N. ribis*
179	S1-179	Group A	*N. australe*		
180	S1-180	Group A	*N. australe*		
LUPP006	S2-6	Group A	*N. australe*		
LUPP093	S2-93	Group A	*N. australe*		
LUPP094	S2-94	Group A	*N. australe*		
LUPP084	S2-84	Group A	*N. australe*		
LUPP085	S2-85	Group A	*N. australe*		
LUPP086	S2-86	Group A	*N. australe*		
LUPP087	S2-87	Group A	*N. australe*		
LUPP089	S2-89	Group A	*N. australe*		
LUPP090	S2-90	Group B			*N. ribis*
LUPP096	S2-96	Group A	*N. luteum*		
LUPP091	S2-91	Group A	*N. luteum*		
LUPP092	S2-92	Group A	*N. luteum*		
LUPP129	S2-129	Group A	*N. australe*		

B.14 *Neofusicoccum ribis* isolates used for UP-PCR from Survey 2009/2010

Freezer No	Additional No	Farm code	Region isolated
83	S1-83	HBF	Hamilton
88	S1-88	HBF	Hamilton
92	S1-92	HBF	Hamilton
96	S1-96	HOF	Hamilton
106	S1-106	HOF	Hamilton
150	S1-150	OBC	Ohaupo
158	S1-158	OBF	Ohaupo
159	S1-159	OBF	Ohaupo
175	S1-175	OBF	Ohaupo

B.15 First report of stem blight on blueberries induced by *Pestalotiopsis* species in New Zealand

Materials and methods

Sampling of necrotic tissues in blueberry farms to identify Botryosphaeria dieback disease carried out in New Zealand from June 2013 to January 2014, consistently found a fungus for which the conidia resembled *Pestalotiopsis* species. They were found alone or coexisting with Botryosphaeriaceae species in some of the dieback and canker samples and in asymptomatic plants from blueberry nurseries. In the early stages of the survey, the *Pestalotiopsis* species incidences were not recorded, nor were these isolates stored. However in 2013/2014, *Pestalotiopsis* species isolated from dieback symptoms from two farms, one farm in Canterbury and one farm in the Waikato region were retained for identification using morphological and molecular methods and to test their pathogenicity on blueberry plants.

The 12 isolates recovered from a Waikato blueberry farm and one isolate recovered from a Canterbury farm were grown on PDA at 25°C until they oozed dark conidiomata. Conidial suspensions were spread onto water agar and incubated for 2 days at 25°C, when single

spore colonies were selected for transfer onto PDA. Cultures were incubated at 25°C in the dark as described by Ismail *et al.* (2013) and cultural morphology was examined after 7 days. Conidia from the conidiomata in the centres of the colonies were used to make suspensions in sterile water and these conidia were examined by light microscope at 600x magnification.

Five representative isolates were used for molecular identification (P10-2, P5-3, H1, P1-1 and P1-3). Isolates were grown in PDA for 5-7 days at 25°C in the dark. A tuft of aerial mycelium was removed from each culture for DNA extraction using the REDExtract-N-Amp™ Plant PCR Kit (Sigma Aldrich, Missouri, USA) was conducted as instructed by the manufacturer. Amplification of the ribosomal DNA was conducted using the REDExtract-N-Amp™ Plant PCR ready mix, with primers ITS-4 (TCCTCCGCTTATTGATATGC) and ITS-5 (GGAAGTAAAAGTCGTAACAAGG). For each sample, the reaction mixture contained 10 µL of the REDExtract-N-Amp™ Plant PCR ready mix, 1 µL of each primer (5 µM), 4 µL of sterile nanopure water (SNW) and 4 µL of DNA extract. Negative controls contained nanopure water instead of the DNA extract. The thermal cycle consisted of initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 55 s at 52°C and extension for 1 min at 72°C, followed by a final extension at 72°C for 7 min. To identify isolates the PCR products were sequenced using primer ITS4 at the Lincoln University Sequencing facility using an ABI PRISM® 310 Genetic Analyser (Applied Biosystems, Foster City, California). However, none of the resulting sequences of the isolates were 100% homologous with any of the accessions in GenBank. Therefore the β- tubulin gene was selected for amplification and sequencing. The extracted DNA was amplified using primers Bt2a and Bt2b as described above and the PCR product sequenced. None of the resulting sequences of the isolates were 100% homologous with any of the accessions in GenBank.

In a further attempt to resolve species identity, the tef1 gene was amplified using the primer pairs EF1-526F (5-GTCGTYGTYATYGGHCAYGT-3) and EF1-1567R (5-ACHGTRCCRATACCACCRATCTT-3) (Rehner, 2001). The PCR was performed as described by Maharachchikumbura *et al.* (2012), an initial step of 5 min at 94°C, 10 cycles of 30 s at 94°C, 55 s at 63°C or 66°C (decreasing 1°C per cycle), 90 s at 72°C, plus 36 cycles of 30 s at 94 °C, 55 s at 53°C or 56°C, 90 s at 72 °C, followed by 7 min at 72°C.

The five isolates for which identities were confirmed with molecular methods were used for pathogenicity studies. Two shoots per plant for soft green and hard green shoots of 2 year old blueberry plants (6 plants per isolate) cultivar 'Dolce Blue' were surface sterilised by swabbing with 70% ethanol, superficially wounded with a sterile scalpel (~1-2 mm deep and 6 mm in diameter) and inoculated with a 3 mm diameter mycelial plugs cut from the edges of 5-day-old PDA cultures of the isolates. Sterile agar was used for the negative control. The plants were immediately covered with new individual plastic bags, which were misted inside with water and left for 24 h to provide high humidity. Plants were placed in the shadehouse and allowed to develop lesions in a shade house at the Lincoln University Nursery. Soft shoots developed brown colour lesions after 5-8 days and were harvested after 15 days. Hard green shoots developed brown lesions after 10-12 and were harvested after 30 days. Lesion lengths were measured with a digital caliper.

Results

Cultures were allocated into two groups based on colony growth rate and colony colour. Group A isolates took 4-7 days to fill the entire plate and Group B took 10-15 days to fill the entire plate. The mycelium of Group A cultures was pinkish gold in colour on the underside of the plate with faint concentric markings (Figure B.1). The aerial mycelium was whitish cream, with fluffy clumps near the centre and flat extending mycelium at the edges. Small shiny, black acervular conidiamata were present in the centre. Out of 13 isolates four isolates were placed into Group A (isolates P1-3, P12-3, P10-1 and P1-1) and nine isolates into Group B (isolates P2-3, P11-2, P3-3, P9-3, P4-1, P8-1, P5-3, P7-3 and H1). The mycelium of Group B cultures (Figure B.1), was creamy beige in colour with greenish central areas and greenish concentric markings on the undersides of the plates. The aerial mycelium was white and fluffy, with some mycelial ridges in irregular concentric circles. Small, shiny black acervular conidiamata were present in the centre.

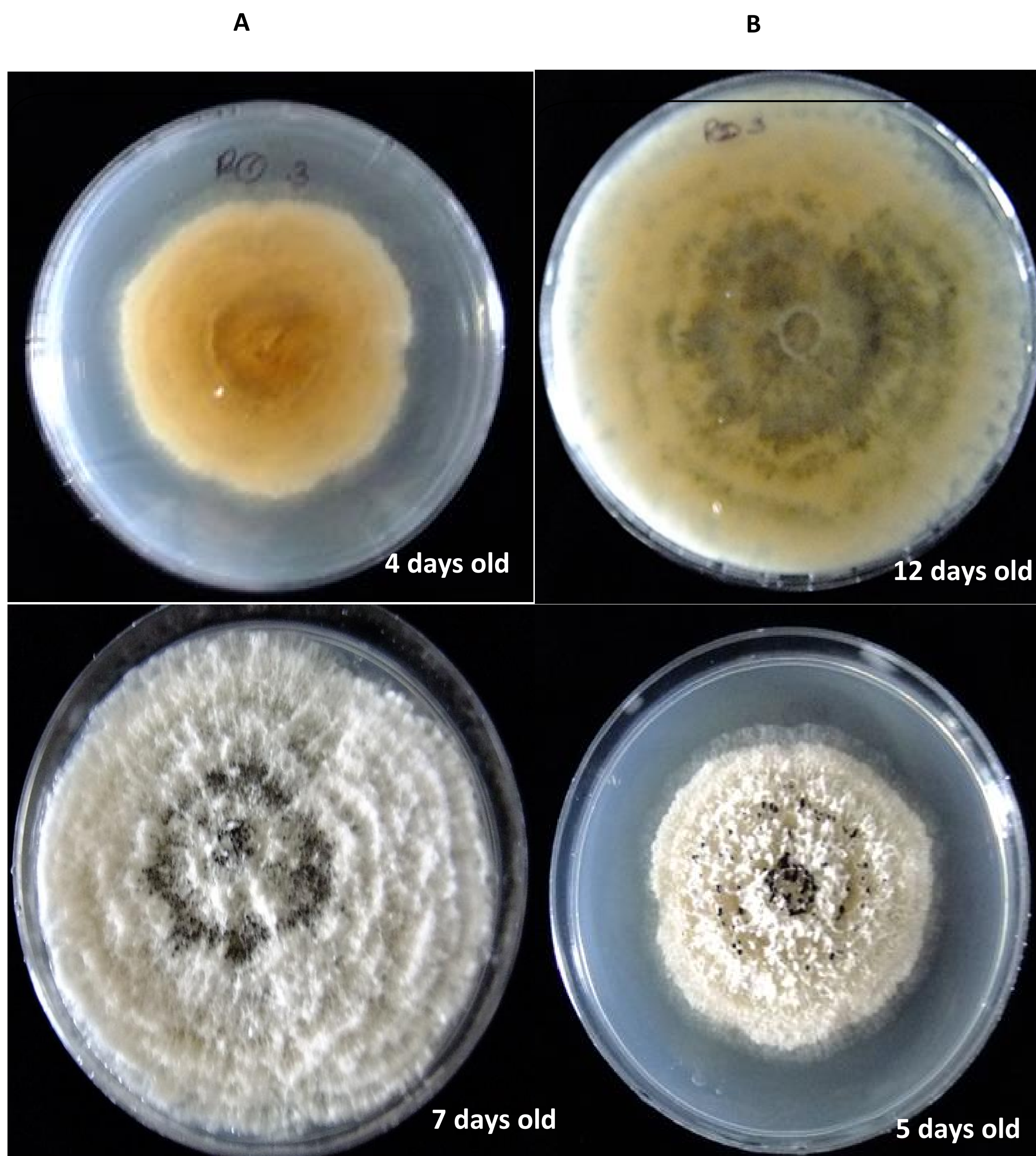

Figure B.1: Culture morphology of *Pestalotiopsis* species cultured on PDA and incubated in the dark at 25°C. Groups A and B represent the two different morphologies observed based on colony colour and growth characteristics.

The two groups appeared to have similar conidia (Figure B.2); they were fusiform to clavate, most commonly with five cells. Apical and basal cells were colourless, while the middle cells were darker, of which the upper two middle cells were darker than the lower one. There were two to four hyaline filamentous appendages attached to each apical cell, and one short hyaline appendage attached to each basal cell.

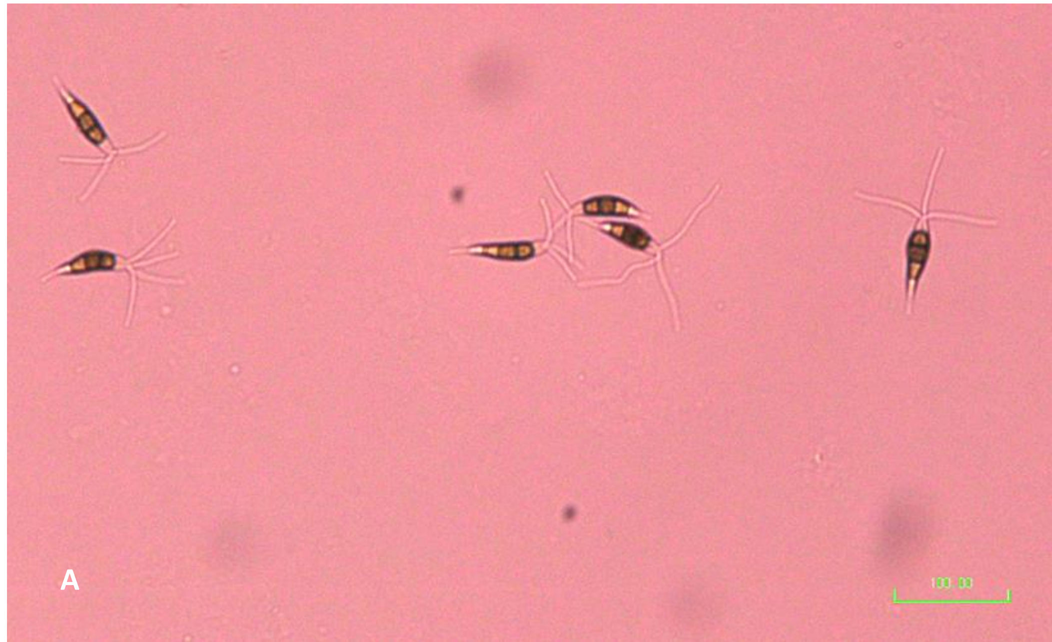

Figure B.2: Commonly observed conidia from *Pestalotiopsis* spp. represented by isolate P10-1 (Figure B.2.A) and isolate P3-3 (Figure B.2.B) (×600)

Based on sequences of the tef gene (Appendix F) isolates P1-3 and P1-1 showed 99% sequence identity to *Pestalotiopsis trachicarpicola* (The holotype for this is GenBank accession code JQ845946.1 work done by Zhang *et al.,* 2012). Isolates H1, P5-3 and P10-2 showed 99% sequence identity to *Pestalotiopsis clavispora* (GenBank accession code JX 399044.1 work done by Maharachchikumbura *et al*., 2012).

Soft green shoots started to develop brown colour lesions 5-8 days after inoculation. Mean lesion lengths after 15 days were 18.0 and 12.0 mm, respectively, for isolates P1-3 and P1-1 (*P. trachicarpicola*), 13.0, 10.9 and 67.0 mm, respectively, for isolates P10-2, P5-3 and H1 confirmed as *P. clavispora*. Hard green shoots lesions started to develop lesions 10-12 days after inoculation. Mean lesion lengths after 30 days were 26.0 and 22.0 mm, respectively, for isolates P1-3 and P1-1 (*P. trachicarpicola*), 40.0, 30.0 and 80.0 mm, respectively for isolates P10-2, P5-3 and H1 (*P. clavispora*). Control plants produced no lesions. These pathogens were consistently reisolated from the inoculated plants while none were found in negative control plants.

Appendix C

C.1 ANOVA results for pathogenicity of (lesion lengths) *Neofusicoccum* species isolates on detached soft green and semi hard blueberry shoots

A) Isolate effect on detached soft green shoots

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block stratum	5	839.3	167.9	0.67	
Isolates	15	31403.9	2093.6	8.30	<0.001
Residual	75	18916.8	252.2		
Total	95	51159.9			

B) Species effect on detached soft green shoots

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block stratum	5	839.3	167.9	0.67	
Species	3	21866.3	7288.8	28.90	<0.001
Residual	75	18916.8	252.2		
Total	95	51159.9			

C) Isolate effect on detached hard green shoots

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block stratum	5	115.26	23.05	0.27	
Isolates	15	5515.29	367.69	4.30	<0.001
Residual	75	6416.86	85.56		
Total	95	12047.40			

D) Species effect on detached hard green shoots

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	5	115.26	23.05	0.27	
Species	3	3189.45	1063.15	12.43	<0.001
Residual	75	6416.86	85.56		
Total	95	12047.40			

C.2 ANOVA results for pycnidia and conidia production of the *Neofusicoccum* species isolates

A) Pycnidia production of the isolates

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	5	26396	5279	0.86	
Isolates	15	295634	19709	3.20	<0.001
Residual	75	461642	6155		
Total	95	783671			

B) Pycnidia production of the species

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	5	26396	5279	0.86	
Species	3	89566	29855	4.85	0.004
Residual	75	461642	6155		
Total	95	783671			

C) Conidia production of the isolates

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	5	7.448	1.490	1.47	
Isolates	15	39.093	2.606	2.57	0.004
Residual	75	76.166	1.016		
Total	95	122.706			

D) Conidia production of the species

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	5	7.448	1.490	1.47	
Species	3	15.860	5.287	5.21	0.003
Residual	75	76.166	1.016		
Total	95	122.706			

C.3 ANOVA results for conidial infection of *N. ribis* in bark and wood

A) Pathogen progression after 1 day incubation

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Wounding	1	1805.00	1805.0 0	13.37	0.002
Type (bark vs wood)	1	5.00	5.00	0.04	0.850
Wounding. type	1	125.00	125.00	0.93	0.350
Residual	16	2160.00	135.00		
Total	19	4095.00			

B) Pathogen progression after 2 days incubation

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Wounding	1	405.00	405.00	16.20	<0.001
Type (bark vs wood)	1	125.00	125.00	5.00	0.040
Wounding. type	1	125.00	125.00	5.00	0.040
Residual	16	400.00	25.00		
Total	19	1055.00			

C) Pathogen progression after 3 days incubation

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Wounding	1	180.00	180.00	3.79	0.069
Type (bark vs wood)	1	80.00	80.00	1.68	0.213
Wounding. type	1	80.00	80.00	1.68	0.213
Residual	16	760.00	47.50		
Total	19	1100.00			

D) Pathogen progression after 7 days incubation

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Wounding	1	245.00	245.00	2.80	0.114
Type (bark vs wood)	1	5.00	5.00	0.06	0.814
Wounding. type	1	45.00	45.00	0.51	0.484
Residual	16	1400.00	87.50		
Total	19	1695.00			

E) Pathogen progression after 10 days incubation

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Wounding	1	180.0	180.0	1.80	0.198
Type (bark vs wood)	1	20.0	20.0	0.20	0.661
Wounding. type	1	500.0	500.0	5.00	0.040
Residual	16	1600.0	100.0		
Total	19	2300.0			

C.4 ANOVA results for pathogenicity of (lesion lengths) *Neofusicoccum* species isolates on attached soft green and hard blueberry shoots

A) Attached soft green shoots

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	22541.20	11270.60	21.63	<0.001
Residual	27	14069.40	521.10		
Total	29	36610.6			

B) Attached hard green shoots

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	2663.27	1331.63	15.21	<0.001
Residual	27	2363.47	87.54		
Total	29	5026.74			

C.5 ANOVA results for pathogenicity of *Neofusicoccum* species isolates on attached wounded and non-wounded soft green and hard blueberry shoots

A) Lesion development in soft green shoots

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	1	2523.00	2523.00	27.84	<0.001
Residual	10	906.25	90.62		
Total	11	3429.25			

B) Lesion development in hard green shoots

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	1	1333.52	1333.52	13.43	0.004
Residual	10	993.21	99.32		
Total	11	2326.73			

C) Pathogen progression in wounded and non-wounded soft green shoots

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	**1**	3333.30	3333.30	19.05	<0.001
Wounding treatment	1	3333.30	3333.30	19.05	<0.001
Tissue type (bark vs wood)	1	1408.30	1408.30	8.05	0.007
Species. wounding	1	675.00	675.00	3.86	0.057
Species. tissue type	1	33.30	33.30	0.19	0.665
Wounding treatment. tissue type	1	133.30	133.30	0.76	0.388
Species. wounding	1	8.30	8.30	0.05	0.828
Residual	40	7000.00	175.00		
Total	47	15925.00			

D) Pathogen progression in wounded and non- wounded hard green shoots

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Species	**1**	1302.10	1302.10	6.55	0.014
Wounding treatment	1	2002.10	2002.10	10.07	0.003
Tissue type (bark vs wood)	1	168.80	168.80	0.85	0.362
Species. wounding	1	352.10	352.10	1.77	0.191
Species. tissue type	1	468.80	468.80	2.36	0.132
Wounding treatment. tissue type	1	2.10	2.10	0.01	0.919
Species. wounding	1	102.10	102.10	0.51	0.478
Residual	40	7950.00	198.80		
Total	47	12347.90			

C.6 ANOVA results for susceptibility of different blueberry tissues to *Neofusicoccum* species infection and disease progression through different blueberry tissues

A) Incidence in non-wounded and wounded fruits

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Species	2	5177.5	2588.8	15.77	<0.001
Residual	27	4432.5	164.2	1.16	
Wound/non-wound	1	4681.7	4681.7	33.16	<0.001
Species ×Wound/non-wound	2	30.8	15.4	0.11	0.897
Residual	27	3812.5	141.2		
Total	59	18135.0			

B) Incidence in non-wounded and wounded buds

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Species	2	2830.8	1415.4	5.59	0.009
Residual	27	6831.2	253.0	1.65	
Wound/non-wound	1	3920.4	3920.4	25.62	<0.001
Species ×Wound/non-wound	2	10.8	5.4	0.04	0.965
Residual	27	4131.2	153.0		
Total	59	17724.6			

C) Pycnidia development in fruits

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Species	2	2751.7	1375.8	8.81	0.001
Residual	27	4215.0	156.1		
Total	29	6966.7			

D) Incidence of fruits infected through fruit stem inoculation

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Species	2	1431.7	715.8	7.10	0.003
Residual	27	2722.5	100.8		
Total	29	4154.2			

E) Lesion lengths in trunks

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	1389.03	694.52	8.00	0.002
Residual	27	2344.05	86.82	3.00	
Upward.downward	1	98.82	98.82	3.41	0.076
Species.upward.downward	2	123.23	61.62	2.13	0.139
Residual	27	781.45	28.94		
Total	59	4736.58			

F) Pathogen progression in the crown

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	6925.3	3462.6	19.67	<0.001
Residual	27	4754.1	176.1		
Total	29	11679.4			

G) Pathogen progression in the trunk

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	3440.0	1720.0	9.09	<0.001
Residual	27	5110.0	189.3		
Total	29	8550.0			

H) Pathogen progression in the roots from the inoculated crown

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	1190.6	595.3	2.42	0.108
Residual	27	6634.6	245.7		
Total	29	7825.2			

I) Pathogen progression in the shoots

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	4365.3	2182.6	3.86	0.034
Residual	27	15270.1	565.6		
Total	29	19635.4			

C.7 ANOVA results for susceptibility of soft green shoots and hard green shoots of different blueberry cultivars to *Neofusicoccum* infection

A) Soft green shoots

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	1	118767.1	118767.1	243.82	<0.001
Cultivar	6	20285.5	3380.9	6.94	<0.001
Treatment ×cultivar	6	27962.8	4660.5	9.57	<0.001
Residual	70	34097.7	487.1		
Total	83	201113.1			

B) Hard green shoots

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	1	14649.36	14649.36	225.52	<.001
Cultivar	6	4120.34	686.72	10.57	<.001
Treatment ×cultivar	6	3118.47	519.74	8.00	<.001
Residual	70	4547.06	64.96		
Total	83	26435.23			

C.8 ANOVA results for susceptibility of fruits, flower buds and leaf buds of different blueberry cultivars to *Neofusicoccum* infection

A) Incidence in fruits

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	6	81.155	13.526	1.92	0.081
Species	1	8.595	8.595	1.22	0.271
Wounding	1	57.167	57.167	8.13	0.005
Cultivar.species.wounding	19	110.821	5.833	0.83	0.669
Residual	140	984.333	7.031		
Total	167	1242.071			

B) Incidence in flower buds

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	6	75.702	12.617	2.99	0.009
Species	1	176.095	176.095	41.74	<.001
Wounding	1	74.667	74.667	17.70	<.001
Cultivar.species.wounding	19	104.655	5.508	1.31	0.189
Residual	140	590.667	4.219		
Total	167	1021.786			

C) Leaf buds

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cultivar	6	96.476	16.079	4.83	<.001
Species	1	121.720	121.720	36.56	<.001
Wounding	1	26.720	26.720	8.02	0.005
Cultivar.species.wounding	19	104.768	5.514	1.66	0.051
Residual	140	466.167	3.330		
Total	167	815.851			

C.9 Identification of Botryosphaeriaceae isolates from farm plant materials collected during 2009 farm sampling, which used for pathogenicity studies

Freezer no	Additional isolate no	Species/Group based on the restriction digest of rDNA			
		Taq I	SacII	Hae III	NciI
131	S1-131	GroupA	*N. australe*		
174	S1-174	GroupA	*N. australe*		
111	S1-111	GroupA	*N. australe*		
90	S1-90	GroupA	*N. australe*		
142	S1-142	GroupA	*N. luteum*		
130	S1-130	GroupA	*N. luteum*		
167	S1-167	GroupA	*N. luteum*		
118	S1-118	GroupA	*N. luteum*		
58	S1-58	GroupB		*N. parvum*	
173	S1-173	GroupB		*N. parvum*	
98	S1-98	GroupB		*N. parvum*	
138	S1-138	GroupB		*N. parvum*	
110	S1-110	GroupB			*N. ribis*
175	S1-175	GroupB			*N. ribis*
158	S1-158	GroupB			*N. ribis*
150	S1-150	GroupB			*N. ribis*

Appendix D

D.1 ANOVA results for effect of relative humidities (RH) under different temperatures on the number of pycnidia oozing and conidia production from stem lesions of three *Neofusicoccum* species.

A) 1. Pycnidia oozing at 15°C in *Neofusicoccum* isolates

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	3	12941	4314	2.50	
Humidity	2	4067	2034	1.18	0.316
Isolates	5	59895	11979	6.95	<0.001
Humidity ×Isolates	10	21728	2173	1.26	0.278
Residual	51	87922	1724		
Total	71	186553			

2. Pycnidia oozing at 15°C in *Neofusicoccum* species

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	3	12941	4314	2.50	
Humidity	2	4067	2034	1.18	0.316
Species	2	24758	12379	7.18	0.002
Species× Humidity	4	5851	1463	0.85	0.501
Residual	51	87922	1724		
Total	71	186553			

B) 1. Conidia production at 15°C in *Neofusicoccum* isolates

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block stratum	3	25.765	8.588	2.21	
Humidity	2	9.742	4.871	1.25	0.294
Isolates	5	61.388	12.278	3.16	0.015
Humidity ×Isolates	10	26.206	2.621	0.67	0.742
Residual	51	198.017	3.883		
Total	71	321.119			

2. Conidia production at 15°C in *Neofusicoccum* species

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block stratum	3	25.765	8.588	2.21	
Humidity	2	9.742	4.871	1.25	0.294
Species	2	48.727	24.363	6.27	0.004
Species× Humidity	4	4.462	1.116	0.29	0.885
Residual	51	198.017	3.883		
Total	71	321.119			

C) 1. Pycnidia production at 20°C in *Neofusicoccum* isolates

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block stratum	3	9755	3252	1.93	
Humidity	2	1344	672	0.40	0.673
Isolates	5	57574	11515	6.84	<0.001
Humidity ×Isolates	10	48851	4885	2.90	0.006
Residual	51	85876	1684		
Total	71	203400			

2. Pycnidia production at 20°C in *Neofusicoccum* species

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block stratum	3	9755	3252	1.93	
Humidity	2	1344	672	0.40	0.673
Species	2	43428	21714	12.90	<0.001
Species× Humidity	4	38400	9600	5.70	<0.001
Residual	51	85876	1684		
Total	71	203400			

D) 1. Conidia production at 20°C in *Neofusicoccum* isolates

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block stratum	3	11894	3965	2.37	
Humidity	2	7138	3569	2.13	0.129
Isolates	5	85962	17192	10.26	<0.001
Humidity ×Isolates	10	19222	1922	1.15	0.348
Residual	51	85464	1676		
Total	71	209681			

2. Conidia production at 20°C in *Neofusicoccum* species

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block stratum	3	11894	3965	2.37	
Humidity	2	7138	3569	2.13	0.129
Species	2	81204	40602	24.23	<0.001
Species× Humidity	4	5143	1286	0.77	0.552
Residual	51	85464	1676		
Total	71	209681			

E) 1. Pycnidia production at 25°C in *Neofusicoccum* isolates

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block stratum	3	7587	2529	1.09	
Humidity	2	60104	30052	12.91	<0.001
Isolates	5	118613	23723	10.19	<0.001
Humidity ×Isolates	10	56790	5679	2.44	0.018
Residual	51	118678	2327		
Total	71	361772			

2. Pycnidia production at 25°C in *Neofusicoccum* species

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block stratum	3	7587	2529	1.09	
Humidity	2	60104	30052	12.91	<0.001
Species	2	114831	57416	24.67	<0.001
Species× Humidity	4	52648	13162	5.66	<0.001
Residual	51	118678	2327		
Total	71	361772			

F) 1. Conidia production at 25°C in *Neofusicoccum* isolates

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block stratum	3	22900	7633	2.44	
Humidity	2	37541	18770	5.99	0.005
Isolates	5	245186	49037	15.65	<0.001
Humidity ×Isolates	10	60157	6016	1.92	0.064
Residual	51	159818	3134		
Total	71	525602			

2. Conidia production at 25°C in *Neofusicoccum* species

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	3	22900	7633	2.44	
Humidity	2	37541	18770	5.99	0.005
Species	2	161312	80656	25.74	<0.001
Species× Humidity	4	41851	10463	3.34	0.017
Residual	51	159818	3134		
Total	71	525602			

G) 1. Pycnidia production at 30°C in *Neofusicoccum* isolates

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	3	402.4	134.1	0.16	
Humidity	2	19576.9	9788.4	11.56	<0.001
Isolates	5	118669.3	23733.9	28.03	<0.001
Humidity ×Isolates	10	58466.5	5846.6	6.90	<0.001
Residual	51	43187.6	846.8		
Total	71	240302.6			

2. Pycnidia production at 30°C in *Neofusicoccum* species

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	3	402.4	134.1	0.16	
Humidity	2	19576.9	9788.4	11.56	<0.001
Species	2	108149.8	54074.9	63.86	<0.001
Species× Humidity	4	50863.5	12715.9	15.02	<0.001
Residual	51	43187.6	846.8		
Total	71	240302.6			

H) 1. Conidia production at 30°C in *Neofusicoccum i*solates

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	3	2254	751	0.12	
Humidity	2	10429	5214	0.83	0.443
Isolates	5	190370	38074	6.05	<0.001
Humidity ×Isolates	10	101154	10115	1.61	0.132
Residual	51	321184	6298		
Total	71	625391			

2. Conidia production at 30°C in *Neofusicoccum* species

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	3	2254	751	0.12	
Humidity	2	10429	5214	0.83	0.443
Species	2	183458	91729	14.57	<0.001
Species× Humidity	4	35348	8837	1.40	0.246
Residual	51	321184	6298		
Total	71	625391			

D.2 ANOVA results for the effect of different number of conidia of *N. ribis* and *N. parvum* on lesions length on different stem tissues

A) Lesion length on soft green shoots inoculated with different spore concentrations of *N. ribis* and *N. parvum*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	1	3519.2	3519.2	11.26	0.002
Concentration	3	25491.3	8497.1	27.18	<0.001
Species×Concentration	3	3401.3	1133.8	3.63	0.021
Residual	40	12505.8	312.6		
Total	47	44917.5			

B) Lesion length on hard green shoots inoculated with different spore concentrations of *N. ribis* and *N. parvum*

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Species	1	845.88	845.88	24.01	<0.001
Concentration	3	2192.47	730.82	20.75	<0.001
Species×Concentration	3	65.77	21.92	0.62	0.605
Residual	40	1409.12	35.23		
Total	47	4513.24			

C) Lesion length on hard green shoot wood inoculated with different spore concentrations of *N. ribis* and *N. parvum*

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Species	1	3032.13	3032.13	54.20	<0.001
Concentration	3	941.18	313.73	5.61	0.003
Speciesn×Concentration	3	14.18	4.73	0.08	0.968
Residual	40	2237.88	55.95		
Total	47	6225.37			

D.3 ANOVA results for lesion length on different stem tissue after inoculation of wounds of different ages with of *N. ribis*

A) Soft green shoots

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Wound age	3	22061.1	7353.7	11.10	<0.001
Residual	20	13252.2	662.6		
Total	23	35313.3			

B) Hard green shoots

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Wound age	2	502.57	251.28	9.98	0.002
Residual	15	377.85	25.19		
Total	17	880.42			

C) Woody trunk

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Wound age	2	321.41	160.71	8.53	0.003
Residual	15	282.47	18.83		
Total	17	603.88			

D.4 ANOVA results for pathogen movement on different stem tissues after inoculation wounds of different ages with *N. ribis.*

A) Soft green shoots

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Wound age	6	41547.6	6924.6	45.16	<0.001
Residual	35	5366.7	153.3		
Total	41	46914.3			

B) Hard green shoots

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Wound age	6	15100.0	2516.7	22.68	<0.001
Residual	35	3883.3	111.0		
Total	41	18983.3			

C) woody trunk

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Wound age	6	10823.8	1804.0	16.47	<0.001
Residual	35	3833.3	109.5		
Total	41	14657.1			

D.5 ANOVA results on the effect of relative humidity at either 20°C or 25°C on infection of wounded or non-wounded hard green shoots by *N. ribis*.

A) Lesion length which developed in wounded hard green shoots incubated at 20°C

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
RH	1	1850.08	1850.08	41.59	<0.001
Residual	10	444.83	44.48		
Total	11	2294.92			

B) Lesion length which developed in wounded hard green shoots incubated at 25°C

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
RH	1	120.3	120.3	0.85	0.379
Residual	10	1423.3	142.3		
Total	11	1543.7			

C) Pathogen movement in the hard green shoots incubated at 20°C

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Wounded/nonwounded	1	1837.50	1837.50	32.91	<0.001
RH	1	4537.50	4537.50	81.27	<0.001
Wounded/nonwounded×RH	1	704.17	704.17	12.61	0.002
Residual	20	1116.67	55.83		
Total	23	8195.83			

D) Pathogen movement in the hard green shoots incubated at 25°C

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Wounded/nonwounded	1	1837.5	1837.50	13.05	0.002
RH	1	2204.2	2204.2	15.65	<0.001
Wounded/nonwounded ×RH	1	4.2	4.2	0.03	0.865
Residual	20	2816.7	140.8		
Total	23	6862.5			

D.6 ANOVA results for the effect of wounding at different times of the year on susceptibility to *N. ribis.*

A.1 Pathogen progression in the bark and wood of hard green shoots inoculated in the summer

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Tissue	1	3008.3	3008.3	9.76	0.011
Residual	10	3083.3	308.3		
Total	11	6091.7			

A.2 Infection incidence in the bark and wood of hard green shoots inoculated in the summer

Fixed term	**Wald statistic**	**n.d.f.**	**F statistic**	**d.d.f.**	**F pr.**
Position	3.89	1	3.89	9.0	0.080

A.3 Pathogen progression in the bark and wood of trunks inoculated in the summer

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Tissue	1	0.0	0.0	0.00	1.000
Residual	10	7566.7	756.7		
Total	11	7566.7			

A.4 Infection incidence in the bark and wood of trunks inoculated in the summer

Fixed term	**Wald statistic**	**n.d.f.**	**F statistic**	**d.d.f.**	**F pr.**
Position	1.07	1	1.07	9.0	0.327

B.1 Pathogen progression in the bark and wood of hard green shoots inoculated in the autumn

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Tissue	1	1408.3	1408.3	1.93	0.195
Residual	10	7283.3	728.3		
Total	11	8691.7			

B.2 Infection incidence in the bark and wood of hard green shoots inoculated in the autumn

Fixed term	**Wald statistic**	**n.d.f.**	**F statistic**	**d.d.f.**	**F pr.**
Position	3.89	1	3.89	9.0	0.080

B.3 Pathogen progression in the bark and wood of trunks inoculated in the autumn

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Tissue	1	33.3	33.3	0.05	0.823
Residual	10	6333.3	633.3		
Total	11	6366.7			

B.4 Infection incidence in the bark and wood of trunks inoculated in the autumn

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr.
Position	1.07	1	1.07	9.0	0.327

C.1 Pathogen progression in the bark and wood of hard green shoots inoculated in the winter

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Tissue	1	833.33	833.33	14.71	0.003
Residual	10	566.67	56.67		
Total	11	1400.00			

C.2 Infection incidence in the bark and wood of hard green shoots inoculated in the winter

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr.
Position	4.13	1	4.13	10.0	0.069

C.3 Pathogen progression in the bark and wood of trunks inoculated in the winter

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Tissue	1	133.3	133.3	0.57	0.467
Residual	10	2333.3	233.3		
Total	11	2466.7			

C.4 Infection incidence in the bark and wood of trunks inoculated in the winter

Fixed term	**Wald statistic**	**n.d.f.**	**F statistic**	**d.d.f.**	**F pr.**
Position	0.00	1	0.00	10.0	1.000

D.7 ANOVA results for the effect of herbicide injuries on disease development by *N. ribis*.

A) The lesion length in the wood with herbicide treatment of shoots

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Treatment	3	78.23	26.08	1.21	0.331
Residual	20	430.33	21.52		
total	23	508.56			

B) Pathogen progression on the bark

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Treatment	3	54.00	18.00	1.70	0.200
Residual	20	212.00	10.60		
total	23	266.00			

C) Pathogen progression on the wood

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Treatment	3	67.12	22.38	1.72	0.194
Residual	20	259.50	12.97		
total	23	326.62			

Appendix E

E.1 ANOVA results for fungicide effect on mycelial growth of different *Neofusicoccum* species

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	3	34.7339	11.5780	28.13	<0.001
Fungicide	8	738.4338	92.3042	224.30	<0.001
Species. Fungicide	24	137.0090		13.87	<0.001
Residual	288	118.5177	0.4115		
Total	323	1028.6943			

E.2 ANOVA results for fungicide effect on conidia germination of different *Neofusicoccum* species

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	3	151.1843	50.3948	249.83	<0.001
Fungicide	8	199.0318	24.8790	123.34	<0.001
Species. Fungicide	24	473.7545	19.7398	97.86	<0.001
Residual	72	14.5234	0.2017		
Total	107	838.4940			

E.3 ANOVA results for fungicide effect on conidia germ tube growth of different *Neofusicoccum* species

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	3	53.71454	17.90485	235.55	<0.001
Fungicide	8	126.54288	15.81786	208.09	<0.001
Species. Fungicide	24	87.03532	3.62647	47.71	<0.001
Residual	72	5.47299	0.07601		
Total	107				

E.4 ANOVA results for biocontrol agents' effect on mycelial growth of different *Neofusicoccum* isolates and species

A) Effect on isolates

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	11	409.41	37.22	2.35	0.021
treatment	1	654.88	654.88	41.33	<0.001
Isolate. treatment	11	988.66	89.88	5.67	<0.001
Residual	48	760.54	15.84		
Total	71	2813.49			

B) Effect on species

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	3	196.03	65.34	2.15	0.102
treatment	1	654.88	654.88	21.56	<0.001
Species. treatment	3	18.37	6.12	0.20	0.895
Residual	64	1944.22	30.38		
Total	71	2813.49			

E.5 ANOVA results for copper hydroxide, phosphorous acid and cyprodinil+ fludioxonil effect on mycelial growth of different *Neofusicoccum* isolates

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	11	1017.1505	92.4682	355.95	<0.001
Fungicide	2	5.6743	2.8371	10.92	<0.001
Isolate. Fungicide	22	2145.9126	97.5415	375.48	<0.001
Residual	72	18.7039	0.2598		
Total	107	3187.4413			

E.6 ANOVA results for copper hydroxide, phosphorous acid and cyprodinil+ fludioxonil effect on mycelial growth of different *Neofusicoccum* species

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	3	332.99	111.00	5.53	0.002
Fungicide	2	5.67	2.84	0.14	0.868
Species. Fungicide	6	920.92	153.49	7.64	<0.001
Residual	96	1927.86	20.08		
Total	107	3187.44			

E.7 ANOVA results for copper hydroxide, phosphorous acid and cyprodinil+ fludioxonil effect on conidial germination of different *Neofusicoccum* species

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	3	43.9432	14.6477	120.84	<0.001
Fungicide	2	114.1134	57.0567	470.72	<0.001
Species. Fungicide	6	69.7555	11.6259	95.91	<0.001
Residual	24	2.9091	0.1212		
Total	35	230.7212			

E.8 ANOVA results for copper spray, phosphorous acid and cyprodinil+ fludioxonil effect on germ tube length of different *Neofusicoccum* species

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	3	13.67343	4.55781	407.46	<0.001
Fungicide	2	58.95816	29.47908	2635.37	<0.001
Species. Fungicide	6	36.97243	6.16207	550.88	<0.001
Residual	24	0.26846	0.01119		
Total	35	109.87249			

E.9 ANOVA results for effect of fungicides on infection of wounded and non-wounded buds by *Neofusicoccum ribis*

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Fungicide	4	33767.3	8441.8	55.86	<0.001
Wounded×Non-wounded	1	258.4	258.4	1.71	0.197
Fungicide× Wounded×Non-wounded	4	368.7	92.2	0.61	0.657
Residual	50	7556.8	151.1		
Total	59	41951.1			

E.10 ANOVA results of effect of fungicides and paint on *Neofusicoccum ribis* infection incidence in wounded soft green shoots

Fixed term	**Wald statistic**	**n.d.f.**	**F statistic**	**d.d.f.**	**F pr.**
Treatment	27.32	5	5.46	30.0	<0.001

E.11 ANOVA results of effect of fungicides and paint on *Neofusicoccum ribis* progression in wounded soft green shoots

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Treatment	5	43747.22	8749.44	88.98	<0.001
Residual	30	2950.00	98.33		
Total	35	46697.22			

E.12 ANOVA results of effect of fungicides on *Neofusicoccum ribis* infection incidence in wounded and non-wounded soft green shoots

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr.
Fungicide	20.75	4	5.19	49.0	<0.001
Wounded×Non-Wounded	5.43	1	5.43	49.0	0.024
Fungicide×Wounded ×Non- Wounded	5.27	4	1.32	49.0	0.277

E.13 ANOVA results of effect of fungicides on *Neofusicoccum ribis* progression in wounded and non-wounded soft green shoots

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Fungicide	4	42756.67	10689.17	114.53	<0.001
Wounded×Non-Wounded	1	3226.67	3226.67	34.57	<0.001
Fungicide× Wounded× Non-Wounded	4	455.83		4.88	0.002
Residual	50	93.33			
Total	59				

E.14 ANOVA results of effect of fungicides and paint on *Neofusicoccum ribis* infection incidence in wounded hard green shoots

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Treatment	11.64	5	2.33	30.0	0.067

E.15 ANOVA results of effect of fungicides and paint on *Neofusicoccum ribis* progression in wounded hard green shoots

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	5	28855.56	5771.11	62.58	<0.001
Residual	30	2766.67	92.22		
Total	35	31622.22			

E.16 ANOVA results of effect of fungicides on *Neofusicoccum ribis* infection incidence in wounded and non-wounded hard green shoots

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr.
Fungicide	14.20	4	3.55	49.0	0.013
Wounded× Non-wounded	0.30	1	0.30	49.0	0.587
Fungicide× Wounded× Non-wounded	0.49	4	0.12	49.0	0.973

E.17 ANOVA results of effect of fungicides on *Neofusicoccum ribis* progression in wounded and non-wounded hard green shoots

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Fungicide	4	46626.67	11656.67	136.60	<0.001
Wounded× Non-wounded	1	540.00	540.00	6.33	0.015
Fungicide× Wounded× Non-wounded	4	626.67	156.67	1.84	0.137
Residual	50	4266.67	85.33		
Total	59	52060.00			

E.18 ANOVA results for the effect of fungicides after inoculation on *Neofusicoccum ribis* infection

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	3	8083.33	2694.44	30.50	<0.001
Residual	20	1766.67	88.33		
Total	23	9850.00			

E.19 Effect of fungicides for Botryosphaeriaceae infection incidence under natural conditions

	Value	df	*P*-value
Pearson Chi-square	4.80	3	0.187

E.20 ANOVA results for the effect of copper hydroxide, phosphorous acid and biocontrol agents on *Neofusicoccum ribis* infection of bark and wood tissue

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	4	10290.0	2572.5	13.40	<0.001
Position	1	26.7	26.7	0.14	0.711
Fungicide. Position	4	256.7	64.2	0.33	0.854
Residual	50	9600.0	192.0		
Total	59	20173.3			

E.21 Fungicides and biocontrol agents tested against botryosphaericeous species

Active ingredient	Trade name	Chemical class	Manufacturer	Field rate /100L (water)
Flusilazole	Nustar®	Triazole	Ei Du Pont de Nemours and Co Inc,Wilmington	10 g
Carbendazim	Protek®	Benzimidazole	Tapuae partnership, NZ	50 mL
Mancozeb	Dithane	Dithiocarbamates	Dow Agro Sciences NZ Ltd	210 g
Tebuconazole	Hornet™ 430SC	Triazole	Nufarm Ltd	23 mL
Pyraclostrobin	Cabrio® WG	Strobilurin	BASF New Zealand Ltd	50 g
Captan *	Captan 800 WG	Cyclic imide	Arysta Lifesciences Corporation USA	125 g
Iprodione	Rovral® FLO	Dicarboximide	Etec Crop Solutions Ltd	100-150 mL
Fludioxonil	Maxim®	Phenylpyrrole	Syngenta Group Company	9-10 L
Thiram	Thiram 40F	Disulphide	Nufarm Ltd	150-200 g
Mono+ di potassium salts	Foschek ™	Phosphorus acid	Tapuae partnership, NZ	500 mL*
Copper hydroxide	DuPont™ Kocide® Opti™	Inorganics	DuPont (New Zealand) Limited	130 g*
Cyprodinil+ Fludioxonil	Switch®	Anilinopyrimidine	Syngenta Group Company	80 g *
Bacillus subtilis	Serenade® MAX		AgraQuest Inc	250-400 g
Trichoderma atroviride	Vinevax™		Agrimm Technologies Ltd	1000 g

* Registered for blueberries for stem blight, leaf spot and botryitis

Appendix F

F.1 Nucleotide sequence of ITS region obtained from molecular identification using ITS4 primer of representative Botryosphaeriaceae species isolates from the 2009-2014 surveys

N. australe

Isolate N113

TGAGAAATTCAAAGGTTCGTCCGGCGGGCGACGCCATGCGCTCCAAAGCGAGGTGTTTTCTACTACGCTTGAGGCAAGAC
GCCACCGCCGAGGTCTTCGAGGCGCGTCCGCGGAGGACGGAGCCCAATACCAAGCAGAGCTTGAGGGTTGAAATGACG
CTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATT
CACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAGTTTATTAAC
TTGTTTCTCAGACTGCGACGTTTACTGACTGGAGTTTTGTGGTCCTCTGGCGGGCGCTGGCCGGCCCCCGAAGGGGGTCG
GGGCGGAGGACCCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATGGGGTGGGAGAGTCGAGCCGGAGCTCGAAT
CAA

Isolate N342

ACGCCATGCGCTCCAAAGCGAGGTGTTTTCTACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTCGAGGCGCGTCCG
CGGAGGACGGAGCCCAATACCAAGCAGAGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAG
GGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCA
TCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAGTTTATTAACTTGTTTCTCAGACTGCGACGTTTACTGACTGG
AGTTTTGTGGTCCTCTGGCGGGCGCTGGCCGGCCCCCGAACGGGGGTCGGTGCGGAGGACCGCGGCCCGCCAAAGCAA
CAGAGGTAGGTACACATGGG

Isolate N435

TCGTACTGATCCGAGGTCACCTTGAGAAATTCAAAGGTTCGTCCGGCGGGCGACGCCATGCGCTCCAAAGCGAGGTGTTT
TCTACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTCGAGGCGCGTCCGCGGAGGACGGAGCCCAATACCAAGCAG
AGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGA
TGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTG
TTGAAAGTTTTAGTTTATTAACTTGTTTCTCAGACTGCGACGTTTACTGACTGGAGTTTTGTGGTCCTCTGGCGGGCGCTGG
CCGGCCCCCGAACGGGGGTCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATG

Isolate N465

CGAGGTCAACCTTGAGAAAAATTCAAAGGTTCGTCCGGCGGGCGACGCCATGCTGCTCCAAAGCGAGGTGTTTTCTACTA
CGCTTGAGGCAAGACGCCACCGCCGAGGTCTTCGAGGCGCGTCCGCGGAGGACGGAGCCCAATACCAAGCAGAGCTTG
AGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGAATTCGATGATT
CACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAA
AGTTTTAGTTTATTAACTTGTTTCTCAGACTGCGACGTTTACTGACTGGAGTTTTGTGGTCCTCTGGCGGGCGCTGGCCGG
CCCCCGAATCGGGGGTCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACCAGAGGTAGGGTACACATGGGGTGGGGAA
AACCGAACCGGACCCC

Isolate N471

GAGGTCAACCTTGAGAAAAATTCAAAGGTTCGTCCGGCGGGCGACGCCATGCGCTCCAAAGCGAGGTGTTTTCTACTACG
CTTGAGGCAAGACGCCACCACCGAGGTCTTCGAGGCGCGTCCGCGGAGGACGGAGCCCAATACCAAGCAGAGCTTGAG
GGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATTGTGCGTTTCAAAGATTCGATGATTCA
CTGAATTCTGCAATTCACATTACTTATTCGCATTTTCGCTGCGGTTCTTCATCGATTGCCAGAACCAAGAGATCCGTTGTTG
AAAGTTTTAGTTTATTAACTTGTTTCTCAGACTGCGACGTTTACTGACTGGAGTTTTGTGGTCCTCTGGCGGGCGCTGGCC
GGCCCCCGAACGGGGGTCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATGGGGTGGGAGAG
TCGAGCCGA

Isolate N496

CCTACCTGATCCGAGGTCACCTTGAGAAATTCAAAGGTTCGTCCGGCGGGCGACGCCATGCGCTCCAAAGCGAGGTGTTT
TCTACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTCGAGGCGCGTCCGCGAAGGACGGAGCCCAATACCAAGCAG
AGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCTTTTCGGAATATCAAGGGGCTCAATGTGCGTTCAAAGATTCGAT
GATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTG
TTGAAAGTTTTAGTTTATTAACTTGTTTCTCAGACTGCGACGTTTACTGACTGGAGTTTTGTGGTCCTCTGGCGGGCGCTGG
CCGGCCCCCGAACGGGGGTCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATGGGGTGGGAG
AGTCGAGCCGGAGCTCGAAT

Isolate N4103

TCGTACCTGATCGAGGTCACCTTGAGAAATTCAAAGGTTCGTCCGGCGGGCGACGCCATGCGCTCCAAAGCGAGGTGTTT
TCTACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTCGAGGCGCGTCCGCGGAGGACGGAGCCCAATACCAAGCAG
AGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCTTTCAAAGATTCGAT
GATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGT
TGAAAGTTTTAGTTTATTAACTTGTTTCTCAGACTGCGACGTTTACTGACTGGAGTTTTGTGGTCCTCTGGGCGGGCGCTG
GCCCGGCCCCCCAAACGGGGGTCGGTGCGGGGGGACCGCGGCCCCGCCAAAAGCACCAGAGGTAGGGT

Isolate 526

ATACCTGATCGAGGTCACCTTGAGAAATTCAAAGGTTCGTCCGGCGGGCGACGCCATGCGCTCCAAAGCGAGGTGTTTTC
TACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTCGAGGCGCGTCCGCGGAGGACGGAGCCCAATACCAAGCAGAG
CTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATG
ATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTT
GAAAGTTTTAGTTTATTAACTTGTTTCTCAGACTGCGACGTTTACTGACTGGAGTTTTGTGGTCCTCTGGCGGGCGCTGGC
CGGCCCCCGAACGGGGGTCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATGGGGTGGGAGA
GTCGAGCCGGAGCTCGAATCAACTCGGTAATGATCCTTCCGCAGGTTCACCT

Isolate S1-140

GTACCTGATCGAGGTCACCTTGAGAAATTCAAAGGTTCGTCCGGCGGGCGACGCCTGCGCTCCAAAGCGAGGTGTTTTCT
ACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTCGAGGCGCGTCCGCGAAGGACGGAGCCCAATACCAAGCAGAGC
TTGAGGGTTGAAATGACGCTCGAACAGGCATGCTCCTCGGAATATCAAGGAGCGCTAAGTTCTTTCAAAGAATCGATTAT
TCACTGAATTCTGCACTAACATTACTTATCGATTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGGCAG
TTTTAGTTTATTAACTTGTTTCTCAGACTGCAACGTTTACTGACTGGAGTTTTGTGGTCACTGGCGGGCGCTGGCCGGCCCC
CGAAGGGGGTCGGGCGAAGGACCGCGCCCCGCAAAGCACAGAGTAGGTCACATGGGGGGGAGAGTCGAGCCGAGCTC
GAATCATTCGGTAAGATCCTCCCGCCGGTTCACCTACGAAAACCTTTGGT

Isolate S1-148

CTCTCCCACCCCATGTGTACCTACCTCTGTTGCTTTGGCGGGCCGCGGTCCTCCGCACCGACCCCCGTTCGGGGGCCGGCC
AGCGCCCGCCAGAGGACCACAAAACTCCAGTCAGTAAACGTCGCAGTCTGAGAAACAAGTTAATAAACTAAAACTTTCAA
CAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAAT
CATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCT
CTGCTTGGTATTGGGCTCCGTCCTCCGCGGACGCGCCTCGAAGACCTCGGCGGTGGCGTCTTGCCTCAAGCGTAGTAGAA
AACACCTCGCTTTGGAGCGCATGGCGTCGCCCGCCGGACGAACCTTTGAATTTTTCTCAAGGTTGACCTCGGATCAGGTA
GGGATACCCGCTGAACTTAAGCATATCA ATAAGCGGAG GAAAAGAAAC CAACAGGGAT

Isolate S2-84

ATGTCCGGATCAGTCGGCACCAGACTGGCCGAAGACAAAGTTGTCGGGGCGGAAGAGCTGGCCGAAGGGGCCGGCGCG
GACGGCATCCATGGTGCCGGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTCGACGCCTGCTGCGCG
TTCAGAAGATTGCCATACTTTACGTGTTTGTGCGATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGAGC
TGGAGGTCGGAGGTGCCATTGTAGCTGCTGCGGGTCAGCCATTGCCATTCGCGCAAAGAACGGCGCAGACTTACACGCC
AGAGCCGTCCAGGCCGTGCTCGCCAGAAATGGTCTGCCTGCGGTGTCGATTCGCGTCAGCGGGGGCGCGGGAGCGGC
GGTGTTTTGGCAACAAACCAGAAAGCAGCACCATT

Isolate S2-93

TTGAGAAAAATTCAAAGGTTCGTCCGGCGGGCGACGCCATGCGCGCTCCAAAGCGAGGTGTTTTCTACTACGCTTGAGGC
AAGACGCCACCGCCGAGGTCTTCGAGGCGCGTCCGCGGAGGACGGAGCCCAATACCAAGCAGAGCTTGAGGGTTGAAA
TGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTG
CAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAGTTTA
TTAACTTGTTTCTCAGACTGCGACGTTTACTGACTGGAGTTTTGTGGTCCTCTGGCGGGCGCTGGCCGGCCCCCGAACGGG
GGTCGGTGGCGGAGGACCGCGGCCCGCCAAAGCAACAG
AGGTAGGTACACATGGGGTGGGAGAGTCGAGCCGGAGCTC

N. luteum

Isolate 7151

GTACTGATCGAGGTCACCTTGAGAAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGTTTTCT
ACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTCGAGGCGCGTCCACAGAGGACGGAGCCCAATACCAAGCAGAGC
TTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGA
TTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTG
AAAGTTTTAGTTTATTAACTTGTTTCTCAGACTGCGACGTTTACTGACTGGAGTTTTGTGGTCCTCTGGCGGGCGCTGGCC
GGCCCCCCGAACGGGGGTCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACAAG

Isolate S1-137

TACCTGATCGAGGTCACCTTGAGAAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGTTTTCT
ACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTCGAGGCGCGTCCACAGAGGACGGAGCCCAATACCAAGCAGAGC
TTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGA
TTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTG
AAAGTTTTAGTTTATTAACTTGTTTCTCAGACTGCGACGTTTACTGACTGGAGTTTTGTGGTCCTCTGGCGGGCGCTGGCC
GGCCCCCCGAACGGGGGTCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATGGG

Isolate S1-141

CTACCTGATCCGAGGTCAACCTTGAGAAAAATTCAAAGGTTCGTCCGGCGGGCGGCGCCGTGCGCTCCAAAGCGAGGTG
TTTTCTACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTCGAGGCGCGTCCACAGAGGACGGAGCCCAATACCAAGC
AGAGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTC
GATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGT
TGTTGAAAGTTTTAGTTTATTAACTTGTTTCTCAGACTGCGACGTTTACTGACTGGAGTTTTGTGGTCCTCTGGCGGGCGCT
GGCCGGCCCCCCGAACGGGGGTCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATGGGGGGG
GAGAGGCGACCCGGAGCTCGAATCAACTCGGTAATGATCCTTCCGCAGGTT

N. parvum

Isolate N414

TACTGATCGAGGTCACCTTGAGAATAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGTTTTC
TACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTTAAGGCGCGTCCGTGGAGGACGGGGCCCAATACCAAGCAGAG
CTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATG
ATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTT
GAAAGTTTTAGTTTATTAACTTGTTTTTCAGACTGCGAAGTTCACTGACTGGAGTTTTATGGTCCTCTGGCGGGCGCTGGC
CAGCCCCTCGAAGGGCGCCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATTGG

Isolate N454

AGAAATAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGTTTTCTACTACGCTTGAGGCAAGA
CGCCACCGCCGAGGTCTTTAAGGCGCGTCCGTGGAGGACGGGGCCCAATACCAAGCAGAGCTTGAGGGTTGAAATGACG
CTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATT
CACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAGTTTATTAAC
TTGTTTTTCAGACTGCGAAGTTCACTGACTGGAGTTTTATGGTCCTCTGGCGGGCGCTGGCCAGCCCCCCGAAGGGCGCC
GGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATAGGGTGGGAGAGTCGAGC

Isolate S1-93

AGGTCACCTTGAGAATAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGTTTTCTACTACGCT
TGAGGCAAGACGCCACCGCCGAGGTCTTTAAGGCGCGTCCGTGGAGGACGGGGCCCAATACCAAGCAGAGCTTGAGGG
TTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGA
ATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTT
AGTTTATTAACTTGTTTTTCAGACTGCGAAGTTCACTGACTGGAGTTTTATGGTCCTCTGGCGGGCGCTGGCCAGCCCCTC
GAAGGGCGCCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATTGGGGGGGGAGAGTCGAGCC
GGAGCTCAAATCAACTCGGTAATGA TCCTTCCGCAGGTTCACCTACGGAAAGTTC

Isolate S1-101

CTGGATCCCGAGGTCAACCTTGAGAATAATTCAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGTTTT
CTACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTTAAGGCGCGTCCGTGGAGGACGGGGCCCAATACCAAGCAGA
GCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGAT
GATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGT
TGAAAGTTTTAGTTTATTAACTTGTTTTTCAGACTGCGAAGTTCACTGACTGGAGTTTTATGGGTCCTCTGGCGGGAGCTG
GCCAGCCCCCCGAAGGGCGCCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATAGGGTGGGA
GAGTCGAGCCGGAGCTCGAATCAACTCGGTAATGATCCTTCCGCAGGTTCAC

N. ribis

Isolate S1-83

AGAAATAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGTTTTCTACTACGCTTGAGGCAAGA
CGCCACCGCCGAGGTCTTTAAGGCGCGTCCGTGGAGGACGGAGCCCAATACCAAGCAGAGCTTGAGGGTTGAAATGACG
CTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATT
CACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAGTTTATTAAC
TTGTTTTTCAGACTGCGAAGTTCACTGACTGGAGTTTTATGGTCCTCTGGCGGGCGCTGGCCAGCCCCCCGAAGGGCGCC
GGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTGGGTACACATTGGGTGGGAGAGTCGAGCCGGAGCTCGAA
TC

Isolate S1-91

TACCTGATCGAGGTCACCTTGAGAATAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGTTTT
CTACTACGCTTGAGGCAAGACGCCACCGCCGAGGTCTTTAAGGCGCGTCCGTGGAGGACGGAGCCCAATACCAAGCAGA
GCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGAT
GATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGT
TGAAAGTTTTAGTTTATTAACTTGTTTTTCAGACTGCGAAGTTCACTGACTGGAGTTTTATGGTCCTCTGGCGGGCGCTGGC
CAGCCCCCCGAAGGGCGCCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTGGGTACACATTGGGTGGGAGA
GTCGAGCCGGAGCTCGAAATC

Isolate S1-109

TCACCTTGAGAAATAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGTTTTCTACTACGCTTG
AGGCAAGACGCCACCGCCGAGGTCTTTAAGGCGCGTCCGTGGAGGACGGAGCCCAATACCAAGCAGAGCTTGAGGGTT
GAAATGACGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAAT
TCTGCATTTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAG
TTTATTAACTTGTTTTTCAGACTGCGAAGTTCACTGACTGGAGTTTTATGGTCCTCTGGCGGGCGCTGGCCAGCCCCCCCG
AAGGGCGCCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTAGGTACACATTGGGTGGGAGAGTCGAGCCGG
AGCTCGAATCAACTCGGTAATGA

F.2 Nucleotide sequence of β-tubulin gene of representative Botryosphaeriaceae species isolates from the 2009-2014 surveys

N. australe

Isolate N113

ACGGTCTGGACAGACTGGCCGAAGACAAAGTTGTCGGGGCGGAAGAGCTGGCCGAAGGGGCCGGCGCGGACGGCATC
CATGGTGCCGGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTCGACGCCTGCTGCGCGTTCAGAAGA
TTGCCATACTTTACGTGTTTGTGCGATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGAGCTGGAGGTC
GGAGGTGCCATTGTAGCTGCTGCGGGTCAGCCATTGCCATTCGCGCAAGAAACGGCGCAGACTTACACGCCAGAGCCGT
CCAGGCCGTGCTCGCCAGAAATGGTCTGCCTGCGGTGTCGATTC GCGTCAGCGG GAGCGCGGG AGCGGCGTTT
TTTGGCCAC AAACCAGAAAGCAGCTCCGATTTTGGTACA

Isolate N342

ATTTTCGGACAGTTCGGCACCAGACTGGCCGAAGACAAAGTTGTCGGGGCGGAAGAGCTGGCCGAAGGGGCCGGCGCG
GACGGCATCCATGGTGCCGGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTCGACGCCTGCTGCGCG
TTCAGAAGATTGCCATACTTTACGTGTTTGTGCGATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGAGC
TGGAGGTCGGAGGTGCCATTGTAGCTGCTGCGGGTCAGCCATTGCCATTCGCGCAAGAAACGGCGCAGACTTACACGCC
AGAGCCGTCCAGGCCGTGCTCGCCAGAAATGGTCTGCCTGCGGTGTCGATTCGCGTCAGCGGGGGCGCGGGAGCGGCG
TGTTTGGCAACAAACCAGAAAGCAGCACCATTTGGTTACAAA

Isolate N435

CTGGACTGGCCGAAGACAAAGTTGTCGGGGCGGAAGAGCTGGCCGAAGGGGCCGGCGCGGACGGCATCCATGGTGCC
GGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTCGACGCCTGCTGCGCGTTCAGAAGATTGCCATAC
TTTACGTGTTTGTGCGATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGAGCTGGAGGTCGGAGGTGCC
ATTGTAGCTGCTGCGGGTCAGCCATTGCCATTCGCGCAAGAAACGGCGCAGACTTACACGCCAGAGCCGTCCAGGCCGTG
CTCGCCAGAAATGTCTCCTGCGTGCGATCGAGCCGCGGAGCCGACGCGGGTTTTGCCACAGCAGAAGAACACATTTTGTA
CCTCGCAACTAGGCGTGACGCTTCCGCGAGCTGAGATGCCGTGCGCGTGGGGGTTGAGCTAGCAGCAAATAGAAGTGCT
CGCGCCCGGAGTTACCCCACACATCGGGAGATACGGGTTACGGTATAATAAAAAAAAGAAAAGCCGG

Isolate N465

TTTTTCCGGCTCAGTTCGGCACCAGACTGGCCGAAGACAAAGTTGTCGGGGCGGAAGAGCTGGCCGAAGGGGCCGGCG
CGGACGGCATCCATGGTGCCGGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTCGACGCCTGCTGCG
CGTTCAGAAGATTGCCATACTTTACGTGTTTGTGCGATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGA
GCTGGAGGTCGGAGGTGCCATTGTAGCTGCTGCGGGTCAGCCATTGCCATTCGCGCAAGAAACGGCGCAGACTTACACG
CCAGAGCCGTCCAGGCCGTGCTCGCCAGAAATGGTCTGCCTGCGGTGTCGATTCGCGTCAGCGGGGGCGCGGGAGCGG
CGGTGTTTTGGCAACAAACCAGAAAGCAGCACCATTTGGTTACAA

Isolate N471

ACGGTCGGCACCAGACTGGCCGAAGACAAAGTTGTCGGGGCGGAAGAGCTGGCCGAAGGGGCCGGCGCGGACGGCAT
CCATGGTGCCGGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTCGACGCCTGCTGCGCGTTCAGAAG
ATTGCCATACTTTACGTGTTTGTGCGATTAGGGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGAGCTGGAGGT
CGGAGGTGCCATTGTAGCTGCTGCGGGTCAGCCATTGCCATTCGCGCAAGAAACGGCGCAGACTTACACGCCAGAGCCG
TCCAGGCCGTGCTCGCCAGAAATGGTCTGCCTGCGGTGTCGATTCGCGTCAGCGGGGGCGCGGGAGCGGCGGTGTTTTG
G CAACAAACCAG AAAGCAGCACA ATTTGGTTACAA

Isolate N496

CTCGGTCGGACAGACTGGCCGAAGACAAAGTTGTCGGGGCGGAAGAGCTGGCCGAAGGGGCCGGCGCGGACGGCATC
CATGGTGCCGGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTCGACGCCTGCTGCGCGTTCAGAAGA
TTGCCATACTTTACGTGTTTGTGCGATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGAGCTGGAGGTC
GGAGGTGCCATTGTAGCTGCTGCGGGTCAGCCATTGCCATTCGCGCAAGAAACGGCGCAGACTTACACGCCAGAGCCGT
CCAGGCCGTGCTCGCCAGAAATGGTCTGCCTGCGGTGTCGATTCGCCTCAGCGGGGCGCGGGGACGCGGGTGTTTTGGC
AACAAACAAGAAAGCAGCACCATTTGGTTACAA

Isolate N4103

CGGTCGGACAGACTGGCCGAAGACAAAGTTGTCGGGGCGGAAGAGCTGGCCGAAGGGGCCGGCGCGGACGGCATCCA
TGGTGCCGGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTCGACGCCTGCTGCGCGTTCAGAAGATT
GCCATACTTTACGTGTTTGTGCGATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGAGCTGGAGGTCGG
AGGTGCCATTGTAGCTGCTGCGGGTCAGCCATTGCCATTCGCGCAAGAAACGGCGCAGACTTACACGCCAGAGCCGTCCA
GGCCGTGCTCGCCAGAAATGGTCTGCCTGCGGTGTCGATTCGCGTCAGCGGGGGCGCGGGAGCGGCGGTGTTTGGCAA
CAAACCAGAAAGCAGCACCATTTGGTTACAAA

Isolate 526

ACGGTCGGCACCAGACTGGCCGAAGACAAAGTTGTCGGGGCGGAAGAGCTGGCCGAAGGGGCCGGCGCGGACGGCAT
CCATGGTGCCGGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTCGACGCCTGCTGCGCGTTCAGAAG
ATTGCCATACTTTACGTGTTTGTGCGATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGAGCTGGAGGT
CGGAGGTGCCATTGTAGCTGCTGCGGGTCAGCCATTGCCATTCGCGCAAGAAACGGCGCAGACTTACACGCCAGAGCCG
TCCAGGCCGTGCTCGCCAGAAATGGTCTGCCTGCGGTGTCGATTCGCGTCAGCGGGGGCGCGGGAGCGGCGGTGTTTTG
GCAACAAACCAGAAAGCAGCACCATTTGGTTACAA

Isolate S1-140

CGGTCGGCACCAGACTGGCCGAAGACAAAGTTGTCGGGGCGGAAGAGCTGGCCGAAGGGGCCGGCGCGGACGGCATC
CATGGTGCCGGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTCGACGCCTGCTGCGCGTTCAGAAGA
TTGCCATACTTTACGTGTTTGTGCGATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGAGCTGGAGGTC
GGAGGTGCCATTGTAGCTGCTGCGGGTCAGCCATTGCCATTCGCGCAAGAAACGGCGCAGACTTACACGCCAGAGCCGT
CCAGGCCGTGCTCGCCAGAAATGGTCTGCCTGCGGTGTCGATTCGCGTCAGCGGGAGCGCGGGAGCGGCGGTGTTTTG
GCAACAAACCAAAAAAAGCCC

Isolate S1-148

CTTTTTCGGGACGGTTCGGACAGACTGGCCGAAGACAAAGTTGTCGGGGCGGAAGAGCTGGCCGAAGGGGCCGGCGCG
GACGGCATCCATGGTGCCGGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTCGACGCCTGCTGCGCG
TTCAGAAGATTGCCATACTTTACGTGTTTGTGCGATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGAGC
TGGAGGTCGGAGGTGCCATTGTAGCTGCTGCGGGTCAGCCATTGCCATTCGCGCAAGAAACGGCGCAGACTTACACGCC
AGCGCCGTCCAGGCCGTGCTCGCCAGAAATGGTCTGCCTGCGGTGTCGATTCGCGTCAGCGGGAGCGCGGGAGCGGCG
GTGTTTTGGCAACAAACAAAAGAGGCCGTTTGTCCAA

Isolate S2-84

ATGTCCGGATCAGTCGGCACCAGACTGGCCGAAGACAAAGTTGTCGGGGCGGAAGAGCTGGCCGAAGGGGCCGGCGCG
GACGGCATCCATGGTGCCGGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTCGACGCCTGCTGCGCG
TTCAGAAGATTGCCATACTTTACGTGTTTGTGCGATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGAGC
TGGAGGTCGGAGGTGCCATTGTAGCTGCTGCGGGTCAGCCATTGCCATTCGCGCAAAGAACGGCGCAGACTTACACGCC
AGAGCCGTCCAGGCCGTGCTCGCCAGAAATGGTCTGCCTGCGGTGTCGATTCGCGTCAGCGGGGGCGCGGGAGCGGC
GGTGTTTTGGCAACAAACCAGAAAGCAGCACCATT

Isolate S2-93

ATGGTCGGGCTCAGTCGGACAGACTGGCCGAAGACAAAGTTGTCGGGGCGGAAGAGCTGGCCGAAGGGGCCGGCGCG
GACGGCATCCATGGTGCCGGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTCGACGCCTGCTGCGCG
TTCAGAAGATTGCCATACTTTACGTGTTTGTGCGATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGAGC
TGGAGGTCGGAGGTGCCATTGTAGCTGCTGCGGGTCAGCCATTGCCATTCGCGCAAGAAACGGCGCAGATTTACACGCC
AGAGCCGTCCAGGCCGTGCTCGCCAGAAATGGTCTGCCTGCGGTGTCGATTCGCGTCAGCGGGAGCGCGGGAGCGGCG
TTTTTTGGCAACAAACCAGACAGCAGCACCGATTTTGTACACA

N. luteum

Isolate 7151

GTCGGTTCGGCACCAGACTGGCCGAAGACAAAGTTGTCGGGGCGGAAGAGCTGGCCGAAGGGGCCGGCGCGGACGGC
ATCCATGGTGCCGGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTCGACGCCTGCTGCGCGTTCAGA
AGATTGCCATACTTTACGTGTTCGTGCGATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGAGCTGGAG
GTCGGAGGTGCCATTGTAGCTGCTGCGGGTCAGCCATTGCCATTCGCGCAAGAAACGGCGCAGACTTACACGCCAGAGC
CGTCCAGGCCGTGCTCGCCAGAAATGGTCTGCCTGCGGTGTCGATTCGCGTCAGCGGGCCCCGGGACCACGGTCTTTGGC
CAAAAACAGGAAAAACACGTTTGGTACCAA

Isolate S1-137

CACGGATGGACAGACTGGCCGAAGACAAAGTTGTCGGGGCGGAAGAGCTGGCCGAAGGGGCCGGCGCGGACGGCATC
CATGGTGCCGGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTCGACGCCTGCTGCGCGTTCAGAAGA
TTGCCATACTTTACGTGTTCGTGCGATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGAGCTGGAGGTC
GGAGGTGCCATTGTAGCTGCTGCGGGTCAGCCATTGCCATTCGCGCAAGAAACGGCGCAGACTTACACGCCAGAGCCGT
CCAGGCCGTGCTCGCCAGAAATGGTCTGCCTGCGGTGTCGATTCGCGTCAGCGGGGGCGCGGGAGCGGCAGTGTTTTGG
CAACAAACCAGAAAGCGCCCATTTTTTCCCAA

Isolate S1-141

ATGAGGTCTACGCGTCCCCTGACAGCCCCGCGTCTTGCCCCCCGGCTACGACAACGGGGTCAACACCGCTGCGCACTCGT
GCTAACGTCGTCTTTTCGCATCCATAGGTTCACCTCCAGACCGGCCAATGCGTAAGTCTCCTCACATCCGCTGGAATCGCT
GCATCGCGCTGACTTTGCCCAGGGTAACCAAATCGGTGCTGCCTTCTGGTTTGTTGCCAAAACACTGCCGCTCCCGCGCCC
CCGCTGACGCGAATCGACACCGCAGGCAGACCATTTCTGGCGAGCACGGCCTGGACGGCTCTGGCGTGTAAGTCTGCGC
CGTTTCTTGCGCGAATGGCAATGGCTGACCCGCAGCAGCTACAATGGCACCTCCGACCTCCAGCTCGAGCGCATGAACGT
CTACTTCAACGAGGTACTCTCTCACTAATCGCACGAACACGTAAAGTATGGCAATCTTCTGAACGCGCA

N. parvum

Isolate N414

GTTTTTCCGGCTCGGTCGGCGCCAGACTGACCGAAGACGAAGTTGTCAGGGCGGAAGAGCTGGCCGAAGGGGCCGGCG
CGGACGGCATCCATGGTGCCGGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTGGACGCCTGCTGCG
CGTTCAGAAGATTGCCATACTTTACGTGTTTGTGCAATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGA
GCTGCAGGTCGGAGGTGCCATTGTAGCTGCTGCGGGTCAGCCATTGCCATTCGCGCGGGAAACGGCGCAGACTTACACG
CCAGAGCCGTCCAGGCCGTGCTCGCCAGAAATGGTCTGCCTGTGGTGTCGATTCGCGTCAGCGGGGGCGCGGGAGCGG
GAGTGTTTTGGCAACAAACCAGAAAGCAGCACCGATTTGGTTCACAA

Isolate N454

GGCTCTACTCTGCATGACATAGCCCGCGGTCTGGCCCCCCGGCTACGACAACGGGGTCAACACCGCTGCG
CCACTCCTGCTAACATCGTCTTTTCGCATCCATAGGTTCACCTCCAGACCGGCCAATGCGTAAGTCTCCTCGCATCCGCTGC
ACTCGCTGCACCGCGCTGACTTTGCCCAGGGTAACCAAATCGGTGCCGCCTTCTGGTTTGTTGCCAAACACTCCCGCTCCC
GCGCCCCCGCTGACGCGAATCGACACCACAGGCAGACCATTTCTGGCGAGCACGGCCTGGACGGCTCTGGCGTGTAAGT
CTGCGCCGTTTCCCGCGCGAATGGCAATGGCTGACCCGCAGCAGCTACAATGGCACCTCCGACCTGCAGCTCGAGCGCAT
GAACGTCTACTTCAACGAGGTACTCTCTCACTAATTGCACAAACACGTAAAGTATGGCAATCTTCTGAACGCGCAGCAGGC
GTCCAACAACAAGTACGTTCCTCGTGCCGTCCTCGTCGACCTCGAG

Isolate S1-93

CCGGTCGGCGCCAGACTGACCGAAGACGAAGTTGTCAGGGCGGAAGAGCTGGCCGAAGGGGCCGGCGCGGACGGCAT
CCATGGTGCCGGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTGGACGCCTGCTGCGCGTTCAGAAG
ATTGCCATACTTTACGTGTTTGTGCAATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGAGCTGCAGGTC
GGAGGTGCCATTGTAGCTGCTGCGGGTCAGCCATTGCCATTCGCGCGGGAAACGGCGCAGACTTACACGCCAGAGCCGT
CCAGGCCGTGCTCGCCAGAAATGGTCTGCCTGTGGTGTCGATTCGCGTCAGCGGGGGCGCGGGAGCGGGAGTGTTTTGG
CAACAAACCAGAAAGCAGCACCGATTTGGTACA

Isolate S1-101

GCAGACTGACCGAAGACGAAGTTGTCAGGGCGGAAGAGCTGGCCGAAGGGGCCGGCGCGGACGGCATCCATGGTGCC
GGGCTCGAGGTCGACGAGGACGGCAGGAGGAACGTACTTGTTGTTGGACGCCTGCTGCGCGTTCAGAAGATTGCCATAC
TTTACGTGTTTGTGCAATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGAGCTGCAGGTCGGAGGTGCC
ATTGTAGCTGCTGCGGGTCAGCCATTGCAATTCGCGCGGGAAACGGCGCAGACTTACACGCCAGAGCCGTCCAGGCCGT
GCTCGCCAGAAATGGTCTGCCTGTGGTGTCGATTCGCGTCAGCGGGGGCGCGGGAGCGGGAGTGTTTTGGCAACAAACC
AGAAAGCAGCACCGATTTGTCCC

N. ribis

Isolate S1-83

GGTTCGGCGCCAGACTGACCGAAGACGAAGTTGTCAGGGCGGAAGAGCTGGCCGAAGGGGCCGGCGCGGACGGCATC
CATGGTGCCGGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTGGACGCCTGCGCGCGTTCAGAAGAT
TGCCATACTTTACGTGTTTGTGCAATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGAGCTGCAGGTCG
GAGGTGCCATTGTAGCTGCTACGGGTCAGCCATTGCCATTCGCGCGGGAAACGGCGCAGACTCACACGCCAGAGCCGTC
CAGGCCGTGCTCGCCGGAAATGGTCTGCCTGTGGTGTCGATTCGCGTCAGCGGGGGCGCGGGAGCGGGAGTGTTTTGGC
AACAAACCAGAAAGCAGCACCGATTTGGTTCACAA

Isolate S1-91

ATTTCCGGCGCGGTCGGCGCCAGACTGGCCGAAGACGAAGTTGTCGGGGCGGAAGAGCTGGCCGAAGGGGCCGGCGC
GGACGCCATCCATGGTGCCGGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTGGACGCCTGCTGCGC
GTTCAGAAGATTGCCATACTTTACGTGTTTGTGCAATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGAG
CTGCAGGTCGGAGGTGCCATTGTAGCTGCTGCGGGTCAGCCATTGCCATTCGCGCGGGAAACGGCGCAGACTCACACGC
CAGAGCCGTCCAGGCCGTGCTCGCCGGAAATGGTCTGCCTGTGGTGTCGATTCGCGTCAGCGGGGGCGCGGGAGCGGG
AGTGTTTTGGCAACAAACCAGAAGGCGGCACCGATTTGTATACAA

Isolate S1-109

TATTTTCGGGATCATTCGGCGCCAGACTGGCCGAAGACGAAGTTGTCGGGGCGGAAGAGCTGGCCGAAGGGGCCGGCG
CGGACGGCATCCATGGTGCCGGGCTCGAGGTCGACGAGGACGGCACGAGGAACGTACTTGTTGTTGGACGCCTGCTGCG
CGTTCAGAAGATTGCCATACTTTACGTGTTTGTGCAATTAGTGAGAGAGTACCTCGTTGAAGTAGACGTTCATGCGCTCGA
GCTGCAGGTCGGAGGTGCCATTGTAGCTGCTGCGGGTCAGCCAATGCCATTCGCGCGGGAAACGGCGCAGACTCACACG
CCAGAGCCGTCCAGGCCGTGCTCGCCGGAAATGGTCTGCCTGTGGTGTCGATTCGCGTCAGCGGGGGCGCGGGAGCGG
GAGTGTTTTGGCAACAAACCAGAAAGCAGCCCATTTTGTTCACAA

F.3 Nucleotide sequence of *tef1* gene of representative *Pestalotiopsis* species isolates

H1- *P. clavispora*

EF1-526F

ATCTACAGTGCGGTGGTATCGACAAGCGTACCATCGAGAAGTTCGAGAAGGTTAGTCATCTATTGATTCCCATCATCATTC
CCCTTCACTTCAGCGTCATGATTTTCAACATACGTGTTGAAAATTATTTTCGCTCCTTCCACACTTTTTTTCGCTGGTTACCCC
GCCGCGAGGCACCCGCACGACCCCGCGGTGCAAACGAAAAATTTCTTATCACAGCCCCACCTTGCACAAGCAACCATGCA
TTACTCATGAGACCCACTTTGAACAATTGCTAATTCCTTCATTCAGGAAGCCGCCGAGCTCGGTAAGGGTTCCTTCAAGTA
CGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACCATCGATATCGCTCTCTGGAAGTTCGAGACCA
ACGAGTACAATGTCACCGTCATTGGTCAGTACCCTTCTACCTATGCCATGTACTGCTCCATAAGACACTTGACTAACCTTGC
TTCATAGACGCTCCCGGTCACCGTGATTTCATCAAGAACATGATTACTGGTACTTCCCAGGCCGACTGCGCCATTCTCATCA
TTGCCGCTGGTACTGGTGAGTTCGAGGCTGGTATCTCCAAGGATGGCCAGACTCGTGAGCACGCTCTGCTCGCTTTCACCC
TCGGTGTCAAGCAGCTCATCGTTGCCATCAACAAGATGGA

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TTGTCGGTAGGACGGCTAGGGGGGTTGATGGCGTCAATGGCCTGGAAGAGGGTCTTGCCCTCCTTCTTGACACCCTTGAC
CTCCTTCTCCCAGCCCTTGTACCAGGAAGCGTTGGTGGTCTCCTCCAACATGTTGTCACCGTTGAAACCGGAGATGGGGAC
GAAGGGGACCTCCTTGGGGTTGTAGCCAACCTTCTTGATGAAGTTGGAGGTCTCCTTGATGATCTCCTTGTAACGGGCCTC
GGACCACTTGGCAGTGTCCATCTTGTTGATGGCAACGATGAGCTGCTTGACACCGAGGGTGAAAGCGAGCAGAGCGTGC
TCACGAGTCTGGCCATCCTTGGAGATACCAGCCTCGAACTCACCAGTACCAGCGGCAATGATGAGAATGGCGCAGTCGGC
CTGGGAAGTACCAGTAATCATGTTCTTGATGAAATCACGGTGACCGGGAGCGTCTATGAAGCAAGGTTAGTCAAGTGTCT
TATGGAGCAGTACATGGCATAGGTAGAAGGGTACTGACCAATGACGGTGACATTGTACTCGTTGGTCTCGAACTTCCAG
AGAGCGATATCGATGGTGATACCACGCTCACGCTCGGCCTTGAGCTTGTCAAGAACCCAGGCGTACTTGAAGGAACCCTT
ACCGAGCTCGGCGGCTTCCTGAATGAAGGAATTAGCAATTGTTCAAAGTGGGTCTCATGAGTAATGCAT

P1-1 *P. trachicarpicola*

EF1-526F

TGATCTACAGTGCGGTGGTATCGACAAGCGTACCATCGAGAAGTTCGAGAAGGTTAGTCATCCTCGCAATCCCATCATCCT
CATCATCATCATCATCACCACCTCGCAAACGTTGCCACACCGGTGCCGAAAATCTGGTTTTCGCACCTGCCCATTTTCCCAG
ACACTTACCCCGCCGCACGACCCCGCGGTGCAAACGAAAAATTTCTTATCATAGCCCCACATCACACAAACATTTTGGCAG
CCACGCACTTTGCATGACCCACAATGAACAATTGCTGACCCCGCCAAATAGGAAGCCGCCGAGCTCGGAAAGGGTTCCTT
CAAGTACGCATGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACCATTGATATCGCTCTCTGGAAGTTCG
AGACCAACGAGTACAATGTCACCGTCATTGGTTAGTATCCCTGTCCACAACATGTGTCATGTCTCCAAACTCAAGACTAAC
CTTGCAATACAGACGCTCCCGGTCACCGTGATTTCATCAAGAACATGATTACTGGTACTTCCCAGGCCGACTGCG
CCATTCTCATCATTGCCGCCGGTACTGGTGAGTTCGAGGCTGGTATCTCCAAGGATGGCCAGACTCGTGAGCACGCTCTG
CTTGCCTTCACCCTGGGTGTCAAGCAGCTCATCGTTGCCATCAACA

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AGTCTTGCCCTCCTTCTTGACACCCTTGACCTCCTTCTCCCAACCCTTGTACCAGGGGGCGTTGGTGGTCTCGTC
CAACATGTTGTCACCGTTGAAACCGGAGATGGGGACGAAGGGGACCTCCTTGGGGTTGTAGCCAACCTTCTTGAT
GAAGTTGGAGGTCTCCTTGATGATCTCCTTGTAACGGGCCTCGGACCACTTGGCAGTGTCCATCTTGTTGATGGC
AACGATGAGCTGCTTGACACCCAGGGTGAAGGCAAGCAGAGCGTGCTCACGAGTCTGGCCATCCTTGGAGATACC
AGCCTCGAACTCACCAGTACCGGCGGCAATGATGAGAATGGCGCAGTCGGCCTGGGAAGTACCAGTAATCATGTT
CTTGATGAAATCACGGTGACCGGGAGCGTCTGTATTGCAAGGTTAGTCTTGAGTTTGGAGACATGACACATGTTG
TGGACAGGGATACTAACCAATGACGGTGACATTGTACTCGTTGGTCTCGAACTTCCAGAGAGCGATATCAATGGT
GATACCACGCTCACGCTCGGCCTTGAGCTTGTCAAGAACCCATGCGTACTTGAAGGAACCCTTTCCGAGCTCGGC
GGCTTCCTATTTGGCGGGGTCAGCAATTGTTCATTGTGGGTCATGCAAAGTGCGTGGCTGCCAAAATGTTTGTGT
GATGTG

P1-3 *P. trachicarpicola*

EF1-526F

TCTACAGTGCGGTGGTATCGACAAGCGTACCATCGAGAAGTTCGAGAAGGGAGTCATCCTCGCAATCCCATCATCCTCAT
CATCATCATCATCACCACCTCGCAAACGTTGCCACACCGGTGCCGAAAATCTGGTTTTCGCACCTGCCCATTTTCCCAGACA
CTTACCCCGCCGCACGACCCCGCGGTGCAAACGAAAAATTTCTTATCATAGCCCCACATCACACAAACATTTTGGCAGCCA
CGCACTTTGCATGACCCACAATGAACAATTGCTGACCCCGCCAAATAGGAAGCCGCCGAGCTCGGAAAGGGTTCCTTCAA
GTACGCATGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACCATTGATATCGCTCTCTGGAAGTTCGAGA
CCAACGAGTACAATGTCACCGTCATTGGTTAGTATCCCTGTCCACAACATGTGTCATGTCTCCAAACTCAAGACTAACCTTG
CAATACAGACGCTCCCGGTCACCGTGATTTCATCAAGAACATGATTACTGGTACTTCCCAGGGCCGACTGCGCCATTCTCA
TCATTGCCGCCGGTACTGGTGAGTTCGAGGCTGGTATCTCTAAGGATGGCCAGACTCGTGAGCACGCTCTGCTT

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AGTCTTGCCCTCCTTCTTGACACCCTTGACCTCCTTCTCCCAACCCTTGTACCAGGGGGCGTTGGTGGTCTCGTC
CAACATGTTGTCACCGTTGAAACCGGAGATGGGGACGAAGGGGACCTCCTTGGGGTTGTAGCCAACCTTCTTGAT
GAAGTTGGAGGTCTCCTTGATGATCTCCTTGTAACGGGCCTCGGACCACTTGGCAGTGTCCATCTTGTTGATGGC
AACGATGAGCTGCTTGACACCCAGGGTGAAGGCAAGCAGAGCGTGCTCACGAGTCTGGCCATCCTTGGAGATACC
AGCCTCGAACTCACCAGTACCGGCGGCAATGATGAGAATGGCGCAGTCGGCCTGGGAAGTACCAGTAATCATGTT
CTTGATGAAATCACGGTGACCGGGAGCGTCTGTATTGCAAGGTTAGTCTTGAGTTTGGAGACATGACACATGTTG
TGGACAGGGATACTAACCAATGACGGTGACATTGTACTCGTTGGTCTCGAACTTCCAGAGAGCGATATCAATGGT
GATACCACGCTCACGCTCGGCCTTGAGCTT

P5-3 *P. clavispora*

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GCGGTGGTATCGACAAGCGTACCATCGAGAAGTTCGAGAAGGTTAGTCATCTACTGTTTCCCGTCATCATTCTCCTTCACT
TCAGCGTCATGATTTTCAACCTACGTGCTGAAAATTATTTTCGCTCCTTCCACACTTTTTTCGCTGGTTACCCCGCCGCGAGG
CACCCGCACGACCCCGCGGTGCAAACGAAAAATTTCTTATCACAGCCCCACCTTGCATAAGCAACCATGCATTGCTCATGA
GATCCACTTTGAACAATTGCTAATGCCTTCGTACAGGAAGCCGCCGAGCTCGGTAAGGGTTCCTTCAAGTACGCCTGGGTT
CTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACCATCGATATCGCTCTCTGGAAGTTCGAGACCAACGAGTACAA
TGTCACCGTCATTGGTCAGTACCCCTCCACCTATGCCATGTGCTGCTCCATAAGACACTTGACTAACCTTGCTTCATAGATG
CTCCCGGTCACCGTGATTTCATCAAGAACATGATTACTGGTACTTCCCAGGCCGACTGCGCCATTCTCATCA
TTGCCGCTGGTACTGGTGAGTTCGAGGCTGGTATCTCCAAGGATGGCCAGACTCGTGAGCACGCTCTGCTCGCTTTCACCC
TCGGTGTCAAGCAGCTCATCGTTGCCATCAACAAGATGGACACTGCCAAGTGGTCCGAGGCCCGTTACAAGGAGATCATC
AAGGAGACCTCCAACTTCATCAAGAAGGT

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CTTGTCGGTAGGACGGCTAGGGGGGTTGATGGCGTCAATGGCCTGGAAGAGGGTCTTGCCCTCCTTCTTGACACCCTTGA
CCTCCTTCTCCCAGCCCTTGTACCAGGAAGCGTTGGTGGTCTCCTCCAACATGTTGTCACCGTTGAAACCGGAGATGGGGA
CGAAGGGAACCTCCTTGGGGTTGTAGCCAACCTTCTTGATGAAGTTGGAGGTCTCCTTGATGATCTCCTTGTAACGGGCCT
CGGACCACTTGGCAGTGTCCATCTTGTTGATGGCAACGATGAGCTGCTTGACACCGAGGGTGAAAGCGAGCAGAGCGTG
CTCACGAGTCTGGCCATCCTTGGAGATACCAGCCTCGAACTCACCAGTACCAGCGGCAATGATGAGAATGGCGCAGTCGG
CCTGGGAAGTACCAGTAATCATGTTCTTGATGAAATCACGGTGACCGGGAGCATCTATGAAGCAAGGTTAGTCAAGTGTC
TTATGGAGCAGCACATGGCATAGGTGGAGGGGTACTGACCAATGACGGTGACATTGTACTCGTTGGTCTCGAACTTCCA
GAGAGCGATATCGATGGTGATACCACGCTCACGCTCGGCCTTGAGCTTGTCAAGAACCCAGGCGTACTTGAAGGAACCCT
TACCGAGCTCGGCGGCTTCCTGTACGAAGGCATTAGCAATTGTTCAAAGTGGATCTCATGAGCAATGCATGGTTGCTTAT
GCAAGGTGGGGCTGTGATAG

P10-2 *P. clavispora*

EF1-526F

CTTGATCTACAGTGCGGTGGTATCGACAAGCGTACCATCGAGAAGTTCGAGAAGGTTAGTCATCTATTGATTCCCATCATC
ATTCCCCTTCACTTCAGCGTCATGATTTTCAACATACGTGTTGAAAATTATTTTCGCTCCTTCCACACTTTTTTTCGCTGGTTA
CCCCGCCGCGAGGCAGCCGCACGACCCCGCGGTGCAAACGAAAAATTTCTTATCACAGCCCCACCTTGCACAAGCAACCA
TGCATTGCTCATGAGACCCACTTTGAACAATTGCTAATTCCTTCATTCAGGAAGCCGCCGAGCTCGGTAAGGGTTCCTTCA
AGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACCATCGATATCGCTCTCTGGAAGTTCGAG
ACCAACGAGTACAATGTCACCGTCATTGGTCAGTACCCCTCCACCTATGCCATGTGCTGCTCCATAAGACACTTGACTAACC
TTGCTTCATAGACGCTCCCGGTCACCGTGATTTCATCAAGAACATGATTACTGGTACTTCCCAGGCCGACTG
CGCCATTCTCATCATTGCCGCTGGTACTGGTGAGTTCGAGGCTGGTATCTCCAAGGATGGCCAGACTCGTGAGCACGCTCT
GCTCGCTTTCACCCTCGGTGTCAAGCAGCTCATCGTTGCCATCAACAAGATGGA

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TTGTCGGTAGGACGGCTAGGGGGGTTGATGGCGTCAATGGCCTGGAAGAGGGTCTTGCCCTCCTTCTTGACACCCTTGAC
CTCCTTCTCCCAGCCCTTGTACCAGGAAGCGTTGGTGGTCTCCTCCAACATGTTGTCACCGTTGAAACCGGAGATGGGGAC
GAAGGGAACCTCCTTGGGGTTGTAGCCAACCTTCTTGATGAAGTTGGAGGTCTCCTTGATGATCTCCTTGTAACGGGCCTC
GGACCACTTGGCAGTGTCCATCTTGTTGATGGCAACGATGAGCTGCTTGACACCGAGGGTGAAAGCGAGCAGAGCGTGC
TCACGAGTCTGGCCATCCTTGGAGATACCAGCCTCGAACTCACCAGTACCAGCGGCAATGATGAGAATGGCGCAGTCGGC
CTGGGAAGTACCAGTAATCATGTTCTTGATGAAATCACGGTGACCGGGAGCGTCTATGAAGCAAGGTTAGTCAAGTGTCT
TATGGAGCAGCACATGGCATAGGTGGAGGGGTACTGACCAATGACGGTGACATTGTACTCGTTGGTCTCGAACTTCCAG
AGAGCGATATCGATGGTGATACCACGCTCACGCTCGGCCTTGAGCTTGTCAAGAACCCAGGCGTACTTGAAGGAACCCTT
ACCGAGCTCGGCGGCTTCCTGAATGAAGGAATTAGCAATTGTTCAAAGTGGGTCTCATGAGCAATGCATG

F.4 Nucleotide sequence of ITS region obtained from molecular identification using ITS4 primer of isolate S1-110 (*N. ribis*)

GAGAAATAATTCAAAGGTTCGTCCGGCGGGCGACGCCGTGCGCTCCAAAGCGAGGTGTTTTCTACTACGCTTGAGGCAA
GACGCCACCGCCGAGGTCTTTAAGGCGCGTCCGTGGAGGACGGAGCCCAATACCAAGCAGAGCTTGAGGGTTGAAATGA
CGCTCGAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAA
TTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAGTTTATTA
ACTTGTTTTTCAGACTGCGAAGTTCACTGACTGGAGTTTTATGGTCCTCTGGCGGGCGCTGGCCAGCCCCCCCGAAGGGC
GCCGGTGCGGAGGACCGCGGCCCGCCAAAGCAACAGAGGTGGGTACACATTGGGTGGGAGAGTCGAGCCGGAGCTCG
AATCAACTCGGTAATGATCCTTCCGCAGGTTCACC

F.5 Nucleotide sequence of 16S rRNA gene amplified using the primers F27 and R1494 primers of *Bacillus subtilis*

Primer F 27

GCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGCGTGAGTAACACGTGGGTAACCTGCCTG
TAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGG
CTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTA
GCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATC
TTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAG
GGAAGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGA
TGTGAAAGCCCCCGGCTCAACCGGGGGAGGGTCATTG

Primer R1494

TTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCC
GCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCT
TAACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGA
CGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGG
TTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCG
AAGGGGACGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCAC
ATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGC