Characterising the pathogenicity of *Cylindrocarpon pauciseptatum* associated with black foot disease on grapevines

---

A thesis

submitted in partial fulfilment

of the requirements for the Degree of

Master of Applied Science

at

Lincoln University

by

Yu Sheng

Lincoln University

2014

Abstract of a thesis submitted in partial fulfilment of the
requirements for the Degree of Master of Applied Science.

Characterising the pathogenicity of *Cylindrocarpon pauciseptatum* associated with black foot disease on grapevines

by

Yu Sheng

Black-foot disease caused by *Cylindrocarpon/Ilyonectria* species is a widespread disease of grapevines. In a 2005 survey of symptomatic plants in New Zealand, 95% of the pathogenic species recovered were "*Cylindrocarpon" destructans*, *I. liriodendri* and *"C". macrodidymum*. However, 11 *C. pauciseptatum* isolates were also recovered. In this study, the 11 isolates of *C. pauciseptatum* were identified by morphology and DNA sequences, their pathogenicity investigated and the activity of secreted laccase measured.

Phylogenetic analyses of individual ITS, β – tubulin, EF1-α and histone datasets showed all 11 isolates grouped into well-supported clades along with voucher specimens. The 11 isolates formed a monophyletic unit in the rRNA gene and histone gene trees, while they were paraphyletic based on β – tubulin, EF1-α phylogenies. For the NJ tree generated from β-tubulin sequences eight isolates, including the avocado isolate were identical to a New Zealand isolate recovered from *Erica melanthera* (CBS100819) and grapevine (CBS113550), but did not group with voucher isolates recovered from other countries. There were 9 bp differences between these seven isolates and the other four isolates which had grouped with international voucher specimens in clade 1. The genetic diversity analysis of the 11 *C. pauciseptatum* isolates showed that they were genetically diverse. A total of 65 bands (loci) were produced by five primers, with 46% polymorphism. Two major groups within the dendrogram were generated by the UP-PCR data for the 11 *C. pauciseptatum* isolates. Group 1 contained five isolates, whereas the other group contained 6 isolates. No clonal isolates were detected with all 11 isolates having unique genotypes. Each genetic group was composed of isolates from different geographic regions and vineyards. Vegetative compatibility groups showed that 64% of the paired reactions were compatible despite the distinct genotypes of the isolates. Two main VCGs were identified between the pairings of *C. pauciseptatum* isolates. In addition, 3 sub-groups which overlapped in a complicated manner with each other were observed. No correlation was found between VCGs and genotypic diversity based on NJ tree. Microscopic analysis of interactions between *C. pauciseptatum*

isolates showed that there were anastomoses and hyphal fusions within the actively growing hyphae of isolates in compatible reactions.

Pathogenicity tests with eight isolates on detached root assay of three rootstock cultivars showed that isolates Mar6a, Mar14b, Ack2b Ack2e CO6g and Hb3b were present in lesions and that isolates Mar6a, Mar14b, CO6g and Hb3b could move endophytically within the root. No isolates were recovered from inoculated potted vines. There was no relationship between the genetic group determined by UP-PCR/ β–tubulin and pathogenicity of the *C. pauciseptatum* isolates. Unfortunately, a high degree of contamination by botryosphaeriaceous fungi interfered with the recovery of the inoculated pathogen from infected plant tissue in both assays.

The preliminary study on *in vitro* production of laccase by *C. pauciseptatum* isolates showed both type of laccase activity (PPO-I and PPO-II) were measurable for all isolates with varied levels. There was no relationship between pathogenicity and laccase activity, but a trend could be observed for some isolates. Some of the isolates produced laccase levels similar to the positive controls. Using degenerate PCR the gene encoding *lcc1* laccase was isolated. Only 40% of the putative laccase gene was isolated and sequenced. The active sites and the specific Cu-oxidase domain were not amplified. There were no polymorphic amino acid residues observed between the *C. pauciseptatum* isolates. There were 13 amino acid differences observed between *I. liriodendra/I.novozelandicai* and *C. pauciseptatum*, in which twelve were non-conservative changes and only one was a conservative substitution (Δ73I→V).

Overall this study identified the 11 isolates as *C. pauciseptaum,* confirmed that they were present in lesions formed on detached roots and showed that they produced laccase *in vitro*. This study has improved understanding of this species in New Zealand and its ability to infect grapevines.

Keywords: Black foot, *Cylindrocarpon*, *Cylindrocarpon pauciseptatum*, Grapevine, UP-PCR, Genetic diversity, Pathogenicity, Enzymes, Laccase

Acknowledgements

First of all, I would like to express my sincere gratitude to my belovely parents far away in China who have been encouraging and supporting me to initiate this acadamic journey and be hopeful to complete the task, and my mother in law who has been helping me and supporting me with her care and love. Frankly to say, I was really not confident enough at the beginning to reach to the current stage due to a few reasons. The mother of three children is the most critical hurdle to slow down the process at any time. Fortunately, my husband Dr Malik Altaf Hussain, as the other important role I would like to appreciate, always cooperate with me by looking after kids and managing housekeeping works whenever I am in need. It was him placing me on this platform, lifting me whenever I got lost from the tough experiment, accompanying me until the end of this process. Without these important persons in my life, I wouldn't be able to achieve this milestone to fulfill my dream.

The most greatest appreciation from me to Lincoln University will be offered to Dr Hayley Ridgway, my main supervisor, for her consistant and patient guidance, enthusiastic encouragement and useful critiques of this research work. Her continual encouragement, perfect advice and determination in pushing me to achieve my goals always play an essential role in my academic achivement. Her broad knowledge on fungal molecular detection really impressed me and also inspired me to possibly stay in this field. I would also like to thank my co-supervisor Dr Eirian Jones for her timely advice, endless caring and assistance in keeping my progress on schedule. Her excellent experience on fungal pathogenesis made my experiment on fungal mophological identification smoother and easier. As a matter of fact, these two supervisors' patience and compassion have been a tremendous source of the inspiration for me to face every difficuty on the way.

My special thanks are extended to lab manager Candice Barclay, for her incredible patience in helping through my lab work and lots of useful technical experience in completing experiment effeciently; nursery manager Brent Richards, for his great help and guildance in my greenhouse experiments; Emma Gambles, for her kind help on hundreds of DNA sequencing. My grateful thanks are also especially extended to Mr Heng Wee Tan and Ms Wendy Liu for their endless help with kind patience on teaching me how to use related data analysis softwares. Last but not the least, Ms Megan Anne Outram, Mrs Dalin Brown, Ms Celine Blond, Mrs Jackie Sammonds and Mr Wisnu Wicaksono should also receive a special gratitude for their kind help and assistance.

My final dedication will be given to my three lovely kids, Afeef, Hassan and Awa. Their naughtiness, their company, their demand, their everthing make this master degree meaningful, hopeful, and promising. Thank you very much for being with me.

Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction

1.1 Viticulture, wine industry and grapevine rootstock varieties in New Zealand

Viticulture is the science of growing grapes, with grapes grown in every continent except for Antarctica (Johnson, 1989). Grape plants are woody climbing vines producing clinging tendrils that allow the growing stems to attach themselves to upright supports. North America and East Asia are the primary centres of species diversity and each of the many varieties of grapes has its own unique characteristics. In New Zealand, the majority of grapes are grown for wine production. The major areas for grape growing are Auckland, Bay of Plenty, Canterbury, Central Otago, Gisborne, Hawkes Bay, Marlborough, Nelson, Northland, and Wellington, (Pathrose, 2012). Only 50 grape varieties are grown in New Zealand out of more than 8,000 known grape cultivars. In 2013, Sauvignon Blanc was the most common type planted (68% of the total producing vineyard area), followed by Pinot Noir (9.4%), Chardonnay (8.1%) and Pinot Gris (6.8%) (New Zealand Winegrowers Statistical Annual Report, 2013, Figure 1.1). New vine varieties have dramatically increased the range of products and improved the quality of the grapes and wine produced in New Zealand.

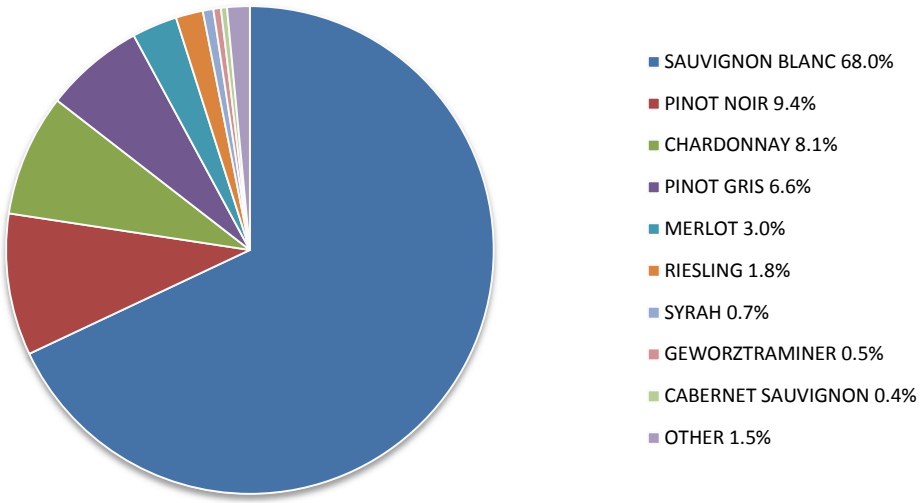

Figure 1.1 National plantings of grapevine varieties in 2013 (Data was obtained from New Zealand Winegrowers Statistical Annual Report, 2013).

Over the past two decades, New Zealand has experienced a viticulture boom with a 173% increase in the number of wineries (Dodds *et al*., 2013). New Zealand is now the world's 8th largest exporter by value. The export value in 2012 increased 3% from the previous years' with a record of $1.21B. In 2013 the grapevine harvest achieved a tonnage of 345,000

tonnes and subsequently, around 250 million litres of wine was produced in this year (New Zealand Winegrowers Statistical Annual Report, 2013, Figure 1.2). Figure 1.2 illustrates the rapid growth of grapevine production over the past decade.

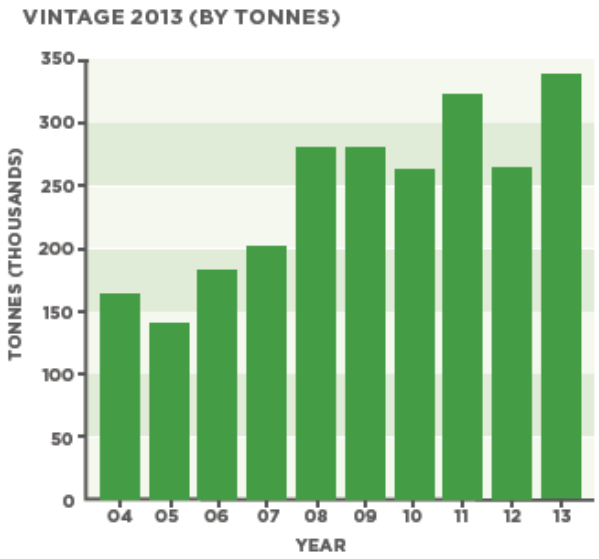

Figure 1.2 Production of grapevines in New Zealand, 2013 (Obtained from New Zealand Winegrowers Statistical Annual Report, 2013).

The major reason to use plants where the scion is grafted onto rootstocks is that it is the only effective method to combat phylloxera and nematodes. In the early 20th century, government viticulturist Romeo Bragato introduced grafted grapevines into New Zealand when root louse phylloxera spread throughout New Zealand. Most of the rootstocks used in New Zealand are derived from three American species – *Vitis riparia, V. berlandieri* and *V. rupestris*, or their interspecific *Vitis* hybrid (Morton, 1995). The North American *Vitis* species have provided some phylloxera resistant hybrids suitable for use as rootstocks to be grafted to *Vitis vinifera* fruiting varieties. In 2010, New Zealand Winegrowers Statistical Annual (2010) reported that the grafted vines accounted for 94% of the grapevines in New Zealand vineyards. Consequently, rootstock selection has been becoming one of the most influential factors for matching to soil type, obtaining the appropriate vigour and achieving phylloxera tolerance (Billones-Baaijens *et al*., 2014). A wide range of rootstock types are available in New Zealand. At present, Riparia Gloire, 3309, Schwartzman, 101-14, 5C and SO4 are comparatively popular rootstock varieties used in New Zealand grapevine nurseries (Jaspers *et al*., 2007).

1.2 Black foot disease

Quoted by Halleen *et al*. (2006a), "*Black foot disease caused by Cylindrocarpon spp. is a global devastating disease of grapevines in nurseries and young vineyards*". Over the last

decade its incidence has increased significantly in all major viticulture regions, including South Africa, New Zealand, Italy, Spain, Portugal, Australia, North America and Brazil (Halleen *et al*., 2004; Petit and Gubler, 2005; Alaniz *et al*., 2009b, Cabral *et al*., 2012a, Úrbez-Torres *et al*., 2013; dos Santos *et al*., 2014). The disease occurs in young and mature vines, losses usually occur during the first 5 years, sometimes even during the first year after planting (Halleen *et al*., 2006b). Infected vines typically show a range of decline symptoms, including delayed or absence of budding, sparse foliage and small leaves with interveinal chlorosis, reduced vigour, slower growth, shortened internodes and necrosis. In the latter stages, there is wilting and dieback, followed by death of the vines (Figure 1.3 A, B, C) (Grasso, 1984; Oliveira *et al.,* 2004; Halleen *et al.,* 2006a). The disease causes decay of the roots, development of secondary root crowns with growth parallel to the soil surface and necrotic root crowns. Removal of the rootstock bark may reveal brown/purple to black discoloration due to wood necrosis which develops from the trunk base of the rootstock. Cross-section of the wood shows severe necrosis extending from the bark to the pith; while obvious dark vascular streaking is seen in longitudinal sections (Figure 1.3 D, E, F). Black foot disease of grapevine is caused by a complex of soilborne fungi. The most common and virulent species causing black foot disease are *Phaeomoniella chlamydospora, Phaeoacremonium* species and *Cylindrocarpon* species*,* which are found across all major grape growing regions of the world (Oliveira *et al.*, 2004*).*

In recent years, a large number of *Cylindrocarpon* or *Cylindrocarpon*-like species (teleomorph = *Ilyonectria* species) causing black foot disease symptoms have been described, but *I. liriodendri* J.D. MacDonald & E.E. Butler and members of the *I. macrodidyma* complex Schroers, Halleen & Crous, were identified as the major causal agents of black foot disease (Alaniz *et al.*, 2009). Other species with a more limited distribution and uncertainty regarding their pathogenicity include *"C." destructans, C. obtusisporum, Campylocarpon fasciculare, C. pseudofasciculare* and *C. pauciseptatum.*

It was found that vine decline, black foot and Petri disease were expressed more commonly on rootstocks tolerant to phylloxera. For instance, Scheck *et al*. (1998) reported an increase in grapevine death caused by root diseases since the introduction of rootstocks resistant to phylloxera in California. Halleen *et al.* (2006b) concluded that the susceptibility to infection by *Cylindrocarpon* spp. varied among cultivars but little information is currently available regarding rootstock susceptibility. Alaniz *et al.* (2010) evaluated the susceptibility of the most commonly used Spanish grapevine rootstocks towards "C." *liriodendri* and *"C." macrodidymum*. Results showed all rootstocks inoculated with these two pathogens were affected to some degree with the most susceptible rootstock being 110-R, a rootstock with high tolerance to drought. Jaspers *et al*. (2007) stated that grapevine rootstock Riparia Gloire had low susceptibility and 101-14 moderate to high susceptibility towards "*C." destructans*.

Based on their experimental results, rootstocks were demonstrated to vary in their susceptibility to *Cylindrocarpon* spp.

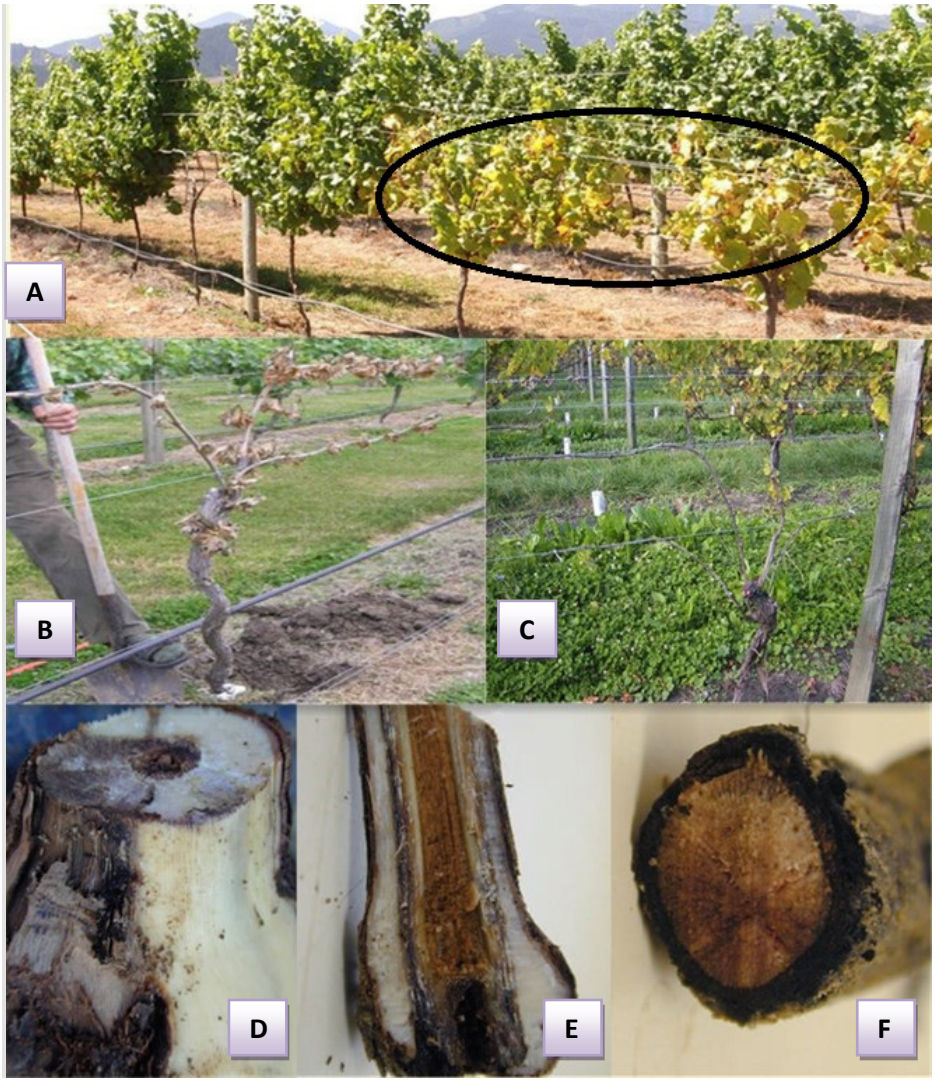

Figure 1.3 Black foot symptoms on grapevines: Above ground symptoms: (A) Slow growth and reduced vigour (indicated by circle); (B) Dead vine; (C) Retarded/absent budding, abnormal and weak vegetation, sparse and chlorotic foliage; Internal symptoms: (D) Cross-section with severe necrosis extending from the bark to the pith; (E) Discoloration and brown to dark streaks in wood; (F) Cross-section with dark vascular streaking. (A-C: retrieved from Probst, 2011; D-F: retrieved from Halleen *et al*., 2006a).

1.3 Taxonomy of *Cylindrocarpon* species

The genus *Nectria* Willkommiotes Wollenweber, which is characterised by usually superficial, brightly colored perithecia with articulated ascospores and phialidic anamorphs belonging to the genus *Cylindrocarpon,* was suggested by Fries in 1849 (Hirooka and Kobayashi, 2007). By the mid 20th century, Booth (1966) divided this genus into four groups on the basis of the presence of microconidia or chlamydospores. *Cylindrocarpon magnusianum* (Sacc.) Wollenw, *C. cylindroides* Wollenw., members of *Cylindrocarpon* spp. connected with teleomorphs of *Nectria mammoidea* W. Phillips and Plowr. Group, and *Cylindrocarpon*

obtusiusculum. Mantiri *et al.* (2001) and Brayford *et al.* (2004) moved representatives of all "*Nectria*" groups with *Cylindrocarpon* anamorphs into *Neonectria*. The teleomorphs of *Cylindrocarpon* spp. responsible for black foot on grapevines have rarely been observed: *Ilyonectria radicicola* (Gerlach and Nilsson) Mantiri and Samuels (teleomorph of "*C*." *destructans*[1]) was observed on grapevines in France (Maluta and Larignon, 1991); *Ilyonectria macrodidyma* Halleen, Schroers and Crous (teleomorph of "*C*." *macrodidymum*) and *Ilyonectria liriodendri* Halleen, Rego and Crous (teleomorph of "*C*." *liriodendri)* were obtained *in vitro* (Halleen *et al.,* 2004; Halleen *et al*., 2006b).

Around 125 *Cylindrocarpon* spp. and sub-species have been described (Mantiri *et al*., 2001). Recently, it was reported that the colony morphology of isolates from one species were variable (Halleen *et al*., 2006a). Molecular identification using sequences of the partial nuclear large subunit ribosomal RNA gene, internal transcribed spacers (ITS) 1 and 2 of the ribosomal RNA genes and partial β-tubulin gene introns and exons, led Halleen *et al.* (2004) to observe that *Neonectria /Cylindrocarpon* species segregated into three groups: the *Neonectria radicicola* "*C*." *destructans* complex, "*C*." *macrodidymum* and *C. cylindroides*. Two groups excluded from this monophyletic group were members of the *Neonectria mammoidea* complex and a new genus found in South Africa named "*Campylocarpon*", with two species, *Campylocarpon fasciculare* Schroers, Halleen and Crous and *Campylocarpon pseudofasciculare* Halleen, Schroers and Crous, which were recovered from vines symptomatic of black foot disease. This genus is similar to *Cylindrocarpon,* however, it lacks microconidia. In 2006, Halleen *et al.* (2006b) recovered *I. liriodendri* sp. nov. from diseased grapevine isolates and identified it using ITS and partial β-tubulin gene sequencing. Based on these results, *I. liriodendri* was identified as another species derived from the ex-type strain of *Neonectria radicicola*.

An advanced phylogenetic study conducted by Chaverri *et al*. (2011) focusing on morphological characters and molecular phylogenetic analyses developed a taxonomic revision dividing the Neonectria complex into five informal genera: *Ilyonectria* (Booth's group 3), *Neonectria/ Cylindrocarpon sensu stricto* (Booth's groups 1 and 4), *Rugonectria, Thelonectria* (Booth's group 2), and the anamorph genus *Campylocarpon*. In this study, a group of pleomorphic fungi as one clade was proposed a single generic name, which means that the *Cylindrocarpon*-like anamorphs of *Ilyonectria, Rugonectria* and *Thelonectria* species were put into one teleomorph genera. Therefore, *Cylindrocarpon*-like anamorphs of known species (e.g., "*C*." *liriodendri* and "*C*." *macrodidymum*) associated with black-foot disease were called *Ilyonectria* species.

Cylindrocarpon pauciseptatum and "*C*." *macrodidymum* complex are monophyletic and are closely related to other species of *Cylindrocarpon sensu stricto* including members of the *"C." destructans* (teleomorph, *Ilyonectria radicicola*) species complex, which accommodates "*C."*

liriodendri (teleomorph, *Ilyonectria liriodendri*), "*C*." *destructans* var. *crassum* and *C. austrodestructans* (teleomorph, *Neonectria austroradicicola* comb. nov.) (Schroers *et al*., 2008).

Cabral and co-workers applied a multigene analysis techniques (based on ITS ribosomal RNA gene, the β-tubulin, translation elongation factor 1-α and histone H3) along with colony and conidia morphology to reveal the existence of genetic diversity in a wide collection of isolates of *Ilyonectria radicicola* ("*C*." *destructans*) and *I. macrodidyma*-like isolates from grapevine and other hosts (Cabral *et al*., 2012b). Morphological characters, such as conidial size and molecular data enabled the distinction of six monophyletic *Ilyonectria* species within the *I. macrodidyma* complex, in which four were named *I. alcacerensis, I. estremocensis, I. novozelandica,* and *I. torresensis.* In the *Ilyonectria radicicola* complex, 12 new taxa were discriminated within this species, namely *I. anthuriicola, I. cyclaminicola, I. europaea, I. gamsii, I. liliigena, I. lusitanica, I. mors-panacis, I. panacis, I. pseudodestructans, I. rufa, I. venezuelensis* and *I. vitis* (Cabral *et al*., 2012a).

In New Zealand a survey of eight major grape growing regions isolated 174 isolates of *Cylindrocarpon* species and the majority (95%) were identified as belonging to the "*C*." *destructans, "C." macrodidymum* and *"C." liriodendri* (Bleach *et al*., 2006). Eleven isolates of *C. pauciseptatum* were also isolated both alone and in combination with other species from grapevine (Pathrose, 2012). However, the relative virulence of this species was not investigated.

1.4 Disease cycle and Pathogenesis

Cylindrocarpon species are ubiquitous soilborne and opportunistic pathogens that commonly occur as saprobes on dead plant substrata, or infect roots and stems of a range of hosts through wounds or openings, or through the crown of rootstocks (Probst *et al*., 2012). The fungus then gradually invades lignified tissues of the plant, producing root lesions and subsequently plugging the xylem by fungal tissue, gums and tyloses. Scheck and his colleagues found young plants died very rapidly once attacked, while infection of mature vines results in a more gradual decline till death that may take 1 year or more to happen (Scheck *et al*., 1998). Death appears to be unavoidable if vines are infected when they are less than 10 year-old, and older vines can also succumb to the disease.

Infected propagation materials have a role as a primary source of inoculum of black-foot pathogens in vineyards (Agustí-Brisach *et al*., 2013a). Agustí-Brisach *et al*. (2013b) demonstrated for the first time that inoculum of *Ilyonectria* spp. was detected at the different stages of the grapevine nursery propagation process using multiplex nested-PCR, such as *I. liriodendri* and *I. macrodidyma-complex* found in hydration tanks, Ω-cut grafting machines, scissors and/or callusing peat from two different nurseries. Soil should also be considered as

a source of inoculum since most species of these genera produce chlamydospores which enable the pathogen to survive in the soil (Halleen *et al*., 2004). In recent work on South African strains of "*C*." *destructans*, it was found that drip-irrigation among relatively warm and dry viticultural areas was able to keep the roots and the belowground region of the stem moist for long periods, which created conditions favourable for disease development (Halleen *et al*., 2004). Halleen *et al*. (2003) discovered the percent infection in grafted grapevines after 7 months growth was more than 50% in nursery field sites in South Africa. Agustí-Brisach *et al.* (2013) isolated and analysed *I. alcacerensis*, *I. macrodidyma, I. novozelandica* and *I. torresensis* from roots of seedlings grown in a series of soil samples collected from commercial vineyards and nursery fields, which again confirms the black-foot pathogens are present in the soils of nursery and vineyard.

To some extent, weeds are another source of infection. In the experiment of Agustí-Brisach *et al*. (2011), "*C*." *macrodidymum* was successfully isolated from the roots of weeds from 17 of the 32 field sites, 15 of 19 weed families and 26 of 52 weed species. Pathogenicity tests showed all isolates obtained from the survey were pathogenic to seedlings obtained from grapevines with typical black-foot disease symptoms. In addition, the isolates obtained from non-grapevine hosts were found to be virulent to grapevines underlined the cross-infection potential of these pathogens (Cabral *et al*., 2012c).

An investigation of the phylogenetic identification, morphological characterisation, and pathogenic variation among *Cylindrocarpon* species isolates associated with black foot disease of grapevine in California was carried out by Petit and Gubler (2005). Pathogenicity of the *Cylindrocarpon* isolates was assessed based on re-isolation of the pathogen from symptomatic roots four months after inoculation of the vines. All Californian "C." *destructans* and "C." *macrodidymum* isolates tested were pathogenic to grapevines with, no significant variation between different isolates or different species found except for significant leaf symptoms caused by one of the '*C. destructans'* isolates. In contrast, Tewoldemedhin *et al.* (2011) reported that isolates of the same *Cylindrocarpon* spp. yielded different phenotypic characters, such as longevity in the field, host range, aggressiveness, pathogenicity and virulence, and susceptibility toward different disease control treatments. In addition to this finding, they also reported significant differences in pathogenicity among isolates of four species "*C*." *destructans, "C." liriodendri, "C." macrodidymum* and *C. pauciseptatum* on apple seedlings. All isolates, except for one isolate from *C. pauciseptatum*, were able to cause lesion development on seedling roots. More than half (57%) of the isolates reduced seedling weight and/or height. Interestingly, Cabral *et al*. (2012c) reported in a recent study that grapevine isolates from newly described species such as *I. lusitanica, I. estremocensis* and *I. europaea* are more virulent to grapevine than the species previously accepted to represent the main causal agents of black foot disease, including *I. liriodendri* and *I. macrodidyma*.

A complete understanding of the factors affecting pathogenicity is essential to enable the development of better prevention and management strategies. Young grapevines in nurseries and vineyards may be at a higher risk of disease due to stress conditions. The stress factors can be from vineyard management practices and the environment, including soil compaction, poor drainage, poor nutrition, wild cropping of young vines, extreme high or low temperatures and pH, impacts of pests and pathogens as well as inadequate planting holes, which can cause poor root development (Probst *et al*., 2012). Two common and potentially stressful factors occurring during grapevine propagation (cold storage and pathogenic infection) were investigated by Probst *et al.* (2011) to determine their influence on black foot incidence. The experimental results supported the hypothesis that longer cold storage times increased the incidences and severity of "*C.*" *liriodendri* and "*C.*" *destructans* to grapevine cuttings. Additionally, infection by *Phaeomoniella chlamydospora* increased the incidence of "*C*." *liriodendri* infection of grapevines.

In Spain, a study on the effects of pH, temperature and water potential on mycelial growth, sporulation and chlamydospore production of "*C*." *liriodendri*, "*C*." *macrodidymum* and *C. pauciseptatum* isolated from grapevines was undertaken recently (Agustí-Brisach and Armengol, 2012). According to the experimental results, most of the *Cylindrocarpon* spp. isolates sporulated at all temperatures, pH and water potentials tested. Across all the studied conditions, "*C*." *liriodendri* had the greatest sporulation capacity as compared with "*C*." *macrodidymum* and *C*. *pauciseptatum*. In general, chlamydospore production was not affected by temperature, pH and water potential as compared with mycelial growth. Brown *et al*. (2012) evaluated the effects of partial defoliation on disease severity, incidence and dry weight accumulation on "*C*." *destructans* inoculated young grapevines in New Zealand. Assessments after eight months showed that although disease incidence was not affected by the defoliation treatments, disease severity was significantly higher for the highly defoliated plants, and root dry weight of highly defoliated plants was reduced more than moderately defoliated and undefoliated plants.

1.5 Cell wall degrading enzymes produced during pathogenesis

Fungi produce a variety of enzymes to degrade the plant cell wall, namely endopolygalacturonases, cellulases, pectin lyase, xylanase, β-galactosidase, laccases and proteases. These enzymes degrade plant polysaccharides to facilitate intercellular fungal growth by release of nutrients and destabilization of host cell integrity (ten Have *et al*., 2004). So far, more than 100 enzymes have been purified from fungal cultures and characterized in terms of their biochemical and catalytic properties.

Cellulases, are one of the most widely studied cellulolytic enzymes and are able to hydrolyse cellulose to provide fungi with a carbon source. Liu *et al*. (2012) evaluated the *in vitro*

production of three enzymes involved in cellulose degradation, β-glucosidase, endoglucanase, and exoglucanase, in four fungal species namely *Aspergillus niger, Trichoderma viride, T. koningii,* and *T. reesei*. The concentration of furfural residues which was used as a substrate in the experiment was observed to decrease indicating that this material was largely digested by the fungi with certain variations. Xylanases are enzymes that hydrolyse xylan, which is the other major polysaccharide in the secondary maturation stage of plant cell walls. King *et al*. (2011) compared fungal plant pathogens with *T. reesei*, a well-known industrial microbial source for commercially degrading cellulases, xylanases and other cell wall enzymes, on the hydrolytic activities tested on grass cell walls. Result illustrated that many pathogenic fungi had high xylanase activity, including *C. didymium,* having greater activity than *T. reesei.* Pectinases or pectinolytic enzymes are the enzymes that degrade pectic substances that also make up a large portion of primary cell walls. A number of plant pathogens produce these enzymes to attack cell walls, including *"C." destructans*, which has been extensively studied especially with regards to being a pathogen of ginseng which is a cash crop in many Asian countries (Sathiyaraj *et al*., 2011).

Laccases are the largest subclass of blue polyphenol oxidases containing four copper atoms in their active sites, and well known as lignolytic enzymes. Laccases are ubiquitous among ligninolytic enzymes found in the soil environment responsible for lignin degradation (Arora and Sharma, 2010). As well as lignin degradation, these enzymes can play numerous roles, such as host-pathogen interactions, sporulation, stress defence and morphogenesis (Thurston, 1994). Laccase activity has been demonstrated and characterised in many fungal species. For example, it has been reported that the ascomyceteous fungi, *Mauginella* sp. (Palonen *et al*., 2003) and *Botryosphaeria rhodina* MAMB-05 (Dekker *et al*., 2007), can produce laccase to degrade lignified plant cell walls. In the study of Cañero and Roncero (2008), the role of laccases in pathogenesis in tomato by the vascular wilt pathogen, *Fusarium oxysporum,* which causes disease in a variety of crop plants, was examined. Reverse transcription-polymerase chain reaction (RT-PCR) was applied to confirm that laccase genes lcc1, lcc3, and lcc9 were expressed in roots and stems of infected plants.

Apart from laccases, proteolytic enzymes play an important role in plant-pathogen interactions on the molecular, cellular, tissue, or inter-organ levels (Bueno *et al*., 2012). Proteases play a variety of functions in the organism including activating or inactivating physiologically active proteins and peptides to alter biological activity (Dunaevskii *et al*., 2007). They also assist host penetration and colonization of plant cell walls by degrading structural proteins and enzymes that are embedded in the plant cell wall (Rolland *et al*., 2009). However, there is variation on the specific functions of catalysing the proteins between different groups of proteases.

Previous work done by Pathrose (2012) showed that the three main species of *Cylindrocarpon* associated with black foot in New Zealand ("*C.*" *destructans, "C." macrodidymum* and *I. liriodendri*) produce laccase *in vitro*. Additionally, the results of the biochemical analyses showed clear variation between species and isolates. However, laccases have not been studied in other species such as *C. pauciseptatum.*

1.6 *Cylindrocarpon pauciseptatum*

Cylindrocarpon pauciseptatum, to date, has been reisolated from symptomatic grapevines alone or in combination with other predominant *Cylindrocarpon* species in many countries. In Slovenia, Schroers *et al.* (2008) recovered it from necrotic regions on roots, wood and trunk bases of infected *Vitis* rootstocks and from the roots of dying plants with similar disease symptoms to black foot disease caused by other *Cylindrocarpon*-like species. Furthermore, the authors reported that strains from Slovenia and New Zealand were distinguishable based on the sequences of the β-tubulin gene. Alaniz *et al.* (2009a) identified *C. pauciseptatum* as a black foot pathogen by using species-specific primers. It was also identified from necrotic lesions on the roots of 110R grapevine rootstocks. Abreo *et al*. (2010) recovered *C. pauciseptatum* from the roots of symptomatic plants and plantlets from commercial vineyards in Uruguay. Martin *et al.* (2011) also isolated *C. pauciseptatum* from grapevine roots with decline symptoms in Spain and this species was able to produce necrotic lesions on roots of inoculated 110R rootstock. Cabral *et al.* (2012a) confirmed the distinction of New Zealand *C. pauciseptatum* strains from Slovenian and Portuguese *C. pauciseptatum* strains by multi-genes analysis (ITS of the rRNA, part of the β-tubulin, translation elongation factor 1- α and histone H3 genes). In the same year, *C. pauciseptatum* isolates from both grapevine and non-grapevine hosts were reported as being equally virulent on grapevines (Cabral *et al.,* 2012c).

Recently, Reis *et al*. (2013) assessed the inter- and intra-specific variability among isolates of *I. estremocensis, I. europaea, I. liriodendri, I. macrodidyma, I. torresensis, I. vitis* and *C. pauciseptatum* from grapevines on the basis of morphological, cultural and biomolecular characteristics. The first attempt to identify and characterize the fungal pathogens associated with black foot disease of grapevines in British Columbia (Canada) was conducted using DNA analyses of the internal transcribed spacer region (ITS1-5.8S-ITS2) of the rRNA, and part of the β-tubulin and translation elongation factor 1- α genes. The results identified five different black foot fungi associated with declining young vines, namely *Cylindrocarpon pauciseptatum*, *Ilyonectria liriodendri, Ilyonectria macrodidyma, Ilyonectria robusta,* and *Ilyonectria torresensis* (Úrbez-Torres *et al*., 2014). dos Santos *et al*. (2014) used multi-gene DNA sequencing methodology (rRNA-ITS, β-tubulin, and histone H3) to identify two isolates of *C. pauciseptatum* obtained from grapevines in Brazil.

Cylindrocarpon pauciseptatum has also been recovered from other hosts. In South Africa, Tewoldemedhin *et al.* (2011) investigated the pathogenesis and virulence of *C. pauciseptatum* causing apple replant disease. In this study, four species of *Cylindrocarpon* including *Cylindrocarpon pauciseptatum* (3 strains) were selected for *in vitro* pathogencity tests on apple. Two isolates were confirmed as being pathogenic to apple causing a significant reduction in seedling weight and/or height, with corresponding root rot also observed. Yaseen *et al*. (2012) reported a low incidence of *C. pauciseptatum* associated with root rot and decline in peach. In Turkey, Erper *et al.* (2013) collected a wide group of fungal isolates from kiwifruit trees showing symptoms of root rot in the Black Sea region. It was the first study confirming the pathogenicity of *C. pauciseptatum* to kiwifruit. '*Cylindrocarpon' destructans* and *C. pauciseptatum* have also been recovered from blackberry decline sites in Western Australia (Aghighi *et al*., 2014).

Isolates of *C. pauciseptatum* that have been studied are quite homogeneous in their micro- and macro- morphology. Although DNA sequencing analysis is able to differentiate this species from other *Cylindrocarpon* spp., there is very limited knowledge of intra-species variation and the specific role of this *Cylindrocarpon* spp. as a root pathogen in New Zealand.

1.7 Morphology of *Cylindrocarpon pauciseptatum*

Before the development of molecular tools to accurately identify fungi species, morphological characteristics of colony as well as conidial size and shape were used to differentiate among isolates. For instance, Booth (1966) segregated more than 30 species of *Cylindrocarpon* into four groups based on morphological details relating to occurrence of microconidia and chlamydospores, conidium size, shape and number of septa.

Pure cultures of *C. pauciseptatum* on PDA are white to greyish brown or golden-brown due to the formation of chlamydospores and felty due to aerial mycelium or in dark orange to brownish hues in sectors without aerial mycelium, particularly when arranged in strands (Figure 1.4) (Schroers *et al*., 2008). *Cylindrocarpon* is within hypocrealean genera which are characterised by 1- to multiseptate macroconidia often with non-septate microconidia and brownish chlamydospores (Booth, 1966; Halleen *et al*., 2004). Chlamydospores are golden-brown, formed in intercalary chains or in aggregates, globose to ellipsoidal (Figure 1.5 A-C). Macroconidia of *C. pauciseptatum* are straight with rounded ends, up to 10 mm in width with 3-septate (Figure 1.5 E–G). Simple conidiophores are sparsely formed, arising laterally or terminally from aerial mycelium, solitary, unbranched or sparsely branched, consisting only of phialides or up to three supporting cells bearing 1–3 phialides (Figure 1.5 H-O). The macroconidia for *C. pauciseptatum* can be distinguished from those of *C. fraxini* based on the length: width ratio being 5–10 for *C. fraxini*, compared with 4-6 for *C. pauciseptatum* (Schroers *et al*., 2008). Furthermore, unlike *I. liriodendri*, *C. pauciseptatum* does not form

ellipsoidal microconidia, with these forming in heads, 0-septate, subglobose to ovoidal mostly with a visible, centrally located or slightly laterally displaced hilum (Figure 1.5 P-Q).

Although morphological characters are often useful for initial groupings of isolates they are insufficient for accurate species identification. Schroers *et al. (*2008) reported from the re-examinations of dried herbarium cultures that *C. pauciseptatum* and *C. austrodestructans* were morphologically similar and hard to distinguish. These species have common characteristics of straight and predominantly 3-septate macroconidia as well as chlamydospores (but not in *C. coprosmae* and *C. macroconidialis*) and colonies containing brownish pigment. From multi-gene DNA sequencing data, the authors also found the close relationship between *C. pauciseptatum* and "*C." macrodidymum* and both of these species are similar to members of the "*C." destructans* species complex including *C. macroconidialis*, *C. coprosmae*, "*C." liriodendri* and "*C*." *destructans* var. crassum. The three-septate macroconidia formed by *C. austrodestructans* are largely impossible to differentiate in size and shape from those of *C. pauciseptatum* except for a minor difference for higher and more variable numbers of septa in macroconidia, lack of chlamydospores and comparatively slower growth at 20°C (Samuels and Brayford, 1990).

In conclusion, the size of conidia of different species often overlaps and maturity level also affects conidial pigmentation and septation. Consequently, species identification based on anamorph or telemorph morphology alone is unreliable (Halleen *et al*., 2009). The most commonly used strategy is to combine both morphological characteristic and molecular tools (Alaniz *et al*., 2009).

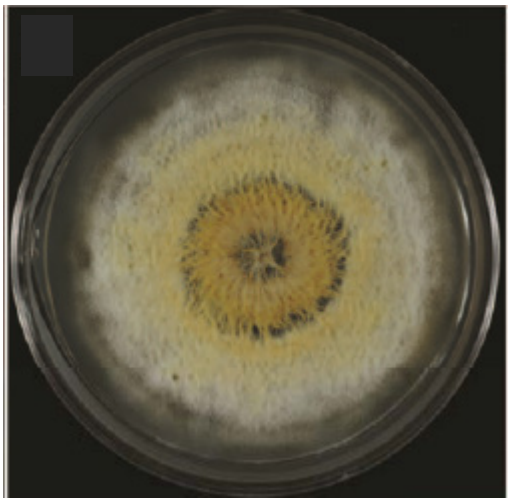

Figure 1.4 Colony of *Cylindrocarpon pauciseptatum* on PDA showing white to greyish brown or golden-brown colouration and felty aerial mycelium (Image retrieved from Úrbez-Torres *et al*., 2014).

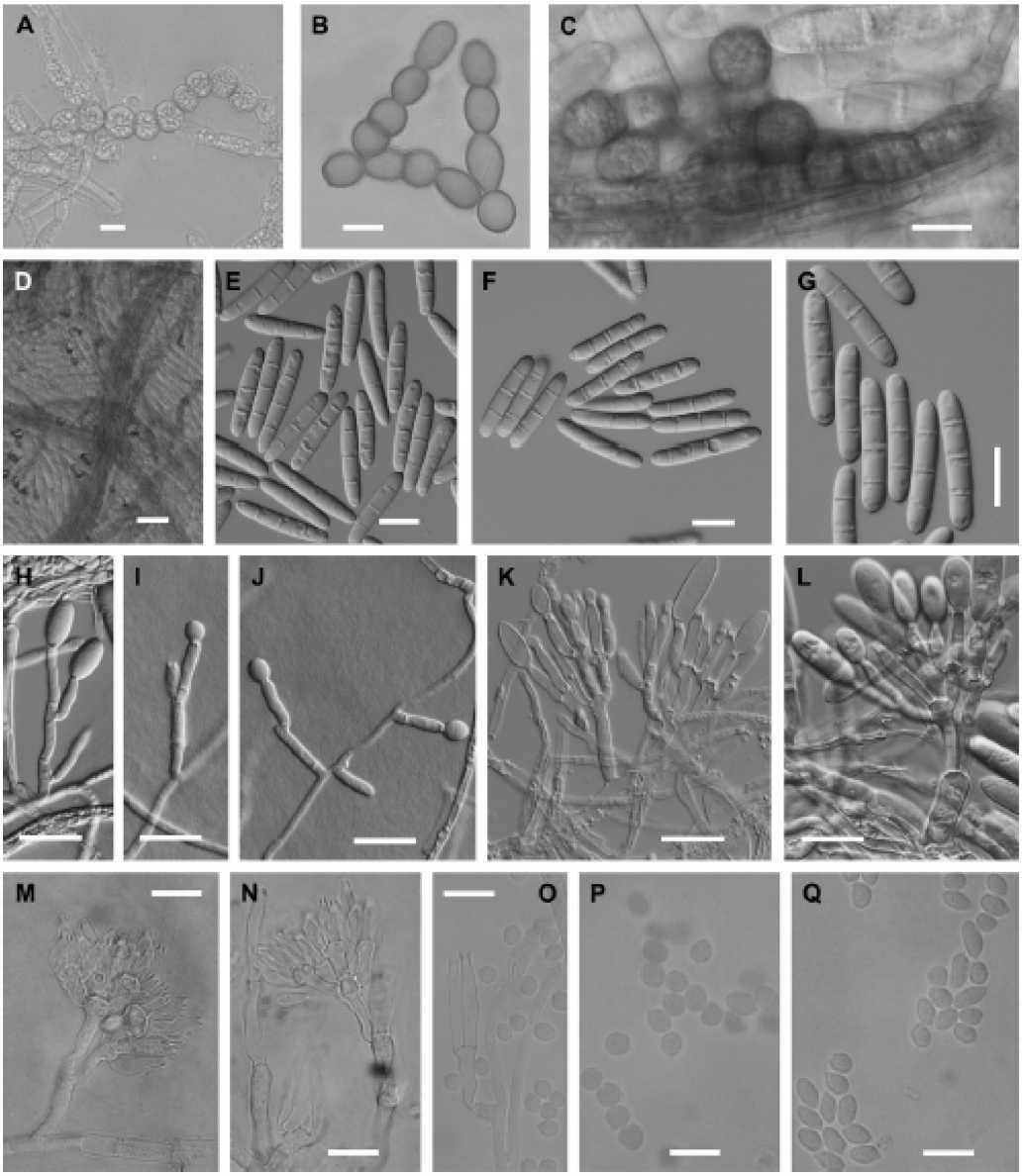

Figure 1.5 *Cylindrocarpon pauciseptatum*. (A–C) Chlamydospores. (D) Hyphal strands of aerial mycelium. (E–G) Macroconidia. (H–J) Simple, sparsely branched conidiophores of the aerial mycelium forming macroconidia. (K–L) More frequently branched, ter- to quinqueverticillate conidiophores of sporodochia forming macroconidia. (M–Q) Penicillate or sparsely branched conidiophores (M–O) forming subglobose to ovoidal microconidia (P–Q). (Pictures obtained from Schroers *et al*., 2008)

1.8 Molecular identification of *Cylindrocarpon* spp.

Isolates of different *Cylindrocarpon* species have variable phenotypes which overlap among species (Alaniz *et al*., 2009). Consequently, phenotype characterisation is not a reliable strategy to distinguish isolates. In contrast, DNA sequence analysis is a reliable method for detection and identification of fungi (Schroers *et al*., 2008). Genetic techniques that can quickly and easily identify the DNA sequence of fungal species play a leading role amongst diagnostic methods (Fox and Narra, 2006).

The most commonly targeted gene region for identification of fungi are the nuclear large and small subunits encoding the ribosomal RNA genes (5.8S ribosomal DNA or rDNA) and internally transcribed spacers (ITS) (Fox and Narra, 2006). These sequences are often present in many copies and conserved within a species which dramatically improves the sensitivity of amplification. However, for closely related organisms with low or no variations in the ITS sequences, genes encoding β-tubulin and translation elongation factor 1α (tef-1α) are widely used as alternatives (Schmitt *et al*., 2009). Schroers *et al*. (2008) reported a novel species, *C. pauciseptatum,* which was associated with diseased roots of *Vitis* spp. in Slovenia and New Zealand. Phylogenetic inferences in this study were conducted by amplification of DNA sequence data of the ITS regions 1 and 2 plus the 5.8S rDNA and the partial β-tubulin gene. Cabral *et al.* (2012a) employed a multigene analysis strategy based on the ITS on both sides of the 5.8S nuclear ribosomal RNA gene, β-tubulin, translation elongation factor 1α and histone H3 to distinguish a collection of 68 *I. radicicola sensu stricto* isolates from grapevine and other hosts with root rot disease symptoms. Histone H3 sequences was the most informative across the four genes. In the same year, Cabral *et al.* (2012b) recognised six new morphologically similar species of *Ilyonectria* (*I. alcacerensis, I. estremocensis, I. novozelandica, I. torresensis, Ilyonectria* sp. 1 and *I*. sp. 2) and *I. macrodidyma* sensu stricto, in the *I. macrodidyma*-complex by using the same four informative genes.

1.9 Detection of genetic diversity for *Cylindrocarpon* spp.

1.9.1 Molecular techniques

Besides molecular tools for genetic identification, PCR-based techniques can be used to investigate intra-species biodiversity. Microsatellite loci, short tandemly repeated motifs of 1–6 bases, which is also called simple sequence repeats (SSR), are broadly used as genetic markers in both animals and plants, but few reports have been published for their use in fungal studies (Zane *et al*., 2002). However, they usually need to be isolated *de novo* from each species, which may consume long time and lots of money. Secondly, Dutech *et al*. (2007) found that in comparison with other organisms, it is harder to isolate fungal microsatellite that is highly polymorphic. The cross-species transferability of fungal microsatellites is also lower than in other phylogroups. Inter Simple Sequence Repeat (ISSR) also amplifies repetitive DNA regions using primers complementary to the repetitive sequences and this method does not need sequence information or prior genetic studies. Alaniz *et al.* (2009) investigated the biodiversity of 87 "*C*." *liriodendri* and "*C*." *macrodidymum* isolates and successfully achieved 21 different genotypes from seven ISSR groups using four ISSR primers ($(GT)_7$ $(CCA)_5$ $(CGA)_5$ $(TCG)_5$). This is not as robust as microsatellites due to the repetitive sequences in the primers.

Other techniques involve the random priming of areas of the fungal genome to create DNA fingerprints containing randomly amplified bands. These methods include random amplification of polymorphic DNA (RAPD), universally primed PCR (UP-PCR) fingerprinting methods (Bulat *et al*., 1998) and amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995). Amplified fragment length polymorphism (AFLP) is based on selective amplification of restricted fragments from the digested genomic DNA. AFLPs have been widely used to study the genetic variability of a range of fungal species. Comont *et al.* (2010) used up to 10 AFLP markers to study the genetic structure of 74 isolates *of Phaeomoniella chlamydospora,* one of the main causal agents of Petri disease and esca on grapevines in France. The mean overall haplotypic diversity was as high as 0.97. Using 15 loci (randomly chosen among the 22 loci) was sufficient to discriminate 90% of the multilocus haplotypes in the dataset. Although this technique is robust the utilisation of several steps, including restriction enzymes, adapter ligation and amplification makes this technique a complicated and time consuming process.

UP-PCR is another robust random priming method. UP-PCR was specifically developed for fungi and has many advantages over the other two commonly used techniques. UP-PCR employs longer primers (15-20 nucleotides) than RAPDs with a higher primer annealing temperature (52-56°C) resulting in a higher degree of reproducibility and the generation of more bands. This allows more amplification of bands (Bulat *et al.,* 1998). UP-PCR also preferentially samples the intergenic and correspondingly more polymorphic areas of the fungal genome, while the genetic locus that RAPD primers bind to is unpredictable (Gramaje *et al*., 2013). This technique is simpler to apply than AFLP as it is a simple PCR process without any requirement for restriction digestion or adaptor ligation and it is able to distinguish a large number of genotypes to discriminate intra-species.

Genetic variation within populations of grapevine trunk disease pathogens has been extensively studied using UP-PCR technique during the past few years. In the recent work done by Gramaje *et al*. (2013), seven UP-PCR primers were used to screen 58 *Toginia minima* isolates causing Petri disease covering 11 provinces in Spain. A total of 49 polymorphic bands (60.5% of polymorphism) and 54 different genotypes were obtained among the isolates studied. In Billones' (2011) PhD thesis, analysis of genetic variability was carried out using UP-PCR and results showed that different isolates of botryosphaeriaceous species causing decline, dieback and death of grapevines were genetically diverse. Pathrose *et al*. (2014) generated a neighbour joining tree generated from UP-PCR data which showed high inter- and intra-vineyard diversity with few clonal isolates identified for each tested species, namely *I. liriodendri, I. marcrodidymum* and *C. destructans* grapevine isolates.

1.9.2 Vegetative compatibility groupings

In many filamentous fungi, physiologically distinct individuals (genotypes) of the same species can fuse asexually to form a heterokaryon. Such individuals are considered to be in one vegetative compatibility group (VCG). Vegetative compatibility in fungi reveals phenotypic differences or similarity among individuals of a species (Leslie, 1993). The mycelia of different isolates of one species that do not fuse or produced unstable heterokaryons with the presence of a barrage-like reaction at the meeting point on the plate are referred to as vegetatively incompatible (Figure 1.6 B). When mycelia of the two isolates merged together uniformly without distinction, they are called vegetatively compatible (Figure 1.6 A). In this case, fusions between hyphae of paired fungi growing closely are continuously formed, yielding a network of interconnected hyphae.

The molecular, genetic, and physiological control of hyphal anastomosis is still not well understood. Isolates that are different at one or more vegetative incompatibility (*vic*) loci are genetically distinct, and the heterokaryotic fusion cells are often compartmentalized and result in death by a lytic process (Garnjobst and Wilson, 1956). Vegetative incompatibility may reduce the risks of transmission of infectious cytoplasmic elements, such as virus-like dsRNAs, and of exploitation by aggressive genotypes (Glass *et al.*, 2000). Isolates belonging to the same VCG are presumed to be more genetically similar to each other. Cortesi and Milgroom (1998) reported that different fungal species vary in the number of *vic* loci responsible for the VCG genotypes. For fungal species with coloured or dark mycelium in culture, such as *Botryosphaeriaceae* and *Cryphonectriaceae*, a thick barrage line at the juncture point between incompatible paired isolates is observed on agar plates. However, it is very difficult to discern the barrage zones if the fungal mycelium is light in colour. In such cases, the production of nit mutants is necessary to define the individuals in culture (Swift, 2002). Since VCGs approach was established, this method has been successfully applied to understand fungal population structure. Pathrose (2012) did the first VCGs study in New Zealand *Cylindrocarpon* species. Three VCGs for seven "*C*." *destructans* isolates and two VCGs for both nine *"C*." *liriodendri* isolates and eight "*C*." *macrodidymum* isolates were produced. Some of the pairings of "*C*." *destructans* isolates were characterised as partially incompatible but the genetic basis of this reaction was not determined.

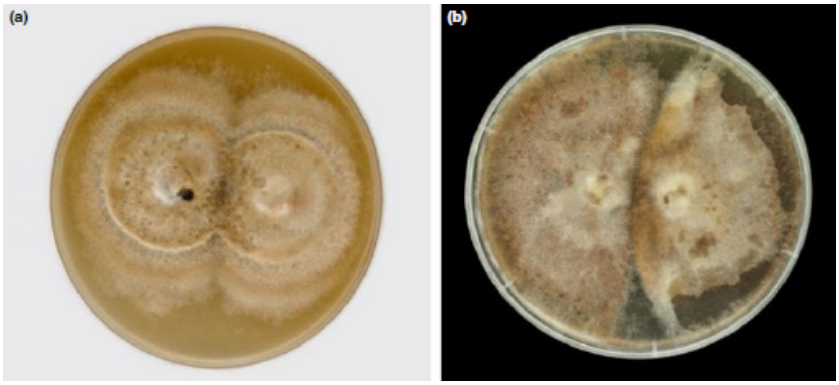

Figure 1.6 Vegetatively compatible (a) and incompatible (b) reactions formed by *I. liriodendri* isolates (Images retrieved from Pathrose *et al.,* 2014).

1.10 Rationale and objectives of this research

Cylindrocarpon spp. are unable to be accurately identified and characterized using morphological methods alone. The identity of isolates from New Zealand vineyards putatively identified as *C. pauciseptatum* will be confirmed by sequencing taxonomically informative genes, with the genetic diversity of these isolates determined using UP-PCR and vegetative compatibility groupings. Both *in vitro* methods and *in vivo* methods will be used to determine the pathogenicity of *C. pauciseptatum* isolates isolated from symptomatic NZ grapevines. Since cell wall degrading enzymes are important in the pathogenicity, the relative ability of these isolates to produce laccases will be determined. Degenerate primers, which have been developed in a previous study, will be used to amplify the genes for laccase and to investigate any sequence differences between isolates.

The specific objectives are listed below:

1. To use a multi-gene phylogeny to accurately identify the New Zealand *C. pauciseptatum* isolates
2. To investigate the genetic diversity of the New Zealand *C. pauciseptatum* isolates
3. To establish the relative pathogenicity of *C. pauciseptatum* on common grapevine rootstocks
4. To investigate laccase production by *C. pauciseptatum*

Chapter 2

Taxonomy and Genetic Diversity of *Cylindrocarpon paciseptatum*

2.1 Introduction

Cylindrocarpon pauciseptatum Schroers & Crous is a fungal species associated with black foot disease. It has been characterised by morphology, molecular tools, and pathogenicity assays throughout the world, including Spain (Martin *et al*., 2011), Turkey (Erper *et al*., 2013), Canada (Úrbez-Torres *et al*., 2014), Brazil (dos Santos *et al.,* 2014), and Italy (Aiello *et al*. 2014). Schroers *et al.* (2008) reclassified the *I. radicicola* complex into "*C." destructans*, "*C." destructans* var. crassum, *I. coprosmae*, *I. liriodendri, N. austroradicicola* and *N. macroconidialis* by the analysis of ITS rRNA gene sequences. For *Cylindrocarpon* spp. it has recently been demonstrated that a four gene phylogeny is required for accurate identification. Cabral *et al*. (2012b) revealed the existence of polymorphism in a wide collection of isolates previously identified as *"C." macrodidymum* by using the ITS region, translation elongation factor 1-α, the β-tubulin, and histone H3 gene regions. As a result, six new species of *Ilyonectria* and *I. macrodidyma* sensu stricto, which are morphologically similar, were identified. The histone H3 nucleotide sequence has become increasingly useful to identify pathogens associated with black foot disease. In Portugal, a large collection of fungal isolates morphologically identified as belonging to the *Ilyonectria* genera and *C. pauciseptatum* that had been collected from vineyards was analysed by DNA sequencing of the histone H3 gene, which largely confirmed the morphological identification. So far *C. pauciseptatum* strains isolated from New Zealand (Tauranga and Keesbury Estate) were identified by Schroers *et al*. (2008) using phenotypical characteristics and DNA sequence data of the ITS and partial β-tubulin gene. There has been no analysis using a four gene phylogeny to identify this species in New Zealand.

Genetic diversity of isolates of *C. pauciseptatum* has not been studied. In New Zealand, the genetic diversity of isolates of *Ilyonectria liriodendri, I. macodidyma* complex and *I. radicicola* complex collected from a survey of grapevines symptomatic for black foot has been done using UP-PCR (Pathrose *et al*., 2014). The results showed that these species were genetically diverse with a high level of intra- and inter-vineyard genetic variation detected. Although eleven isolates morphologically identified as *C. pauciseptatum* were also isolated in the survey their genetic diversity was not assessed. Apart from molecular tools, vegetative compatibility groupings (VCGs) have also been extensively applied to study the genetic relationships in anamorphic species of plant pathogenic fungi (Leslie, 1993). Leslie also stated that this method can demonstrate the propensity for fungi that reproduce asexually to

exchange their genetic material through parasexual recombination. Pathrose (2012) did the first and only VCGs study of *Cylindrocarpon* species which revealed three VCGs for "*C*." *destructans* isolates and two VCGs for both "*C*." *macrodidymum* and *I. liriodendri*.

The aim of this study was to i) confirm the identification of the eleven putative *Cylindrocarpon pauciseptatum* by a combination of culture morphology, conidial characteristics and a multigene phylogeny; ii) establish the genetic diversity of these isolates using UP-PCR, and iii) investigate the vegetative compatibility groups formed by these isolates.

2.2 Materials and Methods

2.2.1 Fungal Cultures

Cylindrocarpon pauciseptatum isolates were obtained from the Lincoln University culture collection where they had been stored as mycelia plugs (5-10) in sterile vials containing 20% glycerol at -80°C (Thermo Scientific, Forma 900 series). All isolates were subcultured by transferring a plug from the glycerol stock onto a potato dextrose agar (PDA; Difco) plate and grown at 20°C 12 h light:dark conditions for 7 days. Of the 11 *C. pauciseptatum* isolates originally stored only eight remained viable and could be recovered from storage onto agar (Appendix A.1).

2.2.2 Identification of *C. pauciseptaum* based on colony and conidium morphology

Preliminary identification of the eight *C. pauciseptatum* isolates (Hb3b, Mar5a, Mar14b, Mtb1a, CO6g, Ack2b, Mar6a and Ack2e) was carried out by the comparison of the colony morphology on potato dextrose agar (PDA; Difco) and anamorph characteristics to those reported in the literature (Schroers *et al*., 2008). All *C. pauciseptatum* isolates were inoculated onto duplicate PDA plates and incubated in 12 h dark:light conditions at 20°C for 7-18 days. The colony morphology was observed by eye under standard fluorescent light after 5, 11 and 18 days. Pigments of colonies and surfaces profile of colonies were photographed and described, and colony diameter was measured by digital caliper in two perpendicular directions from which an average was obtained. Results had been analysed using one-way ANOVA by GenStat version 16, with significant differences between treatments means determined using Fisher's protected least significant difference (LSD). Conidial characteristics were examined under a microscope (at ×40 and ×60) and digitally imaged using an Olympus BX51 microscope with a built-in Olympus DP12 camera (Olympus optical, Japan). Conidial suspension was prepared by scraping the surface of culture plate with a microscope slide after flooding with 5-10 mL of sterile distilled water (SDW). Slides were prepared by placing a drop of conidial suspension from an 18-d-old culture onto microscope slide. The length and width of 5 conidia per type per isolate were measured using the CellSens software Version 1.9 (Olympus optical, Japan).

2.2.3 Genomic DNA

DNA samples from all 11 isolates had been prepared previously by Dr Blessy Pathrose (Pathrose, 2012) and stored at 4°C. DNA concentration was measured by spectrophotometry with a NanoDrop-ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA). DNA samples of 20-25 ng /µL was prepared by diluting the original DNA stock solutions with sterile distilled water (SDW) for PCR. The DNA sample of an isolate collected by Dr Eirian Jones from a symptomatic avocado plant, which had been identified by DNA sequencing of the β – tubulin gene as *C. pauciseptatum,* was also included in the molecular identification assay.

2.2.4 Molecular identification of *C. pauciseptatum*

2.2.4.1 Standard PCR protocol

The identity of all 11 *C. pauciseptatum* isolates was confirmed by DNA sequencing of four taxonomically informative genes, which are the internally transcribed spacer region (ITS), the translation elongation factor 1-α, β – tubulin and the histone H3 as described by Cabral *et al*. (2012a). The ribosomal DNA was amplified using the universal primers ITS4 and ITS1F (White *et al*., 1990), the elongation factor 1-α gene using primers EF1 and EF2 (Carbone *et al*., 1999), the β-tubulin gene using primers T1 and Bt2b (Cabral *et al*., 2012a) and the histone gene using primers CYLH3F and CYLH3A (Crous *et al*., 2004) (Table 2.1). Each 25 µl PCR contained 1× PCR buffer (Roche Diagnostics, Basel, Switzerland), 200 µM each of dGTP, dCTP, dATP and dTTP, 5 pmol of each primer, 1.25 U FastStart Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany), 20-25 ng template DNA. A negative control where 1 µL SDW was added instead of DNA was prepared to make sure no contamination was present. A master mix was prepared in Class II Bio-safety hood as a DNA free environment. PCR amplifications were performed on a Veriti Thermal cycler (Biorad Laboratories, California, USA). For the ITS region the thermal cycle consisted of an initial step of 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at the 55°C for 30 s, and elongation at 72°C for 1 min. A final extension was performed at 72°C for 7 min. The reaction conditions were modified slightly for each primer pair with the modifications as described in Appendix A.4.

Table 2.1 Primers and sequence used for amplification of target gene for taxonomic identification.

Target Gene	Name of Primer	Primer Sequence
ITS	ITS4	5'TCCTCCGCTTATTGATATGC3'
	ITS1F	5'CTTGGTCATTTAGAGGAAGTAA3'
The translation elongation factor 1-α	EF1	5'ATGGGTAAGGARGACAAGAC3'
	EF2	5'GGARGTACCAGTSATCATGTT3'
β – tubulin	T1	5'AACATGCGTGAGATTGTAAGT3'
	Bt2b	5'ACCCTCAGTGTAGTGACCCCTTGGC3'
Histone	CYLH3F	5' AGGTCCACTGGTGGCAAG3'
	CYLH3R	5' AGCTGGATGTCCTTGGACTG3'

2.2.4.2 Agarose gel electrophoresis

All PCR products were separated by agarose gel electrophoresis. A 1% agarose gel was made by adding 1 g agarose powder to 100 mL 1×Tris-Acetate EDTA buffer (40 mM Tris acetate, 2 mM Na_2EDTA, pH 8.5). The agarose was dissolved by heating to boiling and cooled to approximately 60°C before being poured into a gel tray (10 cm × 20 cm) of a horizontal E-C® Gel Electrophoresis Apparatus (Thermo Scientific, NY, USA) and a comb inserted. The comb was removed from the set gel after 20 min, and the gel was then placed into an electrophoresis chamber and covered with TAE. Five µL of PCR product was loaded into the wells after combining with 3 µL loading dye (40% (w/v) sucrose; 0.25% bromophenol blue; 0.25% xylene cyanol). Three µL of 1kb plus DNA ladder (0.1 ng/µl; Invitrogen) was used as a molecular weight marker by combining with 3 µL loading dye and this was run alongside the PCR products. The gel was run at 10 V/cm for 45 min before being transferred into a container with ethidium bromide solution (0.5 µg /mL, AMRESCO®, OH, USA) for staining for 20 min. The gel then was destained by rinsing with tap water three times and placed in transilluminator (UVITEC Cambridge Imaging System, Total Lab Systems Ltd.), where it was photographed under UV light using Firereader 16.04 (UVITEC Cambridge Imaging System, Total Lab Systems Ltd.).

2.2.4.3 DNA sequencing and phylogenetic analysis of *C. pauciseptatum*

The PCR products were sequenced using the ABI PRISM 310 Genetic analyser (Applied Biosystem, Foster city, California) automated sequencer in the Bio-Protection Research Centre Sequencing Facility. The sequence data was analysed using Sequence Scanner software (Version: 1.0; Applied Biosystems) and the ambiguous area at the beginning and end of the sequence was trimmed using DNAMAN (Version: 4.0a; Lynnon BioSoft). The sequences were then submitted to a blastn search (http://www.ncbi.nlm.nih.gov/BLAST/) using the basic local alignment search tool (BLAST) function to confirm matching species in

the GenBank database. The sequences were aligned with closely related species using the CLUSTALW function of Mega version 5.05 (Tamura *et al*., 2011). A phylogenetic tree was produced using maximum likelihood based on the neighbour-joining method (Saitou & Nei, 1987) and 1000 bootstrap replications.

2.2.5 Genetic diversity using UP-PCR

2.2.5.1 UP-PCR

Eleven different UP-PCR primers were used for the detection of genetic diversity. The sequences of the UP-PCR primers and their respective annealing temperatures are listed in Table 2.2. Each 25 μL PCR reaction contained 1× PCR buffer (Roche Diagnostics, Basel, Switzerland), 200 μM each of dGTP, dCTP, dATP and dTTP), 20 pmol of UP-PCR primer, 2.5 mM $MgCl_2$, 1.25 U FastStart Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany) and 20-25 ng template DNA. A negative control where 1 μL SDW was used instead of the DNA was prepared to ensure no contamination in the PCR reagents. PCR amplifications were performed on a Veriti Thermal Cycler-200. The program consisted of an initial step of 3 min at 94°C, followed by 5 cycles of denaturation at 94°C for 30 s, annealing at the appropriate temperature (Table 2.2) for 30 s, and elongation at 72°C for 1 min. The next step was 34 cycles of 94°C for 30 s, annealing at the appropriate temperature (Table 2.2) for 90 s and 1 min primer extension at 72°C. A final extension was performed at 72°C for 10 min.

2.2.5.2 Agarose gel electrophoresis

All UP-PCR amplification products were separated by agarose gel electrophoresis as previously described in Section 2.2.4.2. Except that 2 g of agarose powder was added into 200 mL TAE buffer to make 1% agarose gel. A gel tray (20 cm × 20 cm) of a horizontal E-C® Gel Electrophoresis Apparatus (Thermo Scientific, NY, USA) was used instead of small tray for standard PCR. Eight μL of PCR product and 3 μL loading dye, 5 μL 1kb DNA ladder (Invitrogen) and 3 μL loading dye were combined, respectively, before loading into the wells. The gel was run at 10 V/cm for 2 h 45 min before being transferred into a container with an ethidium bromide and stained and photographed as described in Section 2.2.4.2.

Table 2.2 Nucleotide sequence and respective annealing temperatures of the UP-PCR primers.

Primer Name	Primer Sequence	Annealing Temperature (°C)	Reference
L45	5'GTAAAACGACGGCCAGT3'	51	Bulat *et al*., 1994
AA2M2	5'CTGCGACCCAGAGCGG3'	50	Lübeck *et al*., 1998
AS4	5'TGTGGGCGCTCGACAC3'	55	Lübeck *et al*., 1998
AS15	5'GGCTAAGCGGTCGTTAC3'	55	Bulat *et al*., 1994
AS15 Inv	5'CATTGCTGGCGAATCGG3'	52	Cumagun *et al*., 2000
Fok 1	5'GGATGACCCACCTCCTAC3'	52	Lübeck *et al*., 1998
L15	5'GAGGGTGGCGGTTCT3'	52	Tyson *et al*., 2002
L15/AS19	5'GAGGGTGGCGGCTAG3'	52	Lübeck *et al*., 1998
3-2	5'TAAGGGCGGTGCCAGT3'	52	Bulat *et al*., 1994
0.3-1	5'CGAGAACGACGGTTCT3'	50	Bulat *et al*., 1994
L21	5'GGATCCGAGGGTGGCGGTTCT3'	58	Lübeck *et al*., 1998

2.2.5.3 Scoring of DNA fingerprints

UP-PCR profiles from the five primers that produced the most bands and the most polymorphic bands were analysed from prints of the agarose gels. Bands from each primer that were strong and reproducible were counted and scored in the analysis. Faint or poorly amplified fragments were excluded from the analysis. Bands was scored "1" if present and "0" if absent. Isolates with similar banding patterns were regarded genetically similar and bands of the same molecular weight were assumed homologous.

2.2.5.4 Neighbour joining tree generated from DNA fingerprinting

The binomial matrix generated using Microsoft Excel 2010 was converted to a ".nex" file using Mesquite 2.75 program (Mesquite Software Inc., Oregon State University and University of British Columbia, USA). The converted file was analysed using PAUP V. 4.0b10 (Swofford, 2002) to generate a neighbour joining tree to determine the relationships between genotypes of *C. pauciseptatum* isolates. Figtree V1.4.2 software (BEAST, UK) was used to view dendrogram pattern of neighbour joining tree.

2.2.6 Vegetative compatibility groupings

2.2.6.1 Confirming the optimal agar medium for vegetative compatibility groupings

Half-strength PDA (19.5 g PDA /L plus 7.5 g agar), full- strength PDA (39 g PDA /L; Difco Laboratories, USA) as well as Czapek dox agar (45.4 g CDA/L; Oxoid Microbiology, UK) were initially tested to determine the best media for assessing vegetative compatibility. Three *C. pauciseptatum* isolates (Ack2b, Mar5a, Mtb1a) from different branches of the neighbour joining tree were selected for the pilot study. A 3 mm diameter mycelial plug was taken from the edge of a 5-7 day old colony, and placed on PDA, ½PDA and CDA, respectively, and incubated at 20ºC under 12/12 h light:dark conditions for 7 days prior to the experiment set up. In terms of pairing two isolates in one Petri dish, a mycelial plug from each of the two isolates were co-cultured 2.5 cm apart in the middle of the Petri dish containing the appropriate agar medium. All isolates were tested in pairs against each other (self versus non-self) and themselves (self versus self). Each combination of isolates was tested in duplicate. Colonies were observed and imaged using a digital camera after 7, 10 and 13 days' incubation at 20ºC under 12/12 h light:dark conditions. The agar media displaying the clearest profile with hyphal fusion and barrage zone was chosen for further study of all eight isolates.

The colonies on Petri dishes were visualized on both the upper and reverse side and scored as: (C) - vegetatively compatible, when mycelia of the two isolates merged together uniformly without distinction, (IC) - vegetatively incompatible, when mycelia of the two isolates grew to a meeting point on the plate but remained separated by a barrage zone formed along the line of contact between paired isolates; (PI) - partially incompatible, which is any other intermediate reactions different from self versus self reaction, such as no merge without a barrage line.

2.2.6.2 Vegetative compatibility groupings assay set up

All 8 isolates of *C. pauciseptatumpauciseptatum* were paired on CDA medium for vegetative compatibility groupings analysis using the method described in Section 2.2.6.1.

2.2.6.3 Microscopic observation of vegetative compatibility

Following Aimi's *et al*. (2002) vegetative compatibility groups protocol, 5 mm diameter plugs of 1/8 strength PDA media, which contained 1% agar, were placed 1 cm apart on a sterile glass microscope slide. A 2 mm cube of mycelium colonized agar obtained from a 3-4 day old culture on PDA was placed on the top of the agar. Each pair of selected isolates were paired against each other and themselves on separate microscope slides. Each slide containing paired inoculated agar plugs was placed on a sterile plastic straw in a Petri dish lined with moist filter paper, covered and sealed with cling film (Figure 2.1). Three replicates

were prepared for every pair of isolates. Finally, all inoculated slides were incubated at 20ºC under 12 light:12 dark conditions until the hyphae from each isolate began to merge at the centre of the glass slide. Changes in hyphal morphology were subsequently observed for pairings of isolates from each compatibility reaction on microscope slides. Prior to observations under the microscope, the agar cubes were removed and a drop of sterile water added onto the centre of the slide and covered with a cover slip. The hyphal merging zone between the isolates was observed at different magnifications (×100, 400 and 600). The leading hyphae from each isolate colony were traced under the microscope to identify the anastomoses between the isolates and digitally imaged using an Olympus BX51 microscope with a built-in Olympus DP12 camera (Olympus optical, Japan).

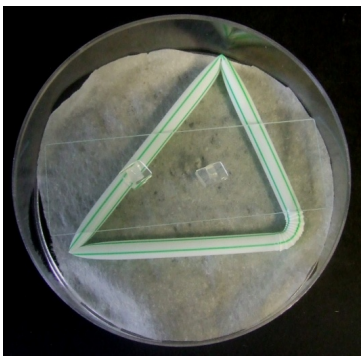

Figure 2.1 Experimental setup for the microscopic observation of vegetative compatibility groupings of isolate pairs.

2.3 Results

2.3.1 *Cylindrocarpon* species colony morphology

The initial characterisation of isolates was achieved by colony observation including mycelial growth, the production of yellow pigment and presence of aerial mycelium on PDA. Except for Mtb1a, all other isolates produced a yellowish to brown pigment, among which Mar5a produced the greatest amount (most intense colour). Mtb1a produced a white to greyish pigment. Mar5a produced a brown pigment with smooth surface at the edge of cottony margins on day 18 on PDA (Figure 2.2). All eight isolates produced felty aerial mycelium. Mtb1a produced more aerial mycelium than others (Figure 2.2). On the reverse side of colony, all isolates showed a brown colour with light brown at the edge due to the formation of chlamydospores except for Mtb1a which was dark brown colour with black ray like branches. Except for Mar5a, Mar6a and Mtb1a, the hues in growing sectors were clearly observed for the eight colonies on the reverse side.

The average diameter of colonies on PDA plates is presented in Figure 2.3 (Appendix A.5). Mtb1a grew the fastest with an average diameter on day 5, 11 and 18 of 35.0, 73.0 and 81.8 mm, respectively. CO6g had the smallest average diameter on day 5 (14.0 mm), whereas

the average diameter of Mar6a was the least on day 11 (41.7 mm). Hb3b had the smallest diameter (61.0 mm) on day 18. There was significant difference ($P < 0.001$) between the growth rate of different isolates over time.

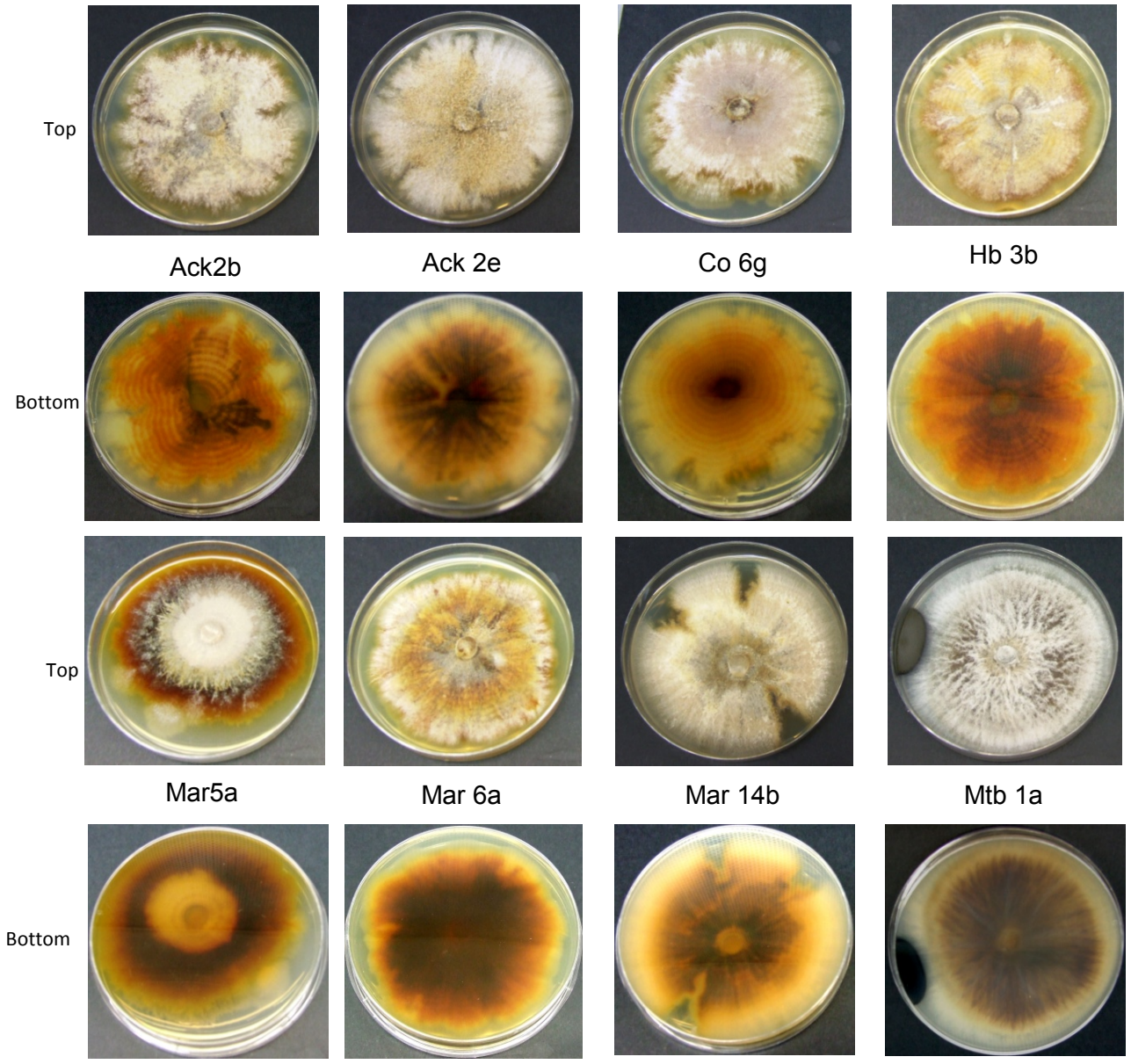

Figure 2.2 Colony morphologies of *Cylindrocarpon pauciseptatum* isolates after 18 days' incubation on potato dextrose agar.

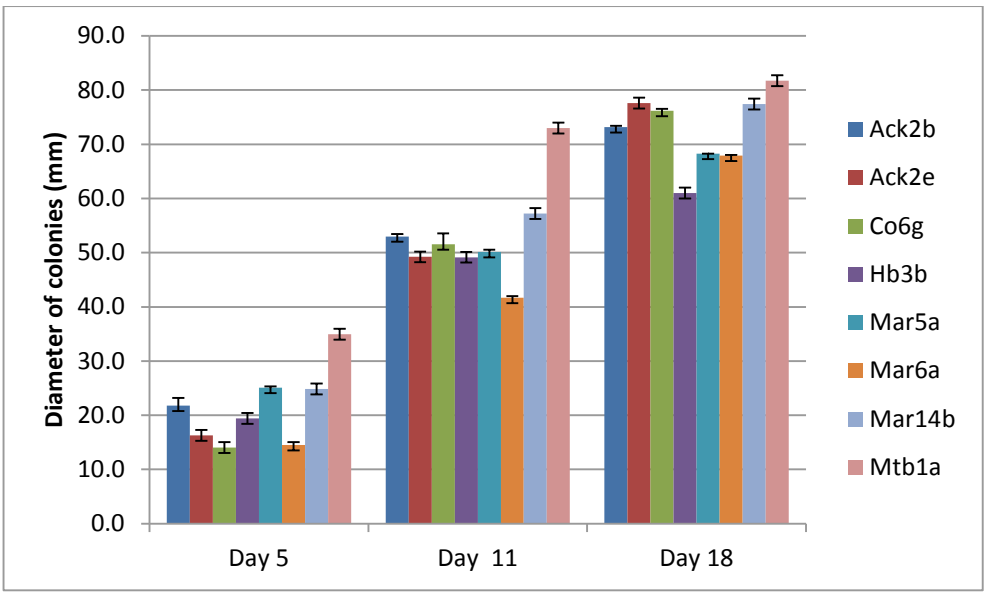

Figure 2.3 Average colony diameters for each isolate of *Cylindrocarpon pauciseptatum* grown on PDA on days 5, 11 and 18. Measurements are in mm.

Conidial characteristics, such as, shape and size were used to further identify the species (Figure 2.4). Macroconidia formed on sporodochial fascicles predominantly with 3-septate, hyaline, which was very slightly and regularly curved with broadly rounded ends. Length×width was 41.4–45.6–53.1×7.8–8.0–11.5 µm. Microconidia were rarely observed from most of the isolates' colonies except for Hb3b, which was hyaline, formed by simple, sparsely branched conidiophores on the aerial mycelium as subglobose to ovoidal shape. This finding was the same as the description of Schroers and co-workers (Schroers *et al*., 2008). Measurement was 5.2–7.2×3.3–4.7 µm. When compare to the macroconidia (50–70×7–11 µm) illustrated and described by Booth (1966), the length of macroconidia in this study was shorter while width was within the reported range.

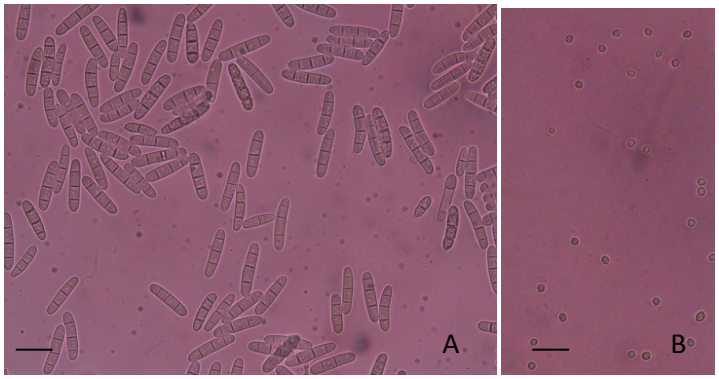

Figure 2.4 Macroconidia (A, isolate Ack2e) and microconidia (B, isolate Hb3b) of *Cylindrocarpon pauciseptatum* isolates. Bar represents 50µm.

2.3.2 Molecular identification using four taxonomically informative genes

PCR amplification of selected regions of ITS, β – tubulin, EF1-α and histone produced the expected fragments of approximately 600, 600, 700 and 500 bp, respectively (Figure 2.5) (Appendix A.2).

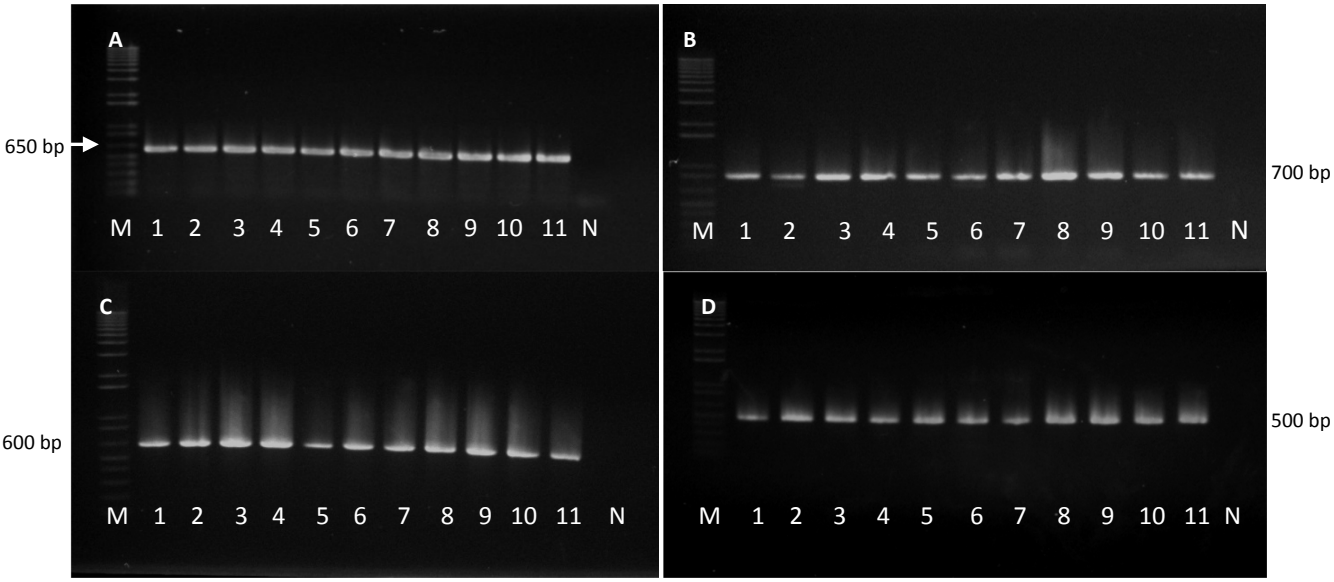

Figure 2.5 Representative photograph of the amplified fragments of specific DNA for 11 *C. pauciseptatum* isolates using A: ITS; B: Translation elongation factor 1-α; C: β – tubulin; D: Histone. Lane 1 to 11 contains representative isolates (1. CO6g, 2. Mar5A, 3. HB6b, 4. HB3b, 5. MTB1a, 6. Mar17d, 7. Mar17e, 8. Mar14b, 9. Ack2b, 10. Mar6a, 11. Ack2e.). N: negative control. M: 1 kb plus DNA ladder. Numbers on the far left and right sides denote molecular weights.

Around 500 bp usable sequences of the isolates were used to produce four neighbour joining (NJ) trees (Figures 2.6, 2.7, 2.8 and 2.9). All of the GenBank isolates used for comparison had been cited in the taxonomy work of Cabral *et al*. (2012a) or were of New Zealand origin (Appendix A.3). The ITS NJ tree grouped the 11 isolates into one clade (100% bootstrap support) with sequences from six voucher specimens of *C. pauciseptatum* (Cy217, Cy238, CBS113550, CBS120171, CBS120173, and CBS100819). This clade was distinct from the two other *Cylindrocarpon* species used as outgroups (*I. liriodendri* and *C. theobromicola*) (Figure 2.6). The translation elongation factor 1-α grouped the isolates into 3 clades with low bootstrap value (Figure 2.7). Clade 1, comprising 6 isolates with the entire representative international strains (34% bootstrap support). CO6g and Mar6a (43% bootstrap support) formed another clade. Mar17d and Mtb1a were placed in one group (59% bootstrap support). All the rest isolates in this study were displayed as individual branch in the group of *C. pauciseptatum*. The NJ tree generated from β – tubulin sequences placed the 11 isolates into two clades (Figure 2.8). The first clade (Mar17d, Mar17e, Mar6a, CO6g) was highly supported (86%) that New Zealand isolates clustered with five *C. pauciseptatum* sequences obtained from GenBank including the voucher isolates Cy238, CBS120171 and Cy217. The

other clade contained seven isolates and was moderately supported (78%). It contained a sub-clade containing a single isolate (Mtb1a), while the other six isolates clustered (64% bootstrap support) as sub-clade 1 with two *C. pauciseptatum* voucher isolates (CBS100819 and CBS113550) previously collected from New Zealand (Schroers *et al*., 2008). The six isolates in clade 2 were identical to each other but had 9 single base pair substitutions compared with isolates in clade 1. Isolate Mtb1a contained a further 2 bp difference from the other six isolates in clade 2. The isolate recovered from a symptomatic avocado plant was also placed into the sub-clade of clade 2 and clustered with Ack2e as one further sub-clade (62% bootstrap value). This further sub-clade contrained 1 bp substitution from the other 4 isolate in sub-clade 1. The sequences of the histone H3 gene, unfortunately, didn't distinguish C. pauciseptatum isolates and published representative international strains at all. For the trees generated by ITS, β - tubulin, and elongation factor 1-α, the same two *Cylindrocarpon* spp. (*C. theobromicola* and *"C." liriodendri*) from GenBank were used as outgroups, but for histone *I. radicicola* and *"C.". liriodendri* were used as outgroups. The sequences of two New Zealand isolates (CBS113550 and CBS100819) were placed in the NJ trees accordingly.

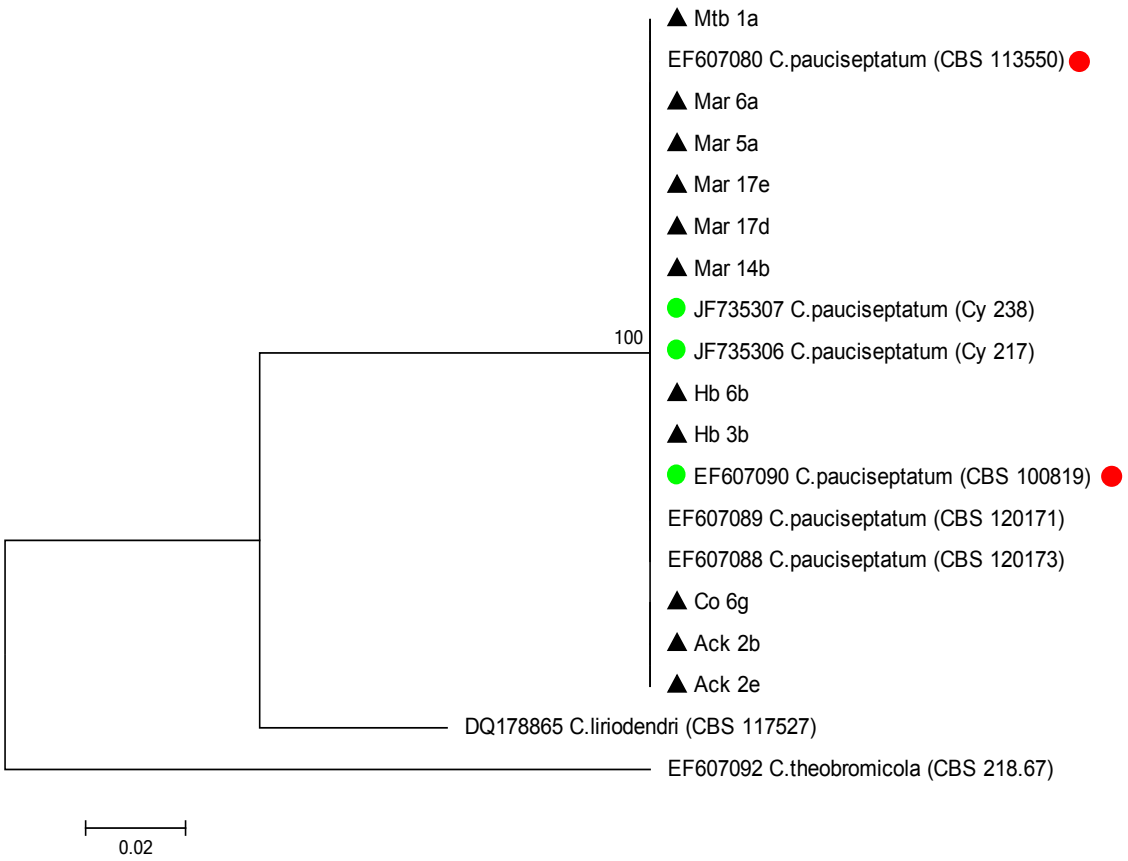

Figure 2.6 The neighbour joining tree with 1000 replicates of bootstrap values generated by Kimura 2-parameter model of MEGA 5.05 using ITS sequences of *Cylindrocarpon pauciseptatum* isolates from New Zealand vineyards and additional sequences of *C. pauciseptatum* from GenBank with accession numbers. Isolates with a green dot represent the highly identified voucher specimens based on ITS gene. Isolates highlighted with a black triangle represent New Zealand *C. pauciseptatum* isolates from the present study. Isolates with a red dot at right side are published New Zealand strains.

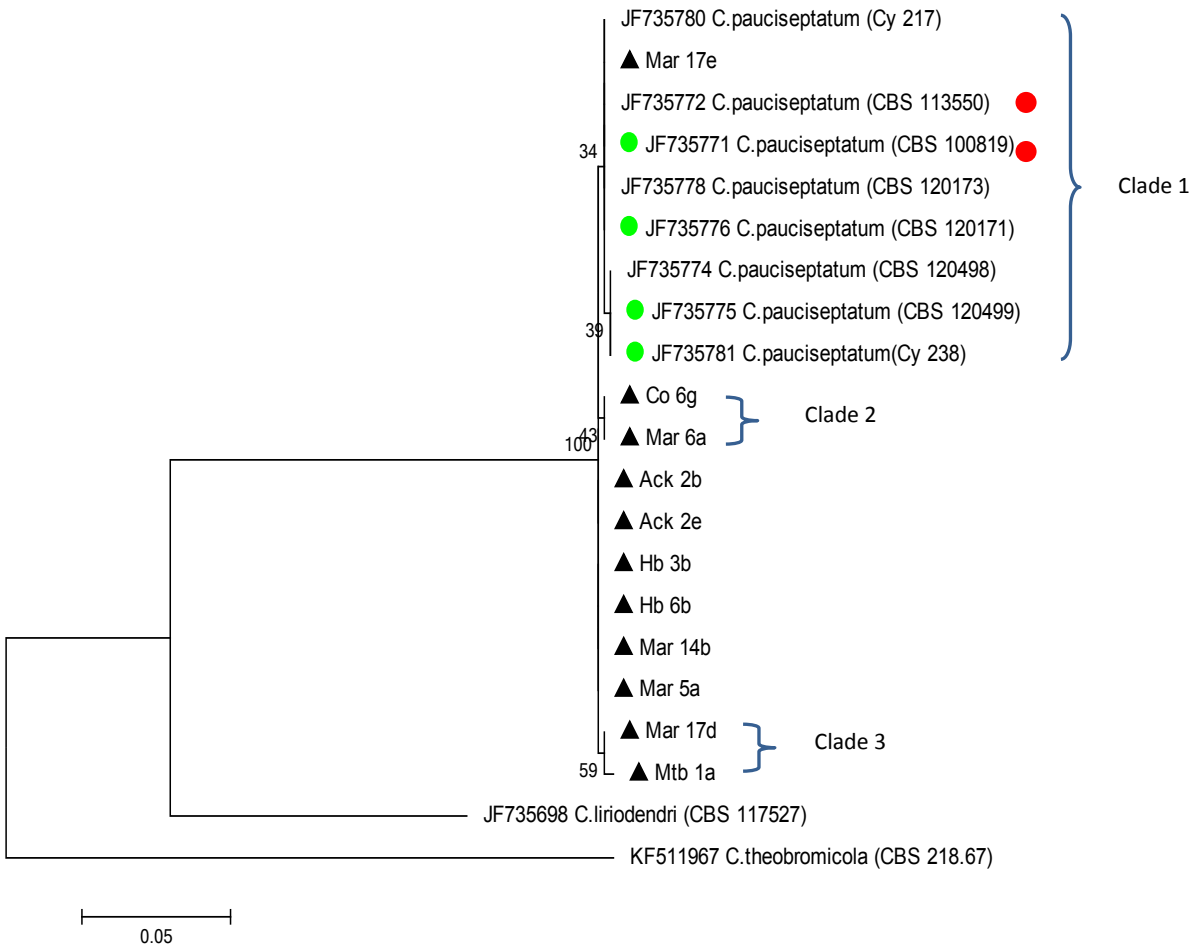

Figure 2.7 The neighbour joining tree with 1000 replicates of bootstrap values generated by Kimura 2-parameter model of MEGA 5.05 using the translation elongation factor 1-α sequences of *Cylindrocarpon pauciseptatum* isolates from New Zealand vineyards and from GenBank with accession numbers. Isolates with a green dot represent the highly identified voucher specimens based on TEF gene. Isolates highlighted with a black triangle represent New Zealand *C. pauciseptatum* isolates from the present study. Isolates with a red dot at right side are published New Zealand strains.

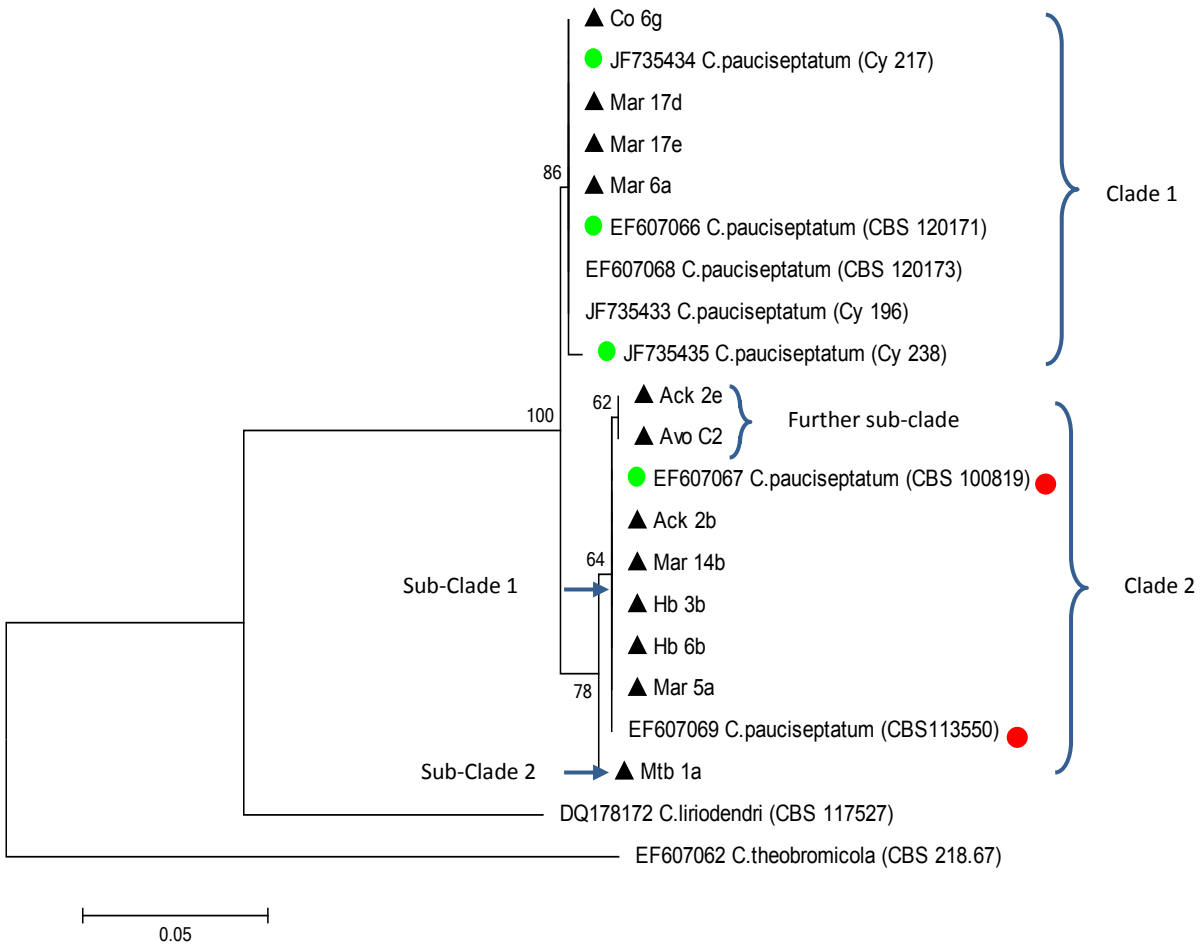

Figure 2.8 The neighbour joining tree with 1000 replicates of bootstrap values generated by Kimura 2-parameter model of MEGA 5.05 using β – tubulin sequences of *Cylindrocarpon pauciseptatum* isolates from New Zealand vineyards and from GenBank with accession numbers. Isolates with a green dot represent the highly identified voucher specimens based on β – tubulin gene. Isolates highlighted with a black triangle represent New Zealand *C. pauciseptatum* isolates from the present study. Isolates with a red dot at right side are published New Zealand strains.

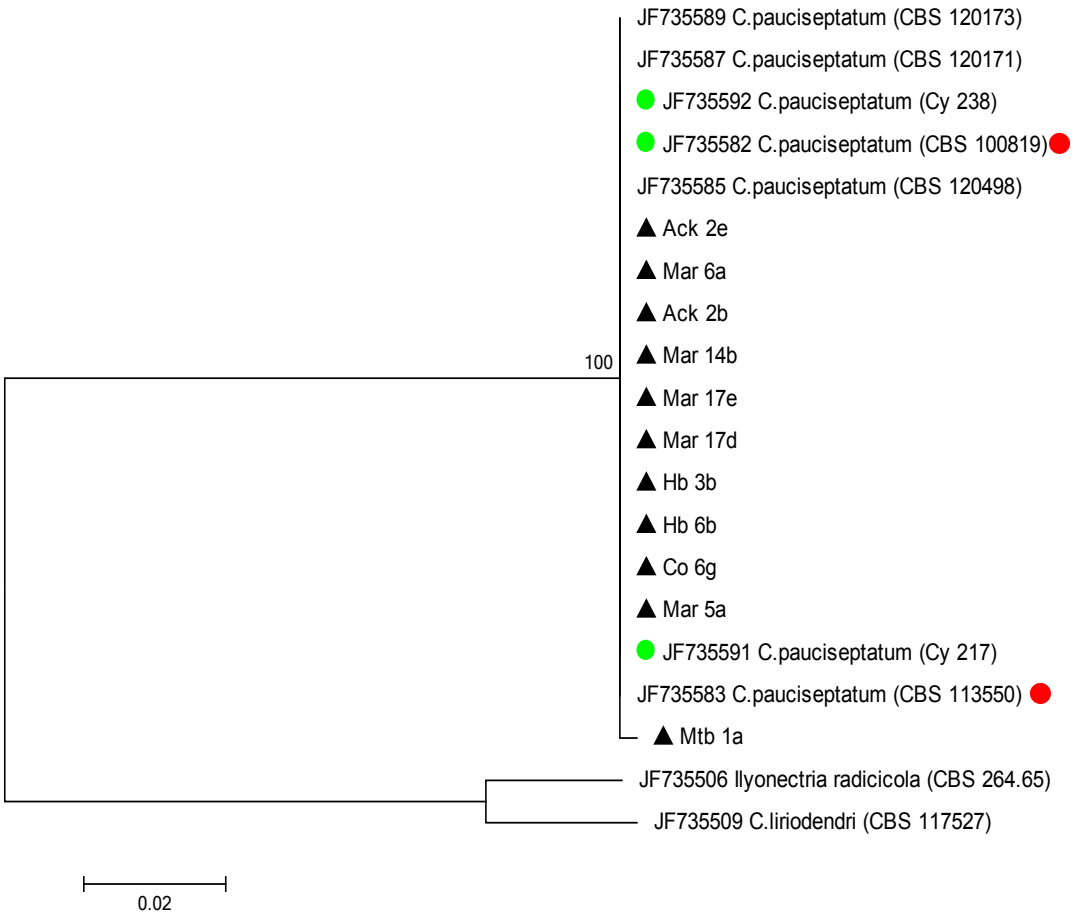

Figure 2.9 The neighbour joining tree with 1000 replicates of bootstrap values generated by Kimura 2-parameter model of MEGA 5.05 using histone sequences of *Cylindrocarpon pauciseptatum* isolates from New Zealand vineyards and from GenBank with accession numbers. Isolates with a green dot represent the highly identified voucher specimens based on histone gene. Isolates with black triangle represent *C. pauciseptatum* samples used in this study. Isolates with a red dot at right side are published New Zealand strains.

2.3.3 Genetic diversity study of *C. pauciseptatum*

The bands produced by UP-PCR ranged in size from 200 to 2000 bp (Figure 2.10). Five UP-PCR primers were selected from which to generate the NJ tree (Table 2.3). Among these five selected primers, the greatest number of UP-PCR bands was generated by primer AS15inv (17 bands from 11 isolates), which also gave the highest number of polymorphic bands (n=10) and highest percentage of polymorphic bands (59%). Primer AS15 produced the least bands (10), while primer 3-2 generated the least percentage of polymorphic bands (23%). A total of 65 scorable bands were generated by these five primers, of which 46% were polymorphic.

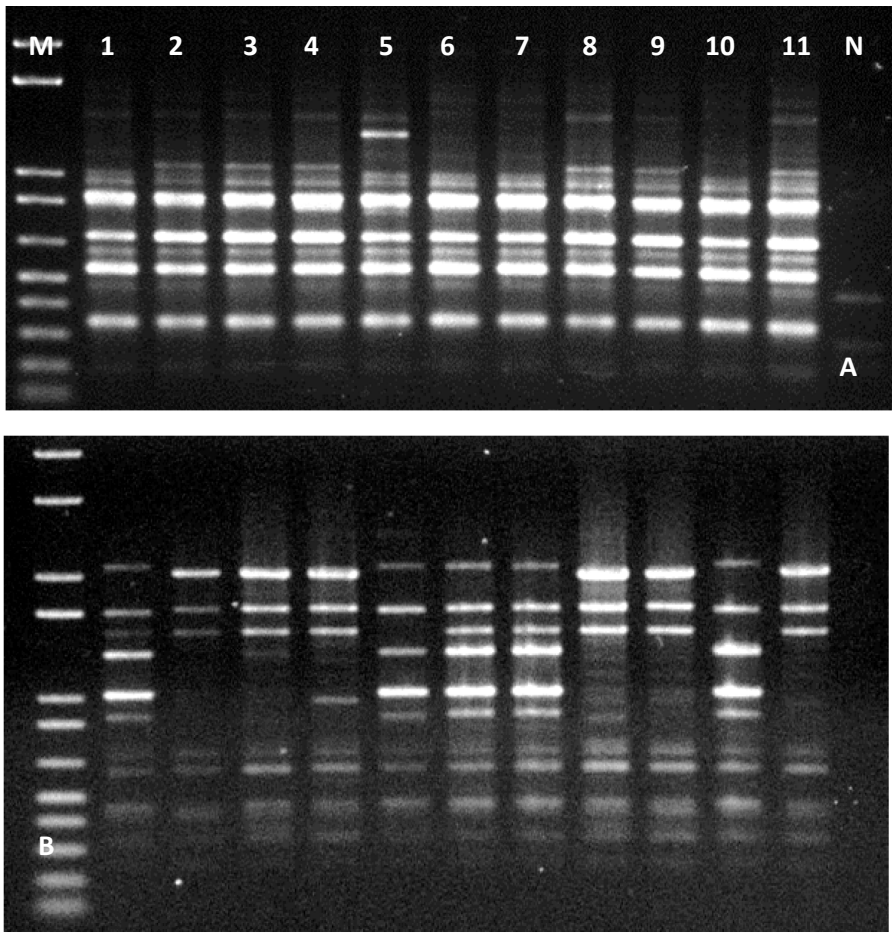

Figure 2.10 Examples of 1% agarose gels presenting bands amplified by UP-PCR primers, A: L15As19; B: As15. Lanes 1-11 denote representative isolates (1-CO6g; 2-Mar5a; 3-Hb6b; 4-Hb3b; 5-Mtb1a; 6-Mar17d; 7-Mar17e; 8-Mar14b; 9-Ack2b; 10-Mar6a; 11-Ack2e). M: 1Kb plus ladder marker. N: negative control.

Table 2.3 The number of bands (NB), polymorphic bands (PB) and percentage of polymorphic bands (P%) for 11 UP-PCR primers. Primers in red represent the primers selected for the genetic variation analysis.

Primer	NB	PB	P%
L 15/As 19	14	8	57
L 15	11	3	27
Fok 1	7	5	71
3-2	13	3	23
As 15	10	5	50
As 15inv	17	10	59
AA2M2	11	4	36
0.3-1	2	1	50
L 45	9	5	56
L 21	8	2	25
As 4	9	3	33

An arbitrary line drawn at 1 change from the root of the NJ tree showed that the population studied in this project was divided into 2 clusters (Figure 2.11). These clusters matched the

distinction of isolates shown by the β-tubulin NJ tree. Each genetic group was composed of a range of isolates from different geographic regions and vineyards. The isolates Mar×× obtained from different vineyards in the same region (Marlborough) were placed in genetic groups 1, and 2 which indicated genetic diversity of *C. pauciseptatum* between the vineyards (inter-vineyard genetic diversity). The isolates Ack2x and Mar17x were obtained from the same vineyard in Auckland and Marlborough, respectively, and were located in the same genetic groups respectively showed there was little intra-vineyard genetic diversity. No genetically identical (clonal) isolates were found within these 11 specimens as each of them was located on a unique branch.

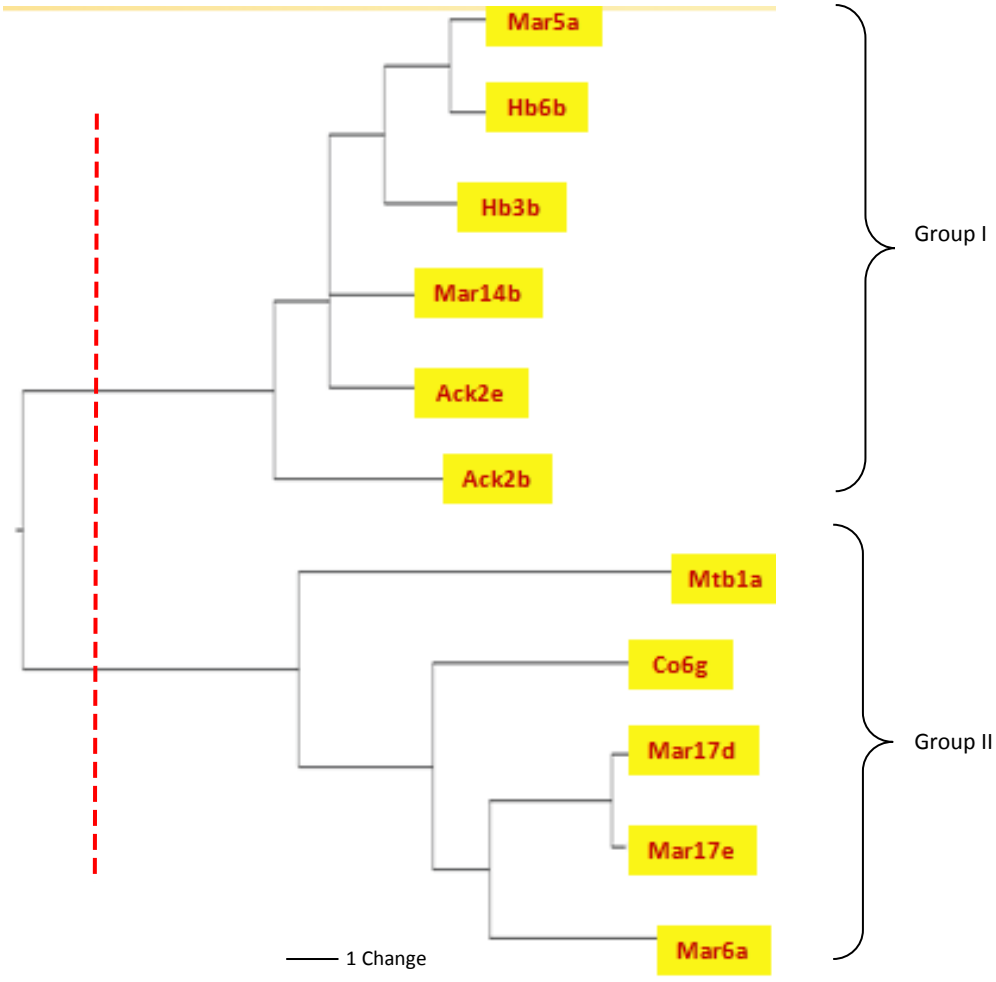

Figure 2.11 Neighbour joining tree generated by PAUP 4.0b 10 using UP-PCR fingerprinting data. A red dotted line indicates the arbitrary line to recognize two genetic groups. Groups are indicated on the right side.

2.3.4 Optimisation of agar medium for VCG test

On ½ PDA and PDA, there was little distinction between colonies (Figure 2.13). The colonies grew rapidly on CDA plates for both compatible and incompatible pairs. Furthermore, CDA produced a clear barrage line between two incompatible isolates in contrast to PDA plates

(Figure 2.13, C). Consequently, CDA medium was selected to analyse the vegetative compatibility among *C. pauciseptatum* isolates.

2.3.5 Vegetative compatibility groups

The eight *C. pauciseptatum* isolates paired on CDA produced three types of reaction (Figure 2.14). Most (64%) of the paired reactions were compatible as the hyphae merged along the contact zone. The incompatible pairs which formed clear zones between the growing edges of the paired isolates accounted for 17% of the interactions (Table 2.4). Seven pairs of isolates were partially incompatible as they yielded either a mild barrage line with an unclear dark coloured line at the colony meeting point or a partial (but not complete) fusion of mycelia as seen from the reverse side (Figure 2.14). The partial incompatible pairs were considered as compatible pairs for the designation of VCGs (Figure 2.12) as it is possible that some fusion was occurring. The eight isolates were placed into 2 groups, and each group was composed of 3 sub-groups which overlapped in a complicated manner with each other. Mtb1a and Ack2b were compatible with all other isolates. There was no correlation between VCGs and genetic diversity assessed by UP-PCR (Figure 2.11) since incompatible pairs were from the same genetic group and only 22% of compatible pairs were from the same clades.

Table 2.4 Vegetative compatibility results of *Cylindrocarpon pauciseptatum*.

Isolates	Hb3b	Mar5a	Mar14b	Mtb1a	CO6g	Ack2b	Mar6a	Ack2e
Hb3b	C	I	PI	C	PI	C	PI	I
Mar5a		C	I	C	PI	PI	PI	I
Mar14b			C	C	C	C	C	I
Mtb1a				C	C	C	C	C
CO6g					C	PI	I	C
Ack2b						C	C	C
Mar6a							C	C
Ack2e								C

C: Compatible; I: Incompatible; PI: Partially Incompatible

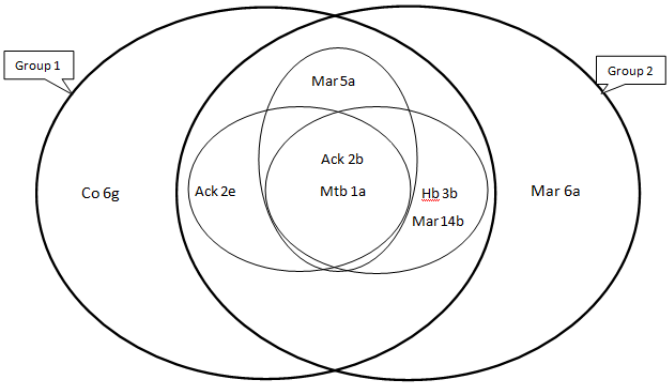

Figure 2.12 VCGs generated from vegetative compatibility test.

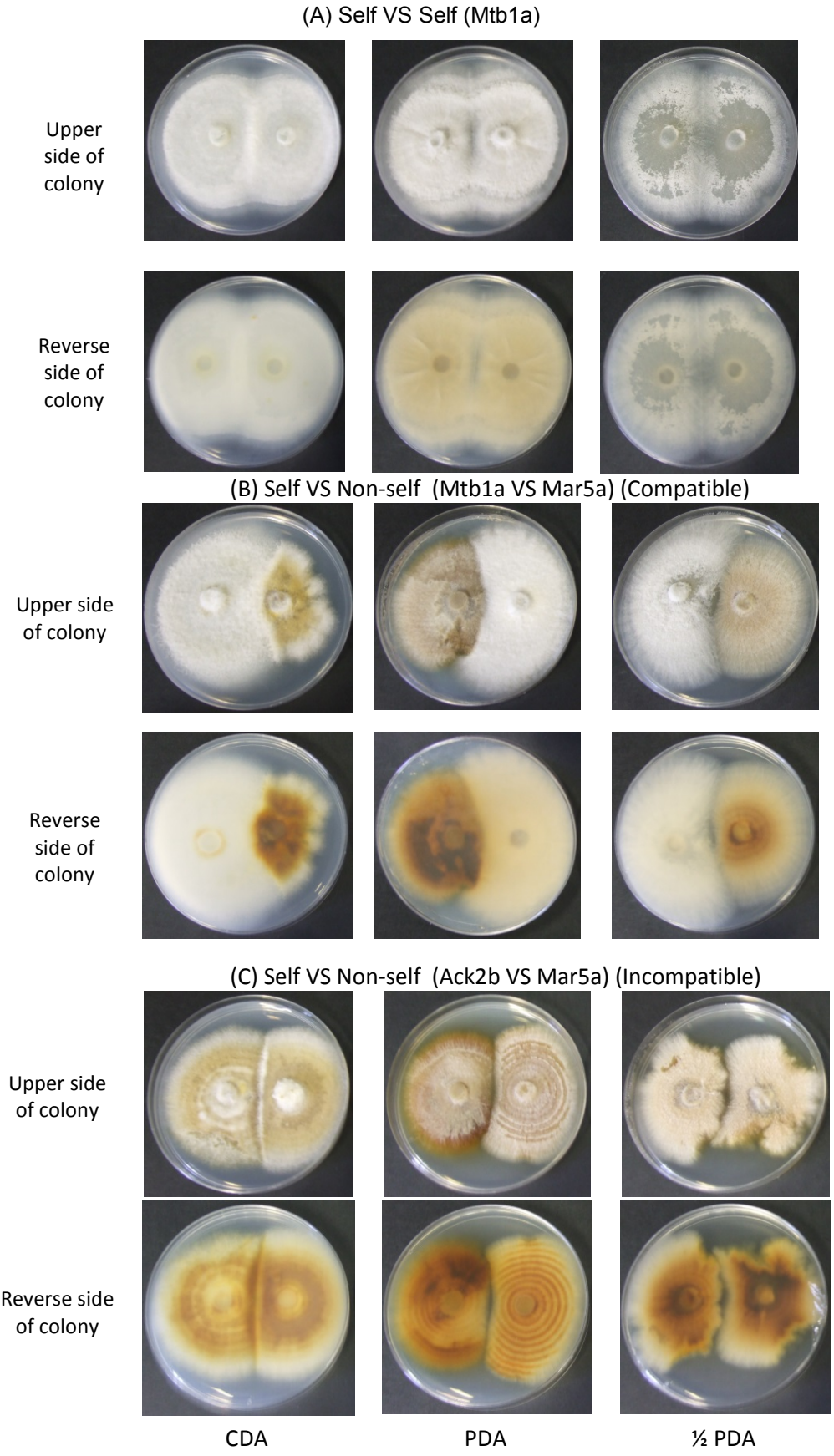

Figure 2.13 Comparison of different agars used for vegetatively compatible and incompatible reactions produced by pairs of *Cylindrocarpon pauciseptatum* isolates after 10 days' incubation. A)

Self-self pairing of isolate Mtb1a; B) compatible reaction (between isolates Mtb1a and Mar5a; and C) incompatible reaction between isolates Ack2b and Mar5a.

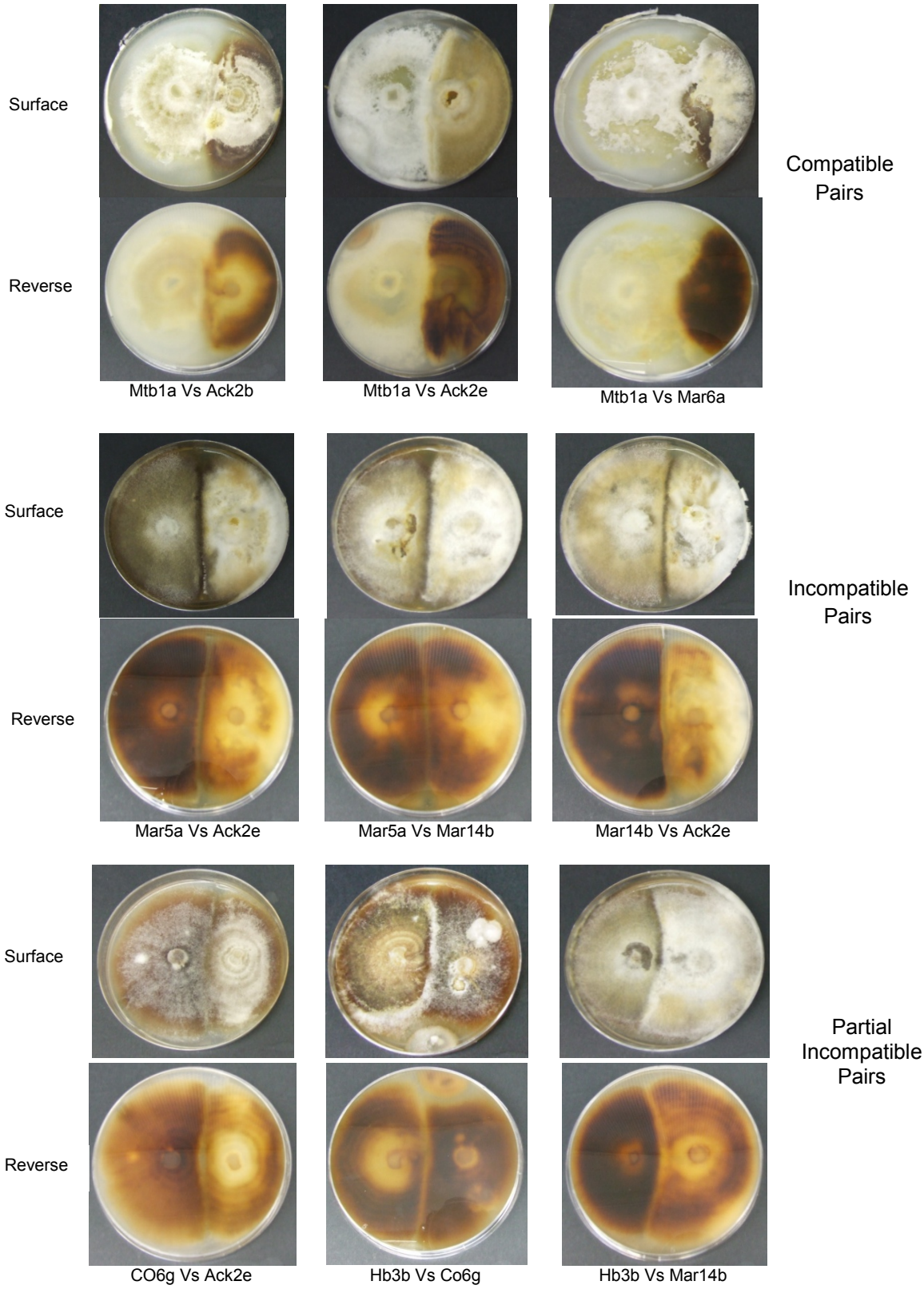

Figure 2.14 Vegetative compatibility and incompatibility reactions produced by pairing *C. pauciseptatum* isolates after 10 days' incubation.

2.3.6 Examination of the hyphal reactions of different VCGs

Anastomoses (hyphal fusions) were observed under the microscope on self-self isolate pairings and non-self isolate pairings (Figure 2.15). Two types of anastomosis were observed (1) hyphal wall to wall fusion both in distal and proximal sections of the colony (Figure 2.15A) and (2) fusion of growing hyphal tips (Figure 2.15B). The complicated hyphal network formed by non-self paired colonies made the identity of individual hyphae hard (Figure 2.15C, D). Most of the hyphae from both colonies thickly merged in the middle between the two inoculation plugs.

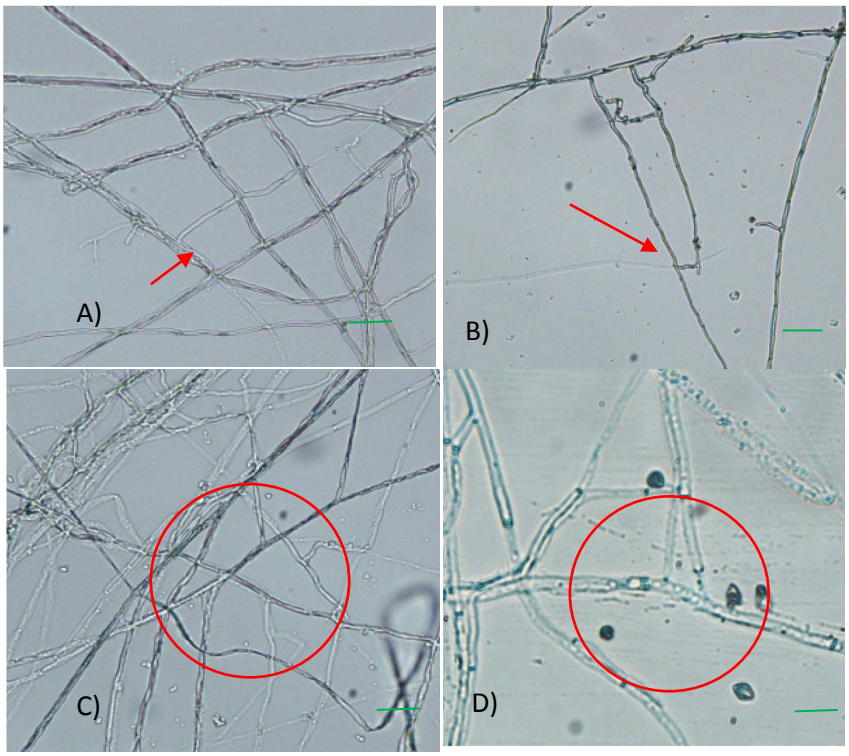

Figure 2.15 Microscopic examination of compatible vegetative compatibility groupings of *C. pauciseptatum* isolates. A) Self-self pairing of Mar 6a , B) Mtb1a vs Ack2e, C) Mtb1a vs Ack2b, D) Mtb1a vs Mar5a. Arrows and circles indicate hyphal anastomosis types. Bars represent 100.00 µm.

In the incompatible interactions, no hyphal merging was observed and the hyphal tips were often observed to be dead (Figure 2.16A, B). Partially incompatible pairings showed healthy hyphae in the interaction zone (Figure 2.16C). Only a few partially incompatible pairs were observed to have merging hyphae (Figure 2.16C) and the anastomoses were only found within each colony but not at the interaction zone.

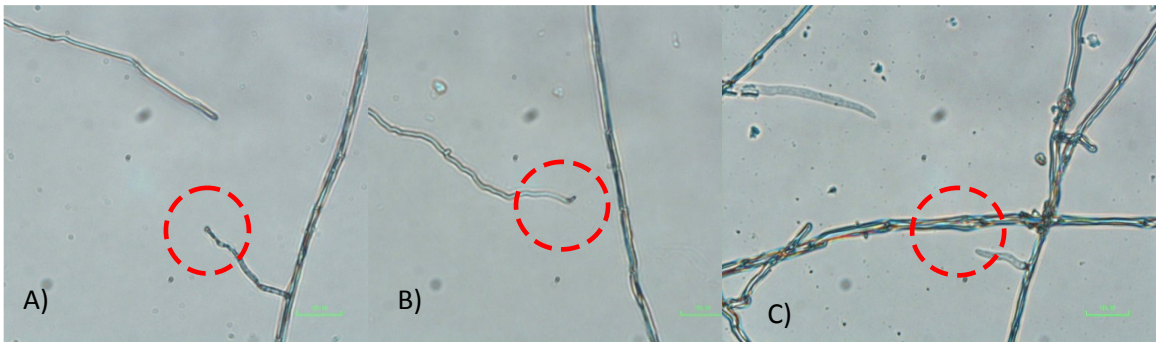

Figure 2.16 Microscopic examination of incompatible and partial incompatible vegetative compatibility groupings of *Cylindrocarpon pauciseptatum* isolates. A) Mar14b vs Mar5a (Incompatible), B) Mar5a vs Ack2e, (Incompatible) C) CO6g vs Ack2b (Partial Incompatible). Red broken circles indicate the growth condition of hyphae. Bars represent 100.00 µm.

2.4 Discussion

This is the first study using a combination of sequencing of four taxonomically informative gene regions (ITS, β – tubulin, translation elongation factor and histone) to identify 11 putative *C. pauciseptatum* isolates that had been isolated from symptomatic grapevines in New Zealand. Culture and conidial morphological characterisation was used for preliminary identification by comparing with published description of *C. pauciseptatum* (Schroers *et al*., 2008). UP-PCR showed that the isolates were genetically diverse. VCGs analysis was conducted for eight isolates and showed that hyphal anastomoses could form between the isolates providing a potential mechanism for genetic exchange.

The *C. pauciseptatum* isolates originated from a large collection of *Cylindrocarpon*-like isolates that had been recovered from symptomatic grapevines demonstrating typical characteristics of black foot disease. The colony characteristics used in initial differentiation was the formation of brown pigments in cultures on PDA. The colony morphology described here confirmed those original descriptions for eight isolates (Pathrose, 2012). When the conidia produced by these eight isolates were examined, their morphology and their dimensions (length and width) measured in this study were within the range described by Schroers *et al*. (2008) being cylindrical, multiseptate macroconidia and (37–) 42–45–47(–54)×(7.0–)8.5–9.0–9.5(–10.0) µm). In addition, the length: width ratio of macroconidia was between 4 and 6 µm. The finding that for the majority of macroconidia for all isolates was 3 septate also matched the general profile of *C. pauciseptatum*. Conidial morphology of "*C.*" *destructans* var. crassum and *C. theobromicola* overlap with *C. pauciseptatum* in terms of length and width and number of septa (Schroers *et al*., 2008). Some macroconidia of the *C. pauciseptatum* isolates in this study were very slightly curved and had rounded ends which distinguished them from *C. theobromicola,* which has consistently curved macroconidia as described by Schroers *et al*. (2008). *Cylindrocarpon pauciseptatum* has been characterised

by the lack of microconidia, which was also shown in this study. The subglobose to ovoidal microconidia formed by HB3b in this study differed from the ellipsoidal microconidia known in many other *Cylindrocarpon* species, such as *I. liriodendri* (Halleen *et al*., 2006). Although phenotypic characters were valuable for the preliminary identification of *C. pauciseptatum* in this study, it was still necessary to conduct molecular analyses to accurately identify the species.

As demonstrated by Cabral *et al*. (2012a, b), multi-gene sequence analyses using the ITS, β–tubulin (BT), EF1-α, and histone H3 are necessary to distinguish *Ilyonectria/Cylindrocarpon* species. In that work they showed that among these four target genes, sequences generated from the ITS were the least informative, while histone H3 sequences provided the most informative loci screened. Therefore, the multi-gene sequence analysis was applied in this study to accurately identify the New Zealand isolates. Phylogenetic analyses of individual ITS, β – tubulin, EF1-α and histone datasets showed all 11 isolates grouped into well-supported clades along with voucher specimens. The 11 isolates formed a monophyletic unit in the rRNA gene tree which was expected as this gene generally shows least variation in *Cylindrocarpon* species. The histone gene also generated the same result. However, the isolates were paraphyletic based on β – tubulin, EF1-α phylogenies. Sequences of these regions were both 99% similar, or identical to C. pauciseptatum strains CBS100819 and CBS113550 (accessions numbers ITS [EF607090; EF607080]; BT [EF607067; EF607069], EF1-α [JF735771; JF735772], and histone H3 [JF735582; JF735583]). *Cylindrocarpon pauciseptatum* strain CBS100819 had been recovered from symptomatic roots of *Erica melanthera* in Tauranga, (1998) and CBS113550 had been isolated from symptomatic grapevines in Keesbury Estate (2003) (Schroers *et al.,* 2008). These two reference strains were identical to each other for the four sequenced regions and both were deposited at the CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands (CBS) (Cabral *et al*. 2012).

The sequencing result of rRNA gene is similar to the work of Schroers *et al.* (2008) who reported that *C. pauciseptatum* strains from New Zealand and Slovenia are identical to each other by ITS rRNA sequences but using other genes they differ slightly from other *C. pauciseptatum* isolates from elsewhere. dos Santos *et al*. (2014) achieved similar results for isolates used in their multi-gene DNA sequence analysis showing that the sequences of three regions (rDNA-ITS, β–tubulin, and histone H3) had 99, 99, and 97% similarity with the type specimen *C. pauciseptatum* isolate Cy238. From the New Zealand isolates the NJ tree generated from β-tubulin sequences produced two clades. Eight isolates, including the avocado isolate were identical to the New Zealand isolate recovered from *Erica melanthera* (CBS100819) and grapevine (CBS113550), but did not group with other voucher isolates recovered from other countries. There were 9 bp differences between these seven isolates

and the other four isolates which had grouped with international voucher specimens in clade 1. For EF1-α, only isolate Mar17e was identical to the voucher specimens, including isolate CBS100819. The other 10 isolates contained 1 bp substitution from the voucher specimens.

Small amounts of sequence divergence are permissible within species and the sequence variation generated from EF1-α and histone shown by New Zealand *C. pauciseptatum* isolates is less than that shown between closely related species of *Ilyonectria*. For example, Cabral *et al*. (2012b) aligned the β-tubulin, EF1-α and histone H3 sequences of CBS 112615, the holotype of *I. macrodidyma,* with those of *I. torresensis, I. alcacerensis* and *I. novozelandica*. Taking the nucleotide differences between the discriminated species *I. macrodidyma* and *I. torresensis* as an example*,* 2 bp, 11 bp, and 6 bp differences were seen between the β-tubulin, histone H3 and EF1-α sequences, respectively. However, the 9 bp substitutions between the two clades generated by the sequences of β-tubulin is much greater than that observed by Cabral *et al*. (2012b) and may indicate a species divergence in *C. pauciseptatum* similar to recent reclassification within the *I. macrodidyma* and *I. radicicola* complexes (Cabral *et al*., 2012a,b). This is further supported by the genetic diversity analysis in which the isolates from each (β-tubulin) clade are found in different groups. It is also interesting to note that isolate Mtb1a is consistently located on a separate branch from other isolates on the NJ trees based on sequences of the β-tubulin, EF1-α gene. The placement of one isolate from avocado in the β-tubulin NJ tree in one of the genetic groups containing grapevines isolates suggest the possibility of cross infection between grapevine and other non-grapevine hosts for *C. pauciseptatum*. Future studies based on more isolates and additional molecular characters plus refined taxonomical classification may determine the possible intra- or interspecific variation within *C. pauciseptatum* strains.

This is the first study to assess genetic diversity of *C. pauciseptatum* isolates in New Zealand. Using the UP-PCR method the results showed that the isolates were genetically diverse. All 11 UP-PCR primers produced different number of bands with the range of 2 – 17 in the 11 *C. pauciseptatum* isolates tested and this is similar to the range of bands produced for other fungi eg *Ilyonectria* spp. (Pathrose, 2012), *Phaeomoniella chlamydospora* (Pottinger *et al*. 2002) and *Neofusiccocum* spp. (Baskarathevan, 2012). A total of 65 bands (loci) were produced by five primers, with 46% polymorphism. When compared to the results for other grapevine pathogens, this result is more polymorphic than the results for *P. chlamydospora* obtained by Pottinger *et al*. (2002) as five out of nine UP-PCR primers they used did not produce polymorphic bands. Another New Zealand study on the olive leaf spot pathogen *Spilocaea oleagina* used five UP-PCR primers and all produced polymorphic bands (Obanor *et al*., 2010). Baskarathevan *et al*. (2012) achieved 82 bands (loci) from 61 *Neofusicoccum parvum* isolates by eight UP-PCR primers, in which 73 (89.0%) were polymorphic bands and 56 genotypes were generated. In the study of Pathrose *et al*. (2014) on the genetic diversity

of *I. liriodendri*, five UP-PCR primers generated a total of 66 informative loci with 59% polymorphic, 84% of the 57 New Zealand isolates were identified as unique genotypes. The results of this study investigated a similar number of loci. The high reproducibility of UP-PCR indicated that it was an appropriate method to study the genetic diversity of *C. pauciseptatum*. However, Martín *et al*. (2014) used AFLP to analysis the genetic distance for 17 single-spore isolates of *Phaeoacremonium aleophilum* and three main groups were generated. Using five primers they amplified 358 loci, of which 309 (86.3%) were polymorphic. Thus, AFLP could be used to generate a larger number of loci and may be useful for future studies on the genetic diversity of *C. pauciseptatum*.

Two major groups within the dendrogram were generated by the UP-PCR data for the 11 *C. pauciseptatum* isolates and these matched those generated by the β-tubulin phylogeny. Group 1 contained five isolates, whereas the other group contained 6 isolates. No clonal isolates were detected with all 11 isolates having unique genotypes indicating that the isolates were genetically diverse. This result is similar to that reported by Pathroses *et al*. (2014), where high diversity was observed in *Ilyonectria liriodendri*associated with black foot. Genetically different individuals were observed between vineyards of the same region but not from the same vineyards. Each genetic group is composed of a range of isolates from different geographic regions and vineyards. In some cases, more than one genotype within a vineyard was encountered. In the study by Pathrose (2012) it was later shown that the main branches of the dendogram generated from the UP-PCR data for "*C.*" *macrodidymum* and *"C." destructans* aligned with the new species designations described by Cabral *et al* (2012a, b). UP-PCR has also been used to delineate species in previous studies on *Trichoderma* (Lübeck *et al*., 2000) and the *Rhizoctonia solani* complex (Lübeck and Poulsen, 2001). When combined with the separation of the isolates in the β-tubulin NJ tree this may further suggest the potential for separation of the two isolate groups.

Genetic diversity of *C. pauciseptatum* may arise through the introduction of new genotypes or recombination of existing genotypes (Pathrose *et al*. 2014). On the other hand, the free movement of fungal species between the regions and vineyards may be via being carried in non-symptomatic grapevine propagation materials and this mode of travel was demonstrated for botryosphaeriaceous species in nursery surveys (Billones-Baaijens *et al*., 2009; Reis *et al.,* 2013). Last but not the least, one isolate recovered from an infected avocado plant was placed in one of the genetic groups derived from the β-tubulin gene but not in a separate branch suggest the possibility of cross infection among different hosts. As a supporting case, Schroers *et al*. (2008) found the *C. pauciseptatum* strains CBS100819 and CBS113550 which were isolated from different hosts in New Zealand were identical to each other in the sequence of taxonomic genes. In contrast, Pathrose *et al*. (2014) included international *I. liriodendri* isolates from apple and 'Traminer' grapevines in their study and they were placed

in a separate group from New Zealand isolates. This indicated that pathogens on different hosts are not necessarily genetic similarity. Overall many more isolates on *C. pauciseptatum* are required to make definitive statements about genetic diversity within and between hosts in New Zealand.

This work is the first investigation of VCGs in *C. pauciseptatum*. The work showed that 64% of the paired reactions were compatible despite the distinct genotypes of the isolates. Partially incompatible reactions were treated as compatible pairs for the convenience of designating VCG. Two main VCGs were identified between the pairings of *C. pauciseptatum* isolates. In addition, 3 sub-groups which overlapped in a complicated manner with each other were shown. The incompatible reactions resulted from the pairing of isolates either from different genetic groups or same genetic groups, which indicated no correlation between VCGs and genotypic diversity based on NJ tree. Pathrose *et al.* (2014) obtained two VCGs among the pairings of *I. liriodendri* isolates which was delimited by two incompatible isolates. In their study incompatible isolates were also identified from the same group in the NJ tree. However, a difference between this study and the work of Pathrose *et al*. (2014) is that all her isolates were compatible to each other in the overlapping region.

Parasexual recombination has been proven to play a major role in the genetic diversity of a fungal species which mainly reproduce asexually (Zeigler, 1998). Parasexual recombination can only occur between members of the same VCG and therefore determination of VCGs in a fungal population is necessary for the investigation of the potential parasexuality (Leslie, 1993). Microscopic analysis of interactions between *C. pauciseptatum* isolates showed that there were anastomoses within the actively growing hyphae of isolates in compatible reactions. This suggests that *C. pauciseptatum* can undergo genetic recombination which may play a role in increasing genetic diversity. However, it will be interesting to further investigate the role of these hyphal fusions in increasing genetic diversity. This could be done by comparing the DNA fingerprint profiles of the parent isolates with the DNA fingerprints of the isolates from the interaction zone using the DNA fingerprinting methods such as UP-PCR, RAPD and AFLP. Zeigler *et al.* (1997) recovered the isolates from the interaction zone of *Me. Grisea,* which yielded different fingerprints to the parent isolates. There is no other specific information on recombination in *C. pauciseptatum* species to compare with this study.

In summary, the multi-gene phylogeny conclusively identified 11 putative *C. pauciseptatum* isolates that had been isolated from symptomatic grapevines in New Zealand according to current phylogenetic placement. Minor polymorphism was noted within the EF1-α and histone gene sequences but these were unlikely to signify a new species. The 9 bp substitutions between two clades in β-tubulin NJ tree correlating with the identification of two

genetic groups detected by UP-PCR suggested that *C. pauciseptatum* may be subject to reclassification in the future. In addition, UP-PCR showed that the isolates were genetically diverse and this was further demonstrated by VCGs. Future studies should include more isolates and apply other molecular methods to strengthen these results that have demonstrated genetic diversity within New Zealand *C. pauciseptatum* strains.

Chapter 3

Pathogenicity of *Cylindrocarpon pauciseptatum*

3.1 Introduction

Cylindrocarpon species have been extensively reported as pathogenic on grapevines and other hosts throughout the world. Alaniz *et al*. (2009b) reported isolate differences and observed a correlation between genotype and pathogenicity among isolates of *I. liriodendri* and members of the *"C." macrodidyma* complex. They showed that isolates from two main clades were significantly more pathogenic than isolates placed in other clades. In New Zealand, isolates of *I. liriodendri,* the *I. macrodidyma* complex and the *I. radicicola* complex were found in a nationwide survey of symptomatic plants (Bleach *et al.* 2006). Pathrose (2012) showed that these species were pathogenic on grapevine although there was significant variation between individual isolates.

As part of the New Zealand survey by Bleach *et al*. (2006) eleven isolates of *C. pauciseptatum* were recovered. However, they were not included in pathogenicity studies and their ability to infect and cause symptoms in grapevines is unknown. In other countries researchers have studied *C. pauciseptatum* isolates recovered from woody plants. dos Santos *et al*. (2014) carried out research on *C. pauciseptatum* isolates associated with black foot disease of grapevines in Brazil. Four month old rooted cuttings of *V. labrusca* cv. Bordô were inoculated with two isolates (Cy12UFSM and Cy13UFSM), which had been identified by a multi-gene DNA sequencing methodology (rRNA-ITS, β-tubulin, and histone H3), by immersing them in a conidial suspension (10^6/mL) for 60 min. Thirty days after inoculation, vines were re-inoculated with 40 ml of a 10^6 conidia ml^{-1} suspension to ensure root infection. After 4 months, the inoculated plants had reduced root mass reduction, root and crown necrosis, vascular lesions, shoot decline, and vine mortality.

In South Africa, four species ("*C." destructans, "C." liriodendri, "C." macrodidymum and C. pauciseptatum*) were identified using β-tubulin gene sequencing and phylogenetic analysis from isolates recovered from apple roots (Tewoldemedhin *et al*. 2011). Isolates within each of the four species were variable in their pathogenicity towards apple seedlings. All isolates were able to induce lesion development on seedling roots except for one isolate of *C. pauciseptatum* (STE-U6630), which did not cause a significant amount of root rot or reduction of weight and height. This isolate was considered non-pathogenic. Erper *et al*. (2013) tested the pathogenicity of selected isolates from a group identified as *Cylindrocarpon* and *Ilyonectria* species, including a *C. pauciseptatum* isolate from kiwifruit. Results showed that 10 out of 11 isolates, including the isolate of *C. pauciseptatum*, were able to induce

typical root rot disease symptoms, affecting plant development and leading to the death of some plants.

In this chapter, the overall goal was to establish whether the *C. pauciseptatum* isolates recovered from symptomatic grapevines were able to infect through wounded roots and to estimate the relative pathogenicity of genetically distinct isolates. Two methods were used, an *in vitro* detached root method in which lesions could be measured and an *in vivo* pot experiment in which whole plants were inoculated. These methods had been developed and used successfully for other *Cylindrocarpon* species pathogenic on grapevines (Pathrose, 2012; Probst 2011). To complete Koch's postulates, pathogens re-isolated from infected material were identified morphologically and by molecular methods.

3.2 Materials and Methods

3.2.1 Fungal isolates

Eight isolates of *C. pauciseptatumpauciseptatum* (Co6g, Mar5a, Hb3b, Mtb1a, Mar14b, Ack2b, Mar6a, Ack2e) were obtained from the Lincoln University culture collection. As positive controls, one isolate of *I. liriodendri* (CO1b), *I. macrodidyma* (Hb2b) and *I. europaea* (Gis1b) each were selected as representative isolates from species previously demonstrated to be pathogenic on grapevines (Pathrose, 2012). The fungi had been stored as single spore isolates on colonised agar plugs in 15% glycerol solution at -80°C in cryovials (1.5 mL capacity). Prior to use, a small plug of the colonized agar from each cryovial was transferred to potato dextrose agar (PDA) plates for spore generation. Cultures were placed in the 20°C incubator (12 h light and 12 h dark) for 7-14 days.

3.2.2 Growth medium

Half strength PDA was used throughout experiment. Nineteen and half g of potato dextrose agar (Oxoid Ltd, Basingstoke, UK) and 7.5 g of agar No.3 (Oxoid) were added to 1 L of tap water before autoclaving for 20 min at 121°C and 15 psi.

3.2.3 Detached root assay

This was done as described by Pathrose *et al*. (2014). A mycelial plug was taken from the edge of a colony grown on PDA using a cork borer and subcultured onto ½ PDA for another 7 days' growth at 20°C under 12:12 h dark:light conditions. A mycelial disc from these plates was used to inoculate the detached root assays. Young feeder roots (Figure 3.1 A) of similar age, diameter (1.5-2.0 mm) and pigment were randomly selected from one year old rootstock plants from three cultivars (Riparia Gloire, 3309, Schwarzman), trimmed by cutting off the lateral roots and washed with tap water to remove soil. They were cut into 6 cm long lengths (Figure 3.1 B, C). Each root was placed into a deep Petri dish (90 × 25 mm) containing 30 g

of sterile silica sand (Fulton Hogan, New Zealand) wetted with 9 mL of sterile water. The basal cut end of each root was inserted through a Parafilm™ cap into a 1.75 mL tube filled with sterile water (Figure 3.1 D). The apical end of each root was cut across using a sterile scalpel blade and a mycelial plug taken from the growing edge of a fungal colony placed against the cut end (Figure 3.1 E). As negative controls, the roots were inoculated with a plug of sterile ½PDA which was done before inoculation of pathogens onto other roots to minimise the chance of contamination. The Petri dishes were sealed with clingfilm. Eight replicates were completed for each *Cylindrocarpon/Ilyonectria* isolate. Finally, the treated dishes were arranged in a complete randomised block and incubated at room temperature without light for 8 weeks. Measurement of lesion length using a digital calliper (Mitutoyo, U.K Ltd) was done 4 weeks and 8 weeks after inoculation. This experiment was repeated but only using rootstocks Riparia Gloire and 3309.

Observations were made on the type/colour of any lesions which developed on the inoculated roots by eyes and under the stereo microscope. The experiment was repeated twice. The lesion length data was analysed by analysis of variance (ANOVA) using GenStat version 16, with significant differences between treatments means determined using Fisher's protected least significant difference (LSD).

To determine Koch's postulates it is necessary to confirm the identity of the pathogen species by re-isolation from the lesions which develop on the inoculated roots. Infected roots were surface sterilised by placing in 0.35% sodium hypochlorite (bleach) for 3 min and washed with sterile water twice for 2 min. Then 0.3 cm length of roots at 0, 0.5 and 1.5 cm from the lesion margin were taken individually and placed separately onto PDA plates amended with chloramphenicol to prevent from bacterial growth (Figure 3.2). The plates were incubated at 20°C under 12:12 h dark:light growing condition for 1 week before any colonies growing from the roots were identified based on colony and conidial morphology. Representative cultures growing from the detached roots were subcultured onto fresh PDA plates.

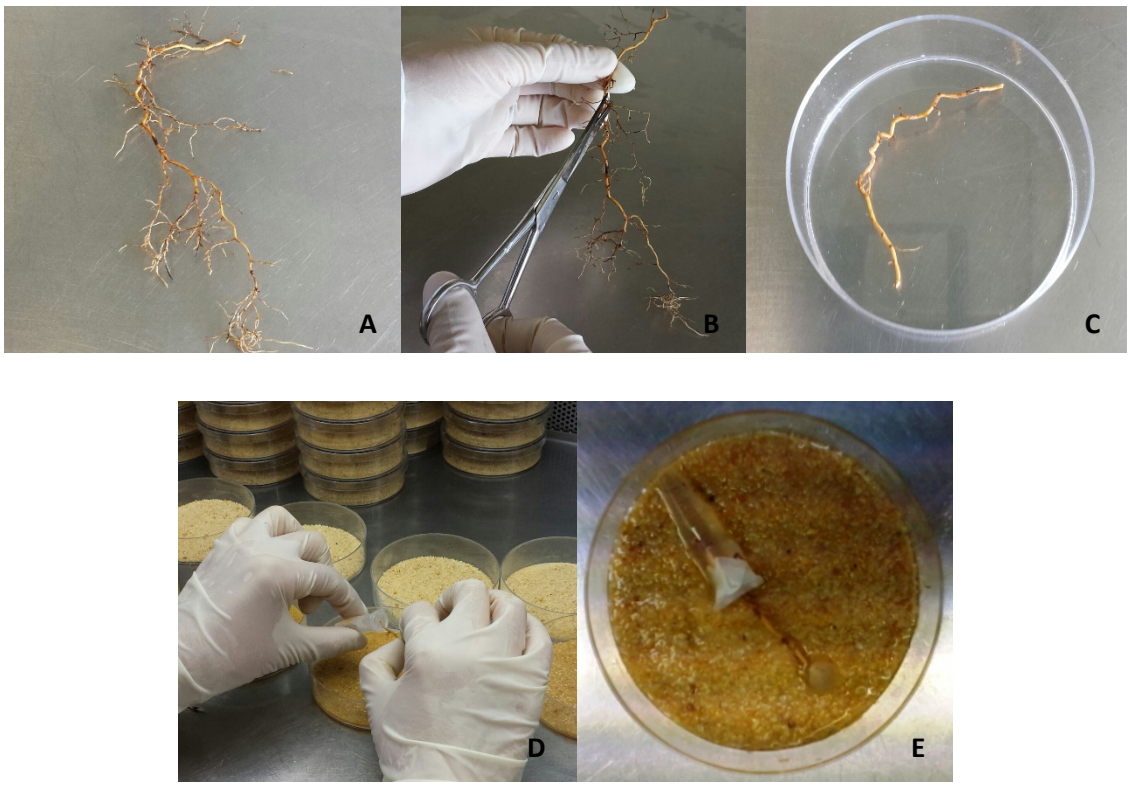

Figure 3.1 The detached root assay set-up: A-Selected individual grapevine roots ready to be trimmed; B- Root trimming with clean scissors; C- Trimmed root section; D- Inserting basal end of trimmed root into tubes filled with sterile water; E- The apical end of a root was placed under a mycelial plug on wetted silica sand.

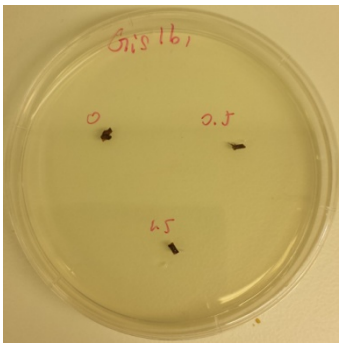

Figure 3.2 Placement of three segments from grapevine roots on a PDA plate. 0, 0.5 and 1.5 represent the location of sample collection from edge of the growing lesion.

3.2.4 Whole plant pot experiment

3.2.4.1 Rootstocks preparation

Ninety grapevine canes each of three cultivars (Riparia Gloire, 3309, Schwarzman) obtained from Corbans Viticulture (Auckland) were placed in trays containing wetted pumice (Atiamuri Sand and Pumice Co., New Zealand) after cutting the bottom end and placed on a heating pad to induce root formation. The plants were grown in a shade house on a heat pad at 25°C for 4-6 weeks until a good root system has developed (Figure 3.3).

Figure 3.3 Grapevine canes inserted into pumice and placed on heat pad in Lincoln University glasshouse to induce rooting.

3.2.4.2 *Cylindrocarpon* spp. inoculum production

Four *C. pauciseptatum* isolates (CO6g, Mar6a, Hb3b, and Ack2e) were selected from different branches of the NJ tree generated by UP-PCR and to represent isolates from different geographic locations (Chapter 2). One isolate each of *I. europaea* (Gis1b), *I. liriodendri* (Co1b) and *I. macrodidyma* (Hb2b) was used as positive control. Each isolate of the *Cylindrocarpon* spp. to be tested was subcultured onto PDA with 10 replicates as described in Section 3.2.1. The colonies were grown for 3-4 weeks to allow conidial formation. The conidial inoculum for each isolate was prepared by scraping the surface of culture plate with a sterile slide after flooding with 5-10 mL of sterile distilled water (SDW). A drop of tween 80 (BDH Chemicals Ltd Poole, England) was added into 1L tap water before autoclaving to prepare SDW in order to allow conidia to be suspended in the solution. A sterilized 150 µm pore size sieve was used to sieve the solution to remove mycelia fragments. The conidial concentration for each isolate was then adjusted to 1×10^4 conidia/mL based on the counts determined using a haemocytometer. The spore suspensions were used within 3 h of preparation to ensure the viability of the inocula prior to inoculation.

3.2.4.3 Inoculation of whole grapevines

For each of the three rootstock cultivars (Riparia Gloire, 3309, Schwarzman) rooted grapevines were inoculated with each of four *C. pauciseptatum* isolates (Co6g, Mar6a, Hb3b, Ack2e) , and one each of *I. europaea* (Gis1b), *I. liriodendri* (Co1b) and *I. macrodidyma* (Hb2b). A negative control without pathogen inoculation was also included for each cultivar. Inoculation was done as follows. Firstly, the rooted plants were carefully removed from the trays containing pumice and their roots were lightly trimmed before inoculation to create wounds. Then the plant base (about 15 cm) was soaked in the conidial suspension (2-3 L) of each isolate for 30 min (Figure 3.4, A). Plants were potted into 1.5 L pots containing potting mix (Media: 80% composted bark, 20% pumice, 2 kg/m^3 Osmocote® extract; Fertilisers: horticultural lime 1.5 kg/m^3, Hydraflo® 500 g/m^3 [granular wetting agent, Scott Product New

Zealand Ltd]). The negative control vine roots were soaked in tap water instead of conidial suspension. The plants were placed in the greenhouse on metal tables in a randomised complete block design (RCBD) consisting of 10 replicates for each isolate/cultivar treatment. All plants were carefully watered daily for 4 months (Figure 3.4, B). During the 4 months growing period, any dead plants were harvested and the stem base plated onto agar to determine whether infection by *Cylindrocarpon* species was present, and pots left were not re-randomised. After 4 months all plants were harvested and infection of the stem base by *Cylindrocarpon* species was assessed.

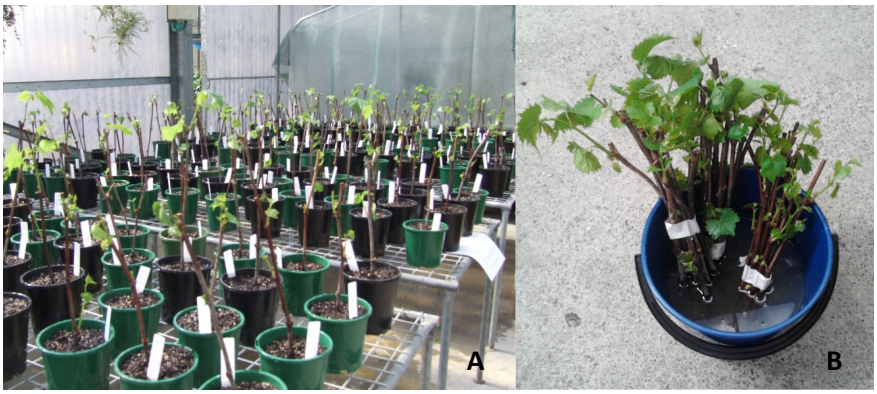

Figure 3.4 A: Experimental set-up for rooted cuttings of three varieties of rootstocks in a glass house at the Lincoln University nursery. Plants were arranged in a completely randomised design. B: Inoculation process with about 15 cm from plants base was soaked in the conidial suspension (3-4 L) contained in plastic bucket.

3.2.4.4 Re-isolation assessment

The stem base was plated as follows. Initially, the roots of the plants were removed from the stem base. Seven cm of the trunk from stem base was kept for further processing. The 7 cm stems were thoroughly washed with running tap water and air-dried before immersing in the sterilisation tanks. The process of sterilisation comprised of 30 s in 70% ethanol, 5 min in 0.35% sodium hypochlorite and finally 30 s in 70% ethanol. Sterilized stems were then placed into a sterile plastic bag for the next step (tissue re-isolation).

Tissue re-isolation was done in a laminar flow cabinet. Firstly, the root crown was removed using ethanol sterilised secateurs (Figure 3.5 B). For any plants that died prior to the end of the 4 month growth period a 1-2 mm transverse section of the stem base was placed as a whole piece onto PDA amended with chloramphenicol. For the plants harvested at the end of the 4 month duration of the experiment, a 1-2 mm transverse section (Figure 3.5 A) from the stem base (0 cm) and another 0.5 cm above the base of stem were each cut into four equal pieces of about 3 mm width and placed equidistant around the edge of a PDA plate amended with chloramphenicol (Figure 3.5 C). An additional 1-2 mm transverse section at 1 and 2 cm above the stem base was also sliced and placed together onto a PDA plate containing

chloramphenicol as whole pieces with a 2 cm gap in between them (Figure 3.5 D). All plates containing isolated tissues were incubated for 1 week at 20°C with 12:12 h dark: light. In addition, shoot and root dry weights were recorded for each surviving plant harvested at 4 months after inoculation.

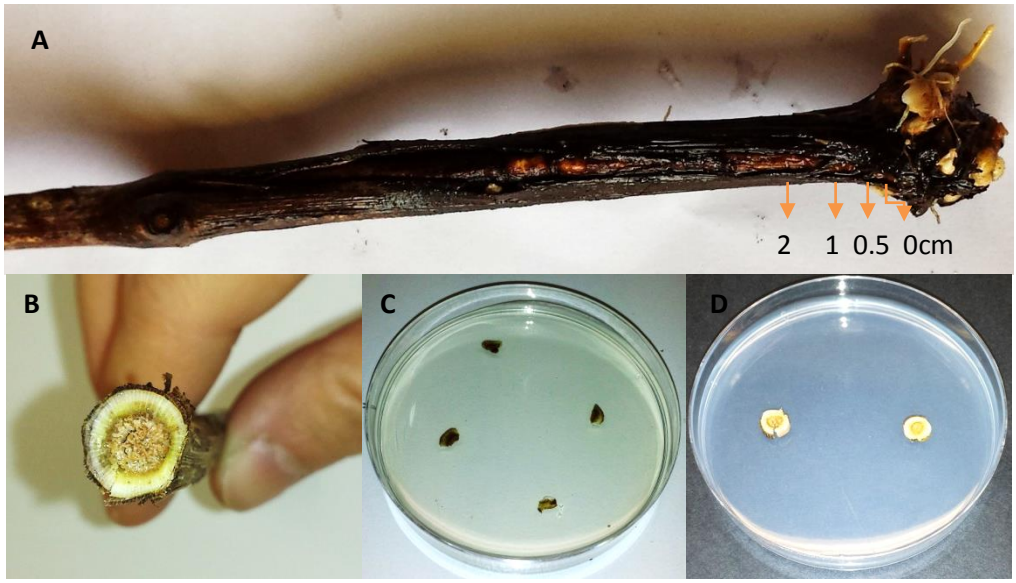

Figure 3.5 Tissue re-isolation process from the stem base of *Cylindrocarpon pauciseptatum* inoculated potted grapevines: A-Selected sampling site on the stem; B- Transverse section of stem base after cutting off the crown; C-Four equal pieces of transverse section at stem base were placed around the edge of a PDA plate with equal distance between them; D-Transverse sections at 1 and 2 cm above the stem base were placed together on one PDA plate as a whole piece 2 cm apart.

As described in the re-isolation for the detached root assay, any colonies growing from the root segments were identified based on the morphology of the colony and conidia. Representative cultures growing from the stem base were purified by subculturing onto PDA. The number of root pieces infected with each of the respective *Cylindrocarpon* species was counted and the incidence of recovery from tissues at the stem bases and each of the four distances above the base of stem was recorded. Data from lesions lengths, pathogen recovery onto agar and grapevine growth parameters from the pot experiment were independently analysed by one-way analysis of variance (ANOVA) conducted using GenStat version 16. Treatment means were compared using Fisher's protected least significant difference test at 5% significant level.

3.2.5 Molecular identification of recovered colonies

3.2.5.1 DNA sequencing of the rRNA gene region

The rRNA gene region from colonies that were morphologically identified as "*Cylindrocarpon*-like" recovered from the detached root or stem base isolations was sequenced. DNA was extracted directly from the mycelium taken from the growing edge of a colony on PDA using

the Extract-N-Amp™ Plant PCR kit (Sigma) with small modifications for fungi as follows. A 2-3 mm diameter portion of mycelium was removed using a sterile pipette tip from the aerial mycelium growing at the edge of the colony, added to 100 µL Extraction solution and incubated at 95ºC for 10 min. Then 100 µL of the Dilution solution was added and vortexed briefly. This solution was stored at –20°C until PCR. Each 20 µl PCR contained 10 µL Extract N AMP™ solution, 1 µL of a 5 µM solution of each primer (ITS4 and ITS1F), 4 µL extracted DNA and 4 µL water. A negative control where 4 µL of a 50:50 mix of Extraction and Dilution solution was added instead of DNA to make sure no contamination was present. PCR amplification cycle, gel electrophoresis and sequencing process were as detailed in Section 2.2.3. Finally, the sequences were submitted to a blastn search (http://www.ncbi.nlm.nih.gov/BLAST/) using the basic local alignment search tool (BLAST) function to confirm matching species in the GenBank database.

3.2.5.2 UP-PCR of recovered colonies suspected as isolates of *C. pauciseptatum*

For the colonies identified as members of the genus *Cylindrocarpon/Ilyonectria* and having typical morphological profiles of *C. pauciseptatum*, genomic DNA was extracted from ground frozen mycelium using the Puregene® system (Gentra systems, USA). Mycelia were prepared for DNA extraction by growing pure cultures on potato dextrose broth (PDB) at 20°C in 12/12 h light/dark conditions for 4–6 days. Mycelium was harvested onto sterile Miracloth™ (Calbiochem), squeezed between paper towels to remove excess moisture, immediately wrapped with aluminium foil, clearly labelled and transferred into liquid nitrogen to snap freeze. Harvested mycelium was stored at –80°C until use for DNA extraction. About 100 mg of frozen mycelium was ground to a fine powder in a cooled mortar and pestle then used for DNA extraction according to the manufacturer‘s instructions. The resulting DNA pellet was rehydrated in 50 µL DNA hydration solution. To check DNA quality a 2 µL aliquot of purified DNA was separated by electrophoresis in a 1% agarose gel in 1 × TAE buffer at 10 V/cm for 45 min. Gels were stained with ethidium bromide for 20 min and visualized on a UV transilluminator (UVITEC Cambridge Imaging System (Total Lab Systems Ltd.)) and photographed. The concentration of the DNA solution was measured by spectrophotometry (NanoDrop, NanoDrop technologies, USA). All genomic DNA samples were diluted to 20–30 ng/µL and stored at −20 °C for the use in PCR.

UP-PCR primers As15inv and As15 were selected for differentiating strains as they produced distinctive banding patterns for the isolates used which consisted of multiple well separated bands according to the results of genetic diversity study for *C. pauciseptatum* isolates in Chapter 2. UP-PCR was done as detailed in Section 2.2.4. UP-PCR profiles were analysed from the fingerprints on the agarose gels. *Cylindrocarpon pauciseptatum* isolates were

identified based on their similarity to the banding patterns previously produced from the pure gene of particular *C. pauciseptatum* isolates.

3.3 Results

3.3.1 Detached root assay

3.3.1.1 Experiment 1

Rootstocks Riparia Gloire and 3309 were discarded due to severe contamination of the control treatment in which most detached roots were completely necrotic.

Lesion size on rootstock variety Schwarzman was measured at 1 month (Table 3.1, Appendix B.1). For rootstock variety Schwarzman the control roots produced minor lesions (average size 0.91 mm) (Figure 3.6, A). All roots inoculated with a pathogen had dark brown lesions that were clearly distinct from the remaining healthy root (Figure 3.6, B). The pathogenicity of the *C. pauciseptatum* isolates within and between each genetic group generated by UP-PCR analysis was variable. *Cylindrocarpon pauciseptatum* isolates CO6g and Mar14b produced lesions that were significantly different from the control (*P*=0.05) with the longest mean lesion length produced by isolate CO6g (14.67 mm) followed by Mar14b (13.94 mm). The remaining six isolates of *C. pauciseptatum* were not significantly different from the control. The positive control CO1b (*I. liriodendri*) produced the largest mean lesion overall (36.4 mm). In contrast, inoculation with *I. macrodidyma* isolate Hb2b did not produce a lesion different from the control treatment. *Cylindrocarpon* species were not re-isolated from control roots as observed by colony morphology. However, typical colonies of *Botryosphaeriaceous* species with characteristic pycnidia were recovered on many plates (Figure 3.6, C, D). These contaminant colonies were also frequently recovered from root pieces that had been inoculated. The contaminant fungus was fast growing and often overgrew the *Cylindrocarpon*-like colonies making recovery of these more difficult. Colonies with typical morphology of *Cylindrocarpon* species were also re-isolated from the lesion margin of some inoculated roots. Percent recovery of *Cylindrocarpon*-like species varied between 37.5% for Mtb1a and 75% for Ack2e, Ack2b and Mar5a (n=8 replicates each) (Table 3.1). Due to contamination of the control and the inoculated detached roots by botryosphariaceous species the experiment was repeated.

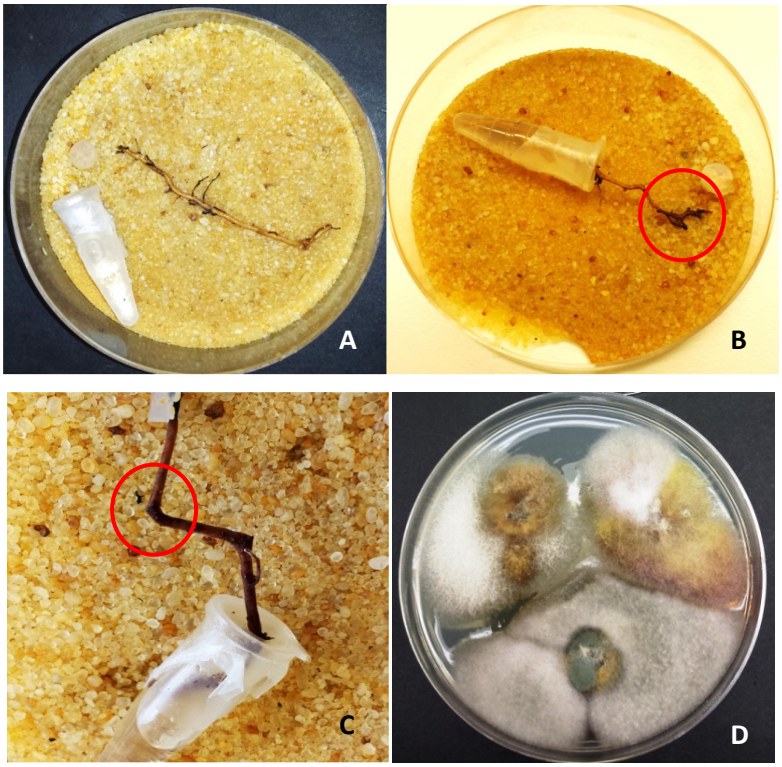

Figure 3.6 Comparison between control root and *Cylindrocarpon* spp. inoculated roots after one month. A: Root inoculated with sterile PDA agar (healthy root); B: Root inoculated with *C. pauciseptatum* Mar14b with a dark lesion occurring at apical end (red circle); C: Control root contaminated with *Botryosphaeria* sp. (red circle); D: Contaminant species re-isolated from inoculated detached roots.

Table 3.1 Mean lesion lengths (mm) produced on detached roots of rootstock Schwarzman measured after 1 month incubation and proportion of *Cylindrocarpon* species recovered from the lesion margin of inoculated root tissues. Isolates in red are from second genetic group by UP-PCR.

Species	Isolate	Mean lesion length (mm)	Proportion of *Cylindrocarpon* species recovered from lesion margin (%) (8 replicates)
I. macrodidyma	Hb2b	8.73 ab	62.5
I. liriodendri	CO1b	36.40 c	50.0
C. pauciseptatum	Mar6a	0.18 a	50.0
	Mtb1a	3.64 ab	37.5
	CO6g	14.67 b	62.5
	Ack2e	0.50 a	75.0
	Ack2b	1.58 a	75.0
	Mar5a	2.04 a	75.0
	Hb3b	3.84 a	50.0
	Mar14b	13.94 b	62.5
	Control	0.91 a	0
LSD		11.32	
***P*-Value**		<0.001	

Values with different letters for mean lesion length represent significantly different obtained by Fishers protected LSD test ($P<0.05$).

3.3.1.2 Experiment 2

This experimental repeat was only on two of the three rootstocks used in Experiment 1. All isolates produced longer lesions than the untreated control (Table 3.2, Appendix B.1) on rootstock variety Riparia Gloire and 3309 at 1 month.

For Riparia Gloire, lesion length after 1 month was significantly different (*P*=0.022) between isolates of *Cylindrocarpon* species (Table 3.2). For isolates of *C. pauciseptatum* only Ack2e and Mtb1a were significantly different from the control and the longest mean lesion was produced by Mtb1a (14.52 mm). For the positive controls *I. liriodendri* CO1b and *I. macrodidyma* Hb2b, only *I. liriodendri* isolate CO1b was different from the control. Two months after inoculation, the lesions were measured again. The lesions were not significantly different from each other or the negative control (*P*=0.062).

Colonies with typical morphology of *Cylindrocarpon* species were recovered from the lesion margin and endophytically at different proximity to the lesion on the inoculated roots after 2 months. No *Cylindrocarpon*-like colonies were isolated from the control roots. The highest proportion of *Cylindrocarpon*-like isolates recovered on the lesion margin was from plants inoculated with Mar14b (87.5%, n=7/8) as compared to the lowest one which was plants inoculated with Mar5a (0%, n=0/8). *Cylindrocarpon*-like colonies were recovered from plants inoculated with isolate Mar14b at 0.5 cm beyond the lesion margin (75%, n=6/8) and at 1 cm beyond the lesion margin (75%, n=6/8) at the same frequency. Of the plants inoculated with *C. pauciseptatum* isolates, for all except those inoculated with isolate Ack2e more *Cylindrocarpon*-like colonies were recovered from lesion margin than 0.5 cm beyond the lesion margin. The positive control showed a lower frequency of *Cylindrocarpon*-like colonies at the lesion margin and beyond the lesion.

For rootstock 3309, there was no significant difference between inoculated roots and the control when lesions were measured at either 1 month or after two months (*P*=0.384 and 0.058, respectively) (Appendix B.1, B.2).

There was a high degree of contamination from a botryosphaeriaceous fungus when root pieces were plated, however, colonies morphologically similar to *Cylindrocarpon* species were obtained from all treatments except the control. Again plants inoculated with isolate Mar14b showed the highest proportion of *Cylindrocarpon*-like colonies recovered from lesion margin, and colonies were recovered from root material 1 cm beyond the lesion margin for plants inoculated with all isolates except for Ack2b and Hb2b (positive control). These colonies were purified by sub culturing on agar and analysed by molecular techniques to confirm species and isolate identity (Section 3.3.1.4 and Section 3.3.3.5).

Table 3.2 Mean lesion lengths (mm) produced on detached roots of rootstock Riparia Gloire measured after 1 month and 2 months incubation and proportion of *Cylindrocarpon* species recovered from root tissues. Isolates in red are from second genetic group by UP-PCR.

Species	Isolate	Mean lesion length (mm)		Percentage of *Cylindrocarpon-like* colonies recovered from root tissues (%) at each distance from lesion margin after 2 month		
		1 month	2 months	0 cm	0.5 cm	1 cm
I. macrodidyma	**Hb2b**	6.95 abcd	13.59	0 (0/7) *	0 (0/7)	0 (0/6)
I. liriodendri	**CO1b**	12.52 cd	17.39	50 (3/6)	16.7 (1/6)	0 (0/6)
C. pauciseptatum	**Mar6a**	2.23 ab	2.31	75 (6/8)	62.5 (5/8)	50 (4/8)
	Mtb1a	14.52 d	15.15	75 (6/8)	75 (6/8)	62.5 (5/8)
	CO6g	1.95 ab	3.19	75 (6/8)	37.5 (3/8)	25 (2/8)
	Ack2e	9.09 bcd	16.81	33.3 (2/6)	33.3 (2/6)	66.7 (4/6)
	Ack2b	4.31ab	5.18	75 (7/8)	25 (2/8)	12.5 (1/8)
	Mar5a	3.09 ab	8.72	0 (0/8)	0 (0/8)	0 (0/8)
	Hb3b	6.63 abcd	18.58	75 (6/8)	25 (2/8)	28.6 (2/7)
	Mar14b	6.20 abc	24.04	87.5 (7/8)	75 (6/8)	75 (6/8)
	Control	0.72 a	1.12	0 (0/8)	0 (0/8)	0 (0/8)
LSD		8.078	5.68			
***P*-Value**		0.022	0.062			

Values with different letters for mean lesion length for each column represent significantly different obtained by Fishers protected LSD test ($P<0.05$).
"*" represents the numbers of *Cylindrocarpon-like* colonies vs numbers of samples examined.

3.3.1.3 Morphological characteristics

For all samples of root pieces from inoculated and uninoculated detached roots some colonies produced morphologies typical of *Cylindrocarpon* spp. (Figure 3.7). However, a fast growing fungus with grey to black colony colour and aerial mycelium was also frequently recovered (Figure 3.8). This was morphologically identified as a botryosphaeriaceous species.

Of the 288 isolates recovered from the detached root experiment with rootstock Riparia Gloire, 92 isolates (32%) produced *Cylindrocarpon*-like colonies with light to dark brown or yellow pigment after 7 days when grown on PDA. For rootstock 3309, 86 (43%) out of 199 isolates produced *Cylindrocarpon*-like fungal colonies (Figure 3.7). Multiple species were likely to be present based on the large variation in colony appearance observed. White fluffy aerial mycelium was observed covering the top of colonies for some isolates, while others yielded flat mycelium. Some colonies had a great deal of growth ring demarcation observed on the top side of the culture. Amber to light brown colony margins were observed on some colonies. However, most of the colonies recovered from root pieces inoculated with the same isolate but different replicates generally resembled each other.

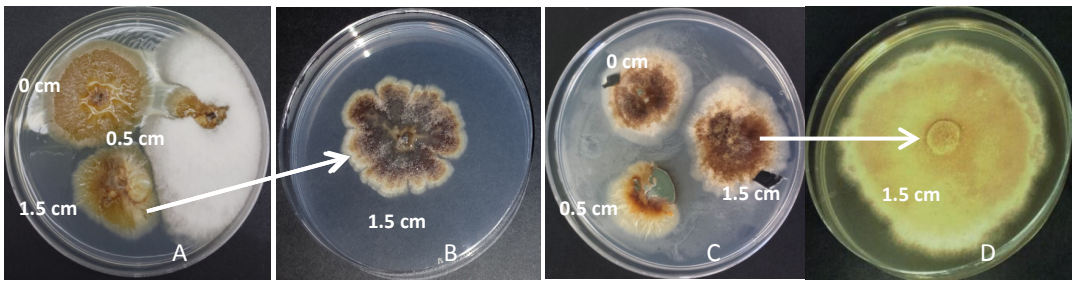

Figure 3.7 *Cylindrocarpon*-like colonies growing from root pieces collected from detached root assay after 5 days inoculation. A: Original colonies of isolate CO6g from rootstock 3309; B: Subculture of the colony from 1.5 cm above lesion for A; C: Original colonies of isolate CO6g from rootstock Riparia Gloire; D: Subculture of the colony from 1.5 cm above lesion for C.

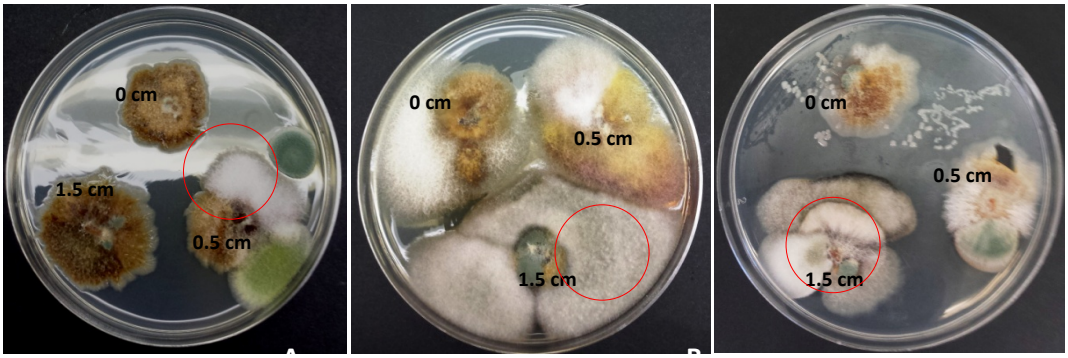

Figure 3.8 Colonies suspected of being a *Botryosphaeriaceous* species (red circles) with grey to black colour aerial mycelium growing from root pieces collected from detached root assay after 5 days inoculation; A: Isolates recovered from root pieces from rootstock 3309 plants inoculated with isolate Mar14b; B: Isolates recovered from root pieces from3309 rootstock plants inoculated with isolate CO6g; C: Isolates recovered from root pieces from rootstock Riparia Gloire plants inoculated with of isolate CO6g.

3.3.1.4 Molecular characterization by DNA sequencing

For Experiments 1 and 2 a high degree of contamination by botryosphaeriaceous species made the confirmation of pathogen presence difficult. Thus, *Cylindrocarpon*-like isolates recovered from the plates were purified and analysed by molecular techniques to confirm species and isolate identity (Section 3.2.5.1 and Section 3.2.5.2). Subculturing of recovered *Cylindrocarpon*-like colonies was repeated at least twice to achieve a clean culture. Any subcultures that either 1) did not resemble Cylindrocarpon species after subculturing or 2) remained severely contaminated by other fungi or bacteria were excluded for molecular identification. From the 176 isolates initially recovered only 24 isolates produced clean cultures of *Cylindrocarpon*-like species. A subsample of nine representing different isolates, rootstocks and distances from the lesion were selected for sequencing. The ITS region of the rRNA gene was sequenced for four isolates from rootstock Riparia Gloire and five isolates from rootstock 3309. The ITS1F and ITS4 primers yielded a product of approximately 650 bp for each isolate (Figure 3.9). The sequence quality was poor and approximately 350 bp of

unambiguous sequence was used for comparison with sequences on GenBank. This data is summarised in Table 3.3. According to comparison with the GenBank database, all nine isolates originating from the detached root experiment that were sequenced were 98-100% identical to members of the genus *Cylindrocarpon/Ilyonectria*. For Riparia Gloire roots inoculated with isolate Mar14b a colony identified as *Cylindrocarpon/Ilyonectria* spp. was recovered endophytically 1.5 cm from the lesion margin. For roots inoculated with Ack2b a colony identified as *Cylindrocarpon/Ilyonectria* was recovered at the lesion margin, while for rootstocks inoculated with CO6g no colonies of *Cylindrocarpon* spp. were recovered. In contrast, in rootstock 3309 roots inoculated with isolate CO6g produced colonies identified as *Cylindrocarpon/Ilyonectria* spp. that were recovered endophytically 1.5 cm beyond the lesion, and at 1 cm and the margin edge for rootstocks inoculated with the other two isolates (Mar14b and Hb3b).

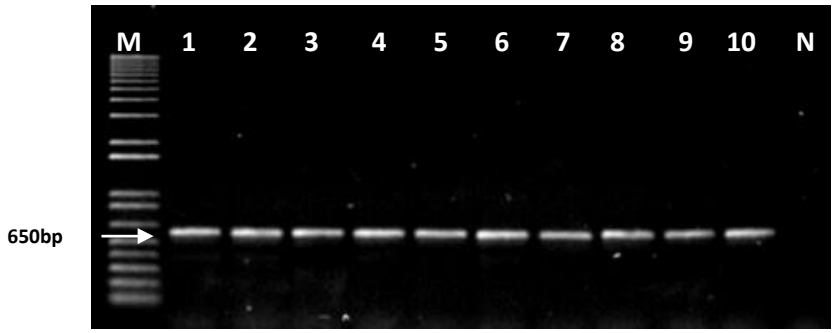

Figure 3.9 1% agarose gel showing the ~600 bp PCR product amplified from colonies of suspected *Cylindrocarpon* species recovered from the detached root experiment. Lane 1 – 10 denote amplimers from *Cylindrocarpon*-like colonies; M represents 1Kb plus DNA ladder (Invitrogen); N represents negative control; numbers on far left denote molecular weights of the bands of the 1Kb plus ladder.

Table 3.3 Identity of isolates recovered from detached roots of grapevine rootstocks at different proximity to the lesion after 2 months incubation.

Rootstock	Isolate	Proximity to the lesion from which the isolate was recovered			Identity of the recovered isolate	Percent Identity (%)
		0cm	0.5cm	1.5cm		
Riparia Gloire	Mar14b		1		*Cylindrocarpon/Ilyonectria* sp.	99
				1	*Cylindrocarpon/Ilyonectria* sp.	99
	Ack2b	1			*Cylindrocarpon/Ilyonectria* sp.	99
	CO6g			INC		
3309	Mar14b	1			*Cylindrocarpon/Ilyonectria* sp.	99
			1		*Cylindrocarpon/Ilyonectria* sp.	98
	Hb3b	1			*Cylindrocarpon/Ilyonectria* sp.	100
			1		*Cylindrocarpon/Ilyonectria* sp.	100
	CO6g			1	*Cylindrocarpon/Ilyonectria* sp.	100

Numbers of sequenced isolates are shown for specific sampling position; “INC” indicates inconclusive result.

3.3.1.5 Molecular characterization by UP-PCR

A total of 18 isolates from clean subcultures (13 for rootstock Riparia Gloire and 5 for rootstock 3309) from the detached root experiment were selected as representatives and assessed for their similarity to the UP-PCR fingerprints of the inoculated isolates after couple of times of subculturing.

The UP-PCR was completed with two primers, AS15inv and AS15, and yielded band between 500 to 2000 bp for each isolate (Figure 3.10, 3.11). Fourteen of the 18 isolates that had originated from the detached root assay produced fingerprints that were identical to the inoculated isolates of *C. pauciseptatum*. These were *C. pauciseptatum* isolates Mar6a (n=5), Ack2e (n=2), Ack2b (n=1) and Mar14b (n=1) from rootstock Riparia Gloire, CO6g (n=2), Mar6a (n=2) and Mar14b (n=1) from rootstock 3309 (Table 3.4). These results confirmed that these isolates were present in the lesion margin and that Mar6a could move endophytically up to 0.5 cm from the lesion margin. All isolates inoculated onto both rootstocks were confirmed at the lesion margin except for isolate CO6g for which no colonies had been recovered. Two isolates from Riparia Gloire were shown not to be the inoculants strains; these were isolates from plants inoculated with Mtb1a recovered from the margin edge and 0.5 cm above it, and the colony recovered from roots inoculated with isolate CO6g at 0.5 cm beyond the lesion.

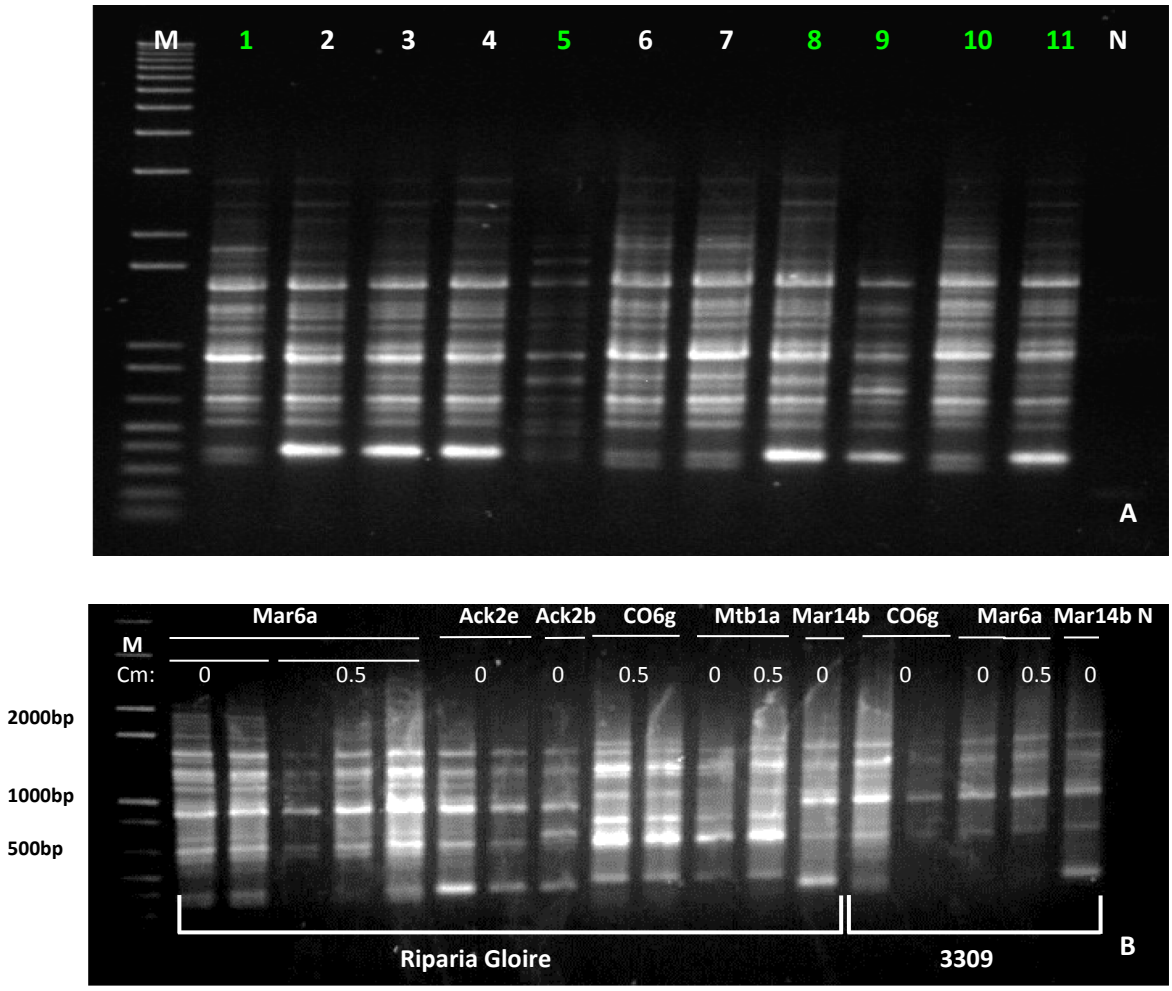

Figure 3.10 A: Fingerprint profile generated by primer As15inv. Lanes 1-11 denote representative isolates (1-CO6g; 2-Mar5a; 3-Hb6b; 4-Hb3b; 5-Mtb1a; 6-Mar17d; 7-Mar17e; 8-Mar14b; 9-Ack2b; 10-Mar6a; 11-Ack2e).The isolates number highlighted with green are inoculated isolates. Discriminating bands are the doublet or single band between 300 and 400 bp. M: 1Kb plus ladder marker. N: negative control. B: Genotyping of isolates morphologically identical to *C.pauciseptatum* recovered from detached root assay using UP-PCR primer As15inv. Above the gel are the isolate names and proximities to the lesion margin. M for each gel denotes 1 Kb plus ladder and numbers on far left denote molecular weights of the bands of the 1Kb plus ladder and N represents negative control. Riparia Gloire and 3309 represent rootstocks.

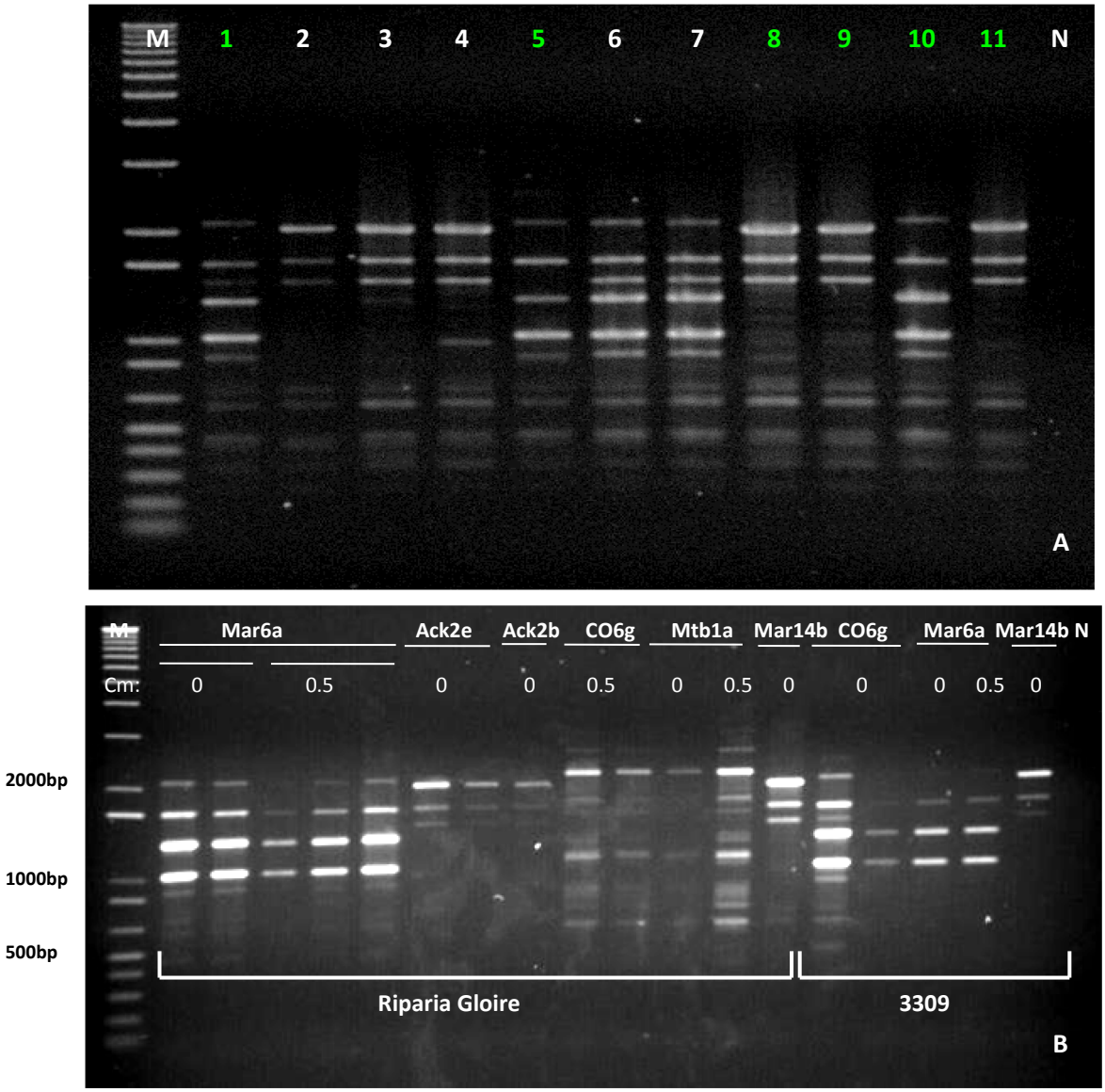

Figure 3.11 A: UP-PCR fingerprint profile generated by primer As15. Lanes 1-11 denote representative isolates (1-CO6g; 2-Mar5a; 3-Hb6b; 4-Hb3b; 5-Mtb1a; 6-Mar17d; 7-Mar17e; 8-Mar14b; 9-Ack2b; 10-Mar6a; 11-Ack2e). Discriminating bands are between 1000 and 2000 bp. The isolates number highlighted with green are inoculants. M: 1Kb plus ladder marker. N: negative control. B: Genotyping of colonies morphologically identical to *C. pauciseptatum* recovered from detached root assay by UP-PCR using primer As15. Each lane is denoted with isolate names and proximities to the lesion. M for each gel denotes 1 Kb plus ladder and numbers on far left denote molecular weights of the bands of the 1Kb plus ladder and N represents negative control. Riparia Gloire and 3309 represent rootstocks.

Table 3.4 Genotypes of recovered *Cylindrocarpon*-like isolates obtained from the specific root tissues at different proximity to the lesion after 2 months inoculation for rootstock Riparia Gloire and 3309 associated with detached root assay based on UP-PCR analysis.

Rootstock	Isolate	Proximity to the lesion from which the isolate was recovered					
		0 cm			0.5 cm		
		Isolate No.	As15 inv	As 15	Isolate No.	As15 inv	As 15
Riparia Gloire	**Mar6a**	2	+, +	+, +	3	INC, +, +	+, +, +
	Ack2e	2	+, +	+, +			
	Ack2b	1	+	+			
	CO6g				2	-, -	-, -
	Mtb1a	1	-	INC	1	-	INC
	Mar14b	1	+	+			
3309	**Co6g**	2	+, INC	+, INC			
	Mar6a	1	+	+	1	+	+
	Mar14b	1	+	+			

+: the fingerprint was exactly the same as the original banding profile of the original inoculums;
-: the fingerprint didn't resemble to the bands pattern of original inoculum;
INC: indicates inconclusive results.

3.3.2 The pathogenicity of different *Cylindrocarpon* isolates achieved from potted vine experiment

3.3.2.1 *Cylindrocarpon* species and isolate effects

The pathogenicity of *C. pauciseptatum* was further assessed with a potted vine experiment using four isolates of *C. pauciseptatum* and one isolate each of *I. liriodendri* (CO1b), *I. macrodidyma* (Hb2b) and *I. europaea* (Gis1b) as the positive controls. Most (64%) plants including both inoculated and uninoculated plants of rootstock Schwarzman were visibly dead by 1 week likely due to transplanting shock as these plants had very small root systems at potting. Consequently, rootstock Schwarzman was discarded from analysis.

For rootstock Riparia Gloire, plant death due to transplant shock within the first week after potting left only 40% and 50%, respectively of plants inoculated with *C. pauciseptatum* isolates Hb3b and Ack2e alive (Appendix B.3). Thus, these treatments were discarded from the experiment. For the plants inoculated with the other three isolates, 70%, 80% and 80% of plants for isolates *I. macrodidyma* Hb2b, *C. pauciseptatum* CO6g and *I. liriodendri* CO1b remained alive after 1 week, respectively (Table 3.5).

At harvest, there was a significant effect (P=0.002) of inoculation on lower stem base infection, with inoculation with the *C. pauciseptatum* isolate (CO6g) yielded infection at the lower stem base that was significantly higher (P=0.05) than that of the uninoculated control (Table 3.5). There was no significant difference between plants inoculated with either of the positive control isolates and the uninoculated control. No *Cylindrocarpon*-like colonies were

recovered at >1 cm above the trunk base for inoculated plants except for *I. macrodidyma* Hb2b. For the uninoculated control treatments *Cylindrocarpon*-like colonies were recovered up to 2 cm above the stem base.

For rootstock 3309, the proportion of live plants was higher for untreated control (100%) than for all inoculated plants (60-70%), which were similar to each other (Table 3.6). At harvest, there was a significant effect (*P*=0.015) of inoculation on infection at the stem base (0 cm) with more *Cylindrocarpon*-like colonies recovered from the uninoculated control and plants inoculated with the *C. pauciseptatum* isolate CO6g than plants inoculated by any other isolates including the positive controls. At 1 cm and 2 cm above the trunk base, incidence was 0 for most of the isolates except for plants inoculated with isolate CO6g (10%) at 1 cm above trunk base and the negative control at both assessment points.

Colonies typical of botryosphaeriaceous species were frequently recovered from plated plant tissues at each of the four sampling points for all rootstocks. For Riparia Gloire, the presence of botryosphaeriaceous species as a percent recovery from stem base for plants inoculated with different isolates including negative control varied between 22.5% (CO1b) and 32.5% (CO6g) (Table 3.5). Plants inoculated with CO6g had the highest incidence for botryosphariaceous species at 0.5 cm above trunk base. The severity of contamination by *Botryosphaeria* sp. at ≥1 cm above trunk base was between 20 and 80% for all plants. Contamination by *Botryosphaeria* sp. on rootstock 3309 was similarly high. The lowest amount was present in root pieces from the stem base of plants inoculated with *I. liriodendri* isolate CO1b (0%) and the highest at 1 cm from the stem base for plants inoculated with isolate *I. europaea* Gis1b (40%) (Table 3.6).

Table 3.5 Proportion of live plants and incidence of recovery of *Cylindrocarpon* species from tissue at the stem bases (0 cm) and 0.5, 1, and 2 cm above the stem base investigated from 4-month-old grapevine rootstock Riparia Gloire inoculated with *Cylindrocarpon* species. *Ilyonectria liriodendri* (CO1b) and *I. macrodidyma* (Hb2b) are positive control.

Isolates	Proportion of live plant (%)	Proportion of *Cylindrocarpon*-like colonies recovered from plant tissues (%)				Proportion of *Botryosphaeria*-like colonies recovered from plant tissues (%)			
		0 cm	0.5 cm	1 cm	2 cm	0 cm	0.5 cm	1 cm	2 cm
Co1b	80	2.5 a	2.5 a	0	0	22.5	27.5	80	20
Hb2b	70	17.5 b	17.5 b	10	0	30	20	40	40
CO6g	80	30 c	15 b	0	0	32.5	47.5	70	60
Control	90	10 ab	15 b	10	20	30	32.5	70	40
LSD		10.66	7.54						
***P*-Value**	0.584	0.002	0.006						

Table 3.6 Proportion of live plants and incidence of recovery of *Cylindrocarpon* species and *Botryspharia* species from tissue at the stem bases (0 cm) and 0.5, 1, and 2 cm above the stem base investigated from 4-month-old grapevine rootstock 3309 inoculated with *Cylindrocarpon* species. *I. europaea* (Gis1b), *I. macrodidyma* (Hb2b) and *I. liriodendri* (CO1b) are used as positive control inoculums.

Isolates	Propotion of live plant (%)	Proportion of *Cylindrocarpon*-like colonies recovered from plant tissues (%)				Propotion of *Botryosphaeria*-like colonies recovered from plant tissues (%)			
		0 cm	0.5 cm	1 cm	2 cm	0 cm	0.5 cm	1 cm	2 cm
Gis1b	70	2.5 a	2.5	0	0	17.5	22.5	40	30
Hb2b	70	2.5 a	5	0	0	20	20	30	20
CO1b	60	2.5 a	2.5	0	0	0	12.5	10	10
Ack2e	60	2.5 a	5.2	0	0	15	12.5	30	10
CO6g	60	7.5 b	17.5	10	0	30	5	10	60
Control	100	10 b	7.5	10	10	12.5	20	30	20
LSD		4.961	12.81						
***P*-Value**		0.015	0.185						

Values within a column with the same letters are not significantly different according to Fishers LSD test (P<0.05).

3.3.2.2 Measurement of root and shoot dry weight

Table 3.7 showed the mean root and shoot dry weight of rootstock Riparia Gloire after 4 months inoculation. For Riparia Gloire there was significant difference for both mean root and shoot dry weight (*P*=0.011 and *P*=0.039, respectively) (Appendix B.5). Vines inoculated with isolates of the positive controls CO1b and Hb2b had greater root dry weights than those inoculated with *C. pauciseptatum* isolate CO6g (9.04 g) and control plants (9.04 g). Mean shoot dry weights of CO6g (14.61 g) and untreated plants (14.20 g) were also significantly lower than plants inoculated with isolate CO1b (18.7 g). For rootstock 3309 there was no significant difference in dry weight between inoculated or control plants for either shoot (*P*=0.527) or root (*P*=0.527) dry weight (Appendix B.4).

Table 3.7 Mean root and shoot dry weights (g) of plants 4 months after inoculation with three *Cylindrocarpon* isolates for rootstock Riparia Gloire. Isolates in red are from *I.marcrodidym*a (Hb2b) and *I. liriodendri* (CO1b) as positive control.

Isolates	Mean root dry weight (g)	Mean shoot dry weight (g)
Hb 2b	11.69 b	15.72 ab
CO 1b	11.06 b	18.70 b
CO6g	9.04 a	14.61 a
Control	9.04 a	14.20 a
***P*-value**	0.011	0.039
LSD	1.854	3.273
SED	0.892	1.574

3.3.2.3 Morphological characteristics

Cylindrocarpon-like species accounted for 78 (18%) and 43 (9%) out of the 430 isolates for Riparia Gloire and 460 isolates for 3309, respectively, recovered from plates onto which the stem base and progressive sections from the stem base had been plated. Similar colony morphologies for *Cylindrocarpon*-like colonies as described for the detached root experiment were observed (Figure 3.12). However, the same contaminating fungus, as found in the detached root experiment, was found here. It had grey to black colony colour and aerial mycelium and grew very fast. The grey to black pigment was also visible from the reverse side of the Petri dishes (Figure 3.13). Any subcultures with severe contamination or unclear morphological characters were excluded from molecular identification.

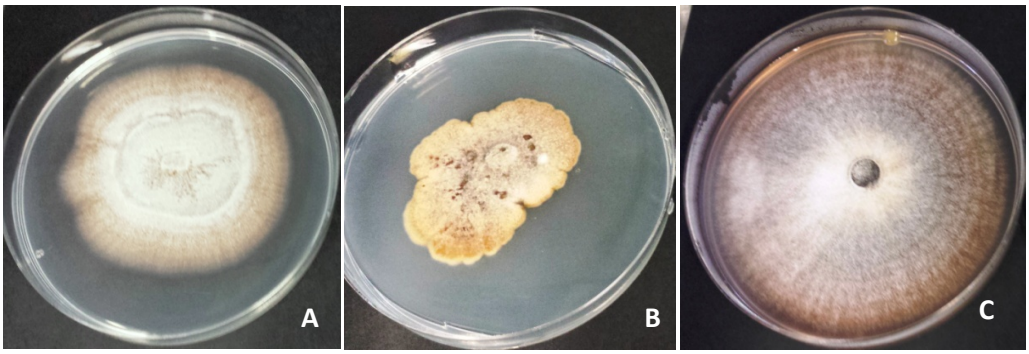

Figure 3.12 *Cylindrocarpon*-like colonies growing from stem piece collected from potted vines after 5 days inoculation. A: Subculture of isolate Hb3b recovered at 0.5 cm above trunk base of rootstock 3309; B: Subculture of the colony of isolate CO6g re-isolated at 0.5 cm above stem base of rootstock 3309; C: Subculture of isolate Ack2e at 2 cm above stem base of rootstock Riparia Gloire.

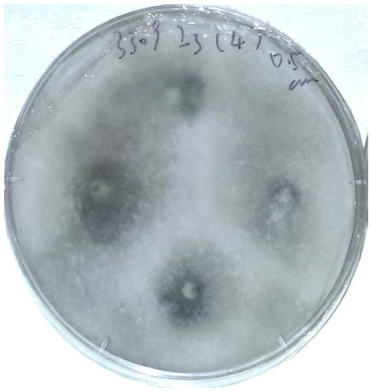

Figure 3.13 Colonies suspected of being a *Botryosphaeriaceous* species with grey to black colour aerial mycelium growing from root pieces collected from potted vines after 5 days inoculation.

3.3.2.4 Molecular characterization by DNA sequencing

The ITS1F and ITS4 primers produced the expected 650 bp amplimer for each isolate (Figure 3.14). Sequencing of isolates suspected as *C. pauciseptatum* that had been recovered from whole potted plants produced poor sequence quality with only 300-400 bp of usable sequence. The ITS region of the rRNA gene was sequenced for 18 isolates from

rootstock Riparia Gloire and 16 isolates from rootstock 3309, respectively. All isolates of rootstock Riparia Gloire were identified as belonging to the genus *Ilyonectria/Cylindrocarpon*. For rootstock 3309, the isolates originating from plants inoculated with the two positive controls *I. macrodidyma* isolate Hb2b and *I. liriodendri* isolate Co1b at 0.5 cm above stem base were 99% identical to the *Ilyonectria/Cylindrocarpon* species (GenBank accessions HQ703420 and KF512001, respectively). Furthermore, one isolate from the stem base and two from 0.5 cm above the stem base for the uninoculated vine were also identical to species from the genus *Ilyonectria/Cylindrocarpon.*

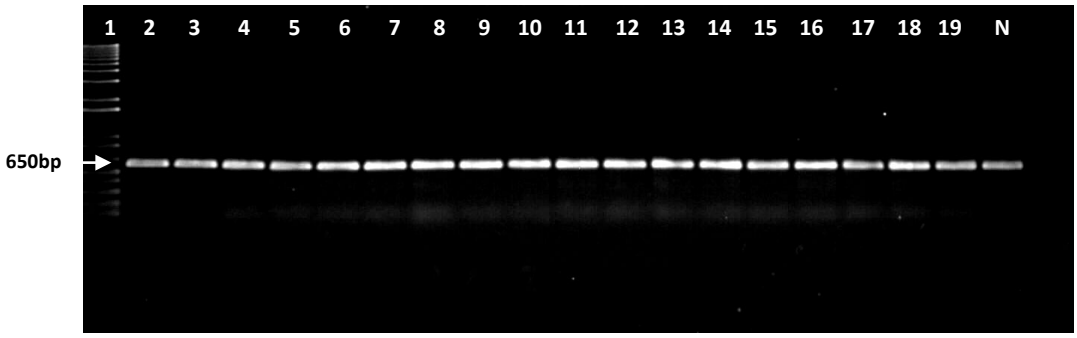

Figure 3.14 Example of product 1% agarose gel showing the ~600 bp PCR product amplified from colonies of suspected *Cylindrocarpon* species recovered from the potted vine. Lane 1 – 19 denote amplimers from *Cylindrocarpon*-like colonies; M represents 1Kb plus DNA ladder (Invitrogen); N represents negative control; numbers on far left denote molecular weights of the bands of the 1Kb plus ladder.

3.3.2.5 Molecular characterization by UP-PCR

A total of 15 isolates were randomly selected from the potted vine experiment (11 for rootstock Riparia Gloire and 4 for rootstock 3309) and assessed for their similarity to the UP-PCR fingerprints of the inoculated isolates (Figures 3.15, 3.16). The results from primer As15inv were inconclusive for *C. pauciseptatum* isolates CO6g, Ack2e and Hb3b. Using primer As15, most of the *Cylindrocarpon*-like colonies recovered from the two rootstocks, four out of five of isolates from plants inoculated with CO6g and all of those inoculated with Ack2e and Hb3b isolates at stem base plus those inoculated with isolate of CO6g re-isolated from 0.5 cm above stem base were not the original inoculant strain (Table 3.8). Since UP-PCR analysis of the positive control isolates were not carried out in this study, there is no UP-PCR fingerprint for the pure culture for the positive control *I. macrodidyma* Hb2b, so the identity of the recovered isolates in this treatment remains unconfirmed.

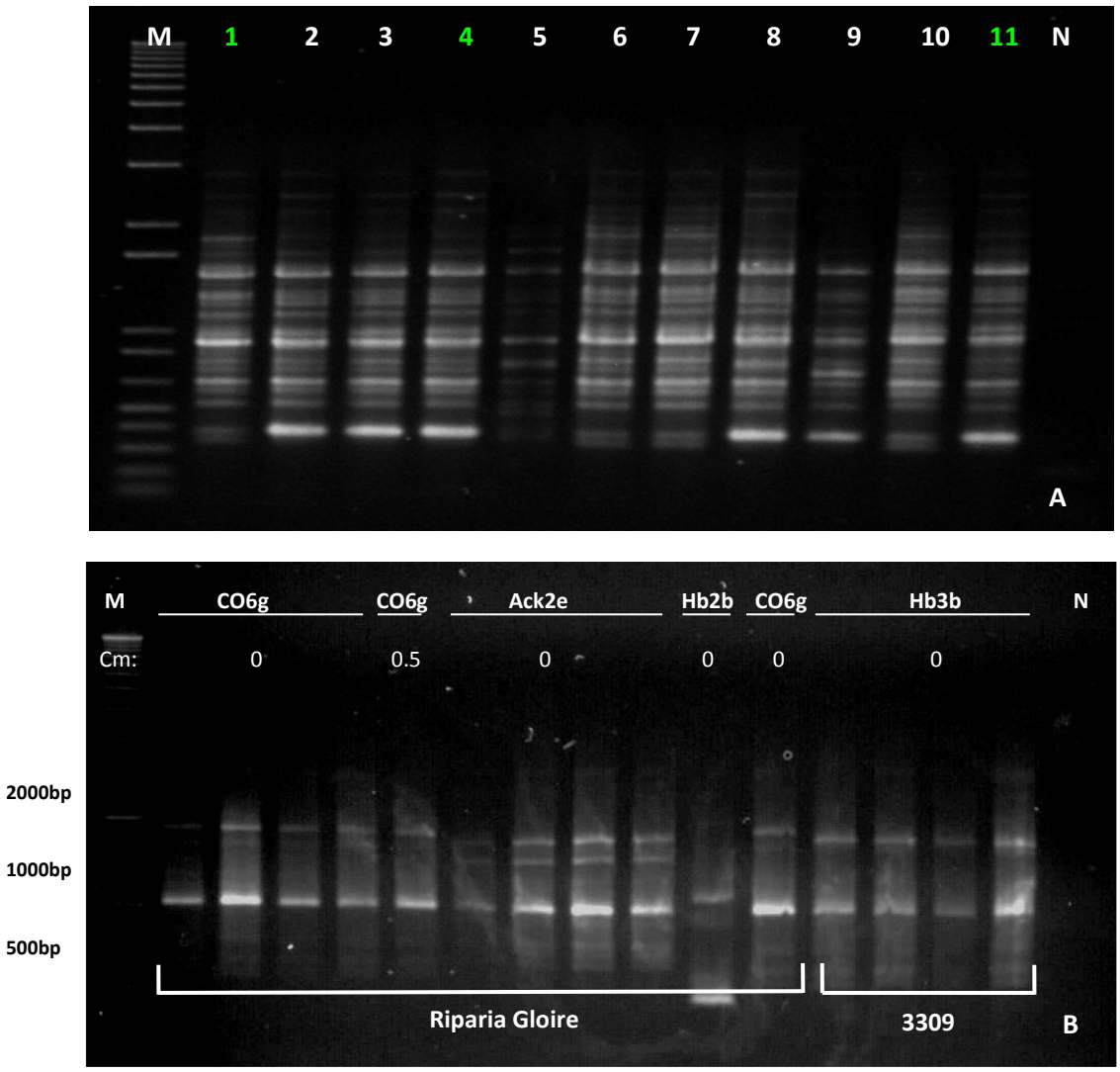

Figure 3.15 A: Original UP-PCR fingerprint profile generated by primer As15inv. Lanes 1-11 denote representative isolates (1-CO6g; 2-Mar5a; 3-Hb6b; 4-Hb3b; 5-Mtb1a; 6-Mar17d; 7-Mar17e; 8-Mar14b; 9-Ack2b; 10-Mar6a; 11-Ack2e).The isolates number highlighted with green are inoculums. M: 1Kb plus ladder marker. N: negative control. B: Genotyping of morphologically identical to *C.pauciseptatum* isolates recovered from pot trial assay generated by UP-PCR using primer As 15 inv. Each lane is denoted with isolate names and proximities to the stem base. M for each gel denotes 1 Kb plus ladder and numbers on far left denote molecular weights of the bands of the 1Kb plus ladder and N represents negative control. Two rootstocks are denoted as Riparia Gloire and 3309.

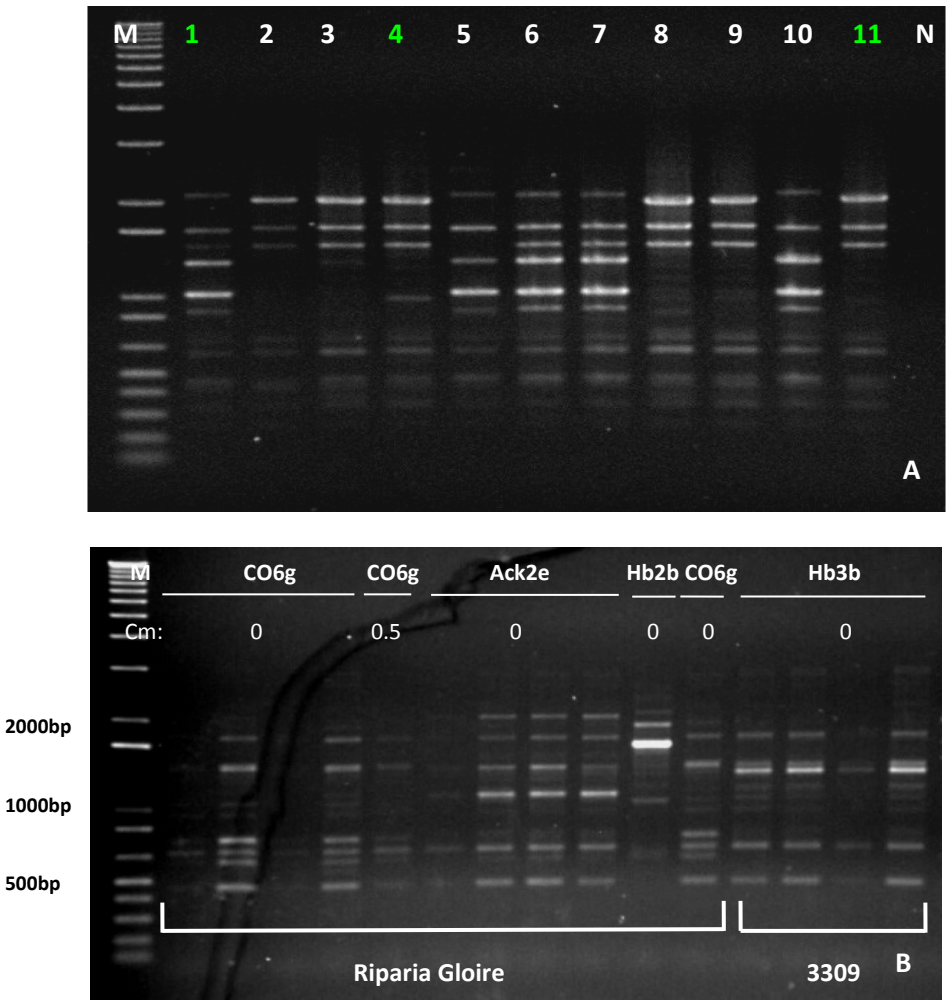

Figure 3.16 A: Original UP-PCR fingerprint profile generated by primer As15. Lanes 1-11 denote representative isolates (1-CO6g; 2-Mar5a; 3-Hb6b; 4-Hb3b; 5-Mtb1a; 6-Mar17d; 7-Mar17e; 8-Mar14b; 9-Ack2b; 10-Mar6a; 11-Ack2e).The isolates number highlighted with green are inoculums. M: 1Kb plus ladder marker. N: negative control. B: Genotyping of morphologically identical to *C.pauciseptatum* isolates recovered from pot trial assay generated by UP-PCR using primer As 15. Each lane is denoted with isolate names and proximities to the stem base. M for each gel denotes 1 Kb plus ladder and numbers on far left denote molecular weights of the bands of the 1Kb plus ladder and N represents negative control. Two rootstocks are denoted as Riparia Gloire and 3309.

Table 3.8 Genotypes of recovered *Cylindrocarpon*-like isolates obtained from tissue at different proximity to the stem base investigated from 4-month-old grapevine rootstock Riparia Gloire and 3309 associated with pot trial assay based on UP-PCR analysis.

Rootstock	Isolate	Proximity to the stem base from which the isolate was recovered					
		0 cm			0.5 cm		
		Isolate No.	As15 inv	As 15	Isolate No.	As15 inv	As 15
Riparia Gloire	CO6g	4	I, I, I, I, I	-, -, -, -, I	1	I	-
	Ack2e	4	I, I, I, I	-, -, -, -			
	Hb2b	1	I	I			
3309	Hb3b	4	I, I, I, I	-, -, -, -			

+": the fingerprint was exactly the same as banding profile of the original inoculum; "-": the fingerprint didn't resemble the banding pattern of the original inoculums; "I": It was inconclusive to decide.

3.4 Discussion

The aim of this chapter was to investigate the pathogenicity of different isolates of *C. pauciseptatum* compared to pathogenic isolates from *Ilyonectria* species known to cause black foot disease. The pathogenicity was investigated using both detached root material and whole plants on three common grapevine rootstocks (Riparia Gloire, 3309, Schwarzman). A high degree of contamination by a botryosphaeriaceous fungus interfered with the recovery of the inoculated pathogen from infected plant tissue in both studies. Therefore, molecular tools were used to try and confirm infection by *C. pauciseptatum*.

The detached root assay was done twice, once on three rootstocks (3309, Riparia Gloire and Schwarzman) and a second time on two rootstocks (Riparia Gloire and 3309). Only rootstock Schwarzman from experiment 1 and Riparia Gloire in experiment 2 gave meaningful results due to contamination of the control treatments for the other rootstocks. For Schwarzman, the inoculated roots showed black discolouration as typical infection symptoms at one and two month assessment times. The mean lesion lengths produced on detached roots varied between isolates of *C. pauciseptatum* among two rootstocks (Schwarzman and Riparia Gloire) after 1 month inoculation (0.18–24.04 mm) and 2 months inoculation (2.31–24.04 mm). Isolates Mar14b and CO6g were pathogenic and showed the longest mean lesion length (13.94 and 14.67 mm, respectively) on inoculated detached roots of Schwarzman in experiment 1. Isolates Ack2e and Mtb1a were pathogenic (9.09 mm and 14.52 mm, respectively) for Riparia Gloire in experiment 2. In experiment 1 the lesion was approximately half the size (36.40 mm) produced by the positive control, *I. liriodendri* isolate CO1b. In experiment 2 the lesion size of *C. pauciseptatum* isolates Mtb1a/Ack2e and the positive control CO1b (12.52 mm) were similar to each other, however, the lesion from the positive control was unexpectedly small compared to previous work (Pathrose 1012). The generally smaller lesion length of *C. pauciseptatum* in comparison with Pathrose's result (Pathrose, 2012) and positive control (Experiment 1) suggested that *C. pauciseptatum* is weakly pathogenic towards grapevines. The positive controls gave variable results in this study. *Ilyonectria liriodendri* isolate CO1b as positive control produced a bigger lesion on rootstock Schwarzman than Riparia Gloire. In contrast, the other positive control *I. macrodidyma* isolate Hb2b did not produce lesions significantly different from *C. pauciseptatum* isolates on either rootstock. In contrast, Pathrose (2012) achieved a much longer lesion length at 2 months assessment time for the isolate Hb2b (30.9 mm), although the length at 1 month was similar to the results presented here. This variation in pathogenicity could be explained by two reasons. Firstly, these fungal pathogens have been stored since 2006 and may have lost pathogenicity in storage. Richter *et al.* (2004) reported that frequent transfer of fungal cultures on agar plates can change of fungal characteristics such as pathogenicity, virulence, and growth rate. For example, Richter and Bruhn (1989) revived 35 out of 37 isolates of

saprotrophic fungi stored for 8-48 months in sterile cold water (5°C) (95% viability), while only 53 out of a total of 98 isolates of mycorrhizal fungi were viable (54%), demonstrating species variation in viability following storage. Herath Mudiyanselage *et al.* (2013) tested the viability of boysenberry downy mildew pathogen *Peronospora sparsa* after 6 months storage at different temperatures (-20°C and -80°C). They found that storage time and temperature significantly affected spore viability and ability to infect plants. Only ≤5% of *P. sparsa* spores germinated after 4 months storage as compared to that (60%) after 1 month storage period, and very low infection rates were observed from spores after 3 months storage. A second possible reason for variation in pathogenicity in the infection studies described in this chapter in the confounding influence of the existing infection by the botryosphaeriaceous contaminant which may have excluded the inoculated *Cylindrocarpon* spp. from infection sites. In this study, there was a high degree of contamination from a botryosphaeriaceous fungus on the root pieces plated on PDA plate which was confirmed morphologically. The colonies morphologically similar to botryosphaeria were obtained from the lesion of fungal inoculated roots and uninoculated control roots. Thus, the lesions may be caused by multiple pathogens and may not be solely due to inoculation with the *Cylindrocarpon* species. This may have been exacerbated by the *C. pausciseptatum* being a weak pathogen meaning that pathogenicity in a background of infected material would have been even more difficult to quantify.

In the study of potted vines, the potted vines did not display any signs of decline within 4 months. More than half of the total 270 plants including uninoculated plants were dead in 1 week time due to transplant shock. The rootstock Schwarzmann was completely discarded due to more severe mortality than for the other rootstocks Riparia Gloire and 3309. Therefore, most of the isolates were obtained from symptomless plant, which further supports the categorisation of *C. pauciseptatum* as opportunistic saprophytes or weak pathogens in grapevines (Phillips, 1998). Plant death is likely to have been due to the stresses imposed on newly rooted grapevines in the glasshouse, such as high temperatures or inadequate humidity. During summer in the glasshouse the temperature often reached 28°C and the pots had limited temperature buffering due to surface area and volume, unlike field soil. Small root and vascular system of plants would not be able to supply enough water to compensate for the higher transpiration rates.

The morphological recovery of isolates from both the detached roots and the potted vines was unreliable due to the presence of contaminants. The source of these contaminants is unclear. The contaminants might have come with the purchased vine. Normally rootstock suppliers do not apply any treatment to rootstock cuttings, which increase the opportunities of being infected by fungal pathogens prior to the experiments. The work of Billones-Baaijens (2013) showed frequent infection by botryosphaeriaceous species in nursery stock including

grade 1 and 2 plants. This infection could not be completely removed by hot water treatment and/or fungicides and was likely to have originated from infected mothervines. It has also been shown that *Botryosphaeriaceae* and *Ilyonectria* species might occur together in young grapevines in nurseries (Oliveira *et al*., 2004). Whiteman *et al*. (2007) also reported that the sources of infection for *Phaeomoniella chlamydospora* in young vines included the infected propagation material. Thus, although botryosphaeriaceous species appeared to be the dominant contaminant, this may simply be due to the fast growth of these colonies and it is possible that this material was infected by multiple pathogens. This was further shown by the recovery of *Cylindrocarpon*-like colonies that were ultimately shown not to be the inoculated isolates. Multiple infections may have further stressed the young plants contributed to the early death as Probst *et al.* (2012) reported that prior infection of grapevine rootstocks by *P. chlamydospora* increased the the severity and incidence of "*C". liriodendri*. The results showed that, for rootstock Riparia Gloire, *C. pauciseptatum* isolate CO6g had more *Cylindrocarpon*-like colonies at the stem base compared to the positive and negative controls but was not significantly different from the negative control for rootstock 3309. This demonstrates the high degree of natural infection with a large number of *Cylindrocarpon* colonies recovered from the negative control. The transplanted rootstocks were placed under high temperatures in the glasshouse and this may also have made the potted vines more susceptible to these latent infections. In addition to the purchase of infected plant material it is also possible that multiple infections occured among the plants by splash dispersal from the inoculant conidia of a nearby plant or soil surface. In this experiment plants were watered from above by nursery staff and in future work, the cross contamination of pot cultures could be minimised if a splash-free drip irrigation system was established.

Molecular tools were used in an attempt to overcome the contamination evident in the detached roots. In this study, the ITS gene region of all of the re-isolated *Cylindrocarpon*-like colonies obtained at stem base or 0.5/1.5 cm above it from detached roots and potted vines were sequenced. They were 98-100% identical to members of the genus *Cylindrocarpon/Ilyonectria*. However, only approximately 300-400 bp of usable sequence was produced which does not cover the entire ITS region. In addition the use of the ITS region did not identify to species level and the histone H3 gene could have produced more discriminatory sequence information at the species level. Pathrose (2012) used a combination of species-specific PCR with primers designed to amplify the ITS, β–tubulin, EF1-α and histone to identify a large collection of *Ilyonectria*-like isolates. Unfortunately, amplification of the histone H3 and β–tubulin were not applied in this study. It is strongly recommended to add histone, β–tubulin and species-specific primers in the future work for the better resolution between the closely related species.

The isolates recovered from the detached roots were further examined using UP-PCR to compare with the genotypes of the inoculated isolate. Two primers were used with primer As15 producing a clearer fingerprint for comparison of genotype variation. For the detached roots 14 out of 18 isolates of *C. pauciseptatum* were identical to the inoculated isolates of *C. pauciseptatum* by UP-PCR. All identical isolates were present in the lesion margin and Mar6a from rootstock Riparia Gloire had moved endophytically up to 0.5 cm from the lesion margin. This suggested that pathogen could infect the detached roots and move inside the roots endophytically without damaging the plant tissue. This was also shown for other highly pathogenic species, such as *I. liriodendri*, and members of the *I. macrodidyma* and *I. radicicola* complexes (Pathrose, 2012). The UP-PCR method used in the current research was effective in identifying the re-isolated colonies of *C. pauciseptatum*. For potted vines UP-PCR was also used to try and confirm pathogenicity. This method could not confirm any of the *C. pauciseptatum* isolates had invaded the potted rootstocks. However, it did confirm that the *Cylindrocarpon*-like colonies recovered from the two rootstocks were not the original inoculant strain. This further demonstrated that the plant material was likely infected by the pathogen either prior to planting or during the experiment.

Symptomless vines in this study may also be due to the combination of weak virulence of *C. pauciseptatum* and lower concentration used in this study. The conidial concentration applied in this study was 1×10^4 conidia/mL which was 10 times lower than that of Pathrose (2012) (1×10^5 conidia/mL). In that study, the typical infection symptoms of black discolouration were observed within a month, and the pathogen was recovered from the stem base after 3 months. Researchers have reported that the application of increased inoculum concentrations to plants generally resulted in increased disease severity under artificial conditions (Pegg and Dixon, 1969; Shaw *et al*., 1997; Sippell and Hall, 1982). In both of these studies, the plants were grown in potting mix which does not reflect the natural environment of the *Cylindrocarpon* species in vineyards, where the micro flora environment is comparatively different to the microorganisms present in the soil. Future work could repeat the potted vine study using higher concentrations of conidia.

For the detached root results there appeared to be no relationship between pathogenicity and genetic group. This was similar to the results of Pathrose *et al*. (2014) who also concluded no correlation between the genetic group that the isolates belonged to in the neighbour joining tree and their pathogenicity. This is probably due to both the weakly pathogen isolates and the limitations of UP-PCR primers, which are designed to target intergenic and non-coding areas of the genome (Bulat *et al*., 1998). Pathogenicity is likely to be regulated by single or multiple genes (Agrios, 2005) which may not be targeted by the UP-PCR primers. Therefore, genotyping tool would be considered to target these special

genes, such as AFLP (McDonald, 1997), which is more random and may sample coding gene regions in addition to non- coding areas.

As compared to potted vine assay which usually takes 3 to 6 months, the detached root assay is much faster in terms of disease development. Three main reasons can explain this fact: 1) the young soft roots selected for the bioassay are more vulnerable to pathogen attack; 2) the plant defence responses are omitted due to the detachment of root from the plant (Elżbieta *et al*., 2013); 3) there is much less competing microbial flora which would naturally interact with *C. pauciseptatum* isolates in the soil. The detached root can also be set up at anytime of the year witihin a controlled environment including light, temperature and humidity. Thus, this may have been why pathogenicity was expressed on the detached roots and not the potted vines; strengthening the conclusion that *C. pauciseptatum* is a weak pathogen.

Overall the results demonstrate that *C. pauciseptatum* is a weak pathogen. Its presence in symptomatic vines may be due to being a secondary coloniser or being synergistic with the other pathogens recovered in the original survey. Pitt *et al*. (2010) reported the observation of multiple botryosphaeriaceous species from the grapevines in a single vineyard. Dual root/basal stem inoculation with *I. macrodidyma* and botryosphaeriaceous species *Diplodia seriata*, by Whitelaw-Weckert *et al*. (2013), caused a further decrease in total leaf dry weight (63%), total number of leaves (21%), root health rating (29%) and fine root rating (49%) compared to inoculation with *I. macrodidyma* alone. The authors explained the mechanism is likely due to the *Ilyonectria* spp. disrupting root function and retarding early plant development, then *Botryosphaeriaceae* fungi gradually invading the xylem to cause decline and eventual death of the plant. However, this is one of a very limited number of detailed reports describing co-infection causing greater pathogen severity and the reasons for this. Consequently, co-inoculation of some *C. pauciseptatum* isolates with other *Cylindrocarpon* spp. may be valuable to try in future work.

In conclusion, this study suggests that *C. pauciseptatum* is weakly pathogenic towards grapevines with lesions only apparent in detached root material. In the detached root assay endophytic movement of pathogen occurred. However, there was no relationship between the genetic group developed by UP-PCR and pathogenicity of the *C. pauciseptatum* isolates. There was no infection of potted vines. Future work should repeat the detached root and potted vine assays. The potted vine experiment could be improved by using sanitised plant material, higher inoculum concentrations and a longer time period and include the molecular analyses to clearly discriminate the infecting isolates.

Chapter 4

Characterisation of Laccases Produced by *Cylindrocarpon pauciseptatum*

4.1 Introduction

The plant cell wall is comprised of cellulose, hemicelluloses, pectic substances and proteinaceous compounds. Fungi produce a number of enzymes that degrade plant tissue incuding endopolygalacturonases, cellulases, pectin lyase, xylanase, β-galactosidase, laccases and proteases (Jorgensen *et al*., 2007). These enzymes play multiple roles including participating in morphogenesis, fungal plant-pathogen/host interaction, stress defence, pigmentation, sporulation, detoxification and lignin degradation (Thurston, 1994, Moreira *et al*., 2005). Laccases, which are classified as multi-copper oxidases (p-diphenol:oxygen oxidoreductase, EC 1.10.3.2), are a family of fungal enzymes that have been implicated in pathogenesis and the degradation of lignin (Vasconcelos *et al*., 2001). They are widely distributed among fungi associated with wood decay, basidiomycetes and ascomycetes alike, and are involved in pathogenicity towards plants (Barbosa *et al*., 2007). This family of enzymes is also called blue copper oxidases as they contain four catalytic copper atoms (Hakulinen *et al*., 2002). Laccase production and enzymatic properties have been studied for different fungi associated with varied hosts, such as *Aureobasidium pullulans* associated with painted wood (Rich *et al*., 2013), *Fusarium oxysporum* f. sp*. lycopersici* in tomato (Cañero and Roncero, 2008), *Podospora anserina* as a white-rotof wood (Xie *et al.,* 2013). To date, little has been documented on the variation of enzymatic properties of laccases produced by *Cylindrocarpon* spp.

Generally, extracellular laccases are of two types (PPO-I and PPO-II), which can be induced in the presence of veratryl alcohol on Vogel‘s minimum salt medium (Vasconcelos *et al*., 2000). Veratryl (3,4-dimethoxybenzyl) alcohol, a secondary metabolite synthesised *de-novo* by several fungi, specifically stimulates the transcription of genes for laccases (Dekker *et al*. 2001). Dekker *et al*. (2001) explored a new physiological role for veratryl alcohol by studying laccases of *Botryosphaeria* spp. involved in the degradation of starch and lignocelluloses. The authors stated that it could induce the synthesis of laccases and regulate the laccase activity by the finding of 2-fold increase in the laccase activity over non-induced cultures. Fungi, including both ascomycetes and basidiomycetes, generally produce multiple laccase isoenzymes encoded by complex multi-gene families (Giardina *et al*., 2009). *Pleurotus ostreatus,* as white-rot fungus*,* was reported as having nine laccase genes *lcc1, lcc2, lcc3, lcc4, lcc6, lcc7, lcc8, lcc10* and *lcc12* (Pezzella *et al*., 2013). Seven laccase genes *lcc1, lcc2,*

lcc3, lcc4, lcc5, lcc6, lcc7 were identified for *Auricularia auricula-judae* strain Au916 (Fan *et al.*, 2014). Seventeen different laccase genes were detected for litter-degrading ink-cap mushroom *Coprinopsis cinerea* as one of the largest groups of laccase genes ever described for a fungus (Kilaru *et al*., 2006a). Rühl *et al*. (2013) did further study on *Coprinopsis cinerea,* and discovered that *lcc1* and *lcc5* are the main laccases. Generally *Lcc1* is the most abundant secretory product of most of the ascomycetes and basidiomycetes.

Rahman and Punja (2005) studied "*C." destructans* isolates which were pathogenic towards ginseng and their results showed that highly virulent isolates produced significantly higher laccase activity. More recently, laccase production by isolates of three *Cylindrocarpon* spp. were assessed *in vitro* by Pathrose (2012). Among the three species, "*C." macrodidymum* produced 10 times higher laccase activity than the other two species ("*C." destructans* and *"C." liriodendri*). However, it was not determined whether the greater activity was due to a higher enzyme concentration or to a more active enzyme isoform. The laccase discovered by Pathrose (2012) was shown to be a member of the *lcc1* group by a phylogenetic study.

In the previous chapter, the result from detached roots and whole plants inoculated with the *C. pauciseptatum* isolates suggested that this species was a weak pathogen and indicated that there was isolate variability in pathogenicity. Pathogenicity may be exerted by the secretion of plant cell wall degrading enzymes such as laccases. Thus, by determining whether *C. pauciseptatum* produces laccase and comparing the amino acid similarity of this enzyme to those produced by other *Cylindrocarpon* species the pathogenicity of this species may be further clarified. The aims of this chapter were to 1) demonstrate laccase secretion by *C. pauciseptatum,* 2) to isolate the gene encoding *lcc1* and 3) to compare the amino acid sequence of *C. pauciseptatum lcc1* to other fungal species.

4.2 Materials and Methods

4.2.1 Enzymatic assays

Laccase activity is commonly determined spectrophotometrically and is based on the ability of this enzyme to oxidize–colorize specific aromatic compounds. Laccase activity was measured using two substrates, ABTS (2,2,1-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid; Sigma) and DMP (2,6-dimethoxyphenol, Global). Oxidation of ABTS by laccase will result in a green–blue coloured radical cation (ABTS+•) measurable at 420 nm (Figure 4.1). While the absorbance of DMP reaction solution was measured at 468 nm.

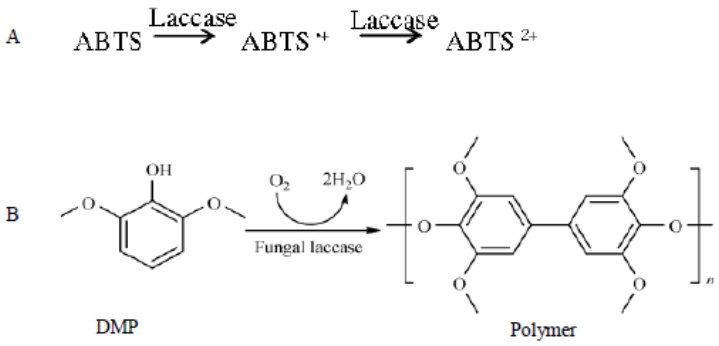

Figure 4.1 Oxidation of A) ABTS by laccase results in a green–blue coloured radical cation (ABTS+•) and B) 2, 6-dimethoxyphenol by fungal laccases.

For eight *C. pauciseptatum* (CO6g, Mar5a, Hb3b, Mtb1a, Mar14b, Ack2b, Mar6a and Ack2e), and one isolate each of *I. macrodidyma* (Hb2b) and *I. liriodendri* (Hb2a) as positive controls, a 5 mm diameter mycelia colonized agar plug was cut from the growing edge of a 14 day old colony on a PDA plate and used to inoculate 25 mL of sterilized minimal medium (pH 6.0, comprising Vogel minimal salts medium and glucose (1% w:v) as the carbon source) (Appendix 4.E) in 100 mL flasks and sealed using cotton wool plug. Three replicates were set up for each isolate. A 119 µL aliquot of veratryl alcohol (Sigma 3,4-dimethyozybenzyl alcohol, 96%) was added immediately after the three colony plugs . Cultures were incubated at 20°C, in a rotatory shaker (Chiltern Scientific) at 140 rpm under 12:12 light:dark condition for 7 days.

After 5 and 7 days incubation, cell-free culture fluid obtained after removal of mycelium by centrifugation (12,500 ×*g* for 20 min) was used as the source of extracellular enzyme extract. Laccase activity of PPO-I (polyphenol oxidase-1) was assayed with 0.05 mL 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma) (50 mM, in water) as a substrate at pH 3.0 using 0.15 mL citrate–phosphate buffer (120 mM) (Appendix 4.F). Five hundred µL cell-free enzyme supernatant was added in this solution and sterilized water was added to make it up to 1 mL as the final volume. The absorbance was measured at 420 nm using a spectrophotometer after 5 min heating at 50°C (Barbosa *et al*. 1996). Laccase activity of PPO-II (polyphenol oxidase-2) was assayed with 2,6-dimethoxyphenol (DMP; Aldrich) as a substrate at pH 6.5 by incubating a mixture containing substrate (0.15 mL aliquot of 10 mM DMP in deonised water), 170 mM citrate–phosphate buffer (0.15 mL, pH 6.5) (Appendix 4.F), and enzyme supernatant (0.7 mL), in a final volume of 1.0 mL by adding sterilized water at 45°C for 5 min, and the absorbance was measured at 468 nm using a spectrophotometer (Model 6305; Jenway Ltd, England) (Vasconcelos *et al*., 2000). Laccase activity was expressed in units as the number of micromoles of the oxidised product formed per minute per millilitre of enzyme solution for each laccase substrate. Both types of laccase produced

by the eight *C. pauciseptatum* isolates were quantified and compared between the isolates, and finally the laccase activity were statistically analysed by ANOVA.

This assay was repeated. Laccase activity was measured 5 days after inoculation in experiment 1, and after 5 days and 7 days inoculation in experiment 2.

4.2.2 Gene discovery

4.2.2.1 Pilot study of degenerate PCR for confirming appropriate annealing temperature

Degenerate PCR was used to isolate and sequence the gene encoding laccase enzyme in *C. pauciseptatum.* Genomic DNA of one *C. pauciseptatum* isolate (Hb3b) and one *I. novozelandica* isolate (Gis4a) (provided by Ms Megan Anne Outram) as a positive control was obtained as described in Section 2.2.3. Each PCR was conducted in a 25 µL reaction volume containing 1× buffer, 200 µM of each dNTP, 20 pmol of primer F1 (5'YGA TGC VA CTG GGG HGA CA3') and R1 (5'GYV YNK CHH TSC AYT GGC AYG G3') (Outram, 2013), respectively, 1.25 U Faststart Taq DNA polymerase (Roche Molecular Biochemicals, New Zealand) and 20 ng of DNA template. Negative control with sterile water in place of DNA was also included in each set of reactions to confirm there was no contamination. The amplification cycle was 3 min at 94°C followed by 40 cycles of denaturation at 94°C for 30 s, annealing temperature was either 54, 57, 60, 63, 66°C for 45 s and extension at 72°C for 30 s, ending with extension at 72°C for 7 min. PCR products were mixed with loading dye and separated by electrophoresis as described in Section 2.2.4.2. The same process was done for three other randomly selected *C. pauciseptatum* isolates (Mar17e, Mar6a and Hb6b) and isolate Gis4a was used as a positive control using annealing temperature of 63°C to achieve robust single band of amplified base pairs.

The amplified bands of three *C. pauciseptatum* isolates (Mar17e, Mar6a and Hb6b) at 63°C were sequenced using the forward degenerate primer F1 at the Lincoln University Sequencing Facility as described in Section 2.2.4.3. The sequences produced from primer F1 from the three isolates were aligned using the computer program DNAMAN version 4.0a (Lynnon Biosoft®) and used to design an internal primer for sequencing to improve sequence quality. The consensus sequences generated from alignment were then submitted to a blastx search (http://www.ncbi.nlm.nih.gov/BLAST/) in the GenBank database for identification.

4.2.2.2 Amplification and sequencing of the *Cylindrocarpon* laccase genes

The genomic DNA of all 11 *C. pauciseptatum* isolates (CO6g, Mar5a, Hb3b, Mtb1a, Mar14b, Mar17d, Mar17e, Mar14b, Ack2b, Mar6a and Ack2e) were amplified using F1and R1 primers. Each PCR was conducted as described in Section 4.2.2.1 with an annealing temperature of 63°C for 45 s. The PCR products were separated as described in Section 2.2.4.2.

The amplified laccase genes from all 11 isolates were sequenced using the newly designed internal forward primer CPLaccaseF (5'TCA GGT TCC TGA CAT TAA CGC3') (Invitrogen™) at the Lincoln University Sequencing Facility. Resultant sequences were edited to remove any ambiguities at both ends by Sequence Scanner software (Version: 1.0; Applied Biosystems). The sequences were submitted to a blastx search (http://www.ncbi.nlm.nih.gov/BLAST/) in the GenBank database to confirm their identity. The sequences obtained plus the sequence of *I. novozelandica* isolate Gis4a, and *I. liriodendri* isolate Hb2a were then aligned using DNAMAN version 4.0a (Lynnon Biosoft®) to identify any differences within and between species.

The DNA sequences of laccase from the 11 *C. pauciseptatum* isolates were translated into predicted amino acid sequences which were then submitted to a blastp search (http://www.ncbi.nlm.nih.gov/BLAST/) in the GenBank database for identification and alignment of the domain structures in the protein (Hakulinen *et al*., 2002). The amino acid sequences of laccase from *C. pauciseptatum* isolates, *I. novozelandica* isolate Gis4a, *I. liriodendri* isolate Hb2a and other plant pathogens, industrially important ascomycetes and basidiomycetes, were used to produce a phylogenetic tree in MEGA version 5.05 (Tamura *et al*., 2011) using maximum likelihood based on the neighbour-joining method (Saitou and Nei, 1987) and 1000 bootstrap replications. Representatives of different members of the laccase family (*lcc1, lcc2, lcc3, lcc5, lcc9*) were included. The protein sequence of laccase from the bacterium of *Bacillus* spp. was used as an out-group. The aligned sequences were analysed to find the best model, using maximum likelihood with 1000 bootstrap replications.

4.3 Results

4.3.1 Laccase activity of *C. pauciseptatum*

4.3.1.1 Experiment 1

In experiment 1, there was a significant isolate effect on the laccase activity for both types of laccase (PPO-I: P=0.04; PPO-II: P<0.001) within isolates of *C. pauciseptatum* (Appendix 4.A). However, the experiment was repeated due to the unexpectedly lower PPO-I laccase activity of the positive control *I. macrodidyma* isolate Hb2b (0.063 U mL^{-1}) than *C. pauciseptatum* isolates and when compared to the result of Pathrose, (2012) where it was 0.766 U mL^{-1}.

4.3.1.2 Experiment 2

Laccase activity was measured at 5th and 7th day after inoculation during experiment 2 for the evaluation of laccase. For PPO-I, a significant difference amongst isolates and species was observed at both sampling times (5th day: P=0.04; 7th day: P<0.001). The relative ranking of isolates changed between day 5 and day 7 (Table 4.1). The *I. liriodendri* isolate Hb2a, as the

positive control, produced the highest activity with a mean of 0.240 U mL^{-1} of PPO-I after 7 days incubation. The positive control was not significantly different from *C. pauciseptatum* isolates Mar5a, Mar14b and Mtb1a at 5 days incubation, but only Mar5a and Mtb1a remained similar by day 7. For *C. pauciseptatum* isolates, Mtb1a yielded the highest mean laccase activity on day 5 with 0.138 U mL^{-1} and 0.214 U mL^{-1} after 7 days. CO6g and Ack2e yielded the least laccase activity at both sampling times compared to the other isolates tested, which was ~25% of the laccase activity produced by *I. liriodendri* Hb2a after 7 days incubation. The PPO-I type laccase activity of all isolates including positive control Hb2a increased between 5 and 7 days of inoculation.

All isolates, including one positive control, produced measureable levels of PPO-II laccase. There was variability in the mean activity between isolates of *C. pauciseptatum* and the positive control *I. liriodendri* isolate Hb2a at both measuring times (P<0.001, P=0.019, respectively) (Table 4.1). PPO-II production by *I. liriodendri* isolate Hb2a was the highest at both sampling times, and had peaked on day 5 and was decreasing by day 7, whereas all *C. pauciseptatum* isolates increased between day 5 and 7. On day 5 PPO-II levels of *C. pauciseptatum* isolates were low and not significantly different from each other. On day 7 PPO-II production by *I. liriodendri* isolate Hb2a was 0.120 U mL^{-1} which was significantly different from all other isolates except *C. pauciseptatum* isolates Mar6a and Mar14b, while the least production was yielded by Mtb1a (0.000 U mL^{-1}).

Table 4.1 Experiment 2 - Mean PPO-I and PPO-II type laccase activity (U mL^{-1}) produced by isolates of *Cylindrocarpon pauciseptatum* and one isolate of *I. liriodendri* as a positive control.

	Isolate	PPO-I (U mL^{-1})		PPO-II(U mL^{-1})	
		5 days	7 days	5 days	7 days
C.pauciseptatum	Mar5a	0.093 bcd	0.210 cde	0.004 a	0.022 ab
	Mar6a	0.035 ab	0.147 b	0.015 a	0.043 abc
	Mar14b	0.070 abc	0.172 bcd	0.013 a	0.053 bc
	Mtb1a	0.138 d	0.214 de	0.010 a	0.000 a
	CO6g	0.007 a	0.056 a	0.006 a	0.020 ab
	Ack2b	0.011 a	0.135 b	0.012 a	0.011 ab
	Ack2e	0.013 a	0.056 a	0.009 a	0.015 ab
	Hb3b	0.024 a	0.166 bc	0.006 a	0.018 ab
I. liriodendri	Hb2a	0.107 cd	0.240 e	0.120 b	0.080 c
***P*-Value**		0.004	<0.001	<0.001	0.019
LSD		0.067	0.043	0.023	0.044

4.3.2 Amplification and sequencing of the laccase (*lcc1*) gene

The laccase degenerate primers F1 and R1 produced the expected 1100 bp for isolate Hb3b of *C. pauciseptatum* at all annealing temperatures. For annealing temperature 54°C, multiple bands were produced with an additional band of approximately 450 bp, while a fainter band

of 450 bp appeared at 57°C. These primers produced fainter bands at 66°C as compared with other temperatures. Thus, annealing temperatures 63°C was selected for amplification of all 11 isolates as it produced a bright single band of the expected 1100 bp (Figure 4.2). A strong single band of 1100 bp was amplified at annealing temperature 63°C with multiple bands still evident at 60°C for the three randomly selected isolates (Mar17e, Mar6a and Hb6b) by primers F1 and R1.

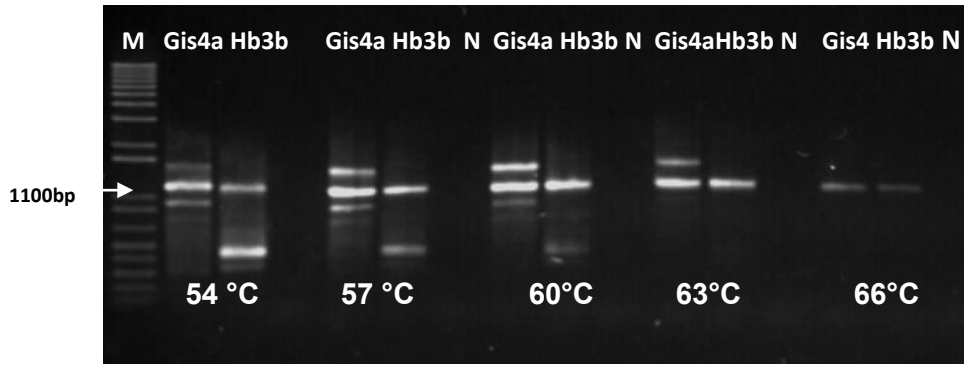

Figure 4.2 Agarose gel (1%) of the laccase gene amplified from randomly selected isolates of *C. pauciseptatum* and one isolate of *I. novozelandica* (Gis4a) using annealing temperatures of 54, 57, 60, 63 and 66°C and the laccase degenerate primers F1 and R1. M indicates 1Kb plus ladder. N indicates negative control. Each lane is denoted with isolate name. The numbers on far left denote molecular weights of the bands of the 1Kb plus ladder.

All 11 isolates of *C. pauciseptatum* produced a strong and single band of approximately 1100 bp using 63°C as annealing temperature (Figure 4.3). Sequencing of the 1100 bp bands with the internal sequence primer produced 750-820 bp of unambiguous sequence that was used for BLAST after trimming ambiguities from both ends of sequence. Comparison with known DNA sequences in the GenBank database showed 73% similarity to *Fusarium solani* laccase gene (GenBank accession# AHD26939), 69% similarity to *Metarhizium acridum* CQMa 102 *lcc1* (GenBank accession# XM007813230), 68% similarity to *Fusarium oxysporum lcc1* (GenBank accession# ABS19938), and 69% similarity to Fusarium oxysporum lcc2 (GenBank accession# ENH66075). No polymorphic bases amongst these 11 isolates of *C. pauciseptatum* were found (Appendix 4.B). The *C. pauciseptatum* isolates had 83.1% similarity to *I. novozelandica* isolate Gis4a, and 88.0% similarity to *I. liriodendri* isolate Hb2a.

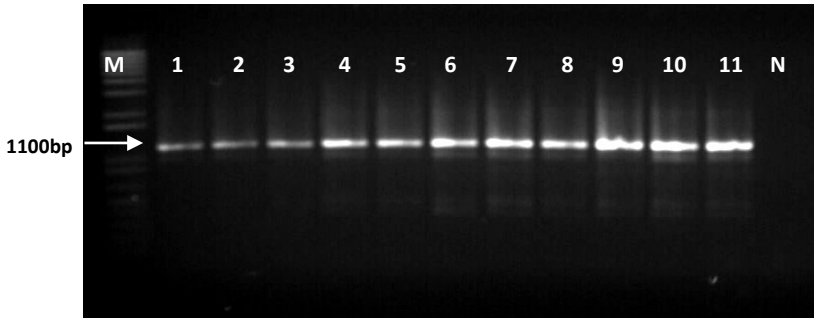

Figure 4.3 Agarose gel (1%) of the laccase gene amplified from all 11 isolates of *C. pauciseptatum* at 60°C using the laccase degenerate primers F1 and R1. M indicates 1Kb plus ladder. N indicates

negative control. Lane 1 to 11 contains representative isolates. (1. CO6g, 2. Mar5a, 3. HB6b, 4. HB3b, 5. Mtb1a, 6. Mar17d, 7. Mar17e, 8. Mar14b, 9. Ack2b, 10. Mar6a, 11. Ack2e.) The numbers on far left denote molecular weights of the bands of the 1Kb plus ladder.

An approximately 200 amino acid sequence was yielded by the translation of 600 bp of the putative laccase gene sequence. The aligned amino acid sequences of *C. pauciseptatum* isolates had no polymorphism between each other and 79.8% similarity to isolate Gis4a and 72.8% similarity to isolate Hb2a. The aligned protein sequence of the *C. pauciseptatum* isolates and that of *Metarhizium acridum* CQMa 102 *lcc1* (GenBank accession# XM007813230) showed 91.2% similarity. The portion of the protein recovered for *C. pauciseptatum* corresponded to approximately 40% of the total amino acid sequence encoded by the *lcc*1 gene of *Metarhizium acridum* CQMa 102 and was located in the middle relative to that gene. The alignment of amino acid sequences showed that the non-specific Cu-oxidase domain (pfam00394) (amino acids 206-366), cupredoxin2-Ma-LCC like domain (amino acids 209-382) and cupredoxin3-Ma-LCC like domain (amino acids 403-532) were present (Appendix 4.B).

The alignment of amino acid sequences of laccase genes from isolates of *C. pauciseptatum* and the positive controls *I. novozelandica* Gis4a and *I. liriodendri* Hb2a is shown in Figure 4.4. The conserved amino acids are marked with asterisks; three β-strands are highlighted with purple. There were 13 amino acid differences between *C. pauciseptatum* isolates and the positive controls (Table 4.2). One was a conservative substitution to an amino acid with similar properties (Δ73I→V; both Isoleucine (I) and Valine (V) are non-polar residues). In the 12 remaining non-conservative substitutions, three (positions 4, 5, 9) were solely between *C. pauciseptatum* and the other two species.

Table 4.2 Amino acid polymorphism in the translated sequences from isolates of *C. pauciseptatum*, positive control Gis4a of *I. novozelandica* (in red) and Hb2a of *I. liriodendri* (in green).

Isolates	3	4	5	6	7	9	23	25	27	25	48	56	73
Ack2b	S	R	P	P	F	Q	T	A	D	N	T	A	I
Ack2e	S	R	P	P	F	Q	T	A	D	N	T	A	I
CO6g	S	R	P	P	F	Q	T	A	D	N	T	A	I
Hb3b	S	R	P	P	F	Q	T	A	D	N	T	A	I
Hb6b	S	R	P	P	F	Q	T	A	D	N	T	A	I
Mar5a	S	R	P	P	F	Q	T	A	D	N	T	A	I
Mar6a	S	R	P	P	F	Q	T	A	D	N	T	A	I
Mtb1a	S	R	P	P	F	Q	T	A	D	N	T	A	I
Mar14b	S	R	P	P	F	Q	T	A	D	N	T	A	I
Mar17d	S	R	P	P	F	Q	T	A	D	N	T	A	I
Mar17e	S	R	P	P	F	Q	T	A	D	N	T	A	I
Gis4a	G	G	L	A	F	D	T	A	D	A	T	V	V
Hb2a	R	G	L	V	I	D	A	P	T	Q	V	S	V

Conservative substitutions are with grey shade. The letters in the column 3-73 denotes the amino acids. Amino acid with polar residues (T – Threonine, S – Serine, G – Glycine, Q – Glutamine, N – Asparagine); Non-polar residues (I – Isoleucine, L – Leucine, V – Valine, A – Alanine, F – Phenylalanine, P – Proline (α-imino acid); Acidic (D – Aspartic acid,); Basic (R – Arginine).

Figure 4.4 Alignments of multiple amino acid sequences of laccase from isolates of *C. pauciseptatum*, positive control Gis4a of *I. novozelandica* and Hb2a of *I. liriodendri,* retrieved from Genbank. All amino acids (1-75) are in the domain B. Conserved amino acids are marked with asterisks. β-strands are shaded purple. Amino acid substitutions in species were noticed with "#" on top.

4.4 Phylogenetic study of laccase (*lcc1*) gene

A phylogenetic tree was produced for the amino acid sequence generated from the gene encoding the laccase of *C. pauciseptatum* isolates and other representative laccase isoenzymes including *lcc2, lcc3, lcc5* and *lcc9* from other fungal species. WAG, gamma distributed and invariant sites substitution model (WAG+G+I model) was selected as the best model to describe the substitution pattern.

The neighbour joining tree produced two clades (Figure 4.5). The laccase produced by *Bacillus subtilis* was clearly distinct from fungal laccases. The *C. pauciseptatum* isolates grouped together in one clade (Clade 1) accompanied by three reference strains of comparatively high similarity which were chosen from the BLAST result and one *lcc5* retrieved from the GenBank database (Figure 4.5). The positive control *I. novozelandica* Gis4a and *I. liriodendri* Hb2a were placed in this clade and these laccases had already been confirmed as *lcc1 (*Outram, 2013; Pathrose*,* 2012). For *Fusarium solani* (AHD26939) the laccase sequence was not designated to a specific laccase type. *Coprionopsis cinerea* (AAS38574) was included to represent basidiomycete laccases and shared the clade with *Fusarium oxysporum* (ABS19943) *lcc3 and Fusarium oxysporum* (ABS19942) *lcc9* genes.

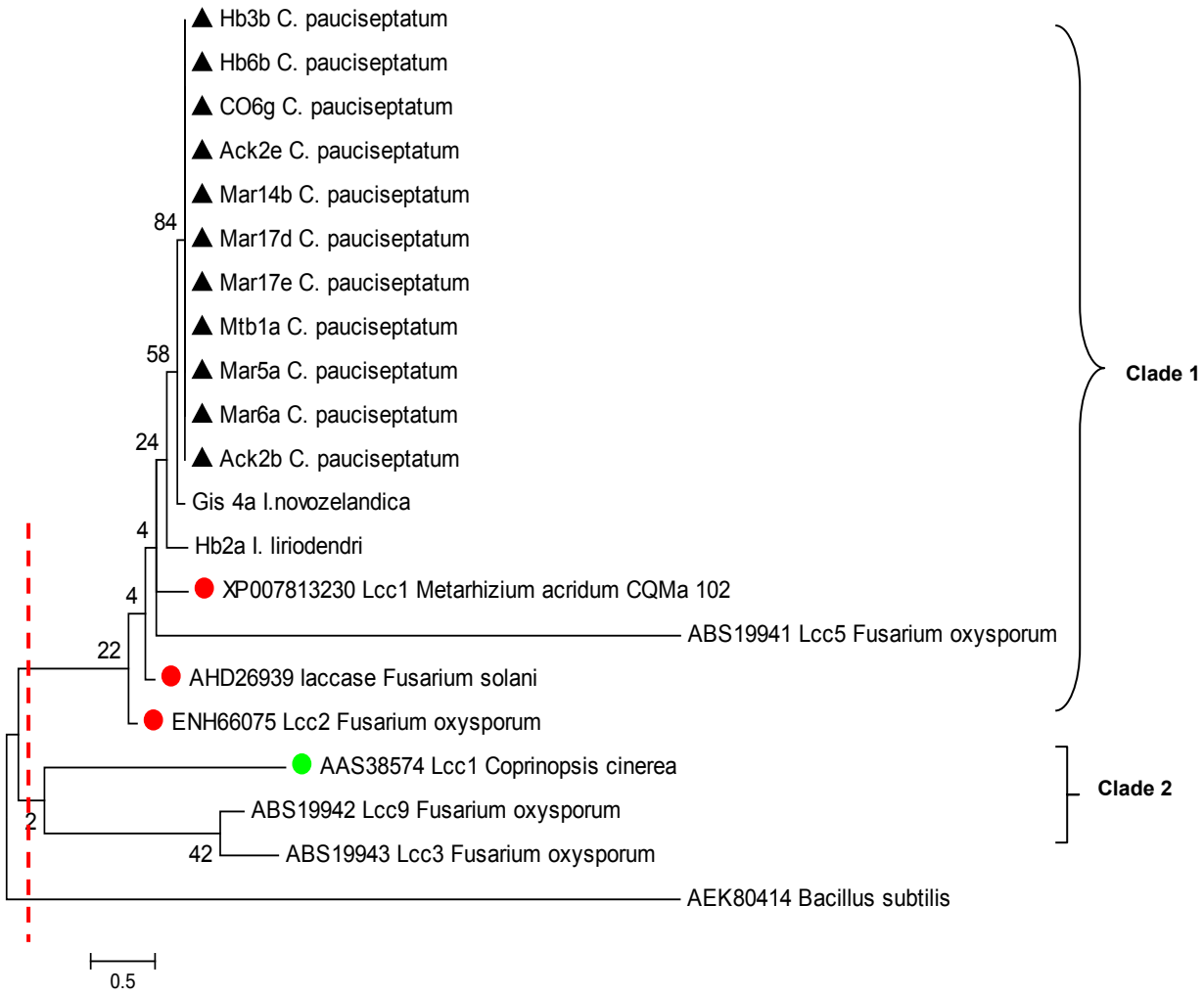

Figure 4.5 Neighbour joining tree produced for the laccase enzyme of *C. pauciseptatum* isolates and other types of laccase of ascomycetes and basidiomycetes with the out-group *Bacillus subtilise*, using the best protein model with MEGA version 5.05. The distance bar represents the Kimura distance. A red dotted line indicates the arbitrary line to recognize 2 genetic groups. Isolates with black triangle represent *C. pauciseptatum* samples used in this study. Isolates with a red dot are the most identical strains based on blastp of GenBank database. Isolate with green dots belong to basidiomycete fungi.

4.5 Discussion

This is the first report of laccase production by *C. pauciseptatum.* This chapter aimed to evaluate the relative activity of the laccase, as an important cell wall degrading enzyme, by determining differences in laccase activity (PPO-I and PPO-II) between isolates of *C. pauciseptatum* and in comparison to a highly pathogenic isolate of *I. liriodendri* as a positive control. The gene encoding laccase *lcc*1 was also investigated to determine whether any predicted amino acid sequence variation correlated with measured differences in enzyme activity. The results showed a significant difference between *C. pauciseptatum* isolates and the positive control isolate for both PPO-I and PPO-II laccase activity at both day 5 and 7. However, there was substantial variation within *C. pauciseptatum* isolates and some produced activity that was similar to the positive control. The translated sequence of the

subsequently isolated gene encoding *lcc*1 showed that there was no polymorphism between isolates of *C. pauciseptatum*.

Both type of laccase activity (PPO-I and PPO-II) were measurable for all tested isolates including the positive control. This shows that *C. pauciseptatum* is capable of producing both types of laccase. Although the biochemistry and molecular characterisation of specific PPOs of some fungi have been studied, the individual function of them is still unclear. In this work no relationship between PPO-I and PPO-II activity was found although PPO-I activity was generally always substantially higher than the PPO-II produced by most of the isolates except for Ack 2b of *C. pauciseptatum* and Hb2a from *I. lriodendri*. Pathrose (2012) and Vasconcelos *et al*. (2001) also found a lower activity level of PPO-II than PPO-I secreted by *Cylindrocarpon* spp. and *B. ribis,* respectively. This comparison suggests that although these two isozymes of laccase can be found in several fungal species, the levels of expression vary between species.

The amount of activity produced by *C. pauciseptatum* isolates varied. Isolates Mtb1a, Mar5a, Mar14b produced PPO-I levels equivalent to the positive control on either day 5 or 7 or both. Mtb1a is from genetic group 1 generated by UP-PCR assay, while the latter two are from the other genetic group. Ack2e in contrast yielded the least laccase activity. Interestingly, Mtb1a produced the longest lesion size on detached root for rootstock Schwarzman, and Mar14b also produced a long lesion for rootstock Riparia Gloire and was confirmed at the lesion margin by molecular detection. The lesion length of Ack2e was the shortest for rootstock Schwarzman. Although no isolates matched the positive control for PPO-II production on day 5, Mar6a and Mar14b were equivalent to the positive control on day 7. These two isolates are not from the same genetic group. Coincidently, Mar6a did not produce a long lesion on either rootstock but could move endophytically up to 0.5 cm from the lesion margin as detected by UP-PCR. It is likely that multiple enzymes are involved and therefore a good correlation with a single enzyme type may be unlikely. The higher laccase activity by some isolates could explain the greater degree of degradation of the grapevine roots observed in the detached root assay. Some isolates had lower laccase activity (PPO-I) and were observed to move inside the root endophytically without causing substantial deterioration of the root tissue (eg. CO6g). In a similar study done by Pathrose (2012), the isolates with higher PPO-I laccase activity were more virulent than others in the detached root assay and this trend was also observed for a few isolates in potted vines. The positive control, Hb2a produced much higher PPO-II enzymatic activity on day 5 than day 7. The higher pathogenicity of this species could, therefore, be due to the combined action of high PPO-1 and PPO-II in the early phase of infection. Consequently, further study should monitor this enzyme over time to improve understanding of their role in pathogenesis using a larger number of *C. pauciseptatum* isolates from genetic groups.

The difference in enzyme activity observed between isolates of *Cylindrocarpon* spp. may be either due to the different efficiencies of enzymes or the different amounts of the enzyme secreted extracellularly. Variation in activity between species of the same genus with similar modes of infection has been reported previously. Eight isolates of *L. theobromae* had variable laccase activity when studied by Saldanha *et al*. (2007), "*C." liriodendri* and *"C." destructans* was shown to have lower laccase activity compared to *"C." macrodidymum* stated by Pathrose (2012), and the study on a wide array of laccase activities detected variation within the botryosphaeriales fungi (Esteves *et al*., 2014). However, there were no polymorphic amino acid residues observed within the *C. pauciseptatum* isolates. Thus, this suggests that the variation is due to differences in extracellular secretion, although this cannot be determined completely as only ~40% of the gene was amplified.

The mechanism of secretion of this enzyme is poorly understood in the *Cylindrocarpon/Ilyonectria* genus. Previous work has indicated that the secretion of this enzyme is generally dependent on physico-chemical factors such as agitation (Leisola *et al*., 1984), temperature and pH (Linko, 1992), and addition of veratryl alcohol (Vasconcelos *et al*., 2001), nutrient-limiting conditions and biomass growth, etc. Compounds other than veratryl alcohol can also stimulate the laccase secretion, including copper ions (Shikha, 2012), ethanol (Vázquez-Garcidueñas *et al*., 2012) and isopropanol (Crowe and Olsson, 2001). For example, Shikha (2012) reported Cu^{2+} (1.0 mM) and veratryl alcohol induced the maximum laccase production. Wei *et al*. (2013) found that different metal ions, such as Cu^{2+} and Fe^{2+} as well as the aromatic compounds such as tannic acid, syringic acid, cinnamic acid, gallic acid and guaiacol with different structure could stimulate laccase gene transcription and laccase synthesis in *Trametes velutina*. Thus, it is unknown if the laccase secretion by isolates in this study was under optimal conditions. Significant work would need to be done to understand the role of nutrient and environmental variables on laccase secretion by *C. pauciseptatum*. Further work should include an estimation of the protein concentration in the filtrate to determine whether the variable activity between isolates was due to variable rates of extracellular secretion. This could be improved by filtration of specific target proteins using a system such as column chromatography.

The contamination which confounded the detached root and potted vine assays has made it very difficult to relate the enzyme activity observed *in vitro* to the pathogenicity *in planta*. Whilst some isolates were confirmed in root lesions their relative pathgenicty rank was not determined. The difficulty in achieving this is likely confounded by the weak pathogenicity of *C. pauciseptatum* which is supported by the generally lower enzyme activities measured in this chapter. A similar study done by Pathrose (2012) using the same methodology previously showed a good relationship between virulence and laccase activity with "*C". macrodidymum* having both the highest level of laccase activity and being the most virulent

among all tested *Cylindrocarpon* spp. She also found a relationship between laccase activity and virulence for individual isolates. Baskarathevan (2011) did the same enzymatic assay with only three isolates of the grapevine pathogen *Neofusicoccum parvum*. Although these varied in the amount of both PPO-I and PPO-II laccase produced, no correlation with pathogenicity was achieved. Consequently, it would be ideal to redo the pathogenicity of *C. pauciseptaum* isolates on clean detached root and potted vine material with isolates selected based on their measured laccase activities rather than genotypes. The role of PPO-I and PPO-II laccase in virulence could also be further investigated using gene knock-outs and over expression mutants to determine whether PPO-I, PPO-II, a combination or either, are pivotal to pathogenicity of this species.

One of the interesting observations was that for both *C. pauciseptatum* isolates and positive control the activity of laccase changed over time. Laccase production by all *C. pauciseptatum* isolates increased between day 5 and 7, but laccase produced by the positive control isolate peaked by day 5 followed by a drop at day 7. This indicated a possible differentiation of secretion timeframes between species. Osma *et al*. (2013) studied laccase production and the growth morphology of different white-rot fungi by measuring PPO-I laccase activity over 16 days incubation. They concluded that there was a high similarity in laccase production by different species over that time profile. In this study only two times were sampled and further work using a time course approach would be required to show if secretion profiles between and within species were similar.

This study was the first to sequence and reveal interspecies variation between the gene encoding the enzyme *lcc1* of *C. pauciseptatum* and *I. liriodendri* and *I. novozelandica*. In Pathose's (2012) study, analysis of the translated DNA sequence of three *Cylindrocarpon* species showed variation of the predicted amino acid sequences between species and isolates. In this study, degenerate primers were able to produce an 1100 bp amplicon for isolates of *C. pauciseptatum*. Comparison of the unambiguous 750-820 bp sequence with DNA sequences in the GenBank database showed approximately 69% similarity to the *lcc1* genes of other related ascomycetes. Phylogenetic analysis of the predicted laccase amino acid sequence showed high similarity to the *lcc1* enzymes produced by other ascomycetes and grouped together with these voucher sequences into one clade. The other laccase enzymes (*lcc3, lcc5* and *lcc9*) plus one of basidiomycetes were placed in separate clades. These genomic results indicate that the *C. pauciseptatum* laccase gene encoding in the enzyme is probably the *lcc1* gene that was targeted by the degenerate PCR. Comparison of the ~200 amino acid sequence, translated from the *C. pauciseptatum lcc1,* with laccase enzymes from other ascomycetes showed the presence of the non-specific Cu-oxidase domain, cupredoxin2-Ma-LCC like domain, and cupredoxin3-Ma-LCC like domain (Gao *et al*., 2011). As compared to the *Metarhizium acridum* CQMa 102 *lcc1* gene this was

approximately 40% of the expected protein and was located in approximately the middle of the polypeptide. As only 40% of the whole genome of the laccase gene was isolated and sequenced, active sites and the specific Cu-oxidase domain were not amplified. Thus, there is a chance that important polymorphisms occur in the unsequenced region which could be responsible for differences in enzymatic activity.

The positive control Hb2a from *I. lriodendri*, in this study, produced higher activity of PPO-I and PPO-II on both assessment times when compared to the majority of *C. pauciseptatum* isolates. The *C. pauciseptatum* amino acid sequence had only 72.84% similarity with the positive control Hb2a from *I. liriodendri*. There were 13 amino acid differences observed between *I. liriodendri* and *C. pauciseptatum*, in which twelve were non-conservative changes and only one was conservative substitution (Δ73I→V). The non conservative substitutions are likely to have changed the three dimensional structure of the *C. pauciseptaum* laccase relative to those from *I. lriodendri* and *I. novozelandica* and may have altered active or catalytic sites. Thus, these changes may explain the lower laccase activity observed in general for *C. pauciseptatum*. Where isolates of *C. pauciseptatum* reached similar levels of laccase activity to *I. liriodendri/I. novozealandica* it may have been due to greater extracellular secretion of the enzyme. Clearly, further work to separate the cause of the measured differences in laccase activity is required and questions remain as to the relative input of enzyme concentration to the measured activity. It is also possible that the laccase activity measured is the total from the family of enzymes and not simply the product of *lcc*1. It would be beneficial to sequence the remainder of the gene and study the crystal structure of the different laccase enzymes to shed more light on the relationship of different isoforms to activity. Further work could also be done to investigate the relative regulatory regions upstream of the laccase gene to determine whether the enzymes of isolates are differentially regulated, thus, contributing to the different levels measured.

In summary, this study has for the first time demonstrated laccase activity by *C. pauciseptatum* and characterised a portion of the gene encoding *lcc*1. The results showed intra-species variation of laccase activity but it is unclear the relative contribution of the different members of the laccase family to this measurement. No intra-species differences were revealed by the alignment of the predicted amino acid sequence from the portion of the gene isolated. Further work is required to understand the secretion of laccase over time and the relationship of this enzyme to pathogenicity.

Chapter 5

Conclusions and future work

Cylindrocarpon pauciseptatum was first recovered from necrotic regions on roots, wood and trunk bases of infected *Vitis* rootstocks and dying plants with similar disease symptoms to black foot disease by Schroers *et al. (*2008*).* To date, It has been characterised by morphology, molecular tools, and pathogenicity assays as a grapevine and non-grapevine pathogen in many countries, including Spain, Turkey, Canada, Brazil, and Italy (Erper *et al*., 2013; Úrbez-Torres *et al*., 2014; dos Santos *et al*., 2014; Aiello *et al*. 2014; Martin *et al*., 2011). As part of the New Zealand survey by Bleach *et al*. (2006) eleven isolates of *C. pauciseptatum* were recovered. However, they were not included in the genetic diversity and pathogenesis studies. The overall aim of this study was to accurately identify the New Zealand *C. pauciseptatum* isolates, investigate their genetic diversity and to study sub-species variation in pathogenicity and correlate that with activity of laccase.

A multi-gene sequence analysis was applied in this study to accurately identify the New Zealand isolates. Phylogenetic analyses of individual ITS, β – tubulin, EF1-α and histone datasets showed all 11 isolates grouped into well-supported clades along with voucher specimens. The 11 isolates formed a monophyletic unit in the rRNA gene and histone gene tree, while they were paraphyletic based on β – tubulin and EF1-α phylogenies. For the NJ tree generated from β-tubulin sequences eight isolates, including the avocado isolate were identical to the New Zealand isolate recovered from *Erica melanthera* (CBS100819) and grapevine (CBS113550), but did not group with other voucher isolates from other countries. There were 9 bp differences between these seven isolates and the other four isolates which had grouped with international voucher specimens in clade 1. For EF1-α, only isolate Mar17e was identical to the voucher specimens, including isolate CBS100819. The other 10 isolates contained 1 bp substitution from the voucher specimens. It is also interesting to note that isolate Mtb1a was consistently located on a separate branch from other isolates on the NJ trees based on sequences of the β-tubulin and EF1-α gene. The substantial sequence difference in the β-tubulin gene may suggest that isolates with these substitutions, which have only been found in New Zealand, are a different and as yet unrecognised species. The *Cylindrocarpon/Ilyonectria* genus is a complex group which has undergone several reclassifications over the last decade (Agustí-Brisach and Armengol, 2013c). The placement of one isolate from avocado in the β-tubulin NJ tree in the genetic groups containing grapevines isolates suggests the possibility of cross infection between grapevine and other non-grapevine hosts for *C. pauciseptatum*. Future studies based on more isolates and additional molecular characters plus refined taxonomic classification may clarify the possible intra- or interspecific variation within *C. pauciseptatum* strains.

The genetic diversity analysis of these 11 isolates of *C. pauciseptatum* showed that they were genetically diverse in New Zealand. A total of 65 bands (loci) were produced by five primers, with 46% polymorphism. When compared to the results for other grapevine pathogens, this result is more polymorphic than the results for *P. chlamydospora* obtained by Pottinger *et al*. (2002) but similar to those of Pathrose (2012) for other *Cylindrocarpon* species and Baskarathevan (2011) for botryosphariaceous species. Although not yet identified it may be indicative that a sexual stage of *C. pauciseptatum* is present or that there is a strong parasexual cycle in this species. To more fully characterise this species in New Zealand a much larger number of isolates (>100) is required. Other molecular methods such as AFLP could be used to generate a larger number of informative loci and may be useful for future studies on the genetic diversity of *C. pauciseptatum*.

Two major groups within the dendrogram were generated by the UP-PCR data for the 11 *C. pauciseptatum* isolates. Group 1 contained five isolates, whereas the other group contained 6 isolates. Analysis of a greater number of isolates could help determine the relative frequency of members of each of the genetic groups which correlated with the separation of the isolates in the β-tubulin NJ tree. Each genetic group was composed of isolates from different geographic regions and vineyards. In some cases, more than one genotype within a vineyard was encountered. Overall many more isolates on *C. pauciseptatum* from New Zealand and overseas are required to make definitive statements about genetic diversity within and between hosts and provide more details about the relationship between these populations and possibly identify the likely path of entry of this species into New Zealand.

Although the mechanism which generates the relatively high genetic diversity of *C. pauciseptatum* is unknown, microscopic analysis of interactions between *C. pauciseptatum* isolates showed that there were anastomoses and hyphal fusions within the actively growing hyphae of isolates in compatible reactions. This suggests that *C. pauciseptatum* can undergo parasexual recombination which may play a role in increasing genetic diversity. However, further study is required to investigate the role of these hyphal fusions in increasing genetic diversity. This could be done by comparing the DNA fingerprint profiles of the parent isolates with the DNA fingerprints of the isolates from the interaction zone using the DNA fingerprinting methods such as UP-PCR, RAPD and AFLP.

Pathogenicity tests with the 11 isolates on detached root assay and potted vines of three cultivars and comparison with the pathogenicity of representative isolate of three other predominant species (*I. europaea, I. macrodidyma* and *I. liriodendri*) suggested that *C. pauciseptatum* was weakly pathogenic towards grapevines with lesions only apparent in detached root material .There was no relationship between the genetic group shown by UP-PCR/β–tubulin gene and pathogenicity of the *C. pauciseptatum* isolates. However, a high

degree of contamination by a botryosphaeriaceous fungus interfered with the recovery of the inoculated pathogen from infected plant tissue in both assays and thus the relative pathogenicity of the two genetic groups remains unresolved. Numerous sources of these contaminants were hypothesised and may include 1) contaminant was already in the purchased vine; 2) Potted vines were more susceptible to the latent infections due to stress caused by transplanting; 3) multiple infections occurred among the plants by splash dispersal from the inoculant conidia of a nearby plant/soil surface. Therefore, although necrotic lesions were observed in detached roots/inoculated plants this may not be solely due to inoculation with the orginal inocula. Molecular tools proved very useful for confirming infection by *C. pauciseptatum* despite the presence of the contaminating pathogens.

In future work, the cross contamination of pot cultures could be minimised by appropriate sanitisation of plant materials and splash-free drip irrigation system. Furthermore, future work should repeat the detached root and potted vine assays using clean plant material and include the molecular analyses of histone to discriminate the infecting isolates with better resolution. Co-inoculation of *C. pauciseptatum* isolates with other *Cylindrocarpon* spp. may also be valuable to try in the future work to determine if they are synergistic with other pathogens. *Cylindrocarpon pauciseptatum* may also be more pathogenic on hosts other than grapevines, such as avocado. It may be possible that a survey of more hosts with symptoms characteristic of black foot may increase the culture collection and the corresponding pathogenicity assays may demonstrate that this species is more pathogenic on other hosts.

The preliminary study on *in vitro* production of laccase by *C. pauciseptatum* isolates showed both type of laccase activity (PPO-I and PPO-II) were measurable for all isolates with varied levels. Although there was no relationship between pathogenicity and laccase activity, this was not unexpected given the difficulties faced in the pathogenicity assays. The higher laccase activity by some isolates could explain the greater degree of degradation of the grapevine roots observed in the detached root assay. Some isolates had lower laccase activity (PPO-I) and were observed to move inside the root endophytically without causing substantial deterioration of the root tissue. However, overall there were too few isolates and too little clear data from the pathogenicity experiments to make firm conclusions.

The enzyme analysis was complemented by successful amplification of a portion of the laccase gene (40%). Although there were no amino acid polymorphisms between C. pauciseptatum isolates in theis region there is a chance that important polymorphisms occured in the unsequenced region and these could be responsible for differences in enzymatic activity. It would be beneficial to sequence the remainder of the gene and study the crystal structure of *Cylindrocarpon* laccase enzymes. It was also unresolved whether the isolates with higher level of laccase activity produced more enzyme or a more active

enzyme. Further work should include an estimation of the protein concentration in the filtrate to determine whether the variable activity between isolates was due to variable rates of extracellular secretion. The laccase extract obtained from isolates of *Cylindrocarpon* spp. could be purified and diluted to the same concentration prior to being tested in similar assays. Addition of the purified enzyme preparations to plant roots, followed by an assessment of the level of maceration produced could be used to investigate the role of this enzyme in pathogenicity There were 13 amino acid differences observed between *I. liriodendri* and *C. pauciseptatum*, so the higher pathogenicity of the *I. liriodendri* could be due to the changes altering the three dimensional structure of the protein and thus activity. Further work could use gene knock-outs based on transgenic methods and over expression mutants to determine whether PPO-I, PPO-II, a combination or either are pivotal to pathogenicity of this species. Future studies that measure the laccase, protease and cellulase activity would also improve the knowledge of how these enzymes act together in pathogenicity.

In summary, this study has identified the 11 *C. pauciseptatum* isolates and shown that they fall into two genetic groups. *Cylindrocarpon pauciseptatum* was identified in the lesion produced on detached roots but not in potted vines, however, the contamination of these experiments by other pathogens made conclusions about the relative pathogenicity of isolates difficult. The isolates were shown to produce laccase in extracellular filtrate and the gene encoding the lcc1 laccase was similar but not identical to that produced by other pathogenic *Cylindrocarpon/Ilyonectria* species. Increasing the number of isolates in the collection and analysing their pathogenicity on a range of “clean” host material would improve understanding of the relative pathogenicity of this species. This work has shown that the inclusion of molecular tools to verify isolates recovered from infection studies is essential given the natural background of these pathogens and others found in planta.

References

Abreo, E., Martínez, S., Betucci, L., and Lupo, S. (2010). Morphological and molecular characterization of Campylocarpon and Cylindrocarpon spp. associated with black foot disease of grapevines in Uruguay. *Australasian Plant Pathology*, 39, 446–452.

Aghighi, S. Fontanini, L., Yeoh, P.B., St.J.Hardy, G.E., Burgess, T.I., and Scott, J.K. (2014). A conceptual model to describe the decline of European blackberry (*Rubus anglocandicans*), a weed of national significance in Australia. *Plant Disease*, 98(5), 580-589.

Agrios, G.N. (2005). Plant Pathology (5th ed.). California, USA: Academic Press.

Agustí-Brisach, C., and Armengol, J. (2012). Effects of temperature, pH and water potential on mycelia growth, sporulation and chlamydospore production in culture of *Cylindrocarpon* spp. associated with black foot of grapevines. *Phytopathologia Mediterranea*, 51(1), 37−50.

Agustí-Brisach, C., and Armengol, J. (2013c). Black-foot disease of grapevine: an update on taxonomy, epidemiology and management strategies. *Phytopathologia Mediterranea,* 52(2), 245−261.

Agustí-Brisach, C., Gramaje, D., García-Jiménez, J., and Armengol, J. (2013a). Detection of black-foot and Petri disease pathogens in soils of grapevine nurseries and vineyards using bait plants. *Plant Soil,* 364, 5–13.

Agustí-Brisach, C., Gramaje, D., García-Jiménez, J., and Armengol, J. (2013b). Detection of black-foot disease pathogens in the grapevine nursery propagation process in Spain. *European Journal of Plant Pathology*, 137, 103–112.

Agustí-Brisach, C., Gramaje, D., León, M., García-Jiménez, J., and Armengol, J. (2011). Evaluation of vineyard weeds as potential hosts of black-foot and petri disease pathogens. *Plant Disease*, 95 (7), 803-810.

Aiello, D., Guarnaccia, V., Epifani, F., Perrone, G., and Polizzi, G. (2014). *'Cylindrocarpon'* and *Ilyonectria* species causing root and crown rot disease of potted Laurustinus plants in Italy. *Journal of Phytopathology*, DOI: 10.1111/jph.12306.

Aimi, T., Yotsutani, Y., and Morinaga, T. (2002). Vegetative incompatibility in the ascomycete *Rosellinia necatrix* studied by fluorescence microscopy. *Journal of Basic Microbiology*, 42(3), 147-155.

Alaniz S., Armengol, J., García-Jiménez, J., Abad-Campos, P., and Léon, M. (2009a). A multiplex PCR system for the specific detection of *Cylindrocarpon liriodendri*, *C. macrodydimum* and *C. pauciseptatum* from grapevine. *Plant Disease,* 93, 821–825.

Alaniz, S., Armengol, J., Leo´ n, M., Garci´a-jime´nez, J., and Abad-campos, P. (2009b). Analysis of genetic and virulence diversity of *Cylindrocarpon liriodendri* and *C. macrodidymum* associated with black foot disease of grapevine. *Mycological Research*, 113, 16-23.

Alaniz, S., García-Jiménez, J., Abad-Campos, P., and Armengol, J. (2010). Susceptibility of grapevine rootstocks to *Cylindrocarpon liriodendri* and *C. macrodidymum*. *Scientia Horticulturae,* 125, 305–308.

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

Arora, D.S., and Sharma, R.K. (2010). Lignolytic fungal laccases and their biotechnological applications. *Applied Biochemistry and Biotechnology,* 160, 1760–88.

Baldrian, P. (2006). Fungal laccases-occurrence and properties. *FEMS Microbiology Review*, 30, 215–242.

Ballingall, J., and Schilling, C. (2009). Economic impact of the New Zealand wine industry (An NZIER report to New Zealand Winegrowers).

Barbosa, A.M., Dekker, R.F.H., and St.Hardt, G.E. (1996). Veratryl alcohol as an inducer of laccase by an ascomycete, *Botryosphaeria* sp., when screened on the polymeric dye Poly R-478. *Letters in Applied Microbiology*, 23(2), 93-96.

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

Baskarathevan, J., Jaspers, M.V., Jones, E.E, Cruickshank, R.H., and Ridgway, H.J. (2012). Genetic and pathogenic diversity of *Neofusicoccum parvum* in New Zealand vineyards. *Fungal Biology*, 116(2), 276–288.

Billones, R. (2011). *Botryosphaeria* infections in New Zealand grapevine nurseries: Inoculum sources and infection pathways. PhD thesis, Lincoln University, New Zealand.

Billones, R., Jones, E.E., Ridgeway, H.J., & Jaspers, M.V. (2009). Distribution of *Botryospheria* spp. infection in different grapevine nursery materials in New Zealand (Abstract). *New Zealand Plant Protection,* 62, 406.

Billones-Baaijens, R., Jones, E.E., Ridgway, H.J., and Jaspers, M.V. (2014). Susceptibility of common rootstock and scion varieties of grapevines to *Botryosphaeriaceae* species. *Australasian Plant Pathology*, 43(1), 25-31.

Billones-Baaijens, R.G., Ridgway, H.J., Jones, E.E. and Jaspers, M.V. (2013). Prevalence and distribution of *Botryosphaeriacea* species in New Zealand grapevine nurseries. *European Journal of Plant Pathology,* 135 (1), 176-185.

Bleach, M.C., Jones, E.E., and Jaspers, M.V. (2006). Survey for black foot decline in New Zealand vineyards. Paper presented at the Proceedings of the 4th Australasian Soil borne Diseases Symposium.

Bock, C.H., Endalew, T.T., Biswas, B.K. Yadav, A.K., Sitther, V., Hotchkiss, M.W., Stevenson, K.L., and Wood, B.W. (2014). Comparison of UP-PCR and RAPD markers to study genetic diversity of *Fusicladium effusum* (G. Winter), cause of pecan scab. *Forest Pathology*, 44, 266–275.

Booth, C. (1966). The genus *Cylindrocarpon*. *Mycological Papers*, 104, 56.

Brayford, D. (1993). *Cylindrocarpon*. In 'Methods for research on soilborne phytopathogenic fungi.' (Singleton, L.L., Mihail, J.D., and Rush, C.M. (Eds)), *American Phytopathology Society*, St Paul, USA.

Brown, D., Jones, E.E., Ridgway, H.J., and Jaspers, M.V. (2012). Effect of partial defoliation on *Cylindrocarpon destructans* infection of grapevines. *New Zealand Plant Protection*, 65, 256-261.

Bueno, E.A., Oliveira, M.B., Andrade, R.V., Junior, M.L., and Petrofeza, S. (2012). Effect of different carbon sources on proteases secreted by the fungal pathogen *Sclerotinia sclerotiorum* during *Phaseolus vulgaris* infection. *Genetics and Molecular Research*, 11(3), 2171-2181.

Bulat, S.A., Lübeck, M., Mironenko, N., Jensen, D.F., and Lübeck, P.S. (1998). UP-PCR analysis and ITS1 ribotyping of strains of *Trichoderma* and *Gliocladium. Mycological research,* 102 (8), 933-943.

Burgess, T., Bihon, W., Wingfield, M.J., and Wingfield, B.D. (2009). A simple and rapid method to determine vegetative compatibility groups in fungi. *Inoculum*, 60 (6).

Burgess, T., Wingfield, B.D., and Wingfield, M.J. (2001). Comparison of genotypic diversity in native and introduced populations of *Sphaeropsis sapinea* isolated from *Pinus radiata*. *Mycological Research*, 105, 1331-1339.

Cabral, A., Groenewald, Z.J., Rego, C., Oliveira, H., and Crous, W.P. (2012a). *Cylindrocarpon* root rot: multi-gene analysis reveals novel species within the *Ilyonectria radicicola* species complex. *Mycological Progress*, 11(3), 655-688.

Cabral, A., Rego, C., Crous, W.P., and Oliveira, H. (2012c). Virulence and cross-infection potential of *Ilyonectria* spp. to grapevine. *Phytopathologia Mediterranea*, 51(2), 340−354.

Cabral, A., Rego, C., Nascimento, T., Oliveira, H., Groenewald, J.Z., and Crous, P. (2012b). Multi-gene analysis and morphology reveal novel *Ilyonectria* species associated with black foot disease of grapevines. *Fungal Biology*, 116, 62-80.

Carbone, I., Anderson, J.B., and Kohn, L.M. (1999). Patterns of descent in clonal lineages and their multilocus fingerprints are resolved with combined gene genealogies. *Evolution*, 53, 11-21.

Cardoso, M., Diniz, I., Cabral, A., Rego, C., and Oliveira, H. (2012). Unveiling inoculum sources of Black foot pathogens in a commercial grapevine nursery. *Phytopatologia Mediterranea*, 51, 442–443.

Castilho, F., Torres, R., Barbosa, A., Dekker, R. and Garcia, J. (2009). On the diversity of the laccase gene: A phylogenetic perspective from *Botryosphaeria rhodina* (Ascomycota: Fungi) and other related taxa. *Biochemical Genetics*, 47(1), 80-91.

Catalano, V., Vergara, M., Hauzenberger, J., Seiboth, B., Sarrocco, S., Vannacci, G., Kubicek, C., and Seidl-Seiboth, V. (2011). Use of a non-homologous end-joiningdeficient strain (delta-ku70) of the biocontrol fungus *Trichoderma virens* to investigate the function of the laccase gene lcc1 in sclerotia degradation. *Current Genetics*, 57(1), 13-23.

Chaverri, P., Salgado, C., Hirooka, Y., Rossman, A.Y., and Samuels G.J. (2011). Delimitation of *Neonectria* and *Cylindrocarpon* (*Nectriaceae, Hypocreales, Ascomycota*) and related genera with *Cylindrocarpon*-like anamorphs. *Studies in Mycology*, 68, 57–78.

Comont, G., Corio-Costet, M.F., Larignon, P., and Delmotte, F. (2010). AFLP markers reveal two genetic groups in the French population of the grapevine fungal pathogen *Phaeomoniella chlamydospora*. *European Journal of Plant Pathology*, 127, 451–464.

Conesa, A., Punt, P.J., van Luijk, N., and van den Hondel, C.A. (2001). The secretion pathway in filamentous fungi: a biotechnological view. *Fungal Genetics and Biology*, 33, 155-171.

Cortesi, P., and Milgroom, M.G. (1998). Genetics of vegetative incompatibility in *Cryphonectria parasitica*. *Applied and Environmental Microbiology*, 64 (8), 2988-2994.

Crous, P.W., Groenewald, J.Z., Risede, J.M., and Hywel-Jones, N.L. (2004). *Calonectria* species and their *Cylindrocladium* anamorphs: species with sphaeropedunculate vesicles. *Studies in Mycology*, 50, 415–429.

Crowe, J. D., and Olsson, S. (2001). Induction of laccase activity in *Rhizoctonia solani* by antagonistic *Pseudomonas fluorescens* strains and a range of chemical treatments. *Applied and Environmental Microbiology*, 67(5), 2088-2094.

Cullen, D. (1997). Recent advances on the molecular genetics of ligninolytic fungi. *Journal of Biotechnology*, 53, 273–89.

Dekker, R.F.H., Barbosa, A.M., Giese, E.C., Godoy, S.D.S., and Covizzi, L.G. (2007). 'Influence of nutrients on enhancing laccase production by *Botryosphaeria rhodina* MAMB-05'. *International Microbiology*, 10, 177-186.

Dekker, R.F.H., Vasconcelos, A.D., Barbosa, A.M. Giese, E.C., and Paccola-Meirelles, L. (2001). A new role for veratryl alcohol: regulation of synthesis of lignocellulose-degrading enzymes in the ligninolytic ascomyceteous fungus, *Botryosphaeria* sp.; influence of carbon source. *Biotechnology Letters*, 23, 1987–1993.

Dodds, R., Graci, S., Ko, S., and Walker, L. (2013) "What drives environmental sustainability in the New Zealand wine industry? An examination of driving factors and practices", *International Journal of Wine Business Research*, 25(3), 164 – 184.

dos Santos, R.F., Blume, E., Muniz, M.F.B., Steckling, S.M., and Burtet, G.W. (2014). First report of "*Cylindrocarpon" pauciseptatum* associated with black foot disease of grapevine in Brazil. *American Phytopathological Society,* 98(4), 567.

Dubrovski, S., and Fabritius, A. L. (2007). Occurrence of *Cylindrocarpon* spp. in nursery grapevines in California. *Phytopathologia Mediterranea*, 46, 84–86.

Dunaevski, Y.E., Matveeva, A.R., Fatkhullina, G.N., Belyakova, G.A., Kolomiets, T.M., Kovalenko, E.D., and Belozersky, M.A. (2008). Extracellular Proteases of Mycelial Fungi as Participants of Pathogenic Process. *Bioorganicheskaya Khimiya*, 34 (3), 317–321.

Dutech, C., Enjalbert, J., Fournier, E., Delmotte, F., Barre`s, B., Carlier, J., Tharreau, D., and Giraud, T. (2007). Challenges of microsatellite isolation in fungi. *Fungal Genetics and Biology*, 44, 933–949.

Elżbieta, O., Briardo, L., and Cristina, C. (2013). Plant integrity: An important factor in plantpathogen interactions*. Plant Signaling and Behavior*, 8(1), e22513.

Erper, I., Agustí-Brisach, C., Tunali, B., and Armengol, J. (2013). Characterization of root rot disease of kiwifruit in the Black Sea region of Turkey. *European Journal of Plant Pathology,* 136, 291–300.

Esteves, A.C., Saraiva, M., Correia, A., and Alves, A. (2014). Botryosphaeriales fungi produce extracellular enzymes with biotechnological potential. *Canadian Journal of Microbiology*, 60(5), 332-342.

Fan, X.Z., Zhou, Y., Xiao, Y., Xua, Z.Y., and Bian, Y.B. (2014). Cloning, expression and phylogenetic analysis of a divergent laccase multigene family in *Auricularia auricula-judae. Microbiological Research,* 169(5–6), 453–462.

Fox, R.T.V., and Narra, H.P. (2006). Plant disease diagnosis. In the epidemiology of plant diseases (2nd ed.), Cooke, B.M., Jones, D.G., and Kaye, B. (Eds). Springer, Dordrecht, the Netherlands.

Gao,Q., Jin,K., Ying,S.H., Zhang,Y., Xiao,G., Shang,Y., Duan,Z., Hu,X., Xie,X.Q., Zhou,G., Peng,G., Luo,Z., Huang,W., Wang,B., Fang,W., Wang,S., Zhong,Y., Ma,L.J., St Leger,R.J., Zhao,G.P., Pei,Y., Feng,M.G., Xia,Y. and Wang,C. (2011). Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. *PLoS Genetics*, 7(1), E1001264.

Garnjobst, L., and Wilson, J.F. (1956). Heterocaryosis and protoplasmic incompatibility in *Neurospora crassa*. *Proceedings of the National Academy of Science, USA*, 42, 613–618.

Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S., and Sannia, G. (2009). Laccases: a never-ending story. *Cellular and Molecular Life Science*, 67:369–38.

Glass, N.L., Jacobson, D.J., and Shiu, P.K.T. (2000). The genetics of hyphal fusion and vegetative incompatibility in filamentous ascomycete fungi. *Annual Review of Genetics*, 34, 165–186.

Gordon, C.L., Khalaj, V., Ram, A.F., Archer, D.B., Brookman, J.L., Trinci, A.P., Jeenes, D.J., Doonan, J.H., Wells, B., Punt, P.J., van den Hondel, C.A., and Robson, G.D. (2000).

Glucoamylase: green fluorescent protein fusions to monitor protein secretion in *Aspergillus niger*. *Microbiology*, 146, 415–426.

Gramaje, D., Alaniz, S., Abad-Campos, P., Garc´ıa-Jime´ nez, J., and Armengol, J. (2010). Effect of hot-water treatments in vitro on conidial germination and mycelial growth of grapevine trunk pathogens. *Annals of Applied Biology*, 156 (2), 231-241.

Gramaje, D., and Armengol, J. (2011). Fungal trunk pathogens in the grapevine propagation process: Potential inoculum sources, detection, identification, and management strategies. *Plant Diseases*, 95, 1040-1055.

Gramaje, D., Armengol, J., and Ridgway, H.J. (2013). Genetic and virulence diversity, and mating type distribution of *Togninia minima* causing grapevine trunk diseases in Spain. *European Journal of Plant Pathology*, 135, 727–743.

Grasso, S. (1984). Infections of *Fusarium oxysporum* and *Cylindrocarpon destructans* associated with a decline of young grapevine plants in Sicily. *Informatore Fitopatologico*, 34, 59-63.

Gubler, W.D., Baumgartner, K., Browne, G.T., Eskalen, A., Latham, S.R., Petit, E., and Bayramian, L.A. (2004). Root diseases of grapevines in California and their control. *Australasian Plant Pathology*, 33(2), 157-165.

Hakulinen, N., Kiiskinen, L.L., Kruus, K., Saloheimo, M., Paananen, A., Koivula, A., and Rouvinen, J. (2002). Crystal structure of a laccase from *Melanocarpus albomyces* with an intact trinuclear site. *Nature Structural Biology*, 9, 601-605.

Halleen, F., Crous, P.W., and Petrini, O. (2003). Fungi associated with healthy grapevine cuttings in nurseries, with special reference to pathogens involved in the decline of young vines. *Australasian Plant Pathology*, 32, 47-52.

Halleen, F., Fourie, P.H., and Crous, P.W. (2006a). A review of black foot disease of grapevine. *Phytopathologia Mediterranea*, 45, S55–S67.

Halleen, F., Fourie, P.H., and Crous, P.W. (2007). Control of black foot disease in grapevine nurseries. *Plant Pathology*, 56(4), 637–645.

Halleen, F., Schroers, H.J, Groenewald, J.Z., and Crous, P.W. (2004). Novel species of *Cylindrocarpon (Neonectria*) and *Campylocarpon* gen. nov. associated with black foot disease of grapevines (*Vitis* spp.). *Studies in Mycology*, 50, 431–455.

Halleen, F., Schroers, H.J., Groenewald, J.Z., Rego, C., Oliveira, H., and Crous, P.W. (2006b). *Neonectria liriodendri* sp. nov., the main causal agent of black foot disease of grapevines. *Studies in Mycology,* 55, 227–234.

Harvey, I., and Jaspers, M.V. (2006). Susceptibility of grapevine rootstock varieties to *Cylindrocarpon* spp: the cause of blackfoot disease. New Zealand Winegrowers Research Council, New Zealand.

Herath Mudiyanselage, A.M., Jaspers, M.V., Ridgway, H.J., Walter, M., Langford, G.I., and Jones, E.E. (2013). Evaluation of methods for long term storage of the boysenberry downy mildew pathogen *Peronospora sparsa*. *New Zealand Plant Protection*, 66, 254-258.

Hirooka, Y., and Kobayashi T. (2007). Taxonomic studies of nectrioid fungi in Japan. I: The genus *Neonectria*. *Mycoscience*, 48(1), 53-62.

Hoegger, P.J., Kilaru, S., James, T.Y., Thacker, J.R., and Kues, U. (2006). Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences. *FEBS Journal,* 273, 2308–2326.

Hoegger, P.J., Navarro-Gonzalez, M., Kilaru, S, Hoffmann, M., Westbrook, E.D., and Kues, U. (2004). The laccase gene family in *Coprinopsis cinerea* (*Coprinus cinereus*). *Current Genetics*, 45, 9–18.

Hoksbergen, T. (2006). New clonal material and varieties – prospects for the New zealand wine industry. Romeo Bragato Conference.

Hu, J., Eysden, J. V., and Quiros, C. F. (1995). Generation of DNA-based markers in specific genome regions by two-primer RAPD reactions. *Genome Research*, 4, 346-351.

Janusz, G., Kucharzyk, K.H., Pawlik, A., Staszczaka, M., and Paszczynski, A.J. (2013). Fungal laccase, manganese peroxidase and lignin peroxidase: Gene expression and regulation. *Enzyme and Microbial Technology,* 52, 1–12.

Jaspers, M.V., Bleach, C.M., and Harvey, I.C. (2007). Susceptibility of grapevine rootstocks to *Cylindrocarpon* disease. *Phytopathologia Mediterranea*, 46, 114.

Johannes, C., and Majcherczyk, A. (2000). Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems. *Applied Environmental Microbiology, 66, 524–528.*

Johnson, H. (1989). Vintage: The Story of Wine. *Simon and Schuster*, 17–19.

Jones, P.E. (2001). The development of diagnostic tools for the grapevine pathogen *Eutypa lata*: a thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Genetics at Massey University, Palmerston North, New Zealand.

Jorgensen, H., Vibe-Pedersen, J., Larsen, J., and Felby, C. (2007.) Liquefaction of lignocellulose at high-solids concentrations. *Biotechnology Bioengineering*, 96(5), 862–870.

Kallio, J. P., Gasparetti, C., Andberg, M., Boer, H., Koivula, A., Kruus, K., Rouvinen, J., and Hakulinen, N. (2011). Crystal structure of an ascomycete fungal laccase from Thielavia arenaria-common structural features of asco-laccases. *FEBS Journal*, 278(13), 2283-2295.

Keegstra, K. (2010). Plant cell walls. *Plant Physiology,* 154, 483–486.

Kilaru, S., Hoegger, P.J., and Kües, U. (2006). The laccase multi-gene family in *Coprinopsis cinerea* has seventeen different members that divide into two distinct subfamilies. *Current Genetics*, 50, 45–60.

King, B.C., Waxman, K.D., Nenni, N.V., Walker, L.P., Bergstrom, G.C., and Gibson, D.M. (2011). Arsenal of plant cell wall degrading enzymes reflects host preference among plant pathogenic fungi. *Biotechnology for Biofuels,* 4, 4.

Kosalková, K., García-Estrada, C., Barreiro, C., Flórez, G.M., Jami, S.M., Paniagua, A.M., and Martín, F.J. (2012). Casein phosphopeptides drastically increase the secretion of extracellular proteins in *Aspergillus awamori*. Proteomics studies reveal changes in the secretory pathway. *Microbial Cell Factories,* 11, 5.

Larignon, P., and Dubos, B. (2001). Black dead arm a new disease - not to be confused with esca. *Phytoma*, (538), 26-29.

Leisola, M.S.A., Ulmer, D.C., Waldner, R., and Fiechter, A. (1984). Role of veratryl alcohol in lignin degradation by *Phanerochaete chrysosporium. Journal of Biotechnology*, 1, 331–339.

Leslie, J.F. (1993). Fungal vegetative compatibility. *Annual Review of Phytopathology*, 31, 127-150.

Leslie, J.F. (1996). Fungal vegetative compatibility - Promises and prospects. *Phytoparasitica*, 24, 1-4.

Linko, S. (1992). Production of *Phanerochaete chrysosporium* lignin peroxidise. *Biotechnology Advances*, 10, 191–236.

Liu, H.Q, Feng, Y., Zhao, D.Q., and Jiang, J.X. (2012). Evaluation of cellulases produced from four fungi cultured on furfural residues and microcrystalline cellulose. *Biodegradation*, 23 (3), 465-472.

Lübeck, M., and Poulsen, H. (2001). UP-PCR cross blot hybridization as a tool for identification of anastomosis groups in the *Rhizoctonia solani* complex. *FEMS Microbiology Letters,* 201(1), 83–89.

Lübeck, M., Poulsen, S., Lübeck, P.S., Jensen, D.F., and Thrane, U. (2000). Identification of *Trichoderma* strains from building materials by ITS1 ribotyping, UP-PCR fingerprinting and UP-PCR cross hybridization. *FEMS Microbiology Letters,* 185(2), 129–134.

Lübeck, P.S., Alekhina, I.A., Lübeck, M., and Bulat, S.A. (1998). UP-PCR genotyping and rDNA analysis of *Ascochyta pisi Lib*. *Jounal of Phytopathology*, 146, 51–55.

Maluta, D.R., and Larignon, P. (1991). Pied-noir: Mieux vaut prevenir. *Viticulture*, 11, 71-72.

Mantiri, F.R., Samuels, G.J., Rahe, J.E., and Honda, B.M. (2001). Phylogenetic relationships in *Neonectria* species having *Cylindrocarpon* anamorphs inferred from mitochondrial ribosomal DNA sequences. *Canadian Journal of Botany*, 79(3), 334-340.

Martín, L., Sáenz de Miera, L.E., and Martín, M.T. (2014). AFLP and RAPD characterization of *Phaeoacremonium aleophilum* associated with *Vitis vinifera* decline in Spain. *Journal of Phytopathology*, 162(4), 245–257.

Martin, M.T., Martin, L., Cuesta, M.J., and García-Benavides, P. (2011). First report of *Cylindrocarpon pauciseptatum* associated with grapevine decline from Castilla y León, Spain. *Plant Disease*, 95, 361.

McDonald, B.A. (1997). The population genetics of fungi: tools and techniques. Paper presented at the 87th Annual Meeting of the American Phytopathological Society, August 16, 1995, Pittsburgh, PA, USA.

Mohammadi, H., Alaniz, S., Banihashemi, Z., and Armengol, J. (2009). Characterization of *Cylindrocarpon liriodendri* associated with black foot disease of grapevine in Iran. *Journal of phytopathology,* 157 (10), 642–645.

Moreira, F.G., Reis, S.d., Costa, M.A.F., Souza, C.G.M., and Peralta, R.M. (2005). Production of hydrolytic enzymes by the plant pathogenic fungus *Myrothecium verrucaria* in submerged cultures. *Brazilian Journal of Microbiology*, 36, 07-11.

Morton, L. (1995). Recommendations for the study of grape rootstocks in New Zealand. *New Zealand Journal of Crop and Horticultural Science*, 23(4), 403-405.

Mugnai, L., Graniti, A., and Surico, G. (1999). Esca (black measles) and brown woodstreaking: two old and elusive diseases of grapevines. *Plant Disease*, (83), 404−416.

New Zealand Winegrowers Statistical Annual Report. (2010). Wine Institute of New Zealand Inc., New Zealand.

New Zealand Winegrowers Statistical Annual Report. (2013). Wine Institute of New Zealand Inc., New Zealand.

O'Gorman D.T., and Haag, P. (2011). Trunk diseases of wine grapes in Okanagan Valley. *Canadian Plant Disease Survey*, 91, 152–154.

Obanor, F.O., Walter, M., Jones, E.E., Candy, J., and Jaspers, M.V. (2010). Genetic variation in *Spilocaea oleagina* populations from New Zealand olive groves. *Australasian Plant Pathology*, 39(6), 508-516.

Oliveira, H., Rego, C., and Nascimento, T. (2004). Decline of young grapevines caused by fungi. *Acta Horticulturae,* 652, 295–304.

Outram, M.B. (2013). Identification of the individual species within the *Ilyonectria macrodidyma* complex that cause black foot disease of grapevines in New Zealand.Thesis of Bachelor of Science (honours), Lincoln University, New Zealand.

Palonen, H., Saloheimo, M., Viikari, L., and Kruus, K. (2003). Purification, characterization and sequence analysis of a laccase from the ascomycete *Mauginiella* sp. *Enzyme and Microbial Technology,* 33, 854–862.

Pathrose, B. (2012). Characterising sub-species variation in New Zealand *Cylindrocarpon* species that cause black foot of grapevines. PhD thesis, Lincoln University, New Zealand.

Pathrose, B., Jones, E.E., Jaspers, M.V., and Ridgway, H.J. (2011). Species composition and genetic diversity of *Cylindrocarpon* species found commonly infecting New Zealand grapevines. Paper presented at the 4th Asian Conference on Plant Pathology concurrent with the 18th Biennial Australasian Plant Pathology Society Conference, Darwin, Australia.

Pathrose, B., Jones, E.E., Jaspers, M.V., and Ridgway, H.J. (2014). High genotypic and virulence diversity in *Ilyonectria liriodendri* isolates associated with black foot disease in New Zealand vineyards. *Plant Pathology*, 63, 613–624.

Pegg, G.F. and Dixon, G.R. (1969). The reactions of resistant and susceptible tomato cultivars to strains of *Verticillium albo-atrum*. *Annals of Applied Biology*, 63(3), 389-400.

Petit, E., and Gubler, W.D. (2005). Characterization of *Cylindrocarpon* species, the cause of black foot disease of grapevine in California. *Plant Disease,* 89(10), 1051–1059.

Pettitt, T.R., Wainwright, M.F., Wakeham, A.J., and White, J.G. (2011). A simple detached leaf assay provides rapid and inexpensive determination of pathogenicity of Pythium isolates to 'all year round' (AYR) chrysanthemum roots. *Plant Pathology*, 60(5), 946–956.

Pezzella, C., Lettera, V., Piscitelli, A., Giardina, P., and Sannia, G. (2013). Transcriptional analysis of *Pleurotus ostreatus* laccase genes. *Applied Microbiology and Biotechnology*, 97, 705–717.

Phillips, A.J.L. (1998). *Botryosphaeria dothidea* and other fungi associated with excoriose and dieback of grapevines in Portugal. *Journal of Phytopathology*, 146(7), 327-332.

Pitt, W.M., Huang, R., Steel, C.C., and Savocchia, S. (2010). Identification, distribution and current taxonomy of *Botryosphaeriaceae* species associated with grapevine decline in New South Wales and South Australia. *Australian Journal of Grape and Wine Research*, 16(1), 258-271.

Pottinger, B., Stewart, A., Carpenter, M., and Ridgway, H. J. (2002). Low genetic variation detected in New Zealand populations of *Phaeomoniella chlamydospora*. *Phytopathologia Mediterranea*, 41, 199-211.

Probst, C., Jones, E.E., Ridgway, H.J., and Jaspers, M.V. (2012). *Cylindrocarpon* black foot in nurseries—two factors that can increase infection. *Australasian Plant Pathology*, 41, 57–163.

Probst, C.M. (2011). *Cylindrocarpon* black foot disease in grapevines identification and epidemiology. PhD thesis, Lincoln University, New Zealand.

Rahman, M., and Punja, Z. K. (2005). Factors influencing development of root rot on ginseng caused by *Cylindrocarpon destructans*. *Phytopathology*, 95, 1381-1390.

Reis, P., Cabral, A., Nascimento, T., Oliveira, H., and Rego, C. (2013). Diversity of *Ilyonectria* species in a young vineyard affected by black foot disease. *Phytopathologia Mediterranea*, 52(2), 335−346.

Rich, J.O., Leathers, T.D., Anderson, A.M., Bischoff, K.M., and Manitchotpisit, P. (2013). Laccases from *Aureobasidium pullulans. Enzyme and Microbial Technology,* 53, 33– 37.

Richter, D.L. (2008). Revival of saprotrophic and mycorrhizal basidiomycete cultures after 20 years in cold storage in sterile water. *Canada Journal of Microbiology*, 54(8), 595-599.

Richter, D.L., and Bruhn, J.N. (1989). Revival of saprotrophic and mycorrhizal basidiomycete cultures from cold storage in sterile water. *Canada Journal of Microbiology*, 35, 1055-1060.

Richter, D.L., Laks, P.E., Larsen, K.M., and Stephens, A.L. (2004). Comparison of isolates and strains within the brown rot fungus genus *Gloeophyllum* using the soil block decay method. *Forest Products Journal*, 55(1), 72-75.

Rodriguez, E., Ruiz-Duenas, F.J., Kooistra, R., Ram, A., Martinez, A.T., and Martinez, M.J. (2008). Isolation of two laccase genes from the white-rot fungus *Pleurotus eryngii* and heterologous expression of the pel3 encoded protein. *Journal of Biotechnology*, 134, 9–19.

Rossetto, M. (2001). Sourcing of SSR markers from related plant species. In: Henry, R.J. (Ed.), Plant Genotyping: the DNA Fingerprinting of Plants. CABI Publishers, New York, 211–224.

Rühl, M., Majcherczyk, A., and Kües, U. (2013). Lcc1 and Lcc5 are the main laccases secreted in liquid cultures of *Coprinopsis cinerea* strains. *Antonie van Leeuwenhoek,* 103, 1029–1039.

Saitou, N., and Nei, M. (1987). The neighbour-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4, 406-425.

Samuels, G.J., and Brayford, D. (1990). Variation in *Nectria radicicola* and its anamorph, *Cylindrocarpon destructans*. *Mycological Research*, 94, 433–442.

Samuels, G.J., and Brayford, D. (1993). Phragmosporous *Nectria* species with *Cylindrocarpon* anamorphs. *Sydowia*, 45, 55–80.

Sathiyaraj, G., Srinivasan, S., Kim, H.B., Subramaniyam, S., Lee, O.R., Kim, Y.J., and Yang, D.C. (2011). Screening and optimization of pectin lyase and polygalacturonase activity from ginseng pathogen *Cylindrocarpon destructans. Brazilian Journal of Microbiology,* 42, 794-806.

Scheck, H., Vasquez, S., Fogle, D., and Gubler, W.D. (1998). Grape growers report losses to black-foot and grapevine decline. *California Agriculture*, 52(4), 19-23.

Schmitt, I., Crespo, A., Divakar, P.K., Fankhauser, J.D., Herman-Sackett, E., Kalb, K., Nelsen, M.P., Nelson, N.A., Rivas-Plata, E., Shimp, A.D., Widhelm, T., and Lambsch, H.T.

(2009). New primers for promising single-copy genes in fungal phylogenetics and systematics. *Persoonia*, 23, 35-40.

Schroers, H.J., Zerjav, M., Munda, A., Halleen, F., and Crous, P.W. (2008). *Cylindrocarpon pauciseptatum* sp. nov., with notes on *Cylindrocarpon* species with wide, predominantly 3-septate macroconidia. *Mycological Research,* 112, 82–92.

Shaw, D.V., Gubler, W.D., and Hansen, J. (1997). Field resistance of Californian strawberries to *Verticillium dahliae* at three conidial inoculum concentrations. *HortScience*, 32(4), 711-713.

Sippell, D.W., and Hall, R. (1982). Effects of pathogen species, inoculum concentration, temperature and soil moisture on bean root rot and plant growth. *Canadian Journal of Plant Pathology*, 4(1), 1-7.

Stamp, J.A. (2001). The contribution of imperfections in nursery stock to the decline of young vines in California. *Phytopathologia Mediterranea*, 40, 369–375.

Swift, C.E. (2002). Vegetative compatibility groups of *Fusarium oxysporum* f. sp. *cepae* from onion in Colorado. *Plant Disease,* 86, 606-610.

Swofford, D. L. (2002). PAUP. Phylogenetic Analysis Using Parsimony (and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28(10), 2731-2739.

ten Have, A., Dekkers, E., Kay, J., and Phylip, L.H. (2004). An aspartic proteinase gene family in the filamentous fungus *Botrytis cinerea* contains members with novel features. *Microbiology*, 150, 2475-2489.

Tewoldemedhin, Y.T., Mazzola, M., Mostert, L., and McLeod, A. (2011). *Cylindrocarpon* species associated with apple tree roots in South Africa and their quantification using real-time PCR. *European Journal of Plant Pathology*, 129, 637–651.

Thurston, C.F. (1994). The structure and function of fungal laccases. *Microbiology*, 140, 9–26.

Torres, J.M.O., and dela Cruz, T.E.E. (2013). Production of xylanases by mangrove fungi from the Philippines and their application in enzymatic pretreatment of recycled paper pulps. *World Journal of Microbiology and Biotechnology*, 29 (4), 645-655.

Úrbez-Torres, J.R., Haag, P., Bowen, P., and O'Gorman, D.T. (2013). Grapevine trunk diseases in British Columbia: incidence and characterization of the fungal pathogens associated with black foot disease of grapevine. *Plant Disease*, 98(4), 456-468.

Uzan, E., Nousiainen, P., Balland, V., Sipila, J., Piumi, F., and Navarro, D. (2010). High redox potential laccases from the ligninolytic fungi *Pycnoporus coccineus* and *Pycnoporus sanguineus* suitable for white biotechnology: from gene cloning to enzyme characterization and applications. *Journal of Applied Microbiology*, 108, 2199–213.

Vasconcelos, A.F.D., Barbosa, A.M., Dekker, R.F.H., Scarminio, I.S., and Rezende, M.I. (2000). Optimisation of laccase production by *Botryosphaeria* sp. in the presence of veratryl alcohol by the response-surface method. *Process Biochem*istry, 35, 1131–1138.

Vasconcelos, A.F.D., Dekker, R.F.H., Barbosa, A.M., and Paccola-Meirelles, L. (2001). Comparison of the laccases, molecular marker proteins, and induction of pycnidia by three species of botryosphaeriaceous fungi. *Mycoscience*, 42(6), 543-548.

Vázquez-Garcidueñas, M.S., Guzmán, I.M., and Vázquez-Marrufo, G. (2012). Molecular genetic identification and extracellular laccase activity of two wild fungal isolates from Mexico. *African Journal of Biotechnology*, 11(4), 829-837.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., and Kuiper, M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research.* 23 (21), 4407–4414.

Ward, E., Foster, S.J., Fraaije, B.A., and McCartney, H.A. (2004). Plant pathogen diagnostics: immunological and nucleic acid-based approaches. *Annals of Applied Biology*, 145 (1), 1-16.

White, T.J., Bruns, T., Lee, S., and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: a guide to methods and applications. (Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., eds). *Academic Press*, New York, USA, 315–322.

Whitelaw-Weckert, M.A., Rahman, L., Appleby, L.M., Hall, A., Clark, A.C., Waite, H., and Hardie, W.J. (2013). Co-infection by *Botryosphaeriaceae* and *Ilyonectria* spp. fungi during propagation causes decline of young grafted grapevines. *Plant Pathology*, (2013) 62, 1226–1237.

Whiteman, S.A., Stewart, A., Ridgway, H.J., and Jaspers, M.V. (2007). Infection of rootstock mother-vines by *Phaeomoniella chlamydospora* results in infected young grapevines. *Australasian Plant Pathology*, 36(2), 198-203.

Xie, N., Leclerc, F.C., Silar, P., and Robert, G.R. (2014). Systematic gene deletions evidences that laccases are involved in several stages of wood degradation in the filamentous fungus *Podospora anserine. Environmental Microbiology,* 16(1), 141–161.

Yang, Y., Wei, F., Zhuo, R., Fan, F., Liu, H., Zhang, C., Ma, L., Jiang, M., and Zhang, X. (2013). Enhancing the laccase production and laccase gene expression in the white-rot fungus *Trametes velutina* 5930 with great potential for biotechnological applications by different metal ions and aromatic compounds. *PLOS ONE*, 8(11), e79307.

Yaseen, T., Ahmed, Y., D'Onghia, A.M., and Digiaro, M. (2012). First report of *cylindrocarpon pauciseptatum* associated with root rot and decline of peach in southern Italy (Apulia region). *Plant Disease*, 96 (5), 764.3 - 764.3.

Yaver, D.S., and Golightly, E.J. (1996). Cloning and characterization of three laccase genes from the white-rot basidiomycete *Trametes villosa*: genomic organization of the laccase gene family. *Gene,* 181, 95–102.

Yaver, D.S., Overjero M.D.C., Xu, F., Nelson, B.A., Brown, K.M., Halkier, T., Bernauer, S., Brown, S.H., and Kauppinen, S. (1999). Molecular characterization of laccase genes from the basidiomycete *Coprinus cinereus* and heterologous expression of the laccase lcc1. *Applied Environmental Microbiology*, 65(11), 4943-4948.

Yeh, F.C., Yang, R.C., and Boyle, T. (1999). POPGENE 32-version 1.31. *Population Genetics Software*. http.//www.ualberta.ca/~fyeh/fyeh/.

Zane, L., Bargelloni, L., and Patarnello, T. (2002). Strategies for microsatellite isolation: a review. *Molecular Ecology*, 11, 1–16.

Zeigler, R.S., Scott, R.P., Leung, H., Bordeos, A.A., Kumar, J., and Nelson, R.J. (1997). Evidence of parasexual exchange of DNA in the rice blast fungus challenges its exclusive clonality. *Phytopathology*, 87(3), 284-294.

Appendix A

A.1 List of isolates and codes and numbers used to name the isolates in this study. All isolates were recovered from symptomatic grapevines.

No	Code	Location recovered from
1	Ack2b	Auckland
2	Ack2e	Auckland
3	CO6g	Central Otago
4	Hb3b	Hawkes Bay
5	Hb6b	Hawkes Bay
6	Mar5a	Marlborough
7	Mar6a	Marlborough
8	Mar14b	Marlborough
9	Mar17d	Marlborough
10	Mar17e	Marlborough
11	Mtb1a	Martinborough

For each code, the letters before number represent sample collection place; number denotes vineyard number; the letter at the end of the code represents location within vineyard and isolate. List of isolates and codes and numbers used to name the isolates in this study.

A.2 DNA sequences from the *C. pauciseptatum* isolates: ITS, elongation factor 1-α, β-tubulin and histone genes.

A.2.1 ITS sequences of *C. pauciseptatum* isolates

Ack 2b

```
1       GGGGGTTTTA CGGCGTGGCC GCGCTGTTTC CCAGTGCTAA GTGTGCTACT ACGCAGAGGA
61      AGTTACAGCG TGACCGCCAC TAAATTTAGG GGACGGCGGG CGCTGAGGCT CGCCGATCCC
121     CAACACCAAG CCCGGGGGCT TGAGGGATGT AATGACGCTC GAACAGGCAT GCCCGCCAGA
181     ATACTGGCGG GCGCAATGTG CGTTCAAAGA TTCGATGATT CACTGAATTC TGCAATTCAC
241     ATTACTTATC GCATTTCGCT GCGTTCTTCA TCGATGCCAG AACCAAGAGA TCCGTTGTTG
301     AAAGTTTTGA TTGATTTAAT CGGTTACTCA GAAGACTCAC TCAAGGAAGT TTGGGTTTGG
361     GGTCCTCTGG CGGGCTGTCG GAACAGGCAC CGCCGAGGCA ACAAAATGGT ATGTTCACAG
421     GGGTTTGGGA GTTGAAAACT CGGTAATGAT CCCTCCGCTG GTTCACCAAC GGAGACCTTG
```

Ack 2e

```
1       GGGGGTTTTA CGGCGTGGCC GCGCTGTTTC CCAGTGCTAA GTGTGCTACT ACGCAGAGGA
61      AGTTACAGCG TGACCGCCAC TAAATTTAGG GGACGGCGGG CGCTGAGGCT CGCCGATCCC
121     CAACACCAAG CCCGGGGGCT TGAGGGATGT AATGACGCTC GAACAGGCAT GCCCGCCAGA
181     ATACTGGCGG GCGCAATGTG CGTTCAAAGA TTCGATGATT CACTGAATTC TGCAATTCAC
241     ATTACTTATC GCATTTCGCT GCGTTCTTCA TCGATGCCAG AACCAAGAGA TCCGTTGTTG
301     AAAGTTTTGA TTGATTTAAT CGGTTACTCA GAAGACTCAC TCAAGGAAGT TTGGGTTTGG
361     GGTCCTCTGG CGGGCTGTCG GAACAGGCAC CGCCGAGGCA ACAAAATGGT ATGTTCACAG
421     GGGTTTGGGA GTTGAAAACT CGGTAATGAT CCCTCCGCTG GTTCACCAAC
```

CO6g

```
1       TTCGAGGTCA ACGTTCAAAA GTTGGGGGGT TTTACGGCGT GGCCGCGCTG TTTCCCAGTG
61      CTAAGTGTGC TACTACGCAG AGGAAGTTAC AGCGTGACCG CCACTAAATT TAGGGGACGG
121     CGGGCGCTGA GGCTCGCCGA TCCCCAACAC CAAGCCCGGG GGCTTGAGGG ATGTAATGAC
181     GCTCGAACAG GCATGCCCGC CAGAATACTG GCGGGCGCAA TGTGCGTTCA AAGATTCGAT
241     GATTCACTGA ATTCTGCAAT TCACATTACT TATCGCATTT CGCTGCGTTC TTCATCGATG
301     CCAGAACCAA GAGATCCGTT GTTGAAAGTT TTGATTGATT TAATCGGTTA CTCAGAAGAC
361     TCACTCAAGG AAGTTTGGGT TTGGGGTCCT CTGGCGGGCT GTCGGAACAG GCACCGCCGA
421     GGCAACAAAA TGGTATGTTC ACAGGGGTTT GGGAGTTGAA AACTCGGTAA TGATCCCTCC
481     GCTGGTTCAC CAACGGAGAC CTTGTTACGA CTTTTACTTC CTCTA
```

Hb3b

```
1       GGGGTTTTAC GGCGTGGCCG CGCTGTTTCC CAGTGCTAAG TGTGCTACTA CGCAGAGGAA
61      GTTACAGCGT GACCGCCACT AAATTTAGGG GACGGCGGGC GCTGAGGCTC GCCGATCCCC
121     AACACCAAGC CCGGGGGCTT GAGGGATGTA ATGACGCTCG AACAGGCATG CCCGCCAGAA
181     TACTGGCGGG CGCAATGTGC GTTCAAAGAT TCGATGATTC ACTGAATTCT GCAATTCACA
241     TTACTTATCG CATTTCGCTG CGTTCTTCAT CGATGCCAGA ACCAAGAGAT CCGTTGTTGA
301     AAGTTTTGAT TGATTTAATC GGTTACTCAG AAGACTCACT CAAGGAAGTT TGGGTTTGGG
361     GTCCTCTGGC GGGCTGTCGG AACAGGCACC GCCGAGGCAA CAAAATGGTA TGTTCACAGG
421     GGTTTGGGAG TTGAAAACTC GGTAATGATC CCTCCGCTGG TTCACCAACG GAGACCTTGT
481     TACGACTTTT ACTTCCTCTA
```

Hb6b

```
1       TCAAAAGTTG GGGGGTTTTA CGGCGTGGCC GCGCTGTTTC CCAGTGCTAA GTGTGCTACT
61      ACGCAGAGGA AGTTACAGCG TGACCGCCAC TAAATTTAGG GGACGGCGGG CGCTGAGGCT
121     CGCCGATCCC CAACACCAAG CCCGGGGGCT TGAGGGATGT AATGACGCTC GAACAGGCAT
181     GCCCGCCAGA ATACTGGCGG GCGCAATGTG CGTTCAAAGA TTCGATGATT CACTGAATTC
241     TGCAATTCAC ATTACTTATC GCATTTCGCT GCGTTCTTCA TCGATGCCAG AACCAAGAGA
301     TCCGTTGTTG AAAGTTTTGA TTGATTTAAT CGGTTACTCA GAAGACTCAC TCAAGGAAGT
361     TTGGGTTTGG GGTCCTCTGG CGGGCTGTCG GAACAGGCAC CGCCGAGGCA ACAAAATGGT
421     ATGTTCACAG
```

Mar5a

```
1       GGGGGTTTTA CGGCGTGGCC GCGCTGTTTC CCAGTGCTAA GTGTGCTACT ACGCAGAGGA
61      AGTTACAGCG TGACCGCCAC TAAATTTAGG GGACGGCGGG CGCTGAGGCT CGCCGATCCC
121     CAACACCAAG CCCGGGGGCT TGAGGGATGT AATGACGCTC GAACAGGCAT GCCCGCCAGA
181     ATACTGGCGG GCGCAATGTG CGTTCAAAGA TTCGATGATT CACTGAATTC TGCAATTCAC
241     ATTACTTATC GCATTTCGCT GCGTTCTTCA TCGATGCCAG AACCAAGAGA TCCGTTGTTG
301     AAAGTTTTGA TTGATTTAAT CGGTTACTCA GAAGACTCAC TCAAGGAAGT TTGGGTTTGG
361     GGTCCTCTGG CGGGCTGTCG GAACAGGCAC CGCCGAGGCA ACAAAATGGT ATGTTCACAG
421     GGGTTTGGGA GTTGAAAACT CGGTAATGAT CCCTCCGCTG GTTCACCAAC GGAGACCTTG
481     TTACGACTTT
```

Mar6a

```
1       GGGGTTTTAC GGCGTGGCCG CGCTGTTTCC CAGTGCTAAG TGTGCTACTA CGCAGAGGAA
61      GTTACAGCGT GACCGCCACT AAATTTAGGG GACGGCGGGC GCTGAGGCTC GCCGATCCCC
121     AACACCAAGC CCGGGGGCTT GAGGGATGTA ATGACGCTCG AACAGGCATG CCCGCCAGAA
181     TACTGGCGGG CGCAATGTGC GTTCAAAGAT TCGATGATTC ACTGAATTCT GCAATTCACA
241     TTACTTATCG CATTTCGCTG CGTTCTTCAT CGATGCCAGA ACCAAGAGAT CCGTTGTTGA
301     AAGTTTTGAT TGATTTAATC GGTTACTCAG AAGACTCACT CAAGGAAGTT TGGGTTTGGG
361     GTCCTCTGGC GGGCTGTCGG AACAGGCACC GCCGAGGCAA CAAAATGGTA TGTTCACAGG
421     GGTTTGGGAG TTGAAAACTC GGTAATGATC CCTCCGCTGG TTCACCAACG GAGACCTTGT
```

Mar14b

```
1       GGGGGTTTTA CGGCGTGGCC GCGCTGTTTC CCAGTGCTAA GTGTGCTACT ACGCAGAGGA
61      AGTTACAGCG TGACCGCCAC TAAATTTAGG GGACGGCGGG CGCTGAGGCT CGCCGATCCC
121     CAACACCAAG CCCGGGGGCT TGAGGGATGT AATGACGCTC GAACAGGCAT GCCCGCCAGA
181     ATACTGGCGG GCGCAATGTG CGTTCAAAGA TTCGATGATT CACTGAATTC TGCAATTCAC
241     ATTACTTATC GCATTTCGCT GCGTTCTTCA TCGATGCCAG AACCAAGAGA TCCGTTGTTG
301     AAAGTTTTGA TTGATTTAAT CGGTTACTCA GAAGACTCAC TCAAGGAAGT TTGGGTTTGG
361     GGTCCTCTGG CGGGCTGTCG GAACAGGCAC CGCCGAGGCA ACAAAATGGT ATGTTCACAG
421     GGGTTTGGGA GTTGAAAACT CGGTAATGAT CCCTCCGCTG GTTCACCAAC GGAGACCTTG
```

Mar17d

```
1       CAAAAGTTGG GGGGTTTTAC GGCGTGGCCG CGCTGTTTCC CAGTGCTAAG TGTGCTACTA
61      CGCAGAGGAA GTTACAGCGT GACCGCCACT AAATTTAGGG GACGGCGGGC GCTGAGGCTC
121     GCCGATCCCC AACACCAAGC CCGGGGGCTT GAGGGATGTA ATGACGCTCG AACAGGCATG
181     CCCGCCAGAA TACTGGCGGG CGCAATGTGC GTTCAAAGAT TCGATGATTC ACTGAATTCT
241     GCAATTCACA TTACTTATCG CATTTCGCTG CGTTCTTCAT CGATGCCAGA ACCAAGAGAT
301     CCGTTGTTGA AAGTTTTGAT TGATTTAATC GGTTACTCAG AAGACTCACT CAAGGAAGTT
361     TGGGTTTGGG GTCCTCTGGC GGGCTGTCGG AACAGGCACC GCCGAGGCAA CAAAATGGTA
421     TGTTCACAGG GGTTTGGGAG TTGAAAACTC GGTAATGATC CCTCCGCTGG TTCACCAACG
481     GAGACCTTGT
```

Mar17e

```
1       GGGGTTTTAC GGCGTGGCCG CGCTGTTTCC CAGTGCTAAG TGTGCTACTA CGCAGAGGAA
61      GTTACAGCGT GACCGCCACT AAATTTAGGG GACGGCGGGC GCTGAGGCTC GCCGATCCCC
121     AACACCAAGC CCGGGGGCTT GAGGGATGTA ATGACGCTCG AACAGGCATG CCCGCCAGAA
181     TACTGGCGGG CGCAATGTGC GTTCAAAGAT TCGATGATTC ACTGAATTCT GCAATTCACA
241     TTACTTATCG CATTTCGCTG CGTTCTTCAT CGATGCCAGA ACCAAGAGAT CCGTTGTTGA
301     AAGTTTTGAT TGATTTAATC GGTTACTCAG AAGACTCACT CAAGGAAGTT TGGGTTTGGG
361     GTCCTCTGGC GGGCTGTCGG AACAGGCACC GCCGAGGCAA CAAAATGGTA TGTTCACAGG
421     GGTTTGGGAG TTGAAAACTC GGTAATGATC CCTCCGCTGG TTCACCAACG GAGACCTTGT
```

Mtb1a

```
1       GGGGGTTTTA CGGCGTGGCC GCGCTGTTTC CCAGTGCTAA GTGTGCTACT ACGCAGAGGA
61      AGTTACAGCG TGACCGCCAC TAAATTTAGG GGACGGCGGG CGCTGAGGCT CGCCGATCCC
121     CAACACCAAG CCCGGGGGCT TGAGGGATGT AATGACGCTC GAACAGGCAT GCCCGCCAGA
181     ATACTGGCGG GCGCAATGTG CGTTCAAAGA TTCGATGATT CACTGAATTC TGCAATTCAC
241     ATTACTTATC GCATTTCGCT GCGTTCTTCA TCGATGCCAG AACCAAGAGA TCCGTTGTTG
301     AAAGTTTTGA TTGATTTAAT CGGTTACTCA GAAGACTCAC TCAAGGAAGT TTGGGTTTGG
361     GGTCCTCTGG CGGGCTGTCG GAACAGGCAC CGCCGAGGCA ACAAAATGGT ATGTTCACAG
421     GGGTTTGGGA GTTGAAAACT CGGTAATGAT CCCTCCGCTG GTTCACCAAC GGAGACCTTG
481     TTACGACTTT TACTTCCTCT
```

A.2.2 Elongation factor 1-α genes sequences of *C. pauciseptatum* isolates

Ack2b

```
1       CGCCACACAT GCCTCGCCGT GACAGCTTGC TGACCTGCCT CCCAGGGGTT CGTACCACCA
61      CAACACTCTT CGAGTTATTA CCAAACAGCT TGCTGACACC CTCGACAGCC ACGTCGACTC
121     TGGCAAGTCG ACCACCGTGA GTATTCCCGC TACCATGACC GTCTTATCTC GGTTGCATTA
181     ACCCGCCATG ACCAGCGCGG GGTATTTCTA TCACGGCTTA CTGACATTCA TCAATAGACT
241     GGTCACTTGA TCTACCAGTG CGGTGGTATC GACAAGCGAA CCATCGAGAA GTTCGAGAAG
301     GTTGGTCCTC TTTTTTCCGA TTTTATCCAC CTCGATCGTC GGTCGATTCC CACGTTGCGC
```

```
361     TGCTTATGCG CTCGACGCAT ATACTCATCC CTCGATCAAT TTTTTTTTCC CACACACTGC
421     ATTGTTTTTT TGGTGGGGCG AATTTTACCC CTCCACACAA TCGTGGTCGA AATTTGCCCC
481     ACCCCACCCC AGCAACACTG AACCACCAAC CCGAGCGTCG TCACATGCTA
```

Ack2e

```
1       CTCGCCGTGA CAGCTTGCTG ACCTGCCTCC CAGGGGTTCG TACCACCACA ACACTCTTCG
61      AGTTATTACC AAACAGCTTG CTGACACCCT CGACAGCCAC GTCGACTCTG GCAAGTCGAC
121     CACCGTGAGT ATTCCCGCTA CCATGACCGT CTTATCTCGG TTGCATTAAC CCGCCATGAC
181     CAGCGCGGGG TATTTCTATC ACGGCTTACT GACATTCATC AATAGACTGG TCACTTGATC
241     TACCAGTGCG GTGGTATCGA CAAGCGAACC ATCGAGAAGT TCGAGAAGGT TGGTCCTCTT
301     TTTTCCGATT TTATCCACCT CGATCGTCGG TCGATTCCCA CGTTGCGCTG CTTATGCGCT
361     CGACGCATAT ACTCATCCCT CGATCAATTT TTTTTTCCCA CACACTGCAT TGTTTTTTTG
421     GTGGGGCGAA TTTTACCCCT CCACACAATC GTGGTCGAAA TTTGCCCCAC CCCACCCCAG
```

CO6g

```
1       TCGCCACACA TGCCTCGCCG TGACAGCTTG CTGACCTGCC TCCCAGGGGT TCGTACCACC
61      ACAACACTCT TCGAGTTATT ACCAAACAGC TTGCTGACAC CCTCGACAGC CACGTCGACT
121     CTGGCAAGTC GACCACCGTG AGTATTCCCG CTACCATGAC CGTCTTATCT CGGTTGCATT
181     AACCCGCCAT GACCAGCGCG GGGTATTTCT ATCACGGCTT ACTGACATTC ATCAATAGAC
241     TGGTCACTTG ATCTACCAGT GCGGTGGTAT CGACAAGCGA ACCATCGAGA AGTTCGAGAA
301     GGTTGGTCCT CTTTTTTCCG ATTTTATCCA CCTCGATCGT CGGTCGATTC CCACGTTGCG
361     CTGCTTATGC GCTCGACGCA TATACTCATC CCTCGATCAA TTTTTTTTCC CCACACACTG
421     CATTGTTTTT TTGGTGGGGC GAATTTTACC CCTCCACACA ATCGTGGTCG AAATTTGCCC
481     CACCCCACCC CAGCAACACT GAACCACCAA CCCGAGCGTC GTCACATGCT ATGCACAGAA
541     TACTGACAGA GACGTTTGCA GGAAGCCGCC
```

Hb3b

```
1       GCTCATCGCC ACACATGCCT CGCCGTGACA GCTTGCTGAC CTGCCTCCCA GGGGTTCGTA
61      CCACCACAAC ACTCTTCGAG TTATTACCAA ACAGCTTGCT GACACCCTCG ACAGCCACGT
121     CGACTCTGGC AAGTCGACCA CCGTGAGTAT TCCCGCTACC ATGACCGTCT TATCTCGGTT
181     GCATTAACCC GCCATGACCA GCGCGGGGTA TTTCTATCAC GGCTTACTGA CATTCATCAA
241     TAGACTGGTC ACTTGATCTA CCAGTGCGGT GGTATCGACA AGCGAACCAT CGAGAAGTTC
301     GAGAAGGTTG GTCCTCTTTT TTCCGATTTT ATCCACCTCG ATCGTCGGTC GATTCCCACG
361     TTGCGCTGCT TATGCGCTCG ACGCATATAC TCATCCCTCG ATCAATTTTT TTTTCCCACA
421     CACTGCATTG TTTTTTTGGT GGGGCGAATT TTACCCCTCC ACACAATCGT GGTCGAAATT
481     TGCCCCACCC CACCCCAGCA ACACTGAACC ACCAACCCGA GCGTCGTCAC ATGCTATGCA
541     CAGAATACTG ACAGAGACGT TTGCAGGAAG CCGCCGAGCT
```

Hb6b

```
1       CACACATGCC TCGCCGTGAC AGCTTGCTGA CCTGCCTCCC AGGGGTTCGT ACCACCACAA
61      CACTCTTCGA GTTATTACCA AACAGCTTGC TGACACCCTC GACAGCCACG TCGACTCTGG
121     CAAGTCGACC ACCGTGAGTA TTCCCGCTAC CATGACCGTC TTATCTCGGT TGCATTAACC
181     CGCCATGACC AGCGCGGGGT ATTTCTATCA CGGCTTACTG ACATTCATCA ATAGACTGGT
241     CACTTGATCT ACCAGTGCGG TGGTATCGAC AAGCGAACCA TCGAGAAGTT CGAGAAGGTT
301     GGTCCTCTTT TTTCCGATTT TATCCACCTC GATCGTCGGT CGATTCCCAC GTTGCGCTGC
361     TTATGCGCTC GACGCATATA CTCATCCCTC GATCAATTTT TTTTTCCCAC ACACTGCATT
421     GTTTTTTTGG TGGGGCGAAT TTTACCCCTC CACACAATCG TGGTCGAAAT TTGCCCCACC
481     CCACCCCAGC AACACTGAAC CACCAACCCG AGCGTCGTCA CATGCTATGC ACAGAATACT
541     GACAGAGACG TTTGCAGGAA GCCGCCGAGC
```

Mar5a

```
1       ACACATGCCT CGCCGTGACA GCTTGCTGAC CTGCCTCCCA GGGGTTCGTA CCACCACAAC
61      ACTCTTCGAG TTATTACCAA ACAGCTTGCT GACACCCTCG ACAGCCACGT CGACTCTGGC
121     AAGTCGACCA CCGTGAGTAT TCCCGCTACC ATGACCGTCT TATCTCGGTT GCATTAACCC
181     GCCATGACCA GCGCGGGGTA TTTCTATCAC GGCTTACTGA CATTCATCAA TAGACTGGTC
```

```
241      ACTTGATCTA CCAGTGCGGT GGTATCGACA AGCGAACCAT CGAGAAGTTC GAGAAGGTTG
301      GTCCTCTTTT TTCCGATTTT ATCCACCTCG ATCGTCGGTC GATTCCCACG TTGCGCTGCT
361      TATGCGCTCG ACGCATATAC TCATCCCTCG ATCAATTTTT TTTTCCCACA CACTGCATTG
421      TTTTTTTGGT GGGGCGAATT TTACCCCTCC ACACAATCGT GGTCGAAATT TGCCCCACCC
481      CACCCCAGCA ACACTGAACC ACCAACCCGA GCGTCGTCAC ATGCTATGCA CAGAATACTG
541      ACAGAGACGT TTGCAGGAAG CCGCCGAGCT
```

Mar6a

```
1        CACACATGCC TCGCCGTGAC AGCTTGCTGA CCTGCCTCCC AGGGGTTCGT ACCACCACAA
61       CACTCTTCGA GTTATTACCA AACAGCTTGC TGACACCCTC GACAGCCACG TCGACTCTGG
121      CAAGTCGACC ACCGTGAGTA TTCCCGCTAC CATGACCGTC TTATCTCGGT TGCATTAACC
181      CGCCATGACC AGCGCGGGGT ATTTCTATCA CGGCTTACTG ACATTCATCA ATAGACTGGT
241      CACTTGATCT ACCAGTGCGG TGGTATCGAC AAGCGAACCA TCGAGAAGTT CGAGAAGGTT
301      GGTCCTCTTT TTTCCGATTT TATCCACCTC GATCGTCGGT CGATTCCCAC GTTGCGCTGC
361      TTATGCGCTC GACGCATATA CTCATCCCTC GATCAATTTT TTTCCCCACA CACTGCATTG
421      TTTTTTTGGT GGGGCGAATT TTACCCCTCC ACACAATCGT GGTCGAAATT TGCCCCACCC
481      CACCCCAGCA ACACTGAACC ACCAACCCGA
```

Mar14b

```
1        CTGGGGGGTA TCGCCAGTCG ACGCTCATCG CCACACATGC CTCGCCGTGA CAGCTTGCTG
61       ACCTGCCTCC CAGGGGTTCG TACCACCACA ACACTCTTCG AGTTATTACC AAACAGCTTG
121      CTGACACCCT CGACAGCCAC GTCGACTCTG GCAAGTCGAC CACCGTGAGT ATTCCCGCTA
181      CCATGACCGT CTTATCTCGG TTGCATTAAC CCGCCATGAC CAGCGCGGGG TATTTCTATC
241      ACGGCTTACT GACATTCATC AATAGACTGG TCACTTGATC TACCAGTGCG GTGGTATCGA
301      CAAGCGAACC ATCGAGAAGT TCGAGAAGGT TGGTCCTCTT TTTTCCGATT TTATCCACCT
361      CGATCGTCGG TCGATTCCCA CGTTGCGCTG CTTATGCGCT CGACGCATAT ACTCATCCCT
421      CGATCAATTT TTTTTTCCCA CACACTGCAT TGTTTTTTTG GTGGGGCGAA TTTTACCCCT
481      CCACACAATC GTGGTCGAAA TTTGCCCCAC
```

Mar17d

```
1        CGCCGTGACA GCTTGCTGAC CTGCCTCCCA GGGGTTCGTA CCACCACAAC ACTCTTCGAG
61       TTATTACCAA ACAGCTTGCT GACACCCTCG ACAGCCACGT CGACTCTGGC AAGTCGACCA
121      CCGTGAGTAT TCCCGCTACC ATGACCGTCT TATCTTGGTT GCATTAACCC GCCATGACCA
181      GCGCGGGGTA TTTCTATCAC GGCTTACTGA CATTCATCAA TAGACTGGTC ACTTGATCTA
241      CCAGTGCGGT GGTATCGACA AGCGAACCAT CGAGAAGTTC GAGAAGGTTG GTCCTCTTTT
301      TTCCGATTTT ATCCACCTCG ATCGTCGGTC GATTCCCACG TTGCGCTGCT TATGCGCTCG
361      ACGCATATAC TCATCCCTCG ATCAATTTTT TTTTCCCACA CACTGCATTG TTTTTTTGGT
421      GGGGCGAATT TTACCCCTCC ACACAATCGT GGTCGAAATT TGCCCCACCC CACCCCAGCA
```

Mar17e

```
1        GCCACACATG CCTCGCCGTG ACAGCTTGCT GACCTGCCTC CCAGGGGTTC GTACCACCAC
61       AACACTCTTC GAGTTATTAC CAAACAGCTT GCTGACACCC TCGACAGCCA CGTCGACTCT
121      GGCAAGTCGA CCACCGTGAG TATTCCCGCT ACCATGACCG TCTTATCTCG GTTGCATTAC
181      CCCGCCATGA CCAGCGCGGG GTATTTCTAT CACGGCTTAC TGACATTCAT CAATAGACTG
241      GTCACTTGAT CTACCAGTGC GGTGGTATCG ACAAGCGAAC CATCGAGAAG TTCGAGAAGG
301      TTGGTCCTCT TTTTTCCGAT TTTATCCACC TCGATCGTCG GTCGATTCCC ACGTTGCGCT
361      GCTTATGCGC TCGACGCATA TACTCATCCC TCGATCAATT TTTTTTTCCC ACACACTGCA
421      TTGTTTTTTT GGTGGGGCGA ATTTTACCCC TCCACACAAT CGTGGTCGAA ATTTGCCCCA
```

Mtb1a

```
1        CATGCCTCGC CGTGACAGCT TGCTGACCTG CCTCCCAGGG GTTCGTACCA CCACAACACT
61       CTTCGAGTTA TTACCAAACA GCTTGCTGAC ACCCTCGACA GCCACGTCGA CTCTGGCAAG
121      TCGACCACCG TGAGTATTCC CGCTACCATG ACCGTCTTGT CTTGGTTGCA TTAACCCGCC
181      ATGACCAGCG CGGGGTATTT CTATCACGGC TTACTGACAT TCATCAATAG ACTGGTCACT
```

```
241      TGATCTACCA GTGCGGTGGT ATCGACAAGC GAACCATCGA GAAGTTCGAG AAGGTTGGTC
301      CTCTTTTTTC CGATTTTATC CACCTCGATC GTCGGTCGAT TCCCACGTTG CGCTGCTTAT
361      GCGCTCGACG CATATACTCA TCCCTCGATC AATTTTTTTT TC
```

A.2.3 β-tubulin genes sequences of *C. pauciseptatum* isolates

Ack2b

```
1        TTGTAAGTTG CCTGCCTCCT GCTGTGCTTG GCTCAGTCTC GACGACGCGG CTGTCGTTAC
61       CTCTGGGTGC TGCCCCTGAT TCTACCCCGC TGCAGCATTT CCACCGCCTC GAGCAAAACA
121      AAGCAGCGGC TTCAACCACG ACGCGATTCT GGGACATAAT GGCTAATATG ACTTCTTCTG
181      CAACATAGGT CCACCTCCAG ACCGGCCAGT GCGTAAGTGG TCCTTCTTCT TCCTCGGCCG
241      CACGGCAGAG ACTCTAACAA CGCGTGGATA GGGTAACCAA ATCGGTGCTG CTTTCTGGCA
301      GACCATCTCC GGCGAGCATG GTCTTGACAG CAATGGTGTC TACAACGGCA CTTCCGAGCT
361      CCAGCTCGAA CGCATGAGCG TCTACTTCAA CGAGGTACGT GAATAAACTC TGCTGCCTGC
421      TCGGCCGCTG GAAGCACGAA ACTCACACCA CCCAGGCTTC TGGCAACAAG TATGTCCCTC
481      GCGCCGTCCT CGTCGATCTC GAGCCCGGTA CCATGGACGC CGTCCGTGCC GGCCCCTTCG
541      GCCAGCTCTT CCGTCCCGAC AAC
```

Ack2e

```
1        AGATTGTAAG TTGCCTGCCT CCAGCTGTGC TTGGCTCAGT CTCGACGACG CGGCTGTCGT
61       TACCTCTGGG TGCTGCCCCT GATTCTACCC CGCTGCAGCA TTTCCACCGC CTCGAGCAAA
121      ACAAAGCAGC GGCTTCAACC ACGACGCGAT TCTGGGACAT AATGGCTAAT ATGACTTCTT
181      CTGCAACATA GGTCCACCTC CAGACCGGCC AGTGCGTAAG TGGTCCTTCT TCTTCCTCGG
241      CCGCACGGCA GAGACTCTAA CAACGCGTGG ATAGGGTAAC CAAATCGGTG CTGCTTTCTG
301      GCAGACCATC TCCGGCGAGC ATGGTCTTGA CAGCAATGGT GTCTACAACG GCACTTCCGA
361      GCTCCAGCTC GAACGCATGA GCGTCTACTT CAACGAGGTA CGTGAATAAA CTCTGCTGCC
421      TGCTCGGCCG CTGGAAGCAC GAAACTCACA CCACCCAGGC TTCTGGCAAC AAGTATGTCC
481      CTCGCGCCGT CCTCGTCGAT CTCGAGCCCG GTACCATGGA CGCCGTCCGT GCCGGCCCCT
541      TCGGCCAGCT CTTCCGTCCC GACAACT
```

CO6g

```
1        TGTAAGTTGC CTGCCTCCTG CTGTGCTTGG CTCGGTCTCG ACGACGCGGC TGTCGTTACC
61       TCTGGGCGCT GCCCCTGATT CTACCCCGCT GCAGCATTTC CACCGCCTCG AGCAAAACAA
121      AGCAGCGGCT TCAACCACGA CGCGATTCTG GGACATGATG GCTAATATGA CTTCTTCTGC
181      AACATAGGTC CACCTCCAGA CCGGCCAGTG CGTAAGTGCT CCTTCTTTTT CCTCGACGGC
241      ACGGCAGAGA CTCTAACAAC GCGTGGATAG GGTAACCAAA TTGGTGCTGC TTTCTGGCAG
301      ACCATCTCCG GCGAGCATGG TCTTGACAGC AATGGTGTCT ACAACGGCAC TTCCGAGCTC
361      CAGCTCGAAC GCATGAGCGT CTACTTCAAC GAGGTACGTG AATAAACTCT GCTGCCTGCT
421      CGGCCGCTGG AAGCACGAAA CTCACACCAC CCAGGCCTCT GGCAACAAGT ATGTCCCTCG
481      CGCCGTCCTC GTCGATCTCG AGCCCGGTAC CATGGACGCC GTCCGTGCCG GCCCCTTCGG
541      CCAGCTCTTC CGTCCCGACA A
```

Hb3b

```
1        TTGTAAGTTG CCTGCCTCCT GCTGTGCTTG GCTCAGTCTC GACGACGCGG CTGTCGTTAC
61       CTCTGGGTGC TGCCCCTGAT TCTACCCCGC TGCAGCATTT CCACCGCCTC GAGCAAAACA
121      AAGCAGCGGC TTCAACCACG ACGCGATTCT GGGACATAAT GGCTAATATG ACTTCTTCTG
181      CAACATAGGT CCACCTCCAG ACCGGCCAGT GCGTAAGTGG TCCTTCTTCT TCCTCGGCCG
241      CACGGCAGAG ACTCTAACAA CGCGTGGATA GGGTAACCAA ATCGGTGCTG CTTTCTGGCA
301      GACCATCTCC GGCGAGCATG GTCTTGACAG CAATGGTGTC TACAACGGCA CTTCCGAGCT
361      CCAGCTCGAA CGCATGAGCG TCTACTTCAA CGAGGTACGT GAATAAACTC TGCTGCCTGC
421      TCGGCCGCTG GAAGCACGAA ACTCACACCA CCCAGGCTTC TGGCAACAAG TATGTCCCTC
481      GCGCCGTCCT CGTCGATCTC GAGCCCGGTA CCATGGACGC CGTCCGTGCC GGCCCCTTCG
541      GCCAGCTCTT CCGTCCCGAC AACTT
```

Hb6b

```
1       TTGTAAGTTG CCTGCCTCCT GCTGTGCTTG GCTCAGTCTC GACGACGCGG CTGTCGTTAC
61      CTCTGGGTGC TGCCCCTGAT TCTACCCCGC TGCAGCATTT CCACCGCCTC GAGCAAAACA
121     AAGCAGCGGC TTCAACCACG ACGCGATTCT GGGACATAAT GGCTAATATG ACTTCTTCTG
181     CAACATAGGT CCACCTCCAG ACCGGCCAGT GCGTAAGTGG TCCTTCTTCT TCCTCGGCCG
241     CACGGCAGAG ACTCTAACAA CGCGTGGATA GGGTAACCAA ATCGGTGCTG CTTTCTGGCA
301     GACCATCTCC GGCGAGCATG GTCTTGACAG CAATGGTGTC TACAACGGCA CTTCCGAGCT
361     CCAGCTCGAA CGCATGAGCG TCTACTTCAA CGAGGTACGT GAATAAACTC TGCTGCCTGC
421     TCGGCCGCTG GAAGCACGAA ACTCACACCA CCCAGGCTTC TGGCAACAAG TATGTCCCTC
481     GCGCCGTCCT CGTCGATCTC GAGCCCGGTA CCATGGACGC CGTCCGTGCC GGCCCCTTCG
541     GCCAGCTCTT CCGTCCCGAC AACTTTGTTT CGGTC
```

Mar5a

```
1       ATTGTAAGTT GCCTGCCTCC TGCTGTGCTT GGCTCAGTCT CGACGACGCG GCTGTCGTTA
61      CCTCTGGGTG CTGCCCCTGA TTCTACCCCG CTGCAGCATT TCCACCGCCT CGAGCAAAAC
121     AAAGCAGCGG CTTCAACCAC GACGCGATTC TGGGACATAA TGGCTAATAT GACTTCTTCT
181     GCAACATAGG TCCACCTCCA GACCGGCCAG TGCGTAAGTG GTCCTTCTTC TTCCTCGGCC
241     GCACGGCAGA GACTCTAACA ACGCGTGGAT AGGGTAACCA AATCGGTGCT GCTTTCTGGC
301     AGACCATCTC CGGCGAGCAT GGTCTTGACA GCAATGGTGT CTACAACGGC ACTTCCGAGC
361     TCCAGCTCGA ACGCATGAGC GTCTACTTCA ACGAGGTACG TGAATAAACT CTGCTGCCTG
421     CTCGGCCGCT GGAAGCACGA AACTCACACC ACCCAGGCTT CTGGCAACAA GTATGTCCCT
481     CGCGCCGTCC TCGTCGATCT CGAGCCCGGT ACCATGGACG CCGTCCGTGC CGGCCCCTTC
541     GGCCAGCTCT TCCGTCCCGA CAACTT
```

Mar6a

```
1       TGTAAGTTGC CTGCCTCCTG CTGTGCTTGG CTCGGTCTCG ACGACGCGGC TGTCGTTACC
61      TCTGGGCGCT GCCCCTGATT CTACCCCGCT GCAGCATTTC CACCGCCTCG AGCAAAACAA
121     AGCAGCGGCT TCAACCACGA CGCGATTCTG GGACATGATG GCTAATATGA CTTCTTCTGC
181     AACATAGGTC CACCTCCAGA CCGGCCAGTG CGTAAGTGCT CCTTCTTTTT CCTCGACGGC
241     ACGGCAGAGA CTCTAACAAC GCGTGGATAG GGTAACCAAA TTGGTGCTGC TTTCTGGCAG
301     ACCATCTCCG GCGAGCATGG TCTTGACAGC AATGGTGTCT ACAACGGCAC TTCCGAGCTC
361     CAGCTCGAAC GCATGAGCGT CTACTTCAAC GAGGTACGTG AATAAACTCT GCTGCCTGCT
421     CGGCCGCTGG AAGCACGAAA CTCACACCAC CCAGGCCTCT GGCAACAAGT ATGTCCCTCG
481     CGCCGTCCTC GTCGATCTCG AGCCCGGTAC CATGGACGCC GTCCGTGCCG GCCCCTTCGG
541     CCAGCTCTTC CGTCCCGACA ACTTG
```

Mar14b

```
1       AGATTGTAAG TTGCCTGCCT CCTGCTGTGC TTGGCTCAGT CTCGACGACG CGGCTGTCGT
61      TACCTCTGGG TGCTGCCCCT GATTCTACCC CGCTGCAGCA TTTCCACCGC CTCGAGCAAA
121     ACAAAGCAGC GGCTTCAACC ACGACGCGAT TCTGGGACAT AATGGCTAAT ATGACTTCTT
181     CTGCAACATA GGTCCACCTC CAGACCGGCC AGTGCGTAAG TGGTCCTTCT TCTTCCTCGG
241     CCGCACGGCA GAGACTCTAA CAACGCGTGG ATAGGGTAAC CAAATCGGTG CTGCTTTCTG
301     GCAGACCATC TCCGGCGAGC ATGGTCTTGA CAGCAATGGT GTCTACAACG GCACTTCCGA
361     GCTCCAGCTC GAACGCATGA GCGTCTACTT CAACGAGGTA CGTGAATAAA CTCTGCTGCC
421     TGCTCGGCCG CTGGAAGCAC GAAACTCACA CCACCCAGGC TTCTGGCAAC AAGTATGTCC
481     CTCGCGCCGT CCTCGTCGAT CTCGAGCCCG GTACCATGGA CGCCGTCCGT GCCGGCCCCT
541     TCGGCCAGCT CTTCCGTCCC GACAAC
```

Mar17d

```
1       TGTAAGTTGC CTGCCTCCTG CTGTGCTTGG CTCGGTCTCG ACGACGCGGC TGTCGTTACC
61      TCTGGGCGCT GCCCCTGATT CTACCCCGCT GCAGCATTTC CACCGCCTCG AGCAAAACAA
121     AGCAGCGGCT TCAACCACGA CGCGATTCTG GGACATGATG GCTAATATGA CTTCTTCTGC
181     AACATAGGTC CACCTCCAGA CCGGCCAGTG CGTAAGTGCT CCTTCTTTTT CCTCGACGGC
241     ACGGCAGAGA CTCTAACAAC GCGTGGATAG GGTAACCAAA TTGGTGCTGC TTTCTGGCAG
```

```
301      ACCATCTCCG GCGAGCATGG TCTTGACAGC AATGGTGTCT ACAACGGCAC TTCCGAGCTC
361      CAGCTCGAAC GCATGAGCGT CTACTTCAAC GAGGTACGTG AATAAACTCT GCTGCCTGCT
421      CGGCCGCTGG AAGCACGAAA CTCACACCAC CCAGGCCTCT GGCAACAAGT ATGTCCCTCG
481      CGCCGTCCTC GTCGATCTCG AGCCCGGTAC CATGGACGCC GTCCGTGCCG GCCCCTTCGG
541      CCAGCTCTTC CGTCCCGACA ACTT
```

Mar17e

```
1        TGTAAGTTGC CTGCCTCCTG CTGTGCTTGG CTCGGTCTCG ACGACGCGGC TGTCGTTACC
61       TCTGGGCGCT GCCCCTGATT CTACCCCGCT GCAGCATTTC CACCGCCTCG AGCAAAACAA
121      AGCAGCGGCT TCAACCACGA CGCGATTCTG GGACATGATG GCTAATATGA CTTCTTCTGC
181      AACATAGGTC CACCTCCAGA CCGGCCAGTG CGTAAGTGCT CCTTCTTTTT CCTCGACGGC
241      ACGGCAGAGA CTCTAACAAC GCGTGGATAG GGTAACCAAA TTGGTGCTGC TTTCTGGCAG
301      ACCATCTCCG GCGAGCATGG TCTTGACAGC AATGGTGTCT ACAACGGCAC TTCCGAGCTC
361      CAGCTCGAAC GCATGAGCGT CTACTTCAAC GAGGTACGTG AATAAACTCT GCTGCCTGCT
421      CGGCCGCTGG AAGCACGAAA CTCACACCAC CCAGGCCTCT GGCAACAAGT ATGTCCCTCG
481      CGCCGTCCTC GTCGATCTCG AGCCCGGTAC CATGGACGCC GTCCGTGCCG GCCCCTTCGG
541      CCAGCTCTTC CGTCCCGACA ACT
```

Mtb1a

```
1        GTAAGTTGCC TGCCTCCTGC TGTGCTTGGC TCAGTCTCGA CGACGCGGCT GTCGTTACCT
61       CTGGGTGCTG CCCCTGATTC TACCCCGCTG CAGCATTTCC ACCGCCTCGA GCAAAACAAA
121      GCAGCGGCTT CAACCACGAC GCGATTCTGG GACATAATGG CTAATATGAC TTCTTCTGCA
181      ACATAGGTCC ACCTCCAGAC CGGCCAGTGC GTAAGTGGTC CTTCTTCTTC CTCGACGGCA
241      CGGCAGAGAC TCTAACAACG CGTGGATAGG GTAACCAAAT CGGTGCTGCT TTCTGGCAGA
301      CCATCTCCGG CGAGCATGGT CTTGACAGCA ATGGTGTCTA CAACGGCACT TCCGAGCTCC
361      AGCTCGAACG CATGAGCGTC TACTTCAACG AGGTACGTGA ATAAACTCTG CTGCCTGCTC
421      GGCCGCTGGA AGCACGAAAC TCACACCACC CAGGCTTCTG GCAACAAGTA TGTCCCTCGC
481      GCCGTCCTCG TCGATCTCGA GCCCGGTACC ATGGACGCCG TCCGTGCCGG CCCCTTCGGC
541      CAGCTCTTCC GTCCCGACAA CT
```

A.2.4 Histone genes sequences of *C. pauciseptatum* isolates

Ack2b

```
1        AGGCTGGTGA GTCTCGCCGC GTCATTTAAT TGACGCGTCG CTCCCCCCTA CCGCATCATC
61       GTACTGACCT CGAACCCCAA ACAGCCCGCA AGAGCGCCCC CTCTACCGGT GGTGTCAAGA
121      AGCCTCACCG CTACAAGCCC GGTACCGTCG CTCTCCGTGA GATTCGACGC TACCAGAAGT
181      CGACCGAGCT CCTCATTCGC AAGCTCCCCT TCCAGCGTCT CGTGAGTACC TACCACCACA
241      CATACACAAA GGCACACACG CTAACACGAT CGTGACAGGT CCGTGAGATT GCCCAGGACT
301      TCAAGAGCGA TCTCCGCTTC CAGTCCTCCG CCATCGGCGC TCTCCAGGAG TCCGTTGAGT
361      CGTACCTCGT CTCCCTCTTC GAGGACACCA ACCTTTGCGC CATCCACGCC AAGCGTGTCA
421      CCATCCAGTC C
```

Ack2e

```
1        AGGCTGGTGA GTCTCGCCGC GTCATTTAAT TGACGCGTCG CTCCCCCCTA CCGCATCATC
61       GTACTGACCT CGAACCCCAA ACAGCCCGCA AGAGCGCCCC CTCTACCGGT GGTGTCAAGA
121      AGCCTCACCG CTACAAGCCC GGTACCGTCG CTCTCCGTGA GATTCGACGC TACCAGAAGT
181      CGACCGAGCT CCTCATTCGC AAGCTCCCCT TCCAGCGTCT CGTGAGTACC TACCACCACA
241      CATACACAAA GGCACACACG CTAACACGAT CGTGACAGGT CCGTGAGATT GCCCAGGACT
301      TCAAGAGCGA TCTCCGCTTC CAGTCCTCCG CCATCGGCGC TCTCCAGGAG TCCGTTGAGT
361      CGTACCTCGT CTCCCTCTTC GAGGACACCA ACCTTTGCGC CATCCACGCC AAGCGTGTCA
421      CTCATCCAGT CCA
```

CO6g

```
1       AGGCTGGTGA GTCTCGCCGC GTCATTTAAT TGACGCGTCG CTCCCCCCTA CCGCATCATC
61      GTACTGACCT CGAACCCCAA ACAGCCCGCA AGAGCGCCCC CTCTACCGGT GGTGTCAAGA
121     AGCCTCACCG CTACAAGCCC GGTACCGTCG CTCTCCGTGA GATTCGACGC TACCAGAAGT
181     CGACCGAGCT CCTCATTCGC AAGCTCCCCT TCCAGCGTCT CGTGAGTACC TACCACCACA
241     CATACACAAA GGCACACACG CTAACACGAT CGTGACAGGT CCGTGAGATT GCCCAGGACT
301     TCAAGAGCGA TCTCCGCTTC CAGTCCTCCG CCATCGGCGC TCTCCAGGAG TCCGTTGAGT
361     CGTACCTCGT CTCCCTCTTC GAGGACACCA ACCTTTGCGC CATCCACGCC AAGCGTGTCA
421     CCATCCAGTC CA
```

Hb3b

```
1       AGGCTGGTGA GTCTCGCCGC GTCATTTAAT TGACGCGTCG CTCCCCCCTA CCGCATCATC
61      GTACTGACCT CGAACCCCAA ACAGCCCGCA AGAGCGCCCC CTCTACCGGT GGTGTCAAGA
121     AGCCTCACCG CTACAAGCCC GGTACCGTCG CTCTCCGTGA GATTCGACGC TACCAGAAGT
181     CGACCGAGCT CCTCATTCGC AAGCTCCCCT TCCAGCGTCT CGTGAGTACC TACCACCACA
241     CATACACAAA GGCACACACG CTAACACGAT CGTGACAGGT CCGTGAGATT GCCCAGGACT
301     TCAAGAGCGA TCTCCGCTTC CAGTCCTCCG CCATCGGCGC TCTCCAGGAG TCCGTTGAGT
361     CGTACCTCGT CTCCCTCTTC GAGGACACCA ACCTTTGCGC CATCCACGCC AAGCGTGT
```

Hb6b

```
1       CATGCTCAGG CTGGTGAGTC TCGCCGCGTC ATTTAATTGA CGCGTCGCTC CCCCCTACCG
61      CATCATCGTA CTGACCTCGA ACCCCAAACA GCCCGCAAGA GCGCCCCCTC TACCGGTGGT
121     GTCAAGAAGC CTCACCGCTA CAAGCCCGGT ACCGTCGCTC TCCGTGAGAT TCGACGCTAC
181     CAGAAGTCGA CCGAGCTCCT CATTCGCAAG CTCCCCTTCC AGCGTCTCGT GAGTACCTAC
241     CACCACACAT ACACAAAGGC ACACACGCTA ACACGATCGT GACAGGTCCG TGAGATTGCC
301     CAGGACTTCA AGAGCGATCT CCGCTTCCAG TCCTCCGCCA TCGGCGCTCT CCAGGAGTCC
361     GTTGAGTCGT ACCTCGTCTC CCTCTTCGAG GACACCAACC TTTGCGCCAT CCACGCCAAG
421     CGTGTCA
```

Mar5a

```
1       AGGCTGGTGA GTCTCGCCGC GTCATTTAAT TGACGCGTCG CTCCCCCCTA CCGCATCATC
61      GTACTGACCT CGAACCCCAA ACAGCCCGCA AGAGCGCCCC CTCTACCGGT GGTGTCAAGA
121     AGCCTCACCG CTACAAGCCC GGTACCGTCG CTCTCCGTGA GATTCGACGC TACCAGAAGT
181     CGACCGAGCT CCTCATTCGC AAGCTCCCCT TCCAGCGTCT CGTGAGTACC TACCACCACA
241     CATACACAAA GGCACACACG CTAACACGAT CGTGACAGGT CCGTGAGATT GCCCAGGACT
301     TCAAGAGCGA TCTCCGCTTC CAGTCCTCCG CCATCGGCGC TCTCCAGGAG TCCGTTGAGT
361     CGTACCTCGT CTCCCTCTTC GAGGACACCA ACCTTTGCGC CATCCACGCC AAGCGTGTCA
421     CCATCCAGTC CA
```

Mar6a

```
1       AGGCTGGTGA GTCTCGCCGC GTCATTTAAT TGACGCGTCG CTCCCCCCTA CCGCATCATC
61      GTACTGACCT CGAACCCCAA ACAGCCCGCA AGAGCGCCCC CTCTACCGGT GGTGTCAAGA
121     AGCCTCACCG CTACAAGCCC GGTACCGTCG CTCTCCGTGA GATTCGACGC TACCAGAAGT
181     CGACCGAGCT CCTCATTCGC AAGCTCCCCT TCCAGCGTCT CGTGAGTACC TACCACCACA
241     CATACACAAA GGCACACACG CTAACACGAT CGTGACAGGT CCGTGAGATT GCCCAGGACT
301     TCAAGAGCGA TCTCCGCTTC CAGTCCTCCG CCATCGGCGC TCTCCAGGAG TCCGTTGAGT
361     CGTACCTCGT CTCCCTCTTC GAGGACACCA ACCTTTGCGC CATCCACGCC AAGCGTGTCA
421     CCATCCAGTC CA
```

Mar14b

```
1       AGGCTGGTGA GTCTCGCCGC GTCATTTAAT TGACGCGTCG CTCCCCCCTA CCGCATCATC
61      GTACTGACCT CGAACCCCAA ACAGCCCGCA AGAGCGCCCC CTCTACCGGT GGTGTCAAGA
121     AGCCTCACCG CTACAAGCCC GGTACCGTCG CTCTCCGTGA GATTCGACGC TACCAGAAGT
```

```
181      CGACCGAGCT CCTCATTCGC AAGCTCCCCT TCCAGCGTCT CGTGAGTACC TACCACCACA
241      CATACACAAA GGCACACACG CTAACACGAT CGTGACAGGT CCGTGAGATT GCCCAGGACT
301      TCAAGAGCGA TCTCCGCTTC CAGTCCTCCG CCATCGGCGC TCTCCAGGAG TCCGTTGAGT
361      CGTACCTCGT CTCCCTCTTC GAGGACACCA ACCTTTGCGC CATCCACGCC AAGCGTGTCA
421      CCATCCAGTC CAAGGCC
```

Mar17d

```
1        GCTGGTGAGT CTCGCCGCGT CATTTAATTG ACGCGTCGCT CCCCCCTACC GCATCATCGT
61       ACTGACCTCG AACCCCAAAC AGCCCGCAAG AGCGCCCCCT CTACCGGTGG TGTCAAGAAG
121      CCTCACCGCT ACAAGCCCGG TACCGTCGCT CTCCGTGAGA TTCGACGCTA CCAGAAGTCG
181      ACCGAGCTCC TCATTCGCAA GCTCCCCTTC CAGCGTCTCG TGAGTACCTA CCACCACACA
241      TACACAAAGG CACACACGCT AACACGATCG TGACAGGTCC GTGAGATTGC CCAGGACTTC
301      AAGAGCGATC TCCGCTTCCA GTCCTCCGCC ATCGGCGCTC TCCAGGAGTC CGTTGAGTCG
361      TACCTCGTCT CCCTCTTCGA GGACACCAAC CTTTGCGCCA TCCACGCCAA GCGTGTCACC
421      ATCCAGTCCA
```

Mar17e

```
1        AGGCTGGTGA GTCTCGCCGC GTCATTTAAT TGACGCGTCG CTCCCCCCTA CCGCATCATC
61       GTACTGACCT CGAACCCCAA ACAGCCCGCA AGAGCGCCCC CTCTACCGGT GGTGTCAAGA
121      AGCCTCACCG CTACAAGCCC GGTACCGTCG CTCTCCGTGA GATTCGACGC TACCAGAAGT
181      CGACCGAGCT CCTCATTCGC AAGCTCCCCT TCCAGCGTCT CGTGAGTACC TACCACCACA
241      CATACACAAA GGCACACACG CTAACACGAT CGTGACAGGT CCGTGAGATT GCCCAGGACT
301      TCAAGAGCGA TCTCCGCTTC CAGTCCTCCG CCATCGGCGC TCTCCAGGAG TCCGTTGAGT
361      CGTACCTCGT CTCCCTCTTC GAGGACACCA ACCTTTGCGC CATCCACGCC AAGCGTGTCA
421      CCATCCAGTC CA
```

Mtb1a

```
1        AGGCTGGTGA GTCTCGCCGC CTCATTTAAT TGACGCGTCG CCCCCCCTAC CGCATCATCG
61       TACTGACCTC GAACCCCAAA CAGCCCGCAA GAGCGCCCCC TCTACCGGTG GTGTCAAGAA
121      GCCTCACCGC TACAAGCCCG GTACCGTCGC TCTCCGTGAG ATTCGACGCT ACCAGAAGTC
181      GACCGAGCTC CTCATTCGCA AGCTCCCCTT CCAGCGTCTC GTGAGTACCT ACCACCACAC
241      ATACACAAAG GCACACACGC TAACACGATC GTGACAGGTC CGTGAGATTG CCCAGGACTT
301      CAAGAGCGAT CTCCGCTTCC AGTCCTCCGC CATCGGCGCT CTCCAGGAGT CCGTTGAGTC
361      GTACCTCGTC TCCCTCTTCG AGGACACCAA CCTTTGCGCC ATCCACGCCA AGCGTGTC
```

A.3 Comparison between NZ isolates and sequences present on GenBank data base. First or best match were shown. All matches were to international *C. pauciseptatum* isolates.

Isolate	GenBank Accession Numbers and Percentage Identity			
	ITS	EF1-α	BT	HIS
Co 6g	JF735307, 100%	JF735775, 99%	JF735434, 100%	JF735582, 100%
Mar 5a	JF735307, 100%	JF735771, 99%	EF607067, 100%	JF735582, 100%
Hb 6b	JF735307, 100%	JF735771, 99%	EF607067, 100%	JF735582, 100%
Hb 3b	JF735307, 100%	JF735771, 99%	EF607067, 100%	JF735582, 100%
Mtb 1a	JF735307, 100%	JF735771, 99%	EF607067, 99%	JF735582, 99%
Mar 17d	JF735307, 100%	JF735771, 99%	JF735434, 100%	JF735582, 99%
Mar 17e	JF735307, 100%	JF735771, 100%	JF735434, 100%	JF735582, 99%
Mar 14b	JF735307, 100%	JF735771, 99%	EF607067, 100%	JF735582, 100%
Ack 2b	JF735307, 100%	JF735771, 99%	EF607067, 100%	JF735582, 100%
Mar 6a	JF735307, 100%	JF735771, 99%	JF735434, 100%	JF735582, 100%
Ack 2e	JF735307, 100%	JF735771, 99%	EF607067, 100%	JF735582, 99%

A.4 Thermal cycle for each pair of primers used to amplify taxonomic genes

Target Genes		Initial Stage	Cycles	Denaturation	Annealing	Elongation	Final Extention
ITS	Time	3 min	35	1 min	30 s	1 min	7 min
	T(°C)	94		94	55	72	72
The translation elongation factor 1-α	Time	3 min	40	30 s	1 min	90 s	10 min
	T(°C)	94		94	58	72	72
β – tubulin	Time	5 min	40	30 s	30 s	80 s	10 min
	T(°C)	94		94	52	72	72
Histone	Time	5 min	40	30 s	30 s	80 s	7 min
	T(°C)	94		94	52	72	72

A.5 Average diameter of colonies grown on PDA at 20°C on Day 5, Day 11 and Day 18 (mm). Results had been analysed using one-way ANOVA by GenStat version 16.

Isolate	Day 5	Day11	Day18
Ack2b	21.80 cd	52.99 c	73.18 c
Ack2e	16.30 ab	49.22 b	77.59 d
Co6g	14.03 a	51.54 bc	76.16 d
Hb3b	19.43 bc	49.15 b	61.02 a
Mar5a	25.11 d	50.15 bc	68.24 b
Mar6a	14.54 a	41.68 a	67.92 b
Mar14b	24.85 d	57.2 b	77.40 d
Mtb1a	34.95 e	72.96 e	81.75 e
***P*-value**	<0.001	<0.001	<0.001

Appendix B

B.1 ANOVA for lesion length of *Cylindrocarpon* isolates for each rootstock in detached root assay after one month and two months

Dependent Variable: Lesion lengths (mm) produced on detached roots of rootstock Schwarzman measured after 1 month incubation

Source	df	Sum of Squares	Mean square	F-ratio	P
Isolates	9	9195.2	1021.7	7.93	<.001
Residual	70	9013.2	128.8		
Total	79	18208.4			

Dependent Variable: Lesion lengths (mm) produced on detached roots of rootstock Riparia Gloire measured after 1 month incubation

Source	df	Sum of Squares	Mean square	F-ratio	P
Isolates	11	1606.00	146.00	2.22	0.022
Residual	77	5068.99	65.83		
Total	95	7118.88			

Dependent Variable: Lesion lengths (mm) produced on detached roots of rootstock Riparia Gloire measured after 2 month incubation

Source	df	Sum of Squares	Mean square	F-ratio	P
Isolates	11	5011.0	455.5	1.84	0.062
Residual	77	19101.1	248.1		
Total	95	25142.1			

Dependent Variable: Lesion lengths (mm) produced on detached roots of rootstock 3309 measured after 1 month incubation

Source	df	Sum of Squares	Mean square	F-ratio	P
Isolates	9	4395.0	488.3	1.09	0.384
Residual	70	31457.9	449.4		
Total	79	35852.9			

Dependent Variable: Lesion lengths (mm) produced on detached roots of rootstock 3309 measured after 2 month incubation

Source	df	Sum of Squares	Mean square	F-ratio	P
Isolates	9	4522.7	502.5	1.75	0.058
Residual	26	7448.7	286.5		
Total	38	12162.1			

B.2 Mean lesion lengths (mm) produced on detached roots of rootstock 3309 measured after 1 month and 2 month's incubation and the proportion of *Cylindrocarpon* species re-isolated from specific root tissues at the end of lesion from apical end and 0.5, 1 cm above this point. Isolates in red are from the second genetic group.

Species	Isolates	Mean lesion length (mm)		Percentage of *Cylindrocarpon-like* colonies recovered from root tissues (%) at each distance from lesion margin after 2 month		
		1 month	2 months	0 cm	0.5cm	1cm
I. macrodidyma	**Hb2b**	28.0	30.92	0 (0/4)	0 (0/4)	0 (0/4)
I. liriodendri	**CO1b**	33.03	39.78	66.7 (4/6)	66.7(4/6)	50 (3/6)
C. pauciseptatum	**Mar6a**	10.75	11.12	71.4 (5/7)	71.4 (5/7)	40 (2/5)
	Mtb1a	28.42	29.62	66.7 (4/6)	66.7 (4/6)	66.7 (4/6)
	CO6g	18.37	19.97	57.1 (4/7)	57.1 (4/7)	57.1 (4/7)
	Ack2e	22.36	28.02	75 (3/4)	100 (4/4)	25 (1/4)
	Ack2b	27.57	28.33	0 (0/3)	0 (0/3)	0 (0/3)
	Mar5a	31.12	36.94	16.7 (2/6)	25 (1/4)	25 (1/4)
	Mar14b	11.48	12.84	100 (6/6)	66.7 (4/6)	50 (3/6)
	Control	4.93	5.21	0 (0/3)	0 (0/2)	0 (0/2)
***P*-Value**		0.384	0.058			

"*" represents the numbers of *Cylindrocarpon-like* colonies vs numbers of samples examined.

B.3 Proportion of live plants and incidence of recovery of Cylindrocarpon species from tissue at the stem bases (0 cm) and 0.5, 1, and 2 cm above the stem base investigated from 4-month-old grapevine rootstock Riparia Gloire inoculated with *Cylindrocarpon* species. *I. liriodendri* (Co1b) and *I. macrodidyma* (Hb2b) are positive control.

Isolates	Proportion of live plant	Proportion of *Cylindrocarpon*-like colonies recovered from plant tissues (%)				Proportion of *Botryspharia*-like colonies recovered from plant tissues (%)			
		0 cm	0.5 cm	1 cm	2 cm	0 cm	0.5 cm	1 cm	2 cm
Co 1b	80	2.5 a	2.5	0	0	22.5	27.5	80	20
Hb 2b	70	17.5 b	17.5	10	0	30	20	40	40
Hb 3b	40	17.5 b	17.5	0	10	5	5	40	10
Ack 2e	50	20 bc	20	0	10	15	7.5	40	30
CO6g	80	30 c	15	0	0	32.5	47.5	70	60
Control	90	10 ab	15	10	20	30	32.5	70	40
LSD		10.14	11.09						
P-Value		<0.001	0.053						

B.4 Mean root and shoot dry weights (g) of plants after 4 months inoculation with six *Cylindrocarpon* isolates for rootstock 3309. Isolates in red are from *I. liriodendri* (CO1b) and *I. marcrodidyma* (Hb2b) and *I. europaea* (Gis1b) as positive control.

Isolates	Mean root dry weight (g)	Mean shoot dry weight (g)
Gis 1b	8.43	16.58
Hb 2b	7.87	14.58
Co 1b	6.15	14.15
Ack 2e	7.49	14.77
CO6g	6.97	13.00
Control	7.51	13.47
P-value	0.527	0.527
LSD	2.286	2.408
SED	1.105	1.169

B.5 ANOVA for root and shoot dry weights (g) of plants 4 months after inoculation with three *Cylindrocarpon* isolates for rootstock Riparia Gloire

Dependent Variable: Root dry weights

Source	df	Sum of Squares	Mean square	F-ratio	P
Isolates	3	45.274	15.091	4.74	0.011
Residual	21	66.796	3.181		
Total	31	136.099			

Dependent Variable: Shoot dry weights

Source	df	Sum of Squares	Mean square	F-ratio	P
Isolates	3	99.083	33.028	3.33	0.039
Residual	21	208.064	9.908		
Total	31	390.487			

Appendix C

C.1 Mean PPO-I and PPO-II type laccase activity (U mL^{-1}) produced by isolates of *Cylindrocarpon pauciseptatum* and one isolate of *I. macrodidymum* as positive control after 5 days incubation from experiment 1.

	Isolate	PPO-I (U mL^{-1})	PPO-II(U mL^{-1})
C. pauciseptatum	**Mar5a**	0.399 d	0.041 de
	Mar6a	0.155 abc	0.010 ab
	Mar14b	0.169 abc	0.040 d
	Mtb1a	0.359 cd	0.002 a
	CO6g	0.334 bcd	0.029 cd
	Ack2b	0.130 ab	0.010 ab
	Ack2e	0.136 ab	0.013 ab
	Hb3b	0.231 abcd	0.018 bc
I. macrodidymum	**Hb2b**	0.063 a	0.053 e
***P*-Value**		0.04	<0.001
LSD		0.213	0.126

C.2 Alignment of the laccase (*lcc1*) gene from selected isolates of *Cylindrocarpon pauciseptatum*. Internal primer site is shown in the red block

```
Hb6b     ...GAGTCAGGTTCCTGACATTAACGCGGACGCTCTTGGC     37
Mar6a    ...GAGTCAGGTTCCTGACATTAACGCGGACGCTCTTGGC     37
Mar17e   tggGAGTCAGGTTCCTGACATTAACGCGGACGCTCTTGGC     40
         CPLaccaseF ──────►
Hb6b     CTTGCCGGTGTCCCTGACGTTCCACTGCTCCTCGAAGTCA     77
Mar6a    CTTGCCGGTGTCCCTGACGTTCCACTGCTCCTCGAAGTCA     77
Mar17e   CTTGCCGGTGTCCCTGACGTTCCACTGCTCCTCGAAGTCA     80

Hb6b     TCCTCTCCAAGCTTGGCGAGCAGAAGCGTGGGGCTGTTGT    117
Mar6a    TCCTCTCCAAGCTTGGCGAGCAGAAGCGTGGGGCTGTTGT    117
Mar17e   TCCTCTCCAAGCTTGGCGAGCAGAAGCGTGGGGCTGTTGT    120

Hb6b     AGTTGCCGCGGAAGTCGACGTCGTCGAACTTGAAGAGGGT    157
Mar6a    AGTTGCCGCGGAAGTCGACGTCGTCGAACTTGAAGAGGGT    157
Mar17e   AGTTGCCGCGGAAGTCGACGTCGTCGAACTTGAAGAGGGT    160
```

C.3 Aligned *lcc*1 DNA sequence of all 11 *C.pauciseptatum* isolates

```
Ack2b    ...GTTCCACTGCTCCTCGAAGTCATCCTCTCCAAGCTTG     37
Ack2e    gacGTTCCACTGCTCCTCGAAGTCATCCTCTCCAAGCTTG     40
CO6g     ...GTTCCACTGCTCCTCGAAGTCATCCTCTCCAAGCTTG     37
Hb3b     ...GTTCCACTGCTCCTCGAAGTCATCCTCTCCAAGCTTG     37
Hb6b     ...GTTCCACTGCTCCTCGAAGTCATCCTCTCCAAGCTTG     37
Mar5a    .........CTGCTCCTCGAAGTCATCCTCTCCAAGCTTG     31
Mar6a    ...GTTCCACTGCTCCTCGAAGTCATCCTCTCCAAGCTTG     37
Mar14b   ...GTTCCACTGCTCCTCGAAGTCATCCTCTCCAAGCTTG     37
Mar17d   ...GTTCCACTGCTCCTCGAAGTCATCCTCTCCAAGCTTG     37
Mar17e   ...GTTCCACTGCTCCTCGAAGTCATCCTCTCCAAGCTTG     37
Mtb1a    ...GTTCCACTGCTCCTCGAAGTCATCCTCTCCAAGCTTG     37

Ack2b    GCGAGCAGAAGCGTGGGGCTGTTGTAGTTGCCGCGGAAGT     77
```

```
Ack2e   GCGAGCAGAAGCGTGGGGCTGTTGTAGTTGCCGCGGAAGT     80
CO6g    GCGAGCAGAAGCGTGGGGCTGTTGTAGTTGCCGCGGAAGT     77
Hb3b    GCGAGCAGAAGCGTGGGGCTGTTGTAGTTGCCGCGGAAGT     77
Hb6b    GCGAGCAGAAGCGTGGGGCTGTTGTAGTTGCCGCGGAAGT     77
Mar5a   GCGAGCAGAAGCGTGGGGCTGTTGTAGTTGCCGCGGAAGT     71
Mar6a   GCGAGCAGAAGCGTGGGGCTGTTGTAGTTGCCGCGGAAGT     77
Mar14b  GCGAGCAGAAGCGTGGGGCTGTTGTAGTTGCCGCGGAAGT     77
Mar17d  GCGAGCAGAAGCGTGGGGCTGTTGTAGTTGCCGCGGAAGT     77
Mar17e  GCGAGCAGAAGCGTGGGGCTGTTGTAGTTGCCGCGGAAGT     77
Mtb1a   GCGAGCAGAAGCGTGGGGCTGTTGTAGTTGCCGCGGAAGT     77

Ack2b   CGACGTCGTCGAACTTGAAGAGGGTGATGCCGCTTCCGTT    117
Ack2e   CGACGTCGTCGAACTTGAAGAGGGTGATGCCGCTTCCGTT    120
CO6g    CGACGTCGTCGAACTTGAAGAGGGTGATGCCGCTTCCGTT    117
Hb3b    CGACGTCGTCGAACTTGAAGAGGGTGATGCCGCTTCCGTT    117
Hb6b    CGACGTCGTCGAACTTGAAGAGGGTGATGCCGCTTCCGTT    117
Mar5a   CGACGTCGTCGAACTTGAAGAGGGTGATGCCGCTTCCGTT    111
Mar6a   CGACGTCGTCGAACTTGAAGAGGGTGATGCCGCTTCCGTT    117
Mar14b  CGACGTCGTCGAACTTGAAGAGGGTGATGCCGCTTCCGTT    117
Mar17d  CGACGTCGTCGAACTTGAAGAGGGTGATGCCGCTTCCGTT    117
Mar17e  CGACGTCGTCGAACTTGAAGAGGGTGATGCCGCTTCCGTT    117
Mtb1a   CGACGTCGTCGAACTTGAAGAGGGTGATGCCGCTTCCGTT    117

Ack2b   GACAAAGAACTCGACGTTCATGTCATAAGTGAGGTCAGCA    157
Ack2e   GACAAAGAACTCGACGTTCATGTCATAAGTGAGGTCAGCA    160
CO6g    GACAAAGAACTCGACGTTCATGTCATAAGTGAGGTCAGCA    157
Hb3b    GACAAAGAACTCGACGTTCATGTCATAAGTGAGGTCAGCA    157
Hb6b    GACAAAGAACTCGACGTTCATGTCATAAGTGAGGTCAGCA    157
Mar5a   GACAAAGAACTCGACGTTCATGTCATAAGTGAGGTCAGCA    151
Mar6a   GACAAAGAACTCGACGTTCATGTCATAAGTGAGGTCAGCA    157
Mar14b  GACAAAGAACTCGACGTTCATGTCATAAGTGAGGTCAGCA    157
Mar17d  GACAAAGAACTCGACGTTCATGTCATAAGTGAGGTCAGCA    157
Mar17e  GACAAAGAACTCGACGTTCATGTCATAAGTGAGGTCAGCA    157
Mtb1a   GACAAAGAACTCGACGTTCATGTCATAAGTGAGGTCAGCA    157

Ack2b   GCGGGCAGGGGCAGCTTCATGACCGGGACGGTCAGGGCGA    197
Ack2e   GCGGGCAGGGGCAGCTTCATGACCGGGACGGTCAGGGCGA    200
CO6g    GCGGGCAGGGGCAGCTTCATGACCGGGACGGTCAGGGCGA    197
Hb3b    GCGGGCAGGGGCAGCTTCATGACCGGGACGGTCAGGGCGA    197
Hb6b    GCGGGCAGGGGCAGCTTCATGACCGGGACGGTCAGGGCGA    197
Mar5a   GCGGGCAGGGGCAGCTTCATGACCGGGACGGTCAGGGCGA    191
Mar6a   GCGGGCAGGGGCAGCTTCATGACCGGGACGGTCAGGGCGA    197
Mar14b  GCGGGCAGGGGCAGCTTCATGACCGGGACGGTCAGGGCGA    197
Mar17d  GCGGGCAGGGGCAGCTTCATGACCGGGACGGTCAGGGCGA    197
Mar17e  GCGGGCAGGGGCAGCTTCATGACCGGGACGGTCAGGGCGA    197
Mtb1a   GCGGGCAGGGGCAGCTTCATGACCGGGACGGTCAGGGCGA    197

Ack2b   GGTCGTCATTGACACAGGTTGCCGGGTCGGGGACGTCCCA    237
Ack2e   GGTCGTCATTGACACAGGTTGCCGGGTCGGGGACGTCCCA    240
CO6g    GGTCGTCATTGACACAGGTTGCCGGGTCGGGGACGTCCCA    237
Hb3b    GGTCGTCATTGACACAGGTTGCCGGGTCGGGGACGTCCCA    237
Hb6b    GGTCGTCATTGACACAGGTTGCCGGGTCGGGGACGTCCCA    237
Mar5a   GGTCGTCATTGACACAGGTTGCCGGGTCGGGGACGTCCCA    231
Mar6a   GGTCGTCATTGACACAGGTTGCCGGGTCGGGGACGTCCCA    237
Mar14b  GGTCGTCATTGACACAGGTTGCCGGGTCGGGGACGTCCCA    237
Mar17d  GGTCGTCATTGACACAGGTTGCCGGGTCGGGGACGTCCCA    237
Mar17e  GGTCGTCATTGACACAGGTTGCCGGGTCGGGGACGTCCCA    237
Mtb1a   GGTCGTCATTGACACAGGTTGCCGGGTCGGGGACGTCCCA    237

Ack2b   GGCGGTGGACTTGGGCTGCTTGGTCTGGTCGGCATTGTCG    277
Ack2e   GGCGGTGGACTTGGGCTGCTTGGTCTGGTCGGCATTGTCG    280
CO6g    GGCGGTGGACTTGGGCTGCTTGGTCTGGTCGGCATTGTCG    277
Hb3b    GGCGGTGGACTTGGGCTGCTTGGTCTGGTCGGCATTGTCG    277
Hb6b    GGCGGTGGACTTGGGCTGCTTGGTCTGGTCGGCATTGTCG    277
Mar5a   GGCGGTGGACTTGGGCTGCTTGGTCTGGTCGGCATTGTCG    271
Mar6a   GGCGGTGGACTTGGGCTGCTTGGTCTGGTCGGCATTGTCG    277
Mar14b  GGCGGTGGACTTGGGCTGCTTGGTCTGGTCGGCATTGTCG    277
Mar17d  GGCGGTGGACTTGGGCTGCTTGGTCTGGTCGGCATTGTCG    277
Mar17e  GGCGGTGGACTTGGGCTGCTTGGTCTGGTCGGCATTGTCG    277
Mtb1a   GGCGGTGGACTTGGGCTGCTTGGTCTGGTCGGCATTGTCG    277

Ack2b   TAGTAGATGGCGGCGAGGGCATTGGGCTGGAGGCTCAGGC    317
Ack2e   TAGTAGATGGCGGCGAGGGCATTGGGCTGGAGGCTCAGGC    320
CO6g    TAGTAGATGGCGGCGAGGGCATTGGGCTGGAGGCTCAGGC    317
Hb3b    TAGTAGATGGCGGCGAGGGCATTGGGCTGGAGGCTCAGGC    317
Hb6b    TAGTAGATGGCGGCGAGGGCATTGGGCTGGAGGCTCAGGC    317
Mar5a   TAGTAGATGGCGGCGAGGGCATTGGGCTGGAGGCTCAGGC    311
Mar6a   TAGTAGATGGCGGCGAGGGCATTGGGCTGGAGGCTCAGGC    317
Mar14b  TAGTAGATGGCGGCGAGGGCATTGGGCTGGAGGCTCAGGC    317
Mar17d  TAGTAGATGGCGGCGAGGGCATTGGGCTGGAGGCTCAGGC    317
```

```
Mar17e  TAGTAGATGGCGGCGAGGGCATTGGGCTGGAGGCTCAGGC    317
Mtb1a   TAGTAGATGGCGGCGAGGGCATTGGGCTGGAGGCTCAGGC    317

Ack2b   TGCAGGGGACGGAGATGTTGGCGCGCATCCAGTAGGCGTC    357
Ack2e   TGCAGGGGACGGAGATGTTGGCGCGCATCCAGTAGGCGTC    360
CO6g    TGCAGGGGACGGAGATGTTGGCGCGCATCCAGTAGGCGTC    357
Hb3b    TGCAGGGGACGGAGATGTTGGCGCGCATCCAGTAGGCGTC    357
Hb6b    TGCAGGGGACGGAGATGTTGGCGCGCATCCAGTAGGCGTC    357
Mar5a   TGCAGGGGACGGAGATGTTGGCGCGCATCCAGTAGGCGTC    351
Mar6a   TGCAGGGGACGGAGATGTTGGCGCGCATCCAGTAGGCGTC    357
Mar14b  TGCAGGGGACGGAGATGTTGGCGCGCATCCAGTAGGCGTC    357
Mar17d  TGCAGGGGACGGAGATGTTGGCGCGCATCCAGTAGGCGTC    357
Mar17e  TGCAGGGGACGGAGATGTTGGCGCGCATCCAGTAGGCGTC    357
Mtb1a   TGCAGGGGACGGAGATGTTGGCGCGCATCCAGTAGGCGTC    357

Ack2b   GAGATCGCCGTCGGCCTTGACCAGCACGTCGGTGCGTTGA    397
Ack2e   GAGATCGCCGTCGGCCTTGACCAGCACGTCGGTGCGTTGA    400
CO6g    GAGATCGCCGTCGGCCTTGACCAGCACGTCGGTGCGTTGA    397
Hb3b    GAGATCGCCGTCGGCCTTGACCAGCACGTCGGTGCGTTGA    397
Hb6b    GAGATCGCCGTCGGCCTTGACCAGCACGTCGGTGCGTTGA    397
Mar5a   GAGATCGCCGTCGGCCTTGACCAGCACGTCGGTGCGTTGA    391
Mar6a   GAGATCGCCGTCGGCCTTGACCAGCACGTCGGTGCGTTGA    397
Mar14b  GAGATCGCCGTCGGCCTTGACCAGCACGTCGGTGCGTTGA    397
Mar17d  GAGATCGCCGTCGGCCTTGACCAGCACGTCGGTGCGTTGA    397
Mar17e  GAGATCGCCGTCGGCCTTGACCAGCACGTCGGTGCGTTGA    397
Mtb1a   GAGATCGCCGTCGGCCTTGACCAGCACGTCGGTGCGTTGA    397

Ack2b   CCGATGCCCAAGGTCACGACCTTGGTGTCGTACGGCTGCA    437
Ack2e   CCGATGCCCAAGGTCACGACCTTGGTGTCGTACGGCTGCA    440
CO6g    CCGATGCCCAAGGTCACGACCTTGGTGTCGTACGGCTGCA    437
Hb3b    CCGATGCCCAAGGTCACGACCTTGGTGTCGTACGGCTGCA    437
Hb6b    CCGATGCCCAAGGTCACGACCTTGGTGTCGTACGGCTGCA    437
Mar5a   CCGATGCCCAAGGTCACGACCTTGGTGTCGTACGGCTGCA    431
Mar6a   CCGATGCCCAAGGTCACGACCTTGGTGTCGTACGGCTGCA    437
Mar14b  CCGATGCCCAAGGTCACGACCTTGGTGTCGTACGGCTGCA    437
Mar17d  CCGATGCCCAAGGTCACGACCTTGGTGTCGTACGGCTGCA    437
Mar17e  CCGATGCCCAAGGTCACGACCTTGGTGTCGTACGGCTGCA    437
Mtb1a   CCGATGCCCAAGGTCACGACCTTGGTGTCGTACGGCTGCA    437

Ack2b   CCTGGACAAAGTCGTTGGCGATGATGGTCATGGTGTGCCC    477
Ack2e   CCTGGACAAAGTCGTTGGCGATGATGGTCATGGTGTGCCC    480
CO6g    CCTGGACAAAGTCGTTGGCGATGATGGTCATGGTGTGCCC    477
Hb3b    CCTGGACAAAGTCGTTGGCGATGATGGTCATGGTGTGCCC    477
Hb6b    CCTGGACAAAGTCGTTGGCGATGATGGTCATGGTGTGCCC    477
Mar5a   CCTGGACAAAGTCGTTGGCGATGATGGTCATGGTGTGCCC    471
Mar6a   CCTGGACAAAGTCGTTGGCGATGATGGTCATGGTGTGCCC    477
Mar14b  CCTGGACAAAGTCGTTGGCGATGATGGTCATGGTGTGCCC    477
Mar17d  CCTGGACAAAGTCGTTGGCGATGATGGTCATGGTGTGCCC    477
Mar17e  CCTGGACAAAGTCGTTGGCGATGATGGTCATGGTGTGCCC    477
Mtb1a   CCTGGACAAAGTCGTTGGCGATGATGGTCATGGTGTGCCC    477

Ack2b   GTCGATGGAGAACCGCTGCAAGGCTTCTGCGCCGGCGTTG    517
Ack2e   GTCGATGGAGAACCGCTGCAAGGCTTCTGCGCCGGCGTTG    520
CO6g    GTCGATGGAGAACCGCTGCAAGGCTTCTGCGCCGGCGTTG    517
Hb3b    GTCGATGGAGAACCGCTGCAAGGCTTCTGCGCCGGCGTTG    517
Hb6b    GTCGATGGAGAACCGCTGCAAGGCTTCTGCGCCGGCGTTG    517
Mar5a   GTCGATGGAGAACCGCTGCAAGGCTTCTGCGCCGGCGTTG    511
Mar6a   GTCGATGGAGAACCGCTGCAAGGCTTCTGCGCCGGCGTTG    517
Mar14b  GTCGATGGAGAACCGCTGCAAGGCTTCTGCGCCGGCGTTG    517
Mar17d  GTCGATGGAGAACCGCTGCAAGGCTTCTGCGCCGGCGTTG    517
Mar17e  GTCGATGGAGAACCGCTGCAAGGCTTCTGCGCCGGCGTTG    517
Mtb1a   GTCGATGGAGAACCGCTGCAAGGCTTCTGCGCCGGCGTTG    517

Ack2b   ATGAGGCGCAGGCGATGCGTCTTGCCACGCTTGAACTTGA    557
Ack2e   ATGAGGCGCAGGCGATGCGTCTTGCCACGCTTGAACTTGA    560
CO6g    ATGAGGCGCAGGCGATGCGTCTTGCCACGCTTGAACTTGA    557
Hb3b    ATGAGGCGCAGGCGATGCGTCTTGCCACGCTTGAACTTGA    557
Hb6b    ATGAGGCGCAGGCGATGCGTCTTGCCACGCTTGAACTTGA    557
Mar5a   ATGAGGCGCAGGCGATGCGTCTTGCCACGCTTGAACTTGA    551
Mar6a   ATGAGGCGCAGGCGATGCGTCTTGCCACGCTTGAACTTGA    557
Mar14b  ATGAGGCGCAGGCGATGCGTCTTGCCACGCTTGAACTTGA    557
Mar17d  ATGAGGCGCAGGCGATGCGTCTTGCCACGCTTGAACTTGA    557
Mar17e  ATGAGGCGCAGGCGATGCGTCTTGCCACGCTTGAACTTGA    557
Mtb1a   ATGAGGCGCAGGCGATGCGTCTTGCCACGCTTGAACTTGA    557

Ack2b   ACTTGGAGAGACCAGCGTTGCTGTTGCAGGGGGTGGTGTC    597
Ack2e   ACTTGGAGAGACCAGCGTTGCTGTTGCAGGGGGTGGTGTC    600
CO6g    ACTTGGAGAGACCAGCGTTGCTGTTGCAGGGGGTGGTGTC    597
Hb3b    ACTTGGAGAGACCAGCGTTGCTGTTGCAGGGGGTGGTGTC    597
Hb6b    ACTTGGAGAGACCAGCGTTGCTGTTGCAGGGGGTGGTGTC    597
```

```
Mar5a    ACTTGGAGAGACCAGCGTTGCTGTTGCAGGGGGTGGTGTC    591
Mar6a    ACTTGGAGAGACCAGCGTTGCTGTTGCAGGGGGTGGTGTC    597
Mar14b   ACTTGGAGAGACCAGCGTTGCTGTTGCAGGGGGTGGTGTC    597
Mar17d   ACTTGGAGAGACCAGCGTTGCTGTTGCAGGGGGTGGTGTC    597
Mar17e   ACTTGGAGAGACCAGCGTTGCTGTTGCAGGGGGTGGTGTC    597
Mtb1a    ACTTGGAGAGACCAGCGTTGCTGTTGCAGGGGGTGGTGTC    597

Ack2b    GTCGGCAGCTAGGGTCGAGCAGTTGAAGTTGTTCTTGCCA    637
Ack2e    GTCGGCAGCTAGGGTCGAGCAGTTGAAGTTGTTCTTGCCA    640
CO6g     GTCGGCAGCTAGGGTCGAGCAGTTGAAGTTGTTCTTGCCA    637
Hb3b     GTCGGCAGCTAGGGTCGAGCAGTTGAAGTTGTTCTTGCCA    637
Hb6b     GTCGGCAGCTAGGGTCGAGCAGTTGAAGTTGTTCTTGCCA    637
Mar5a    GTCGGCAGCTAGGGTCGAGCAGTTGAAGTTGTTCTTGCCA    631
Mar6a    GTCGGCAGCTAGGGTCGAGCAGTTGAAGTTGTTCTTGCCA    637
Mar14b   GTCGGCAGCTAGGGTCGAGCAGTTGAAGTTGTTCTTGCCA    637
Mar17d   GTCGGCAGCTAGGGTCGAGCAGTTGAAGTTGTTCTTGCCA    637
Mar17e   GTCGGCAGCTAGGGTCGAGCAGTTGAAGTTGTTCTTGCCA    637
Mtb1a    GTCGGCAGCTAGGGTCGAGCAGTTGAAGTTGTTCTTGCCA    637

Ack2b    TTGATGAGGTTGTTCTGCGAGAAAGGGGGGCGACTGTTAG    677
Ack2e    TTGATGAGGTTGTTCTGCGAGAAAGGGGGGCGACTGTTAG    680
CO6g     TTGATGAGGTTGTTCTGCGAGAAAGGGGGGCGACTGTTAG    677
Hb3b     TTGATGAGGTTGTTCTGCGAGAAAGGGGGGCGACTGTTAG    677
Hb6b     TTGATGAGGTTGTTCTGCGAGAAAGGGGGGCGACTGTTAG    677
Mar5a    TTGATGAGGTTGTTCTGCGAGAAAGGGGGGCGACTGTTAG    671
Mar6a    TTGATGAGGTTGTTCTGCGAGAAAGGGGGGCGACTGTTAG    677
Mar14b   TTGATGAGGTTGTTCTGCGAGAAAGGGGGGCGACTGTTAG    677
Mar17d   TTGATGAGGTTGTTCTGCGAGAAAGGGGGGCGACTGTTAG    677
Mar17e   TTGATGAGGTTGTTCTGCGAGAAAGGGGGGCGACTGTTAG    677
Mtb1a    TTGATGAGGTTGTTCTGCGAGAAAGGGGGGCGACTGTTAG    677

Ack2b    GG                                          679
Ack2e    GG                                          682
CO6g     GG                                          679
Hb3b     GG                                          679
Hb6b     GG                                          679
Mar5a    GG                                          673
Mar6a    GG                                          679
Mar14b   GG                                          679
Mar17d   GG                                          679
Mar17e   GG                                          679
Mtb1a    GG                                          679
```

C.4 Aligned amino acid sequence of laccase of all 11 *C. pauciseptatum* isolates and *Metarhizium acridum* CQMa 102 lcc1. Amino acids with green color represent non-specific Cu-oxidase domain, and those with yellow color indicate cupredoxin3-Ma-LCC like domain. The figures in red denote the number of amino acid.

```
Hb6b           .....................................GPRV      4
Ack2b          ......................................         0
Ack2e          ........................agilgpmvihGPRV     14
CO6g           ....................................hGPRV      5
Hb3b           ....................................hGPRV      5
Mar5a          ......................................         0
Mar6a          ......................................         0
Mar14b         ......................................         0
Mar17d         ......................................         0
Mar17e         ......................................         0
Mtb1a          .....................................PRV      3
XM_007813230   ksytysfeaelygttwyhshysaqyaggivgpmviyGPRt    200
                        206
Hb6b           KRDYDIDLGPVLIGDWYHEEYFDLVEQIMTPNSRPPFSQN     44
Ack2b          ...........................PNSRPPFSQN      10
Ack2e          KRDYDIDLGPVLIGDWYHEEYFDLVEQIMTPNSRPPFSQN     54
CO6g           KRDYDIDLGPVLIGDWYHEEYFDLVEQIMTPNSRPPFSQN     45
Hb3b           KRDYDIDLGPVLIGDWYHEEYFDLVEQIMTPNSRPPFSQN     45
Mar5a          KRDYDIDLGPVLIGDWYHEEYFDLVEQIMTPNSRPPFSQN     40
Mar6a          ................EEYFDLVEQIMTPNSRPPFSQN      22
Mar14b         KRDYDIDLGPVLIGDWYHEEYFDLVEQIMTPNSRPPFSQN     40
Mar17d         KRDYDIDLGPVLIGDWYHEEYFDLVEQIMTPNSRPPFSQN     40
Mar17e         KRDYDIDLGPVLIGDWYHEEYFDLVEQIMTPNSRPPFSQN     40
Mtb1a          KRDYDIDLGPVLIGDWYHEEYFDLVEQIMTPNSRPPFSQN     43
```

```
XM_007813230  aR.YDvDLGPVmlsDWYHrdYyaLVEetMkPNgRPvrSdt    239

Hb6b          NLINGKNNFNCSTLAADDTTPCNSNAGLSKFKFKRGKTHR     84
Ack2b         NLINGKNNFNCSTLAADDTTPCNSNAGLSKFKFKRGKTHR     50
Ack2e         NLINGKNNFNCSTLAADDTTPCNSNAGLSKFKFKRGKTHR     94
CO6g          NLINGKNNFNCSTLAADDTTPCNSNAGLSKFKFKRGKTHR     85
Hb3b          NLINGKNNFNCSTLAADDTTPCNSNAGLSKFKFKRGKTHR     85
Mar5a         NLINGKNNFNCSTLAADDTTPCNSNAGLSKFKFKRGKTHR     80
Mar6a         NLINGKNNFNCSTLAADDTTPCNSNAGLSKFKFKRGKTHR     62
Mar14b        NLINGKNNFNCSTLAADDTTPCNSNAGLSKFKFKRGKTHR     80
Mar17d        NLINGKNNFNCSTLAADDTTPCNSNAGLSKFKFKRGKTHR     80
Mar17e        NLINGKNNFNCSTLAADDTTPCNSNAGLSKFKFKRGKTHR     80
Mtb1a         NLINGKNNFNCSTLAADDTTPCNSNAGLSKFKFKRGKTHR     83
XM_007813230  NmINGKaNFdCSkLpAgDkTPChSNAGiarFKFtRGKTHR    279

Hb6b          LRLINAGAEALQRFSIDGHTMTIIANDFVQVQPYDTKVVT    124
Ack2b         LRLINAGAEALQRFSIDGHTMTIIANDFVQVQPYDTKVVT     90
Ack2e         LRLINAGAEALQRFSIDGHTMTIIANDFVQVQPYDTKVVT    134
CO6g          LRLINAGAEALQRFSIDGHTMTIIANDFVQVQPYDTKVVT    125
Hb3b          LRLINAGAEALQRFSIDGHTMTIIANDFVQVQPYDTKVVT    125
Mar5a         LRLINAGAEALQRFSIDGHTMTIIANDFVQVQPYDTKVVT    120
Mar6a         LRLINAGAEALQRFSIDGHTMTIIANDFVQVQPYDTKVVT    102
Mar14b        LRLINAGAEALQRFSIDGHTMTIIANDFVQVQPYDTKVVT    120
Mar17d        LRLINAGAEALQRFSIDGHTMTIIANDFVQVQPYDTKVVT    120
Mar17e        LRLINAGAEALQRFSIDGHTMTIIANDFVQVQPYDTKVVT    120
Mtb1a         LRLINAGAEALQRFSIDGHTMTIIANDFVQVQPYDTKVVT    123
XM_007813230  LRLINsGcEALQRFtIDGHTMTvIANDFVpVePYhTKVVT    319

Hb6b          LGIGQRTDVLVKADGDLDAYWMRANISVPCSLSLQPNALA    164
Ack2b         LGIGQRTDVLVKADGDLDAYWMRANISVPCSLSLQPNALA    130
Ack2e         LGIGQRTDVLVKADGDLDAYWMRANISVPCSLSLQPNALA    174
CO6g          LGIGQRTDVLVKADGDLDAYWMRANISVPCSLSLQPNALA    165
Hb3b          LGIGQRTDVLVKADGDLDAYWMRANISVPCSLSLQPNALA    165
Mar5a         LGIGQRTDVLVKADGDLDAYWMRANISVPCSLSLQPNALA    160
Mar6a         LGIGQRTDVLVKADGDLDAYWMRANISVPCSLSLQPNALA    142
Mar14b        LGIGQRTDVLVKADGDLDAYWMRANISVPCSLSLQPNALA    160
Mar17d        LGIGQRTDVLVKADGDLDAYWMRANISVPCSLSLQPNALA    160
Mar17e        LGIGQRTDVLVKADGDLDAYWMRANISVPCSLSLQPNALA    160
Mtb1a         LGIGQRTDVLVKADGDLDAYWMRANISVPCSLSLQPNALA    163
XP_007813230  LGIGQRTDVLVaADapLDAYWMRsNISeaCSLagerhAyA    359
                       366
Hb6b          AIYYDNADQTKQPKSTAWDVPDPATCVNDDLALTVPVMKL    204
Ack2b         AIYYDNADQTKQPKSTAWDVPDPATCVNDDLALTVPVMKL    170
Ack2e         AIYYDNADQTKQPKSTAWDVPDPATCVNDDLALTVPVMKL    214
CO6g          AIYYDNADQTKQPKSTAWDVPDPATCVNDDLALTVPVMKL    205
Hb3b          AIYYDNADQTKQPKSTAWDVPDPATCVNDDLALTVPVMKL    205
Mar5a         AIYYDNADQTKQPKSTAWDVPDPATCVNDDLALTVPVMKL    200
Mar6a         AIYYDNADQTKQPKSTAWDVPDPATCVNDDLALTVPVMKL    182
Mar14b        AIYYDNADQTKQPKSTAWDVPDPATCVNDDLALTVPVMKL    200
Mar17d        AIYYDNADQTKQPKSTAWDVPDPATCVNDDLALTVPVMKL    200
Mar17e        AIYYDNADQTKQPKSTAWDVPDPATCVNDDLALTVPVMKL    200
Mtb1a         AIYYDNADQTKQPKSTAWDVPDPATCVNDDLALTVPVMKL    203
XM_007813230  AIYYDgADeTrQPqSqpWDVPDPlTCaNDDLALTkPVMrr    399
                  403
Hb6b          PLPAADLTYDMNVEFFVNGSGITLFKFDDVDFRGNYNSPT    244
Ack2b         PLPAADLTYDMNVEFFVNGSGITLFKFDDVDFRGNYNSPT    210
Ack2e         PLPAADLTYDMNVEFFVNGSGITLFKFDDVDFRGNYNSPT    254
CO6g          PLPAADLTYDMNVEFFVNGSGITLFKFDDVDFRGNYNSPT    245
Hb3b          PLPAADLTYDMNVEFFVNGSGITLFKFDDVDFRGNYNSPT    245
Mar5a         PLPAADLTYDMNVEFFVNGSGITLFKFDDVDFRGNYNSPT    240
Mar6a         PLPAADLTYDMNVEFFVNGSGITLFKFDDVDFRGNYNSPT    222
Mar14b        PLPAADLTYDMNVEFFVNGSGITLFKFDDVDFRGNYNSPT    240
Mar17d        PLPAADLTYDMNVEFFVNGSGITLFKFDDVDFRGNYNSPT    240
Mar17e        PLPAADLTYDMNVEFFVNGSGITLFKFDDVDFRGNYNSPT    240
Mtb1a         PLPAADLTYDMNVEFFVNGSGITLFKFDDVDFRGNYNSPT    243
XM_007813230  rpiApDLTYDMeVglFkNaSnITLwsFggVDFRGdYNrPT    439

Hb6b          LLLAKLGEDDFEEQWN.........................   260
Ack2b         LLLAKLGEDDFEEQWN.........................   226
Ack2e         LLLAKLGEDDFEEQWNv........................   271
CO6g          LLLAKLGEDDFEEQWN.........................   261
Hb3b          LLLAKLGEDDFEEQWN.........................   261
Mar5a         LLLAKLGEDDFEEQ...........................   254
Mar6a         LLLAKLGEDDFEEQWN.........................   238
Mar14b        LLLAKLGEDDFEEQWN.........................   256
Mar17d        LLLAKLGEDDFEEQWN.........................   256
Mar17e        LLLAKLGEDDFEEQWN.........................   256
Mtb1a         LLLAKLGEDDFEEQWN.........................   259
XM_007813230  LLLsaLGnhtFEkQWNvkntqgaksvrvhvinntpvahpm    479
```

C.5 Minimal salt medium

For 50 mL:

1 Solution 1 - 200 g/l K_2HPO_4, 145 g/l KH_2PO_4 - 500 µL

2 Solution 2 - 30 g/l $MgSO_4.7H_2O$, 15 g/l NaCl - 1 mL

3 Solution 3 - 1% w/v $CaCl_2.2H_2O$ - 50 µL

4 Solution 4 - 20% glucose - 500 µL

5 Solution 5 - 0.01% w/v $FeSO_4$ - 500 µL

6 Solution 6 - 20% w/v $(NH_4)_2SO_4$ - 125 µL

C.6 McIlvaine's Buffer System

A citrate/phosphate buffer system that can be volumetrically set for pH in a wide range (2.2 to 8) and is therefore useful for enzyme kinetic studies etc. To prepare 20 mL of the buffer, mix 0.2 M disodium hydrogen phosphate and 0.1 M citric acid as shown below in the Table.

pH required	0.2 M Na_2HPO_4	0.1 M citric acid
	cc.	*cc.*
2.2	0.40	19.60
2.4	1.24	18.76
2.6	2.18	17.82
2.8	3.17	16.83
3.0	4.11	15.89
3.2	4.94	15.06
3.4	5.70	14.30
3.6	6.44	13.56
3.8	7.10	12.90
4.0	7.71	12.29
4.2	8.28	11.72
4.4	8.82	11.18
4.6	9.35	10.65
4.8	9.86	10.14
5.0	10.30	9.70
5.2	1.07	18.93
5.4	11.15	8.85
5.6	11.60	8.40
5.8	12.09	7.91
6.0	12.63	7.37
6.2	13.22	6.78
6.4	13.85	6.15
6.6	14.55	5.45
6.8	15.45	4.55
7.0	16.47	3.53
7.2	17.39	2.61
7.4	18.17	1.83
7.6	18.73	1.27
7.8	19.15	0.85
8.0	19.45	0.55

C.7 ANOVA for the laccase enzyme produced by *Cylindrocarpon* isolates in Experiment 2

Dependent Variable: PPO-I laccase enzyme produced by *Cylindrocarpon* isolates after 5 days incubation

Source	df	Sum of Squares	Mean square	F-ratio	P
Isolates	9	0.058135	0.006459	4.24	0.004
Residual	18	0.027437	0.001524		
Total	29	0.089413			

Dependent Variable: PPO-I laccase enzyme produced by *Cylindrocarpon* isolates after 7 days incubation

Source	df	Sum of Squares	Mean square	F-ratio	P
Isolates	9	0.1280668	0.0142296	21.87	<.001
Residual	18	0.0117101	0.0006506		
Total	29	0.1404142			

Dependent Variable: PPO-II laccase enzyme produced by *Cylindrocarpon* isolates after 5 days incubation

Source	df	Sum of Squares	Mean square	F-ratio	P
Isolates	9	0.0430265	0.0047807	26.67	<.001
Residual	18	0.0032265	0.0001792		
Total	29	0.0464499			

Dependent Variable: PPO-II laccase enzyme produced by *Cylindrocarpon* isolates after 7 days incubation

Source	df	Sum of Squares	Mean square	F-ratio	P
Isolates	9	0.0187181	0.0020798	3.13	0.019
Residual	18	0.0119625	0.0006646		
Total	29	0.0321335			