Cylindrocarpon Black Foot Disease in Grapevines

Identification and Epidemiology

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Chantal Michiko Probst

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This research aimed to improve understanding of the disease development processes in black foot disease on grapevines in New Zealand. The pathogenicity of three isolates from each of *Cylindrocarpon liriodendri*, *C. macrodidymum* and *C. destructans* was investigated by inoculating callused and rooted grapevines from rootstock varieties 101-14 and 5C. Results showed inter- and intra-specific variations, with disease incidences ranging from 32.1% to 82.1%. Callused vines had significantly greater disease incidences than rooted vines (P<0.001), but disease incidences were similar for both rootstock varieties.

Conidia, chlamydospores and mycelium from these species, when added to potting mix, were capable of infecting grapevines through wounded roots and callused basal ends. Conidia caused greater disease incidences than chlamydospores and mycelium, with means of 81.3, 62.5 and 56.3%, respectively, and *C. liriodendri* was consistently the most pathogenic species. The pathogenicity of the different propagules was confirmed in the field where similar results were observed to those in potting mix. When three propagule concentrations were tested for their ability to cause disease, increasing propagule concentrations resulted in increasing disease incidences. However, even the lowest concentrations of 10^2 spores /mL and 1 g of mycelium infested wheat grains were capable of infecting grapevines in potting mix.

Two different PCR based methods were developed to identify the different species and to test infested soils using species specific primers from the beta-tubulin 2 gene. The optimised nested PCR had a detection limit of 1 pg of pure DNA for all species, while the quantitative

PCR was capable of detecting as little as 11.5, 4.8 and 66.0 pg of *C. macrodidymum*, *C. iriodendri* and *C. destructans* pure DNA, respectively. A method was developed to extract DNA from 10 g of soil. The nested PCR was capable of detecting 1×10^5 *Cylindrocarpon* conidia /g of soil for all species while the quantitative PCR was capable of detecting 1.0×10^3 and 1.9×10^3 conidia /g for *C. liriodendri* and *C. macrodidymum*, respectively. The quantitative PCR was tested with soil which was infested with 10^5 conidia /g of soil at 0, 1, 3 and 6 weeks after inoculation. After a week, less than 5% of *C. liriodendri* propagules were detected, the numbers remaining constant over a period of 6 weeks, while *C. macrodidymum* propagules were not detected. The rapid reduction of propagule numbers in soil was also observed when conidia and mycelium were buried in mesh bags in soil. The two propagule types were converted into chlamydospores or degraded.

The effects of different stress factors on disease development were investigated. Infection incidence was increased in heavy soil types (P=0.001) and with waterlogging or dry (75% FC) watering regimes (P=0.022) while the effects of prolonged cold storage, wound age (0 to 8 days) and the presence of *Phaeomoniella chlamydospora* in grapevines did not show an apparent effect on disease development. The presence of wounds on canes, graft-union and trunks was necessary for their infection while disease incidences were similar for wounded or unwounded roots. This study has provided information about the behaviour of propagules of three *Cylindrocarpon* species in potting mix and in soil and the development of methods to detect these pathogens which could potentially be used to assess disease risks.

Keywords: Black foot, *Cylindrocarpon destructans*, *Cylindrocarpon macrodidymum*, *Cylindrocarpon liriodendri*, grapevine, pathogenicity, conidium, mycelium, chlamydospore, quantitative PCR, nested PCR, soil, stress factors, wounding.

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CHAPTER 1

Introduction

1.1. Grapevines

1.1.1. History

Wild and cultivated grapevines belong to the Vitaceae family which includes more than a thousand species that are found mostly in the tropical and subtropical regions of the world, but also in temperate regions (Galet, 2000). Among the nineteen Vitaceae genera identified, *Vitis* (Tournefort) Linneaus includes about sixty inter-fertile species located essentially throughout North America and Asia (This *et al.*, 2006). An Indo-European species from this genus, *Vitis vinifera,* which is believed to have appeared sixty-five million years ago (This *et al.*, 2006), is the most extensively cultivated grape species worldwide (Creasy and Creasy, 2009), and has two subspecies *vinifera* and *silvestris* (This *et al.*, 2006).

Grape fruit provides a broad range of products for daily life: they are consumed fresh or dried, and used to produce juices, preserves and wine (Creasy and Creasy, 2009). The production of wine, which is closely linked to human history (This *et al.*, 2006), is thought to have originated from Asia Minor over 7,000 years ago. The first indication of wine production was discovered in Iran in the Zagros Mountains and was dated around that period. Grapevine cultivation progressively spread with the major civilisations to the bordering regions and then throughout the Mediterranean basin. Vines and winemaking techniques spread with the Romans into the temperate regions of Europe (This *et al.*, 2006) and were brought into the New World by explorers and missionaries (Cooper, 1984), arriving in New Zealand in the nineteenth century (This *et al.*, 2006).

1.1.2. Wine industry in New Zealand

The first vines were planted in New Zealand in 1819 in Northland although the development of the wine industry started only in the late 1960s (Cooper, 1984). Since then the grape industry in New Zealand has grown annually, now stretching from Northland to Central Otago, with total vineyard area reaching 31,964 ha in 2009. Wine is produced in ten major regions: Northland, Auckland, Bay of Plenty, Hawkes Bay, Gisborne, Wellington, Nelson, Marlborough, Canterbury and Central Otago. Marlborough is the largest grape producing region with nearly 18,400 ha, representing 58% of the vineyards in New Zealand (Figure 1.1; New Zealand Winegrowers, 2009).

Figure 1.1. An expanse of vineyards in Blenheim, Marlborough, New Zealand.

In the last 10 years, the area of cultivated grapes has increased by about 20,000 ha and the number of wineries has doubled, reaching 643 wineries in 2009. Sauvignon Blanc is the most commonly grown grape variety, with 13,988 ha, followed by Pinot Noir (4,650 ha) and Chardonnay (3,881 ha), representing 48, 16 and 13% of the cultivated area, respectively (New Zealand Winegrowers, 2009).

New Zealand is still a very young grape-growing country with vineyard areas expanding each year, although it represents less than 1% of the world's viticultural area. The New Zealand wine industry is slowly making its way into the international export market with 1.8% of the market share value in 2006 (FAO, 2009). In 2009, 205.2 million litres of wine were produced in New Zealand, from which 112.6 million litres were exported, representing NZ$ 991.7 million (New Zealand Winegrowers, 2009). In the last 5 years, the production and export of wine has doubled. In 2009, Australia represented 33% of New Zealand's wine market, followed by the United Kingdom (27%), the USA (23%) and Canada (5%; New Zealand Winegrowers, 2009).

1.1.3. Grapevine cultivation

Grapevines are climbing plants with woody stems in the wild, with tendrils that enable them to hang from different supports to capture sunlight (Galet, 2000). In tropical climates, grapevines grow and fruit continuously, whereas in temperate climates, grapevine growth alternates between periods of dormancy (cold temperatures) and periods of active growth (Galet, 2000).

In commercial vineyards, the vines are usually grown in rows with trellis systems to support them. Pruning activities include winter pruning, during which old and diseased or damaged canes are removed, with only enough one year canes or buds being retained to produce the optimum quantity and quality of fruit in the next growing season. In addition, excess shoots are usually removed from the canopy or the trunk during spring (Jackson and Schuster, 1994). In cool climates, summer trimming of shoots and leaves is also a standard practice because it ensures that the canopies remain open and narrow, allowing penetration of sunshine and air circulation which improves fruit ripening and reduces development of diseases on the leaves and fruit.

1.1.4. Grafting of grapevine plants to rootstock varieties

The grapevine pest “phylloxera” (*Daktulosphaira vitifoliae* Fitch.) causes little damage to the North American *Vitis* species but is very damaging to the European *V. vinifera*, on which most wine-grape industries are based. *Daktulosphaira vitifoliae* is a yellow sap-sucking insect that feeds on roots, causing galls which progressively stop nutrient and water flow to the vine (Forneck and Huber, 2009). It is spread with infested soil and plants, by machinery and human activity, and the airborne phase is spread by wind (Wassilieff, 2008).

To combat the pest, different American *Vitis* species, which are naturally resistant to phylloxera, can be used as rootstocks. The rootstock species most commonly grown today are based on hybrids made with North American species (Galet, 2000). In California, the most commonly used rootstocks represent crosses between *V. berlandieri* and *V. riparia*, *V. riparia* and *V. rupestris*, and also *V. berlandieri* and *V. rupestris*, which are resistant to phylloxera biotype B (Gubler *et al.*, 2004). In New Zealand, the phylloxera aphid was first identified in 1885 (Buchanan *et al.*, 2003) however it became a threat only in the late 1980s. Most of New Zealand vineyards have now been planted with phylloxera resistant rootstocks, although about 25% of vines in the Wairarapa region and nearly 40% in Central Otago were ungrafted vines in 2007 (Wassilieff, 2008). In New Zealand, the rootstocks most commonly planted are crosses between *V. rupestris* and *V. riparia* (101-14, 3309 and Schwarzmann) and *V. berlandieri* and *V. riparia* (SO4 and 5C).

Research on rootstock varieties has mainly focused on their resistance to phylloxera and nematodes, as well as their effects on scion vigour, vegetative cycle (effect of cold hardiness, bud break and dormancy), nutrient uptake, starch storage, suitability for soil types, adaptability to high or low soil pH, soil salinity and lime and drought resistance, however little research has considered resistance to soilborne pathogens (Nicholas, 1999; Gubler *et al.*, 2004; Creasy and Creasy, 2009).

1.2. Black foot disease

In the early 1990s, grape growers worldwide started reporting decline symptoms in young grapevines, often followed by their death, and this occurred mainly in the areas where old vineyards were replaced by new vineyard plantings (Oliveira *et al.*, 2004). In California and Brazil, decline was mainly observed on phylloxera-resistant rootstocks (Scheck *et al.*, 1998; Garrido *et al.*, 2004b). The fungi most commonly isolated from symptomatic plants were *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp., which cause Petri disease, and *Cylindrocarpon* spp. which are responsible for black foot disease (Oliveira *et al.*, 2004), with plants frequently being infected by more than one pathogen (Rego *et al.*, 2000; Armengol *et al.*, 2001; Rumbos and Rumbou, 2001; Oliveira *et al.*, 2004; Harvey and Hunt, 2005). Since then, black foot has been increasingly recognised as a threat to grapevines.

1.2.1. Symptoms

Young vines with black foot disease show slow growth, reduced vigour, narrow trunks, retarded or absent sprouting, shortened internodes, sparse and chlorotic foliage with necrotic margins, reduced leaf size, wilting and dieback (Oliveira *et al.*, 2004; Figure 1.2). In addition, they show uneven wood maturity, which is usually associated with rapid desiccation events. Low numbers of feeder roots and sunken, dark, necrotic root lesions may also be present on the infected vines (Scheck *et al.*, 1998). Longitudinal sections made in the trunk bases of affected vines show brown to black vascular streaks from the bark to the pith and large blackened sectors (Oliveira *et al.*, 2004). Halleen *et al.* (2004) also reported necrotic root crowns and the compensatory development of secondary root crowns whose roots grew parallel to the soil surface (Figure 1.3). Similar symptoms occur in mature vines affected by this disease, but decline and death take place more slowly than in young vines (Gubler et *al.*, 2004).

Similar symptoms in rooted vine cuttings have been reported after inoculation with any of the several *Cylindrocarpon* spp., namely *C. obtusisporum* (Cook and Harkness) Wollenweber, *C. macrodidymum* Halleen, Schroers and Crous, *C. liriodendri* Mac Donald and Butler or *C. destructans* (Zinssmeiter) Scholten (Grasso and Magnano di san Lio, 1975; Rego *et al.*, 2001a; Garrido *et al.*, 2004b; Petit and Gubler, 2005; Halleen *et al.*, 2006b). Oliveira *et al.* (2004) reported that by 3 to 6 months after inoculation, the xylem vessels were blocked with gum inclusions that showed brown to black spots or circular necroses in cross section. Sweetingham (1983), who described a dark brown discoloration in the below-ground trunk portions of affected vines that extended up to 15 cm above ground (Figure 1.3), isolated *C. destructans* almost exclusively from the discoloured phloem tissue and found that xylem vessels in the discoloured tissues were mostly blocked with tyloses or a thick brown gum.

However, Halleen *et al.* (2006a) also associated these basal rot symptoms with all the pathogenic *Cylindrocarpon* spp. isolated from grapevines.

Figure 1.2. Above ground black foot symptoms on grapevines: retarded /absent sprouting (A), slow growth and reduced vigour (B), sparse and chlorotic foliage (C), dead vine (D).

Figure 1.3. Below ground black foot symptoms on grapevines: reduced root biomass, few feeder roots, sunken, dark, necrotic root lesions, secondary root crown (A) and longitudinal section: brown to black vascular streaks, from bark to pith (B).

1.2.2. Economic importance

Black foot disease occurs in all the major viticulture regions throughout the world including Italy, Portugal, Spain, South Africa, New Zealand, Chile, Australia and North America (Petit and Gubler, 2005), where it affects young and mature vines from 2 – 10 years old (Gubler *et al.*, 2004). The *Cylindrocarpon* spp. initially identified as the causal agents of black foot disease of grapevine were *C. destructans* from grapevines in France in 1961 (Maluta and Larignon, 1991) and *C. obtusisporum*, first recorded in Sicily in 1975 (Grasso and Magnano di san Lio, 1975). Two other species, *C. macrodidymum* and *C. liriodendri* were later identified as causal agents of black foot on grapevines (Halleen *et al*., 2004; Halleen *et al.*, 2006a).

In young vineyards, black foot disease is considered to be a major problem during the first 3 – 4 years after establishment, causing significant replanting, and so large economical losses (Oliveira *et al.*, 2004). Black foot disease has also been identified as a common cause of poor growth and death of young grafted grapevine plants growing in nursery soils, where it contributes to the annual plant losses of 20 – 40%, which was recently estimated at NZ$25 million in New Zealand. Lost production from failed mothervines was also estimated to be worth about NZ$3 million annually in New Zealand (Bruce Corban, Corbans Viticulture Ltd, pers. comm. 2006).

1.2.3. Taxonomy

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). Later, Wollenweber included species that formed chlamydospores in culture. Booth (1966) divided the genus into four groups depending on the presence or absence of microconidia and chlamydospores. Mantiri *et al.* (2001) and Brayford *et al.* (2004) recently 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: *Neonectria radicicola* (Gerlach and Nilsson) Mantiri and Samuels (teleomorph of *C. destructans*) was observed on grapevines in France (Maluta and Larignon, 1991); *Neonectria macrodidyma* Halleen, Schroers and Crous (teleomorph of *C. macrodidymum*) and *Neonectria liriodendri* Halleen, Rego and Crous (teleomorph of *C. liriodendri*) were obtained *in vitro* (Halleen *et al.*, 2004; Halleen *et al.*, 2006b).

Approximately 125 *Cylindrocarpon* spp. and varieties have been described (Mantiri *et al.*, 2001). Until recently, isolate classification and identification of the *Cylindrocarpon* spp. were based on their morphological characters in pure culture (Brayford, 1992). However significant colony differences between isolates from the same species have been observed in New Zealand, South Africa, France and Australia (Halleen *et al.*, 2006b). 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 beta-tubulin gene introns and exons, led Halleen *et al.* (2004) to observe that *Neonectria* /*Cylindrocarpon* species segregated in principally three groups: strains from the *Neonectria radicicola* /*C. destructans* complex, isolates classified as *C. macrodidymum* and species closely related to *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, both of which were also responsible for black foot disease symptoms (Halleen *et al.*, 2004). This genus is similar to *Cylindrocarpon* however it lacks microconidia.

1.2.4. Species identification

Morphological characterisation of *Cylindrocarpon* spp. on grapevines has led to the misidentification of these species. The use of molecular identification methods based on the sequence comparison of the ITS region of the rRNA and the β-tubulin genes has enabled the re-identification of several isolates worldwide. The common misidentification was between *C. macrodidymum* and *C. obtusisporum* and between *C. liriodendri* and *C. destructans*.

Cylindrocarpon obtusisporum was first reported in Sicily by Grasso and Magnano di san Lio in 1975, then in 1998 in California by Scheck *et al.*, who described the pathogen as growing rapidly and producing abundant micro- and macroconidia and chlamydospores in culture. This species was the most commonly isolated species from grapevines in California. Halleen *et al.* (2006) questioned the identity of these *C. obtusisporum* isolates as they formed perithecia when mated with *C. macrodidymum*. The authors believed the misidentification of *C. macrodidymum* as *C. obtusisporum* originated from the description of the former in 1966 (Booth) as the macroconidia dimensions and shape are similar to those of *C. macrodidymum.* However, Cooke in 1884 described the *C. obtusisporum* macroconidia as having obtuse ends and the dimensions were slightly smaller than those of *C. macrodidymum*. Petit and Gubler (2005) used the ITS and β-tubulin sequences to identify the Californian isolates as *C. macrodidymum* and as a result, *C. obtusisporum* has not been

found on grapevines. Isolates found in Sicily were unavailable therefore the species identity could not be ascertained.

Halleen *et al.* (2006b) re-identified isolates as *C. liriodendri* that were previously known as *C. destructans* using the ITS and β-tubulin genes and by conducting mating studies *in vitro*. *Cylindrocarpon liriodendri* isolates originated from Portugal (Rego *et al.*, 2001a), France, South Africa and New Zealand. Phylogenic trees indicated that these isolates clustered together and the clade was distinct from that of *C. destructans* and *Neonectria radicicola*. Consequently, 26 isolates previously identified as *C. destructans* from Spain were re-identified (Alaniz *et al.*, 2009), while in California, Petit and Gubler (2007) confirmed the need for re-identification of many isolates previously named "*C. destructans*".

A New Zealand survey of declining vines taken from all major grape growing regions in 2005 (Bleach *et al.*, 2006) has resulted in the isolation of 208 *Cylindrocarpon*-like isolates from 87% of the 121 symptomatic vines collected. These isolates were sent to Stellenbosch University in South Africa where 60 isolates were selected for identification by their morphology and their β-tubulin sequences. They were identified by Mostert *et al.* (2006) as: *C. destructans* (35.6%), *C. macrodidymum* (30.5%) and *C. liriodendri* (27.1%) and at least one novel species (6.8%).

1.2.5. Species descriptions

Cylindrocarpon destructans forms microconidia, macroconidia (one to three septate) and chlamydospores in potato dextrose agar (PDA) cultures after 10 days at 20°C in darkness (Rego *et al.*, 2001a). Macroconidia are cylindrical with rounded ends, straight or curved and narrowing slightly towards the base (Booth, 1966), measuring 30 – 40 µm × 5 – 6.5 µm. Microconidia are oval to elliptical (6 – 10 × 3.5 – 4 µm; Figure 1.4-A). Chlamydospores are globose and smooth but often appear rough due to surface deposits. They are hyaline at first, then become brown and have a diameter of 9 – 14 µm. They can be intercalary or terminal in the hyphae, single, in chains or in clumps and can be present in cells of macroconidia (Booth, 1966). Mycelium growth from a single spore on PDA forms a 10 – 20 mm diameter colony after seven days at 25°C (Booth, 1967; Seifert and Axelrood, 1998). Colony texture is cottony or felty with aerial mycelium in the centre or over the entire colony and colour varies from white to cinnamon (Rego *et al.*, 2001a; Figure 1.5-A). *Cylindrocarpon destructans* is widespread (Booth, 1966) and has been found in Africa (South Africa, Nigeria and Tanzania), Asia (Japan, Iran, Pakistan and Indonesia), Australia, New Zealand, Europe, Canada, Venezuela and the USA (Seifert and Axelrood, 1998). It is responsible for seed blights, basal rots and root rots of many host plants (Hirooka and Kobayashi, 2007), including

ginseng (Reeleder and Brammall, 1994), conifers (Bonello and Pearce, 1993), strawberry (Booth, 1967), blackberry (Cedeno *et al.*, 2004), apple trees (Braun, 1995), olive trees (Sánchez-Hernández *et al.*, 1998) and grapevines (Booth, 1966). It may also cause storage rot of carrot (Booth, 1967), young fruit tree rots in cold storage (Traquair and White, 1992) and dry rot of potatoes in storage (Booth, 1966).

Cylindrocarpon obtusisporum produces macroconidia which are one to three septate, curved, cylindrical with a rounded apex and slightly pointed basal cell and are 28 – 50 × 4 – 7.5 µm (Booth, 1966). Microconidia are oval and 7 – 8 × 4 – 5 µm. Chlamydospores are formed singly or in chains, are smooth, globose, hyaline to brown and are 10 – 15 µm in diameter. Mycelium growth on PDA forms a 10 mm colony after 10 days. Colony colour is white or hyaline, becoming tinged with beige and occasionally brown, with a floccose to felted texture. *Cylindrocarpon obtusisporum* has been observed on *Acacia* spp. in the USA, *Apium* spp. in Cyprus, *Beilschmiedia* and *Coprosma* spp. in New Zealand, *Glyceria* and *Tilia* spp. in Britain (Booth, 1966) and *V. vinifera* worldwide (Grasso and Magnano di san Lio, 1975; Scheck *et al.*, 1998) however Halleen *et al.* (2006a) considered that *C. macrodidymum* has been misidentified in *V. vinifera* as *C. obtusisporum*.

Cylindrocarpon macrodidymum produces macroconidia which are predominantly one to three septate, straight or sometimes slightly curved, cylindrical or slightly widening towards the tip (34 – 38 × 5.5 – 6.5 µm) and are abundant on PDA (Halleen *et al.*, 2004; Figure 1.4-C). Microconidia are aseptate or one septate, ovoidal or ellipsoidal, nearly straight and 8 – 10.5 × 4 µm. Chlamydospores are produced rarely on PDA, but occur in short intercalary chains, are hyaline to brown and measure 7 – 12.5 × 6 –10 µm in diameter. Mycelium growth from a single spore on PDA forms a 25 – 35 mm diameter colony after 7 days at 20°C. Colony colour is yellowish and texture is felty with aerial mycelium abundantly formed, covering the whole colony or sectors of it (Halleen *et al.*, 2004; Figure 1.5-C). The optimum temperature for growth is 20 – 25°C.

Petit and Gubler (2005) tried to differentiate "*C. destructans*" and *C. macrodidymum* as both species were commonly isolated from grapevines in California. They observed that macroconidia from *C. macrodidymum* were significantly (P<0.5) larger than those of *C. destructans* and that *C. destructans* isolates grew faster than those of *C. macrodidymum* (respectively 3.4 ± 0.5 and 3.0 ± 0.4 mm /day) at 25°C after 6 days in a 12 h light /dark cycle on PDA. They also observed that the reverse colony colour on 2% malt extract agar (MEA) consistently discriminated *C. destructans* from *C. macrodidymum* the former having a buff reverse whereas the latter had an orange-dark brown reverse after 6 days at 25°C in a 12 h light/dark cycle illuminated by fluorescent strips and near ultraviolet light (366 nm). However,

it is now believed that many *C. liriodendri* isolates were initially misidentified as *C. destructans* (Dr. Paul Fourie pers. comm. 2006).

Cylindrocarpon macrodidymum has only recently been described (Halleen *et al.*, 2004) and to date is known to infect only grapevines (Petit and Gubler, 2005; Figure 1.5-C). It has been found in South Africa, Canada, Australia, USA, New Zealand (Halleen *et al.*, 2004), Chile (Auger *et al.*, 2007) and Spain (Alaniz *et al.*, 2009).

Figure 1.4. Conidia of *C. destructans* (A), *C. liriodendri* (B) and *C. macrodidymum* (C). Photographs courtesy of Carolyn Bleach.

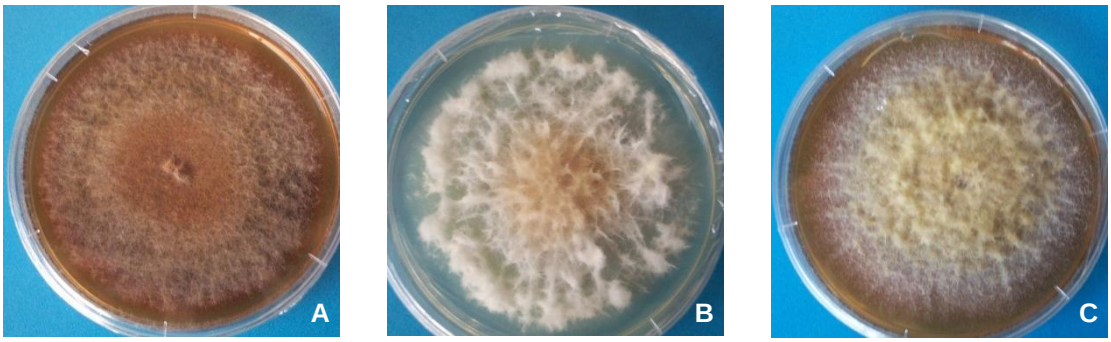

Figure 1.5. *Cylindrocarpon destructans* (A), *C. liriodendri* (B) and *C. macrodidymum* (C) grown on PDA at 20°C for 2 weeks in the dark (Petri dish diameter: 9 cm).

Cylindrocarpon liriodendri was first reported on *Liriodendron tulipifera* L. in California (MacDonald and Butler, 1981). Affected plants appeared severely stunted and the root systems were covered with black, dry, scabby lesions (MacDonald and Butler, 1981). It produced one to three septate macroconidia and chlamydospores but microconidia were reported as lacking, however Halleen *et al.* (2006b) found microconidia in culture. From the DNA sequences of the ITS and β-tubulin gene regions, they subsequently found that many isolates formerly identified as "*C. destructans*" were *C. liriodendri*. Macroconidia are predominantly three septate, straight or sometimes slightly curved, cylindrical and measure 35 – 40 × 5.5 – 6 µm (Figure 1.4-B). Microconidia are aseptate or one septate, ellipsoidal to subcylindrical (5 –15 × 2.5 – 4 µm) and more or less straight, or ovoid (3 – 5 × 3 – 4 µm).

Chlamydospores are medium brown, ovoid to ellipsoid, mostly in short, intercalary chains, and are 10 – 20 × 10 – 17 µm (Halleen *et al.*, 2006b). Colonies grown on PDA at 20°C in the dark are cinnamon to sepia coloured with sparse aerial mycelium and measure 30 – 42 mm diameter after 7 days (Figure 1.5-B). The optimum growth temperature is 20 – 25°C. *Cylindrocarpon liriodendri* has been reported on *V. vinifera* in France, Portugal, New Zealand, South Africa (Halleen *et al.*, 2006b), Australia (Whitelaw-Weckert, 2007) and Iran (Mohammadi *et al.*, 2009), on *Cyclamen* spp. in the Netherlands and on *Liriodendron tulipifera* in California (MacDonald and Butler, 1981). Inoculation of 6 month old potted grapevines with one *C. liriodendri* isolate resulted in the reduction of their root and shoot biomass and in the death of 27.5% of the plants after 60 days (Halleen *et al.*, 2006). Similar results were obtained by Rego *et al.* (2001a).

Campylocarpon fasciculare Schroers, Halleen and Crous produces mostly three to four septate macroconidia which are cylindrical, slightly to moderately curved, with slightly narrowed and obtuse ends, and are 29 – 53 × 6 – 9 µm (Halleen *et al.,* 2004). Microconidia and chlamydospores were not observed. Colonies grown on PDA measure 20 – 30 mm after 7 days. On PDA, aerial mycelium is abundant, white, thick and cottony to felty with white to brown hyphal strands up to 8 mm long and 20 – 70 µm thick, while the colony reverse is brown. The optimum temperature for growth is 30°C. *Campylocarpon fasciculare* was found on roots, rootstock and trunks of grapevines in South Africa (Halleen *et al.* 2004). These authors observed that 4.5 months after inoculation of the young vines (cv. Ramsey) 22.5% had died and there was an overall reduction of root and shoot masses (48 and 17.8%, respectively).

Campylocarpon pseudofasciculare Halleen, Schroers and Crous produces mostly three to five septate macroconidia which are cylindrical, slightly to moderately curved, more narrowed at the base than the apex, with or without an obscure hilum and are 29 – 68 × 6 – 10 µm (Halleen *et al.,* 2004). Microconidia are absent and chlamydospores are sparse, occurring in clusters of three to five, intercalary or on short side branches. They are round to slightly angular and are 8 – 11 × 7 – 8.5 µm. Colonies grown on PDA measure 25 mm after 7 days. On PDA, aerial mycelium is abundant, white to slightly brownish, thickly cottony to felty with white to brown hyphal strands up to 10 mm long and 10 – 80 µm thick, while the colony reverse is brown. The optimum temperature for growth is 30°C. *Campylocarpon pseudofasciculare* was found on roots of asymptomatic nursery grapevines in South Africa. On inoculated grapevines (cv. Ramsey), *C. pseudofasciculare* caused the death of 17.5% of vines 4.5 months after inoculation and an overall reduction of root and shoot masses (40.7 and 22.5%, respectively; Halleen *et al.*, 2004).

1.2.6. Disease cycle

1.2.6.1. Sources of inoculum

Several studies undertaken in different countries have tried to locate the initial sources of inoculum. Since this pathogen infects stem bases, killing the tissue as it expands, it is very unlikely to be present in the canes used for propagation. Rego *et al.* (2001b) rarely found *Cylindrocarpon* spp. in canes of mothervines, which is consistent with the known mode of infection. Halleen *et al.* (2003) found that less than 1% of the grafted plants were infected with *Cylindrocarpon* spp. prior to planting in the nurseries, however at 7 months after planting out in nursery soils more than 50% of the sampled plants were infected, indicating that the pathogen was present in the nursery soils. In Spain, *Cylindrocarpon* spp. were only found in rootstock sections of the grafted vines, mostly after being planted in the nursery field for rooting (23.8 %). In Portugal, severe outbreaks were observed in young vineyards of certain grape cultivars which were linked to the same nursery or the same regional source (Rego *et al.*, 2000). After sampling rootstocks from different nurseries in Portugal, Rego *et al.* (2000) found that a high number of the young plants supplied by nurseries were diseased, with 37% of the plants being infected with *Cylindrocarpon* spp., a conclusion also reached by Armengol *et al.* (2001) in Spain. Standard nursery practice involves growing the grafted plants in the same site continuously or with a 2 year rotation system, whereby cuttings are planted every second year alternated with a cover crop. Repeated use of the same soil can lead to a build-up of soil-borne pathogens such as species of *Cylindrocarpon* (Halleen *et al.*, 2003).

Gubler *et al.* (2004) stated that although black foot disease is linked to nursery production, since they found up to 5% of vines from some nurseries were infected with this pathogen, they believed the presence of *Cylindocarpon* spp. in vineyard soils to be the most common primary inoculum source. This suggestion was also advanced by Oliveira *et al.* (2004), who isolated *Cylindrocarpon* spp. from 87.5% of vines with necrotic rootstock wood tissue that came from a young vineyard in Portugal. It is therefore likely that inoculum of *Cylindrocarpon* spp. can also build up in vineyard sites replanted from grapes or other fruit crops.

Cylindrocarpon isolates from roots of declining apple trees in New Zealand were identified as *C. destructans* and found to be similar to isolates from New Zealand grapevines (Mostert *et al*., unpublished). This suggested that if declining apple orchards were converted to vineyards, they could provide inoculum source for development of black foot (Mostert *et al*., unpublished). Bonfiglioli (2005) postulated that the high rate of black foot infection in New Zealand vineyards could be due to the number of vineyard sites previously planted with apple trees.

1.2.6.2. Behaviour of *Cylindrocarpon* spp. in the soil

Cylindrocarpon spp. are soil-borne fungi that are widespread on every continent, in diverse habitats and on a variety of hosts (Brayford, 1992). They are found in the upper and lower soil horizons because they can grow at low oxygen concentrations (Garrido *et al.*, 2004a), being observed up to 63 cm deep (Matturi and Stenton, 1964). They can also grow rapidly at low nutrient concentrations, enabling them to colonize new substrates before other fungi (Garrido *et al.*, 2004a). They are considered to be pioneer colonizers of young root tips due to their rapid spore germination and mycelial growth, and their ability to use both inorganic and organic nitrogen compounds. The *Cylindrocarpon* spp. present in nursery and vineyard soils can live as saprobes on dead plant material, as root colonizers or pathogens (Brayford, 1992). They could be present as mycelia, conidia or chlamydospores (Figure 1.6), which are believed to survive in the soil for several years. Taylor (1964) showed that a week after placing "*Cylindrocarpon radicicola"* (*C. destructans*) conidia in natural soils, most either did not germinate or had short germ tubes with chlamydospores at the ends. In sterile soils, he observed that conidia germinated and mycelial development was fast with a maximum activity after 2 weeks on easily decomposable organic material. However, after 1 month, hyphae had mostly disappeared and could only be found on the surface of the soil, whereas chlamydospores were common. Matturi and Stenton (1964) reported that when conditions were unfavourable, as in acid and alkaline soils, mycelia and conidia of *Cylindrocarpon* spp. had very short life spans and frequently suffered from lysis possibly due to the activity of bacteria. Under these conditions, the formation of chlamydospores was stimulated (Matturi and Stenton, 1964), and these were believed to stay dormant until they were stimulated by plants or were destroyed by other microorganisms. However, they found that the chlamydospores required a period of dormancy of approximately 12 days before they could be stimulated by plants to germinate (Matturi and Stenton, 1964).

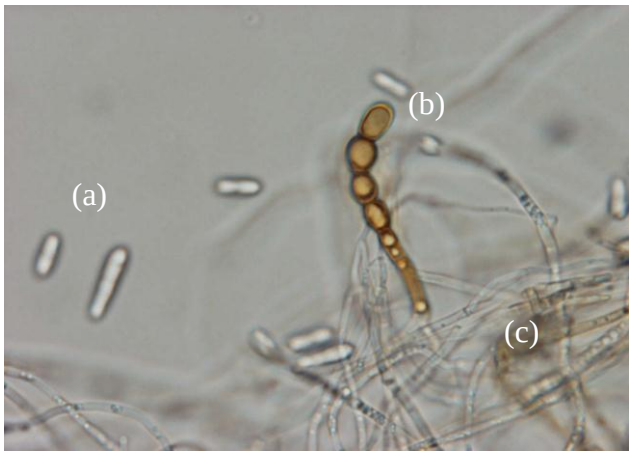

Figure 1.6. *Cylindrocarpon liriodendri*, (a) conidia, (b) chlamydospores, (c) mycelium.

1.2.6.3. Infection and disease development

When the conditions are favourable and possibly through stimulation by root exudates, the chlamydospores germinate, produce hyphae, multiply and grow towards the roots to invade the plants (Horsfall and Dimond, 1960). The growing pathogen probably produces conidia in the soil which germinate, increasing the inoculum potential (Horsfall and Dimond, 1960). *Cylindrocarpon* spp. are believed to infect grapevines through natural openings or through wounds on the roots or other belowground portions of the rootstock such as the uncallused stem base (Scheck *et al.*, 1998). Wounds can be produced by the emergence of secondary roots, activities of insects, or nematodes, or abrasion with soil particles (Horsfall and Dimond, 1960). When Halleen *et al.* (2003) isolated *Cylindrocarpon* spp. from rooted cuttings at different times after planting out in nursery soils (0, 3, 6 and 9 months), the pathogen was often found in the roots after 3 months and was observed to progress into the rootstock, graft-union and scion (although in lower numbers). Infections of roots and stem bases appear to progress into the base of the vine. Sweetingham (1983) frequently isolated *Cylindrocarpon destructans* from the basal parts of trunks and Aroca *et al.* (2006) reported that 72.7% of the *Cylindrocarpon* spp. isolated were from the basal rootstock ends of vine trunks.

As the pathogen moves further into the butt of the vine, the tissues blacken and exhibit a dry cortical rot which rapidly causes vine dieback and death. The xylem usually becomes occluded with fungal tissue, gums and tyloses and the vascular tissue turns black. Sweetingham (1983) observed fungal hyphae mainly in the ray cells of the phloem where starch reserves are stored. When young vines are attacked, they die very quickly but as the vines age, infection results in a more gradual decline, and death may take more than 1 year to occur. Death seems to be inevitable when vines less than 10 years of age are infected with *C. destructans* (Scheck *et al.*, 1998).

Once the grapevine is dead, the pathogen can produce chlamydospores, survive on any organic matter present which provides carbohydrates and cellulosic materials or invade roots from surrounding grapevines. Maluta and Larignon (1991) observed that grapevines with black foot disease in a vineyard were often located in lines or in patches, indicating that infection spreads from one vine to its neighbours, possibly through rain, water, machinery or human activities. Dead vines are usually discarded from the vineyard and replaced by new vines which constitute a new source of carbohydrates for the pathogen.

1.2.7. Effect of stress factors on disease development

Species of *Cylindrocarpon* are generally considered as weak or minor pathogens of limited economic importance on vegetables, small fruits, tree fruits, forage plants, ornamentals and conifers (Mantiri *et al.*, 2001). However, they are believed to incite disease when plants are under stress conditions (Oliveira *et al.*, 2004), which Stamp (2001) maintained is a constant feature of nursery propagation and vineyard establishment in the first years of management.

Reports have indicated that several factors contribute to the development of black foot disease, such as environmental factors, nursery and vineyard practices and biotic stress (Scheck *et al.*, 1998; Stamp, 2001; Oliveira *et al.*, 2004; Halleen *et al.*, 2006b). However, the impact of these factors on the disease has not yet been investigated in depth.

1.2.7.1. Environmental factors

Temperature, water availability and soil components influence the development of grapevines, of which soil appears to be the most complex factor (Galet, 2000). The relative proportions of soil components (silica, clay, limestone and humus) determine the compactness of the soil and the ease with which roots penetrate it. Soil compaction and /or poor soil preparation restrain root development and enhance J-rooting, in which the root system of the young plant is bent to resemble the letter 'J' (Maluta and Larignon, 1991). Soil chemical composition also determines mineral nutrition (Galet, 2000), with insufficient nutrient absorption leading to weakened plants that may exhibit disorders (Galet, 1996). In vineyards, the damage caused by malnutrition depends on the nutrient absorbing qualities of the rootstock variety and can be worsened if the minerals absorbed by the roots are not suitable for the needs of vegetative and fruit development of the scion (Galet, 1996).

The water retention capacity associated with each type of soil affects water circulation. Clay soils are prone to water logging (causing root asphyxiation) and they may contract significantly when water is unavailable, causing deep cracks that can damage roots. Sandy soils do not hold water well and dry up quickly, causing water deficit (Galet, 1996). Water is essential for soil micro-organisms and for the absorption of mineral compounds but in wet conditions the spread of root diseases is enhanced (Galet, 2000), such that losses due to black foot are greater in grapevines grown on heavy, poorly drained soil (Gubler *et al.*, 2004).

High temperatures during summer and dry, violent winds contribute to the expression of root disease (Galet, 1996). In affected plants, the reduced root system and blocked vascular system can mean that the water supplied to the scion is not sufficient to compensate for its heavy transpiration rate (Maluta and Larignon, 1991).

1.2.7.2. Nursery practices

During the different nursery stages, large numbers of wounds are made to the propagation material, providing infection courts for fungal pathogens (Gimenez-Jaime *et al.*, 2006). The grapevine cuttings (scion and rootstock) are harvested from mothervines during winter and put into a cold storage with high humidity to prevent the desiccation of the cuttings and the development of superficial diseases (Gimenez-Jaime *et al.*, 2006; Creasy and Creasy, 2009). The cuttings are often immersed in water for a few hours to days before storage and then again before grafting. In preparation for grafting, the three node rootstock pieces are disbudded, while the scion wood is cut into one node pieces. The rootstock and scion pieces are grafted together and placed into callus boxes in a moist medium at 26 – 28°C for 3 – 5 weeks to heal the graft union and to develop callus on the graft area and stem base. After 3 – 5 day acclimatisation in the shade house, the grafted plants are planted in the field where they develop roots and shoots during 7 – 9 months growth. During the next winter, they are mechanically lifted; roots are trimmed and washed, and the dormant plants are cold-stored for 2 – 3 months before being sent to the growers (Gimenez-Jaime *et al.*, 2006; Creasy and Creasy, 2009).

During each stage of the nursery practices, grapevine cuttings can be infected through the many wounds that are produced, for example by disbudding, grafting, pruning and uprooting (Stamp, 2001). This was demonstrated by Gimenez-Jaime *et al.* (2006) who isolated *Cylindrocarpon* spp. from rootstock cuttings after 2 months of storage at 5 – 6°C at 90% relative humidity (4.8%) and before immersion in water (19.1%). They also isolated them from rootstocks of grafted cuttings after 20 days in callus boxes (4.8%) and from rootstocks of rooted grafted vines in the nursery field, 2 months after being planted (23.8%), after being uprooted (9.5%) and before being planted in vineyards (8.1%). Halleen *et al.* (2003) observed that the proportion of infected vines increased while they were in the nursery field. At the time of planting, callus tissue usually does not cover the entire basal end of the cuttings, leaving them exposed to soil-borne pathogens, and during planting in the field young roots often break, providing entry points for the pathogens (Halleen *et al.*, 2003).

Plants with unhealed wounds, inadequate root systems, undersized trunks with limited carbohydrate reserves and the use of extended cold storage or improper storage, excessive time in containers and inappropriate transportation conditions are more easily stressed and so weakened by the time they are planted out into the field (Stamp, 2001), which may lead to greater incidence of black foot.

1.2.7.3. Vineyard practices

During the first few years after vine establishment, the use of some vineyard practices can cause stress to young plants. These include planting vines in poorly prepared soil and in improper plant holes, use of deficient or excessive irrigation and nutritional deficiencies or excesses. In addition, early cropping and heavy crop loads on young plants can result in diminished carbohydrates reserves that are anecdotally reported to precede many incidences of black foot (Stamp, 2001).

Studies on the influence of seasonal partial defoliation on overwintering carbohydrate reserves showed that shoot growth and total vine pruning weight were decreased in partially defoliated vines where carbohydrate reserves were reduced (Bennett *et al.*, 2005). Dore *et al.* (2009) reported significantly (P<0.05) greater *C. destructans* disease severity in greatly defoliated than in undefoliated young vines, indicating that such practices might make young vines more susceptible to black foot disease.

1.2.7.4. Pest and pathogen effects

The effects of different pathogens can weaken the plants and so enhance incidence or severity of black foot disease. Halleen *et al.* (2003) showed that pathogens such as *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. were often present in rootstock propagation material. These taxa were frequently isolated from vines infected with *Cylindrocarpon* spp. in Portugal (Rego *et al.*, 2000; Oliveira *et al*., 2004), Australia (Edwards and Pascoe, 2004), New Zealand (Harvey and Hunt, 2005), Greece (Rumbos and Rumbou, 2001), Spain (Armengol *et al.*, 2001; Gimenez-Jaime *et al.*, 2006) and South Africa (Gugino and Travis, 2003). *Phaeoacremonium aleophilum*, *Botryosphaeria* spp., *Phomopsis* spp. and other wood fungi were also frequently isolated from vines infected with *Cylindrocarpon* spp. (Oliveira *et al.*, 2004; Gimenez-Jaime *et al.*, 2006). Gubler *et al.* (2004) observed that plants infected with *Cylindrocarpon* spp. and *Phytophthora* spp. showed a more rapid death than plants infected with only one pathogen. Therefore, it seems likely that the presence of *Phytophthora* spp. increases the susceptibility of vines to infection by *Cylindrocarpon* spp. or *vice versa*.

Many different pests can attack roots, wood, shoots and leaves of vines reducing their capacity to absorb nutrients and water, and providing entry points for pathogens such as *Cylindrocarpon* spp. Phylloxera is a cause of serious damage to grape roots allowing pathogens to invade roots through the wounds (Omer *et al.*, 1995). Nematodes like *Meloidogyne* spp., *Tylenchulus semipenetrans* Cobb., *Mesocriconema xenoplax* (Raski) Loof and De Grisse and *Xiphinema* spp. can also be important pests of grapevines, causing decline and making them more susceptible to other diseases (Walker and Grandison, 1994).

The larvae of *Vitacea polistiformis* Harris, which are present in eastern USA, or *Fidia viticida* Walsh and *Bromius obscurus* Linnaeus, which occur in western USA, infest large roots and crowns injuring the vascular system and causing water stress, while the adult forms may feed on leaves.

1.2.8. Management of black foot disease

1.2.8.1. Rootstock varieties

The North American *Vitis* species have provided some phylloxera - and lime - resistant hybrids suitable for use as rootstocks to be grafted to *Vitis vinifera* fruiting varieties. In California, Scheck *et al.* (1998) reported an increase of grapevine death caused by root diseases since the introduction of rootstocks resistant to phylloxera. It has become apparent that vine decline, black foot and Petri disease were being expressed preferentially on the rootstocks planted for phylloxera control (Scheck *et al.*, 1998). Halleen *et al.* (2006b) concluded that there was little information currently available regarding rootstock susceptibility but that susceptibility to infection by *Cylindrocarpon* spp. varied among cultivars. Gubler *et al.* (2004) observed that the most widely planted rootstocks, 3309, 101-14, 5 C and 110 R showed the disease most commonly; however *Vitis riparia*, 039-16 and Freedom had a good degree of resistance to *C. destructans*. Rego *et al.* (2000) observed severe outbreaks of the pathogen with wine cultivars and table grape cultivars grafted onto 99 R, 110 R, 1103 P, 101-14, 140 Ru, 5 BB and 161-49. Two trials carried out at Lincoln by Plantwise Services Ltd in association with Lincoln University in 2006 using 11 rootstock varieties showed that the most tolerant varieties to black foot were Riparia Gloire and 140 Ru. Schwarzmann, 5 BB, Paulsen, 101-14, 3306 and 420 A were the most susceptible rootstock varieties (Harvey and Jaspers, 2006). Current and future grape rootstock research should focus on improving rootstock resistance to soil pests and diseases while maintaining desirable horticultural traits (Gubler *et al.*, 2004).

1.2.8.2. Nursery and vineyard practices

No effective control measures are available to completely prevent infection of young vines by decline diseases but several procedures could be introduced in the nursery to reduce inoculum levels, improve health of young vines and to minimize stress (Oliveira *et al.*, 2004). To reduce potential contamination of grafting wounds, Fourie and Halleen (2006) recommended that equipment be sterilised and hands of the grafting personnel be thoroughly washed. Good hygiene practices need to be implemented in nurseries and vineyards, as well as cultural, biological, physical and chemical control measures to prevent infection and to control disease development.

1.2.8.3. Soil management

As stresses predispose young grapevines to infection, proper management of soil, water, nutrients and other pests is strongly recommended as well as planting clean stock in well-drained soils (Scheck *et al.*, 1998; Oliveira *et al.*, 2004). For new vine planting, heavy soils should be drained (Gubler *et al.*, 2004) and compact soils should be broken up to make the soil accessible to roots (Maluta and Larignon, 1991). Planting holes should be wide and deep enough to allow root development and thus avoid J-rooting (Halleen *et al.*, 2006a).

Suggested methods of reducing levels of soil inoculum include crop rotation and soil fumigation (Damm and Fourie, 2005), however, composting or the use of antagonistic microorganisms could also reduce disease incidence since microorganisms isolated from compost have shown antagonist responses to *C. destructans* (Gugino and Travis, 2003). Whitelaw-Weckert (2004) also observed that 70% of bacteria and actinomycetes from the inter-row vineyard soil treated with herbicide inhibited *C. destructans*.

1.2.8.4. Hot water treatment

Hot water treatment is known to be an effective method for the control of a number of grapevine pests present in dormant cuttings and rooted vines when applied at 50°C for 30 min (Waite and May, 2005) and it may be offered as an additional treatment with nursery procedures. Crous *et al.* (2001) reported an experiment in which dormant young grafted vines (rootstock 101-14 and scion Shiraz 99B) were treated by dipping in a hot water bath at 50°C for 30 min followed by a cold water bath for 30 min and were planted in a nursery for 6 months before being assessed. Shoots from rootstock variety 101-14 were similarly hot water treated and isolations were made from the wood. Hot water treatment completely eliminated fungi from the rootstock cuttings but after 6 month growth in the field, the numbers of isolated fungi in the grafted vines did not differ significantly between the treated plants and the controls. It is probable that the vines became re-infected in the field once planted out. However, this experiment indicated that hot water treatment was effective against grapevine pathogens (Crous *et al.*, 2001) and therefore Halleen *et al.* (2005) recommended hot water treatment of dormant nursery vines prior to planting in vineyards as a proactive method for reducing the incidence of black foot pathogens in vineyards. They also recommended that a biological control agent or a fungicide should be investigated for use when out planting.

1.2.8.5. Biological control agent

*S*oil amendments with *Trichoderma* spp. have been reported to be beneficial to plant growth because these species are antagonistic to soil pathogens, by competing with the pathogens and by inducing the production of defence related compounds in plants that enhance their resistance (Smolinska *et al.*, 2007). Fourie *et al.* (2001) dipped grapevine rootstock material in a *Trichoderma harzianum* conidial suspension directly prior to grafting, and then again after grafting. The plants were callused, planted out into planting holes containing *Trichoderma* spp. conidial pellets and then the root zones of the treated vines were drenched with the suspensions containing *Trichoderma* spp. every month for 6 months. Compared to the controls, root development of these vines was increased by 41.7%, with a decrease of 42.9% in the number of *Cylindrocarpon* isolates and 40% of Petri disease fungi being recovered from the treated vines. Fourie *et al.* (2001) postulated that the *Trichoderma* spp. might have prevented root pathogens from attacking the roots by colonizing the rhizosphere, thereby acting as antagonists or competitors. This experiment demonstrated the potential of *Trichoderma* spp. treatments for reducing infection levels by *P. chlamydospora*, *Phaeoacremonium* and *Cylindrocarpon* spp., and for improving root development.

Studies on chitosan, a polymer obtained from the chitin of crustacean shell wastes, showed that it could inhibit the growth of pathogenic fungi and potentially elicit plant defence responses (Benhamou and Theriault, 1992). Laflamme *et al.* (1999) found that mycelial growth of *C. destructans* (isolated from black spruce) was inhibited by chitosan *in vitro* with growth inhibition of 43 and 71% at concentrations of 1 and 2 mg/mL, respectively. They also observed severe morphological and ultrastructural changes in the pathogen hyphae, ranging from the disorganisation of the cytoplasm to the degradation of the protoplasm. Nascimento *et al.* (2007) found that chitosan could reduce mycelial growth of *C. liriodendri in vitro* but needed a higher concentration (24.65 mg /L) than the one recommended for field use (8.33 mg /L) to reduce growth. However, when applied twice as a foliar spray (7.14 mg /L) onto vines growing on a substrate infested with *C. liriodendri*, chitosan showed efficacy against *C. liriodendri*, with a disease incidence of 32.0% compared to 80.8% with the infected controls, which indicated its potential as an inducer of systemic plant resistance. Its efficacy was similar to that achieved by tebuconazole and the mixtures carbendazim /flusilazole and cyprodinil /fludioxonil, which reduced incidence of *C. liriodendri* to 43.3, 40.8 and 21.7%, respectively. Use of chitosan should be investigated under field conditions, where it may provide an alternative natural product for improving the management of black foot and possibly other grapevine wood diseases (Nascimento *et al.*, 2007).

1.2.8.6. Mycorrhizae

Arbuscular mycorrhizal fungi form symbiotic associations with plants that improve nutrient uptake and stress tolerance of hosts (Chandanie *et al.*, 2009) and may diminish the effects of root pathogens. Bleach *et al.* (2008) studied the effect of two arbuscular mycorrhizae, *Glomus mosseae* and *Acaulospora laevis* on young plants of different rootstock varieties in relation to their susceptibility to three *Cylindrocarpon* spp. previously inoculated into the nursery soils. Both mycorrhizal fungi had some beneficial effects, with those of *G. mosseae* being more consistent than *A. laevis*. After 9 months in the field, treated plants had significantly increased root biomass (214% in Auckland and 22% in Blenheim) but did not have decreased infection incidence. Petit and Gubler (2006) studied whether the presence of *Glomus intraradices* influenced development of black foot disease caused by *C. macrodidymum*, in mycorrhizal plants that had approximately 50% of their root lengths colonised with *G. intraradices*. They reported that foliar symptoms and root rot were reduced by 37 and 40%, respectively compared to non mycorrhizal plants in treatments inoculated with *C. macrodidymum.* The dry shoot and root weights of control plants (no treatments), mycorrhizal plants and mycorrhizal plants with *C. macrodidymum* were higher than the plants only inoculated with the pathogen. Petit and Gubler (2006) hypothesised that *G. intraradices* promoted the growth of soil microbes that are antagonistic to *Cylindrocarpon* spp. and improved the nutrition of the grapevines. Halleen *et al.* (2006a) also suggested that soil amendments with *Trichoderma* spp. and mycorrhizae could effectively enhance plant resistance and thereby reduce disease incidence or severity.

1.2.8.7. Fungicides

Dipping of rootstock cuttings and recently grafted plants (graftlings) in effective fungicides before planting could prevent the infection of vines by *Cylindrocarpon* spp. (Rego *et al.*, 2006). The use of fungicides for protection against *Cylindrocarpon* infection was investigated *in vitro* by Rego *et al.* (2006), who tested the efficacy of 14 fungicides on inhibiting the conidium germination and mycelial growth of "*C. destructans*" (re-identified as *C. liriodendri*). These fungicides were tested with grapevine potted plants grown in "*C. destructans*" infested substrate under greenhouse conditions. An activator of disease resistance, Brotomax, was also tested. Brotomax application gave a similar low disease incidence of *Cylindrocarpon* (1.2%) to the best fungicides compared with the controls (80%; Rego *et al.*, unpublished data, cited in Oliveira *et al.*, 2004). In *in vitro* experiments, Rego *et al.* (2006) showed that mycelial growth of "*C. destructans*" was most inhibited by prochloraz followed by carbendazim /flusilazole, benomyl and cyprodinil /fludioxonil but only cyprodinil /fludioxonil reduced spore germination. Azoxystrobin, tryfloxistrobin and tolyfluanide were effective in reducing spore germination but not mycelial growth.

Results from an *in vivo* study on potted grapevine plants by Rego *et al.* (2006) showed that benomyl, tebuconazole, carbendazim /flusilazole and cyprodinil /fludioxonil significantly improved plant growth and decreased disease incidence compared with non-treated plants. Prochloraz and azoxystrobin failed to reduce disease incidence for half of the *Cylindrocarpon* isolates tested. Halleen *et al.* (2007) also conducted fungicide experiments from which they reported that benomyl and prochloraz were effective fungicides for inhibiting mycelial growth of *C. liriodendri*, *C. macrodidymum*, *Campylocarpon fasciculare* and *Campyl. pseudofasciculare*, but flusilazole and imazalil were effective only against *Cylindrocarpon* spp. However their *in vivo* experiments showed that prochloraz, manganese chloride and imazalil were ineffective, although the benomyl /Nu-Film 17 and benomyl /carnauba wax treatments showed reductions in the incidences of the pathogens in rootstock plants. Overall, the *in vivo* effects of the chemical treatments on incidence of the pathogen were not significant and /or consistent (Rego *et al.*, 2006; Halleen *et al.*, 2007).

Rego *et al.* (2009) evaluated four fungicides in nursery conditions. They found that cyprodinil /fludioxonil significantly decreased the disease incidence and severity of *Cylindrocarpon* spp. in soil with low and high inoculum while pyraclostrobin /metiram and fludioxonil reduced the disease incidence and severity in soil with high inoculum. Further evaluations of fungicides in nursery conditions are needed. In addition better knowledge of the epidemiology of the pathogens might improve strategies for fungicide control.

1.2.9. Molecular diagnostics for plant pathogens

Fast, reliable and sensitive pathogen detection methods are required to identify causal agents, and thereby improve management of plant diseases before development of visible symptoms which could prevent the spread of plant pathogens (McCartney *et al.*, 2003; Ward *et al*., 2004). Traditional methods include the identification of symptoms on the plant, the isolation of the pathogen on media and its morphological identification. However, these methods are problematic as they are time consuming, require expertise in microbiology, are often confounded by faster growing saprophytes and results can be cryptic especially as different pathogens can cause similar symptoms (Ward *et al*., 2004). Closely related species are often difficult to distinguish and distinction between different isolates of the same species is even harder. Diagnostic methods based on the polymerase chain reaction (PCR) have been successfully used for pathogen identification (Fox and Narra, 2006). During PCR amplification, specific DNA sequences are targeted and millions of copies produced. The sensitivity of the method can be increased using a second PCR (nested PCR) and quantification of the target DNA is possible with real-time PCR using either non specific binding dyes or fluorescent probes (Schena *et al.*, 2004).

The DNA encoding the ribosomal subunits and ITS is usually targeted for the development of molecular diagnostic methods as it is often conserved within a species and the high copy number in the genome improves the detection sensitivity of assays (Fox and Narra, 2006). However, for closely related species, variations in the ITS sequences are occasionally very low or nonexistent and the genes encoding β-tubulin, which is a component of microtubules, can be used as an alternative. The elongation factor 1α and the γ-actin gene also constitute commonly used molecular loci in fungal phylogenetics (Schmitt *et al*., 2009). One to five copies of the β-tubulin gene have been reported in fungi but most fungi appear to have only one copy. Insertions and deletions are much less common compared to rRNA genes which often contain gaps that complicate sequence alignments, making sequence alignments using β-tubulin less ambiguous (Thon and Royse, 1999).

Mycologists have exploited molecular techniques for the identification and study of evolutionary relationships among fungi (Thon and Royse, 1999). Thon and Royse (1999) used the β-tubulin sequence to investigate the phylogeny between members of the Basidiomycotina. Groenewald *et al.* (2001) used the ITS and β-tubulin sequences to separate the genus *Phaeomoniella* from *Phaeoacremonium*. Taxonomic studies that included molecular methods have also been conducted more recently to establish the classification and phylogeny of *Cylindrocarpon* spp. and their teleomorphs where present (Seifert *et al.*, 2003; Brayford *et al*., 2004; Halleen *et al*., 2004; Castlebury *et al*., 2006; Schroers *et al*., 2008). Multiple gene phylogenies are usually used in taxonomy.

Species specific primers are being developed for a wide range of plant pathogens and the development of quantitative PCR has proven to be an important tool to identify and quantify pathogens from seeds, plant tissues, water, air and soil samples (Schena *et al.*, 2004), providing information on the interaction between the host and the pathogen, the relationship between yield and loss due to a pathogen and ways to manage the disease (McCartney *et al*., 2003; Ward *et al.*, 2004). To identify pathogens responsible for black foot disease and combat this disease, a rapid and quantitative assay needs to be developed for detection of the *Cylindrocarpon* spp. affecting grapevines in New Zealand.

1.3. Research topic

Despite the importance of black foot disease of grapevines, a comprehensive understanding of the bio-ecology of the causal *Cylindrocarpon* spp. is still lacking (Rego *et al.*, 2006). This knowledge is essential to the development of rational control methods that are able to prevent and manage the disease of young grapevines. However, the pathogen species and strains responsible for this disease differ between regions and countries. Since distinct species may vary in phenotypic characters, including longevity in the field, host range, aggressiveness and susceptibility to different disease control treatments (Petit and Gubler, 2005) the research conclusions from one country cannot be automatically transferred to another country without further evaluation. To combat black foot disease in New Zealand, it is therefore necessary to discover how the New Zealand species and strains of black foot pathogens survive, infect and cause disease in grapevines.

The aim of this project was to identify the *Cylindrocarpon* species inoculum types, concentrations and the bio-ecological conditions that commonly lead to Cylindrocarpon black foot disease in young grapevines in New Zealand. In addition, it aimed to develop robust molecular methods of identifying the *Cylindrocarpon* species and for testing soils for their presence, thereby allowing for avoidance or pre-planting control measures. It also investigates the infection processes and vineyard risk factors.

The objectives of this research were to:

1. determine the pathogenicity of different inoculum types from the New Zealand *Cylindrocarpon* spp. by investigating:
   - the pathogenicity of isolates from *C. liriodendri*, *C. macrodidymum* and *C. destructans*
   - the pathogenicity of propagules from these three species
   - the threshold number of propagules needed for disease development

2. develop a molecular identification method specific for the black foot pathogen species prevalent in New Zealand.

3. apply the infection method developed in (1) to the soil environment for determining
   - the pathogenicity of the propagules in the field
   - the pathogenicity of the propagules in different soil types

4. develop a soil-testing method to identify infested soils using:
- baiting methods
- a molecular method (qPCR) with infested soil samples

5. determine the effects of biological, environmental and cultural factors on Cylindrocarpon black foot disease development:
- water stress
- presence /absence of wounds (pot trial and vineyard)
- cold storage
- prior infection with *Phaeomoniella chlamydospora*

CHAPTER 2

Pathogenicity of different inoculum types from three *Cylindrocarpon* spp. common in New Zealand

2.1. Introduction

Plants are surrounded by microorganisms, not all of which can cause plant disease. Investigations into a plant disease need to prove which of the associated microorganisms is the causal pathogen of the relevant symptoms and this is done by following the postulates of Robert Koch (Strange, 2003). These postulates include the observation of symptoms on a plant, the isolation and description of an organism from the diseased plant, its growth in pure culture and inoculation onto healthy plants, followed by development of the same disease symptoms and then re-isolation of the same organism (Strange, 2003).

The ability of an organism to cause disease depends on the interaction between the host, the pathogen and the environment (Windham and Windham, 2004). However, the types of infective propagules which constitute the inoculum and the minimum quantity needed for infection vary depending on the nature of the host tissues, the pathogen strain and the environment where they interact (Horsfall and Dimond, 1960). Studies of *Cylindrocarpon* spp. pathogenicity on grapevines have generally used conidium suspensions of 10^6 to 10^8 conidia /mL, in which grapevine cuttings were soaked for 30 min, and then grown for 2 – 5 months before being assessed for infection incidence. In Portugal, Rego *et al.* (2000) inoculated rooted cuttings of rootstock variety 99 R by soaking the washed roots of the plants for 30 min in conidium suspensions (10^8 /mL) made from different isolates of *C. destructans*. Two months after inoculation, the pathogen was re-isolated from the plants, of which 16 to 66% developed root symptoms and 33 – 67% died, with differences in symptoms being associated with the different isolates.

Infection of ginseng by *C. destructans* was investigated by Matuo and Miyazawa (1984) who sowed ginseng seeds in pots containing soil mixed with 15 to 20 g of *C. destructans* infested wheat bran; they successfully isolated *C. destructans* from the ginseng plants 7 months after inoculation. Harvey and Jaspers (2006) also used an agar culture slurry made with a mixture of *Cylindrocarpon* spp. to inoculate the potting mix into which rooted grapevine cuttings were grown for 4 months before being assessed. However, this method showed lower disease incidences in the trunk than the root soak method. In this study, mycelia, conidia and

chlamydospores of three *Cylindrocarpon* spp. were tested to determine the pathogenicity of the different inoculum types and then different quantities of these inocula were evaluated.

Isolates of the same *Cylindrocarpon* spp. may differ in their pathogenicity, as shown by Seifert *et al.* (2003) who reported significant differences between isolates of *C. destructans* in ginseng and in conifer and by Alaniz *et al.* (2009) for isolates of *C. macrodidymum* in grapevines. However, when Petit and Gubler (2005) tested Californian *C. destructans* and *C. macrodidymum* isolates for their pathogenicity to grapevine rootstock variety 5C, they found that all isolates caused significant root rot on grapevine, with no significant variation between isolates or species. The same observation was made by Alaniz *et al.* (2009) with Spanish *C. liriodendri* isolates. In New Zealand, although *C. liriodendri*, *C. macrodidymum* and *C. destructans* were found to be equally common throughout the wine regions (Bleach *et al.*, 2006), the relative pathogenicity of the species and isolates to grapevines was unknown. In this study, three isolates of each species, which were isolated from grapevines, were tested to investigate whether all species were pathogenic to grapevines and to indicate any variation in pathogenicity within a species and between species.

Any investigation of a plant fungal disease requires the development of a reliable infection method, with development of symptoms, which are similar to those observed by growers during natural infection. In this study, plants with trimmed roots were used to simulate current planting practices used for vineyard establishment and callused cuttings were used to simulate the nursery practice of planting cuttings directly into the nursery soil. The investigation used two rootstock varieties for planting material and isolates of three *Cylindrocarpon* spp., as well as the different types and quantities of propagules on infection incidence.

2.2. Materials and methods

2.2.1. The pathogenicity of *Cylindrocarpon* species

2.2.1.1. Plant material

Plants of two grapevine rootstock varieties commonly grown in New Zealand vineyards (5C and 101-14) were propagated from the dormant shoots of apparently healthy vines grown in the Lincoln University vineyard. Two node cuttings were grown to rooted plants or callused for use in the infection studies. To obtain rooted plants, the cuttings were placed in trays containing pumice, in a shade house on a heat pad at 25°C for 6 weeks, when the average air temperature was 5.4°C. The rooted plants were carefully removed from the trays and their roots were trimmed to 10 cm before inoculation. To callus the cuttings, they were placed in 20 cm deep vermiculite in a 27°C growth chamber for 4 weeks and then hardened off in a

greenhouse (14 – 28°C) for 1 – 2 weeks before inoculation, which is the standard nursery protocol (B. Corban, pers. comm. 2006). Plants from callused and rooted cuttings are named "callused" and "rooted" plants, respectively, when referring to propagation methods.

2.2.1.2. Inoculum

Three isolates from each of the three different species were randomly selected from the collection of identified *Cylindrocarpon* isolates maintained on Spezieller Nährstoffarmer agar (SNA; Appendix 2) at 4°C in the dark. These isolates were from grapevines from different New Zealand regions (Appendix 1) and had been previously identified as *C.destructans* (isolates referred to as D1, D2 and D3), *C. macrodidymum* (isolates M1, M2 and M3) and *C. liriodendri* (isolates L1, L2 and L3) by molecular methods (Mostert *et al*., 2006). To obtain conidium suspensions, the isolates were grown on PDA (Oxoid Ltd, Basingstoke, UK) at 20°C for 3 weeks in the dark. The conidia were suspended by adding 5 mL of sterile water with 3 drops /L of Tween 80 (polyoxylethylene (20) sorbitan mono-oleate; BDH Chemicals Ltd, Poole, England) to the plate surface, scraping the culture surface with a sterile glass microscope slide and sieving the solution through a sterile sieve of 150 µm mesh. This procedure was repeated twice per culture plate. The final conidium suspension was adjusted to 10^6 conidia /mL using a haemocytometer and sterile water. The viability of the conidia was confirmed by spreading 100 µL of each conidium suspension diluted to 10^2 conidia /mL, onto PDA plates and counting the resulting colonies after 2 days incubation at 20°C in the dark.

2.2.1.3. Inoculation

Plants were inoculated by soaking plant bases for callused plants, or roots for rooted plants, for 30 min in a 5 cm deep conidium suspension (10^6 conidia /mL) of one of the isolates prior to planting or in sterile water (control). They were potted into 1.5 L pots with "3 – 4 month potting mix" (Appendix 2). There were seven replicates of each isolate, plant type and rootstock variety. The plants were laid out on mesh tables in a greenhouse in a randomised block design under high pressure sodium lamps (Son-T Agro 400, Philips), and allowed to grow for 4 months (Figure 2.1). The lights were turned on from 4 am to 12 pm and from 4 pm to 8 pm for the duration of the experiment to ensure plants were under adequate light for 16 h per day. Temperatures varied over 14 – 30°C. Plants were watered daily.

2.2.1.4. Assessment

A few extra plants were infected with the conidium suspension of each isolate. The disease incidences of these vines were assessed at different times (2, 3 and 4 months) to determine the best time for the assessment of the trial. The plants were not watered for 24 h prior to harvesting to facilitate potting mix removal. Plants were lifted out of their pots and their roots were shaken to remove loose potting mix. The roots of each plant were washed under running tap water, removed from the stem base, placed into a paper bag and into an oven

(70°C) at the Field Service Centre at Lincoln University. The roots were dried to constant weight, which was when the dry weights of some randomly selected root systems were equivalent on two consecutive days (7 days), and the weights recorded.

Figure 2.1. Inoculated plants that developed from rooted and callused cuttings of rootstock varieties 101-14 and 5C after 4 months in a greenhouse. Plants were arranged in a completely randomised design.

The main stems (trunks) were thoroughly washed with running tap water and the lower sections were cut to 20 cm to allow for complete vine immersion in the sterilisation tanks. Using the method developed by Halleen *et al.* (2003) to reisolate the pathogen from the infected plants, the lower stem sections were surface sterilized in batches, each time working with plants inoculated with the same pathogen isolate. The stems were soaked in 70% ethanol for 30 s, 0.35% sodium hypochlorite for 5 min and 70% ethanol for 30 s. The stems were wrapped in paper towels to absorb the excess ethanol and placed onto a new plastic bag for transfer to a laminar flow cabinet, where they were air dried for 10 min. Before pathogen isolation, the root crown, comprising the lowest 1 – 2 cm of the stem base was discarded. A 1 – 2 mm transverse piece of tissue was sliced from the basal end of the stem and cut into four pieces of approximately 3 mm^2, which were placed equidistantly and near the edge of a plate containing PDA with chloramphenicol (250 mg /L). A 1 – 2 mm transverse piece of stem was also sliced at 5 cm above the base, cut into two pieces and one piece was placed in the centre of the same plate. It was then sealed with clingfilm and incubated for 7 days at 20°C in the dark. *Cylindrocarpon*-like isolates grown from the wood pieces were identified by comparing colony morphology and conidia with the cultures of the *Cylindrocarpon* isolates used to inoculate the specific plants. The proportion of vines infected

at 1 or 5 cm (disease incidence) and the proportion of infected wood pieces at 1 cm (disease severity) were recorded.

Assessment was based on presence of the pathogen instead of symptoms because tissue discoloration occurs frequently on wounding sites and can lead to confusion when interpreting results. As a result, other researchers (Haleen *et al.*, 2004) have placed most emphasis on their isolation results not the amount of trunk discoloration measures. Gubler *et al.* (2004) stated that grapevines less than 10 years old inevitably died when infected with *Cylindrocarpon* spp. Therefore, colonisation and degree of colonisation of grapevine tissues by *Cylindrocarpon* spp. was interpretated as disease incidence and severity, respectively.

2.2.1.5. Analysis

Data were analysed with the Statistical Package for Social Sciences (SPSS) version 13.0. Although analysis of variance is commonly used to determine disease incidences, the statistician consulted for the different experiments recommended the use of logistic regressions for the binomial data (presence or absence of the pathogen). Therefore disease incidences at 1 and 5 cm above stem bases were analysed using logistic regressions to test all the different effects. This method also provided for pair-wise comparisons between individual treatments within a tested factor, to determine significance of their differences. For the root dry weight and the disease severity, a general linear model was used with terms appropriate to the design and two-way interactions amongst the factors of interest. Where significant main effects or two-way interactions were identified, the significance of differences between related treatments was further explored using Fisher's protected LSD (least significant difference) tests. A *P*-value of ≤0.05 indicated statistical significance.

2.2.2. The pathogenicity of the different pathogen propagules

2.2.2.1. Production of pathogen propagules

Conidia

The most pathogenic of the three isolates of each species was selected from the results of the previous experiment (L1, M1and D2 for respectively *C. liriodendri*, *C. macrodidymum* and *C. destructans*). The conidium suspension for each isolate was obtained as described in Section 2.2.1.2.

Chlamydospores

The method used followed that of Yoo *et al.* (1996) who succeeded in producing abundant chlamydospores of *C. destructans* (5.99×10^6 chlamydospores /mL produced) in Czapek Dox broth (CDB; Sigma Chemicals, St. Louis, USA) grown in shaken culture at 20°C for 30

days. Each autoclaved 500 mL CDB (1/3 strength) contained in a 1 L conical flask was inoculated with five mycelium plugs (5.5 mm diameter) cut from the growing edge of a PDA colony, three flasks for each of the above isolates. The flasks were incubated on a shaker at 100 rev /min at room temperature for 30 days, during which time mycelium grew in the broth and was transformed into chlamydospores. The mycelium from each flask was recovered by sieving with a 150 µm mesh sieve, the mycelium was washed with sterile water and sieved three times to remove conidia. The chlamydospores within the mycelium were harvested by homogenising the mycelium in a kitchen blender for 2 min at high speed and sieving it again to remove the mycelium. The filtrate which contained chlamydospores was adjusted with sterile water using a haemocytometer to give a concentration of 10^6 chlamydospores /mL.

Mycelium

Mycelium of the same isolates was produced by growing them on sterilised wheat grains. The wheat grains (400 g) were placed in 1 L conical flask containing 500 mL of tap water and 250 mg of chloramphenicol and heated to boiling. The grains were left to settle for 10 min then washed three times with tap water and the excess drained off. The grains were autoclaved at 121°C for 15 min at 15 psi on each of two consecutive days. Inoculation onto the wheat grains was with five mycelial discs (7 mm diameter) taken from the margins of *Cylindrocarpon* spp. cultures, as above and incubated at 20°C in the dark for 14 days, at which time they were visually assessed as being well-colonised by mycelium. During that period, the flasks were shaken daily by hand (3 – 5 s) to facilitate colonisation.

2.2.2.2. Inoculation

The cuttings obtained from Corbans Viticulture Ltd were rooted or callused as described in Section 2.2.1.1. The rooted and callused cuttings were inoculated with mycelium by adding 5 g of infested wheat grains or 5 g of autoclaved wheat grains (control) to the potting mix. The 1.5 L pots were half filled with the same potting mix as before, a 5 to 6 cm deep hollow was made, the 5 g of wheat grains were inserted in the hollow (Figure 2.2) and it was covered with 1 – 2 cm of potting mix. The plant was placed above the wheat grains and the pot was filled with potting mix. For the spore inocula, the plants were soaked in the relevant spore suspension for 30 min or sterile water (control) as described in Section 2.2.1.3 and potted with potting mix in 1.5 L pots. The pots were laid out on mesh tables in the same greenhouse as in Section 2.2.1.3 in a split plot design, with 12 replicates for each isolate, propagule type, plant type and rootstock variety. The infected plants were evaluated as in Section 2.2.1.4 after 5 months. In addition, the new shoots that had grown within the 5 months were stripped of their leaves and the shoots were placed into paper bags for drying to a constant level in the 70°C oven (7 days), followed by weight assessment.

2.2.2.3. Analysis

The analysis of the shoot dry weight was done with a general linear model with terms appropriate to the design, to determine effects of the individual factors of interest and the two-way interactions amongst them, using SPSS version 13.0. A *P*-value of ≤0.05 was used to indicate statistical significance. The disease incidences, disease severities and root dry weights were analysed as described in Section 2.2.1.5.

2.2.3. Threshold numbers of pathogen propagules

Callused grapevine cuttings of rootstock variety 101-14, were inoculated with *C. liriodendri* and *C. destructans*, and for each species a mixed inoculum was made from individual inocula of the three previously used isolates (Section 2.2.1.2). The spore concentrations of 0, 10^2, 3.2×10^4 and 10^6 conidia or chlamydospores per mL, were obtained as described in Sections 2.2.1.2 and 2.2.2.1 and diluted with sterile water to the appropriate concentrations. Inoculation was by soaking stem bases in the spore suspensions, as described in Section 2.2.1.3. The mycelium inoculum was prepared as described in Section 2.2.2.1 and pots inoculated by adding 0, 1, 3 or 5 g of the infested wheat grains to a planting hole as described in Section 2.2.2.2. Sterile wheat grains were added to the aliquot of infected grains to ensure a uniform final weight of 5 g per pot (Figure 2.2). The plants were grown in 2 L planting bags (Figure 2.2) with a similar potting mix as before, 14 replicates for each treatment laid out in a split-split plot design.

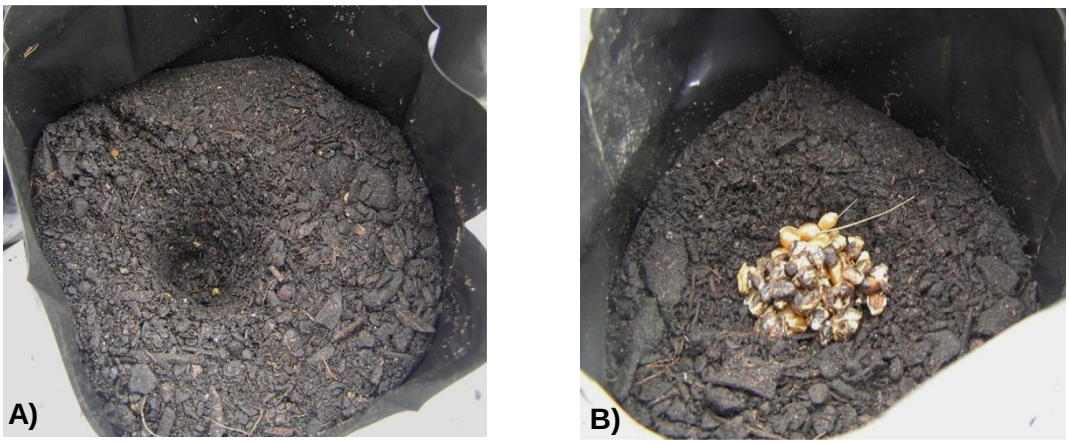

Figure 2.2. Plastic bags (2.5 L) with potting mix, showing a planting hole (A), and a planting hole with infected wheat grains (B).

The experiment was set up in a greenhouse at Cloudy Bay vineyard in Blenheim and the plants were watered with an automatic dripper system each day according to apparent need. The plants were left to grow for 5 months before being assessed. The number of effective

propagules required to induce black foot disease were determined by isolating the fungal pathogens as in Section 2.2.1.4 and the analysis was done as in Section 2.2.1.5.

2.3. Results

2.3.1. The pathogenicity of *Cylindrocarpon* species

The data analysis for this section is reported in Appendix 3 (A3), Section 3.1. Disease incidences at 1 cm above stem bases showed significant differences between isolates (P<0.001; A3.1.1).

Disease incidences at 1 cm varied between 32.1% for D3 and 82.1% for D2 (Figure 2.3). All isolates except D3 and M2 caused significantly greater mean disease incidences than the water control (P≤0.05, A3.1.2). For *C. destructans*, isolate D2 caused greater mean disease incidence than isolates D1 (P=0.010) and D3 (P<0.001). For *C. liriodendri*, the three isolates caused similar mean disease incidences. For *C. macrodidymum*, isolate M3 caused significantly greater mean disease incidence than isolate M2 (P=0.015). When isolates from the same species were grouped together and compared between species (A3.1.3), the species were not significantly different from each other, however *C. liriodendri* caused slightly higher (P>0.05) mean disease incidence (63.0%) than *C. destructans* (55.4%) and *C. macrodidymum* (56.6%).

Disease incidence at 1 cm was higher for callused plants than rooted plants (P<0.001; A3.1.1), with means of 73.5 and 36.0%, respectively (Figure 2.4), but did not differ between rootstock varieties (means of 53.6% for 101-14 and 55.5% for 5C, P=0.610; A3.1.1).

Disease incidences at 5 cm above the stem bases did not differ significantly between isolates (P=0.189; A3.1.4), although they varied widely between isolate means, from 7.1% for D3 to 37.0% for L1 (Figure 2.3). However, only isolates D1, L1 and L2 caused significant differences from the control (P=0.043, 0.016 and 0.052 for D1, L1 and L2, respectively; A3.1.5). For *C. destructans*, isolates D1 and D2 caused greater mean disease incidences than isolate D3 (P=0.022 and P=0.037, respectively). For *C. liriodendri* and *C. macrodidymum*, no significant differences were observed between isolates, however, isolates L1, L2 and M3 caused greater mean disease incidences than isolate D3 (P=0.008, P=0.026 and P=0.049, respectively). When isolates from the same species were grouped together and compared between species, the different species did not cause significantly different mean disease incidences from each other (A3.1.6), with means of 31.3% for *C. liriodendri* and 22.9% for both *C. destructans* and *C. macrodidymum*.

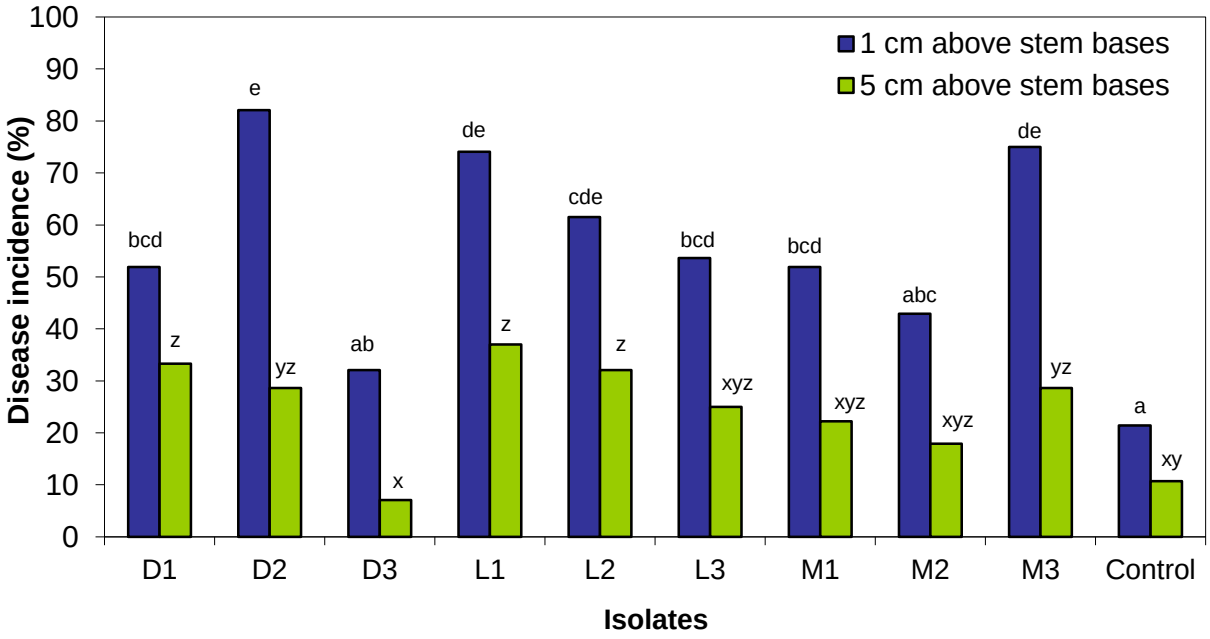

Figure 2.3. Mean disease incidences at 1 and 5 cm above stem bases of young grapevine plants inoculated with nine *Cylindrocarpon* isolates (with isolates D1 to D3: *C. destructans*; L1 to L3: *C. liriodendri*; M1 to M3: *C. macrodidymum*) . For each distance, bars with different letters are significantly different ($P \leq 0.05$), as determined by pair-wise comparisons using logistic regressions.

Disease incidence at 5 cm showed significant differences between propagation methods, with callused plants having greater incidence than rooted plants ($P<0.001$; A3.1.4), means being 34.8 and 13.7%, respectively but did not differ significantly between varieties (mean disease incidences of 26.1 and 22.3% for 101-14 and 5C, respectively).

Disease severities differed significantly between isolates ($P<0.001$; A3.1.7), which varied between 18.7% for D3 and 61.7% for L1 (Table 2.1), All isolates except D3 and M2 caused significantly higher mean disease severities than the control plants. For *C. destructans*, isolate D2 caused greater mean disease severity than isolate D3 ($P \leq 0.05$). For *C. liriodendri* no significant differences were observed between isolates. For *C. macrodidymum*, isolate M3 caused greater mean disease severity than isolate M2 ($P \leq 0.05$). When isolates from the same species were grouped together they did not show significant differences between species (A3.1.8), with means of 50.6% for *C. liriodendri*, 39.8% for *C. destructans* and 40.9% for *C. macrodidymum*.

Table 2.1. Mean percent infected wood pieces (disease severity) at 1 cm above stem bases of plants 4 months after inoculation with nine *Cylindrocarpon* isolates.

Species	Isolates	Disease severity (%)
C. destructans	D1	41.2 bc[1]
	D2	59.8 c
	D3	18.7 ab
C. liriodendri	L1	61.7 c
	L2	47.4 c
	L3	43.7 bc
C. macrodidymum	M1	43.8 bc
	M2	21.4 ab
	M3	58.0 c
Control		12.5 a

[1]Values within columns that are followed by different letters are significantly different ($P \leq 0.05$).

Disease severities differed for propagation methods ($P<0.001$; A3.1.7), with callused plants having greater disease severities than rooted plants ($P<0.001$) with means of 60.6 and 21.1%, respectively. There were no differences between varieties, which had means of 40.6% for 101-14 and 41.1% for 5C ($P=0.892$), but there were significant interactions between propagation methods and varieties ($P=0.039$) and between propagation methods and isolates ($P=0.008$). For callused plants, only isolates D2, L2, L3 and M1 caused greater mean disease severities than rooted plants, the other isolates being similar for the propagation methods. Inoculated callused plants had greater mean disease severities than control callused plants, for all isolates except D3 and M2 and mean severities varied between 33.9% for D3 and 83.9% for D2 (Figure 2.4). For rooted plants, only isolates M3 and L1 caused greater mean disease severities than the control plants and mean severities varied between 3.6% for D3 and 50% for L1 and M3 (Figure 2.4). Disease severities were different between the different plant types from the different rootstock varieties ($P=0.039$), with mean disease severities of callused plants from rootstock varieties 101-14 and 5C (56.1 and 65.1% respectively) being greater than for the rooted plants from these varieties (25.1 and 17.2%, respectively). When callused plants were analysed separately, their disease severities differed between species ($P=0.044$; A3.1.9), with *C. liriodendri* having greater severity than *C. destructans* ($P=0.037$) and *C. macrodidymum* ($P=0.027$; A3.1.10), means being 79.2, 58.9 and 57.3, respectively.

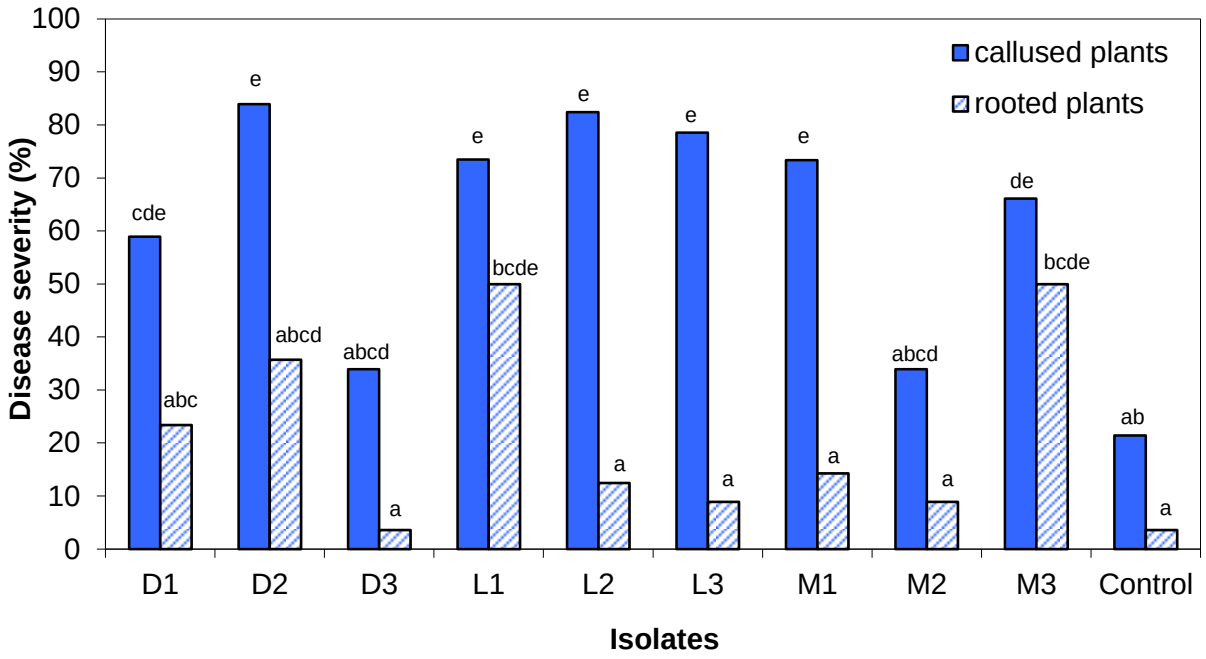

Figure 2.4. Mean disease severities of young grapevine plants inoculated with nine *Cylindrocarpon* isolates (with isolates D1 to D3: *C. destructans*; L1 to L3: *C. liriodendri*; M1 to M3: *C. macrodidymum*). Bars with different letters are significantly different ($P \leq 0.05$).

Root dry weights showed significant differences between isolates ($P=0.030$; A3.1.11), with mean root dry weights varying between 5.1 g for D1, which was significantly less than for the control (6.4 g), and 6.4 g for M2 (Table 2.2). When isolates from the same species were grouped together and the species compared between each other (A3.1.12), no significant differences were observed for the mean root dry weights, with 5.7, 6.1 and 5.6 g for *C. liriodendri*, *C. macrodidymum* and *C. destructans*, respectively.

Table 2.2. Mean root dry weights (g) of plants 4 months after inoculation with nine *Cylindrocarpon* isolates.

Species	Isolates	Mean root dry weight (g)
C. destructans	D1	5.1 a[1]
	D2	5.8 ab
	D3	5.7 ab
C. liriodendri	L1	5.7 ab
	L2	5.4 ab
	L3	6.2 ab
C. macrodidymum	M1	5.8 ab
	M2	6.4 b
	M3	6.2 ab
Control		6.4 b

[1]Values within columns that are followed by different letters are significantly different ($P \leq 0.05$).

The mean root dry weights were higher for callused cuttings than for rooted cuttings, with means of 6.7 and 5.1 g respectively (P<0.001; A3.1.11), and for variety 101-14 (6.3 g) than variety 5C (5.4 g; P<0.001). The interaction between propagation methods and varieties (P=0.023) was shown by the difference in mean root dry weights of callused plants versus rooted plants, which were higher in variety 101-14 (6.9 and 5.7 g, respectively) than in variety 5C (6.4 g and 4.4 g respectively; Table 2.3), with only the mean dry weight of 5C rooted plants differing from the other mean weights (P=0.023).

Table 2.3. Mean root dry weights (g) of rooted and callused plants from rootstock varieties 101-14 and 5 C, 4 months after inoculation with *Cylindrocarpon* isolates.

Propagation methods	Varieties	Mean root dry weight (g)
Callused plants	101-14	6.9 c[1]
	5C	6.4 bc
Rooted plants	101-14	5.7 b
	5C	4.4 a

[1]Values within columns that are followed by different letters are significantly different (P≤0.05).

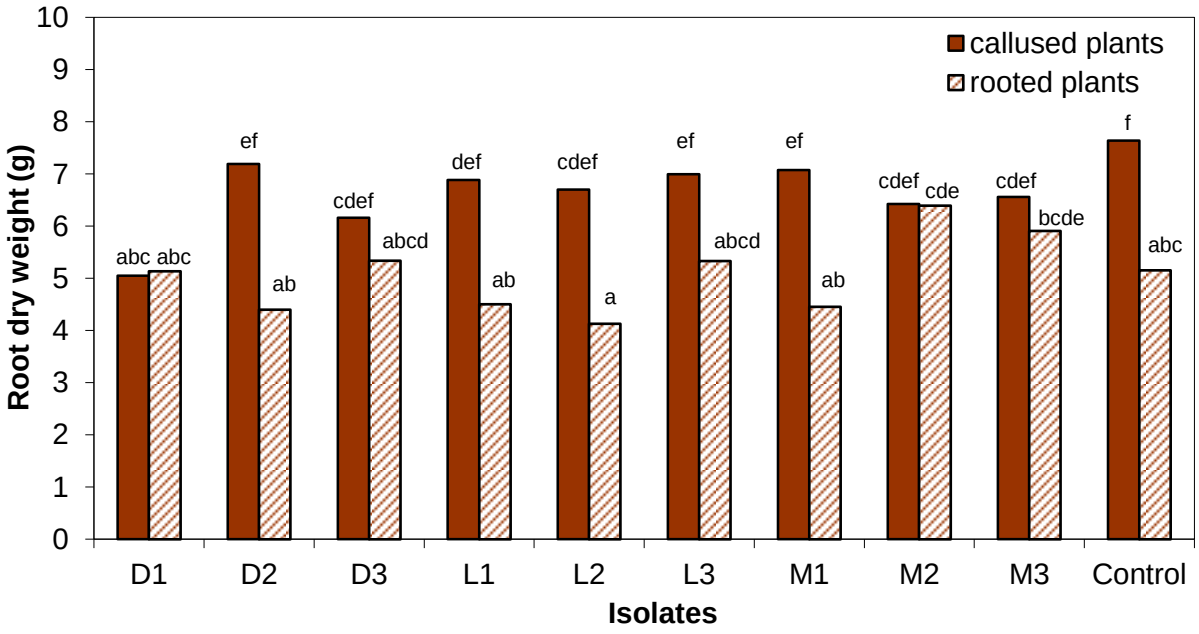

Figure 2.5. Mean root dry weights (g) of plants grown from callused and rooted grapevine cuttings, 4 months after inoculation with nine *Cylindrocarpon* isolates (with isolates D1 to D3: *C. destructans*; L1 to L3: *C. liriodendri*; M1 to M3: *C. macrodidymum*). Bars with different letters are significantly different (P≤0.05).

The interaction between propagation methods and isolates (P<0.001) is shown in Figure 2.5. For the rooted plants, mean root dry weights varied between 4.1 g for L2 and 6.4 g for M2 while for the callused plants, they varied between 5.1 g for D1 and 7.6 g for the control. For

rooted plants, mean root dry weights were not significantly different from the control, while for callused plants D1 caused a lower mean dry weight than the control.

2.3.2. The pathogenicity of the different pathogen propagules

The data analysis for this section is reported in Appendix 3, Section 3.2. Disease incidences at 1 cm above the stem bases showed significant differences between propagule types (P<0.001; A3.2.1), with conidia causing greater mean disease incidence (81.3%) than chlamydospores (62.5%; P=0.001) and mycelium (56.3%; P=0.001; Table 2.4 and A3.2.2). The significant differences between species (P=0.008; A3.1.1) were shown by *C. liriodendri* causing significantly greater mean disease incidence than the two remaining species which had similar mean disease incidences (Table 2.4 and A3.2.2).

Table 2.4. Mean disease incidences at 1 and 5 cm above stem bases of grapevines inoculated with three *Cylindrocarpon* species and three inoculum types.

Treatments	Disease incidence (%) at 1 cm above stem base	Disease incidence (%) at 5 cm above stem base
Species		
C. liriodendri	80.6 b[1]	54.2 n
C. destructans	59.0 a	38.2 mn
C. macrodidymum	60.4 a	30.6 m
Propagules		
Chlamydospores	62.5 i	34.0 y
Conidia	81.3 j	59.7 z
Mycelium	56.3 i	29.2 xy
Control water	33.3 h	6.3 wx
Control wheat	31.3 h	16.7 w

[1]Values within columns followed by different letters are significantly different (P≤0.05).

Incidence at 1 cm showed a significant interaction between types of propagules and the different species (P<0.001; A3.2.3). All treatments inoculated with spores had significantly greater mean disease incidences at 1 cm than with water inoculation (A3.2.4). For chlamydospores (Figure 2.6), there was a trend for greater mean disease incidence for *C. liriodendri* (70.8%) than *C. destructans* (60.4%) and *C. macrodidymum* (56.3%; A3.2.4). For inoculation with conidia, similar trends were observed for the mean disease incidences, with 97.9% for *C. liriodendri*, 77.1%, for *C. destructans* and 68.8% for *C. macrodidymum*. For inoculation with mycelium, *C. liriodendri* caused significantly greater mean disease incidence (72.9%) than *C. macrodidymum* (52.1%) and *C. destructans* (43.8%), although the mean disease incidence for *C. destructans* was not significantly different (P=0.137) to that of the control plants (31.3%).

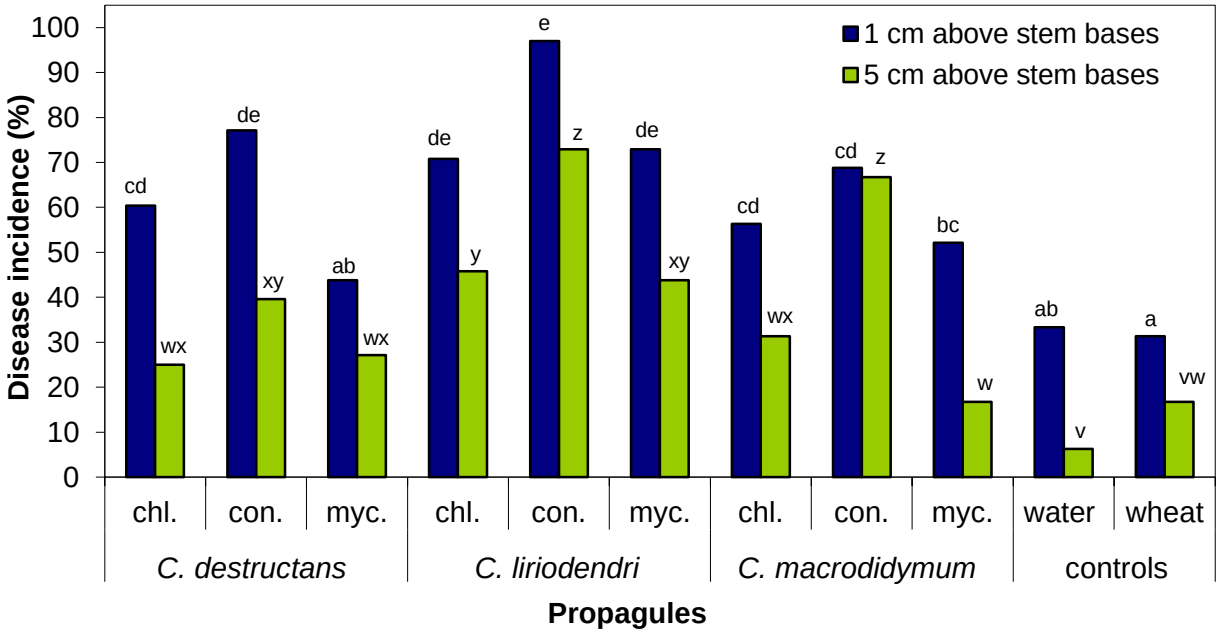

Figure 2.6. Mean disease incidences at 1 and 5 cm above the stem bases of young grapevine plants inoculated with three types of propagules of three *Cylindrocarpon* spp. (con.: conidium inoculum, chl.: chlamydospore inoculum, myc.: mycelium inoculum). For each distance, bars with different letters are significantly different ($P \leq 0.05$).

Callused plants had greater disease incidence than rooted plants ($P<0.001$; A3.2.3), with means of 70.8 and 50.0%, respectively, while disease incidences at 1 cm were not significantly different between plants from the different rootstock varieties (means of 63.6% for 101-14 and 57.2% for 5C, $P=0.087$).

Disease incidences at 5 cm above the stem base showed significant differences between propagule types ($P<0.001$; A3.2.5), with conidia having greater mean disease incidence (59.7%) than chlamydospores (34%; $P=0.001$) and mycelium (29.2%; $P=0.001$; Table 2.4 and A3.2.6). The significant differences between species ($P=0.011$; A3.2.5) were shown by *C. liriodendri* causing significantly greater mean disease incidence than *C. macrodidymum* and *C. destructans* (54.2, 38.2 and 30.6%, respectively; Table 2.4 and A3.2.6).

Disease incidences at 5 cm showed a significant interaction between types of propagules and the different species ($P<0.001$; A3.2.7). All treatments inoculated with spores had significantly greater mean disease incidences than with water inoculation ($P<0.05$; A3.2.8). For inoculation with chlamydospores, *C. liriodendri* caused significantly greater mean disease incidence than *C. macrodidymum* and *C. destructans* (similar) with 45.8, 31.3 and 25.0%, respectively (Figure 2.6). For inoculation with conidia, mean disease incidence in plants inoculated with *C. liriodendri* was similar to that of *C. macrodidymum* and significantly greater than for *C. destructans* with 72.9, 66.7 and 39.6%, respectively. For inoculation with

mycelium, only plants inoculated with *C. liriodendri* had a mean disease incidence significantly greater than for the plants inoculated with sterile wheat grains (*P*=0.004).

Callused plants had greater disease incidences at 5 cm than rooted plants (*P*<0.001; A3.2.7), with means of 46.6 and 24.6%, respectively, but varieties did not differ, with 101-14 and 5C having means of 38.3 and 33.3%, respectively (*P*=0.146).

Disease severity showed significant differences between propagule types (*P*<0.001; A3.2.9), with conidia causing significantly greater mean disease severity (62.7%) than chlamydospores (41%) and mycelium (35.1%; Table 2.5 and A3.2.10). All plants inoculated with propagules showed significantly greater mean disease severities than their respective control (*P*≤0.05). Disease severities also differed between species (*P*<0.001; A3.2.9), with *C. liriodendri* causing greater mean disease severity than *C. macrodidymum* and *C. destructans*, with 63.2, 37.1 and 38.4%, respectively (Table 2.5 and A3.2.10).

Table 2.5. Mean disease severities at 1 cm above stem bases of grapevines inoculated with three *Cylindrocarpon* species and three inoculum types.

Species	Disease severity (%)	Propagules	Disease severity (%)
C. liriodendri	63.2 b[1]	Chlamydospores	41.0 y
C. destructans	38.4 a	Conidia	62.7 z
C. macrodidymum	37.1 a	Mycelium	35.1 y
		Control water	16.1 x
		Control wheat	16.1 x

[1]Values within columns followed by different letters are significantly different (*P*≤0.05).

There was a significant interaction between propagules and species (*P*<0.001; A3.2.11), with all spore inoculation treatments showing significantly greater mean severities than with the water control (*P*≤0.05) except for *C. macrodidymum* chlamydospores (Table 2.6). For chlamydospore inoculation, *C. liriodendri* caused a greater mean disease severity (53.6%) than *C. destructans* (35.4%) and *C. macrodidymum* (33.8%). For conidium inoculation, *C. liriodendri* caused greater disease severity than *C. destructans* and *C. macrodidymum,* with means of 84.9, 52.1 and 51.0%, respectively. For mycelium inoculation, only plants inoculated with *C. liriodendri* infested wheat grains had a significantly greater mean disease severity (51.0%) than the plants inoculated with sterile wheat grains (16.1%; *P*≤0.05).

Table 2.6. Mean disease severity at 1 cm above stem bases of vines, 5 months after inoculation with three propagule types of three *Cylindrocarpon* spp.

Species	Propagules	Disease severity (%)
C. destructans	chlamydospores	35.4 bcd[1]
	conidia	52.1 cd
	mycelium	27.6 ab
C. liriodendri	chlamydospores	53.6 d
	conidia	84.9 e
	mycelium	51.0 c
C. macrodidymum	chlamydospores	33.8 abc
	conidia	51.0 cd
	mycelium	26.6 ab
Controls	water	16.1 a
	wheat	16.1 a

[1]Values within columns that are followed by different letters are significantly different ($P \leq 0.05$).

Disease severity differed between propagation methods ($P<0.001$; A3.2.11), with callused plants having greater disease severity than rooted plants (50.8 and 30.7%, respectively). There were significant interactions between propagation methods, propagules and species ($P=0.004$). For rooted plants, only those inoculated with *C. liriodendri* propagules had significantly greater mean disease severities than the controls ($P<0.05$, Figure 2.7), being 42.7, 76.1 and 44.8% for chlamydospores, conidia and mycelium, respectively, compared to the water and wheat grain controls of 15.6 and 5.2%, respectively. For callused plants, inoculation with conidia of any *Cylindrocarpon* species caused mean disease severities that were significantly greater than for the control plants. Chlamydospores of *C. liriodendri* and *C. destructans* and mycelium from *C. liriodendri* also caused greater disease severities than the relevant controls ($P<0.05$; Figure 2.7).

Disease severities did not differ between rootstock varieties ($P=0.105$), with mean severities being 43.1% for 101-14 and 38.4% for 5C. However, the interactions between varieties, propagules and species ($P=0.004$) and between propagule types, species and varieties ($P=0.007$) was evident. For rootstock variety 5C, mean disease severities of plants inoculated with conidia were significantly greater than for the control (20.8%), being 87.5, 47.9 and 53.1% for *C. liriodendri*, *C. destructans* and *C. macrodidymum*, respectively ($P \leq 0.05$, Figure 2.8). For rootstock variety 101-14, plants inoculated with conidia and chlamydospores from all species and mycelium from *C. liriodendri* had significantly greater mean disease severities than for the relevant controls ($P \leq 0.05$). For both rootstock varieties, plants inoculated with *C. liriodendri* propagules had the greatest mean disease severities, being 68.8, 82.3 and 54.2% for 101-14 and 38.6, 87.5 and 47.9% for 5C, due to inoculation with chlamydospores, conidia and mycelium, respectively.

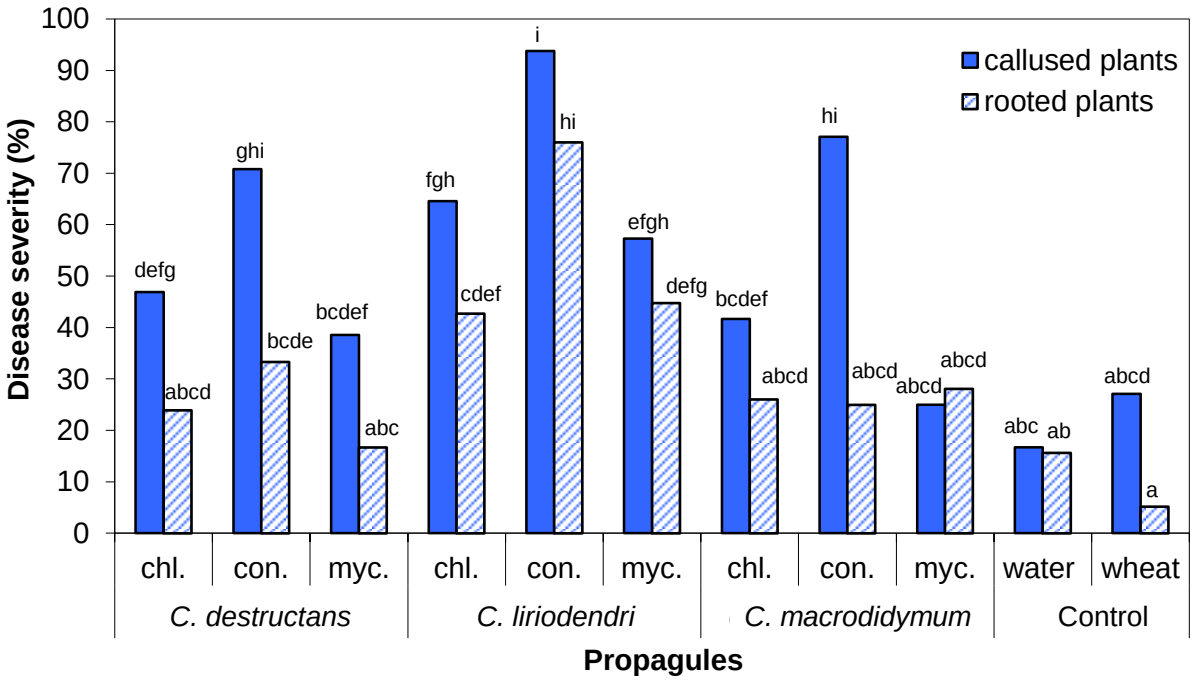

Figure 2.7. Mean disease severities at 1cm above the stem bases of young grapevine plants from two propagation methods inoculated with three propagule types of three *Cylindrocarpon* spp. (con.: conidium inoculum, chl.: chlamydospore inoculum, myc.: mycelium inoculum). Bars with different letters are significantly different ($P \leq 0.05$).

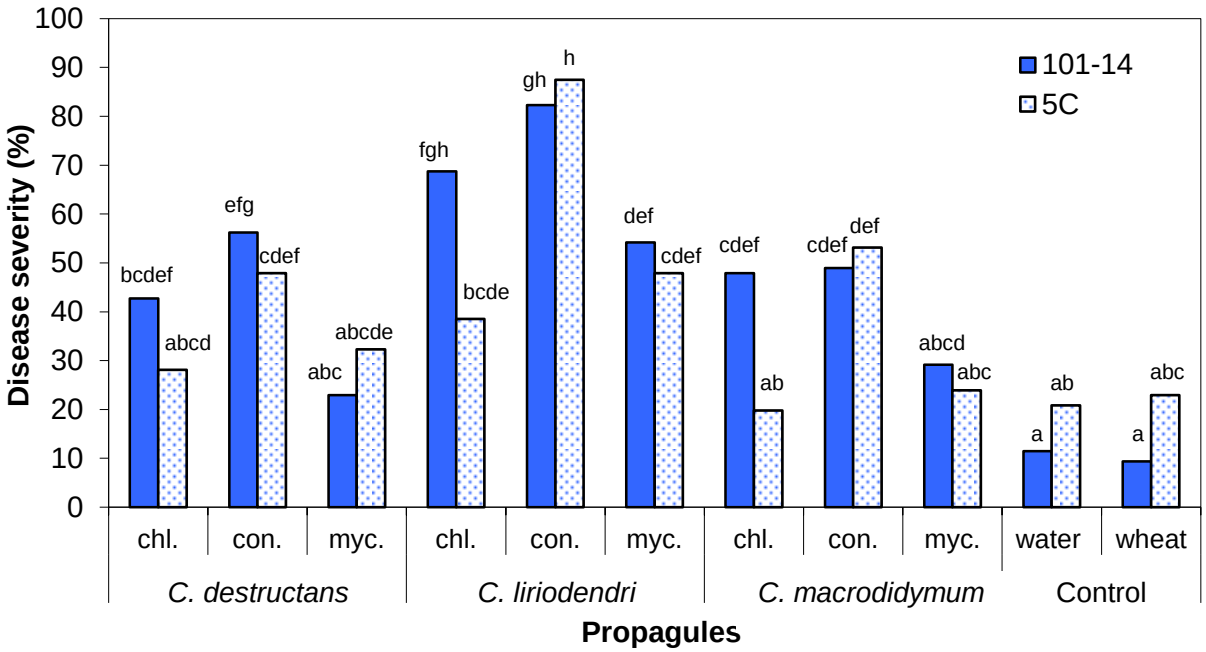

Figure 2.8. Mean disease severities at 1 cm above stem bases of young grapevine plants from rootstock varieties 101-14 and 5C inoculated with three propagule types of three *Cylindrocarpon* spp. (con.: conidium inoculum, chl.: chlamydospore inoculum, myc.: mycelium inoculum). Bars with different letters are significantly different ($P \leq 0.05$).

Root dry weights showed significant differences between propagules (*P*<0.001; A3.2.12), with mean root dry weights of plants inoculated with mycelium being significantly higher (9.7 g) than those inoculated with chlamydospores (8.5 g) and conidia (8.3 g; Table 2.7 and A3.2.13). No significant differences were observed between the inoculated plants and their respective controls. No significant differences in root dry weights were observed between species (*P*=0.754; Table 2.7).

Table 2.7. Mean root dry weights of grapevines inoculated with three *Cylindrocarpon* species and three inoculum types.

Species	Root dry weight (g)	Propagules	Root dry weight (g)
C. liriodendri	8.9 a[1]	chlamydospores	8.5 y
C. destructans	8.9 a	conidia	8.3 y
C. macrodidymum	8.8 a	mycelium	9.7 z
		Control water	8.0 y
		Control wheat	9.8 z

[1]Values within columns that are followed different letters are significantly different (*P*≤0.05).

Root dry weights showed a significant interaction between propagules and species (*P*<0.001; Table 2.8 and A3.2.14). For *C. destructans*, root dry weights were higher for mycelium than for conidium inoculation (*P*≤0.05), but not significantly different between chlamydospore and mycelium inoculations. However for the two remaining species, plants inoculated with mycelium had significantly higher mean root dry weights than plants inoculated with spores (*P*≤0.05).

Table 2.8. Mean root dry weights (g) of young grapevine plants, 5 months after inoculation with three propagule types of three *Cylindrocarpon* spp.

Species	Propagules	Mean root dry weight (g)
C. destructans	chlamydospores	8.8 ab[1]
	conidia	8.3 a
	mycelium	9.5 bc
C. liriodendri	chlamydospores	8.2 a
	conidia	8.5 a
	mycelium	9.9 c
C. macrodidymum	chlamydospores	8.5 a
	conidia	7.9 a
	mycelium	9.8 c
Control	water	8.0 a
	wheat	9.9 c

[1]Values within columns that are followed by different letters are significantly different (*P*≤0.05).

Mean root dry weights were significantly higher for rooted plants than callused plants (*P*≤0.001; A3.2.14) with 9.3 and 8.4 g, respectively and showed significant interactions between propagules, species and propagation methods (*P*≤0.001). For plants inoculated with

conidia from the different species, mean root dry weights were higher for rooted plants than callused plants ($P \leq 0.05$; Figure 2.9), and for those inoculated with mycelium from the different *Cylindrocarpon* spp., the mean root dry weights were similar ($P > 0.05$) for rooted plants and callused plants. For plants inoculated with chlamydospores, all species showed similar mean root weights between rooted and callused plants except for *C. destructans* in which the rooted plants had a greater mean dry weight than callused plants ($P \leq 0.05$). Callused plants inoculated with infested wheat grains had higher mean root dry weights than callused plants inoculated with spore suspensions or water ($P \leq 0.05$).

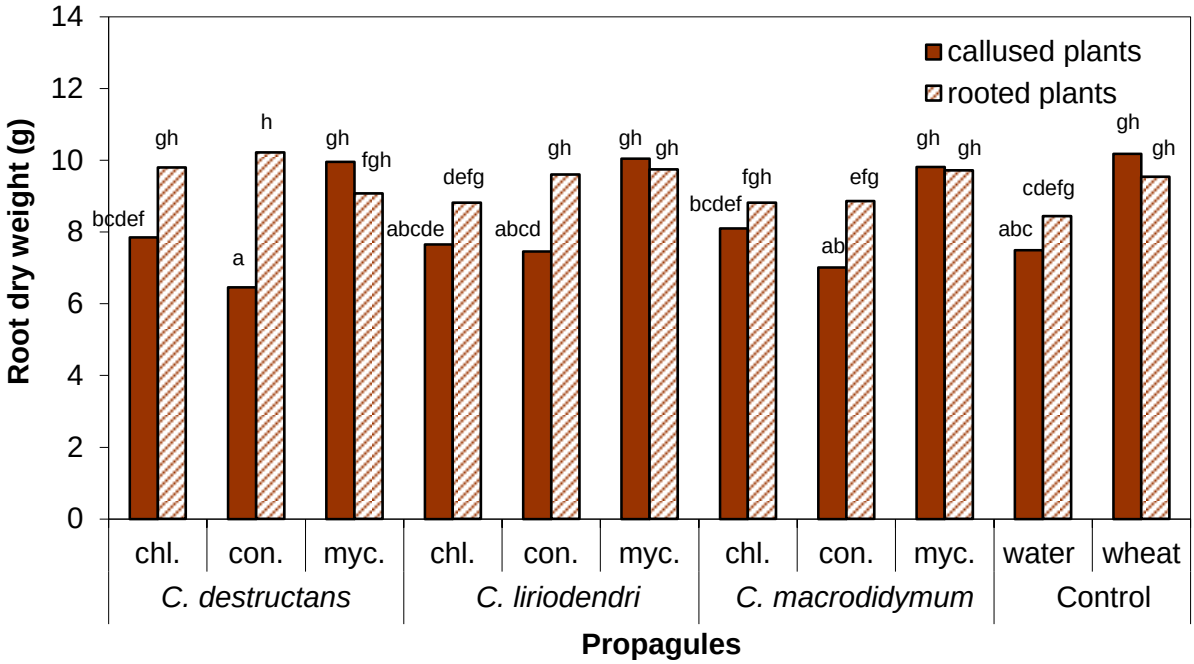

Figure 2.9. Mean root dry weights (g) of callused and rooted plants from rootstock varieties 101-14 and 5C, 5 months after inoculation with three propagule types of three *Cylindrocarpon* spp. (con.: conidium inoculum, chl.: chlamydospore inoculum, myc.: mycelium inoculum). Bars with different letters are significantly different ($P \leq 0.05$).

Rootstock varieties 101-14 and 5C had similar mean root dry weights (8.8 and 9.0 g respectively, $P = 0.154$; A3.2.14) however the significant interaction between rootstock varieties, propagules and species ($P = 0.045$) was evident. In 101-14 plants, those inoculated with *C. liriodendri* and *C. macrodidymum* conidia and chlamydospores and with *C. destructans* conidia had lower mean root dry weights than plants inoculated with mycelium from the different species ($P \leq 0.05$; Figure 2.10) whereas no significant differences were observed with grapevines from rootstock variety 5C.

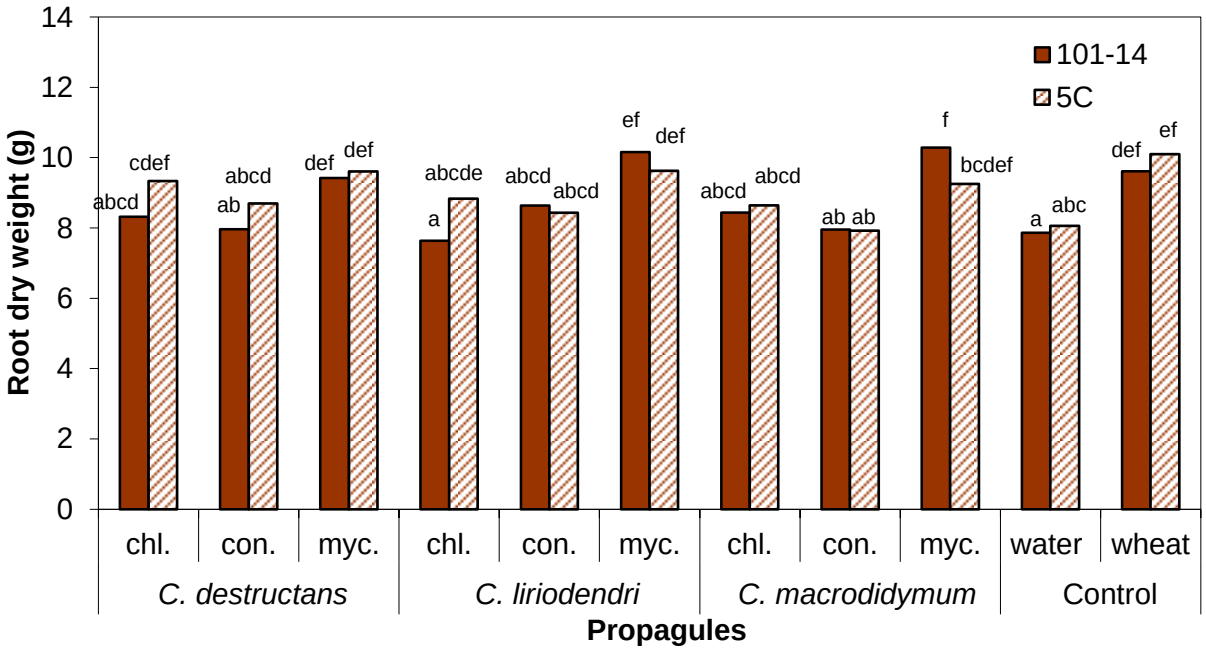

Figure 2.10. Mean root dry weights (g) of callused and rooted plants from rootstock varieties 101-14 and 5C, 5 months after inoculation with three propagule types of three *Cylindrocarpon* spp. (con.: conidium inoculum, chl.: chlamydospore inoculum, myc.: mycelium inoculum). Bars with different letters are significantly different ($P \leq 0.05$).

Shoot dry weights showed significant differences between propagules and species (both $P<0.001$; A3.2.15), with plants inoculated with mycelium having a significantly higher mean shoot dry weight (8.0 g) than those inoculated with chlamydospores (6.4 g) and conidia (6.6 g; A3.2.16). No significant differences were observed between mean shoot dry weights of inoculated plants and their respective controls. Plants treated with water (5.8 g) showed significantly lower mean shoot dry weights than plants treated with wheat grains (8.6 g). For the different species, plants inoculated with *C. macrodidymum* had significantly lower mean shoot dry weights than plants inoculated with the remaining species, being 6.2, 7.4 and 7.5 g for *C. macrodidymum*, *C. liriodendri* and *C. destructans*, respectively (Table 2.9 and A3.2.16).

Table 2.9. Mean shoot dry weights of grapevines inoculated with three *Cylindrocarpon* species and three inoculum types.

Species	Shoot dry weight (g)	Propagules	Shoot dry weight (g)
C. liriodendri	7.4 b[1]	Chlamydospores	6.4 y
C. destructans	7.5 b	Conidia	6.6 y
C. macrodidymum	6.2 a	Mycelium	8.0 z
		Control water	5.8 y
		Control wheat	8.6 z

[1]Values within columns followed by different letters are significantly different ($P \leq 0.05$).

The significant interaction between propagules and species (P<0.001; Table 2.10 and A.3.2.17) was evident in that plants inoculated with chlamydospores of *C. destructans* and with conidia of *C. liriodendri* had significantly higher mean shoot dry weights than the relevant controls. For the plants inoculated with mycelium, *C. macrodidymum* caused significantly lower mean shoot dry weight than the two different species and the wheat control, the latter being higher than for those of plants inoculated with spore suspensions or water.

Table 2.10. Mean shoot dry weights (g) of callused and rooted plants from rootstock varieties 101-14 and 5C, 5 months after inoculation with three propagule types of three *Cylindrocarpon* spp.

Species	Propagules	Mean shoot dry weight (g)
C. destructans	chlamydospores	7.1 b[1]
	conidia	6.6 ab
	mycelium	8.8 c
C. liriodendri	chlamydospores	6.5 ab
	conidia	7.5 b
	mycelium	8.4 c
C. macrodidymum	chlamydospores	5.6 a
	conidia	5.9 a
	mycelium	7.0 b
Control	water	5.8 a
	wheat	8.6 c

[1] Values within columns that are followed by different letters are significantly different (P≤0.05).

The shoot dry weights differed significantly with respect to propagation methods (P<0.001; A3.2.17), being 7.6 g for rooted plants and 6.5 g for callused plants, and the interaction between propagation methods and propagule types (P<0.001; Figure 2.11). Mean shoot dry weights of rooted plants were also greater than for callused plants (P≤0.05) when inoculated with chlamydospores and conidia, but were similar when inoculated with mycelium from the different *Cylindrocarpon* spp.

Shoot dry weights also differed significantly with respect to rootstock variety (P=0.002; A3.2.17) being 7.3 g for 101-14 and 6.8 g for 5C, with a significant interaction (P=0.013) between varieties, propagules, and species. However, only with *C. liriodendri* mycelium inoculation did plants show a rootstock difference, with means of 9.6 g for 101-14 and 7.3 g for 5C (Figure 2.12).

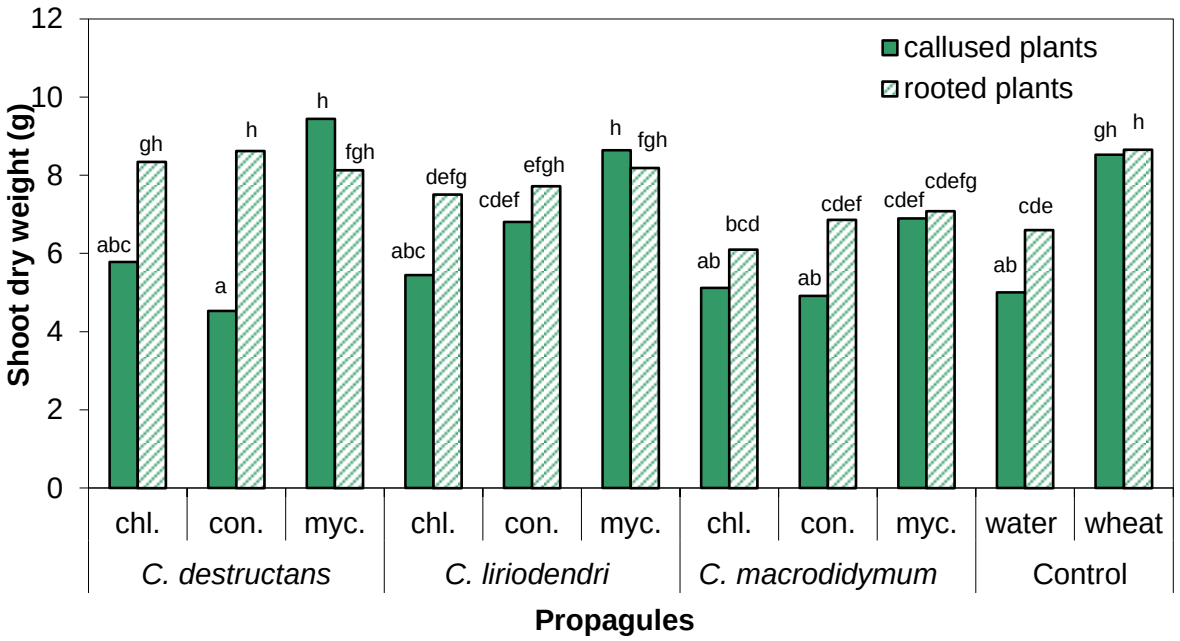

Figure 2.11. Mean shoot dry weights (g) of callused and rooted plants from rootstock varieties 101-14 and 5C , 5 months after inoculation with three propagule types of three *Cylindrocarpon* spp. (con.: conidium inoculum, chl.: chlamydospore inoculum, myc.: mycelium inoculum). Bars with different letters are significantly different (*P*≤0.05).

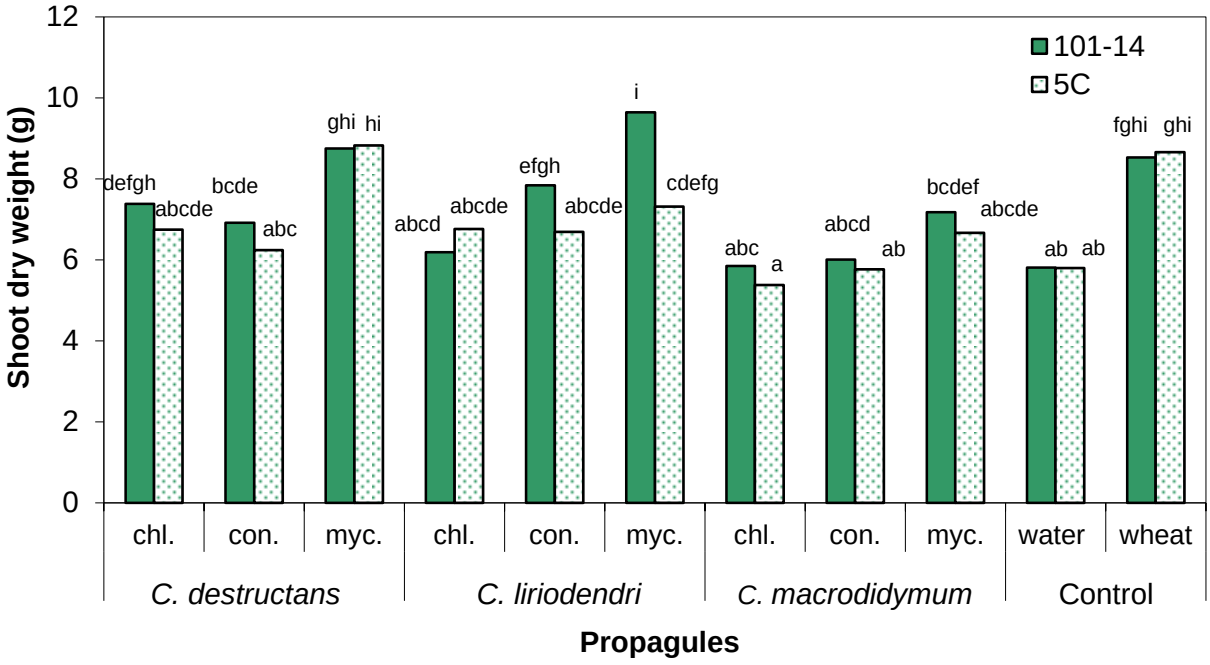

Figure 2.12. Mean shoot dry weights (g) of callused and rooted plants from rootstock varieties 101-14 and 5C , 5 months after inoculation with three propagule types of three *Cylindrocarpon* spp. (con.: conidium inoculum, chl.: chlamydospore inoculum, myc.: mycelium inoculum). Bars with different letters are significantly different (*P*≤0.05).

2.3.3. Threshold numbers of pathogen propagules

The data analysis for this section is reported in Appendix 3, Section 3.3. Disease incidences at 1 cm above the stem bases were significantly affected by propagule concentrations (P<0.001), species (P<0.001), and the interaction between propagule types, concentrations and species (P=0.009; A3.3.1).

Disease incidences at 1 cm increased for the different propagule concentrations of the two *Cylindrocarpon* spp. (Figure 2.13) with concentrations 2 (3.2 x 10^4 spores /mL or 3 g of infested wheat grains) and 3 (10^6 spores /mL or 5 g of infested wheat grains) causing significantly greater incidences than concentrations 0 and 1 (P≤0.05; A3.3.2). Overall, disease incidences were not significantly different between propagule types (P=0.369; A3.3.1) with means of 46.4, 36.9 and 40.5% for chlamydospores, conidia and mycelium, respectively.

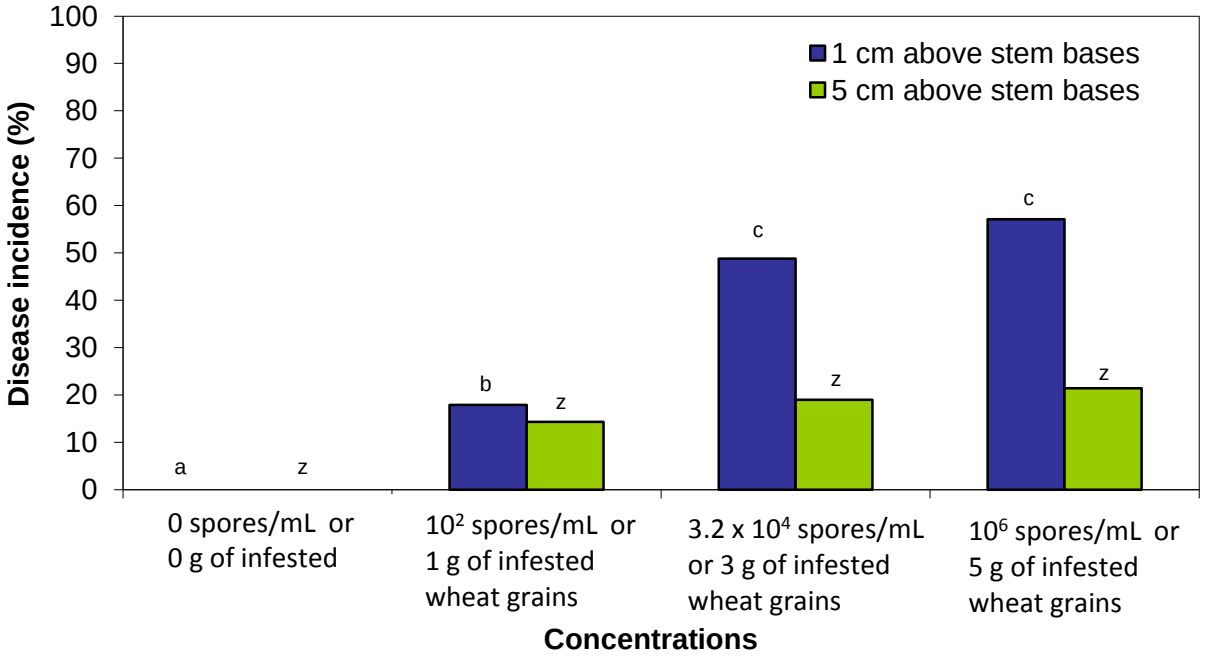

Figure 2.13. Mean disease incidences at 1 and 5 cm above stem bases of young grapevine plants inoculated with four concentrations of three propagules of two *Cylindrocarpon* spp. (For each distance, bars with different letters are significantly different (P≤0.05).

Mean disease incidences of plants inoculated with *C. liriodendri* were significantly greater than for plants inoculated with *C. destructans*, with 53.2 and 29.4%, respectively. The interaction between propagules and concentrations showed a linear increase of disease incidences for all propagules with the concentration (Table 2.11) for *C. destructans* (A3.3.3). For *C. liriodendri*, mean disease incidence increased linearly with the concentration for plants inoculated with mycelium, however, for plants inoculated with conidia and chlamydospores,

the mean disease incidences were slightly greater at a concentration of 3.2×10^4 spores /mL than 10^6 spores /mL, although not significantly different (A3.3.4). At the highest concentration, disease incidences were not significantly different between species.

Table 2.11. Mean disease incidences at 1 cm above stem bases of plants inoculated with four concentrations of *C. destructans* and *C. liriodendri* propagules, 5 months after inoculation.

Species	Propagules	Concentrations[1]			
		0	1	2	3
C. destructans	chlamydospores	0.0 a[2]	7.1 b	21.4 bc	57.1 c
	conidia	0.0 a	7.1 b	21.4 bc	50.0 c
	mycelium	0.0 a	14.3 b	35.7 b	50.0 b
C. liriodendri	chlamydospores	0.0 x	42.9 y	85.7 z	64.3 yz
	conidia	0.0 x	21.4 y	78.6 z	42.9 yz
	mycelium	0.0 x	14.3 y	50.0 yz	78.6 z

[1]Concentrations: 0: 0 spores /mL or 0 g of infected wheat grains, 1: 10^2 spores /mL or 1 g of wheat grains, 2: 3.2×10^4 spores /mL or 3 g of wheat grains and 3: 10^6 spores /mL or 5 g of wheat grains.
[2] Values within rows followed by different letters are significantly different ($P \leq 0.05$).

Disease incidences at 5 cm were not significantly different for the propagule concentrations (P=0.433; Figure 2.11, A3.3.5). Disease incidences were similar between propagule types, with means of 19, 16.7 and 19% for chlamydospores, conidia and mycelium, respectively (P=0.885). However, disease incidences at 5 cm above stem bases showed significant differences between species (P<0.001; A3.3.5), means being 29.4% for *C. liriodendri* and 7.1% for *C. destructans*. Disease incidence at 5 cm above stem bases did not show any significant interaction between species, propagules and concentrations (P=0.105; A3.3.5).

Disease severities at 1 cm above stem bases showed significant differences between propagule concentrations (P<0.001; A3.3.6) and species (P=0.001). Mean disease severities increased for the different propagule concentrations of the two *Cylindrocarpon* spp. with concentrations 1, 2 and 3 causing significantly greater severities than the control and concentrations 2 and 3 greater severities than concentration 1 ($P \leq 0.05$; Table 2.12). Overall, disease severities were not significantly different between propagule types with means of 27.7, 27.4 and 26.8% for chlamydospores, conidia and mycelium, respectively. Plants inoculated with *C. liriodendri* had greater mean disease severities than plants inoculated with *C. destructans* with 35.1% and 19.4%, respectively. Disease severity did not show any significant differences for the interaction between species, propagules and concentrations (P=0.390; A3.3.6).

Table 2.12. Mean disease severity at 1 cm above the stem bases of young grapevine plants inoculated with four concentrations of *C. liriodendri* and *C. destructans* propagules, 5 months after inoculation.

Concentration	Disease severity (%)
0 spores /mL or 0 g of infested wheat grains	0.0 a[1]
10^2 spores /mL or 1 g of infested wheat grains	11.6 b
3.2×10^4 spores /mL or 3 g of infested wheat grains	30.0 c
10^6 spores /mL or 5 g of infested wheat grains	40.2 c

[1]Values within columns followed by different letters are significantly different ($P\leq0.05$).

For the root dry weights, there were no significant differences between treatments (A3.3.7). For the different concentrations, the mean root dry weights were 6.4, 7.0 g, 7.3 g and 6.7 g for the plants treated with the control, 1st, 2nd and 3rd concentrations of propagules, respectively (P=0.285). For the different propagule types, mean root dry weights were 6.7, 6.9 and 7.1 g for chlamydospores, conidia and mycelium respectively (P=0.631). Mean root dry weights were 6.6 and 7.1 g for plants inoculated with *C. liriodendri* and *C. destructans*, respectively (P=0.976).

2.4. Discussion

In this study, two different propagation methods were tested for their ability to simulate *Cylindrocarpon* infection in nurseries and vineyards: grapevine cuttings were either callused at the stem base or rooted and the roots wounded before soaking the plants for 30 min in a spore suspension. Both types of plants became infected, indicating that *Cylindrocarpon* species are capable of infecting grapevines through wounded roots and callused basal ends. The 4 weeks allowed for callusing, as commonly used in nurseries, was not sufficient for the entire basal ends of the rootstock cuttings to become covered by callus, enabling the pathogen to penetrate the vine tissues. Halleen *et al.* (2003) also reported that the basal ends of most grapevine cuttings were only partially callused when they were planted in the nursery soil and therefore were predisposed to infection by soilborne pathogens.

The proportion of plants infected in their stem bases in the two first experiments, at 4 and 5 months after inoculation, was greater for callused plants than rooted plants. Although the extra month's growth period after inoculation affected the proportion of infected plants and degree of penetration, it caused only a slight change to the infection ratios for the plant types. In the experiment that investigated the pathogenicity of the different species, the disease incidences 4 months after inoculation were 73.5 and 34.8% at 1 and 5 cm above stem bases, respectively, for callused plants and 36.0 and 13.7% at 1 and 5 cm above stem bases, respectively, for rooted plants (P<0.001 for all). Infection incidences were a little higher in the experiment that assessed the pathogenicity of the different propagule types at 5 months after inoculation, but the plant types were still significantly different (P<0.001). The disease

incidences for callused plants, at 1 and 5 cm above stem bases, were 70.8 and 50.0%, respectively, and for rooted plants were 46.6 and 24.6%, respectively. *Cylindrocarpon* spp. are known to invade roots and to progressively move through them towards the trunk, a relatively slow process (Halleen *et al.*, 2003). This explains the lower proportion of vine stems infected through root tissues than through callus. In rooted vines, it is likely that in some plants the pathogen development was too slow to have reached the stem bases used for isolations during the few months allowed for plants to grow; a longer growth period would have probably resulted in more disease. This movement towards the stem was illustrated by the work of Halleen *et al*. (2003) on grafted grapevines planted into infested soils from three nurseries. Before planting, isolation attempts at 5 cm above rootstock stem bases found *Cylindrocarpon* spp. only twice in the 40 grafted plants tested, and in only one nursery. After the grafted vines were planted, the pathogen was isolated in progressively greater proportions of tissue pieces at 3, 6 and 9 months after planting, with infection of 15.2, 28.2 and 36.2%, respectively, of root pieces and 0.2, 2.8 and 5.5%, respectively, of pieces at 5 cm above the stem bases. The *Cylindrocarpon* spp. were also isolated from 0, 0.8 and 0.3%, respectively, of pieces from graft-unions, and from scion wood at 2 cm above graft-unions in 0, 0 and 0.2% of infected pieces, respectively. Although no information was provided about disease incidences from the plants at the different sampling regions, Halleen *et al*. (2003) stated that less than 1% of the plants were infected before planting and 50% or more were infected after 9 months. Since callused plants had greater disease incidences in this study, this type of propagation method was used for plants in later experiments, such as the investigation into the threshold number of pathogen propagules.

The experiment that investigated the pathogenicity of three *Cylindrocarpon* spp. showed that all nine isolates were able to infect callused and root-wounded plants. Isolates from each species caused high disease incidences and severities at 1 cm above stem bases, with no differences between species. However, variation between isolates from the same species was observed, the range of incidence at 1 cm being 53.6 to 74.1% for *C. liriodendri*, 32.1 to 82.1% for *C. destructans* and 42.9 to 75.0% for *C. macrodidymum* isolates, 4 months after inoculation. The three *C. liriodendri* isolates tested produced consistently high disease severities, although not significantly different from the other isolates, with averages of 63.0, 55.4 and 56.6% for *C. liriodendri*, *C. destructans* and *C. macrodidymum*, respectively. Rego *et al.* (2001) reported similar variation in pathogenicity of different *Cylindrocarpon* isolates from "*C. destructans*" (later identified as *C. liriodendri* by Alaniz *et al.* in 2007) after 3 month growth of inoculated rooted plants. Alaniz *et al.* (2009) who inoculated 3 – 4 leaf grapevine seedlings with five *C. liriodendri* and 14 *C. macrodidymum* isolates also reported different levels of virulence between *C. macrodidymum* isolates but not between isolates of *C. liriodendri*. They investigated the genetic diversity of these species using inter-simple

sequence repeat analysis and segregated the different genotypes into seven groups. They correlated the observed levels of virulence for *C. macrodidymum* to genetic diversity among isolates, since isolates from two inter-simple sequence repeat groups were significantly more virulent than isolates from other groups. In contrast, Petit and Gubler (2005) failed to observe any variations in pathogenicity between isolates of *C. liriodendri* and *C. macrodidymum* on inoculated 6 month old rooted vines, when assessed 4 months after inoculation.

The variation in pathogenicity of the different isolates could be explained by their enzyme activities or their mycotoxin production. Lyr and Kluge (1968) studied nine isolates of *N. radicicola* which they grouped into three classes according to their degree of pathogenicity against *Pinus sylvestris*. They associated the differences in pathogenicity with toxin production, rather than production of the enzymes, such as pectinase, xylanase, cellulase and beta-glucosidase which are commonly associated with pathogenicity. However, the pathogenic isolates had slightly higher pectinase and cellulase activities than non-pathogenic ones. Evans and White (1966) isolated from "*C. radicicola*" a phytotoxin, initially identified as nectrolide or brefeldin A, which stunted *Eucalyptus pilularis* growth. Sweetingham (1983) also observed that brefeldin A produced by *C. destructans* and *C. didymum* (Harting) Wollenweber reduced germination and root growth of *Pinus radiata* seeds.

Isolate differences in enzyme production were found by Rahman and Punja (2005) who investigated the underlying mechanisms of *C. destructans* virulence on ginseng. After pathogenicity experiments, they separated the isolates into two virulence groups, which comprised highly virulent isolates that could cause lesions on unwounded mature roots and weakly virulent isolates that produced lesions on only wounded roots. The virulence of the isolates was related to pectinase and polyphenoloxidase activities which were higher for highly virulent isolates than weakly virulent isolates. The contribution of pectinases to fungal pathogenicity is widely recognized since they weaken plant cell walls enabling pathogen penetration (Kikot *et al.*, 2009). Ahn and Lee (2001) isolated four different sizes of double-stranded RNAs (dsRNAs) from *Cylindrocarpon destructans* isolates on ginseng and related the presence of one of these dsRNAs to higher levels of virulence, sporulation, pigmentation and laccase activity. They also found that higher proportions of isolates containing that particular dsRNA were present in replanted ginseng fields compared to first cropping of ginseng and hypothesised that the increasing numbers of more pathogenic isolates in the soil could be responsible for ginseng replant failure. To further investigate the underlying mechanisms of pathogenicity of the approximately 200 *Cylindrocarpon*-like isolates available at Lincoln University, from which only nine were selected for the current study, another research program is currently investigating their pathogenicity and capacity to produce enzymes involved in the host infection phase, in relation to genetic variation (Dr. Hayley

Ridgway, pers. comm. 2010). As indicated by Li *et al.* (2009), a better knowledge of the variability of a pathogen would be useful for the selection of isolates to test plant varieties for their resistance against a disease.

The study of the pathogenicity of the different propagule types showed that all three propagule types from three different isolates (different species) were infectious. Conidia caused the highest disease incidence among the different propagules for all species with 81.3, 62.5 and 56.3% for conidia, chlamydospores and mycelium, respectively. Of the different isolates tested, *C. liriodendri* isolate L1 caused the highest disease incidences at 1 cm above stem bases for chlamydospores, conidia and mycelium. Differences between the selected isolates of the species were most evident in the rooted plants, where only propagules from *C. liriodendri* had a disease severity significantly different from the plants treated with water, indicating that it was capable of colonising plant tissues faster than the two other isolates.

Throughout the world, *C. liriodendri* and *C. macrodidymum* seem to constitute the major threat to grapevines as they are the species most commonly reported (Halleen *et al.*, 2004; Halleen *et al.*, 2006b; Auger *et al.*, 2007; Petit and Gubler, 2007; Alaniz *et al.*, 2009). Recently, molecular tools allowed for differentiation and identification of *C. liriodendri* and *C. macrodidymum*, isolates of which had frequently been misidentified as *C. destructans* and *C. obtusisporum*, respectively (Halleen *et al.*, 2006a). In New Zealand, the three *Cylindrocarpon* spp. used in this study appeared to be present in similar proportions (Bleach *et al.*, 2006). Although *C. destructans* does not appear to represent a danger to grapevines in other countries, the conditions in New Zealand are believed to have been favourable for the presence of *C. destructans* inoculum in the soil as vineyards replaced apple and stone fruit orchards which are susceptible to this pathogen (Bonfiglioli, 2005). Since one *C. destructans* isolate (D2) had the highest disease incidence at 1 cm above stem base (82.1%) and severity (83.9%) among the nine isolates tested, *C. liriodendri* and *C. destructans* were selected for the study of the threshold numbers of pathogen propagules.

In this research project, control plants also became infected, with 21.4% incidence at 1 cm in the study with nine isolates of *Cylindrocarpon* spp., while in the study with different propagules, the water and wheat control plants had 33.3 and 31.3% incidence at 1 cm, respectively. This was probably due to cross-contamination caused by splashing dispersal during watering rather than the presence of *Cylindrocarpon* spp. in the potting mix; these experiments were carried out at Lincoln University where the plants were hand watered. This suggestion is supported by the fact that there was no infection of control plants in the threshold experiment, which took place in a Blenheim polyhouse where each pot had a

dripper, thereby preventing the splashing of propagules between pots. Future experiments conducted in the Lincoln University greenhouse were spaced more widely to avoid cross-contamination.

This is the first study reported of the threshold numbers of propagules needed for infection of grapevines by root pathogens, since other studies have all used high concentrations of conidia for inoculation. *Cylindrocarpon liriodendri* and *C. destructans* were able to infect plants at even the lowest concentrations of propagules applied, numbers which are too low for many other disease organisms. For example, a study by Horsfall and Dimond (1960), which investigated the concept of inoculum potential of plant pathogens, reported that the lowest number of propagules needed to cause some soil-borne diseases were: 200 resting sporangia of *Synchytrium endobioticum* /g of soil, 10^4 conidia /mL for *Fusarium nivale* and 7×10^5 conidia /mL for *Fusarium oxysporum* f.sp. *lycopersici*.

A number of researchers have reported that the application of increasing inoculum concentrations to plants generally resulted in increased disease severity (Pegg and Dixon, 1969; Beute, 1971; Sippell and Hall, 1982; Shaw *et al.*, 1997). In the current study, disease incidence and severity at 1 cm increased with the increasing concentrations of the different propagules, except that for plants inoculated with the highest concentrations, 10^6 spores /mL or 5 g of wheat grains, disease incidences at 1 cm above stem bases were not significantly higher than those inoculated with the intermediate concentrations, 3.2×10^4 spores /mL or 3 g of wheat grains. A similar effect was reported by Rahman and Punja (2005), who studied the effect of *C. destructans* inoculum concentration on ginseng by adding 1, 5 and 10% (v/v) of colonised oat grains mixed with field soil to achieve 3.45×10^2, 1.86×10^3 and 3.55×10^3 CFU /g of dry soil. After 30 days, disease severity on the roots had increased with concentration; however, it did not increase above the intermediate concentration of 1.86×10^3 CFU /g of dry soil. In the current study, the lowest concentrations resulted in disease, so additional experiments should investigate the pathogenicity of even lower propagule concentrations in field soils to determine the minimum numbers of propagules needed for infection.

In the different experiments, plants were inoculated and grown in potting mix which does not reflect the natural environment of the *Cylindrocarpon* spp. within vineyards where the pathogen interacts with different soil micro-organisms and the threshold number of propagules needed for infection would probably be higher. This was illustrated with the work of Douglas (1970) who added different concentrations of *Fusarium oxysporum* f. sp. *melonis* to autoclaved soil or non autoclaved soil and grew muskmelons. He observed that a higher number of plants survived in natural soil compared to autoclaved soil at the two highest

concentrations, indicating that the inoculum efficiency was reduced in natural soil. The behaviour of the different propagules in soil was investigated in Chapter 4.

Researchers working on *Cylindrocarpon* spp. on grapevines have generally evaluated the effect of the pathogen on the plant by assessing the root and shoot dry weights, the proportion of dead plants and by rating the root and leaf symptoms. In these experiments, the effects of the different *Cylindrocarpon* spp. on plant growth were determined by assessing the root dry weights. Similar experiments by Halleen *et al.* (2004), which inoculated grapevine cuttings by soaking them in a conidium suspension, led to root dry weight reductions. However, in the experiment reported here, which investigated the pathogenicity of three *Cylindrocarpon* spp., only plants inoculated with *C. destructans* D1 had significantly lower root dry weights than the plants treated with water. The other isolates caused slightly lower root dry weights than the control however differences were not significant and did not differ between species when isolates from the same species were grouped together for analysis. Halleen *et al*. (2004) inoculated rooted vines with *C. destructans* and *C. macrodidymum* isolates, using the same protocol as in this experiment, except that plants were grown in sterilised potting mix for 4.5 months. After that period, plants showed a reduction of 57.5 and 48.0% of their root mass compared to the control for *C. destructans* and *C. macrodidymum* inoculations, respectively. Rego *et al*. (2001) observed that plants inoculated with 8 of the 12 *Cylindrocarpon* isolates tested had a significantly lower number of roots at 3 months after inoculation, but root dry weights were not reported. Their plants were inoculated with a higher conidium concentration (10^8 conidia /mL) than used here and their plants were grown on a mixture of soil, peat and sand which was autoclaved. It is possible that the autoclaved potting mix or soil mixture has enabled the pathogen to infect in a shorter time than in the non-sterile potting mix used in this study. It is also possible that a longer period of time might have given a significant reduction of the root dry weights for the different inoculation treatments as observed by Whitelaw-Weckert *et al.* (2007) who described the disease development as slow with no disease symptoms above ground after 18 months and rotted roots after 3 years. However, Alaniz *et al*. (2009) reported reduced root dry weight and vine death in 3 to 4 leaf seedlings at 2 months after inoculation, and Scheck *et al*. (1998) reported similar effects in 1 month old seedlings, which was probably due their ages since the fleshy roots of such young plants could be quickly invaded.

The root weight results from the experiment reported here, which investigated the pathogenicity of *Cylindrocarpon* isolates, were very similar to those of Mohammadi *et al.* (2009). They inoculated trimmed roots of 1 year old plants with three isolates of *C. liriodendri* and assessed the plants after 4.5 months. However, their isolates caused no differences for the shoot dry weights and only one isolate caused lower root dry weight than the control

plants. Their isolations found significantly greater root disease severity in inoculated plants than in the control, although hardly any root lesions were observed. The conflicting results described above can be explained by the observations of Rahman and Punja (2005) who reported that old roots of ginseng often had a high number of superficial lesions caused by *C. destructans* while younger roots rotted completely.

In the experiment using different types of propagules of the three different species, the root and shoot dry weights of plants soaked for 30 min in a solution (water or spore suspension) were significantly lower than for the plants with wheat grains added to the planting hole. As the dry weight differences observed resided in the inoculation method rather than the presence or absence of *Cylindrocarpon* spp., it is likely that the cooked wheat grains were able to leach nutrients or to be further degraded by micro-organisms which provided nutrients for the plants. In contrast, the experiment with different concentrations of *C. liriodendri* and *C. destructans* did not show any differences between root dry weights. A possible explanation may be associated with the reduced root growth overall in the threshold experiment, mean root weights ranging between 6.4 and 7.4 g compared to the mean root weights in the propagules experiment, which ranged between 7.9 and 9.9 g. In the threshold experiment conducted in a polyhouse, all plants were subjected to high temperatures, which often reached 37°C during the summer of 2007-2008, whereas the plants of the propagule experiment were only subjected to a maximum of 30°C.

In this study, the vines displayed no obvious signs of decline, possibly because they were grown in a greenhouse under conditions that provided the appropriate environment needed for growth, such as temperature, nutrients and water supplies. This could explain why shoot dry weight was not reduced by the inoculated pathogens. The pathogens may also not have had sufficient time to cause vine decline, as the plants were assessed 4 to 5 months after inoculation. A longer growth period would have probably resulted in more severe disease symptoms, as observed by Whitelaw-Weckert *et al.* (2007) who found a 58% decrease in mean vine shoot length by 27 months after inoculation and a 98% decrease in mean root biomass by 31 months after inoculation. However, this project aimed to investigate the factors that affected development of infection, for which pathogen isolation was the assessed outcome, and the time allowed was sufficient for this to occur. If the experiment had continued long enough for vines to become severely diseased or to die, the tissues used for isolation may have become colonised by many other saprophytic microorganisms, with the causal agent sometimes being ousted from the tissue and so absent from isolation plates (Dr. M. Jaspers pers. comm. 2007). Therefore, longer incubation periods were not considered appropriate for this study.

There was no significant difference in susceptibilities of the two rootstock varieties in this study, although there was a trend in some assessments for slightly higher disease levels in 101-14 than in 5C. Selection of the rootstock varieties for this study was based on their relatively common usage in New Zealand (Bruce Corban, pers comm. 2007), their different parentage and their reported susceptibility to black foot disease. Variety 101-14 is the result of a cross between *V. riparia* and *V. rupestris* and 5C a cross between *V. riparia* and V. *berlandieri*. Their relative susceptibility to black foot was found to be high for 101-14 and moderate for 5C (Harvey and Jaspers, 2006), however the results were not statistically analysed. Rego *et al.* (2000), who randomly sampled vines from nurseries around Portugal also found that of the nine different rootstock varieties, 101-14 had a higher disease incidence than SO4 with 47.0 and 33.4% respectively, however the disparity between the relative numbers of their rootstock samples (101-14: 40 plants and SO4: 515 plants) meant that robust statistical analysis was not possible. Rootstock varieties S04 and 5C are from the same parentage and are very similar morphologically (Walker and Boursiquot, 1992), so may be considered equivalent. However, experiments by Dore (2009) found that susceptibility to *C. destructans* was not statistically different between these two rootstock varieties.

Rootstock varieties have different optima for different factors, such as temperature, soil types, water supply, soil pH and vigour. It is therefore possible that the suitability of the selected rootstock to its environment may affect its susceptibility to a range of pathogens or inoculum concentrations. In the experiment using different propagule concentrations, the rootstock variety 101-14 was chosen as it is the rootstock variety most widely used in New Zealand and was shown to be susceptible in the previous experiments. However, the possibility of different rootstock varieties being susceptible to different concentrations and/or propagules of *Cylindrocarpon* spp. should be investigated. Douglas (1970) showed differences between varieties of muskmelons to low concentrations of *Fusarium oxysporum* which were not detected at high concentrations. Mohamed *et al.* (1997) found that 10^3 conidia /g of soil of *Fusarium moniliforme* was needed to reduce shoot and root dry weight of one sorghum cultivar whereas for another cultivar 10 conidia /g of soil was sufficient to reduce shoot and dry weight. In contrast, Pegg and Dixon (1969) found that when 10 day-old tomato seedlings were uprooted and the roots soaked for 15 min in *Verticillium albo-atrum* conidium suspensions of 10, 10^3, 10^5 or 10^7 conidia /mL, the resistant tomato varieties did not show any effect on leaf area at the highest concentration (10^7 conidia /mL), whereas susceptible varieties showed reducing leaf area with increasing inoculum concentration, culminating in a 94% reduction with the highest concentration. Frank *et al.* (1975) also reported that potato varieties known as resistant to *Verticillium albo-atrum* became wilted after inoculation with high conidium concentrations. It is possible that 10^6 spores /mL and 5 g of infested wheat grains used in the experiments that investigated the pathogenicity of

Cylindrocarpon spp. and propagules were too great to allow observation of any differences between varieties.

All three *Cylindrocarpon* species were capable of infecting grapevines through wounded roots or callused basal ends, indicating that the pathogen is capable of invading grapevines either in nurseries or in vineyards. However, these results were obtained with plants grown in potting mix and so the pathogenicity of the propagules in soil was investigated in Chapter 4. The development of methods for identifying these species, which was considered an essential tool for further research objectives, was investigated in Chapter 3, and Chapter 5 further developed these methods for diagnosis of infested soil sites.

CHAPTER 3

Development of a molecular identification method for *Cylindrocarpon* spp. in New Zealand

3.1. Introduction

Traditional identification methods for fungi that cause diseases consist of recognising symptoms on the plant host, isolating the pathogen, and then identifying it by colony morphology and reproductive characters (Fox and Narra, 2006). However, traditional identification methods are frequently beset by practical problems. In many cases, the other pathogens or fast growing saprophytes also present in the tissue may cause confusion or mask the presence of slower growing pathogens. The assessment of symptoms can also be unreliable as symptoms can be influenced by environmental factors, such as seasonal temperature extremes, and different pathogens may show the same symptoms (Fox and Narra, 2006). In grapevines, Scheck *et al.* (1998) concluded that early symptoms caused by *Cylindrocarpon* spp. or by *Phaeoacremonium* spp. on above-ground tissues of young grapevines were almost indistinguishable, and so were classified simply as decline symptoms.

To successfully identify the pathogen by morphology alone, it needs to have specific characteristics, preferably with a low level of phenotypic variation between isolates. The *Cylindrocarpon* spp. studied in this research programme each had identifiable morphological features, but the colony and conidium characteristics of the isolates from one species could display overlap with those of other species (Figure 3.1). Hence isolates from *C. macrodidymum* have been misidentified as *C. obtusisporum* (Halleen *et al.*, 2004; Petit and Gubler, 2005) and isolates from *C. liriodendri* as *C. destructans* (Halleen *et al.*, 2006a; Petit and Gubler, 2007; Alaniz *et al.*, 2009). Conidium sizes from the three different species used in this project overlap as described in Chapter 1, Section 1.2.5. Morphology is often insufficient to differentiate species when used alone (Petit and Gubler, 2005) and gene sequence information is often used to supplement morphological descriptions.

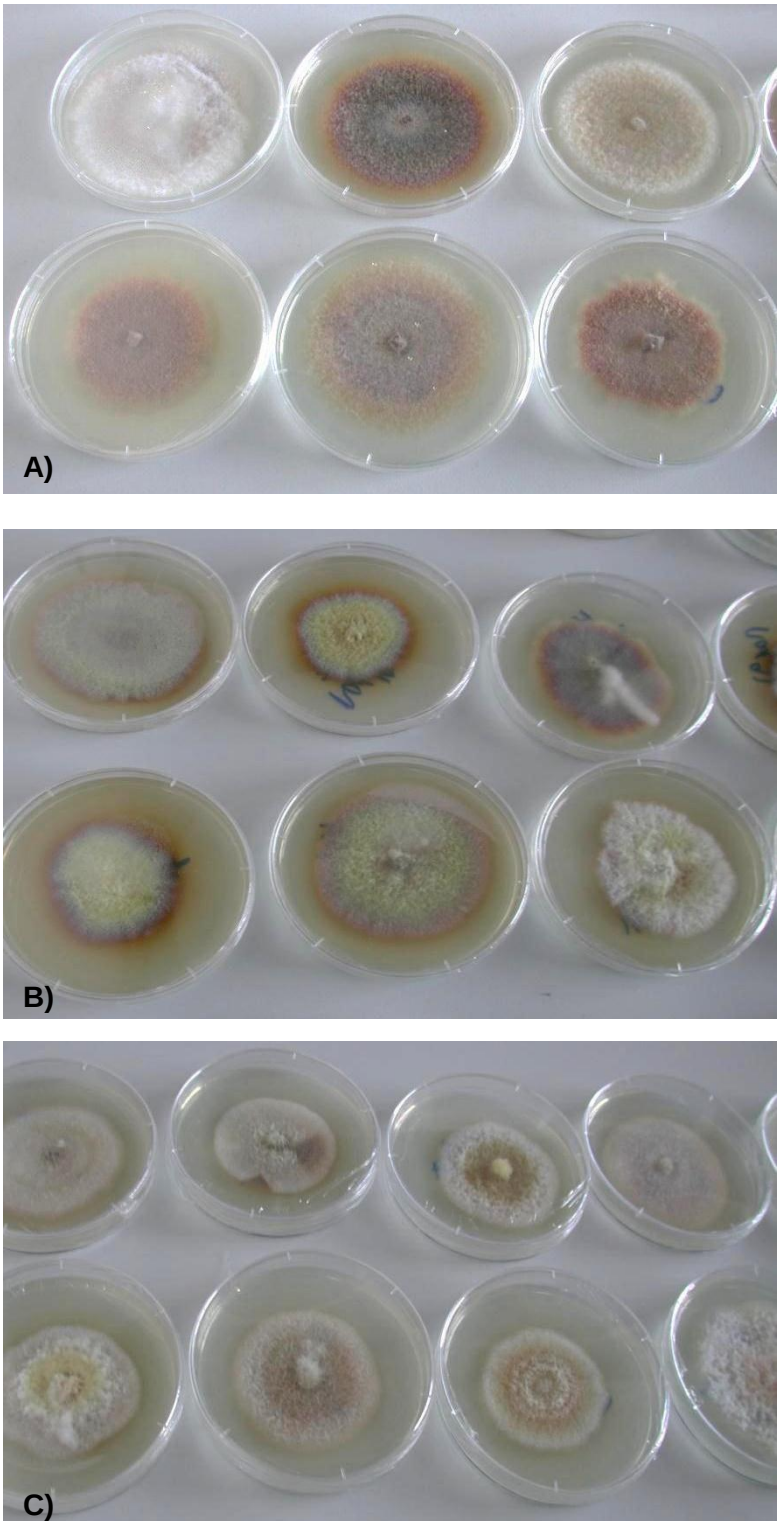

Figure 3.1. *Cylindrocarpon* cultures after 2 weeks growth on PDA at 20°C in the dark. A: *C. destructans* isolates; B: *C. macrodidymum* isolates ; C: *C. liriodendri* isolates.

The ITS region of the rRNA genes has been successfully used to identify the DNA of many fungi, which was extracted from a wide range of sources such as plant tissues and soil (Jones, 2001). Hamelin *et al.* (1996) designed species specific primers (Dest1 and Dest4) which were used to amplify *C. destructans* DNA from conifer seedlings. When Nascimento *et al.* (2001) used these primers to amplify DNA extracted from *C. destructans* cultures obtained from grapevines in Portugal, they obtained the same 400 bp DNA fragment, however, the primers did not discriminate between *C. destructans* and *C. obtusisporum*. To improve the detection sensitivity, they then modified the PCR assay, using universal primers ITS4 and ITS1F in a primary fungus-specific amplification, followed by a secondary amplification with the primers Dest1 and Dest4. Their method was simple and reliable for collective detection of these two *Cylindrocarpon* spp. directly from infected grapevine tissues.

In some cases the rRNA ITS region sequences have had insufficient polymorphism to differentiate between closely related species, and so other DNA regions such as the β-tubulin gene, the elongation factor alpha and the intergenic spacer (IGS) region have been used instead. Aroca and Raposo (2007) used the ITS region to distinguish *Phaeoacremonium* species, but found that *P. viticola* and *P. angustius* could not be discriminated as their rRNA ITS region were 100% identical. Therefore, amplification of the partial β-tubulin gene was essential to differentiate between species (Aroca and Raposo, 2007).

The objective of this chapter was to develop a molecular method to identify the different *Cylindrocarpon* species that cause black foot of young grapevines in New Zealand. This method should provide for identification of isolates and may allow tracking of the pathogen in plant tissues and soil.

3.2. Materials and methods

3.2.1. *Cylindrocarpon* spp. isolates

The Lincoln University culture collection contained *Cylindrocarpon* isolates obtained from symptomatic vines in a 2005 survey conducted throughout New Zealand , which were maintained on SNA (Appendix 2) slopes at 4°C (Bleach *et al.*, 2006). From this collection, 60 isolates had been identified by DNA sequencing by Dr. Lizel Mostert at Stellenbosch University (Mostert *et al*., 2006). For this study, ten isolates of each *Cylindrocarpon* spp. were used (the nine isolates chosen in 2.2.1.2 and seven isolates of each of the three named *Cylindrocarpon* spp. randomly selected), as well as all isolates identified as novel species (Appendix 1).

3.2.2. DNA extraction

The isolates were grown on PDA (Oxoid Ltd, Basingstoke, UK) at 20°C for 2 – 3 weeks in the dark, at which time the conidia were suspended as described in Section 2.2.1.2 and the final concentration adjusted to 100 conidia /mL before spreading 0.1 mL of each suspension onto a PDA plate. The Petri dishes were sealed with a plastic wrap and incubated at 20°C in the dark. After 48 h growth, a colony developing from a single conidium was excised from the medium and placed onto a PDA plate, then incubated in the same conditions for a week. A small piece from the actively growing front of each colony was placed in a deep Petri dish containing potato dextrose broth (PDB; Sigma Chemicals, St. Louis, USA). The cultures were incubated for a week at room temperature, with a 12 /12 h light /dark photoperiod. Mycelium was harvested by filtering the broth through sterile Miracloth™ (Calbiochem®). Excess PDB was removed from the mycelium by pressing the Miracloth™ between paper towels. The mycelium was sealed in aluminium foil wrap and frozen in liquid nitrogen before being stored at -80°C until use.

Prior to DNA extraction, the frozen mycelium was ground to a fine powder in liquid nitrogen using a mortar and pestle and 0.1 g of each isolate's mycelium was used for DNA extraction using a PureGene DNA extraction kit (Qiagen Siences, MD, USA) according to the manufacturer's instructions. The DNA extraction process included an RNAse step to remove any contaminating RNA. To check for presence of DNA, the DNA was run on an agarose gel. The 1% agarose gel was made by adding agarose to a Tris-Acetate EDTA buffer (TAE; Appendix 2). The agarose was dissolved by heating to boiling and cooled to approximately 60°C before being poured into a horizontal E-C® Gel Electrophoresis Apparatus (Thermo Scientific, NY, USA) and a comb inserted. After 30 min, the comb was removed from the set gel, which was placed into an electrophoresis chamber and covered with TAE. The DNA samples were centrifuged at 3000 × *g* for 5 – 10 s, vortexed briefly and loaded into the wells after combining 2 µL of DNA sample with 5 µL of sterile nanopure water (SNW) and 3 µL of loading dye (Appendix 2). For each gel, 4 µL of High DNA Mass Ladder™ (Invitrogen Life Technologies, CA, USA) and 5 µL of a 0.2 µg /µL 1Kb Plus DNA ladder™ (Invitrogen Life Technologies) were loaded; both products were mixed with 5 µL of SNW and 3 µL of loading dye. The gel was run at 10 V /cm for 45 min before being transferred into a container with an ethidium bromide solution (0.5 µg /mL, AMRESCO®, OH, USA) and left to stain for 20 min. The gel was destained by soaking in tap water for 10 min and placed under UV lights, where it was photographed and visualised using a VersaDoc™ Imaging System (Model 3000, Bio Rad, CA, USA).

Genomic DNA quality was assessed by examining the agarose gel under UV light and DNA concentration was measured by spectrophotometry with a NanoDrop-ND-1000 spectrometer (NanoDrop Technologies, DE, USA) and diluted with SNW to 10 ng /µL for PCR.

3.2.3. PCR amplification

Two different PCR methods were tested for their specificity for the *Cylindrocarpon* spp. selected. The first was based on existing methods using a species specific primer for *C. destructans* (Hamelin *et al.*, 1996; Nascimento *et al.*, 2001) from the ITS region and the second one used primers designed by Dr. Lizel Mostert, Stellenbosch University (pers comm., 2007) for the β-tubulin gene regions of the *Cylindrocarpon* spp.

3.2.3.1. Dest1 and Dest4

The PCR method that used species specific primers (Dest1 and Dest4; Table 3.1) for *C. destructans*, which was developed by Hamelin *et al.* (1996) and modified by Nascimento *et al.* (2001), was tested for its reliability and specificity using three isolates each of *C. destructans* (D1, D2 and D3), *C. liriodendri* (L1, L2 and L3) and *C. macrodidymum* (M1, M2 and M3; Appendix 1). Only three isolates of each species were tested because this method was expected to demonstrate limited capabilities in differentiating between species; it was reported as being unable to differentiate between *C. destructans* and *C. obtusisporum*, which was often misidentified as *C. macrodidymum*. Each PCR contained a master mix of: 2.5 µL of 10 × PCR buffer (Roche diagnostics GmbH, Germany), 200 µM of each dNTP (Fermentas Life Sciences, Ontario, Canada), 0.2 µM of each primer (Invitrogen Life Technologies, CA, USA), 1.25 U of FastStart Taq DNA polymerase (Roche diagnostics) and 1 µL of 10 ng /µL DNA, made up to a final volume of 25 µL with SNW. A negative control was used to confirm the absence of contamination by adding 1 µL of SNW to the PCR reaction mix instead of an aliquot of DNA. The thermal cycling conditions were as follows: 94°C for 2 min, 30 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C and a final extension at 72°C for 7 min. The amplification was performed in an Eppendorf® (Hamburg, Germany) Mastercycler® Gradient PCR machine. In addition, the DNA was separately amplified using universal primers ITS4 ($^{5'}$TCCTCCGCTTATTGATATGC$^{3'}$) and ITS1F ($^{5'}$CTTGGTCATTTAGAGGAAGTAA$^{3'}$) as a positive control to demonstrate that the extracted fungal DNA was able to be amplified by PCR. The amplification conditions for the primers ITS4 and ITS1F were similar to those used for Dest1/Dest4 except that an annealing temperature of 50°C was used instead of 60°C. The PCR products were briefly vortexed and 10 µL mixed with 3 µL of loading dye was loaded on a 1.5% agarose gel. The products were separated by electrophoresis in a TAE buffer at 10 V /cm for 45 min as described in Section 3.2.2. The gel was stained in ethidium bromide and visualised under UV light as in Section 3.2.2.

3.2.3.2. Using primers from the beta tubulin sequence

Primer combinations

Specific primer sequences for *C. macrodidymum* (Cyma F1 and Cyma R1) and *C. liriodendri* (Cyli F1 and Cyli R1) were provided by Dr. Lizel Mostert, Stellenbosch University. Specific primer sequences for *C. destructans* were designed at Lincoln University by Dr. Hayley Ridgway (Figure 3.2) using 21 DNA sequences of New Zealand *C. destructans* isolates provided by the University of Stellenbosch. These primer sequences were all specific for the β-tubulin gene and their sequences are listed in Table 3.1. The melting temperature for each primer was calculated using the following equation:

Temperature (ºC) = 59.9 + 41 (%GC) – (675 / primer length) for a 0.05 M salt solution (according to the manufacturer's instructions; Invitrogen™, California, USA).

```
C. liriodendri WPA1e  TCTTCAACGATCCGACGTGCCGGGATTCGCTAACAATGCG   200
C. macrodidymum ACK1a TCTTCctCGtTgacAacacggcGGAgattCTAACAAcGCG   194
C. destructans ACK2d  TCTTCAACGATtCGACGTGCgGGGATTCGCTAACgATcCG   200
C. destructans CO1c   TCTTCAACGATCCGACGTGCgGGcATTCGCTAACgATGCG   200
C. destructans HB4d   TCTTCAtCGATtCGACGTGCgGGGATTCGCTAACgATcCG   200
C. destructans MAR11  TCTTCAACGATCCGACGTGCaGGcATTCGCTAACgATGCG   200
C. destructans MAR15a TCTTCAACGATgCGACGTGCgGGcATTCGCTAACgATGCG   200
C. destructans WPA2a  TCTTCAACGATCCGACGTGCgGGcATTCGCTAACgATGCG   200

                          Cyde F1 small = TGCRGGSATTCGCTAACG

C. liriodendri WPA1e  TACGCG...AAATCTGCTC...TGCCCCTATACATGAAGT   370
C. macrodidymum ACK1a TACGtaatcAAAcCctgctgccTGCtCtgcctCtgGAAGc   370
C. destructans ACK2d  TACGtG...AAATCcaCTCatcTGCCCaTATcCAgGAgGT   373
C. destructans CO1c   TACGtG...gAATCTaCTCatcTGCCCCTATcCAaGAgcT   373
C. destructans HB4d   TACGtG...AAAcCcaCTCatcTGCCCaTATcCAgGAgGT   373
C. destructans MAR11  TACGtG...AAATCTaCcCatcTGCCCCTATcCAaGAgcT   373
C. destructans MAR15a TACGtG...gAATCTaCTCatcTGCCCCTATcCAaGAgcT   373
C. destructans WPA2a  TACGtG...gAATCTaCTCatcTGCCCCTATcCAaGAgcT   373

                                    CydeR2 = CYTGGATAKGGGCAGATG
```

Figure 3.2. Alignment of partial β-tubulin sequences for representative *C. liriodendri, C. macrodidymum* and *C. destructans* isolates from a group of 60 isolates that were sequenced at the University of Stellenbosch. In blue: common nucleotides for *C. destructans* isolates, selected for primers Cyde F1 small and Cyde R2.

Table 3.1. Primer sequences used for this study.

Species	Primer name	Tm[1] (°C)	Sequence (5' to 3')	Source
General tubulin	Tub F	50	CCC CTG ATT CTA CCC CGC	Dr. Hayley Ridgway
	Tub R	48	GCG CGA GGG ACA TAC TTG T	Dr. Hayley Ridgway
C. macrodidymum	Cyma F1	55	CTG GGA CAT GAT GGC TAA TAT GAC TTC TTG	Dr. Lizel Mostert
	Cyma R1	48	GGT GGT GTG AGT TTC GTG C	Dr. Lizel Mostert
C. liriodendri	Cyli F1	58	CTC CTC TTC AAC GAT CCG ACG TGC C	Dr. Lizel Mostert
	Cyli R1	47	GGG GCA GAG CAG ATT TCG	Dr. Lizel Mostert
C. destructans	Cyde F1 small	46	TGC RGG SAT TCG CTA ACG	Dr. Hayley Ridgway
	Cyde R2	45	CYT GGA TAK GGG CAG ATG	Dr. Hayley Ridgway
	Dest1	55	TTG TTG CCT CGG CGG TGC CTG	Hamelin *et al.,* 1996
	Dest4	59	GGT TTA ACG GCG TGG CCG CGC TGT T	Hamelin *et al.,* 1996
universal	ITS4	45	TCC TCC GCT TAT TGA TAT GC	White *et al.,* 1990
	ITS1F	44	CTT GGT CAT TTA GAG GAA GTA A	Gardes and Bruns, 1993

[1]Tm: melting temperature.

Optimising PCR conditions

The PCR protocols supplied with the primers obtained from the Stellenbosch University for *C. macrodidymum* (Cyma F1 and Cyma R1) and *C. liriodendri* (Cyli F1 and Cyli R1) were tested and optimised for use on New Zealand isolates and laboratory conditions. Three isolates from each of the different species were tested with the different optimisation conditions. The test conditions included a gradient of annealing temperatures (56 to 66ºC) and increasing cycle numbers from 32 to 35.

Several primers were initially designed by Dr. Ridgway to be specific for *C. destructans*, each with slightly different length and base content. They were tested for their amplification efficiency and specificity as described in Appendix 4.1. The set which consistently amplified only *C. destructans* isolates was chosen and is shown in Table 3.1. As stated previously (Section 3.2.3.1) the DNA was always amplified with general tubulin primers as a positive control to demonstrate that the PCR reaction was not inhibited. The optimised PCR thermal cycle for the chosen set (Cyde F1 small and Cyde R1) was as follows: 94°C for 3 min, 35 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C and a final extension at 72°C for 7 min. The PCR master mix was identical to that in Section 3.2.3.1.

Assessing the specificity and the sensitivity of the primers

The three sets of species specific primers were assessed for their specificity by testing against 10 isolates each of *C. macrodidymum, C. liriodendri* and *C. destructans* and all of the novel species sequenced at Stellenbosch University (N1, N2, N3 and N4, Appendix 1). For all isolates, 10 ng of purified DNA (Section 3.2.2) was added to the PCR master mix (Section 3.2.3.1) and the optimised PCR described above used to amplify the DNA.

For sensitivity analysis genomic DNA from two isolates from each of *C. macrodidymum* (isolates M2 and M3*), C. liriodendri* (isolates L1 and L2) and *C. destructans* (isolates D1 and D2) was serially diluted with SNW to the following eight concentrations: 10 ng, 1 ng, 0.1 ng 10 pg, 1 pg, 0.1 pg, 50 fg and 10 fg of DNA /µL. For each PCR reaction 1 µL of the diluted DNA was added to the PCR master and amplified with the specific primers to determine the sensitivity of detection. The PCR included a negative control consisting of 1 µL SNW with 24 µL of master mix.

3.2.4. Nested PCR

To increase the sensitivity of the species specific PCR a nested PCR was designed that used the universal β-tubulin primers (Tub F and Tub R, Table 3.1) in the primary PCR. The PCR master mix was the same as in Section 3.2.3.1. The primary PCR conditions were as follows: 94°C for 3 min, 35 cycles of 30 s at 94°C, 30 s at 50°C and 30 s at 72°C and a final extension at 72°C for 7 min. The PCR products and the negative control were briefly vortexed and diluted 1:200 in SNW prior to using 1 µL of each as the templates for the secondary PCR reaction (described in Section 3.2.3.2). A second negative control consisting of the PCR master mix and 1 µL of SNW was included. The same serially diluted concentrations of genomic DNA listed in Section 3.2.3.2 were amplified and the PCR products were visualised on a 1.5% agarose gel as described in Section 3.2.2.

To avoid false positives due to contamination, a number of precautions were taken:the bench was surface sterilised with 70% ethanol; the gloves that were worn had been surface sterilised with 70% ethanol and changed frequently; filter tips were used; pipettes were autoclaved, and the master mix was prepared in a laminar flow hood with dedicated pipettes, pipette tips, tubes and racks, all of which were previously UV irradiated for 20 min. All reagents were subdivided into small single-use quantities in a laminar flow hood to prevent contamination. The negative control of the primary PCR was included as a nested PCR sample to demonstrate that contaminants had been successfully excluded from both PCRs.

3.2.5. Quantitative PCR

A quantitative PCR (qPCR) was used for detection, as an alternative to the nested PCR, and also as a means of quantifying inoculum. DNA was extracted from pure cultures of isolates L1, L2, D1, D2, M2 and M3 (Appendix 1) as described in Section 3.2.2. The DNA extraction process included an RNAse A step to remove any contaminating RNA. The extracted DNA, visualised by electrophoresis on a 1% agarose gel as described in Section 3.2.2., was quantified by spectrophotometry and was serially diluted with SNW to the following concentrations: 30 ng, 3 ng, 0.3 ng, 30 pg, 3 pg and 1 pg of DNA /µL.

Each reaction consisted of 1 × PCR buffer, 200 µM dNTP, 0.1 µM of each species specific primer (Cyde F1 small /Cyde R2 for *C. destructans*, Cyma F1 /Cyma R1 for *C. macrodidymum* and Cyli F1 /Cyli R1 for *C. liriodendri*), 1 U of Taq DNA polymerase, 0.28 µL diluted SYBR Green I (Invitrogen™, California, USA; Appendix 2), 0.4 µL Rox (Invitrogen™) and SNW to a final volume of 19 µL. Each reaction mix was placed into a single well on a 96 PCR well plate (Axygen Scientific, California, USA) on a MicroAmp® splash free 96 well base (Applied Biosystems Inc.) and 1 µL of the appropriate sample DNA or SNW (for the negative control), was added to the mix. Each PCR reaction was done in duplicate. The plate was covered with a PCR plate cover seal and was centrifuged for 3 min at 400 × g. A compression mat was placed onto the plate which was then placed into an ABI Prism 7000 Sequence Detection System (Applied Biosystems Inc.). The PCR conditions were as follows: an initial denaturation at 94°C for 1 min, 40 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s. Following amplification, the melting curves were acquired by heating the samples to 95°C for 1 min, cooling the samples for 1 min to 55°C and slowly increasing the temperature between 65 to 95°C at a rate of 0.5°C every 30 s with continuous fluorescence measurement. The cycle threshold (C_t) was set manually and was used to produce the standard curves for DNA quantification. The melting curves confirmed the presence of a single amplimer of conserved sequence and the absence of primer dimers or other non-specific amplimers.

The qPCR system was optimised by testing a range of primer concentrations (0.2, 0.1 and 0.05 µM), annealing temperatures (between 58 and 62°C) and detection temperatures (between 83 and 86°C) for all species. For qPCR reactions of each *Cylindrocarpon* species, 30 ng of DNA from each of the two other *Cylindrocarpon* species were amplified to check for potential cross-reactivity. Increasing the annealing temperature was expected to increase the specificity of the reactions and decrease non specific binding. The optimal concentration was defined as the concentration that produced no detectable primer dimers, cross reactivity of primers or inhibition of amplification.

The products from any qPCR runs which produced amplimers of unexpected size were sequenced at the Lincoln University Sequencing Facility. The sequences were compared to nucleotide sequences available in GenBank (http:/www.ncbi.nlm.nih.gov/) using the nucleotide BLAST (basic local alignment search tool; Altschul *et al*., 1990) to find the closest match for each sequence based on the maximum identity and E values returned.

3.3. Results

3.3.1. Conventional PCR

3.3.1.1. Dest1 and Dest4 primers

The positive control with fungal primer ITS1F and general primer ITS4 primer (positive control) amplified bands for all DNA samples except for isolate L2 and the negative control. The DNA from isolate L2 was re-extracted and shown to be amplified by primers ITS1F and ITS4 in subsequent PCRs. The bands produced were a strong band of approximately 550 bp, a fainter band of 650 bp and another faint band of 800 bp in some lanes (Figure 3.3 A). When the Dest1 and Dest4 primers were assessed with *C. destructans* DNA, they produced an expected 400 bp band for the three *C. destructans* isolates, however, the same band was also produced for all three *C. macrodidymum* DNA samples (Figure 3.3 B). No bands were observed for *C. liriodendri* isolates and the negative control.

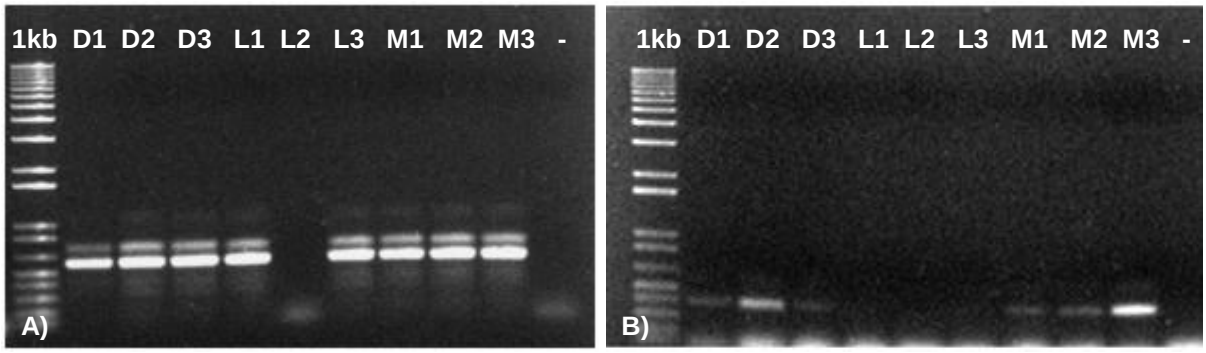

Figure 3.3. PCR products obtained with primers A) ITS1F and ITS4 and B) Dest1 and Dest4 (1 kb: 1Kb Plus DNA ladder™; - : negative control; L1-3: *C. liriodendri* isolates; M1-3: *C. macrodidymum* isolates; D1-3: *C. destructans* isolates).

3.3.1.2. Species specific primers for the beta tubulin region

For the *C. liriodendri* and *C. macrodidymum* species specific primers supplied by Dr. Lizel Mostert (Stellenbosch University), the originally supplied touchdown PCR protocol which specified an annealing temperature of 66°C for 5 cycles, 62°C for 5 cycles and 60°C for 20 cycles did not produce any products (results not shown) despite numerous attempts. Following an optimisation process, the expected 200 bp for *C. liriodendri* and 300 bp band for

C. macrodidymum were produced for three isolates of each species when using an annealing temperature of 58°C for 30 cycles (Figure 3.4 A and B). For any species specific primers, no bands were observed for the two other species or the negative control.

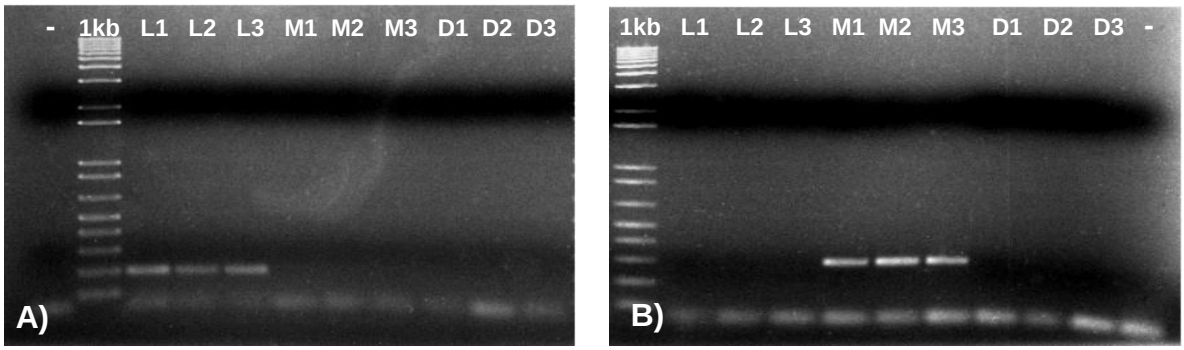

Figure 3.4. PCR products with species specific primers at an annealing temperature of 58°C for 30 cycles (A) primers Cyli F1 and Cyli R1, species specific for *C. liriodendri* (B) Cyma F1 and Cyma R1, species specific for *C. macrodidymum* (1 kb: 1Kb Plus DNA ladder™; - : negative control; L1-3: *C. liriodendri* isolates; M1-3: *C. macrodidymum* isolates; D1-3: *C. destructans* isolates).

The general β-tubulin primers Tub F and Tub R (used as positive controls) amplified a PCR product of 400 bp for the three *C. destructans* isolates tested (Figure 3.5) indicating that the DNA used with the *C. liriodendri* and *C. macrodydimum* specific primers was of amplifiable quality. The primer pair Cyde F1 small /Cyde R2 amplified a 200 bp band from all *C. destructans* isolates at an annealing temperature of 58°C (Figure 3.5). The negative control did not show any bands.

Figure 3.5. PCR products obtained with primer pair Cyde F1 small /Cyde R2 and general tubulin of ten *C. destructans* isolates (1 kb: 1 kb DNA plus ladder; - : negative control; D: *C. destructans*).

A common annealing temperature of 58°C was thus applied in the following experiments for amplification of the three *Cylindrocarpon* species. Amplification of all species could be done using the same reagents concentrations and thermal cycler programme.

Specificity of the primers

Each primer pair was tested for its specificity to the respective *Cylindrocarpon* species for which it had been designed, using the 10 isolates previously selected for each. For the *C. liriodendri* species specific primers, a 200 bp band was observed for the 10 *C. liriodendri* isolates (Figure 3.5 A) and no products were observed for isolates from the other *Cylindrocarpon* species, including novel species (Figure 3.6 B - D).

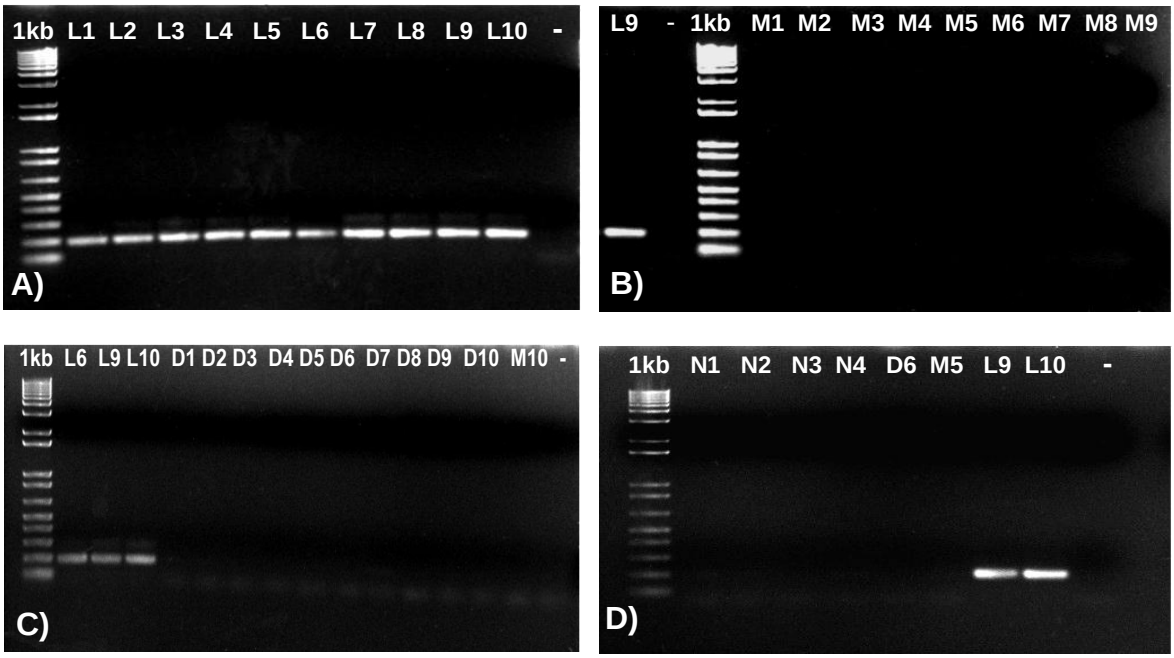

Figure 3.6. PCR products obtained using primers Cyli F1 and Cyli R1 with isolates of *C. liriodendri* isolates (A), *C. macrodidymum* (B), *Cylindrocarpon* novel species (C) and *C. destructans* (D) (1 kb: 1 Kb Plus DNA ladder™; - : negative control; L1-10: *C. liriodendri* isolates; M1-10: *C. macrodidymum* isolates; D1-10: *C. destructans* isolates; N1-4: *Cylindrocarpon* novel species).

The species specific primers for *C. macrodidymum* amplified the predicted band of 300 bp for ten randomly selected isolates of *C. macrodidymum* (Figure 3.7 A) and no bands were observed for isolates from the other *Cylindrocarpon* species (Figure 3.7 B - D).

Ten randomly selected *C. destructans* isolates were all amplified by the specific primer pair and a 200 bp band was observed on the gel (Figure 3.8 A). DNA from *C. macrodidymum*, *C. liriodendri* and the *Cylindrocarpon* novel species was not amplified with the *C. destructans* species specific primers (Figure 3.8 B - D).

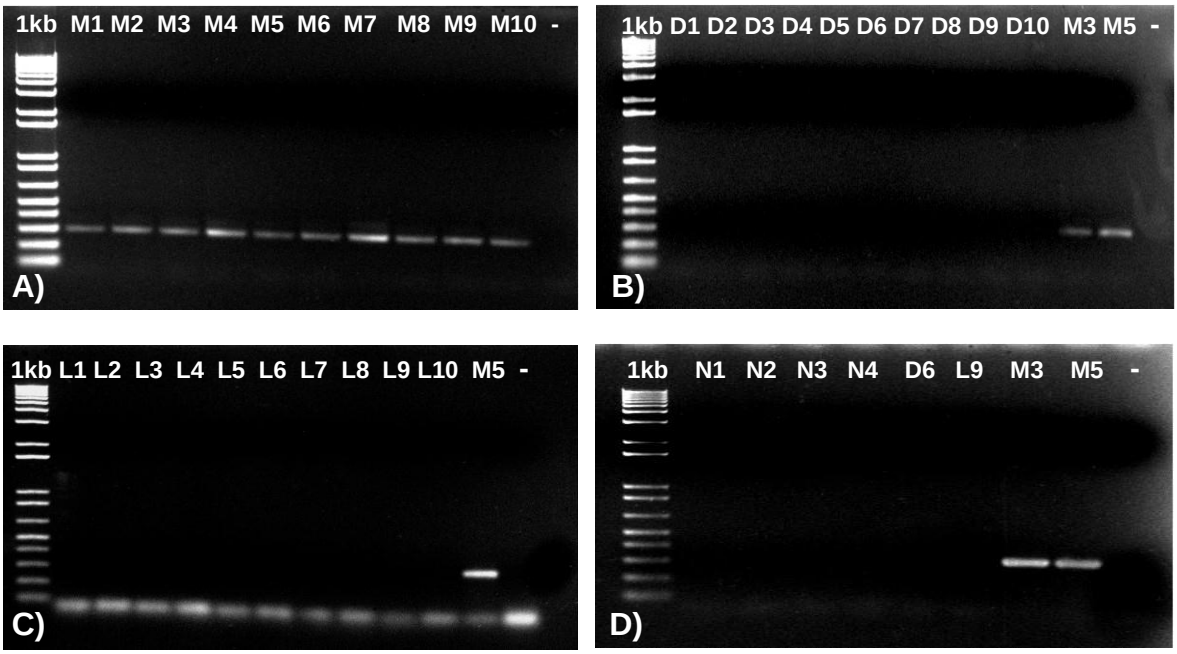

Figure 3.7. PCR products obtained using primers Cyma F1 and Cyma R1 for isolates of *C. macrodidymum* isolates (A), *C. destructans* (B), *C. liriodendri* (C) and *Cylindrocarpon* novel species (D) (1 kb: 1 Kb Plus DNA ladder™; - : negative control; L1-10: *C. liriodendri* isolates; M1-10: *C. macrodidymum* isolates; D1-10: *C. destructans* isolates; N1-4: *Cylindrocarpon* novel species).

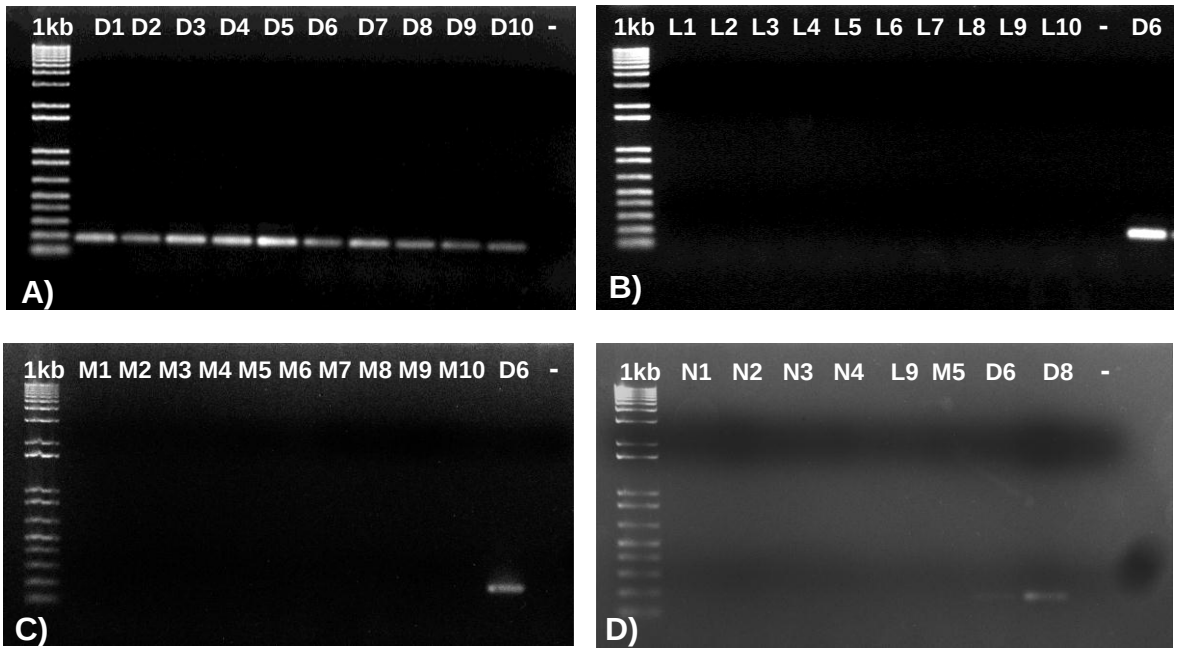

Figure 3.8. PCR products obtained using primers Cyde F1 small and Cyde R2 for isolates of *C. destructans* isolates (A), *C. liriodendri* (B), *C. macrodidymum* (C) and *Cylindrocarpon* novel species (D) (1 kb: 1 Kb Plus DNA ladder™; - : negative control; L1-10: *C. liriodendri* isolates; M1-10: *C. macrodidymum* isolates; D1-10: *C. destructans* isolates; N1-4: *Cylindrocarpon* novel species).

Sensitivity

When the three primer sets were tested for their sensitivity of detection on standard PCR (Figure 3.9), a band was observed for 10, 1 and 0.1 ng of *C. liriodendri* genomic DNA, and for *C. destructans* and *C. macrodidymum*, bands were observed for 10 and 1 ng of DNA.

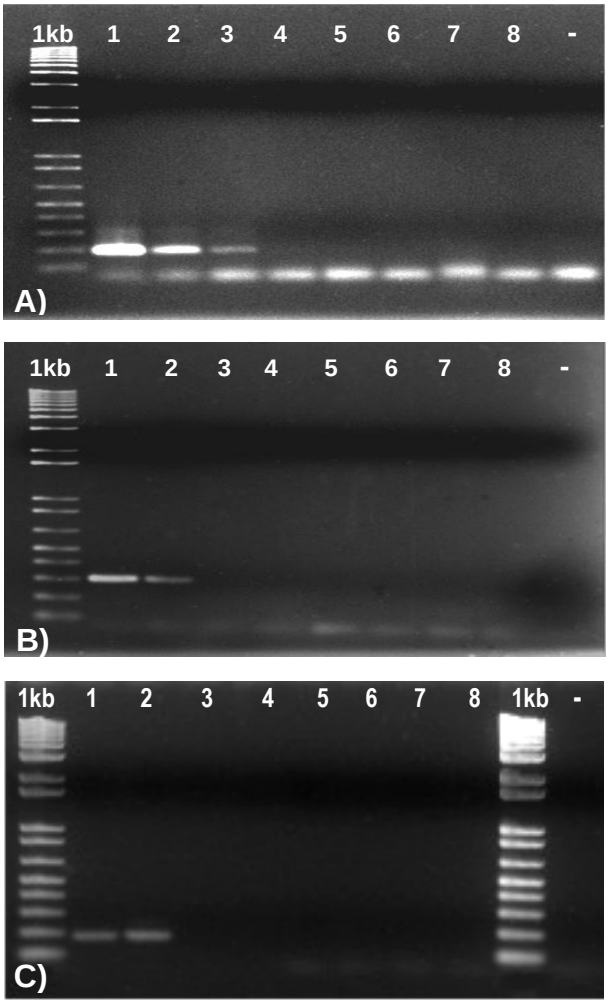

Figure 3.9. PCR products obtained with primers Cyli F1 /Cyli R1, species specific for *C. liriodendri* (A; isolate L1), Cyma F1/Cyma R1, specific for *C. macrodidymum* (B; isolate M3) and Cyde F1 small /Cyde R2, specific for *C. destructans* (C; isolate D2) with eight concentrations of genomic DNA (10 ng, 1 ng, 0.1 ng, 10 pg, 1 pg, 0.1 pg, 50 fg and 10 fg); 1 kb: 1 Kb Plus DNA ladder™; - : negative control.

3.3.2. Nested PCR

The primary PCR amplification using the general β-tubulin primers (Tub F and Tub R) amplified an expected product of approximately 400 bp (Figure 3.10 A). The band was observed at a lowest concentration of 10 pg of template DNA for all the three species after the primary PCR (data only shown for *C. destructans*). The sensitivity was increased to 1 pg

after the second PCR amplification using the species specific primers for all three *Cylindrocarpon* species (Figure 3.10 B, C and D).

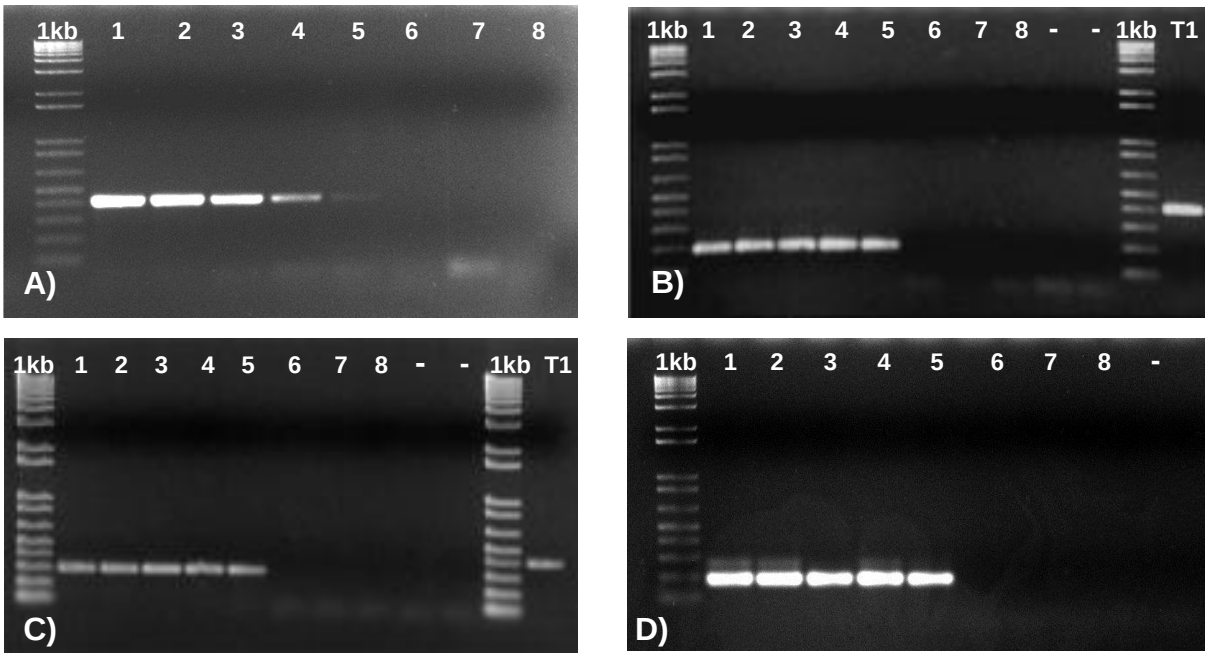

Figure 3.10. PCR products obtained after a nested PCR with primer pairs Tub F /Tub R in a primary PCR (A) and Cyli F1 /Cyli R1, species specific for *C. liriodendri* (B), Cyma F1 /Cyma R1, species specific for *C. macrodidymum* (C) and Cyde F1 small /Cyde R2 species specific for *C. destructans* (D) in a secondary PCR of 8 concentrations of genomic DNA (1 kb: 1 Kb Plus DNA ladder™; - : negative controls; 1 to 8: DNA quantities of 10 ng, 1 ng, 0.1 ng, 10 pg, 1 pg, 0.1 pg, 50 fg and 10 fg). A: isolate L1; B: isolate M3; C and D: isolate D2.

3.3.3. Quantitative PCR

Quantitative PCR of DNA extracted from a pure culture of isolate L1 using species specific primers Cyli F1 and Cyli R1 produced an amplicon using the standard PCR thermal cycle described in Section 3.2.3.2 with template concentrations of 30 ng, 3 ng, 0.3 ng, 30 pg and 3 pg (Figure 3.11 A), which represented a five fold detection range. Gel electrophoresis of the amplicons from DNA concentrations between 30 ng and 3 pg showed that they were of the predicted size (200 bp) but no amplicons were visible on agarose gels for lower DNA concentrations. All threshold cycle values (Ct) above 39 were discarded for all isolates tested, as the ΔRn, which represents the difference between the value of the reaction with the template and the value of the reaction without template, increased abnormally between cycles 39 and 40.

The standard curve showed a linear correlation between the logarithm of the concentration and the threshold cycle (C_t) values with a correlation coefficient (r^2) of 0.996 (Figure 3.11 B). The regression equation of the standard curve was: C_t= -3.65 (log [DNA]) + 28.92.

The dissociative curves showed that all the amplicons had a melting temperature of 87.6°C (Figure 3.11 C). A peak was also visible at a melting temperature of 82.2°C for 3 pg of DNA and NTC (no template control), however this melting temperature was below the detection temperature range of 83 – 86°C.

Quantitative PCR of DNA extracted from pure culture of *C. destructans* isolate D1 using species specific primers Cyde F1 small and Cyde R2 produced an amplicon at an annealing temperature of 58°C for all concentrations tested. The amplicon was of the predicted size (200 bp) after visualisation on a gel electrophoresis for concentrations 30 ng, 3 ng, 0.3 ng, 30 pg and 3 pg.

The standard curve showed a linear correlation between the logarithm of the concentration and the threshold cycle (C_t) values with r^2 of 0.995 and the regression was: C_t= -3.69 (log [DNA]) + 29.25 (Figure 3.12 A). The dissociative curves showed that the amplicons had a melting temperature of 87.6°C (Figure 3.12 B).

Quantitative PCR of DNA extracted from a pure culture of *C. macrodidymum* isolate M3 using species specific primers Cyma F1 and Cyma R1 produced an amplicon which was of the predicted size (300 bp) at an annealing temperature of 58°C for concentrations 30 ng, 3 ng, 0.3 ng, 30 pg and 3 pg tested.

The standard curve showed a linear correlation between the logarithm of the concentration and the threshold cycle (C_t) values with r^2 of 0.988 (Figure 3.13 A). The regression equation of the standard curve was: C_t= -3.66 (log [DNA]) + 30.14. The dissociative curves showed that the amplicons had a melting temperature of 87.6°C (Figure 3.13 B).

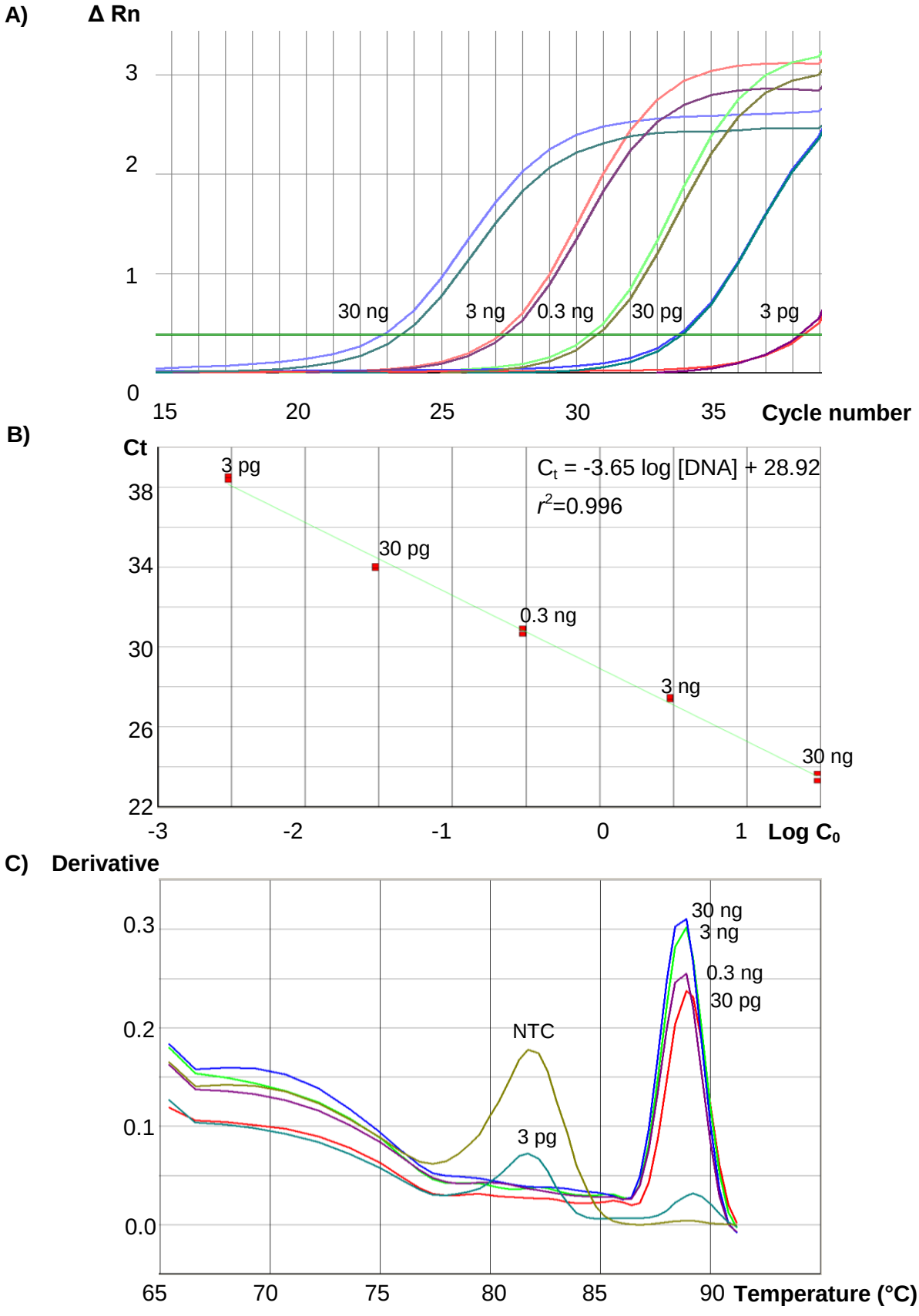

Figure 3.11. Quantitative PCR using 30 ng, 3 ng, 0.3 ng, 30 pg and 3 pg of DNA from isolate L1 and primer pair Cyli F1 /Cyli R1, specific for *C. liriodendri*. A: amplification plot; B: standard curve; C: dissociation curve with NTC (no template control).

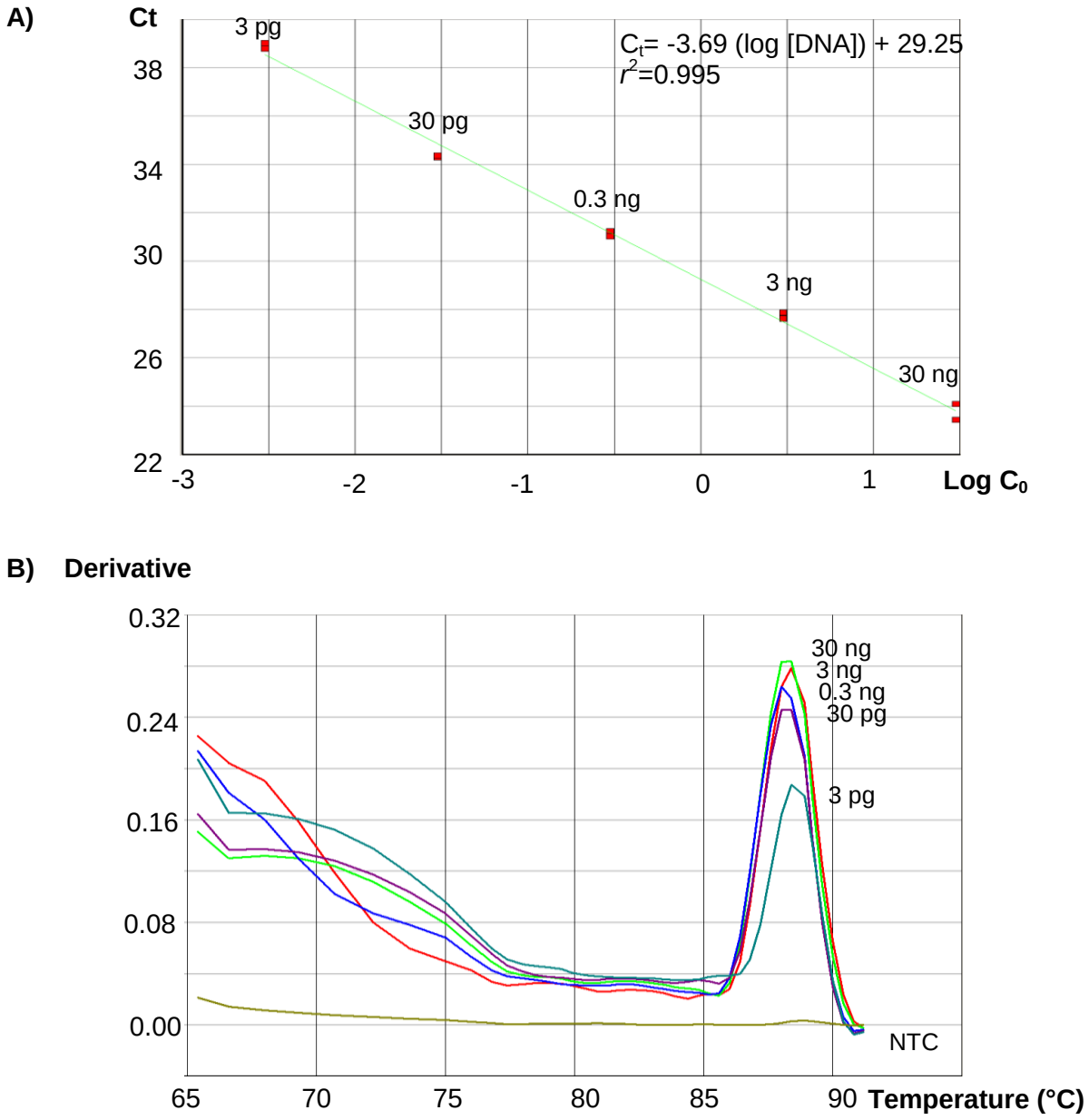

Figure 3.12. Quantitative PCR using 30 ng, 3 ng, 0.3 ng, 30 pg and 3 pg of DNA from isolate D1 and primer pair Cyde F1 small /Cyde R2, specific for *C. destructans*. A: standard curve; B: dissociation curve with NTC: no template control.

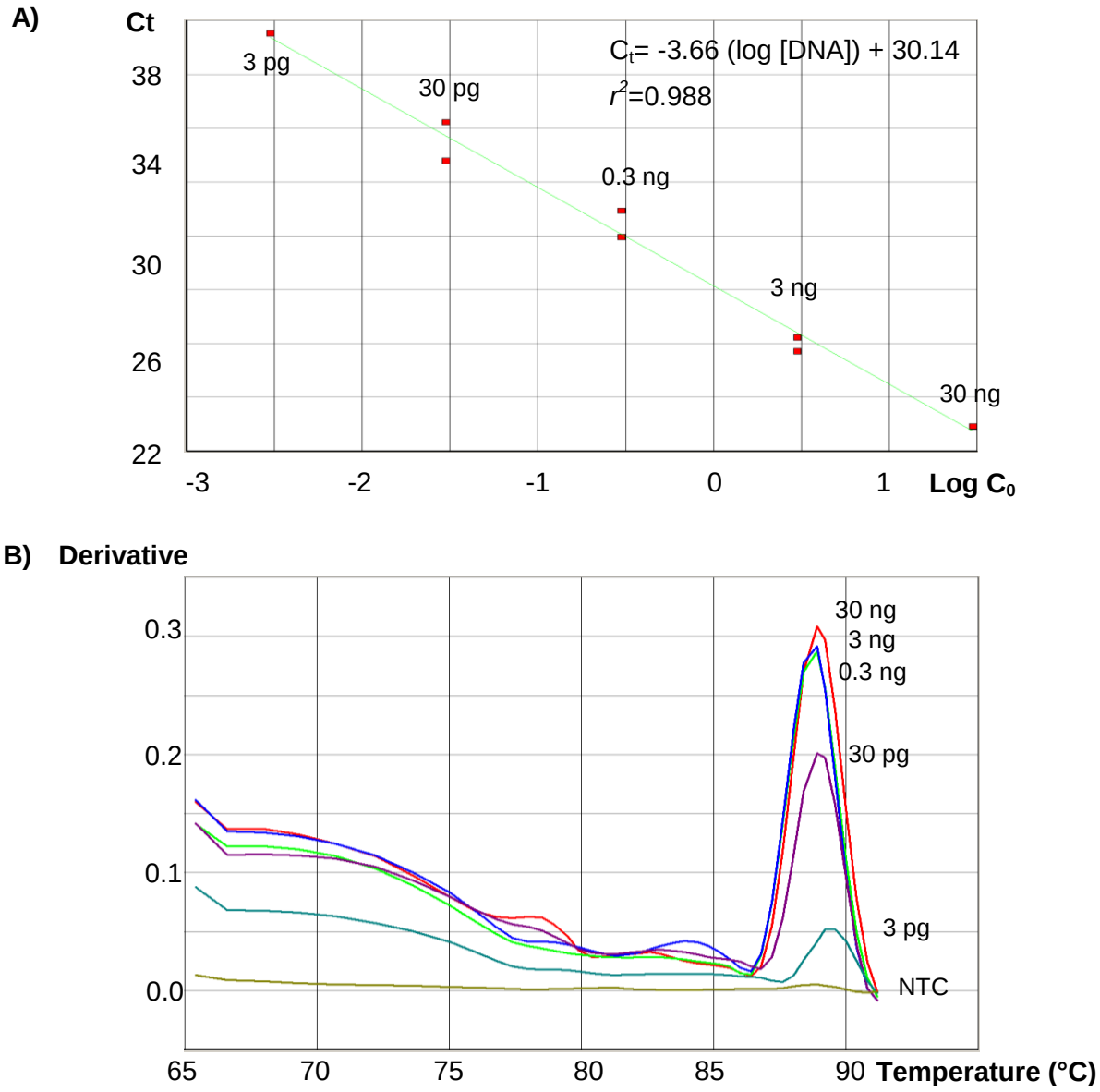

Figure 3.13. Quantitative PCR using 30 ng, 3 ng, 0.3 ng, 30 pg and 3 pg of DNA from isolate M3 and primer pair Cyma F1 /Cyma R1, specific for *C. macrodidymum*. A: standard curve; B: dissociation curve with NTC: no template control.

The results from the cross reactivity test of each species specific primer pair at an annealing temperature of 58°C showed that for each primer pair, a signal was obtained for the two other species. For primer pair Cyli F1 /Cyli R1, C_t values of 23.18, 35.07 and 34.30 were obtained for 30 ng of *C. liriodendri*, *C. destructans* and *C. macrodidymum* DNA, respectively (Table 3.2). For primer pair Cyde F1 small /Cyde R2, C_t values of 22.86 and 33.87 were obtained for 30 ng of *C. destructans* and *C. liriodendri* DNA, respectively, whereas for 30 ng of *C. macrodidymum* DNA, the C_t value was beyond the detection range. For primer pair Cyma F1 /Cyma R1, C_t values of 24.17, 35.83 and 35.05 were obtained for 30 ng of *C. macrodidymum*, *C. liriodendri* and *C. destructans* DNA, respectively.

Dissociation curves could not be obtained for annealing temperatures exceeding 60°C with the ABI Prism 7000 Sequence detection system used for these experiments. For each *Cylindrocarpon* spp., the qPCR detected products for the two other species at annealing temperatures of 58, 60 and 62 (Table 3.2). The qPCR detected a mean C_t value between 35.07 and 37.68 for *C. destructans* and between 34.14 and 35.79 for *C. macrodidymum* with primers Cyli F1 and Cyli R1. For Cyde F1 small and Cyde R2, the mean C_t detected was between 33.32 and 33.64 for *C. liriodendri* and for *C. macrodidymum* a Ct of 36.44 was detected with an annealing temperature of 62°C. Using primers Cyma F1/Cyma R1, the mean C_t detected within the temperature range using *C. destructans* DNA was between 35.55 and 37.88 and with *C. liriodendri* DNA was between 35.08 and 35.19. From these results, it is clear that C_t values above 35 for *C. macrodidymum*, 34 for *C. liriodendri* and 33 for *C. destructans* provide non-specific results as high concentrations of the other species may be detected above these values. On a 1.5% agarose gel, for primer pairs Cyma F1 /Cyma R1, a band was visualised at approximately 600 bp for 30 ng of *C. destructans* DNA. The gel, DNA sequences and DNA sequence alignment matches are shown in Appendices A4.2.1 to A4.2.3. No sequence similarity was found to any sequences on GenBank using BLAST. For *C. destructans* and *C. macrodidymum*, an annealing temperature of 58°C was used for the remaining qPCR optimisation while 60°C was used for the qPCR optimisation of *C. liriodendri*.

The dissociation curves (Appendix A4.2.4) of the three different primer concentrations tested (0.2, 0.1 and 0.05 µM) at an annealing temperature of 58°C for the primer pair specific to *C. macrodidymum* showed decreasing of background fluorescence in the samples with decreasing quantity of primers. As the results from the different primer concentrations were expected to be similar for the two other species to those obtained using species specific primers for *C. macrodidymum*, the different concentrations were only tested for the latter.

Table 3.2. Mean C_t values of the amplicons from the different *Cylindrocarpon* DNA quantities using the species specific primers and annealing temperatures of 58, 60 and 62°C.

species specific primers	DNA quantity (ng)	58°C	60°C	62°C
Cyli F1 /Cyli R1	30 (*C. liriodendri*)	23.18	21.58	23.11
	3 (*C. liriodendri*)	27.26	26.39	27.14
	0.3 (*C. liriodendri*)	30.64	29.87	30.72
	0.03 (*C. liriodendri*)	33.86	34.47	33.59
	NTC	35.10	37.87	36.78
	30 (*C. destructans*)	35.07	36.39	37.68
	30 (*C. macrodidymum*)	34.30	34.81	34.14
Cyde F1 small /Cyde R2	30 (*C. destructans*)	22.86	23.42	23.82
	3 (*C. destructans*)	27.29	27.57	27.91
	0.3 (*C. destructans*)	31.74	31.66	31.59
	0.03 (*C. destructans*)	35.18	35.34	35.49
	NTC	-	-	-
	30 (*C. liriodendri*)	33.87	33.60	33.32
	30 (*C. macrodidymum*)	-	-	36.44
Cyma F1 /Cyma R1	30 (*C. macrodidymum*)	24.17	23.81	23.13
	3 (*C. macrodidymum*)	27.21	26.84	25.82
	0.3 (*C. macrodidymum*)	31.77	30.97	29.69
	0.03 (*C. macrodidymum*)	34.76	35.77	34.07
	NTC	-	-	-
	30 (*C. destructans*)	35.83	35.55	35.69
	30 (*C. liriodendri*)	35.08	35.19	35.13

No qPCR products were detected for a primer concentration of 0.05 µM with primers Cyma F1 and Cyma R1 (Table 3.3). For the standards, the average C_t increased with decreasing primer concentration. To maintain the sensitivity of the reaction, a primer concentration of 0.1 µM was used for all qPCR reactions.

Table 3.3. Mean C_t of the amplicons from the different *Cylindrocarpon* DNA quantities using 0.25, 0.1 and 0.05 µM of the species specific primers Cyma F1 and Cyma R1.

DNA quantity (ng)	Species specific primer concentrations		
	0.2 µM	0.1 µM	0.05 µM
30 (*C. macrodidymum*)	23.83	24.39	26.39
3 (*C. macrodidymum*)	26.66	27.28	30.95
0.3 (*C. macrodidymum*)	30.98	31.37	33.98
0.03 (*C. macrodidymum*)	34.69	35.12	37.94
NTC	33.78	37.98	-
30 (*C. destructans*)	33.95	36.16	-
30 (*C. liriodendri*)	36.07	37.74	-

Since an alternative to primer titration is to quantify the fluorescence above the primer dimer denaturation temperature, the detection temperatures were increased from 72°C to 85°C for *C. macrodidymum*, 86°C for *C. liriodendri* and 84°C for *C. destructans* for 1 min. Cross reactivity was eliminated from the qPCR reactions using a detection temperature of 85°C for *C. macrodidymum*, 86°C for *C. liriodendri* and 84°C for *C. destructans*. The sequencing of the different PCR products showed that the primers were specific to the different species.

The final reaction cycles were as follows:

- for *C. macrodidymum*: 94°C for 1 min, 40 cycles of 94°C for 30 s, 58°C for 30s, 72°C for 30s and 85°C for 1 min, with an effective quantification limit of 0.3 pg of DNA.
  The equation of the standard curve was: C_t= -3.59 (log [DNA]) + 26.1, with r^2=0.996.

- for *C. liriodendri*: 94°C for 1 min, 40 cycles of 94°C for 30 s, 60°C for 30s, 72°C for 30s and 86°C for 1 min, with an effective quantification limit of 0.7 pg of DNA.
  The equation of the standard curve was: C_t= -3.50 (log [DNA]) + 27.93, with r^2=0.994.

- for *C. destructans*: 94°C for 1 min, 40 cycles of 94°C for 30 s, 58°C for 30s, 72°C for 30s and 84°C for 1 min, with an effective quantification limit of 9.5 pg of DNA.
  The equation of the standard curve was: C_t= -3.82 (log [DNA]) + 31.27, with r^2=0.995.

However, the similarity of melting temperatures shows that further work with different real-time PCR machines should be done to confirm the melting temperatures of the amplicons for the different species.

3.4. Discussion

The published Dest1 and Dest4 primers of Hamelin *et al.* (1996) were demonstrated to be not specific for *C. destructans* as they produced the expected 400 bp band for *C. destructans* isolates and for *C. macrodidymum* isolates. These primers were also shown by Rego *et al.* (2001a) to amplify a 400 bp band with *C. obtusisporum* isolates in a nested PCR developed for the detection of *Cylindrocarpon* species in plant tissue. As *C. obtusisporum* had not been reported from grapevines in New Zealand, the primers were considered to have potential for the detection of *C. destructans*. There was also doubt over the identity of the *C. obtusisporum* isolates used by Rego *et al.* (2001a) as they had originated from California where isolates originally identified as *C. obtusisporum* were later confirmed as *C. macrodidymum* after DNA sequencing of taxonomically informative genes (Halleen *et al.*, 2004; Petit and Gubler, 2005). The Portuguese *Cylindrocarpon* isolates used by Rego *et al.* (2001a) were also re-identified by Alaniz *et al.* (2007; 2009) as *C. macrodidymum* and *C. liriodendri*. *In silico* analysis of the DNA showed that Dest1 and Dest4 primers were both 100% homologous to *C. macrodidymum,* that Dest1 had only 2 bp mismatches with *C. obtusisporum* and *C. liriodendri* and that Dest4 was 100% homologous to *C. liriodendri* and had 3 mismatches with *C. obtusisporum*. The two base mismatch at the 3' end of the Dest1 sequence was sufficient to prevent the primers from amplifying *C. liriodendri* and this was also demonstrated by Dubrovsky and Fabritius (2007). Similarly, Foster *et al.* (2004) observed that species specific primers *for Stenotrophomonas maltophilia* did not amplify *Pseudomonas aeruginosa* which showed a two base mismatch at the 3' end of the forward primer while *Xanthomonas* spp. and *Xyllela fastidiosa* which exhibited a single mismatch on the last base at the 3' end of the same primer was amplified. The lack of specificity by primers Dest1 and Dest4 shown in this work and reported by others led to their exclusion as candidates for species specific PCR.

The primers specific for *C. macrodidymum*, *C. liriodendri* and *C. destructans* amplified a 300, 200 and 200 bp band, respectively, only with their designated *Cylindrocarpon* species, in conventional and nested PCR reactions. For these three *Cylindrocarpon* spp., the species specific primers were designed from the β-tubulin 2 gene, which is in contrast to the commonly targeted ribosomal gene region. The ITS regions of the rRNA genes are frequently exploited for PCR detection as DNA sequence variability is usually sufficient inter-specifically (Alaei *et al.*, 2009). The ITS sequences are present in multiple copies (ranging from 50-180 per genome, Ganley and Kobayashi, 2007), which increases the sensitivity of the PCR detection, and are accessible in databases in high numbers, enabling comparison between organisms (Schena *et al.*, 2004; Alaei *et al.*, 2009). However, for some fungi the ITS sequences do not show enough sequence differences for robust species specific primers to be designed. In these instances the β-tubulin gene, another taxonomically useful gene, is

often used. However, it is usually only present as a single or low number of copies within the fungal genome (Ayliffe *et al.*, 2001) and thus is usually less sensitive than PCR of the ITS regions. Within a species, isolates from *C. macrodidymum* and *C. liriodendri* have few differences in their ITS region and partial β-tubulin 2 gene nucleotide sequences. Information from GenBank showed 0 and 1% variation, respectively for *C. macrodidymum,* and with 0.4 and 0.8% variation, respectively for *C. liriodendri*. In contrast, for *C. destructans* isolates, information from GenBank indicated that there was 11 and 18% variability for the ITS region and the partial β-tubulin 2 gene, respectively (the DNA sequences were retrieved from GenBank and aligned using Molecular Evolutionary Genetics Analysis (MEGA version 4; Tamura *et al*., 2007).

The similarities within a species that were observed in nucleotide sequences within the ITS region and partial β-tubulin 2 gene for *C. macrodidymum* and *C. liriodendri* isolates may have led to the success of the primers provided by Dr. Lisel Mostert who designed them for South African isolates. However, the highly divergent nucleotide sequences for the ITS region and the β-tubulin gene among *C. destructans* isolates were previously observed by Seifert *et al.* (2003) who hypothesised the existence of different phylogenetic species among isolates grouped as "*C. destructans*" and its teleomorph "*Neonectria radicicola*". They suggested that the varieties, *Neonectria radicicola* var. *coprosmae* and *N. radicicola* var. *macroconidialis,* should be raised to individual species. *Cylindrocarpon destructans* var. *destructans* and *C. destructans* var. *crassum* are included in the *N. radicicola* complex but in this study, *C. destructans* was not identified at a variety level.

Between species there were consistently greater differences in the β-tubulin 2 gene than in the ITS sequences of the rRNA genes. Information from GenBank showed between *C. macrodidymum* and *C. liriodendri* 9.6 and 15.2% variation for the ITS region and the β-tubulin 2 gene, respectively. Between *C. macrodidymum* and *C. destructans*, information from Genbank showed 0.5 – 8.9% and 12.5 – 14.5% variation for the ITS region and the β-tubulin 2 gene, respectively. Between *C. liriodendri* and *C. destructans*, information from Genbank showed 3.0 – 9.3% and 4.3 – 13.7% variation for the ITS region and the β-tubulin 2 gene, respectively. As nucleotide sequence variability in the ITS region of the rDNA is low between species, the use of species specific primers designed to amplify a portion of the β-tubulin 2 gene seemed more suitable to differentiate the species. Groenewald *et al.* (2001) used the β-tubulin sequences to distinguish the genus *Phaeomoniella* from *Phaeoacremonium* (*Pm*) and to separate *Pm. aleophilum* from *Pm. angustius*. The latter showed sequence variation of 15.5% from each other with partial β-tubulin gene sequences and only 4% variation in their ITS sequences. In another study, a species specific PCR amplification of the tubulin gene was necessary to differentiate *Pm viticola* from *Pm angustius* as the ITS sequences had

100% identity (Aroca and Raposo, 2007). Bilodeau *et al.* (2007) also observed 1.4 and 2.2% of sequence divergence between the two most closely related species *Phytophthora ramorum* and *Phytophthora lateralis* for the ITS region and the β-tubulin gene, respectively. Camele *et al.* (2009) designed species specific primers for *Cylindrocladium pauciramosum* using the β-tubulin gene sequence after preliminary attempts to develop PCR primers using ITS sequences were unsuccessful for this species. Another gene region, the elongation factor 1 α has been increasingly used in species identification (Amatulli *et al*., 2010; Hasegawa *et al*., 2010; Wright and Harmon, 2010) and would probably constitute a more variable gene region for *Cylindrocarpon* spp. identification than the ITS region, however, sequences are not available for an extensive range of species in Genbank in contrast to the ITS and β-tubulin gene sequences.

Following the work presented in this chapter, Alaniz *et al.* (2009) designed species specific primers from the ITS region for *C. liriodendri*, *C. macrodidymum* and *C. pauciseptatum* and could detect as little as 100 pg and 100 fg of DNA depending on the species. They used nested PCR in a multiplex system to improve sensitivity in plant material artificially inoculated with the pathogen. However, sequence alignments on GenBank of the species specific primers for *C. macrodidymum* (Mac1 and MaPa2) and ITS sequences of some *C. destructans* isolates show 100% identity (A4.3.1). The primers should therefore be tested against *C. destructans* isolates in New Zealand to determine whether they are specific. In this study, primers Cyma F1 and Cyma R1 showed seven and five mismatches, respectively for *C. destructans* and *C. liriodendri.* Primers Cyli F1 and Cyli R1 showed two and five mismatches, respectively for *C. destructans* and eleven and eight mismatches, respectively for *C. macrodidymum*. Primers Cyde F1 small and Cyde R2 showed five and eleven mismatches, respectively for *C. macrodidymum* and two and five mismatches, respectively for *C. liriodendri*. These mismatches included at least one base mismatch at the 3' end of each of the primers and were sufficient for their specificity. Sarkar *et al.* (1990) observed that the PCR was inhibited when the last or the penultimate base of the specific primer mismatched the DNA template while the third or forth base from the 3' end of the same primer did not affect amplification. Kwok *et al.* (1990) showed that the PCR efficiency was reduced with a mismatch on the last base of the 3' end of a primer although it depended on the type of mismatch and that PCR efficiency decreased drastically with multiple mismatches at the 3' end. They also observed that the inhibition of a PCR caused by mismatches could be lifted with a lower annealing temperature. In this study, an annealing temperature of 58°C was necessary to prevent non-specific amplification.

Another alternative to the ITS region and β-tubulin gene would be the intergenic spacer (IGS) region of the rRNA which has been reported to evolve faster than the ITS region and is present in multiple copies in the genome (Pramateftaki *et al.*, 2000; Pantou *et al.*, 2003). Chilvers *et al.* (2007) could detect as little as 10 fg of genomic DNA using specific primers targeting the IGS region of *Botrytis aclada*, *B. allii* and *B. byssoidea* with qPCR while Suarez *et al.* (2005) could detect as little as 20 fg of DNA with species specific primers for *B. cinerea*. The latter reported that IGS primers were 1000 times more sensitive than β-tubulin primers however, only limited numbers of IGS sequences are available in GenBank and this is a significant impediment to the development and testing of species specific PCR. Investment in a sequencing regime for multiple isolates from different countries would be required to design species specific primers targeting the IGS region of different *Cylindrocarpon* spp. To develop robust primers this process would have to include a broad range of *Cylindrocarpon* species in addition to those that infect grapevines. Additionally, the IGS region comprises several kilobases (2523 bp reported for *Metarhizium anisopliae* var. *anisopliae* (Pantou *et al*., 2003)) which increases the difficulty of amplifying the sequences and the costs of such a project.

The detection sensitivity of the species specific primers targeted to the nuclear rRNA gene regions of fungi with conventional PCR varies between 100 pg and 0.1 pg for different species (Ippolito *et al.*, 2002; Zeng *et al.*, 2005; Retief *et al.*, 2006; Wang *et al.*, 2007; Alaniz *et al.*, 2009 and Shishido *et al*., 2010). However, the detection sensitivity of the primers that targeted the β-tubulin gene in the standard PCR in this study was between 1 and 0.1 ng, which probably reflects the approximately 100 fold decrease in target copy numbers within the genome. This result is in agreement with that of Yan *et al.* (2008) who observed that specific primers for *Cladosporium fulvum* that targeted the ITS region were 100 fold more sensitive than those targeting the β-tubulin gene. According to Mathur and Utkhede (2004), single copy genes can better clarify species identification but are less sensitive because of their low copy number. When identifying fungi from pure cultures, single or low copy genes can easily detect the species. However, when dealing with propagules in the environment, numbers of propagules are usually low and DNA extraction methods and subsequent PCR can be inhibited by sample impurities. Therefore, the sensitivity of the method needs to be high to detect the presence of the pathogen at low levels and would be of key importance if the assay was to be used to determine the relative success of a sanitation process. This is particularly important for *Cylindrocarpon* spp. which have been found infesting nursery and vineyard soils (Halleen *et al.*, 2003; Gubler *et al.*, 2004; Oliveira *et al.*, 2004).

In this study, the sensitivity of the *Cylindrocarpon* species specific PCR was improved by 100 to 1000 fold with the nested PCR, with as little as 1 pg of genomic DNA visualised on an agarose gel. Nested PCR is often used to increase the sensitivity of standard PCR (Zeng *et*

al., 2005). Alaei *et al.* (2009) obtained a detection limit 100 to 1000 times more sensitive for nested PCR than standard PCR for *Puccinia horiana* using primers targeting the ITS region. Ippolito *et al.* (2002) improved their detection limit 1000 fold from 1 pg to 1 fg of genomic DNA with a nested PCR using *Phytophthora nicotianae* and *Phytophthora citrophthora* specific primers targeted to the ITS sequences. Miyazaki *et al.* (2009) had a detection limit of 50 fg for *Trichoderma harzianum* while Zeng *et al.* (2010) could detect as little as 0.1 fg for *Blumeria graminis* f. sp. *tritici*. However, in this study the nested PCR designed for the different *Cylindrocarpon* species used general tubulin primers in the primary PCR followed by the species specific β-tubulin primers in the secondary PCR to increase the sensitivity. Thus, although sensitivity was increased by a similar proportion to the above studies the ultimate detection level was still substantially less than would be expected for primers designed to target the multicopy rRNA gene region.

Pasquali *et al*. (2006) determined that one nucleus of *Fusarium* spp. weighed approximately 0.0377 pg, So if one hypothesised that *Cylindrocarpon* spp. have approximately the same genome size as *Fusarium* spp, and since one *Cylindrocarpon* conidium has approximately three nuclei they would collectively weigh about 0.1 pg. which means that the detection limit of the qPCR of 3 pg, is equivalent to about 30 conidia. The method could be useful to determine the threshold numbers of *Cylindrocarpon* conidia needed for infection, monitor the concentrations of *Cylindrocarpon* spp. in the soil and determine the movement of the pathogen in plant tissue and in soil. Brake *et al.* (1995) observed that 4.5×10^4 microconidia of *Fusarium oxysporum* f. sp. *cubense* /g of dry soil were required to observed disease symptoms on Cavendish banana plants and Elmer and Lacy (1987) determined that an inoculum density of 42 propagules of *Fusarium oxysporum* f. sp. *apii* /g of soil was necessary to cause vascular discoloration in celery plants. As the method developed in this chapter is able to detect 30 conidia, it would be useful for viticulturists to determine whether the inoculum present in the soil could potentially cause disease.

Although nested PCR is a very sensitive method, it is often prone to false positives. These can result from non-specific primer binding, particularly with closely related species (Nazar *et al.*, 1997). A higher annealing temperature is needed to prevent non stringent hybridisation, whilst still allowing a sufficient amplification level for detection (Nazar *et al.*, 1997). An annealing temperature of 58°C was required for the species specific primers used in this study as they lost their specificity at lower temperatures (data not shown). However, an increase of the annealing temperature results in the decrease of sensitivity. Contamination constitutes another source of false positives and a range of precautions were routinely used in this research and are listed in Section 3.2.4. Hamelin *et al.* (1996) also encountered difficulties with carry over contaminations. Nazar *et al.* (1997) observed that DNA from

organisms studied in the laboratory were present in the work environment, equipments, pipettes, reagents and water and so recommended the use of positive and negative controls for PCR amplification. The risk of contamination is greater with nested PCR than standard PCR as the number of non-specific products present in the primary PCR is increased exponentially by the secondary PCR (Persing, 1991). The use of a single closed tube for nested PCR has been suggested in previous research to significantly reduce this risk (Grote *et al.*, 2002; Retief *et al.*, 2006 and Edwards *et al.*, 2007). Grote *et al.* (2002) compared results from a conventional nested PCR and a one closed tube nested PCR and did not find any differences between the two methods indicating that the sensitivity and the specificity of the method were not affected. An alternative to nested PCR is qPCR. The latter method can have similar detection sensitivity and is less prone to contamination and false positives as a single PCR run is done and gel based detection is absent (Schena *et al*., 2004; Edwards *et al.*, 2007; Alei *et al.*, 2009).

In our experiment, qPCR was able to detect 0.3, 0.7 and 9.5 pg for *C. macrodidymum*, *C. liriodendri* and *C. destructans*, respectively. These results were similar to the sensitivity of the nested PCR limit of 1 pg of genomic DNA. Similar sensitivity was obtained with species specific primers for *Fusarium graminearum* from the β-tubulin gene region using qPCR (Yin *et al.*, 2009). Fraaije *et al.* (2001) used quantitative PCR with SYBR Green I to detect four different pathogens on wheat with species specific primers that targeted the β-tubulin sequence of each pathogen and obtained a sensitivity of between 2 and 60 pg. Alei *et al.* (2009) could detect 10 pg, 10 fg and 5 fg of genomic DNA of *Puccinia horiana* with conventional, nested and quantitative PCR, respectively using the ITS region. Edwards *et al.* (2007) and Vettraino *et al.* (2010) reported that nested PCR was more sensitive than quantitative PCR however the latter was more specific and less prone to false positive results. In this study, 30 cycles were used for the nested PCR amplification however other researchers have used up to 40 cycles which could probably increase the sensitivity of the method (Aroca and Raposo, 2007; Damm and Fourie, 2005).

Quantitative PCR was less time consuming than nested PCR. Each of the two PCR reactions in the nested process required 2 h while a total of 2.5 h was needed for qPCR, which also did not have the additional risk of carry over contamination. Pasquali *et al.* (2006) indicated that quantitative PCR could be performed in 1.5 h while nested PCR required at least 4 h, while Cullen *et al*. (2001) reported 3 h for qPCR and 6 h for nested PCR. Quantitative PCR has the additional advantage of providing DNA quantities in the samples while nested PCR can only determine if the pathogen is present in the sample. Quantification of *Cylindrocarpon* spp. in the soil would provide information on pathogen concentrations and allow monitoring of the pathogen and correlation of DNA quantities with disease incidences.

Different chemistries are available for quantitative PCR. The one used in this chapter was SYBR Green I which is non-specific to sequences as it intercalates between double stranded DNA, while TaqMan, Molecular beacons and Scorpion are sequence specific and use fluorescently labelled probes which bind specifically to the target DNA (Schena *et al.*, 2004). The dye chosen in this study had the advantage of been cheaper than the latter products, although it binds equally well to non specific reaction products and primer dimers, leading to poorer specificity and more false positive results than the alternative technologies (Suarez *et al.*, 2005). Primer dimers and non-specific products were obtained in the experiments with qPCR described in this chapter. These were successfully removed by testing different primer concentrations, annealing and detection temperatures. Protocols for qPCR usually require between 0.1 and 5 µM of primers (Fraaije *et al*., 2001; Filion *et al.*, 2003; Edwards *et al*., 2007; Kernaghan *et al*., 2007) and for this work a final primer concentration of 0.1 µM was used for qPCR of the three *Cylindrocarpon* spp. Use of a detection temperature higher than the primer dimer denaturation temperature has also been reported to eliminate primer dimers in qPCR using SYBR Green I by other researchers (Filion *et al.*, 2003; Yan *et al*., 2008 and Yin *et al*., 2009). This strategy was tested in the current study and final temperatures of 84, 85 and 86°C for *C. destructans*, *C. macrodidymum* and *C. liriodendri*, respectively were able to detect amplimers from the different species and exclude any primer dimers. An alternative to SYBR Green I would be to use TaqMan chemistry however it has been reported to be less sensitive than SYBR Green I, although more reliable (Suarez *et al*., 2005; Edwards *et al.*, 2007; Huang *et al*., 2010; Vettraino *et al*., 2010). An alternative method for identifying the different species is by using high-resolution melting curve analyses. The method consists in detecting differences between curves in small PCR fragments by melting with increasing temperatures and has been successfully used for the identification of *Listeria* species (Wang *et al*., 2010). Erali *et al.* (2006) were capable of distinguishing four *Aspergillus* spp. using a multiplex PCR combined with a melting curve analysis. This method allows simultaneous detection and identification of different species using single or multiple primer sets, does not require gel electrophoresis or additional instrumentation, uses the PCR sample tube and is fast (Reed and Wittwer, 2004). However, it does not allow quantification of the different species.

The molecular methods developed in this chapter are faster and more accurate than conventional morphological identification methods, which required the isolates to be cultured on PDA at 20°C in the dark for 2 to 3 weeks, whereas PCR reduced the time to one week and removed uncertainty in the identification. Similar results have been reported for other fungal species. For example, Miyazaki *et al.* (2009) successfully identified *Trichoderma harzianum* in 2 days with nested PCR compared to 10 days by conventional methods and

Schlenzig *et al.* (2005) reduced the detection time of *Phytophthora fragaria* var. *rubi* from 6 weeks with a bait test to 3 days.

In this study, the three different *Cylindrocarpon* species were successfully detected using species specific primers from the β-tubulin gene region. Two methods were developed: a nested PCR was optimised with a detection limit of 1 pg of pure DNA and a quantitative PCR with a sensitivity of as little as 3 pg of pure DNA. Quantitative PCR has previously been used for the detection of *C. destructans* f. sp. *panacis* (Kernaghan *et al*., 2007) however, this is the first description of a quantitative PCR method for *C. liriodendri* and *C. macrodidymum*. The sensitivity and specificity of the primers developed in this study for identification, discrimination and quantification of *Cylindrocarpon* spp. could potentially be optimised for use in plant tissue and in soil (which was developed in Chapter 5), in order to investigate their epidemiology.

CHAPTER 4

Propagule behaviour in soil environment

4.1. Introduction

The texture of the soil is associated with its physical, chemical and morphological characteristics that indicate its properties (Eash *et al.*, 2008). It also provides the habitat for bacteria, fungi, algae, protozoa, insects, worms and many other organisms that participate in the decomposition of animal and plant remains which subsequently release the nutrients for plant growth (Nautiyal and Dion, 2008). The many microorganisms that interact and compete for nutrient sources are estimated to number approximately 10^8 to 10^9 per gram of soil, of which about 10^5 are fungi found as mycelial or spore structures (Eash *et al.*, 2008).

The development of plant infection by a soilborne pathogen is influenced by the inoculum concentration, the distance to the nutrient and plant roots, the environment and the other microorganisms present (Garrett, 1970). The role of competing microorganisms was demonstrated when wheat seedlings were successfully infected in sterile soils and in sterile sand with *Ophiobolus graminis* ascospores but were not infected in natural soils even at high concentrations of ascospores (Garrett, 1939; Brooks, 1965). In experiments with *Cylindrocarpon* isolates on vines, pathogenicity tests have generally used vines grown in autoclaved soil or potting mixture (Rego *et al.*, 2001; Halleen *et al.*, 2003) which are inoculated with a conidium suspension to prove Koch's postulates. These experiments did not allow for the natural interaction between *Cylindrocarpon* species and other microorganisms present in the soil. Therefore, the first objective of this Chapter was to determine whether the different propagules from the three *Cylindrocarpon* species had similar pathogenicities in soil as in potting mix, by inoculating grapevines in the field.

Cylindrocarpon spp. have been isolated from roots and rootstocks of grapevines in nurseries and in vineyards (Rego *et al.*, 2001; Halleen *et al.*, 2003; Gimenez-Jaime *et al.*, 2006; Dubrovsky and Fabritius, 2007) but have not investigated the associations between soil types and disease incidence. From general observation, Gubler *et al.* (2004) stated that grapevine losses due to *Cylindrocarpon* spp. were greater when the overall soil type in the vineyard was heavy and wet which supports observations from Maluta and Larignon in 1991. The second objective of this Chapter was therefore to investigate the pathogenicity of the different propagules from the three *Cylindrocarpon* spp. in different soil types.

Cylindrocarpon spp. can form mycelia, conidia and chlamydospores in culture but their formation and behaviour in soil have not been investigated. However, Taylor (1964) found that when conidia of "*C. radicicola*" (*C. destructans*) were added to soil, they produced chlamydospores after a week. The third objective of this Chapter was to investigate the fate of conidia and mycelium when placed in soil.

4.2. Materials and methods

4.2.1. Pathogenicity of propagules in field conditions

Grapevine cuttings of the rootstock variety 101-14 were obtained from Corbans Viticulture Ltd and were callused as in 2.2.1.1 for a month in November 2007. The planting site, selected at the Horticulture Research Area of Lincoln University, had a soil type (Wakanui silt loam) that was classified as a mottled immature pallic soil. Prior to planting, the 2.9 × 12.9 m plot of land was ploughed, rotary hoed and three planting rows were prepared, separated from each other by 0.6 m (Figure 4.1). Each planting row constituted two blocks with a 0.5 m inter-block spacing, laid out in a randomised design. Each of the five treatment plots comprised 20 vines planted in a double row with a 0.1 m inter-vine spacing. The different treatment plots were separated by 0.3 m spaces and arranged randomly in each block.

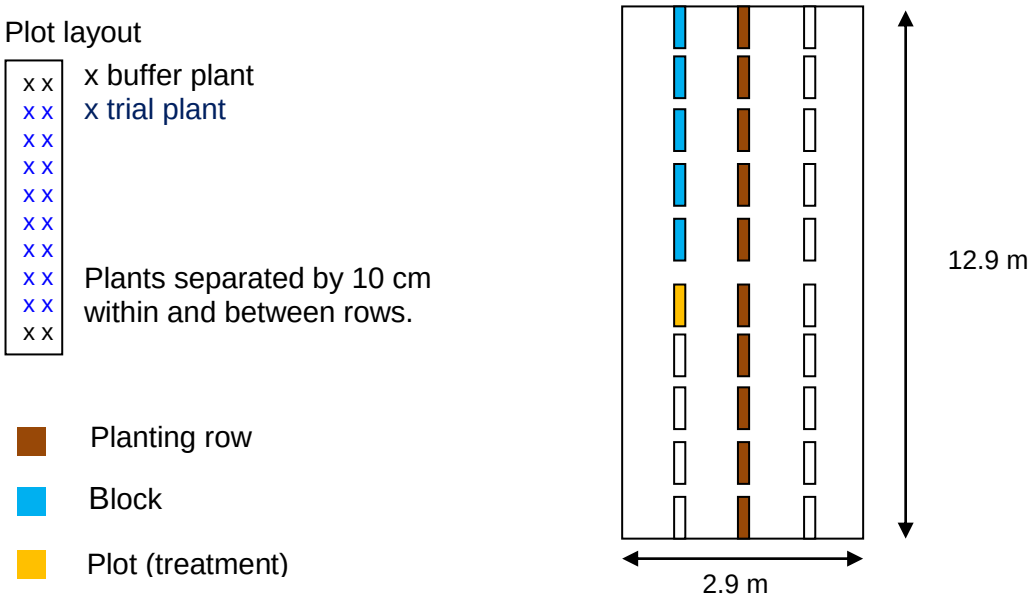

Figure 4.1. Planting layout for the experiment investigating the pathogenicity of *Cylindrocarpon* propagules in field conditions.

Conidium and chlamydospore suspensions (10^6 spores /mL) were prepared as described in Sections 2.2.1.2 and 2.2.2.1. The final composite spore suspensions comprised equal quantities of the nine isolates that were used in Section 2.2.2.1 from the three different species and labelled D1, D2, D3, L1, L2, L3, M1, M2, and M3, for which the isolate details are listed in Appendix 1. For the mycelium inoculum, the same isolates were grown on

autoclaved wheat grains and were mixed together in equal quantities as before (Section 2.2.2.1). For the controls, autoclaved water or autoclaved wheat grains were used.

In December 2007, 7 cm deep planting holes were infested with either 20 mL of conidium or chlamydospore suspensions (10^6 /mL) which were spray injected with a sheep drenching pack and gun (N.J. Phillips Pty Ltd, NSW, Australia) or by adding 5 g of infested wheat grains into the hole. The controls (water or autoclaved wheat grains) were added similarly. The plants were immediately placed into the holes and left to grow for 6 months. Weeds were regularly hand removed until assessment and plants were drip irrigated as indicated by the dryness of the soil during summer 2007 – 2008. The temperature fluctuated between -1.4 and 34.6°C during that period, with an average of 15.8°C. The vines were assessed as described in Section 2.2.1.4. The presence of the three *Cylindrocarpon* species (without distinction) in wood samples at 1 and 5 cm above stem bases was determined. Additionally, the presence of each species at 1 and 5 cm above stem bases was recorded by morphologically identifying each species. The molecular methods described in Chapter 3 had not been fully developed by the time of harvest and so conventional methods were used. The results were statistically analysed as described in Section 2.2.1.5. for the three *Cylindrocarpon* spp. combined and for the individual species.

4.2.2. Pathogenicity of propagules in different soil types

Grapevine cuttings of the rootstock variety 101-14 obtained from Corbans Viticulture Ltd were callused for a month as previously. The three different soil types selected from Lincoln University's surroundings were classified as heavy (Wakanui clay loam), medium (Templeton silt loam) and light (Eyre shallow fine sandy loam). The soils were transported from the different sites to Lincoln University's nursery area in 200 L containers and used the next day.

Conidium and chlamydospore suspensions, infested wheat grains and their respective controls were made as described in Sections 2.2.1.2 and 2.2.2.1, using equal spore and infested wheat grain quantities of the nine isolates used in Section 4.2.1.

In December 2007, 2.5 L pots were filled with each soil type and the 7 cm deep holes were infested with 20 mL of spore suspensions or 5 g of infested wheat grains as described in Section 4.2.1. The callused plants were then placed into the holes and the 20 replicate vines per treatment were laid out on mesh tables in a greenhouse in a split-plot design, and allowed to grow for 6 months. The high pressure sodium lamps (Son-T Agro 400, Philips), lights were turned on from 4 am to 12 pm and from 4 pm to 8 pm for the duration of the experiment to ensure plants were under a 16 h light exposure. Temperatures varied between

14°C and 30°C. Plants were lightly watered daily. The vines were assessed and the results were statistically analysed as decribed in Sections 2.2.1.5 and 4.2.1.

4.2.3. Fate of conidia and mycelium in the soil environment

4.2.3.1. Conidia

For this experiment, one isolate from each of the three *Cylindrocarpon* species were selected: D2, M2 and L1. These isolates were grown on PDA and conidia were harvested from the plates by adding sterile water containing 0.01% Tween 80 as in Section 2.2.1.2. The suspensions were each poured into a 50 mL Falcon tube which was centrifuged at 2000 rpm for 10 min, and the pellet suspended in 1 mL of sterile water. The concentrations of the conidium suspensions were determined using a haemocytometer and the concentration adjusted to 10^8 conidia /mL. Bags (90 × 85 mm) were made of nylon mesh (20 µm pore size; Schweizer Seidengaze-fabrik AG, Thal, Switzerland) and the edges were heat-sealed. After placing 15 g of autoclaved fine dry silica sand (particle size 50 - 500 µm) and 1 mL of a spore suspension into each bag, the sand was wetted with approximately 8 mL sterile water and the open edges of the bags were heat-sealed. The bags were buried in soil (50 /50 mixture of garden soil and 3 to 4 month potting mix) in 4 L ice-cream containers at a depth of 20 mm.

4.2.3.2. Mycelium

The same isolates were used as above. To obtain mycelium, a mycelial plug (5.5 mm diameter) cut from the edge of a growing colony on a plate containing PDA (Oxoid Ltd, Basingstoke, UK) was placed in autoclaved 100 mL PDB (Difco laboratories, Becton Dickinson, USA) contained in a 250 mL conical flask. The flasks were incubated on a shaker at 100 rev /min at room temperature for 4 days. The mycelium was then harvested before the formation of spores. Each mycelium was placed in a bag and the PDB was washed through the nylon mesh with approximately 10 mL of sterile water. Each bag was then heat sealed and buried into the same soil container as used for a bag of conidia. There were six replicates (blocks) for each propagule and isolate and the experiment was laid out in a randomised block design. The soil containers were lightly watered after 1 and 2 weeks.

4.2.3.3. Assessment

Two blocks were randomly selected for examination after two weeks incubation in the soil at room temperature and the remaining four blocks were harvested after three weeks. The contents of each bag were poured into a Petri dish and the bag was rinsed with 5 mL sterile water containing 0.01% Tween 80 and this was added to the Petri dish. The contents of each Petri dish was added to a 100 mL boiling tube containing 50 mL of 5ºC water, swirled 8 – 10 times to mix and allowed to stand for 1 – 2 min. The supernatant was decanted and centrifuged at 805 × *g* for 10 min and the pellet suspended in 1 mL of water. The

suspensions were examined at 100 x magnification under a light microscope and the different structures photographed. The number of conidia and chlamydospores was determined at week 3 by counting the number of propagules present on three randomly chosen 1 mm^2 grids of a haemocytometer viewed at 100 x magnification with a light microscope. The data was analysed using ANOVA with Genstat version 12.1.

4.3. Results

4.3.1. Pathogenicity of propagules in field conditions

The statistical analyses for this section are shown in Section 5.1 of Appendix 5 (A5). Disease incidences at 1 cm above stem bases showed significant differences between propagule types for all *Cylindrocarpon* spp. combined together (P<0.001) and for the individual species, *C. destructans* (P=0.030), *C. liriodendri* (P<0.001) and *C. macrodidymum* (P<0.001; A5.1.1).

For all *Cylindrocarpon* spp. combined together, chlamydospores caused similar disease incidences to conidia (P=0.699; A5.1.2), which were both significantly greater than mycelium, with means of 84.4, 82.3 and 65.5%, respectively (Figure 4.2). All propagules caused mean disease incidences significantly greater than their respective controls. Water treated plants had greater mean disease incidence than plants treated with wheat grains, with 39.6 and 20.8%, respectively. Pairwise comparisons of disease incidence between *Cylindrocarpon* spp. and propagules indicated some differences. For *C. destructans*, conidia caused greater disease incidence (P=0.051; A5.1.3) than chlamydospores whereas mycelium did not cause significantly different disease incidence from chlamydospores and conidia, with means of 33.3, 30.2 and 20.8% for conidia, mycelium and chlamydospores, respectively (Figure 4.3). Conidia and mycelium did not cause significantly different disease incidences from their respective controls whereas chlamydospores caused a lower disease incidence than its control (P=0.025), with means of 20.8 and 35.4% for chlamydospore and water inoculations, respectively (Figure 4.3). For *C. liriodendri*, chlamydospores caused significantly greater disease incidences than conidia and mycelium (P<0.001; A5.1.4), with means of 77.1, 52.1 and 38.5%, respectively. Disease incidences caused by conidia and mycelium were not significantly different (P=0.059). All propagules caused significantly greater disease incidences than their respective controls (P≤0.05). For *C. macrodidymum*, there were significant differences in disease incidences caused by chlamydospores, conidia and mycelium, with means of 6.3, 39.6 and 20.8%, respectively (A5.1.5). Conidia and mycelium caused significantly greater disease incidences than their respective controls whereas disease incidences were not significantly different between chlamydospores and water, with means of 6.3 and 4.2, respectively (Figure 4.3).

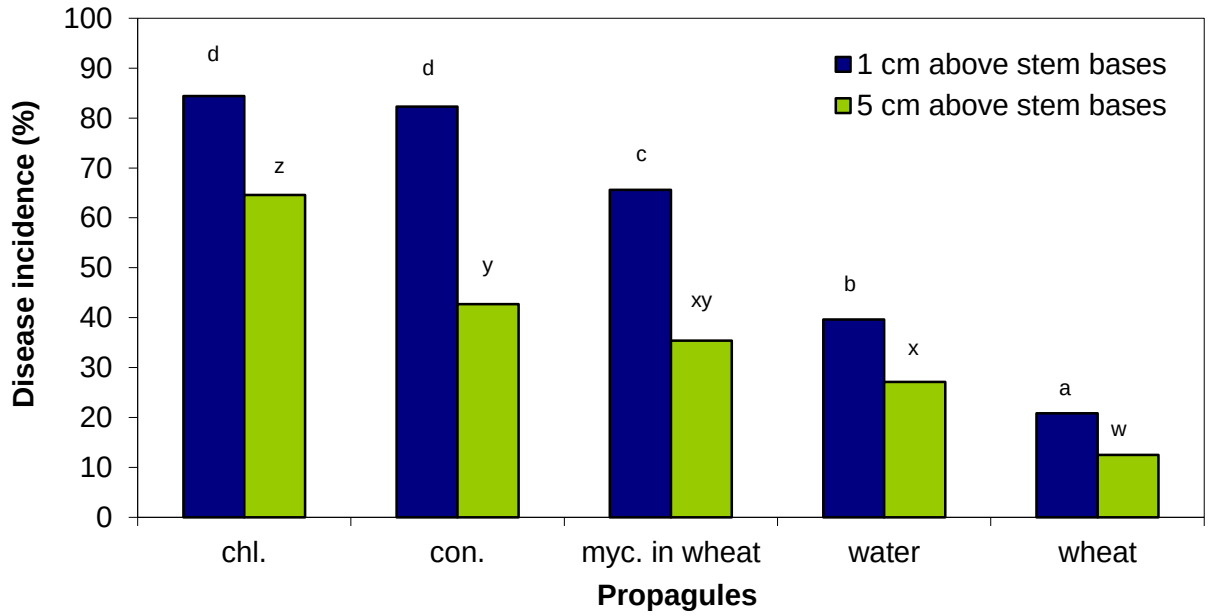

Figure 4.2. Mean disease incidence at 1 and 5 cm above stem bases of 101-14 grapevine plants, 6 months after inoculation with propagules from three *Cylindrocarpon* species in the field (chl.: chlamydospore, con.: conidium, myc.: mycelium). For each distance, bars with different letters indicate values are significantly different ($P \leq 0.05$).

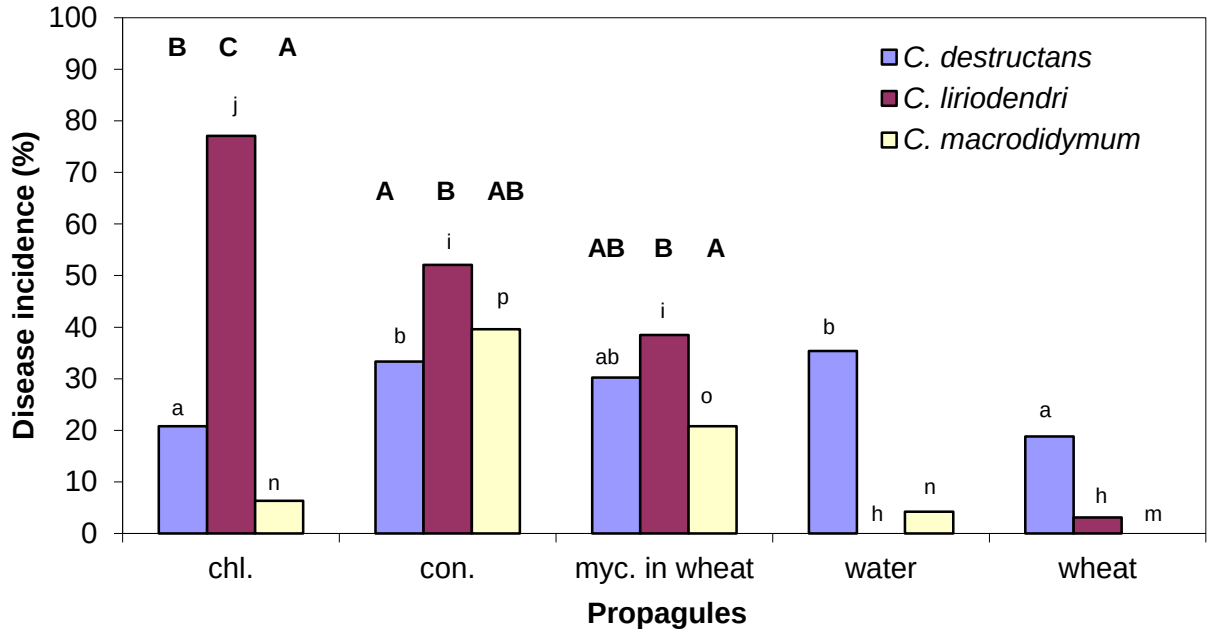

Figure 4.3. Mean disease incidence at 1 cm above stem bases of grapevines, 6 months after inoculation with propagules from three *Cylindrocarpon* species in the field (chl.: chlamydospore, con.: conidium, myc.: mycelium). Bars with different letters indicate values are significantly different between treatments for each species and bars with different capital letters indicate values are significantly different between species for one treatment ($P \leq 0.05$).

For the chlamydospore inoculation, *C. liriodendri* caused significantly greater disease incidences than *C. destructans* and *C. macrodidymum* (Figure 4.3; A5.1.6). For the conidium inoculation, *C. liriodendri* and *C. macrodidymum* caused similar disease incidences which were significantly greater than for *C. destructans*. For the mycelium inoculation, *C. liriodendri* caused significantly greater disease incidences than for *C. macrodidymum* whereas *C. destructans* caused similar disease incidences to the two other species.

Overall, *C. liriodendri* caused significantly greater disease incidences at 1 cm above stem bases than *C. destructans* (P<0.001; A5.1.7) and *C. macrodidymum* (P<0.001), with means of 55.9, 28.1 and 22.2%, respectively (Table 4.1). Colonies of other *Cylindrocarpon* species that grew from control plants were morphologically identified. However the species found in the controls were not included in the overall results.

Table 4.1. Mean disease incidences at 1 and 5 cm above stem bases and disease severities at 1 cm above stem bases of 101-14 grapevine plants, 6 months after inoculation with three *Cylindrocarpon* species in the field.

Treatments	Disease incidence (%) at 1 cm above stem bases	Disease incidence (%) at 5 cm above stem bases	Disease severity (%) at 1 cm above stem bases
C. liriodendri	55.9 b[1]	31.6 n	43.2 z
C. destructans	28.1 a	13.9 m	18.2 y
C. macrodidymum	22.2 a	11.1 m	14.8 y

[1]Within columns, values with different letters indicate values are significantly different (P≤0.05).

Disease incidences at 5 cm above stem bases showed significant differences between propagule types for all *Cylindrocarpon* spp. (P<0.001), *C. liriodendri* (P<0.001) and *C. macrodidymum* (P=0.004; A5.1.8) but not for *C. destructans* (P=0.073).

For all *Cylindrocarpon* spp., chlamydospores caused significantly greater disease incidences at 5 cm above stem bases than conidia (P=0.002; A5.1.9) and mycelium (P<0.001) which had similar effects, with means of 64.6, 42.7 and 35.2%, respectively. All propagules caused significantly greater disease incidences than their respective controls (Figure 4.2). For *C. destructans*, there were no significant differences between propagules (P=0.069; A5.1.10) although plants treated with water had greater disease incidences than plants inoculated with conidia (Figure 4.4). For *C. liriodendri*, chlamydospores caused significantly greater disease incidences than conidia and mycelium, with means of 55.2, 21.9 and 17.7%, respectively (A5.1.11). All propagule treatments caused significantly greater disease incidences than their respective controls. For *C. macrodidymum*, there were no significant differences between conidia and mycelium (P=0.062; A5.1.12), both causing significantly greater disease incidences than their respective controls. Conidia caused significantly greater disease

incidences than chlamydospores (*P*=0.004), with means of 18.8 and 5.2% for conidia and chlamydospores, respectively.

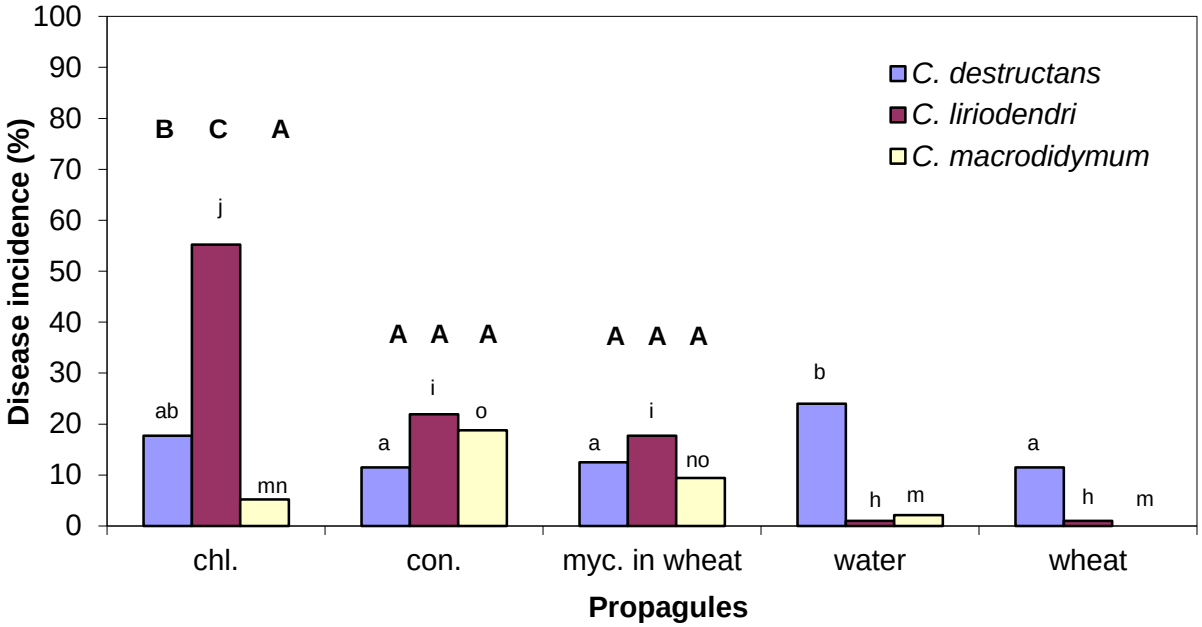

Figure 4.4. Mean disease incidence at 5 cm above stem bases of grapevines, 6 months after inoculation with propagules from three *Cylindrocarpon* species in the field (chl.: chlamydospore, con.: conidium, myc.: mycelium). Bars with different letters indicate values are significantly different between treatments for each species and bars with different capital letters indicate values are significantly different between species for one treatment (*P*≤0.05).

For the chlamydospore inoculation, *C. liriodendri* caused significantly greater disease incidences than *C. destructans* (*P*<0.001; A5.1.13) and *C. macrodidymum* (*P*=0.017; Figure 4.4) whereas for conidium and mycelium inoculations, no significant differences in disease incidences were observed between species.

Overall, *C. liriodendri* caused significantly greater disease incidence at 5 cm above stem bases than *C. destructans* (*P*<0.001; A5.1.14) and *C. macrodidymum* (*P*<0.001), with means of 31.6, 13.9 and 11.1%, respectively (Table 4.1).

Disease severities at 1 cm above stem bases differed significantly between propagule types for all *Cylindrocarpon* spp. (*P*<0.001), *C. destructans* (*P*=0.040), *C. liriodendri* (*P*<0.001) and *C. macrodidymum* (*P*<0.001; A5.1.15).

For all three *Cylindrocarpon* spp., chlamydospores and conidia caused similar disease severities which were significantly greater than for mycelium (A5.1.16), with means of 70.8, 69.3 and 52.4%, respectively (Table 4.2). Disease severities of plants inoculated with propagules were significantly greater than those of plants treated with the respective

controls. For *C. destructans*, the mean disease severities were 13.6, 21.6 and 19.5% for chlamydospores, conidia and mycelium, respectively (Figure 4.5) and were not significantly different from each other (A5.1.17). Chlamydospore inoculations caused significantly lower disease severities than water treatment (P=0.049) and mycelium inoculations caused significantly greater disease severities than autoclaved wheat grain treatments (P=0.052). For *C. liriodendri*, chlamydospores caused significantly greater disease severities than conidia (P<0.001; A5.1.18) and mycelium (P<0.001), with means of 62.8, 39.3 and 27.6%, respectively. Plants inoculated with the different propagules had disease severities significantly greater than their respective controls. For *C. macrodidymum*, conidia caused greater disease severities than mycelium (P=0.044; A5.1.19) and chlamydospores (P<0.001), with means of 5.2, 26.6 and 12.5%, respectively. Plants inoculated with conidia and mycelium had significantly different disease severities from their respective controls whereas chlamydospores caused similar disease severities to water (Figure 4.5).

Table 4.2. Means for the disease severity of grapevines inoculated with three types of propagules of three *Cylindrocarpon* spp., 6 months after planting in the field.

Propagules	Mean disease severity (%)	
Chlamydospores	70.8	c[1]
Conidia	69.3	c
Mycelium in wheat	52.4	b
Water	26.6	ab
Wheat	13.3	a

[1]Values with different letters indicate values are significantly different (P≤0.05).

For the chlamydospore and conidium inoculations, *C. liriodendri* caused significantly greater mean disease severity than *C. destructans* and *C. macrodidymum* (Figure 4.5; A5.1.20). For the mycelium inoculations, *C. liriodendri* caused significantly greater mean disease severity than *C. macrodidymum* (P=0.003).

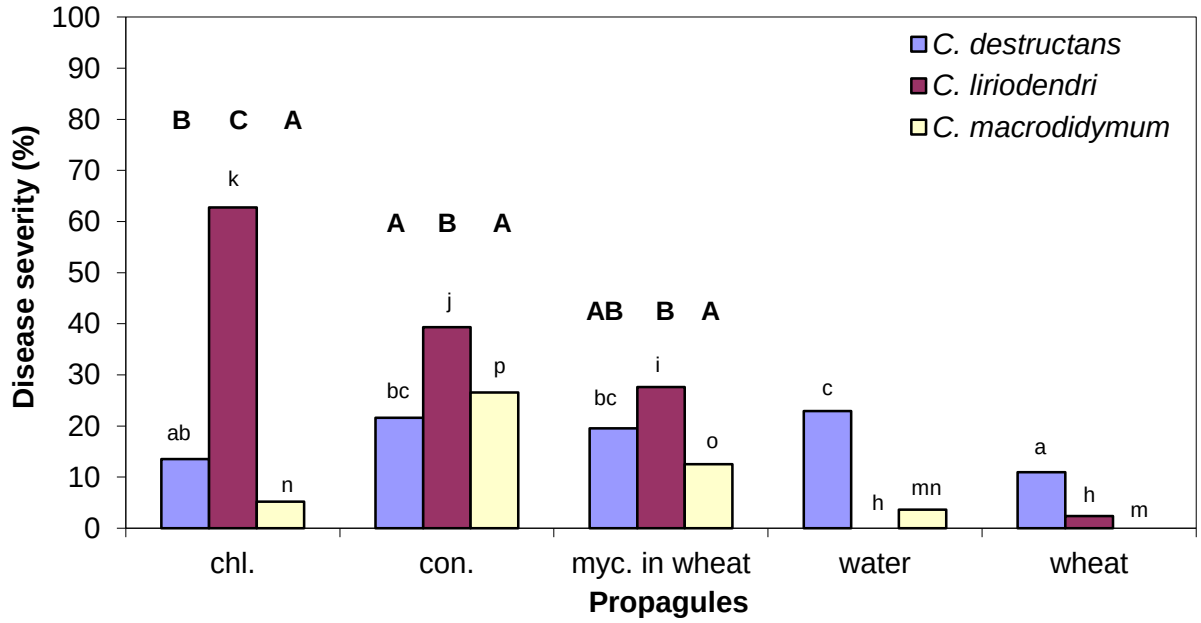

Figure 4.5. Mean disease severity at 1 cm above stem bases of grapevines, 6 months after inoculation with three propagules from three *Cylindrocarpon* species in the field (chl.: chlamydospore, con.: conidium, myc.: mycelium). Bars with different letters indicate values are significantly different between treatments for each species and bars with different capital letters indicate values are significantly different between species for one treatment ($P \leq 0.05$).

Overall, *C. liriodendri* caused significantly greater disease severities at 1 cm above stem bases than *C. destructans* ($P<0.001$; A5.1.21) and *C. macrodidymum* ($P<0.001$), with means of 43.2, 18.2 and 14.8%, respectively (Table 4.1), the two remaining species had similar disease severities.

For the root and shoot dry weights, there were no significant differences between treatments, with $P=0.334$ and $P=0.054$, respectively (A5.1.22). Mean root dry weights ranged between 2.2 g for the plants inoculated with mycelium and 3.5 g for plants treated with water (Table 4.3). The mean shoot dry weights ranged between 1.7 g for plants inoculated with autoclaved wheat grains and 2.5 g for plants inoculated with water (Table 4.3).

Table 4.3. Mean root and shoot dry weights of grapevines inoculated with three types of propagules of three *Cylindrocarpon* spp., 6 months after planting in the field.

Treatments	Mean root dry weight	Standard error	Mean shoot dry weight	Standard error
Chlamydospores	2.9	0.24	2.3	0.17
Conidia	3.2	0.24	2.4	0.17
Mycelium	2.2	0.27	2.1	0.21
Water	3.5	0.24	2.5	0.17
Wheat	2.8	0.30	1.7	0.22

4.3.2. Pathogenicity of propagules in different soils

The statistical analyses for this section are shown in Section 5.2 of Appendix 5. Disease incidences at 1 cm above stem bases showed significant differences between soil types for all *Cylindrocarpon* spp. combined (P=0.001), for *C. destructans* (P<0.001; A5.2.1) and for *C. liriodendri* (P=0.035). For *C. macrodidymum*, no significant differences were observed between soil types with P=0.152.

For all *Cylindrocarpon* spp. combined, mean disease incidences were significantly greater for clay loam (71.0%) than for sandy loam (46.0%) and for silt loam (51.0%), but the last two soils did not differ significantly (Table 4.4; A5.2.2). For *C. destructans*, mean disease incidences were significantly greater for clay loam (53.0%) than for sandy loam (16.0%) and for silt loam (23.0%; Figure 4.6). For *C. liriodendri*, mean disease incidences were significantly greater for clay loam (40.0%) than for sandy loam (27.0%), but both soils did not differ significantly with silt loam (30.0%). For *C. macrodidymum*, disease incidences were similar for soil types, means being 16.0, 9.0 and 10.0% for clay loam, silt loam and sandy loam, respectively.

Table 4.4. Mean disease incidences at 1 and 5 cm above stem bases and disease severity at 1 cm above stem bases of 101-14 grapevine plants, 6 months after inoculation with *Cylindrocarpon* species in three different soil types.

Soil types	Disease incidence at 1 cm above stem bases	Disease incidence at 5 cm above stem bases	Disease severity at 1 cm above stem bases
Sandy loam	46.0 a[1]	30.0 m	31.0 y
Silt loam	51.0 a	37.0 m	38.7 y
Clay loam	71.0 b	54.0 n	61.0 z

[1]Within columns, values with different letters indicate values are significantly different ($P \leq 0.05$).

For sandy loam, *C. liriodendri* caused significantly greater disease incidences than *C. macrodidymum* (Figure 4.6) and disease incidences caused by *C. destructans* were not significantly different from these two species (A5.2.3). For silt loam and clay loam, *C. liriodendri* and *C. destructans* caused similar disease incidences, however, they caused significantly greater disease incidences than *C. macrodidymum*.

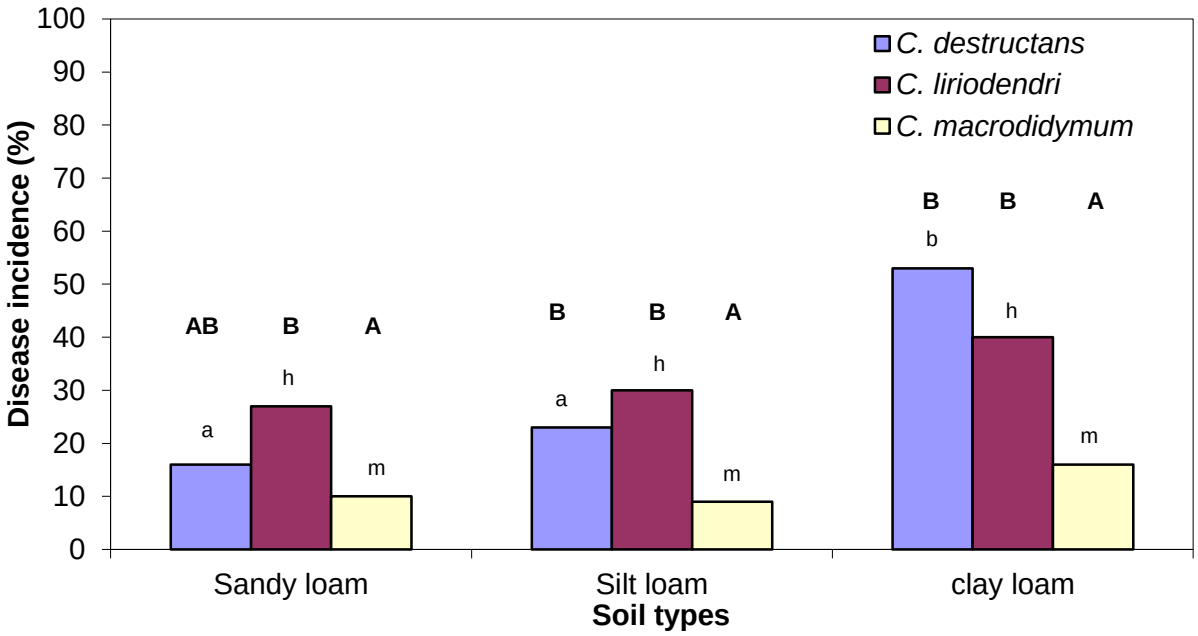

Figure 4.6. Mean disease incidence at 1 cm above the stem bases of 101-14 grapevine plants, 6 months after inoculation with three *Cylindrocarpon* species in three soil types. Bars with different letters indicate values are significantly different between treatments for each species and bars with different capital letters indicate values are significantly different between species for one treatment ($P \leq 0.05$).

Disease incidence at 1 cm above stem base did not show significant differences for the interaction between soil types and propagule treatments for all *Cylindrocarpon* spp. combined (P=0.639), *C. destructans* (P=0.073), *C. liriodendri* (P=0.127) and *C. macrodidymum* (P=0.819; A5.2.4).

Disease incidences at 1 cm above the stem base were significantly affected by the propagule types for all *Cylindrocarpon* spp. combined (P<0.001), *C. destructans* (P=0.004), *C. liriodendri* (P<0.001) and *C. macrodidymum* (P<0.001; A5.2.5).

For all *Cylindrocarpon* spp. combined, disease incidences were not significantly different between propagule types (A5.2.6), with means of 73.3% for chlamydospores, 85.0% for conidia and 78.3% for mycelium (Figure 4.7). All propagule types caused significantly greater disease incidences than their respective controls. For *C. destructans*, conidia caused significantly greater disease incidences than chlamydospores (P=0.027; A5.2.7) but disease incidences were similar for conidia and mycelium (P=0.060), with means of 30.0, 48.3 and 33.3% for chlamydospores, conidia and mycelium, respectively (Figure 4.8). No differences were observed between chlamydospores and water (P=0.416) whereas conidia and mycelium caused greater disease incidences than their respective controls (P=0.004 and 0.011). For *C. liriodendri*, no significant differences were observed in disease incidences

between propagules (A5.2.8), with means of 55.0, 48.3 and 58.3% caused by chlamydospores, conidia and mycelium, respectively, and all propagule types had significantly greater disease incidences than their respective controls. For *C. macrodidymum*, conidia caused greater disease incidence than chlamydospores (P<0.001; A5.2.9) and mycelium (P=0.003), with means of 36.7, 5.0 and 15.0% for conidia, chlamydospores and mycelium, respectively. There were significant differences between conidia and water (P<0.001) and between mycelium and its control (P<0.001) but not between chlamydospores and water (P=0.229).

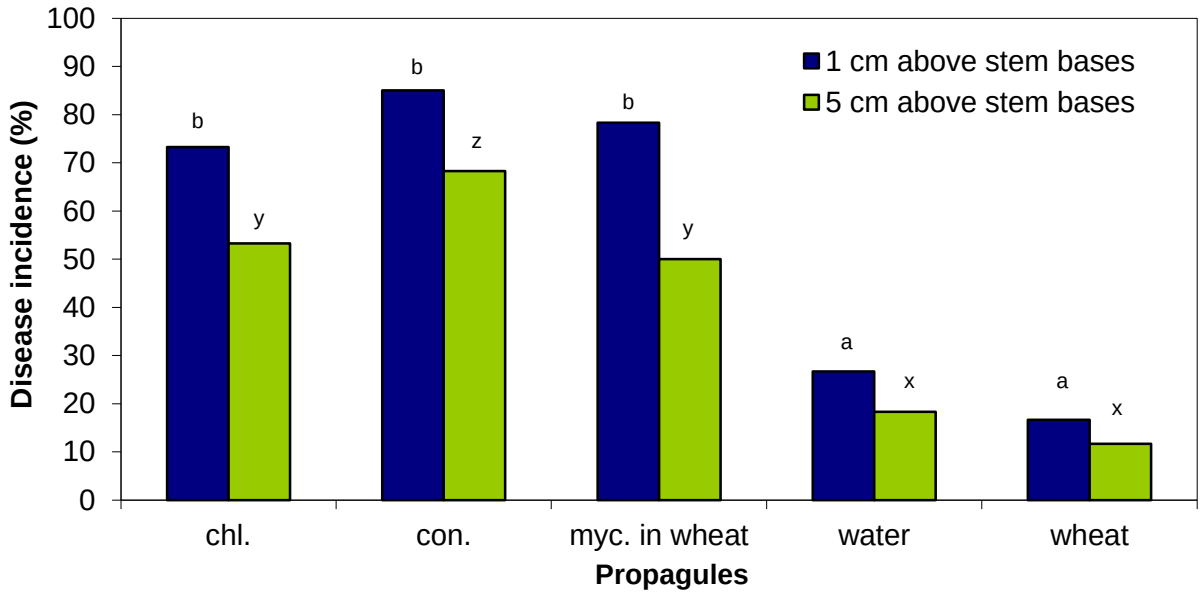

Figure 4.7. Disease incidence at 1 and 5 cm above the stem bases of 101-14 grapevine plants grown on three different soil types, 6 months after inoculation with three propagule types from three *Cylindrocarpon* species. For each distance, bars with different letters indicate values are significantly different from their respective controls (P≤0.05).

For chlamydospore and mycelium inoculations, *C. liriodendri* caused significantly greater disease incidence than *C. destructans* and *C. macrodidymum* (Figure 4.8 and A5.2.10). No significant differences were observed between species for the conidium inoculation.

Overall, *C. liriodendri* caused greater disease incidences at 1 cm than *C. destructans* and *C. macrodidymum*, with means of 53.9, 37.2 and 18.9%, respectively (Table 4.5 and A5.2.11).

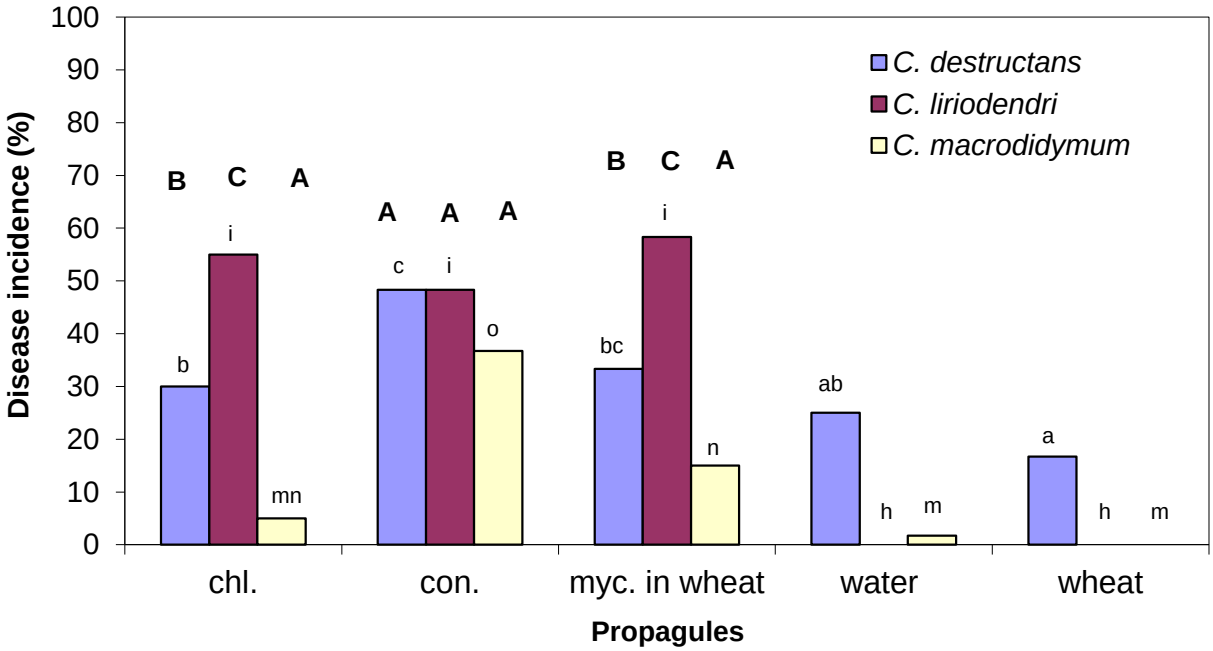

Figure 4.8. Mean disease incidence at 1 cm above the stem bases of 101-14 grapevine plants grown on three soil types, 6 months after inoculation with three propagule types from three *Cylindrocarpon* species (chl.: chlamydospore, con.: conidium, myc.: mycelium). Bars with different letters indicate values are significantly different between treatments for each species and bars with different capital letters indicate values are significantly different between species for one treatment ($P \leq 0.05$).

Table 4.5. Mean disease incidences at 1 and 5 cm above stem bases and disease severity at 1 cm above stem bases of 101-14 grapevine plants, 6 months after inoculation with three *Cylindrocarpon* species, without the controls.

Treatments	Disease incidence (%) at 1 cm above stem base	Disease incidence (%) at 5 cm above stem base	Disease severity (%) at 1 cm above stem base
C. liriodendri	53.9 c[1]	38.9 o	41.5 z
C. destructans	37.2 b	24.0 n	26.7 y
C. macrodidymum	18.9 a	7.2 m	11.7 x

[1]Within columns, values with different letters indicate values are significantly different ($P \leq 0.05$).

Disease incidences at 5 cm above stem bases showed significant differences between soil types for all *Cylindrocarpon* spp. combined ($P<0.001$), *C. destructans* ($P<0.001$) and *C. liriodendri* ($P=0.010$), but not for *C. macrodidymum* ($P=0.898$; A5.2.12).

For all *Cylindrocarpon* spp. combined, the mean disease incidence at 5 cm was significantly greater for clay loam (54.0%) than for sandy loam (30.0%) and for silt loam (37.0%; Table 4.4 and A5.2.13). For *C. destructans*, mean disease incidence was significantly greater for clay loam (34.0%) than for sandy loam (9.0%) and for silt loam (17.0%; Figure 4.9). For *C. liriodendri*, mean disease incidence was significantly greater for clay loam (32.0%) than for sandy loam (19.0%) and for silt loam (19.0%). For *C. macrodidymum*, mean disease

incidences were 5.0, 5.0 and 4.0% for clay loam, silt loam and sandy loam, respectively (Figure 4.9).

For sandy loam, *C. liriodendri* caused significantly greater disease incidences at 5 cm than *C. macrodidymum* (Figure 4.9) but *C. destructans* caused similar disease incidences to the two species (A5.2.14). For silt loam and clay loam, disease incidences were not significantly different between *C. liriodendri* and *C. destructans*, however, they were significantly greater than those caused by *C. macrodidymum.*

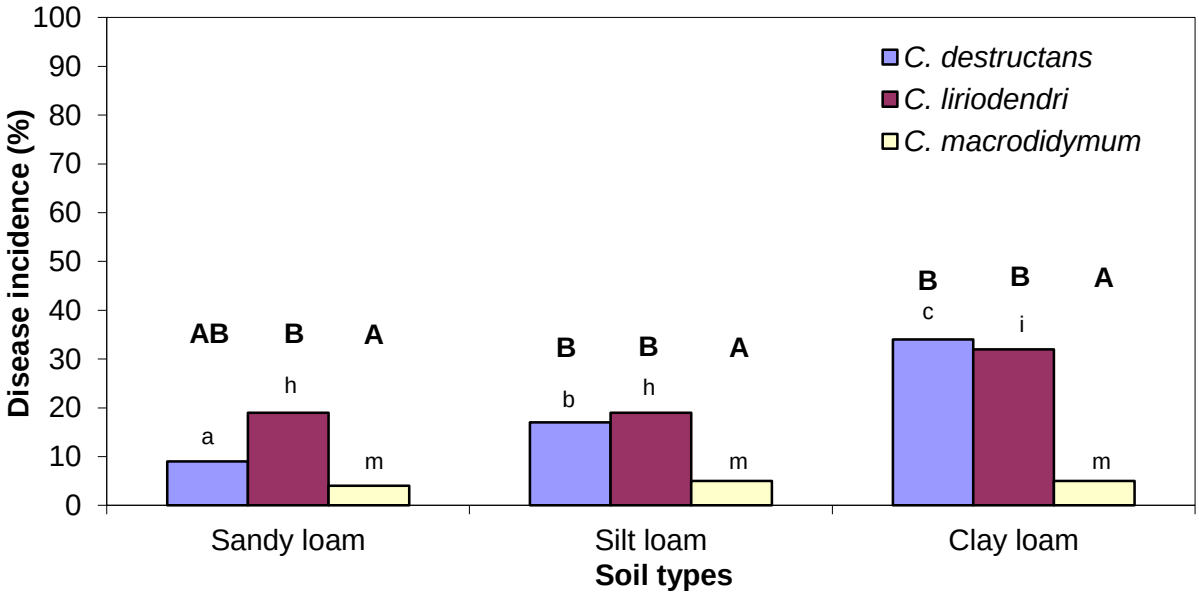

Figure 4.9. Mean disease incidence at 5 cm above the stem bases of 101-14 grapevine plants, 6 months after inoculation with three *Cylindrocarpon* species in three soil types. Bars with different letters indicate values are significantly different between treatments for each species and bars with different capital letters indicate values are significantly different between species for one treatment ($P \leq 0.05$).

Disease incidences at 5 cm above stem bases did not show significant differences for the interaction between soil types and propagule treatments for all *Cylindrocarpon* spp. combined (P=0.649), *C. destructans* (P=0.137), *C. liriodendri* (P=0.565) and *C. macrodidymum* (P=1.000; A5.2.15).

Disease incidences at 5 cm above stem base were significantly different between propagule types for all *Cylindrocarpon* spp. combined (P<0.001), *C. destructans* (P=0.009), *C. liriodendri* (P<0.001) and *C. macrodidymum* (P<0.001; A5.2.16).

For all *Cylindrocarpon* spp. combined, conidia caused greater disease incidences at 5 cm than chlamydospores (*P*=0.047; A5.2.17) and mycelium (*P*=0.015), with means of 68.3, 53.3 and 50.0%, respectively (Figure 4.9). All propagule types caused disease incidences significantly greater than their respective controls. For *C. destructans*, conidia caused significantly greater disease incidences than mycelium (*P*=0.003; A5.2.18) but not chlamydospores (*P*=0.129; Figure 4.10), with means of 35.0, 13.3 and 23.3%, respectively. Only conidia caused significantly different disease incidences from its control. For *C. liriodendri*, no significant differences were observed for disease incidences between propagule types, with means of 40.0, 35.0 and 41.7% for chlamydospores, conidia and mycelium, respectively (A5.2.19). All propagule types caused significantly greater disease incidences than their respective controls. For *C. macrodidymum*, conidia caused significantly greater disease incidences than chlamydospores (*P*=0.011; A5.2.20) and mycelium (*P*=0.014), with means of 16.7, 1.7 and 3.3%, respectively and conidia represented the only propagule type significantly different from the respective control (*P*=0.011), with mean disease incidences of 16.7% for conidia and 1.7% for water (Figure 4.10).

For chlamydospore inoculations, *C. liriodendri* and *C. destructans* had similar disease incidences (*P*=0.076; A5.2.21), but both caused significantly greater disease incidence than *C. macrodidymum* (Figure 4.10). For conidium inoculations, *C. liriodendri* caused significantly greater disease incidence than *C. macrodidymum* (*P*=0.035) however *C. destructans* caused similar disease incidences to the two other species. For mycelium inoculations, *C. liriodendri* caused significantly greater disease incidence than *C. destructans* (*P*=0.002) and *C. macrodidymum* (*P*=0.001) which caused similar disease incidences (*P*=0.109).

Overall, *C. liriodendri* caused greater disease incidences at 5 cm above stem bases than *C. destructans* and *C. macrodidymum*, with means of 24.0, 38.9 and 7.2% for *C. destructans*, *C. liriodendri* and *C. macrodidymum*, respectively (Table 4.5; A5.2.22).

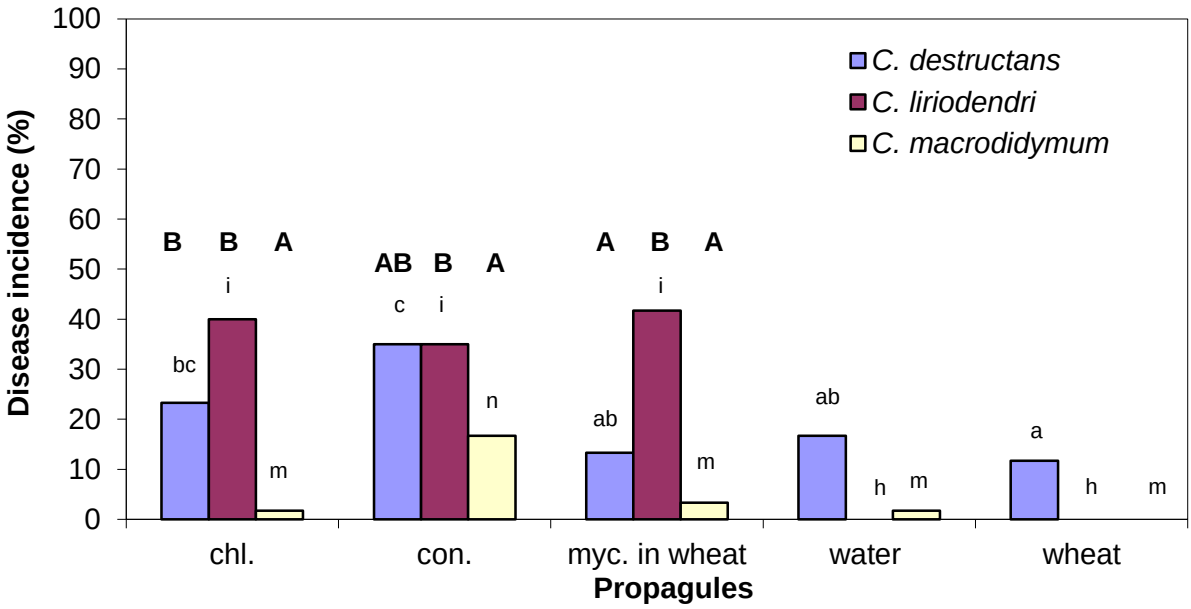

Figure 4.10. Mean disease incidences at 5 cm above the stem bases of 101-14 grapevine plants, 6 months after inoculation with three propagules from three *Cylindrocarpon* species in all soils combined (chl.: chlamydospore, con.: conidium, myc.: mycelium). Bars with different letters indicate values are significantly different between treatments for each species and bars with different capital letters indicate values are significantly different between species for one treatment ($P \leq 0.05$).

Disease severities at 1 cm above stem bases showed significant differences between soil types for all *Cylindrocarpon* spp. combined ($P<0.001$; A5.2.23), *C. destructans* ($P<0.001$) and *C. liriodendri* ($P=0.006$) but not for *C. macrodidymum* ($P=0.168$).

For all *Cylindrocarpon* spp. combined, mean disease severities at 1 cm above stem bases were significantly greater for clay loam (61.0%) than for sandy loam (31.0%) and for silt loam (38.7%; Table 4.4 and A5.2.24). For *C. destructans*, mean disease severity was significantly greater for clay loam (40.8%) than for silt loam (16.0%) which was also significantly greater than for sandy loam (8.3%; Figure 4.11). For *C. liriodendri*, mean disease severity was significantly greater for clay loam (33.5%) than for sandy loam (20.5%) and for silt loam (20.5%). For *C. macrodidymum*, mean disease severities were 10.3, 5.5 and 5.8% for clay loam, silt loam and sandy loam, respectively.

For sandy loam, *C. liriodendri* caused significantly greater disease severities than *C. macrodidymum* and *C. destructans* (Figure 4.11 and A5.2.25). For silt loam, *C. liriodendri* caused significantly greater disease severities than *C. macrodidymum* however *C. destructans* caused similar disease severities to the two other species. For clay loam, disease severities were not significantly different between *C. liriodendri* and *C. destructans* however they were significantly greater than for *C. macrodidymum*.

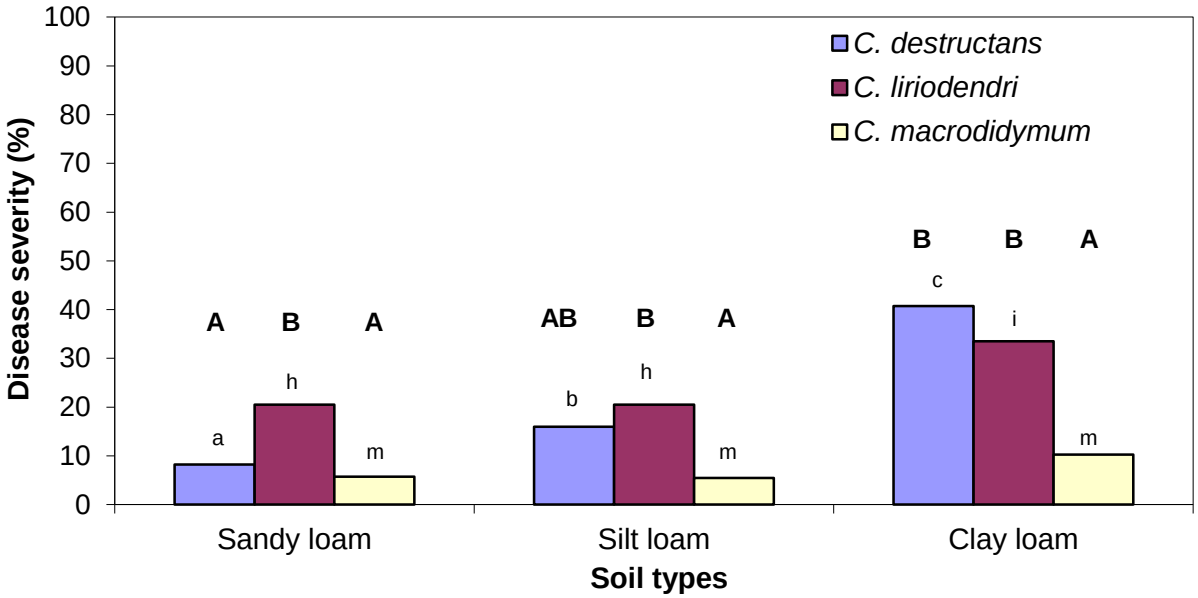

Figure 4.11. Mean disease severities at 1 cm above the stem bases of 101-14 grapevine plants, 6 months after inoculation with three *Cylindrocarpon* species in three soil types. Bars with different letters indicate values are significantly different between treatments for each species and bars with different capital letters indicate values are significantly different between species for one treatment ($P \leq 0.05$).

Disease severities at 1 cm above stem bases showed significant differences between propagule types for all *Cylindrocarpon* species combined ($P<0.001$; A5.2.23), *C. destructans* ($P=0.001$), *C. liriodendri* ($P<0.001$) and *C. macrodidymum* ($P<0.001$).

For all *Cylindrocarpon* species combined, conidia caused greater disease severities than chlamydospores ($P=0.024$; A5.2.26) however mycelium caused similar disease severities to the two other propagules, with means of 55.4, 72.5 and 60.8% for chlamydospores, conidia and mycelium, respectively (Table 4.6). All propagules caused significantly greater disease severities than their respective controls (Table 4.6). For *C. destructans*, conidia caused significantly greater disease severities than mycelium ($P=0.044$) and chlamydospores ($P=0.046$; A5.2.27), with means of 36.3, 22.1 and 21.7% for conidia, mycelium and chlamydospores, respectively (Figure 4.12). No significant differences were observed between chlamydospore (21.7%) and water (16.7%) and between mycelium (22.1%) and autoclaved wheat grain (11.7%) whereas conidia caused a greater mean disease severity (36.3%) than its control (16.7%). For *C. liriodendri*, the propagules caused similar disease severities (A5.2.28), with means of 42.5, 37.9 and 43.8% for chlamydospores, conidia and mycelium, respectively. These disease severities were significantly greater than those of their respective controls. For *C. macrodidymum*, conidia caused significantly greater disease severities than mycelium ($P=0.013$) and chlamydospores ($P<0.001$, A5.2.29), with means of 23.3, 9.6 and 2.1% for chlamydospores, conidia and mycelium, respectively.

Table 4.6. Mean disease severities at 1 cm above stem bases of grapevines inoculated with three propagule types from three *Cylindrocarpon* spp., 6 months after planting.

Treatments	Disease severity (%)
Chlamydospores	55.4 b[1]
Conidia	72.5 c
Mycelium	60.8 bc
Water	17.5 a
Wheat	11.7 a

[1]Values with different letters indicate values are significantly different ($P \leq 0.05$).

For chlamydospore and mycelium inoculations, *C. liriodendri* caused significantly greater disease severities ($P \leq 0.05$) than *C. destructans* and *C. macrodidymum* (Figure 4.12 and A5.2.30). For conidium inoculations, *C. liriodendri* caused significantly greater disease severities than *C. macrodidymum* ($P=0.032$) however *C. destructans* caused similar disease severities to the two other species.

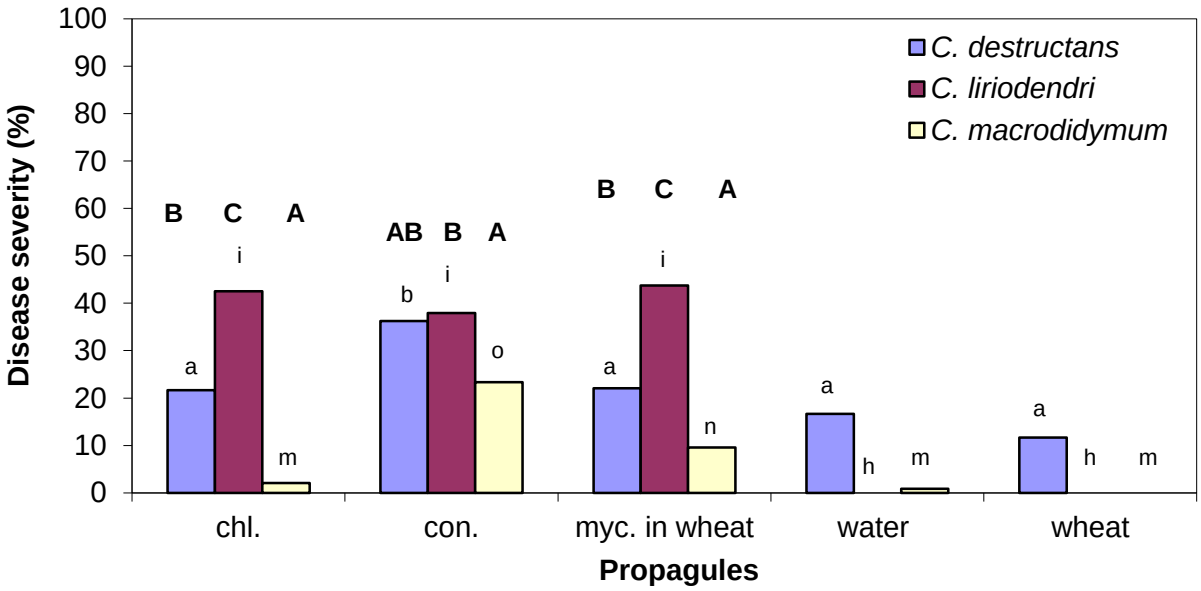

Figure 4.12. Mean disease severities at 1 cm above the stem bases of 101-14 grapevine plants, 6 months after inoculation with three propagules from three *Cylindrocarpon* species in all soils combined (chl.: chlamydospore, con.: conidium, myc.: mycelium). Bars with different letters indicate values are significantly different between treatments for each species and bars with different capital letters indicate values are significantly different between species for one treatment ($P \leq 0.05$).

Overall, *C. liriodendri* caused significantly greater disease severities than *C. destructans* (*P*=0.001) and *C. macrodidymum* (*P*=0.001; A5.2.31), with means of 41.5, 26.7 and 11.7% for *C. liriodendri* and *C. destructans* and *C. macrodidymum*, respectively (Table 4.5).

For the root dry weights, there were significant effects of soil types (*P*<0.001), of the interaction between propagule types and soil types (*P*<0.001) and of propagule types (*P*=0.045; A5.2.32). For the different soil types, the mean root dry weight was greater for plants grown in sandy loam (14.5 g) followed by those grown in clay loam (10.9 g) which were greater than for silt loam (7.4 g; Table 4.7).

Table 4.7. Means for root and shoot dry weights of grapevines inoculated with *Cylindrocarpon* spp., 6 months after planting in three different soil types.

Tissue	Soil types	Dry weight (g)
Roots	Sandy loam	14.5 c[1]
	Silt loam	7.4 a
	Clay loam	10.9 b
Shoots	Sandy loam	2.1 b
	Silt loam	1.0 a
	Clay loam	2.5 b

[1]Values with different letters indicate values are significantly different (*P*≤0.05).

The interaction between soil and propagule types was associated with the similar root dry weights for mycelium inoculations for all soil types but root dry weights differed for other propagules and the controls. The root dry weights varied between 6.0 and 9.8 g for plants grown in silt loam, between 9.2 and 11.4 g for those grown in clay loam and between 11.0 and 17.6 g for those grown in sandy loam (Figure 4.13).

The root dry weights were not significantly different between propagules and their respective controls, however, grapevines inoculated with water had significantly greater mean root dry weight (11.2 g) than plants inoculated with autoclaved wheat grains (9.6 g; Table 4.8).

Table 4.8. Means for root dry weights of grapevines inoculated with three propagules from *Cylindrocarpon* spp., 6 months after planting.

Treatments	Root dry weight (g)
Chlamydospores	11.6 ab[1]
Conidia	11.5 ab
Mycelium	10.6 ab
Water	11.2 b
Wheat	9.6 a

[1]Values with different letters indicate values are significantly different (*P*≤0.05).

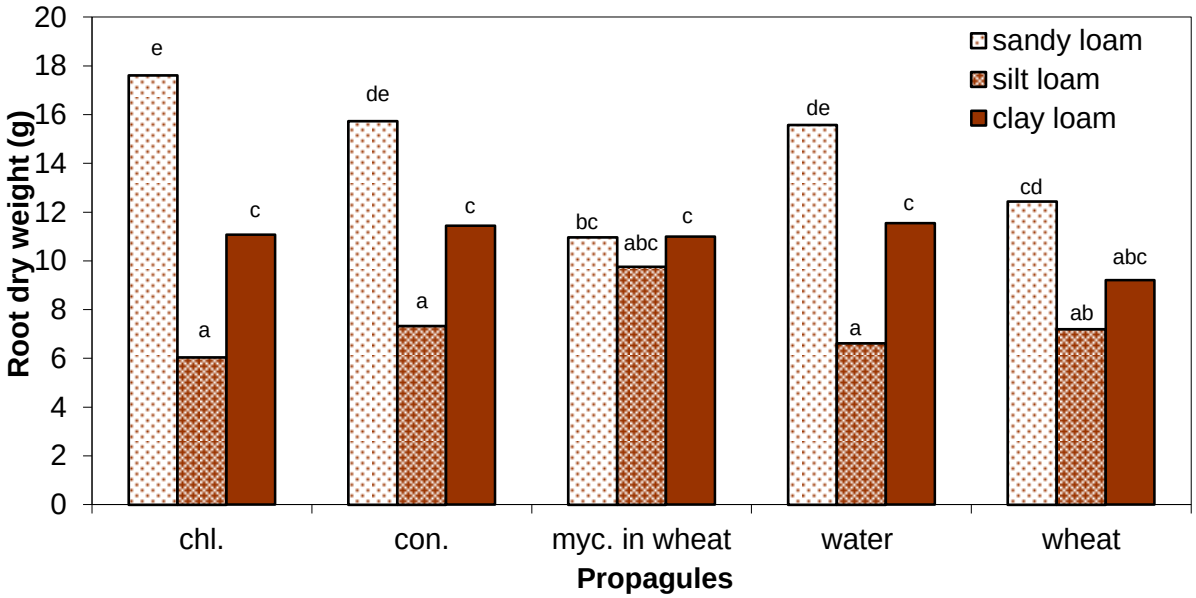

Figure 4.13. Mean root dry weights of 101-14 grapevine plants, 6 months after inoculation with three propagules from *Cylindrocarpon* species in three soil types. Bars with different letters indicate values are significantly different ($P \leq 0.05$).

For the shoot dry weights, there were significant differences between soil types ($P<0.001$) and a significant interaction between soil types and propagule treatments ($P=0.009$; A5.2.33). For the different soil types, the mean shoot dry weights were significantly lower for grapevines grown in silt loam (1.0 g) than those grown in sandy loam (2.1 g) and clay loam (2.5 g; Table 4. 8).

The interaction between soil and propagule types was associated with the similar effect of mycelium in sandy and silt loams, which differed from other propagules The shoot dry weights varied between 0.9 and 1.2 g for plants grown in silt loam, between 2.0 and 2.9 g for those grown in clay loam and between 1.6 and 2.7 g for those grown in sandy loam (Figure 4.14).

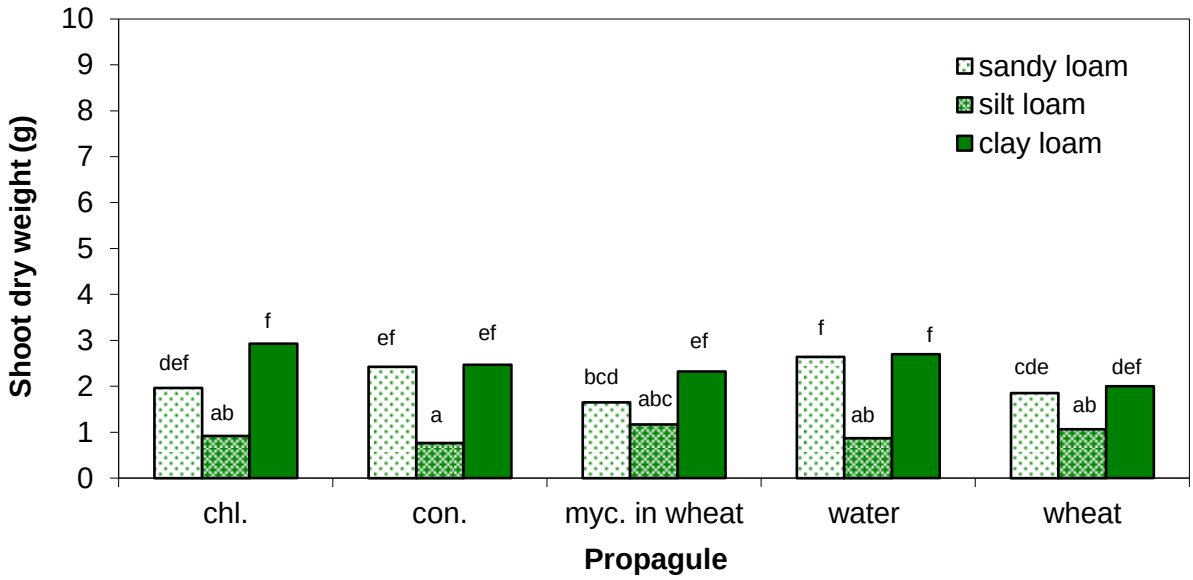

Figure 4.14. Mean shoot dry weights of 101-14 grapevine plants, 6 months after inoculation with three propagules from three *Cylindrocarpon* species in three soil types (same letter indicate values are not significantly different with $P \leq 0.05$).

4.3.3. Fate of conidia and mycelium in soil

Observation of the mycelium and conidia placed in bags in the soil after 2 and 3 weeks showed the presence of conidia, chlamydospores and mycelium (Tables 4.9 and 4.10). The conversion of conidia and mycelium into chlamydospores followed different patterns. The basic chlamydospore structure was a single melanised spherical cell. Chlamydospores originating from mycelium were formed at the terminal end or on the side of a hypha (Figure 4.15 A-a), alone or in a chain which generally comprised three to five cells (Figure 4.15 B).

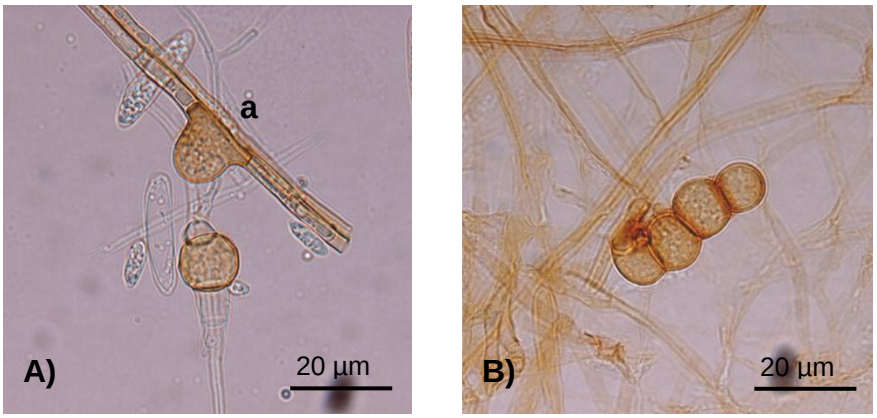

Figure 4.15. Single chlamydospores formed on the side (a) of a hypha (A); chains of chlamydospores (B). Photographs courtesy of Dudley Crabbe.

A single conidium formed one or two adjacent chlamydospores. The latter was formed when the contents of a four-celled conidium moved to two cells on one extremity of the conidium, although the contents occasionally moved to the middle. These cells then started to swell and form melanised walls, resembling the figure eight (Figure 4.16). Chlamydospores in clusters appeared to be formed from either an aggregation of single chlamydospores or the formation of multiple single chlamydospores in a group and were only found in conidium bags for the different *Cylindrocarpon* species

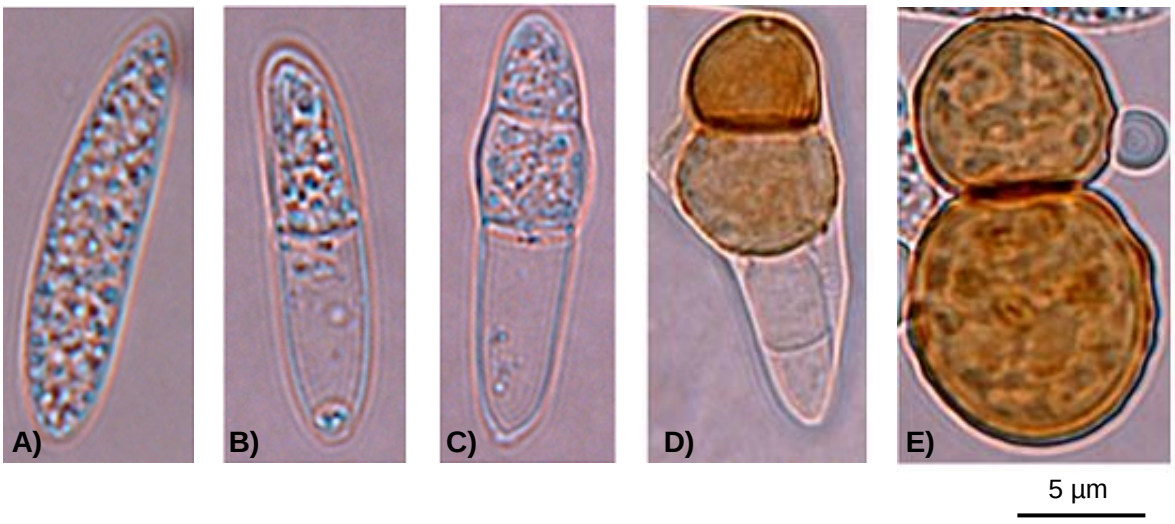

Figure 4.16. Conversion of a conidium into two chlamydospores. A: conidium; B: contents of conidium moving to one extremity; C: cells of conidium are swelling; D: melanisation of the two cells; E: formation of two chlamydospores.

Occasionally, a conidium formed a short germ tube which fused to another conidium and the contents of one conidium were transferred to the other conidium which then formed multiple chlamydospores (Figure 4.17 A and B). The same phenomenon was observed with higher numbers of conidia (Figure 4.17 C and D), indicating that some conidia possibly lacked some elements required to form a complete chlamydospore.

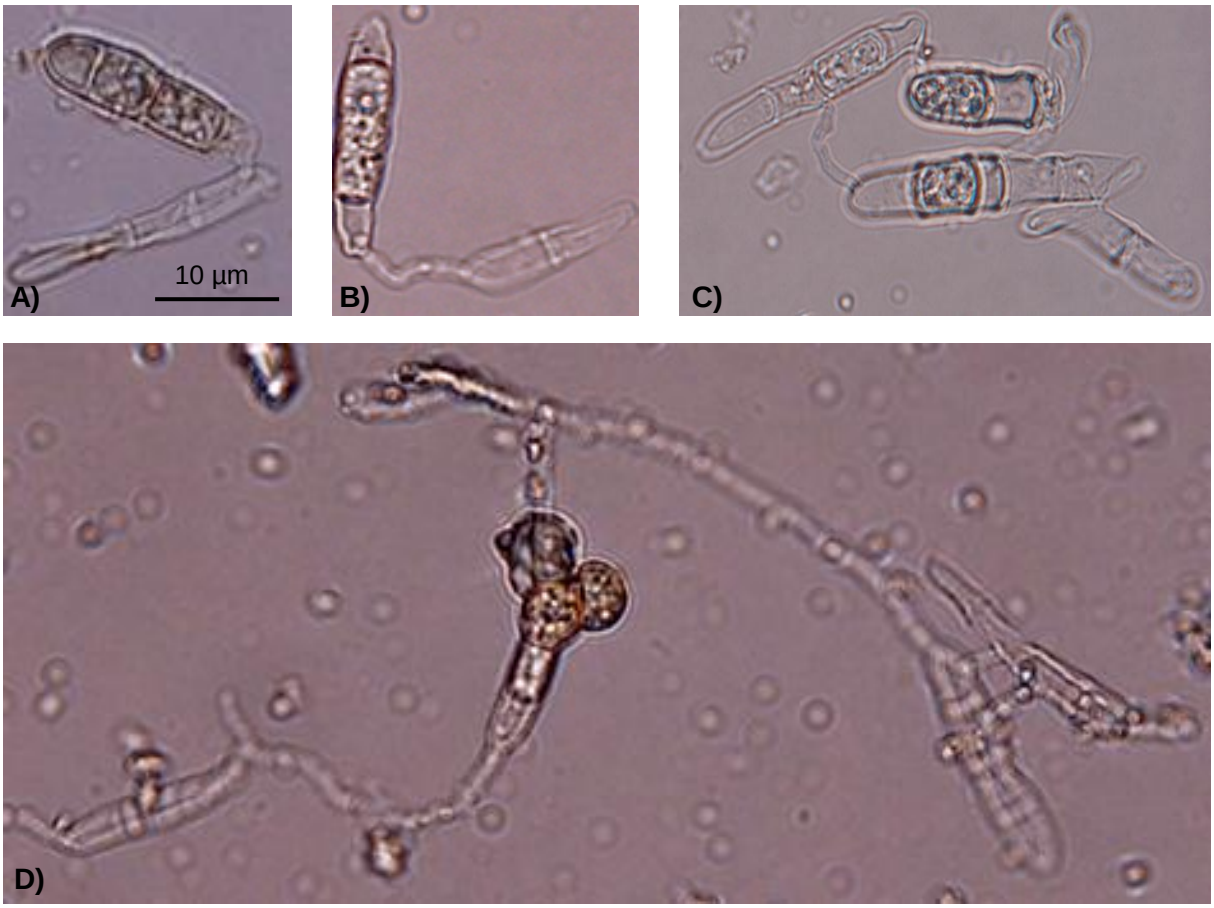

Figure 4.17. Conidia attached with germ tubes and chlamydospore formation. Two conidia with A: chlamydospore formation on extremity of conidium and B: chlamydospore formation in the middle of conidium. Multiple conidia attached with C: formation of single chlamydospores and D: formation of cluster of chlamydospores. Photographs courtesy of Dudley Crabbe.

For *C. destructans* after 2 and 3 weeks, the conidium bags contained conidia (some germinated) and chlamydospores, pairs of chlamydospores and clusters of chlamydospores while single chlamydospores and chlamydospores at the terminal end of a germ tube were observed after 3 weeks (Table 4.7). The mycelium bags showed that conidia and chlamydospores were present after 2 and 3 weeks. Chlamydospores were observed alone, in chains, in pairs or at the terminal end of a germ tube.They were formed at the terminal end of a hypha or on the side of a hypha. Conidium germination was not observed in the mycelium bags (Table 4.10).

For *C. liriodendri*, the observation of the conidium bags after 2 and 3 weeks showed single, clusters and pairs of chlamydospores and conidia, as well as conidia that were empty, forming chlamydospores and completed chlamydospores, while chlamydospores at the terminal ends of germ tubes were observed after 3 weeks (Table 4.9). The observation of the

mycelium bags indicated that after 2 and 3 weeks, conidia, pairs of chlamydospores, single and clusters of chlamydospores were observed. Conidia forming chlamydospores and chains of chlamydospores were observed at week 2 while germinating conidia were observed at week 3 (Table 4.10).

For *C. macrodidymum* after 2 and 3 weeks, the conidium bags had germinating conidia, empty conidia, conidia that formed chlamydospores, clusters of chlamydospores and chlamydospores at the terminal ends of germ tubes, while conidia forming chlamydospores were observed at week 3 and pairs of chlamydospores were observed at week 2 (Table 4.9). The observation of the mycelium bags showed that after 2 and 3 weeks, single chlamydospores and chlamydospores at the terminal ends of hyphae were observed. Conidia forming chlamydospores and chlamydospore clusters were only observed on week 2 (Table 4.10).

Table 4.9. Structures found in conidium bags from the three *Cylindrocarpon* species, 2 and 3 weeks after being buried in the soil.

Structure	*Cylindrocarpon* species		
	C. destructans	*C. liriodendri*	*C. macrodidymum*
Conidia	+[1]	+	+
Germinated conidia	+	+	+
Empty conidia	-[2]	+	+
Conidia forming chlamydospores	-	+	b
Conidia formed chlamydospores	-	+	+
Single chlamydospores	b[3]	+	-
Clusters of chlamydospores	+	+	+
Chains of chlamydospores	-	-	-
Chlamydospore at terminal end of germ tube	b	b	+
Pair of chlamydospores	+	+	a[4]

[1] structure present in week 2 and 3.
[2] structure absent.
[3] structure present in week 3.
[4] structure present in week 2.

Table 4.10. Structures found in mycelium bags from the three *Cylindrocarpon* species, 2 and 3 weeks after being buried in the soil (+ : structure present in week 2 and 3, - : structure absent, a: structure present in week 2 and b: structure present in week 3).

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Structure	*Cylindrocarpon* species		
	C. destructans	*C. liriodendri*	*C. macrodidymum*
Conidia	+[1]	+	a
Germinating conidia	-[2]	b[3]	-
Empty conidia	-	-	-
Conidia forming chlamydospores	-	a[4]	a
Conidia formed chlamydospores	-	-	-
Single chlamydospores	+	+	+
Clusters of chlamydospores	-	+	a
Chains of chlamydospores	+	a	-
Chlamydospore at terminal end of germ tube	+	-	+
Pair of chlamydospores	+	+	-

[1] structure present in week 2 and 3.
[2] structure absent.
[3] structure present in week 3.
[4] structure present in week 2.

After 3 weeks, mean number of conidia present in conidium bags was significantly lower for *C. destructans* (3.5 x10^5) than *C. liriodendri* (1.1 x10^6) and *C. macrodidymum* (4.0 × 10^6; Table 4.11). The mean number of chlamydospores was significantly higher in conidium bags after 3 weeks for *C. liriodendri* (6.0 × 10^5) than *C. destructans* (6.7 × 10^4) and *C. macrodidymum* (2.3 × 10^5) which did not differ significantly. For the mycelium bags, no significant differences were observed between the different species in the numbers of conidia and chlamydospores.

Table 4.11. Conidium and chlamydospore numbers present in conidium and mycelium bags containing three *Cylindrocarpon* spp., 3 weeks after being buried in the soil.

Isolate	Inoculum	Week 0		Week 3			
		Conidia	Chl.[1]	Conidia		Chl.	
C. destructans	Conidia	10^8	0	3.5 × 10^5	a	6.7 × 10^4	i
	Mycelium	0	0	1.7 × 10^5	a	2.1 × 10^5	i
C. liriodendri	Conidia	10^8	0	1.1 × 10^6	b	6.0 × 10^5	j
	Mycelium	0	0	4.9 × 10^4	a	8.7 × 10^4	i
C. macrodidymum	Conidia	10^8	0	4.0 × 10^6	b	2.3 × 10^5	i
	Mycelium	0	0	7.0 × 10^4	a	1.9 × 10^5	i
$LSD_{0.05}$				4.4 × 10^5		3.0× 10^5	

[1]Chl.: chlamydospores.
[2] Values with different letters indicate values are significantly different ($P \leq 0.05$).

4.4. Discussion

The investigation with the different *Cylindrocarpon* propagules in field conditions confirmed their capacity to infect grapevines, as shown in a similar experiment with inoculated plants grown in potting mix (Chapter 2) however the potting mix experiment showed much greater disease incidences at 1 cm above stem bases than the field experiment, except for *C. liriodendri* chlamydospores which caused similar disease incidences of 70.8 and 77.7% for potting mix and soil, respectively. A lower disease incidence for plants grown in the soil environment can result from the interaction of *Cylindrocarpon* spp. with other soil microorganisms. Matturi and Stenton (1964) observed that conidia and hyphae from *Cylindrocarpon* spp. were lysed by bacteria and unknown substances present in the soil environment, which appeared to be associated with *Corynebacterium* spp. and *Bacillus cereus* var. *mycoides*. They also found *Trichoderma viride* parasitizing one of the *Cylindrocarpon* species. Taylor (1964) studied the growth of "*C. radicicola*" (*C. destructans*) in the presence of antagonists in agar and in sand with artificial soil solution and observed that *C. destructans* did not grow with "*Gliocladium roseum*" (*Clonostachys rosea*) in both media, while *C. destructans* growth was reduced with "*Penicillium janthinellum*" (*Penicillium simplicissimum*) and "*Penicillium lilacinum*" (*Paecilomyces lilacinus*). Fu *et al.* (2010) screened bacteria strains for their antagonistic activity towards *C. destructans* on ginseng and isolated ten strains which were detrimental to the pathogen.

The notion of fungistasis was described by Dobbs and Hinson (1953) who observed that spore germination was inhibited in contact with soil and that the inhibition was removed by prolonged drying or autoclaving the soils. The fungistatic nature of soils has been attributed to the presence of high population of antagonistic microorganisms (Tamietti and Prametton, 1990; Toyota *et al*., 1996; Boer *et al.*, 2003), volatile compounds and non volatile compounds (Romine and Baker, 1973; Liebman and Epstein, 1992; Xu *et al*., 2004; Ozer *et al*., 2009), and soil physical and chemical properties (Ko and Hora, 1972; Ko *et al*., 1974). Ozer *et al*. (2009) observed that more than 70% of *Aspergillus niger* spores failed to germinate in 12 out of 27 soils studied and related it to volatile compounds while 12 fungal species were antagonistic against the fungus. In their study, fungistasis was not correlated to physical and chemical characters of soils. Boer *et al.* (2003) correlated fungistasis to the microbial community composition and the presence of pseudomonads. In this study, bacteria and fungi present in the field soil were not studied however Dore (2009) studied the bacterial populations associated with the grapevine rhizospheres from different rootstock varieties using the same soil source. She found that rootstock variety 101-14, which is susceptible to black foot disease (Dore, 2009) had fewer bacteria that were antagonistic towards *C. destructans* in dual culture than did more tolerant rootstocks. Further studies into the

interactions between rhizosphere bacteria population and fungal inhabitants and the different *Cylindrocarpon* species should be conducted. A better knowledge of microorganisms antagonistic towards *Cylindrocarpon* spp. could assist the development of biological controls by enhancing antagonistic microorganisms or applying strains to the soil (Alabouvette, 1999). However, the behaviour of inoculum in different soils is unpredictable due to the great variety of physical and biological conditions and thereby further experiments should investigate common chemical and physical factors known to affect activity of microorganisms.

The experiments investigating the different *Cylindrocarpon* propagules in field conditions and in different soil types in pots showed that chlamydospores caused similar disease incidences at 1 cm above stem bases to conidia when the different species were combined together, with means of 84.4 and 73.3% for the field and soil type experiments, respectively. However, *C. macrodidymum* chlamydospores caused low disease incidence in the field and in the different soil types with 6.3 and 5.0% for the field soil and the different soil types, respectively, while chlamydospores caused a disease incidence of 56.3% for plants grown in potting mix. It seems possible that chlamydospore germination was inhibited or that chlamydospores were damaged or degraded. Matturi and Stenton (1964) observed that chlamydospores needed a period to become mature and that newly formed *C. destructans* chlamydospores were damaged by pea root substances. Their results showed that 87 to 73% of 4 to 8 day old chlamydospores were severly damaged in the presence of roots and failed to germinate while 19% of 30 day old chlamydospores were damaged and 45% germinated. It is possible that *C. macrodidymum* chlamydospores require a longer time to overcome dormancy and infect plants.

As callused plants were used for the different experiments, the plants may have needed to develop roots to stimulate chlamydospores, a process that takes 2 - 10 weeks, depending on soil temperatures. Toussoun and Snyder (1961) reported that most *Fusarium solani* f. *phaseoli* chlamydospores germinated within 3 mm of bean tissue and the number of germinated chlamydospores rapidly decreased with distance from the tissue while no chlamydospore germinated in soil without plant tissue. They stated that the distance of the propagule from the host, physical and nutrient environment and the chlamydospore wall thickness determined the pathogenicity of *Fusarium solani* f. *phaseoli* chlamydospores in soil. Investigation of chlamydospore germination at different distances from grapevine roots and the time required for chlamydospore maturation for the different *Cylindrocarpon* spp. would provide more information on the epidemiology of the *Cylindrocarpon* species.

For the experiment investigating the pathogenicity of the different propagules in three soil types, it appeared that disease incidences caused by the different propagule inoculations

were not influenced by the different soil types as no interaction was observed between propagule treatments and soil types for the different species. However, the different soils affected the pathogenicity of the *Cylindrocarpon* spp. There were greater disease severities and incidences at 1 cm and 5 cm above stem bases in heavy soil than in medium and in light soils. This effect was only significant for *C. destructans* and *C. liriodendri*, not for *C. macrodidymum*. The different types of soil used in this experiment were a sandy loam, a silt loam and a clay loam. Sandy soils dry out and warm up most quickly while clay soils retain more water, dry out and warm up slowly (Bruehl, 1987). Black foot disease has been observed to develop preferentially in poorly drained heavy soils and particularly in areas where plants were waterlogged (Maluta and Larignon, 1991; Gubler *et al.*, 2004; Bonfiglioli, 2005) and this study has provided the evidence that supports those field observations.

The greater prevalence of other root and stem base diseases in heavy or wet soils has also been reported. Azhar *et al.* (2006) observed that *Sclerotium rolfsii* caused greater damping off incidences on chickpea seedlings in clay soils than sandy soils with 94, 82, 78 and 60% disease incidences for clay, clay loam, sandy loam and sandy soils, respectively. Jung *et al.* (2000) determined that oak trees were more susceptible to decline caused by *Phytophthora* spp. when grown on sandy loam to clay soils than sandy to sandy loam soils. However, Graff *et al*. (2005) did not correlate disease incidence and severity of *Spongospora subterranea* on potatoes to soil types but instead with soil moisture and reported that constant wetness of soil caused greater disease incidence and severity than fluctuating moisture. Increased soil moisture may have been responsible for the increase of disease in clay loam since it probably retained more water than the two other soils in this study. Effects of soil types on disease incidence has also been attributed to pH (Agrios, 2005) and *Cylindrocarpon* spp. have been reported to cause higher disease incidence in acid soils rather than alkaline soils, with *C. destructans* causing significantly greater lesions on ginseng at pH 5 than pH 7 (Rahman and Punja, 2005). However, Taylor (1964) observed that pH did not influence "*C. radicicola*" growth on different media although soil pH could influence other microorganisms interacting with the pathogen. In this study, the pH was approximately 6 (Roger McLenaghen pers. comm. 2010) for the three soil types selected. An experiment using different soil moistures, textures and pH would provide more information on the pathogenicity of *Cylindrocarpon* spp.

For the experiment investigating the pathogenicity of propagules in field soil, chlamydospores and conidia from all *Cylindrocarpon* spp. (species data combined) caused significantly higher disease incidence at 1 cm above stem bases than mycelium (84.4, 82.3 and 65.5% for chlamydospores, conidia and mycelium, respectively) while in the experiment investigating the pathogenicity of propagules in different soil types disease incidence was not significantly

different between the propagules (73.3, 85 and 78.3% for chlamydospores, conidia and mycelium, respectively). However, plants grown in potting mix showed differences between propagules, with conidium inoculation causing greater disease incidence (81.3%) than chlamydospore (62.5%) and mycelium (56.3%) inoculations. There were some differences between the species however in the two experiments using soil. Conidia from *C. destructans* and *C. macrodidymum* caused significantly greater disease incidences than chlamydospores and mycelium in the two experiments using soil while for *C. liriodendri*, chlamydospores caused greater disease incidence in the field than conidia and mycelium. However, no differences between propagules were observed with the different soil types. It is possible that conidia infected the plants faster than chlamydospores and mycelium. Matturi and Stenton (1964) observed high conidial germination in acid and alkaline soils in the first week while chlamydospores needed a maturation period. Mycelium growing on autoclaved wheat grains may also need a longer period to infect grapevine roots as the *Cylindrocarpon* spp. are then growing saprophytically. Alternatively, wheat grains might have provided nutrients for other saprophytes which may have diminished the pathogenicity of the mycelium.

The relative pathogenicity of the different species was constant in the field and pot experiment that used three different soil types, with *C. liriodendri* causing significantly greater disease incidences than *C. destructans* and *C. macrodidymum*, which also reflected the results obtained for plants grown in potting mix in Chapter 2. The results in this soil study also seemed to indicate presence of an indigenous population of *C. destructans* in the first field soil study and in the silt loam which originated from the same location. It was responsible for 35.4 and 25% disease incidences at 1 cm above stem bases for the plants inoculated with sterile water and grown in the field and silt loam, respectively. The site was previously used as an apple orchard and so supports the hypothesis that *C. destructans* propagules can accumulate in orchards and so can infect subsequent grapevine crops in New Zealand (Bonfiglioli, 2005).

The root dry weights did not differ between propagule treatments however they were significantly different between soil types, being higher in light soil than in heavy soil and medium soil with 14.5, 10.9 and 7.4 g, respectively. Tomasi *et al.* (2007) also observed that root development in grapevines was greater in sandy soil where the roots needed to search more deeply for water. This principle would indicated lowest root weights for heavy soil, however the soils were well aerated at the beginning of the experiment, which may have allowed greater drainage and accounted for the extensive root systems after 6 months. However, mean root dry weights for plants grown in potting mix for 5 months was similar to those of plants grown in medium soil for 6 months in pots. In contrast, the plants grown in the field exhibited the lowest root dry weights, which was probably due to the fluctuation of the

temperature, a dry summer and the less frequent watering of plants. Shoot dry weights for the different experiments in soil were reduced compared to those of plants grown in potting mix and this was probably due to the same conditions, as well as the lower humidity in the field caused by the wind and high temperature in summer.

The experiment studying the fate of mycelium and conidia in the soil environment showed that both propagules produced chlamydospores. These were reported to form germ tubes either at the terminal ends or on the sides of hyphae, while single conidia formed chlamydospores within their cells or combined the protoplasms of multiple conidia to form multiple chlamydospores. These observations were similar to those of Matturi and Stenton (1964) who described the behaviour of *Cylindrocarpon* spp. in soils with pH 7.4 and 3.8. They reported chlamydospore formation after 3 days incubation of conidia on glass slides in soil, but they did not provide photographs of the processes. The chlamydospores were reported to form germ tubes or hyphae in terminal or intercalary positions and in conidia where one to two cells formed resistant walls. They also reported the transfer of contents of one conidium to a second conidium linked by germ tubes and the formation of two to three cell chlamydospore clusters for "*C. radicicola*" (*C. destructans*) and "*C. album*" (*Neonectria punicea*). Taylor (1964) reported that most of *C. destructans* conidia did not germinate in acid and alkaline soils and a few formed chlamydospores within them while germinated conidia developed short germ tubes which formed chlamydospores. Similar observations were made with *Fusarium solani* f. sp. *phaseoli* (Nash *et al.*, 1961).

In this experiment, chlamydospores were not distinguished by their origin as conidia were also produced in mycelium bags and then formed chlamydospores. However, Matturi and Stenton (1964) recorded the number of chlamydospores that originated from conidia and hyphae and found that chlamydospores were mostly formed from conidia in unfavourable conditions for *C. destructans*, with 31.8 and 90.9% conidial chlamydospores for native soil with pH 7.4 and 3.8, respectively, after 12 days in the soils. Taylor (1964) reported that greater numbers of *C. destructans* chlamydospores was formed in alkaline soil than acid soil and Matturi and Stenton (1964) obtained similar results with four *Cylindrocarpon* species.

The patterns of chlamydospore development seemed to differ between the individual isolates of each species. For *C. destructans*, the transformation of conidia into chlamydospores seemed to occur shortly after conidia were placed in the bags as no intermediate stages of chlamydospore formation were observed at 2 weeks. However, for *C. liriodendri* and *C. macrodidymum*, the different stages of chlamydospore conversion were present in the conidium bags which indicate that the transformation occurred later or progressively. In the mycelium bags, *C. destructans* had the highest number of conidia although not significantly

different from the two other species. This species seemed to produce conidia faster than *C. macrodidymum* and *C. liriodendri*. The presence of chlamydospores that resembled a “figure eight” indicated an early transformation of a conidium into two chlamydospores. *Cylindrocarpon liriodendri* exhibited most stages of chlamydospore formation while only early stages of chlamydospore formation were observed for *C. macrodidymum* suggesting that *C. macrodidymum* produced chlamydospores more slowly than the two other species. Matturi and Stenton (1964) observed that for *C. destructans* chlamydospore formation reached a maximum after 4 days and decreased gradually with time while chlamydospore formation increased gradually for *Cylindrocarpon jantholele* var. *minus* for soil at pH 3.8.

After 3 weeks, the numbers of conidia in the conidium bags was greatly reduced and *C. destructans* had significantly fewer conidia than *C. macrodidymum* and *C. liriodendri*. The reduction of conidium numbers was 99.7, 98.9 and 96.0% for *C. destructans*, *C. macrodidymum* and *C. liriodendri*, respectively. Matturi and Stenton (1964) observed a reduction of the number of *C. destructans* conidia by 70 and 80% for soils at pH 7.4 and 3.8, respectively after 12 days while the decrease was lower for other species. They associated this reduction and the loss of mycelium to bacteria and lysis by undetermined factors and concluded that mycelium and conidia are transient in soil and are rapidly lysed by microorganisms which also activate chlamydospore formation. Chlamydospores remain dormant in soil without organic matter until they are stimulated by plants or destroyed by other microorganisms (Matturi and Stenton, 1964; Taylor, 1964). Taylor (1964) observed that in natural soil hyphal development was restricted and after 1 month, conidia and mycelium were absent while chlamydospores were present. However, in sterilised soil conidial germination and hyphal development were extensive, although mycelium disappeared rapidly after 1 month (Taylor, 1964). Nash *et al.* (1961) also observed a considerable amount of mycelium growth in autoclaved soil for *Fusarium solani* f. sp. *phaseoli* followed by chlamydospore formation.

Another factor contributing to the reduction in the number of conidia is the formation of chlamydospores which results from the combination of one to multiple conidial protoplasms, leaving empty cells. Chlamydospore formation was significantly higher for *C. liriodendri* than *C. macrodidymum* and *C. destructans*, which had the lowest number after 3 weeks. The low number of chlamydospores produced by *C. destructans* might be explained by the fact that the isolate produced mainly one to two celled conidia while *C. liriodendri* and *C. macrodidymum* formed mainly four celled conidia. Since the four-celled conidia were observed to form two chlamydospores each, this indicates that a higher cell number was required to form chlamydospores (Figure 4.15).

Chains of chlamydospores were only observed in mycelium bags however *C. macrodidymum* had not produced them after 3 weeks. This behaviour reflected the observations by other researchers (Halleen *et al*., 2004; Halleen *et al*., 2006) that on media chlamydospores were abundant within 14 days for *C. destructans*, common for *C. liriodendri* and rare for *C. macrodidymum*. The presence of soil may also affect chlamydospore production, as suggested by Reeleder *et al.* (2003) who used soil extracts in their chlamydospore production system with *C. destructans* mycelium, which they believed helped to develop chlamydospores.

This experiment was carried out to determine the fate of mycelium and conidia of *Cylindrocarpon* spp. in soil. However, it did not investigate differences between isolates from each species, different soil types and pH of soil. The use of shorter incubation times in the soils could provide more evidence of the processes and numbers of chlamydospores from mycelium in different soil types and from germinated and ungerminated conidia. Use of multiple isolates of each of the three species could provide more extensive knowledge of the spectrum of conversion behaviour of the different *Cylindrocarpon* spp. Considerably more research is needed into the role of chlamydospores in the development of black foot would be well understood, especially with respect to infection potential of soils over time.

In summary, the present study showed that disease incidences for grapevines grown in natural soils were lower than those grown in potting mix, probably due to the interaction of the pathogen with other microorganisms present in soil. Heavy soils seemed to facilitate *Cylindrocarpon* infection compared to lighter soil types, however, the pathogenicity of the different species was similar to previous experiments (Chapter 2) with *C. liriodendri* isolates being more pathogenic than isolates from the other two species. As with the previous experiments conducted in potting mix, all propagules were able to infect. However, the relatively greater pathogenicity of chlamydospores and mycelium were probably due to the soil environment. Conidia seem to germinate faster than chlamydospores and infect grapevines if present while, in the absence of a suitable host, conidia and mycelium probably produce chlamydospores which can remain dormant in the soil. The following chapter will investigate methods to test soil for presence of the pathogen and propagule numbers.

CHAPTER 5

Development of a soil-testing method to identify *Cylindrocarpon* infested soils

5.1. Introduction

Research investigating the presence of *Cylindrocarpon* spp. in grapevines from nurseries and vineyards suggests that soil constitutes a major source of inoculum (Armengol *et al*., 2001; Rego *et al.*, 2001b; Halleen *et al.*, 2003; Gubler *et al.*, 2004) and that the pathogen is capable of existing for long periods as a saprophyte or as thick walled chlamydospores (Booth, 1967).

To isolate fungal pathogens in the soil, different techniques are available. The most commonly used method is soil dilution plating, which consists of suspending soil in sterile water and plating dilutions of the suspension on an agar medium to isolate the fungi (Davet and Rouxel, 2000). The colonies are then counted and the population density per gram of soil can be deduced by multiplication of the colony numbers and the dilution from which they were obtained. This method is not suitable for *Cylindrocarpon* spp. because they are difficult to identify morphologically and may be outcompeted by other fast growing organisms (Kernaghan *et al.*, 2007). Soil dilution plating is also time-consuming and so large numbers of samples cannot be processed (Luo *et al*., 2009).

An alternative is to use a baiting method. This method has been described as any method using a substrate that stimulates the growth of a species or a group of species in the presence of a natural community of microorganisms (Davet and Rouxel, 2000). The principle of this method is to select the bait best adapted for the detection of the desired fungus (Davet and Rouxel, 2000). Different types of baits are used in plant pathology: inert baits, whole plants or pieces of living plants, and these methods are well known for the isolation of *Phytophthora* spp. and *Pythium* spp. The first aim of this chapter was to develop a baiting method suitable for the detection of *Cylindrocarpon* species in soil. Although baiting methods may not give accurate quantitative results, they should be easily reproducible by winegrowers in their vineyard to determine if the soil is appropriate for planting grapes.

For a soil assay to be useful in routine screening, it needs to be at least semi-quantitative, to be repeatable and specific (Keller *et al.*, 1995). Rapid and reliable methods to detect the pathogen in the soil would be useful for monitoring the efficacy of fungicides, fumigants, soil

amendments and other cropping practices to control *Cylindrocarpon* root rot (Seifert *et al.*, 2003). The same methods would also be helpful for studying the infection cycle of *Cylindrocarpon* spp. and for identifying inoculum potential (Keller *et al.*, 1995; Damm and Fourie, 2005). Nested PCR has been used to detect the presence of soilborne pathogens (Williams *et al*., 2009). This method was more efficient than using a bait for detection of *Phytophthora cinnamomi* (Williams *et al*., 2009). An alternative method is using quantitative PCR (qPCR). This method has been successfully used to quantify fungal pathogen populations from soil (Kernaghan *et al.*, 2007). It relies on the availability of sufficient sequence information to design primers specific for the pathogen species. However, an adequate extraction method to isolate the pathogen DNA from soil is also required in order to apply the qPCR. The second aim of this chapter was to develop a soil-testing method to identify infested soils using nested and qPCR and to use this technique to determine the longevity of spores in the soil.

5.2. Materials and methods

5.2.1. Preliminary experiments to test different baiting methods

5.2.1.1. Apple bait

The apple bait method used by Mwanza and Kellas (1987), which succeeded in isolating *C. destructans* from soil, was tested. Potting mix (1 L) was placed into a double layer of autoclavable bags (305 x 660 mm) and autoclaved at 121°C for 15 min at 15 psi. A conidium suspension from *C. destructans* isolate D2 (Appendix 1) was made as described in Section 2.2.1.2 with a final conidium concentration of 10^6 conidia /mL. The suspension was diluted to 10^5 and 10^4 conidia /mL using sterile distilled water and 1 mL of each of the three conidia suspensions was mixed with 60 g of autoclaved potting mix. Apple (*Malus domestica*) varieties Granny Smith and Braeburn were used as bait with three apples for each treatment. The apples were surface disinfected under a laminar flow hood by spraying 70% ethanol onto their skins until each surface was completely covered. A 3 cm diameter slot cut to a 1 cm depth was made on the surface of each apple and 3 g of infested potting mix was then placed into the slot which was covered with the apple piece. The apples were placed in a 23 × 16 × 8 cm box without a lid, covered with a plastic bag and incubated at 20°C in the dark. After 6 days, 8 pieces (2 × 2 × 2 mm) were cut from the lesion areas in each apple and they were plated onto PDA (Oxoid Ltd, Basingstoke, UK) amended with 250 mg /L of chloramphenicol (Sigma Sigma-Aldrich, St. Louis, USA) within a laminar flow hood. The plates were wrapped with cling film and incubated at 20°C in the dark for 1 – 2 weeks. The number of apples infected with *C. destructans* and other fungal species (out of the three replicate apples) for each concentration and apple variety was recorded.

The experiment was repeated as described previously using the same conidium concentrations mixed with 60 g of non autoclaved garden soil instead of potting mix. Three replicates were used for each time and apples were assessed 5 and 7 days after infestation. Apples incubated for 5 days were assessed as previously described using ten pieces of apple for each replicate, however apples incubated for 7 days were cut in two and ten pieces of apples were taken from lesions in the apple flesh and ten pieces were taken from underneath the apple skin of the lesions. The number of apples infected with *C. destructans* and other fungi for each concentration, apple variety and sample location was recorded. Control plants were not used in these experiments as only the suitability of apple bait to recover *C. destructans* was tested.

5.2.1.2. Seedling bait

A number of vegetable species were selected for their use as seedling bait based on the reported incidence of *Cylindrocarpon* spp. in these hosts and the likely ease of growth by wine growers. Control seedlings were not used for the following experiments as seedlings were being tested for their ability to be infected with *C. destructans*.

A conidium suspension from *C. destructans* isolate D2 was made up as described in Section 2.2.1.2 at a concentration of 10^6 conidia /mL and 100 mL of the suspension was mixed in a 35 × 27 × 7 tray with 6 L of potting mix which was previously autoclaved as described in Section 5.2.1.1. A preliminary experiment used seeds of spinach (*Spinacea oleracea*) hybrid n°7, red clover (*Trifolium pratense*) and pea (*Pisum sativum*) variety Easy Peasy. The seeds were sowed in 3 rows, 3 cm apart in trays with the infested potting mix. The seedling trays were kept for 18 to 26 days at room temperature and were watered frequently, as needed. Symptomatic seedlings were harvested by gently uprooting them, the roots were washed with tap water and the seedlings were placed on paper towels. They were surface sterilised by soaking in 70% of ethanol for 30 s, 0.35% of sodium hypochlorite for 1 min and 70% of ethanol for 30 s under a laminar flow hood. The seedlings were then placed onto a glass Petri dish. Four 2 mm transversal pieces of root and stem were cut and placed onto PDA with chloramphenicol. The plates were sealed with cling film, incubated at 20°C in the dark for 7 days and the proportion of seedlings infected with *C. destructans* and other fungal species was determined.

The experiment was repeated using tomato (*Solanum lycopersicum*) variety Moneymaker, pea and spinach seeds as above. The spinach and tomato seedlings were harvested and assessed as previously after 21 and 43 days, respectively, while pea seedlings were assessed after 18 and 21 days, by taking root pieces as above and four cotyledon pieces. The proportion of seedlings infected with *C. destructans* and other fungal species for the different plant tissues was determined.

The experiment was repeated using tomato, spinach and pea seeds (same varieties as above) sowed in a 50/50 v/v mixture of non autoclaved garden soil and autoclaved potting mix, infested with *C. destructans* and assessed as above.

A second vegetable bait experiment was conducted using a 50 /50 v /v mixture of garden soil and potting mix with seedlings of tomato variety Moneymaker and bean (*Phaseolus vulgaris*) variety Dwarf French Tenderness. The tomato and bean seeds were germinated on filter paper soaked with sterile water in Petri dishes. The seedlings were then grown in vermiculite until roots reached 5 – 10 cm long. A conidium suspension using a mixture of three isolates for each species (*C. liriodendri* isolates L1, L2 and L3 *, C. destructans* isolates D1, D2 and D3*,* and *C. macrodidymum* isolates M1, M2 and M3; Appendix 1) were obtained as in Section 2.2.1.2, with a final concentration of 10^5 conidia /mL. The roots of each seedling were wounded by cutting off root tips before transplanting one seedling per 500 mL pot containing a 50/50 v/v mix of autoclaved garden soil and autoclaved potting mix. Beside each plant stem base, a hole was made in the soil and 1 mL of the spore suspension was poured. The treatments (control, *C. liriodendri, C. destructans* and *C. macrodidymum*) were replicated five times and laid out in a randomised block design. The seedlings were left to grow for a month at room temperature and watered regularly. They were surface sterilised as previously and a 1-2 mm piece was taken from the upper and lower stem parts, the base, upper, intermediate and lower root parts of each plant. The pieces were plated onto PDA with chloramphenicol and incubated as previously. The seedlings were assessed for the presence of *Cylindrocarpon* spp. The infection incidences were analysed using a general linear model with a binomial error distribution with Genstat version 12.

The experiment was repeated with 12 week old tomato plants purchased from a nursery. The plants were wounded and planted in 500 mL pots with a 50/50 mix v/v of non sterile garden soil and potting mix. The soil beside each plant was infested with 1 mL of 10^7 conidia /mL as previously. Five replicates were used for the different species and the control in a randomised block design.The plants were harvested 12 days after inoculation and assessed as above for the presence of *C. destructans*.

5.2.1.3. Parsnip assay

Conidium suspensions of *C. liriodendri* isolates L1, L2 and L3, *C. macrodidymum* isolates M1, M2 and M3 and *C. destructans* isolates D1, D2 and D3 were made as in Section 2.2.1.2 at concentrations of 10^2 and 10^4 conidia /mL. Parsnip roots (*Pastinaca sativa*) were cut transversally into 5 mm deep pieces, which were each placed onto a 10 cm diameter filter paper, for which 10 were arranged on paper towels in a 31 × 42 cm tray. The filter paper and towels were soaked with sterile water. Each parsnip piece was inoculated on its upper cut

surface with 100 µL of conidium suspension. Treatments were replicated 5 times per conidium concentration and laid out in randomised blocks. The trays were covered with a plastic bag containing wet paper towels to maintain high humidity and the parsnips incubated at 20°C in the dark. After 8 days, the parsnip pieces were assessed visually for infection. Samples that visually appeared to be infected by fungi were plated onto PDA with chloramphenicol (250 mg /L) and incubated for 7 days at 20°C in the dark. The plates were assessed for the presence of *Cylindrocarpon* spp.

5.2.2. Soil testing diagnostics

5.2.2.1. DNA extraction method

Two methods for extracting DNA from soil were tested. The first method used the dilution plating principle and the second method was based on the one described by Yeates *et al.* (1998). For both methods, a conidium suspension was obtained as described in Section 2.2.1.2 for isolates L1, D2 and M2 (Appendix 1) with final concentrations of 10^7 conidia /mL. The suspensions were diluted to 10^5 and 10^3 conidia /mL with sterile water. Conidia from each species were mixed with 50 g of field soil in deep Petri dishes to achieve a final concentration of 0, 10^2, 10^4 or 10^6 conidia of each species per 10 g of soil.

Method 1

This method was based on the premise of dilution plating whereby it is assumed that when the soil is mixed with water and agar and the soil is allowed to sediment, all (or most) of propagules in the soil will remain suspended in the liquid suspension. For each treatment, 10 g of inoculated soil was placed in a 250 mL bottle and 90 mL of autoclaved distilled water with 0.01% agar was added. The bottles were placed on a wrist action shaker (Griffin and George Ltd., London, UK) and were shaken for 10 min and then left to stand for 3, 6 or 10 min. Two replicates were used for each treatment combination. After standing, approximately 75 mL of each supernatant was placed into two sterile 50 mL centrifuge tubes. The tubes were centrifuged at 3220 × *g* for 15 min. The supernatant was discarded and the two pellets for each replicate were combined into a single 50 mL centrifuge tube using 5 mL sterile distilled water. The tubes were centrifuged at 3220 × *g* for a further 15 min and the supernatant was discarded. One third of the pellet was used for DNA extraction with the PowerSoil® DNA isolation kit (MoBio Laboratories, Carlsbad, CA) according to manufacturer's instructions. The DNA was suspended in a final volume of 100 µL of TE.

Method 2

This method is based on that of Yeates *et al*. (1998). From the inoculated soil, 10 g was placed in a 100 mL bottle with 10 mL of extraction buffer (Appendix 2) and 10 g of 0.5 mm

diameter glass beads (BioSpec Products, Inc., OK, USA). The bottles were shaken for 8 min on a wrist action shaker (Griffin and George Ltd.) after which, 1 mL of 20% (w/v) sodium dodecyl sulphate (SDS) was added and the bottles were shaken for a further 2 min. The bottles were then incubated at 65ºC for 1 h before the contents were transferred into 50 mL centrifuge tubes. The tubes were centrifuged at 3220 × *g* for 10 min. The supernatant was transferred to a clean 50 mL centrifuge tube. The pellet was re-extracted with 10 mL of extraction buffer and centrifuged for 10 min at 3220 × *g*. The supernatant was again transferred into a 50 mL centrifuge tube to which contained half volume of 30% (v/v) polyethylene glycol /1.6 M NaCl. The solutions were incubated at room temperature for 2 h. They were centrifuged at 3220 × *g* for 25 min, each pellet was suspended in 2 mL of TE (Appendix 2) and sufficient 7.5 M potassium acetate was added to each tube to reach a final concentration of 0.5 M. The solution was placed on ice for 5 min then centrifuged at 3220 × g for 35 min at 4ºC. The aqueous phase was extracted with phenol /chloroform as described by Whiteman (2004). Typically, the supernatant was placed into 15 mL centrifuge tubes and 4 mL each of phenol and chloroform was added and the solutions mixed by inversion five times. The tubes were centrifuged at 600 × *g* for 4 min. The aqueous phase was placed into a new tube and 4 mL each of phenol and chloroform was again added. The solution was centrifuged at 600 × *g* for 4 min and the aqueous phase was put into 15 mL centrifuge tubes. To remove residual phenol one volume of chloroform (approximately 2 mL) was added to each tube and they were centrifuged at 600 × *g* for 4 min. The aqueous phase was placed into a new tube and 1/10 volume of 3 M sodium acetate was added followed by two volumes of ice-cold 100% ethanol. The solution was stored in the freezer (-20ºC) overnight.

Each solution was thawed, separated into two equal portions in 2 mL centrifuge tubes which were centrifuged at -5°C at 20,817 × *g* for 5 min. Each supernatant was discarded and the pellet was washed twice with 1 mL of 70% ethanol. The tubes were centrifuged at 20,817 × *g* for 2 min at -5°C. Each pellet was air dried for 15 min and re-suspended overnight in 75 µL of SNW. The DNA solutions derived from the same frozen solution were recombined at this stage. DNA was visualised by electrophoresis on a 1% agarose gel as described in Section 3.2.2.

5.2.2.2. Nested species specific PCR

An initial PCR using the universal fungal primers ITS4 and ITS1F (Chapter 3, Table 3.1) was conducted to demonstrate that the DNA was suitable for PCR and that it was not inhibited by any contaminants co-purified with the DNA. The PCR reaction mix consisted of 1 × PCR buffer, 200 µM of each dNTP, 0.2 µM of each primer, 1 U of Taq DNA polymerase (Roche), 0.2, 0.5, 1 or 2 µL of the DNA extract and SNW to a final volume of 25 µL.

DNA extracted from soil samples containing no *Cylindrocarpon* spores at the 3 standing times from Method 1 and the 0 conidium concentration in Method 2 was amplified using either 0.2, 0.5, 1 and 2 µL of DNA to determine whether the DNA amplification was inhibited. A negative control with SNW in place of DNA and a positive control (10 ng of DNA extracted from pure culture of isolates M1, L1 or D2) were used. The PCR conditions were as follows: 94°C for 3 min followed by 30 cycles of 30 s at 94°C, 30 s at 50°C and 30 s at 72°C with a final extension at 72°C for 7 min. PCR products were separated by electrophoresis on a 1.5% agarose gel and visualised under UV light using ethidium bromide staining as described in Section 3.2.2.

The nested PCR developed in Section 3.2.4 was employed using the optimal DNA quantity found above in the first PCR using general tubulin primers and the products were diluted 1/100 for the second (species specific) PCR. The PCR products were sequenced to determine the specificity of the primers and the sequences were aligned on GenBank using BLAST as in Section 3.2.5.

5.2.2.3. Quantitative PCR

Quantitative PCR was used to increase the detection sensitivity of the PCR and to quantify the numbers of spores in soil.

5.2.2.3.1. DNA extraction from cultures

DNA from isolates L1, L2, D1, D2, M2 and M3 (Appendix 1) was extracted from pure cultures as described in Section 3.2.2. The extracted DNA was visualised by electrophoresis on a 1% agarose gel as described in Section 3.2.2. The extracted DNA was quantified by spectrophotometry using a Nanodrop-ND-1000 spectrometer and was diluted to 30 ng, 3 ng, 0.3 ng, 30 pg, 3 pg and 1 pg of DNA /µL.

5.2.2.3.2. DNA extraction from conidia

A conidium suspension was made as described in Section 2.2.1.2 with a concentration of 10^6 conidia /mL for isolates L1, L2, M2, M3, D1 and D2. For each isolate, 1 mL of the conidium suspension was placed into a 1.5 mL centrifuge tube and was centrifuged at 20, 817 × g for 15 min. The supernatant was discarded and DNA was extracted using a PowerSoil kit (MO BIO Laboratories Inc.) according to the manufacturer's instructions. The resulting DNA sample was serially diluted 10 fold with SNW to achieve solutions of DNA with the equivalent of 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 conidia per 100 µL.

5.2.2.3.3. DNA extraction from soil over time

A mixed isolate conidium suspension (10^6 conidia /mL) was obtained as described in Section 2.2.1.2 using a three isolate mixture for each species (D1, D2, D3, L1, L2, L3, M1, M2 and M3) and 500 mL of each conidium suspension was mixed with field soil in 5 L pots to achieve a final concentration of 10^5 conidia /g of soil. Controls were treated with a similar quantity of water and three replicates were used per treatment. The 5 L pots were sunk into and levelled with the ground in the Lincoln University vineyard in a randomised design. Once a week two 15 g soil samples were taken from each pot, placed individually into 50 mL tubes and stored at -80°C prior to DNA extraction. DNA was extracted from samples at 0, 1, 2, 3 and 6 weeks after set up using Method 1 as described in Section 5.2.2.1.

5.2.2.3.4. Quantitative PCR

Each reaction consisted of 1 × PCR buffer, 200 µM dNTP, 50 pM of each species specific primer (Cyde F1 small /Cyde R2 for *C. destructans*, Cyma F1 /Cyma R1 for *C. macrodidymum* and Cyli F1 /Cyli R1 for *C. liriodendri*; Chapter 3, Table 3.1), 1 U of Taq DNA polymerase (Roche), 0.28 µL diluted Sybr Green I (Invitrogen™, CA, USA; Appendix 2), 0.4 µL Rox (Invitrogen™) and SNW to a final volume of 19 µL. Each reaction mix was placed into a single well on a 96 PCR well plate (Axygen Scientific, CA, USA) on a MicroAmp® splash free 96 well base (Applied Biosystems Inc, CA, USA) and 1 µL of the appropriate sample DNA or in the case of the negative control, SNW, was added to the mix. In addition to sample DNA, standard curves were developed for DNA extracted from cultures (30 ng to 1 pg) and DNA extracted from conidia (10^6 to 10^1 conidia per mL). Each PCR reaction was conducted in duplicate. The plate was covered with a PCR plate cover seal and was spun in a centrifuge for 3 min at 400 × *g*. A compression mat was placed onto the plate which was then placed into an ABI Prism 7000 Sequence detection system (Applied Biosystems Inc.). The qPCR optimised in Section 3.2.5 was used for each species. The PCR products were sequenced to determine the specificity of the primers and the sequences were aligned on GenBank using BLAST as in Section 3.2.5.

5.3. Results

5.3.1. Baiting soil with susceptible plant material

5.3.1.1. Apple bait

For the apples inoculated with infested autoclaved potting mix, the baiting method resulted in successful isolation of *C. destructans* from both apple varieties. A concentration of 10^6 conidia /mL resulted in the infection of all three apples of both varieties (Table 5.1). With the 10^4 conidia /mL, the pathogen was not recovered from apple variety Braeburn while *C.*

destructans was recovered from one apple from the variety Granny Smith. *Pythium* spp. and *Penicillium* spp. were also isolated from inoculated apples and the number of apples infected with these fungi species is indicated in Table 5.1.

Table 5.1. Number of apples (out of three) from the varieties Granny Smith and Braeburn infected with species of fungi 6 days after inoculation with different concentrations of *C. destructans* conidia mixed with autoclaved potting mix (*C. dest*.: *C. destructans*; *Trichod*.: *Trichoderma spp*.)

Variety	Concentration (conidia /mL)	Apples infected fungal species					
		C. dest.	*Trichod.*	*Rhizopus*	*Penicillium*	*Fusarium*	*Pythium*
Granny Smith	10^4	1	0	0	1	0	3
	10^5	0	0	0	0	0	3
	10^6	3	0	0	1	0	0
Braeburn	10^4	0	0	0	0	0	3
	10^5	0	0	0	1	0	3
	10^6	3	0	0	1	0	0

For the apples infested with conidia mixed with garden soil, *C. destructans* was not recovered from apples after 5 and after 7 days (Tables 5.2 and 5.3, respectively). Other fungal species were present in the apples, such as *Fusarium* spp., *Pythium* spp., *Penicillium* spp. and *Rhizopus* spp. and the number of apples infected with these fungal species is indicated in Tables 5.2 and 5.3.

Table 5.2. Number of apples (out of three) from the varieties Granny Smith and Braeburn, infected with species of fungi 5 days after inoculation with *C. destructans* mixed with garden soil (*C. dest*.: *C. destructans*; *Trichod*.: *Trichoderma spp*.).

Variety	Concentration (conidia /mL)	Applesinfected fungal species					
		C. dest.	*Trichod.*	*Rhizopus*	*Penicillium*	*Fusarium*	*Pythium*
Granny Smith	10^4	0	1	1	1	1	3
	10^5	0	0	1	2	1	3
	10^6	0	0	1	1	0	3
Braeburn	10^4	0	1	2	3	1	3
	10^5	0	0	1	3	1	3
	10^6	0	1	2	3	1	3

Table 5.3. Number of apples (out of three) from the varieties Granny Smith and Braeburn infected with species of fungi 7 days after inoculation with different concentrations of *C. destructans* mixed with garden soil after 7 days and located in the apple flesh (1) or underneath the apple skin (2).

Variety	Concentration (conidia /mL)	Location on apple	Apples infected fungal species		
			C. destructans	*Pythium* spp	*Penicillium* spp.
Granny Smith	10^4	1	0	3	1
	10^4	2	0	3	2
	10^5	1	0	3	0
	10^5	2	0	3	1
	10^6	1	0	3	1
	10^6	2	0	3	2
Braeburn	10^4	1	0	3	3
	10^4	2	0	3	0
	10^5	1	0	3	2
	10^5	2	0	3	2
	10^6	1	0	3	1
	10^6	2	0	3	0

5.3.1.2. Seedling bait

In the preliminary experiment using infested autoclaved potting mix, spinach and peas showed desiccation symptoms 3 weeks after seed sowing. For the spinach, 80 and 78% of the symptomatic tissue samples contained *C. destructans*, 22 and 26 days, respectively, after seed sowing (Table 5.4). For the peas 83.7, 100 and 100% of symptomatic pea tissues contained *C. destructans* 18, 21 and 26 days, respectively, after seed sowing. The red clover did not germinate and thus this treatment was discarded.

The experiment was repeated with tomato, spinach and pea seedlings grown on infested autoclaved potting mix. For the spinach, the pathogen was isolated more frequently from roots (90% of infection) than stems (42.2%; Table 5.5). For the pea seedlings, *C. destructans* was found more frequently in cotyledons (93.3%) than roots and stems which had an identical percent of infected pieces (48.4%). Tomato seedlings did not show external symptoms and were harvested 43 days after planting. Their roots had a higher number of infected pieces (55.6%) than their stems (32.2%).

Table 5.4. Proportion of infected spinach and pea seedlings (%), when grown on autoclaved potting mix infested with *C. destructans*.

Plants	Days after sowing	*C. dest.*	*Penicillium* spp.	*Rhizopus* spp.	*Pythium* spp.	*Fusarium* spp.
Spinach	22	80.0	20.0	0.0	3.3	16.7
	26	78.0	35.0	12.0	4.0	0.0
Pea	18	83.7	30.2	27.8	0.0	0.0
	21	100.0	1.7	23.7	0.0	0.0
	26	100.0	12	48.0	0.0	0.0

Table 5.5. Proportion of infected spinach, pea and tomato seedlings (%), grown on autoclaved potting mix infested with *C. destructans*.

plants	Days after sowing	Tissue	*C. dest.*	*Penicillium* spp.	*Rhizopus* spp.	*Pythium* spp.	*Fusarium* spp.
Spinach	21	roots	90.0	20.0	8.9	0.0	10.0
		stem	42.2	12.2	2.2	3.3	0.0
Pea	18	stem	48.4	0.0	48.4	6.5	0.0
		cotyledons	93.3	0.0	50.0	10.0	0.0
		roots	48.4	0.0	52.2	4.0	0.0
	21	stem	62.8	0.0	36.0	14.0	0.0
		cotyledons	80.0	0.0	16.3	24.0	0.0
Tomato	43	roots	55.6	13.3	56.7	0.0	5.6
		stem	32.2	33.3	32.2	0.0	4.4

Peas, spinach and tomatoes grown on soil infested with *C. destructans* did not show any external infection symptoms and when assessed, the pathogen was not isolated from the different tissues.

Tomato and bean seedlings which were wounded before planting into a 50/50 autoclaved soil and potting mix mixture were found to be infected with the different *Cylindrocarpon* spp. (Table 5.6). The different species were mostly isolated from the lower part of the stem and on the upper parts of the roots. The tomato seedlings showed characteristic symptoms of infection by *Cylindrocarpon* spp. which was associated with pathogen presence. For the tomato seedlings, the number of infected plants was similar for the different pathogen species whereas for the bean seedlings, *C. liriodendri* was isolated more frequently than the two other species. The bean seedlings did not show characteristic symptoms. Tomatoes were therefore tested with infested non-sterile soil.

Table 5.6. Mean proportion of infected tomato and bean seedlings (%) which were wounded before planting into an autoclaved 50/50 potting mix and soil mixture, infested with *C. destructans*.

Plant	Treatment	Stem upper	Stem Lower	Stem Base	Root upper	Root middle	Root lower
Tomato	Control	0.0	0.0	0.0	20.0	0.0	0.0
	C. destructans	60.0	80.0	40.0	40.0	20.0	0.0
	C. macrodidymum	60.0	100.0	80.0	80.0	20.0	0.0
	C. liriodendri	60.0	80.0	80.0	80.0	0.0	20.0
Bean	Control	0.0	20.0	0.0	0.0	0.0	0.0
	C. destructans	20.0	40.0	20.0	20.0	0.0	20.0
	C. macrodidymum	20.0	0.0	20.0	0.0	0.0	0.0
	C. liriodendri	20.0	80.0	60.0	60.0	40.0	20.0

None of the root wounded tomato seedlings grown on infested non sterile soil with potting mix (50/50) showed any decline symptoms and none of the *Cylindrocarpon* spp. were isolated from the plants.

5.3.1.3. Parsnip assay

Parsnip root pieces inoculated with different concentrations of the three *Cylindrocarpon* spp. failed to show any visible rots or lesions and when assessed for the presence of the pathogen, *Cylindrocarpon* spp. were not isolated.

5.3.2. Soil testing diagnosis

5.3.2.1. DNA extraction method

The two DNA extraction methods were shown to successfully extract DNA from the fungal propagules since a portion of the DNA that encoded the rRNA genes encompassing the ITS and 5.8S regions was amplified from the DNA extracts.

As the DNA extracts may have contained impurities that inhibited PCR different volumes (2, 1, 0.5 and 0.2 µL) were tested to determine the minimum volume that could produce consistent amplification. For Method 1 (Figure 5.1 A), PCR products of approximately 550 and 600 bp were visualised on a gel for all samples except for 2 µL of DNA with a standing time of 6 min and for 1 µL with a standing time of 3 min. For Method 2 (Figure 5.1 B), faint bands were present for all DNA samples using 0.2 µL of DNA and for 10^3 conidia /g of soil using 1 µL of DNA whereas bright bands were only observed for 10^5 conidia /g of soil using 1 µL of DNA. The negative controls for both methods did not amplify any products and the positive control which consisted of 1 µL of DNA from isolate M1 showed a band at 550 bp.

From these results, Method 2 was discarded and Method 1 was used for the following DNA extraction from soil.

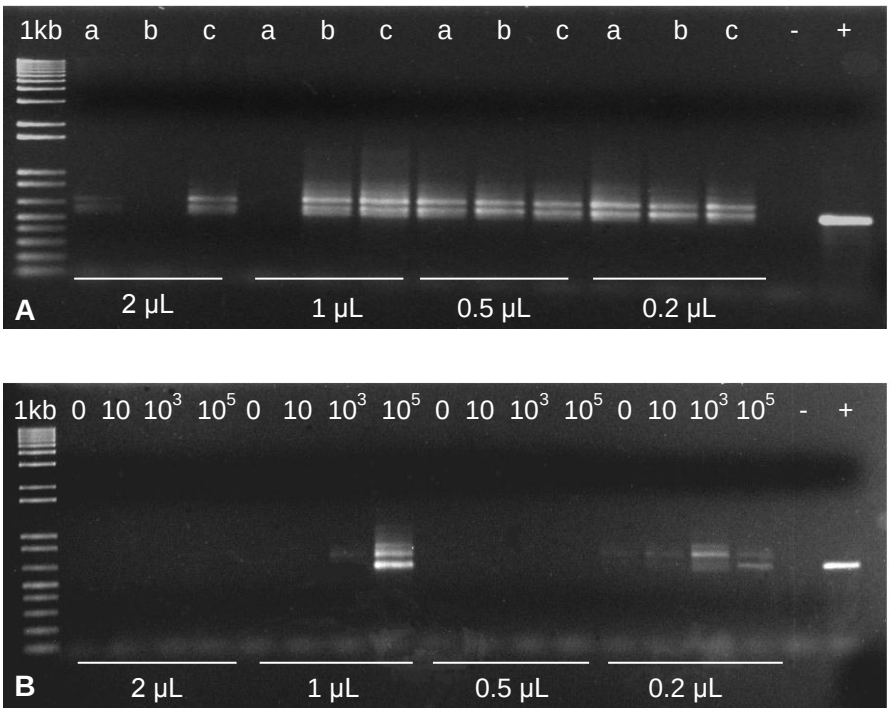

Figure 5.1. PCR products obtained with the universal fungal primers ITS4 and ITS1F, using 2, 1, 0.5 and 0.2 µL of DNA extract from 10 g of soil mixed with 0, 10, 10^3 and 10^5 *C. destructans* conidia /g using two DNA extraction methods (- : negative control; + positive control). A: Method 1 using soil without *Cylindrocarpon* spores (standing times of a: 3 min, b: 6 min and c: 10 min). B: method 2 using the four quantities of spores.1kb – 1 kb plus DNA Ladder (Invitrogen).

5.3.2.2. Nested species specific PCR

When 0.2 µL of DNA was selected for use in the first PCR of the nested PCR, it produced bands of similar brightness for all standing times compared to the 0.5 and 1.0 µL quantities and therefore was the least likely to inhibit the PCR.

The nested PCR using species specific primers for *C. macrodidymum* (Figure 5.2 A) amplified a 300 bp product for DNA extracted from soil containing 10^5 conidia /g of soil with all three standing times. For *C. liriodendri* (Figure 5.2 B), a 200 bp band was amplified for DNA extracted from soil containing 10^5 conidia /g of soil with standing times of 6 min and 10 min. For *C. destructans* (Figure 5.2 C), PCR products were observed on a gel for DNA extracted from soil containing 10^3 and 10^5 conidia /g of soil with standing times of 6 and 10 min. However, a 200 bp band was also present for soil to which *Cylindrocarpon* conidia had not been added. It was very faint with a standing time of 6 min and very strong with a 10 min standing time, possibly indicating either cross contamination or that the soil contained a

resident population of *C. destructans*. None of the negative controls showed a band and all the positive controls amplified a product of the appropriate size. The sequencing result showed that the primers were specific to the different species.

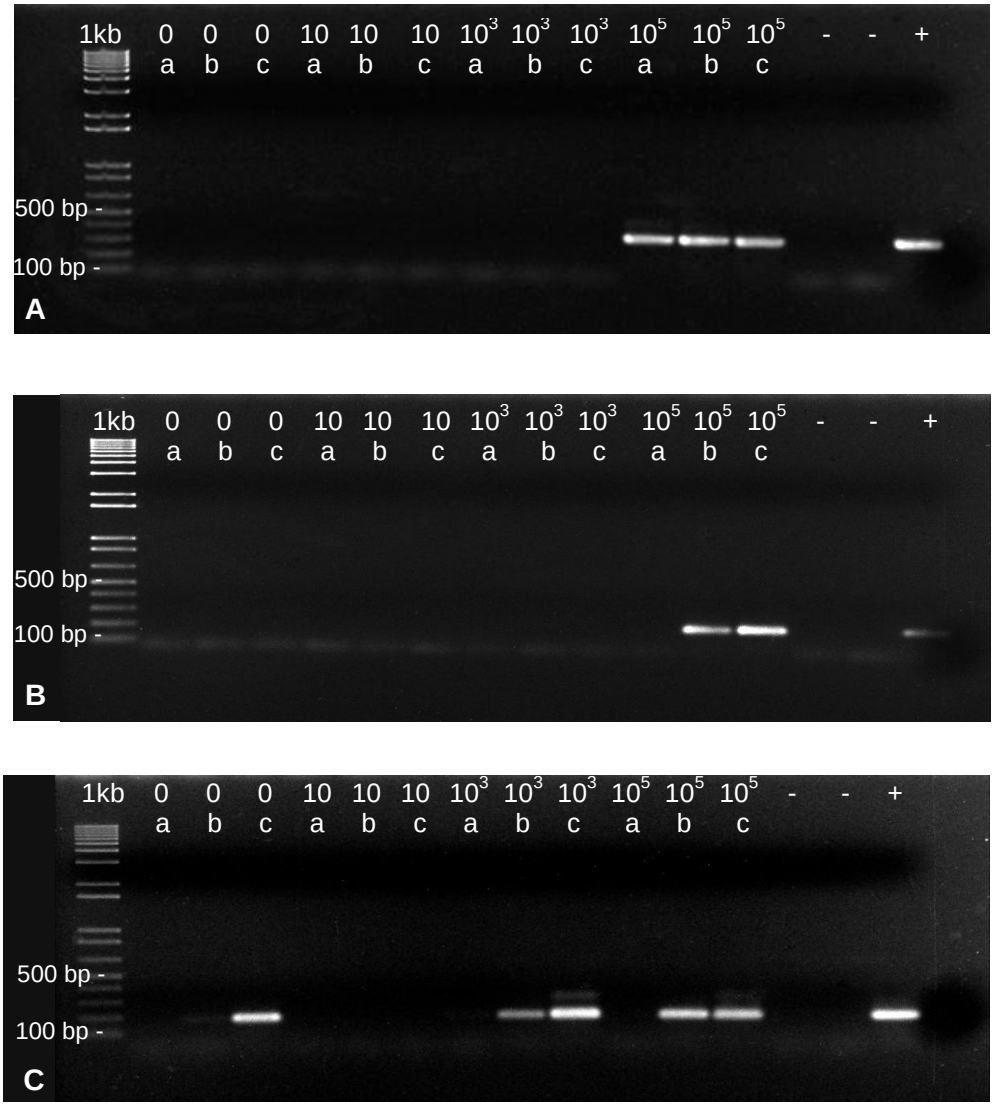

Figure 5.2. PCR products obtained with species specific primers in a nested PCR of DNA extracted from soil mixed with 0, 10, 10^3 and 10^5 *Cylindrocarpon* conidia /g of soil using DNA extraction Method 1 (- : negative control; + positive control; standing times of a: 3 min, b: 6 min and c: 10 min). A: *C. macrodidymum*. B: *C. liriodendri*. C: *C. destructans*. 1kb – 1 kb plus DNA Ladder (Invitrogen).

The pathogens were detected in soil containing conidia at a concentration of 1×10^5 conidia /g of soil. To determine the detection sensitivity of the nested PCR in terms of pg DNA and conidia the following calculations were applied:

To extract DNA using Method 1, 10 g of soil containing 10^6 conidia was initially suspended in autoclaved distilled water with agar and 1/3 of the pellet was used for DNA extraction. Thus, DNA from approximately 3.3×10^5 conidia was extracted and resuspended in 100 µL:

Number of conidia from which DNA was extracted (Y) = (X conidia /10 g soil) × 10 g /3

As only 0.2 µL ($1/500^{th}$) of the resultant DNA extract was used for the first PCR and only the 10^6 concentration produced an amplimer for *C. macrodidymum* and *C. liriodendri*, the DNA from approximately 6.7×10^2 conidia was amplified.

Actual number of conidia whose DNA was amplified by nested PCR = Y/500
(where Y = the number of conidia from which DNA was extracted)

If one *Cylindrocarpon* haploid genome is considered to have approximately the same weight as *Fusarium oxysporum* (both fungi belong to the *Nectriaceae* family) with 0.0377 pg (Pasquali *et al.*, 2006) and one conidium has approximately 3 nuclei, the DNA in one conidium would weigh 0.11 pg.

DNA in one conidium = 0.0377 pg /nucleus × 3 nuclei /conidium

As 6.7×10^2 conidia were able to be detected from each of the different species, the resolution was equivalent to 75.4 pg of DNA.

DNA (pg) amplified = 6.7×10^2 conidia × 0.11 pg DNA.conidium^{-1}

670 conidia or 75.4 pg of DNA were capable of being detected by nested PCR

For soil testing diagnostics using nested PCR, DNA extraction Method 1 with a standing time of 10 min, 0.2 µL of the DNA extracted in a primary PCR using general tubulin primers and the diluted products (1:100) for the secondary PCR using species specific primers was selected.

5.3.2.3. Quantitative PCR

5.3.2.3.1. DNA extraction from cultures

Quantitative PCR of DNA extracted from a pure culture of isolate L1 using species specific primers Cyli F1 and Cyli R1 produced an amplicon using the standard PCR thermal cycle described in Section 3.2.3.2 at template concentrations of 30 ng, 3 ng, 0.3 ng, 30 pg and 3 pg (Figure 5.7). Gel electrophoresis of the amplicons showed that they were of predicted size (200 bp). All threshold cycle values (C_t) above 39 were discarded as described in Section 3.3.4. The standard curve showed a linear correlation between the logarithm of the concentration and the threshold cycle (C_t) values with a correlation coefficient (r^2) of 0.992. The regression equation of the standard curve was: C_t= -4.03 (log [DNA]) + 29.67.

The detection limit of the qPCR was calculated for a C_t value of 39:

log [DNA ng] = (39 - 29.67) /-4.03

= 4.8 pg of genomic DNA

Quantitative PCR of DNA extracted from a pure culture of isolate M3 using species specific primers Cyma F1 and Cyma R1 produced an amplicon which was of predicted size (300 bp) for 30 ng, 3 ng, 0.3 ng and 30 pg of template DNA (Figure 5.8). The standard curve showed a linear correlation between the logarithm of the concentration and the threshold cycle (C_t) values with r^2 of 0.997. The regression equation of the standard curve was: C_t= -4.29 (log [DNA]) + 30.69. The detection limit of the qPCR was 11.5 pg of genomic DNA.

Quantitative PCR of DNA extracted from a pure culture of isolate D2 using species specific primers Cyde F1 small and Cyde R2 produced an amplicon which was of predicted size (200 bp). The standard curve showed a linear correlation between the logarithm of the concentration and the threshold cycle (C_t) values, with r^2 of 0.945. The regression equation of the standard curve was: C_t= -5.00 (log [DNA]) + 33.1. The detection limit of the qPCR was 66 pg of genomic DNA.

5.3.2.3.2. DNA extraction from conidia

For *C. liriodendri*, results from the qPCR indicated an average amount of 321 pg of DNA for 10^4 conidia, 22.1 pg of DNA for 10^3 conidia and 5.7 pg of DNA for 10^2 conidia. No amplicons were detected for 10 conidia and the negative control. When these results were graphed using Excel version 2003, the fitted line showed a linear correlation between the logarithm of the number of conidia and the logarithm of the quantity of DNA, with a correlation coefficient (r^2) of 0.972 (Figure 5.3). The equation of the curve was:

log [DNA quantity] = 0.8 log [conidia number] – 0.85

This equation indicates that DNA quantity = $10^{-0.85}$ for one conidium.

One *C. liriodendri* conidium is equivalent to 0.14 pg of DNA.

If one *Cylindrocarpon* haploid genome is considered to have approximately the same weight as *F. oxysporum* with 0.0377 pg and one conidium is equivalent to 0.14 pg of DNA, one *C. liriodendri* conidium is constituted of approximately 4 nuclei.

As the theoretical detection limit for *C. liriodendri* is 4.8 pg of DNA (Section 5.3.2.3.1) and one conidium contains 0.14 pg of DNA, 4.8 pg of DNA is equivalent to 34.3 conidia. If 34.3 conidia can be detected in 1 µL, then 3430 conidia can be detected in 100 µL. As DNA was extracted from a third of 10 g of soil, approximately 1029 conidia can be detected in 1 g of soil. Therefore, the theoretical detection limit for *C. liriodendri* is 1029 conidia /g of soil (1.0 x 10^3 conidia/g soil).

The products obtained with qPCR were confirmed on a 1.5 % agarose gel. A band at 200 bp was present when DNA extracted from 10^2, 10^3 and 10^4 *C. liriodendri* conidia was amplified (data not shown).

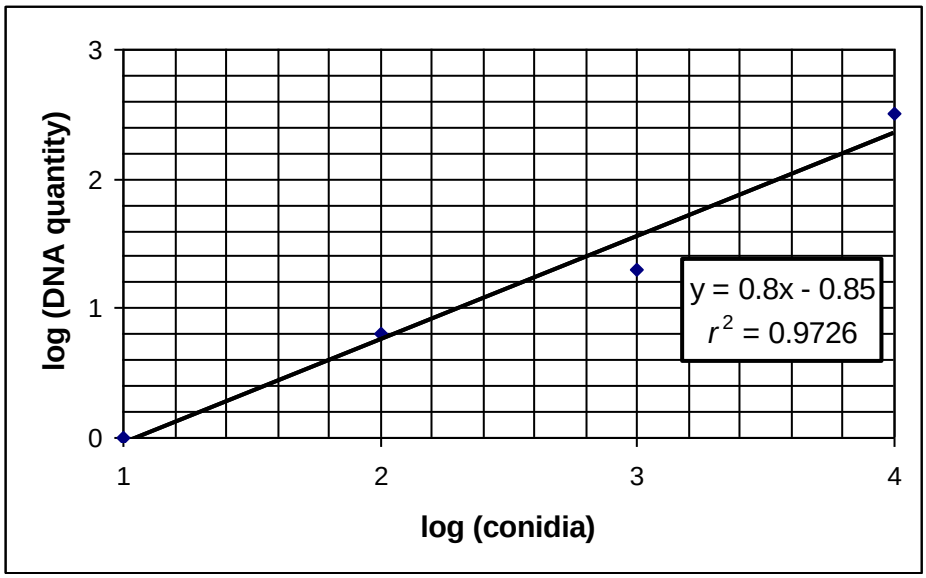

Figure 5.3. Fitted line showing the correlation between number of conidia from which DNA was extracted and DNA quantity estimated by the genomic DNA standard curve for *C. liriodendri*.

For *C. macrodidymum*, results from the qPCR indicated an average amount of 438.5 pg of DNA for 10^4 conidia, 109.5 pg of DNA for 10^3 conidia and 13.3 pg of DNA for 10^2 conidia. No amplicons were detected for 10 conidia and the negative control. When these results were graphed using Excel version 2003, the fitted line showed a linear correlation between the

logarithm of the number of conidia and the logarithm of the quantity of DNA, with a correlation coefficient (r^2) of 0.983 (Figure 5.4). The equation of the curve was:

log [DNA quantity] = 0.87 log [conidia number] – 0.75.

This equation indicates that DNA quantity = $10^{-0.75}$ for one conidium.

One conidium is equivalent to 0.18 pg of DNA.

If one *Cylindrocarpon* haploid genome is considered to have approximately the same weight as *F. oxysporum* with 0.0377 pg and one conidium is equivalent to 0.18 pg of DNA, one *C. macrodidymum* conidium is constituted of approximately 5 nuclei.

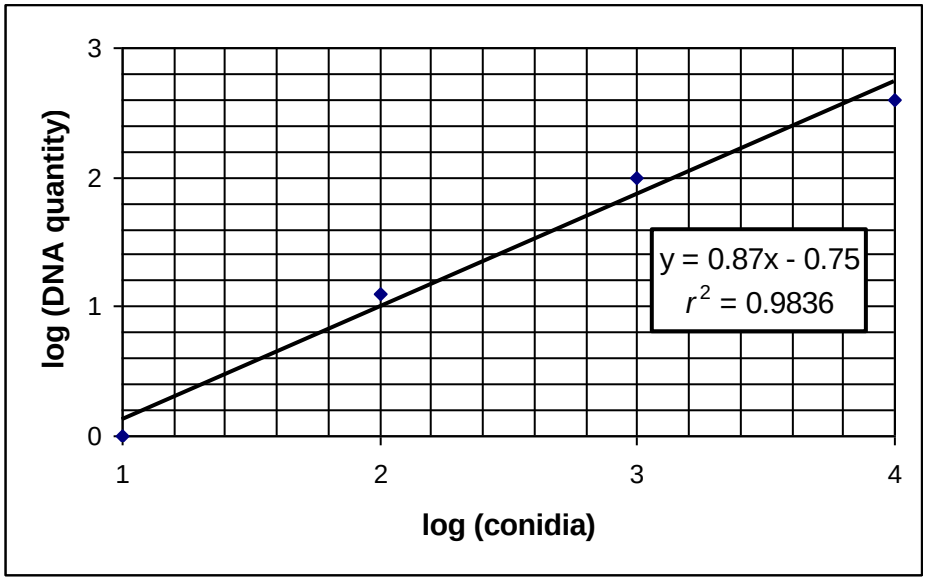

Figure 5.4. Fitted line showing the correlation between number of conidia from which DNA was extracted and DNA quantity estimated by the genomic DNA standard curve for *C. macrodidymum*.

As the theoretical detection limit for *C. macrodidymum* is 11.5 pg of DNA (Section 5.3.2.3.1) and one conidium weighs 0.18 pg of DNA, 11.5 pg of DNA is equivalent to 63.9 conidia. If 63.9 conidia can be detected in 1 µL, then 6390 conidia can be detected in 100 µL. As DNA was extracted from 10/3 g of soil, approximately 1917 conidia can be detected in 1 g of soil. Therefore, the theoretical detection limit for *C. macrodidymum* is 1917 conidia /g of soil (1.9 x 10^3 conidia /g soil).

The products obtained with qPCR were confirmed on a 1.5 % agarose gel (Figure 5.5). A band at 300 bp was present when DNA extracted from 10^2, 10^3 and 10^4 *C. macrodidymum* conidia was amplified.

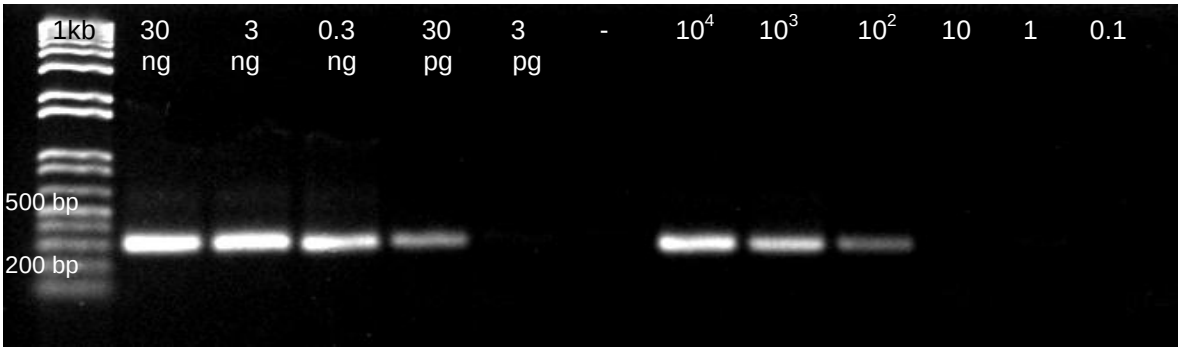

Figure 5.5. PCR products obtained with species specific primers Cyma F1 /Cyma R1 in a qPCR of different DNA quantities extracted from pure culture of *C. macrodidymum* (30 ng to 3 pg) and of DNA extracted from 0.1, 1, 10, 10^2, 10^3 and 10^4 *C. macrodidymum* conidia (- : negative control). 1kb – 1 kb plus DNA Ladder (Invitrogen).

For *C. destructans*, further optimisation of the qPCR was required as the detection limit obtain for this species with pure cultures was 66 pg (Section 5.3.2.3.1) and this limit was likely to increase when the PCR was used with propagules in soil. Therefore, amplification of conidia from this species was not tested.

5.3.2.3.3. DNA extraction from soil over time

DNA from the different soil samples was extracted using a modification of the optimised Method 1. Ten grams of soil were mixed with 45 mL (instead of 90 mL) of sterile water that contained 0.01% of agar and left to stand for 10 min. The agarose gel in Figure 5.6 shows the DNA extracted from the different samples. The amount of DNA extracted from the soil sample taken at time 0 (immediately after inoculation) is higher than any extracted in the following weeks.

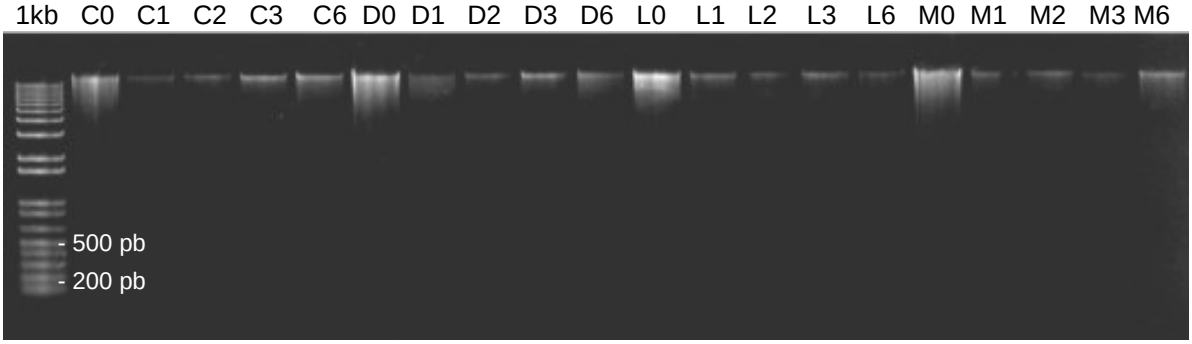

Figure 5.6. Visualisation on 1 % agarose gel of DNA extracted from soil infected with the three *Cylindrocarpon* species and the controls after 0,1,2,3 and 6 weeks (C: control; D: *C. destructans*; M: *C. macrodidymum*; L: *C. liriodendri*; 0 to 6: weeks after soil inoculation). 1kb – 1 kb plus DNA Ladder (Invitrogen).

At time 0, 10 g of soil containing 10^6 conidia was initially suspended in autoclaved distilled water with agar and 1/3 of the pellet was used for DNA extraction, thus DNA from approximately 3.3×10^5 conidia was extracted and suspended in 100 µL. As 1 µL was used for the nested PCR, the DNA from approximately 3.3×10^3 conidia was available for amplification.

For *C. liriodendri*, results from the qPCR indicated an average amount of 57.1 pg of pathogen DNA was detected in the 1 µL of DNA extracted from the soil samples infested with 10^6 *C. liriodendri* conidia /g of soil at time 0.

As the fitted line in Section 5.3.2.3.2 for *C. liriodendri* conidia indicated that

log [conidia number] = (log [DNA quantity] + 0.85) /0.8,

log [conidia number] = (log [57.1]+ 0.85) /0.8

57.1 pg is equivalent to 1.8×10^3 conidia

As 1 µL of DNA extracted from the 100 µL of DNA extracted was used for the qPCR,
1.8×10^3 conidia × 100 µL DNA extraction = 1.8×10^5 conidia were present in 3.3 g of soil.
In 1 g of soil, 1.8×10^5 conidia /3.3 g of soil = 5.5×10^4 conidia were actually detected.
As 1×10^5 conidia /g of soil were initially mixed with the soil, 1.8 times less conidia were detected by qPCR at time 0.

Agarose gel electrophoresis of qPCR amplimers showed that a faint band was obtained for soil samples from 1, 2 and 3 weeks after infestation and these corresponded to 3.9, 3.0 and 3.2 pg of DNA, respectively (equivalent to 64, 46 and 50 conidia using the fitted line in Section 5.3.2.3.2) in 1 µL of DNA extraction. These results are equivalent to 1.9×10^3, 1.4×10^3 and 1.5×10^3 conidia /g, which represent 3.5, 2.5 and 2.7% of the initial conidium number, respectively when considering 5.5×10^4 conidia at time 0.

No amplicons were detected for the different controls or from the inoculated soil at week 6. The molecular weight of the products obtained with the qPCR was confirmed as the correct size on a 1.5 % agarose gel (Figure 5.7), and the sequencing result showed that the primers were specific to the different species.

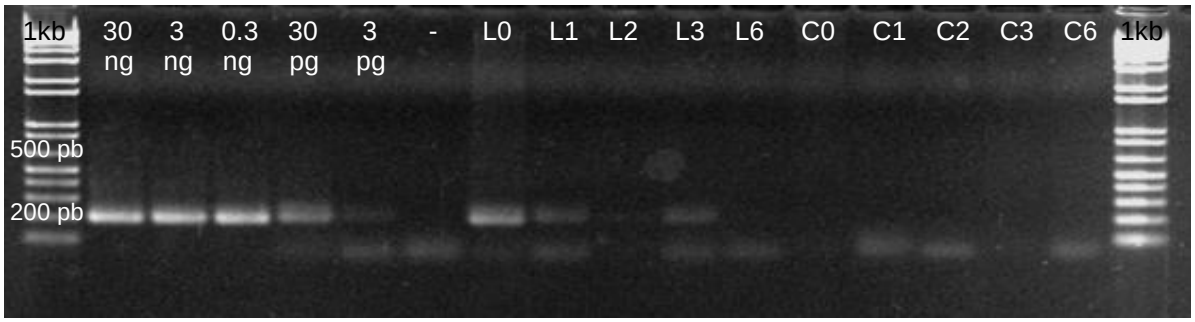

Figure 5.7. PCR products obtained with species specific primers Cyli F1 /Cyli R1 in a qPCR of different DNA quantities extracted from pure culture of *C. liriodendri* and of DNA extracted from soil mixed with 10^6 *C. liriodendri* conidia after 0, 1, 2, 3 and 6 weeks (- : negative control; L: *C. liriodendri*; C: control; 0 to 6: weeks after soil inoculation). 1kb – 1 kb plus DNA Ladder (Invitrogen).

For *C. macrodidymum*, results from the qPCR indicated an average amount of 115.5 pg (equivalent to 1.7×10^3 conidia using the fitted line in Section 5.3.2.3.2 for *C. macrodidymum* conidia) of pathogen DNA was detected in 1 µL of DNA extracted from the soil samples infested with 10^6 *C. macrodidymum* conidia /g of soil at time 0.

As the fitted line in Section 5.3.2.2 for *C. macrodidymum* conidia indicated that

[DNA quantity] = 0.87 log [conidia number] – 0.75,

log [conidia number] = (log [115.5]+ 0.75) /0.87

115.5 pg is equivalent to 1.7×10^3 conidia

As 1 µL of DNA extracted from the 100 µL of DNA extracted was used for the qPCR,
1.7×10^3 conidia × 100 µL DNA extraction = 1.7×10^5 conidia were present in 3.3 g of soil.
In 1 g of soil, 1.7×10^5 conidia /3.3 g of soil = 5.2×10^4 conidia were actually detected.
As 1×10^5 conidia /g of soil were mixed with the soil, 1.9 times less conidia were detected.

No amplicons were detected for infested soil samples or the controls 1, 2, 3 or 6 weeks after infestation. The molecular weight of the products obtained with the qPCR was confirmed as the correct size on a 1.5 % agarose gel (Figure 5.8). A single band at 300 bp was present when the different DNA concentrations from pure culture of *C. macrodidymum* and the soil sample from *C. macrodidymum* at time 0 were amplified. No band was visualised in the controls.

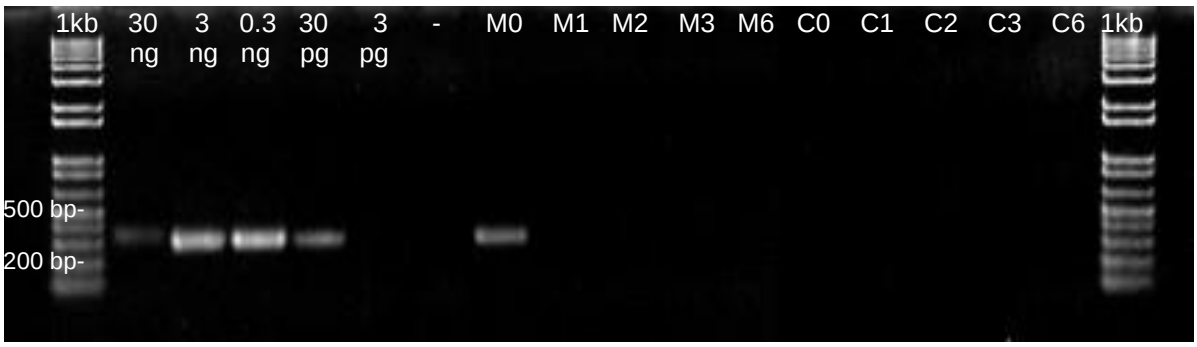

Figure 5.8. PCR products obtained with species specific primers Cyma F1 /Cyma R1 in a qPCR of different DNA quantities extracted from pure culture of *C. macrodidymum* and of DNA extracted from soil mixed with 10^6 *C. macrodidymum* conidia after 0, 1, 2, 3 and 6 weeks (- : negative control; M: *C. macrodidymum*; C: control; 0 to 6: weeks after soil inoculation). 1kb – 1 kb plus DNA Ladder (Invitrogen).

5.4. Discussion

The objective of this chapter was to develop a soil testing method for the detection of *Cylindrocarpon* spp. Different baits were used to isolate the pathogen, however, none were successful with natural soil. *Cylindrocarpon destructans* was successfully isolated from apples inoculated with autoclaved potting mix infested with 5 x 10^4 conidia for both apple varieties and 5 x 10^2 conidia for apple variety Granny Smith. Mwanza and Kellas (1987) isolated *C. destructans* from soil using this method, however, the main fungal species isolated were *Pythium*, *Penicillium* and *Fusarium* as observed in this experiment. Sutherland *et al.* (1966) isolated *Pythium vexans* and *Trichoderma lignorum* using the apple baiting technique. In this soil study, the pathogen may have been masked by the fast growing fungi found on the PDA plates, may not have been able to compete with the microorganisms present in the soil or may have rapidly converted into chlamydospores which did not germinate and invade the apple tissue. In contrast, when placed in autoclaved potting mix the pathogen does not compete with other microorganisms and can multiply in the environment as observed by Taylor (1964) who reported that *Cylindrocarpon* species in contact with sterile soil develop hyphae while in non autoclaved soil, the pathogen produced chlamydospores. As chlamydospores need to be stimulated by the environment to germinate (Matturi and Stenton, 1964), the infection process would be expected to be slow. As a result, this method was discarded.

An alternative to the apple bait method was to use seedlings of different plant species as bait. *Cylindrocarpon* species are known to have a wide host range (Booth, 1966; Brayford, 1992) and *C. destructans* has been recovered from roots of clover (Skipp *et al*., 1986), dwarf bean (Taylor and Parkinson, 1965), pea (Matturi and Stenton, 1964), carrot (Sweetingham, 1983) and parsnip (Channon and Thomson, 1981; Sweetingham, 1983). These plants were selected for their availability, their low cost, the time required for their germination and their susceptibility to *Cylindrocarpon* spp. For spinach and pea seedlings sown into autoclaved potting mix infested with *C. destructans* conidia, the damping off symptoms that developed contained the pathogen, however, when the experiment was repeated with infested garden soil the pathogen was not recovered from the seedlings. Similar observations were reported by Sweetingham (1983) who investigated the pathogenicity of *Cylindrocarpon* species towards *Pinus radiata* seedlings. In autoclaved media, the pathogen caused damping off or root stunting of the pine seedlings while in non sterile media inoculated with *C. destructans*, none of the seedlings were affected (Sweetingham, 1983). The pathogen was clearly not able to infect the seedlings in soil with other microorganisms present.

Since Parkinson and Thomas (1969) reported that the frequency with which "*C. radicicola*" (*C. destructans*) was isolated from dwarf bean root surfaces increased with time (with an incidence of 4.5 and 35.6% after 1 and 6 months, respectively), an experiment that used a longer period of growth might be needed for the pathogen to infect or colonise seedling roots. In Chapter 4, the pathogen was recovered from grapevine stems 6 months after the soil was inoculated with different *Cylindrocarpon* propagules. However, this was not explored as a baiting method suitable for use by grapegrowers should be successful within a reasonable time frame.

As *Cylindrocarpon* species are known to infect plants through wounds (Sweetingham, 1983; Rahman and Punja, 2005), tomato seedlings were root wounded before planting into infested autoclaved and non autoclaved soil. It was hypothesised that the wounded seedlings would facilitate the infection. However, the results were similar to previous observations: the pathogens were isolated from tomato plants grown on autoclaved soil but were absent from those grown on non autoclaved soil.

Parsnip slices were investigated for their susceptibility to *Cylindrocarpon* spp. however *Cylindrocarpon* conidia failed to cause lesions on them. Sweetingham (1983) reported that carrot and parsnip root slices inoculated with plugs from Czapek Dox agar cultures of *C. didymum* induced soft black lesions 7 days after inoculation and wounded carrots inoculated with conidial suspensions developed lesions which increased with conidium concentration. Channon and Thomson (1981) also reported *C. destructans* on parsnip and when isolates

were tested for their pathogenicity towards parsnip, half of the isolates were found weakly pathogenic while the others caused greater lesions. It seems that the isolates used in this chapter were not capable of causing symptoms to parsnip.

As baiting did not show any promising results, the development of a molecular method to detect the three species in soil was investigated. The main issue with DNA extraction from soil samples is the presence of compounds such as humic acids, tannins, pigments, polysaccharides and lignin which can inhibit PCR reactions, decrease the detection sensitivity and underestimate propagule numbers in soils with high DNA binding capacities (Tsai and Olson, 1992; Martin-Laurent *et al*., 2001, Schena *et al*., 2004; Ruano-Rosa *et al*., 2007; Williams *et al*., 2009). To overcome these problems, soil DNA extraction kits contain chemicals and processes designed to minimise the effect of these contaminants and typically use very small soil samples e.g. 0.5 g. This volume is unrealistic for detecting soil pathogens due to the heterogeneity of soil distribution. Two methods were investigated for their ability to extract DNA from large soil samples. A conventional PCR reaction using general primers ITS4 and ITS1F determined that, as expected, the extracts from both methods contained PCR inhibitors based on variations in resultant band intensity when different volumes of the extract were amplified. Method 2 failed to show consistent amplification after testing the addition of different amounts of the DNA extract and was ultimately discarded. In contrast, although Method 1 showed some inhibition using 1 µL of DNA extract it was consistent and efficient using 0.2 µL of DNA extract. Method 1 was developed specifically for this project and allowed extraction of PCR ready DNA from up to 10 g soil. It was based upon the same principles as in soil dilution plating, which is a well accepted microbiological method, and in which the propagules are homogenously suspended in a solution. It provides a new approach to the topic of DNA extraction from soil and could be valuable for detection of other soil-borne diseases. In Method 2, the bead beating method was used, as recommended by Yeates *et al.* (1998) who tested different methods for extracting DNA from soil samples, including sonication, enzymatic lysis and centrifugation. They determined that bead beating was able to lyse all soil organisms with a lysis efficiency greater than 90%. However, in this study, the bead beating method was unable to produce PCR ready DNA.

The substantial interest in detecting soilborne pathogens has underpinned the development of many different DNA extraction methods. Damm and Fourie (2005) investigated the efficacy of low cost DNA extraction methods and an extraction kit. They found that sodium dodecyl sulphate buffer yielded high DNA quantities and PCR inhibitors were removed with bovine serum albumin and a polyvinylpolypyrrolidone (PPV) column, however, the low-cost method they developed used only 0.5 g of soil (Damm and Fourie, 2005). In another study Ruano-Rosa *et al*. (2007) used Sepharose and PPV columns, while Volossiouk *et al.* (1995)

demonstrated the ability of skimmed milk powder to prevent DNA degradation and its adsorption to soil particles during the extraction. Williams *et al.* (2009) also tested three different polymerases and observed that Taq DNA polymerase was more sensitive to soil inhibitors than Tth+ and Taq F1* DNA polymerases. The method developed here may benefit from the addition of one or more of these chemicals. Thus, the iterative testing of these additives to Method 1 would be useful to decrease the presence of inhibitors in the DNA extract and to improve the efficiency of the extraction. However, such an approach would be very time consuming and beyond the scope of this work. In addition, the quantitation of *Cylindrocarpon* DNA from the soil extracts with the three different standing times in Method 1 could provide information that may help improve the recovery of DNA.

In this study, 10 g of soil was used which was similar to that used by Cullen *et al.* (2001) while Reeleder *et al.* (2003) and Kernaghan *et al.* (2007) used a paint shaker to extract DNA from 5 g of soil. However, in most studies the soil samples used for DNA extraction weighed between 0.1 and 1 g (Keller *et al.*, 1995, Ippolito *et al*., 2002; Damm and Fourie, 2005; Wang *et al*., 2007; Williams *et al.*, 2009). Small samples fail to provide accurate data as fungal populations are not uniformly distributed in soils, as reported by Rodriguez-Molina *et al.* (2000) who observed that *Fusarium* population densities were very variable between samples, even at close distances. Therefore a combination of large soil samples and multiple sampling points are needed to reduce variability.

The nested PCR was capable of detecting 75.4 pg in soil which was equivalent to 10^5 conidia /g of soil. This result is much higher than the predicted detection sensitivity of 1 pg of DNA from pure culture obtained in Chapter 3. It is probably due to the fact that intermediate conidium concentrations ranged between 10^5 and 10^3 conidia /g of soil were not tested. Nested PCR only provides information on the presence or absence of the pathogen in soil while qPCR enables to determine DNA quantity of *Cylindrocarpon* spp. present in the soil. The quantitative PCR showed that the detection limit was 4.8 pg for *C. liriodendri*, 11.5 pg for *C. macrodidymum* and 66 pg for *C. destructans* with pure DNA from cultures. The method was capable of detecting 1029 conidia /g of soil for *C. liriodendri* and 1917 conidia /g of soil for *C. macrodidymum*. Similarly, Filion *et al.* (2003) reported a detection limit of 10^4 *Fusarium solani* f. sp. *phaseoli* spores /g of soil using species specific primers targeting the elongation factor 1 α and SYBR Green I chemistry. As reported in Chapter 3, Section 3.4, species specific primers targeting the ITS sequences have a sensitivity approximately 100 fold higher than primers targeting the β-tubulin gene. Thus, Wang *et al.* (2007) were capable of detecting 10 zoospores of *Phytophthora melonis* in 0.5 g of artificially inoculated soil, Cullen *et al.* (2001) detected 1.5 *Helminthosporium solani* spores /g of soil using primers targeting the ITS region of nuclear rRNA gene and Luo *et al.* (2009) were capable of detecting 24

Aspergillus flavus and *A. parasiticus* conidia /g of soil using primers from the ITS region. Additional experiments to determine the threshold number of *Cylindrcarpon* propagules needed for infection are required as suggested in Chapter 2 to establish whether the detection limits of the qPCR developed in this chapter are sufficient for soil detection of minimum pathogen levels that are conducive to disease.

Different strategies to increase the sensitivity of the detection limit of the qPCR should be investigated. Okubara *et al.* (2005) reported that the sensitivity of qPCR is higher with TaqMan chemistry than SYBR Green I, due to the 200 fold increase of fluorescence when TaqMan probes are cleaved compared to the 20 fold increase of fluorescence when SYBR Green I is bound to DNA. In addition, TaqMan chemistry is more specific than SYBR Green I as TaqMan uses a probe which binds to a specific DNA sequence while SYBR Green I binds randomly on double stranded DNA. TaqMan could potentially eliminate problems caused by the non-specific binding observed with SYBR Green I when optimising the qPCR in Chapter 3. A higher number of PCR cycles were used by Filion *et al.* (2003) who used 55 cycles for the detection of *Fusarium solani* f. sp. *phaseoli* using specific primers targeting the elongation factor 1 alpha gene and 70 cycles for *Glomus intraradices* using specific primers from the small subunit rRNA gene. It is possible that the detection limit in this study could be improved by increasing the number of PCR cycles, however, the greater potential for production of spurious amplicons needs to be considered.

In the nested PCR for *C. destructans*, amplimers were also observed at the equivalent of 0 and 6.7 conidia with 5 and 10 min standing times, respectively on a gel. These can either result from cross contamination although precautions were taken as described in Section 3.4 or from indigenous *C. destructans* populations. The latter explanation is more likely as the soil used in the experiment originated from the same location as the field experiment in Chapter 4 where 35% of control plants were infected with *C. destructans*. The soil could have been autoclaved before inoculation, however, soil structure and chemistry is modified by autoclaving which was not suitable for the study. The presence of native microorganisms in the soil was required to determine if their DNA would interfere with the binding of the species specific primers and therefore, steam pasteurisation of the soil was also not a useful alternative. For the three *Cylindrocarpon* species tested, no DNA from other soil microorganisms was amplified which confirms the specificity of the primers. Additionally, the sequencing of the amplicons showed that the primers were specific for each species. Primers based on the β-tubulin gene have been shown to successfully identify fungal species in soil without amplifying other soil microorganisms as this gene region provides sufficient differentiation (Hirsh *et al.*, 2001; Zampieri *et al*., 2009).

For the quantitative PCR, a linear correlation between the logarithm of the DNA quantity and the logarithm of the number of conidia was obtained for *C. liriodendri* and *C. macrodidymum*. From the standard curves, one *C. liriodendri* conidium was equivalent to 0.14 pg of DNA which represents approximately 4 haploid genomes while one *C. macrodidymum* conidium was equivalent to 0.18 pg which represents approximately 5 haploid genomes. The differences in weight between these two species can be related to the propagule type used for DNA extraction. Under a microscope, *C. macrodidymum* conidia were predominantly macroconidia while *C. liriodendri* conidia were a mixture of macroconidia and microconidia.

Quantitative PCR of samples from the time trial conducted in Lincoln University's vineyard with soil infested with 10^5 conidia /g of soil in 2.5 L pots showed that the conidial DNA disappeared rapidly from the soil. At time 0 which actually corresponded to 30 min after the soil was inoculated, the technique detected 57.1 pg of *C. liriodendri* DNA and 115.5 pg of *C. macrodidymum* DNA which was equivalent to 5.5×10^4 and 5.2×10^4 conidia /g of soil, respectively. These results are approximately two times lower than what was expected, probably due to the mixing method employed as the conidia were not distributed evenly, inhibition from compounds present in the soil and possibly lysis. Alternatively the lower than expected DNA concentration may be due to incomplete recovery of the propagules from the soil during the DNA extraction procedure. As previously mentioned further optimisation of the extraction process and standing times following wrist action shaking may improve the efficiency of recovery.

Soil samples taken at 1, 2 and 3 weeks after soil infestation with *C. liriodendri* showed the presence of 3.9, 3.0 and 3.2 pg of DNA while DNA of *C. macrodidymum* failed to be detected. It seems that the number of propagules is substantially reduced in the first week after soil infestation with conidia. For *C. liriodendri* levels stayed relatively constant after that week, but for *C. macrodidymum*, the number of propagules was below the detection limit. The low number of conidia after a week could be associated with the transformation of conidia into chlamydospores as described in Chapter 4. This is accompanied by a substantial reduction in DNA quantities by the conversion of multinucleate conidia to uninucleate chlamydospores and general attrition resulting from failed conversions.

The success rate of the conidia to chlamydospore conversion is not known for *Cylindrocarpon* spp. Matturi and Stenton (1963) observed that *Cylindrocarpon* chlamydospores were formed after 3 days of incubation in the soil while conidia and mycelium were severely lysed. They stated that mycelium and conidia were transient in soil and were replaced by chlamydospores which remained dormant until they were stimulated to germinate, possibly by the presence of a suitable host. However, in this study conidia were

added to the soil environment without a host. Alternatively, degradation by other soil microorganisms or death due to desiccation during a very dry summer could explain the decrease in inoculum. This rapid reduction of propagule numbers over time has been illustrated in Chapter 4 with a conidium reduction of 99.7, 98.9 and 96.0% for *C. destructans*, *C. liriodendri* and *C. macrodidymum*, respectively, 3 weeks after burying conidia in soil. The rapid reduction of propagule numbers over time was illustrated on other pathogens. Harper *et al*. (2002) observed that 40 to 60% of *Sclerotium cepivorum* sclerotia were degraded after 2 months and related this loss to microorganisms and environmental conditions. After that period, the number of sclerotia was reduced less rapidly. Hancock (1981) observed that populations of *Pythium ultimum* decreased exponentially during the first 2 to 3 months with a half life of 30 days and were stabilised after that month. Additional experiments with chlamydospores would provide more information on the conversion rate and longevity of propagules in soil.

Further experiments testing naturally infested soils should determine whether the sensitivities of the qPCR developed in this chapter are maintained when DNA is extracted from different soil types. The soil used for this study was a silt loam. Williams *et al.* (2009) reported that sensitivity of the PCR reaction depended on the type of soil, with soils containing high organic content and potting mix causing higher inhibition than sandy soils while Volossiouk *et al.* (1995) added sand to clay soils to improve sensitivity.

The use of qPCR to quantify *Cylindrocarpon* spp. in soil could help identify sites with high pathogen populations which constitute a risk to grapevines. Damm and Fourie (2005) detected *Cylindrocarpon* spp. in 67% of soil samples tested in a conventional PCR reaction using primers Dest1 and Dest4. However, in order to do this in a biologically meaningful way the correlation between disease incidence and *Cylindrocarpon* DNA quantity should be investigated, especially as the qPCR will detect a range of propagules and potentially DNA from dead organisms (Ridgway *et al*., 2005). Additionally, this technique could be used to monitor the pathogen in the environment, providing a better understanding of the life cycle of the pathogen. Kernaghan *et al.* (2007) correlated qPCR results with disease severity and colony forming units for *C. destructans* f. sp. *panacis* and stated that qPCR was more accurate than bioassay or colony forming unit data. Ruano-Rosa *et al.* (2007) used qPCR to detect *Rosellinia necatrix* in avocado orchard soils and related visible symptoms on trees with root and bark pathogen isolations on media and molecular detection of the pathogen. The extension of this work to include the correlation between disease and DNA quantity could assist vineyards and nurseries in making decisions on planting or managing the pathogen before planting grapevines.

Molecular techniques are unable to discriminate between living and dead propagules although it seems that nucleases present in the soil are capable of degrading the remaining DNA rapidly (Schaad and Frederick, 2002; Schena *et al*., 2004; Ruano-Rosa *et al*., 2007). Ridgway *et al.* (2005) observed that non viable propagules of *Phaeomoniella chlamydospora* could be detected 3 weeks after addition to the soil but not after 8 weeks. An alternative to DNA extraction and quantification directly from soil is to combine a bait with quantitative or nested PCR (Schena *et al*., 2004). Lees *et al.* (2002) used *Chenopodium quinoa* seeds as bait for *Rhizoctonia solani* in soil and performed a quantitative PCR. The sensitivity of their assay was greater than with direct extraction of DNA from 10 g of soil. The baiting step can allow the target fungi to increase in numbers and it eliminates the need for a soil DNA extraction step (Lees *et al.*, 2002). Bonants *et al.* (2004) shortened the time required for a conventional bating system to detect *Phytophthora fragariae* from 5 – 6 weeks to 2 weeks by combining the baiting method with quantitative or nested PCR. The disadvantage of this method is that the result only shows presence or absence of the pathogen and not quantification of the pathogen in soil, and was unreliable in this study.

In this study, a simple DNA extraction method using a relatively large soil sample (10 g) was developed. Conidia from the three *Cylindrocarpon* spp. tested were successfully amplified in a nested PCR and visualised on a gel at a concentration equivalent to 1×10^5 conidia /g of soil. A qPCR was optimised and was capable of detecting 1.0×10^3 and 1.9×10^3 conidia /g for *C. liriodendri* and *C. macrodidymum*, respectively. This method was tested on samples from soil infested with conidia at different times after infestation which showed that after one week, less than 5% of the propagules were detected in the soil, however, the numbers were relatively constant after that period. Different baiting methods were attempted but none was successful. Quantitative PCR would provide information on *Cylindrocarpon* population densities in the soil, however, disease incidence and severity are also influenced by environmental, biological and cultural factors. Some of these stress factors were studied in Chapter 6.

CHAPTER 6

Factors that may affect development of black foot

6.1. Introduction

The 'disease triangle' represents the interaction between the host plant, pathogen and environment, which all need to be optimum for development of significant disease, and need to be considered when investigating the factors which pose a risk to crop health and yield. For root pathogens the soil environment may also impose stresses on host plants, causing reduction of plant growth and yield. The common soil stress factors, which vary with different growing environments and crop management processes, may be physical (e.g. water deficit, flooding, and adverse temperature), chemical (e.g. poor soil fertility, soil pH, soil salinity, pesticides and heavy metals) or biotic (e.g. competition for nutrients, pathogenic micro-organisms and feeding by animals or insects).

For young grapevines in nurseries and in vineyards disease risk is increased by the wounds made during propagation processes and vineyard management, and by the stresses imposed on them, including temperature extremes, prolonged time in containers, poor planting techniques and early fruiting (Scheck *et al.*, 1998; Stamp, 2001; Oliveira *et al.*, 2004). These factors are believed to weaken grapevines, making them more susceptible to pathogens including *Cylindrocarpon* spp. (Scheck *et al.*, 1998). High disease incidences of black foot were associated with abiotic factors such as water logging, soil compaction and heavy soil (Maluta and Larignon, 1991; Gubler *et al*., 2004), however, the effects of the individual factors have not been explored.

Soil water must be adequate for the needs of plants; it provides the medium for mineral absorption and within plants for gas, salt and other solute transportations, as well as for chemical reactions including photosynthesis, and for turgor of cells (Williams, 2000). The water retention capacities of soils differ, causing different levels of risk, through either water logging (root asphyxiation) or water deficit (water stress). In this chapter, the first objective was to determine the effect of three water regimes on disease development.

Wounding may also enhance black foot in grapevines. In ginseng, Rahman and Punja (2005) observed that disease severity was enhanced when the roots were wounded prior to inoculation with *C. destructans*, with half of their isolates being unable to generate lesions

without wounding. The second objective was to determine the effect of wounding on disease development by wounding grapevine roots, canes and trunks in the vineyard.
The susceptibility of different wound ages to pathogen infection was also investigated. When plants are wounded, plant defence systems are believed to be activated since the plant signal generated is similar to that of pathogen invasion, possibly making plants relatively resistant to a pathogen by a few days after wounding (de Bruxelles and Roberts, 2001).

Among the different nursery practices, grapevine cuttings are cold stored prior to grafting and before being sent as grafted plants to the growers (Gimenez-Jaime *et al*., 2006). Since cold storage of plant tissues is known to affect their metabolism, including the starch to sugar conversion within storage tissues, the third objective was to determine the effect of different cold storage times on susceptibility to *Cylindrocarpon* spp.

Plants already under attack by pests and pathogens are also stressed and so more susceptible to diseases. Horsfall and Dimond (1960) stated that root diseases are often the result of infections by more than one pathogen. Halleen *et al.* (2003) showed that pathogens such as *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. were often already present in rootstock propagation material and were frequently isolated with *Cylindrocarpon* spp. Clearwater *et al.* (2000) observed the presence of *P. chlamydospora* in New Zealand and Graham *et al.* (2009) isolated the pathogen from all grapevine cordon samples of rootstock variety 5C examined. Therefore, the possibility of prior infection by *P. chlamydospora* increasing the susceptibility of vines to infection and disease development by *Cylindrocarpon* spp. was investigated as the fourth objective within this chapter.

6.2. Material and methods

The cuttings and grafted plants for the following experiments were obtained from Corbans Viticulture Ltd and inoculation was with the conidium suspensions of the three *Cylindrocarpon* species made as described in Section 2.2.1.2. The suspensions each comprised equal mixtures (10^6 conidia /mL) of the same nine isolates that were studied in Section 2.2.1.2.

6.2.1. Water stress

Grapevine cuttings of rootstock varieties 101-14 and 3309 were callused for a month as in Section 2.2.1.1. The plants were then planted into 2.5 L pots with enough 3 - 4 month potting mix (Appendix 2) to ensure that each pot weighed 2 kg. Three different water regimes were applied for a month: 75% and 100% of field capacity, and water logging. Field capacity (FC) was determined by weighing the pots with dry potting mix and all the water they could hold.

For the water-logged plants, the pots were placed in containers filled with tap water, bringing the water level to the top of the soil level. After a month under these water levels, the grapevines were uprooted, the potting mix was carefully taken off the roots and the roots were either wounded by trimming the terminal ends of the roots or unwounded. The plants were immediately soaked in either a mixed isolate conidium suspension as described in Section 2.2.1.3 or in water (control). The grapevines were then repotted with a Wakanui silt loam from the Horticulture Research Area of Lincoln University and were lightly watered daily while they grew for 6 months on mesh tables in a glasshouse. Since this period extended into winter, high pressure sodium lamps (Son-T Agro 400, Philips), were turned on from 4 am to 12 pm and from 4 pm to 8 pm for the duration of the experiment to ensure plants had 16 h light exposure. The 10 replicates of each treatment were laid out in a split-plot design before being assessed as described in Sections 2.2.1.4 and 4.2.1. The pots were weeded manually as required and the temperature within the greenhouse ranged between 14°C and 30°C.

Statistical analyses were conducted as described in Sections 2.2.1.5 and 4.2.1; however, a first analysis determined whether disease incidences and severities of the inoculated plants were significantly different from the controls. If they were, the data was analysed without the controls as the SPSS was incapable of calculating the logistic regressions for the binomial data when there were a large number of negative results from the control plants.

6.2.2. Presence /absence of wounds

6.2.2.1. Age of root and trunk wounds

Rootstock grapevine varieties 101-14 and 3309 were rooted for a month as in Section 2.2.1.1. They were potted into 1.5 L pots with 3 - 4 month potting mix (recipe in Appendix 2) and left to grow for 12 months, on a gravel floor in a shade house. The plants were then either root or trunk wounded and inoculated with a mixed conidium suspension at 0, 1, 3, 5 and 8 days after wounding. The trunks were wounded with secateurs at 2 cm above the soil level by making a 5 mm deep cut. Each trunk wound was spray-inoculated with 20 mL of a mixed isolate conidium suspension using a sheep drenching pack and gun, and the wound was wrapped with cling film, the wrapping being removed after 1 week. The roots were wounded by driving an asparagus knife into the soil to a depth of 20 cm, at three equidistant points around 10 cm from the trunk. The plants were inoculated by pouring 20 mL of the same conidium suspension into each soil cut, followed by 500 mL of tap water. For the controls, the plants were either trunk or root wounded as above and inoculated with water, or unwounded and inoculated with 20 mL of a mixed isolate conidium suspension. All inoculation treatments were done on the same day. The 12 replicates for each variety, wounding site and wounding time, were laid out in a split-split-plot design in the same shade house in September 2008 and grown for 4 months. The plants were watered daily and the

temperature ranged between 0 and 35°C. Plants were assessed for disease as described in Sections 2.2.1.4 and 4.2.1 and statistical analyses were conducted as described in Sections 2.2.1.5 and 4.2.1.

6.2.2.2. Graft unions

Dormant, grafted vines (101-14 or 3309 grafted onto Sauvignon blanc) that had grown for 8 month in a field nursery, were inoculated onto the graft union by wrapping it with a 5 x 5 cm and 1 cm thick wad of cotton wool soaked with either a mixed isolate conidium suspension or water (control) for 30 min. The graft unions were then wrapped with cling film to prevent the conidia from desiccating, and the cotton and cling film were removed after a week. The grapevines were potted into 3 – 4 month potting mix in October 2007, the six replicates laid out in a randomised design in a shade house on a gravel floor and allowed to grow for 4 months. The plants were watered daily and the temperature in the shadehouse varied between -2 and 30°C. The plants were assessed by surface sterilising the trunks as in Section 2.2.1.4 and isolation of a 2 – 3 mm transverse wood piece cut from the inoculation point, and at 2 cm above and below the inoculation point. Each wood piece was divided into four pieces which were placed equidistantly onto PDA containing chloramphenicol (250 mg /L). The plates were incubated and assessed after 10 – 14 days as in Section 2.2.1.4.

6.2.2.3. Infection progression from trunk and root wounds

Grafted vines (Sauvignon blanc onto Schwarzmann or 101-14) were potted into 2.5 L pots with 8 – 9 month potting mix (Appendix 2) and grown on mesh tables in a greenhouse for 7 months. They were then transferred into 5 L pots with 8 – 9 month potting mix and placed on the gravel floor in a shadehouse in February 2007, where they remained for 5 months. The plants were watered daily and the temperature varied between14 and 30°C in the greenhouse and between -5 and 22°C in the shadehouse. The grapevines were then planted in a site in the Horticultural Research Area at Lincoln University in September 2007. They were planted in two rows 2.8 m apart, in a split-plot design, plants 75 cm apart. When dormant, the plants were pruned to one cane per vine, which was attached to a wire at 1.5 m height with a nylon string (5 mm diameter).

The plants were wounded in September 2008 as described in Section 6.2.2.1 on the 1 year old canes, at 30 cm from the junction with the 2 year old cane or on the scion trunks at 15 cm above the soil level. They were immediately spray-inoculated with 20 mL of a mixed isolate conidium suspension using a sheep drenching pack and gun and wrapped with cling film which was removed after a week. The roots were wounded with an asparagus knife as in Section 6.2.2.2 and inoculated in the same way with 20 mL of the same conidium suspension or water. Unwounded roots, canes and trunks were inoculated with 20 mL of conidium suspension in a similar way. The plants were then watered with 500 mL of tap water each

and left to grow. The vine bases were covered with a vine guard and left to grow for 1 year. During that period, the plants were not watered as rainfall during the spring period was considered adequate. There were 10 replicates for each variety, tissue type, wounding, and inoculation treatments.

The plants were uprooted 6 months after inoculation and assessed. For the root wounds, the roots were cut, washed and 15 root pieces of approximately 5 cm long that displayed necrotic marks, were taken per plant. These root pieces were surface sterilised by soaking the roots in 70% ethanol for 10 s, 0.35% of sodium hypochlorite for 1 min, 70% of ethanol for 10 s, and three times for 1 min each in autoclaved water. The symptomatic tissues on each root piece were then cut out transversally and five 2 – 3 mm pieces were placed onto PDA amended with chloramphenicol (250 mg /L). Each plate contained five root pieces: four at equidistance around the perimeter of the plate and one in the middle. The plates were incubated as in Section 2.2.1.4 and the proportion of roots infected with *Cylindrocarpon* spp. was determined after 10 – 4 days. Additionally, the stems of the root-treated plants were surface sterilised as in Section 2.2.1.4 and a 2 – 3 mm transverse wood piece was cut from each surface sterilised stem at 2.5, 5 and 7.5 cm above the stem base, cut into four pieces which were placed equidistantly onto PDA with chloramphenicol (250 mg /L), and the plates incubated and assessed as in Section 2.2.1.4.

For the trunk and cane wounds, the tissue was cut 9 cm above and below the infection point, was washed and surface sterilised as in Section 2.2.1.4. The remaining wood samples were stored at 1 – 2°C for further assessment if needed. A 2 – 3 mm transverse wood piece was cut from each surface sterilised tissue, at the inoculation point and at 2.5, 5 and 7.5 cm above and below the point, and divided into four pieces which were placed equidistantly onto PDA with chloramphenicol (250 mg /L). The plates were incubated and assessed as in Section 2.2.1.4. Statistical analyses were conducted as described in Sections 2.2.1.5 and 4.2.1.

6.2.3. Effect of cold storage

Cuttings of the rootstock varieties 101-14 and 3309 were stored at 1 - 2°C, with 12 of each being removed after 1, 2, 3, 4 and 5 months. They were soaked for 30 min in a mixed isolate conidium suspension or water (control) before being callused in pumice on a heat pad at 25°C for 3 weeks. They were potted into 1.5 L pots with 3 – 4 month potting mix and laid out in a randomised design on mesh tables in a greenhouse under high pressure sodium lamps (Son-T Agro 400, Philips). The plants were watered daily while they grew for 3 months, during which time the temperature varied in the greenhouse between 14°C and 30°C. They

were assessed for infection as in Section 2.2.1.4 and data analysed as described in Sections 2.2.1.5 and 4.2.1.

6.2.4. Prior infection with *Phaeomoniella chlamydospora*

From 101-14 mothervines known to be infected with *P. chlamydospora,* cuttings were taken. Previous experience had shown that approximately 50% of cuttings from these vines were infected (Dr. Anna Graham, pers. comm. 2007). The cuttings were grafted onto Sauvignon blanc scion cuttings and allowed to grow in the outdoor nursery at Corbans Viticulture Ltd Auckland for 8 months. These vines were then either hot water treated or not and sent to Lincoln University. The plants were soaked in a mixed isolate conidium suspension or water for 30 min and potted with 3 - 4 month potting mix in 2.5 L pots. The 20 replicate plants were placed in a randomised design on the gravel floor in a shadehouse and grown with daily watering for 4 months, when the temperature varied between -5 and 30°C. The plants were assessed as in Section 2.2.1.4 and data analysed as described in Sections 2.2.1.5 and 4.2.1.

6.3. Results

6.3.1. Water stress

The statistical analyses for this section are shown in Section 6.1 of Appendix 6 (A6). Disease incidences at 1 cm above stem bases were significantly affected by the inoculation treatments (P<0.001 with all *Cylindrocarpon* spp. combined, *C. destructans*, *C. macrodidymum* and *C. liriodendri*; A6.1.1). Overall, *C. liriodendri* caused significantly greater incidences at 1 cm above stem bases than *C. destructans* (P=0.010; A6.1.2) while *C. macrodidymum* caused similar incidences to the two other species, with means of 43.3, 28.3 and 31.7%, respectively (Table 6.1).

Table 6.1. Mean disease incidences at 1 cm above stem bases of vines, 6 months after inoculation with three *Cylindrocarpon* spp.

Species	Disease incidence (%)	
	Inoculated vines	Non-inoculated vines
C. destructans	28.3 b[1]	2.5 a
C. liriodendri	43.3 c	1.7 a
C. macrodidymum	31.7 bc	2.5 a
Cylindrocarpon spp. combined	65.0 z	6.7 y

[1]Values with different letters indicate values of inoculated vines are significantly different (P≤0.05). a-c for *Cylindrocarpon* spp. separately and y-z for the combined species.

The following analyses were conducted without the non-inoculated control (water treated vines). Disease incidences at 1 cm above stem bases was significantly affected by the different water regimes for *Cylindrocarpon* spp. combined (P=0.022) and for *C. macrodidymum* (P=0.045) but not for *C. destructans* (P=0.820) or for *C. liriodendri* (P=0.342; A6.1.3).

For all *Cylindrocarpon* spp. combined, the watering treatments significantly affected the disease incidences at 1 cm above stem bases (P=0.022; A6.1.3), with water-logged plants showing significantly greater incidence (P=0.006) than those at the 75% FC water regime, however neither of these two water regimes differed significantly from the 100% FC treatment, with means of 80.0, 62.5 and 52.5%, respectively for waterlogging, 100 and 75% FC treatments (Figure 6.1 and A6.1.4). However, the individual species differed in their responses to the water treatments, with only *C. macrodidymum* being significantly affected by the treatments (P=0.045). The water-logged plants had significantly greater (P=0.012) disease incidences at 1 cm above stem bases than plants with the 75% FC water regime, however neither of these two water regimes differed significantly from the 100% FC treatment, with means of 45.0, 30.0 and 20.0%, respectively for waterlogging, 100 and 75% FC treatments (A6.1.5). For *C. destructans*, mean disease incidences for waterlogging, 100 and 75% FC treatments, respectively, were 30.0, 30.0 and 25.0% and for *C. liriodendri*, they were 47.5, 47.5 and 35.0%, respectively.

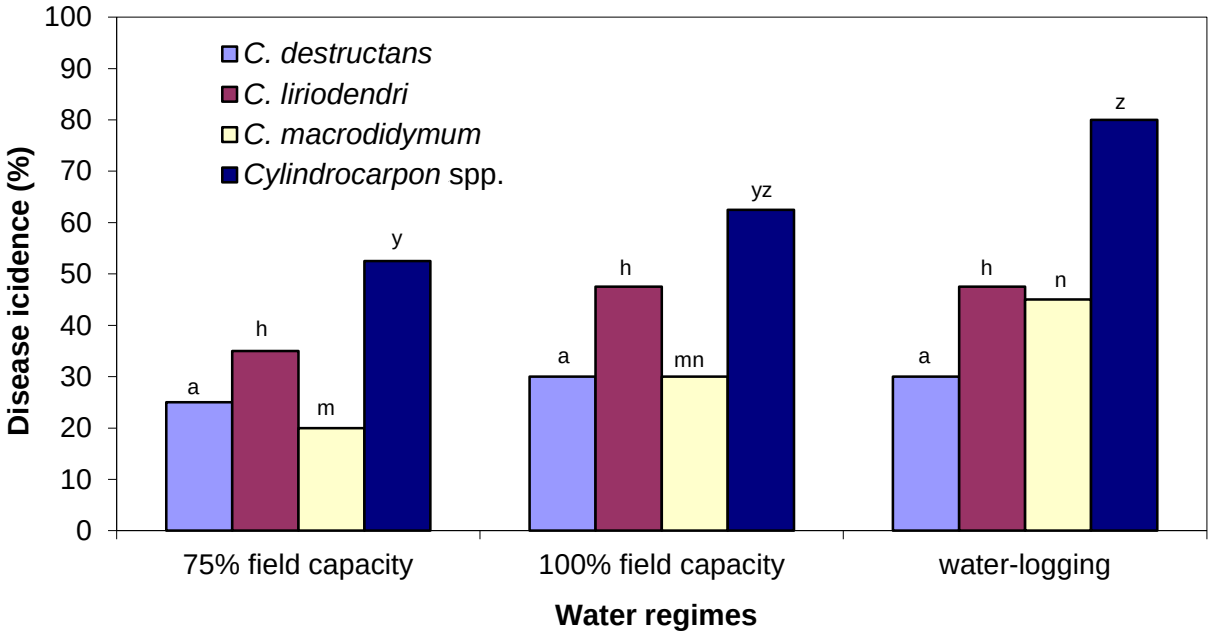

Figure 6.1. Mean disease incidences at 1 cm above stem bases of vines watered with three water regimes for a month, inoculated with three *Cylindrocarpon* spp. and assessed after 6 month growth. For each species, bars with different letters indicate values are significantly different (P≤0.05).

Disease incidences at 1 cm above stem bases did not show any significant effects of root wounding for the different species (A6.1.3 and the results are shown in A6.1.6). There were significant differences between rootstock varieties for all *Cylindrocarpon* spp. combined (*P*<0.001) and for *C. liriodendri* (*P*<0.001) but not for *C. destructans* (*P*=0.081) or for *C. macrodidymum* (*P*=0.208; A6.1.3). For all *Cylindrocarpon* spp. combined, rootstock variety 3309 had greater disease incidences than 101-14, with means of 81.7 and 48.3%, respectively. For *C. liriodendri*, the mean disease incidences for rootstocks 3309 and 101-14 were 61.7 and 25.0%, respectively, for *C. destructans*, they were 35.0 and 21.7%, and for *C. macrodidymum*, they were 36.7 and 26.7%, respectively.

Disease incidences at 5 cm above stem bases were significantly affected by inoculation, with all *Cylindrocarpon* spp. combined (*P*<0.001; A6.1.7), *C. destructans* (*P*=0.008) and *C. liriodendri* (*P*<0.001) but not with *C. macrodidymum* (*P*=0.697; Table 6.2). Overall, *C. liriodendri* caused significantly greater disease incidences at 5 cm above stem bases than *C. destructans* (*P*=0.003; A6.1.8) and *C. macrodidymum* (*P*<0.001), with means of 26.6, 10.8 and 5.8%, respectively (Table 6.2).

Table 6.2. Mean disease incidences at 5 cm above stem bases of vines, 6 months after inoculation with three *Cylindrocarpon* spp.

Species	Disease incidence (%)	
	Inoculated vines	Non-inoculated vines
C. destructans	10.8 b[1]	1.7 a
C. liriodendri	26.7 c	1.7 a
C. macrodidymum	5.8 ab	1.7 a
Cylindrocarpon spp. combined	35.8 z	4.2 y

[1]Values with different letters indicate values of inoculated vines are significantly different (*P*≤0.05). a-c for *Cylindrocarpon* spp. separately and y-z for the combined species.

The following analyses were conducted without the non-inoculated control water treated vines. Disease incidences at 5 cm above stem bases did not show significant effects of the water regimes for the different species (A6.1.9). The results are shown in A6.1.10.

Disease incidences at 5 cm above stem bases did not show significant effects of root wounding for the different species (A6.1.9 and the results are shown in A6.1.11). However, they showed a significant effect due to rootstock varieties for all *Cylindrocarpon* spp. combined (*P*<0.001), with means for 3309 and 101-14 being 55.0 and 16.7%, respectively, for *C. destructans* (*P*=0.042) means being 16.7 and 5.0%, respectively, for *C. liriodendri (P*<0.001) means being 43.3 and 10.0%, respectively, but not for *C. macrodidymum* (*P*=0.196), means being 8.3 and 3.3%, respectively (A6.1.9).

Disease severities at 1 cm above stem bases were not significantly affected by water regimes for the different species (A6.1.12). The results are shown in A6.1.13.

Disease severities at 1 cm above stem bases were not significantly affected by the wounding treatments (the results are shown in A6.1.14). However, they showed a significant effect due to rootstock varieties for all *Cylindrocarpon* spp. combined (*P*<0.001), with means for 3309 and 101-14 being 71.2 and 39.2%, respectively, for *C. destructans* (*P*=0.036) means being 26.7 and 13.3%, respectively, and for *C. liriodendri (P*<0.001) means being 47.9 and 19.2%, respectively, but not for *C. macrodidymum* (*P*=0.154) means being 27.5 and 17.5%, respectively (A6.1.12).

For the root and shoot dry weights, there were no significant effects due to the water regimes, wounding, rootstock varieties and inoculations (A6.1.15 and A6.1.16 for root and shoot dry weights, respectively).

6.3.2. Presence /absence of wounds

6.3.2.1. Investigation of the effect of wounding age

The statistical analyses for this section are shown in Section 6.2 of Appendix 6. Disease incidences at 1 cm above stem bases were significantly different between stem and root wounds for *Cylindrocarpon* spp. combined (*P*<0.001), with means of 61.3 and 39.3%, respectively, and for *C. liriodendri* (*P*<0.001), with means of 46.4 and 23.2%, respectively, but not for *C. destructans*, with means of 15.5 and 14.9%, respectively (*P*=0.872) or *C. macrodidymum*, with means of 16.1 and 8.3%, respectively (*P*=0.863; A6.2.1).

The effects of wounding on stems and roots showed similar trends so the data were combined. Disease incidences at 1 cm above stem bases were significantly affected by wound age for all *Cylindrocarpon* spp. combined (*P*<0.001) and for *C. liriodendri* (*P*<0.001), but not for *C. destructans* (*P*=0.136) or for *C. macrodidymum* (*P*=0.375; A6.2.1). Overall, there were no clear trends that indicated an ongoing effect due to the aging of wounds and the non wounded tissues had similar levels of infection as the wounded tissues (Figure 6.2).

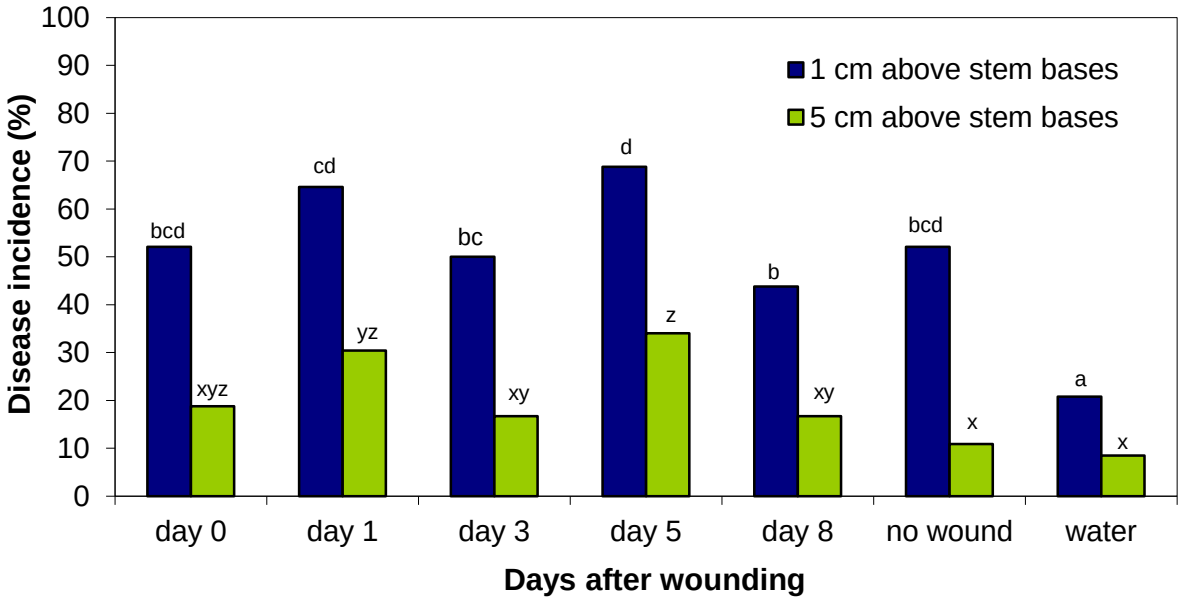

Figure 6.2. Mean disease incidences at 1 and 5 cm above stem bases of vines, 4 months after inoculation with *Cylindrocarpon* spp. at 0, 1, 3, 5 and 8 days after wounding, no wounding and water treatment. For each distance, bars with different letters indicate values are significantly different ($P \leq 0.05$).

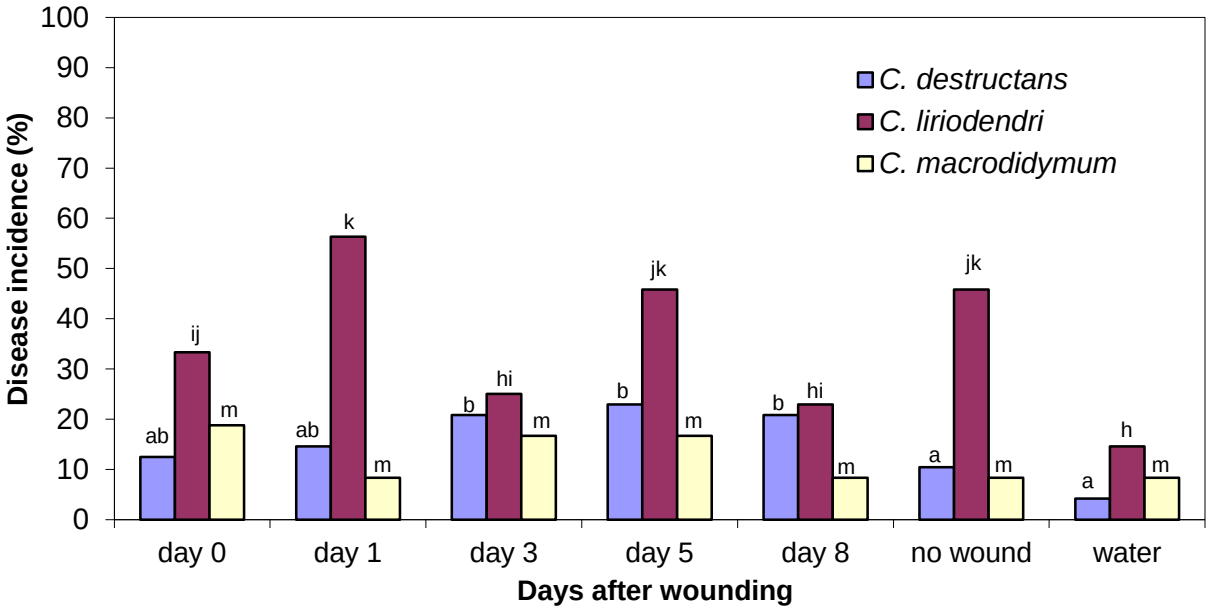

Figure 6.3. Mean disease incidences at 1 cm above stem bases of vines, 4 months after inoculation with *Cylindrocarpon* spp. at 0, 1, 3, 5 and 8 days after wounding, no wounding and no inoculation (water) treatments. For each species, bars with different letters indicate values are significantly different ($P \leq 0.05$).

For all *Cylindrocarpon* spp. combined, plants inoculated 5 days after wounding had significantly greater disease incidences at 1 cm above stem bases than plants inoculated 3 and 8 days after wounding (Figure 6.2 and A6.2.2). All inoculation treatments caused significantly greater disease incidences than water treated plants. For *C. destructans*, mean disease incidences ranged from 10.4 to 22.9% for inoculated plants (Figure 6.3). Pair-wise

comparison between treatments showed that plants inoculated 3, 5 and 8 days after wounding had greater disease incidences than plants inoculated with water (A6.2.3). For *C. liriodendri*, plants inoculated 1 day after wounding had significantly greater disease incidences at 1 cm above stem bases than plants inoculated 0, 3 and 8 days after wounding (A6.2.4). All inoculation treatments had significantly greater disease incidences than water treated plants, except for 3 and 8 days after wounding. For *C. macrodidymum*, mean disease incidences ranged from 8.3 to 18.8% for inoculated plants.

Overall, *C. liriodendri* caused significantly greater disease incidences at 1 cm above stem bases than *C. destructans* (P<0.001) and *C. macrodidymum* (P<0.001) which had similar disease incidences (P=0.295), with means of 34.8, 15.2 and 12.2%, respectively (A6.2.5).

Disease incidences at 1 cm above stem bases were significantly different between rootstock varieties for *C. macrodidymum* (P=0.028; A6.2.1) with mean incidences for 3309 and 101-14 being 16.1 and 8.3%, respectively, but not for all *Cylindrocarpon* spp. (P=0.723), for *C. destructans* (P=0.150) or for *C. liriodendri* (P=0.171; Table 6.3).

Table 6.3. Mean disease incidences at 1 cm above stem bases of vines from rootstock varieties 3309 and 101-14, 4 months after inoculation with *Cylindrocarpon* spp.

Species	Disease incidence (%)	
	101-14	3309
C. destructans	17.9 a[1]	12.5 a
C. liriodendri	31.5 h	38.1 h
C. macrodidymum	8.3 m	16.1 n
Cylindrocarpon spp. combined	49.4 z	51.2 z

[1]Within a row, values with different letters indicate values are significantly different (P≤0.05).

Disease incidences at 5 cm above stem bases were significantly different between stem and root wounds for *Cylindrocarpon* spp. (P<0.001) with means of 27.0 and 12.0%, respectively, and *C. liriodendri* (P=0.002), with means of 17.2 and 6.6%, respectively, but not for *C. destructans* (P=0.175) and for *C. macrodidymum* (P=0.772; A6.2.6 and Table 6.4).

Table 6.4. Mean disease incidence at 5 cm above stem bases of vines, 4 months after inoculation with *Cylindrocarpon* spp. after stem or root wounding.

Species	Disease incidence (%)	
	root wounding	stem wounding
C. destructans	3.6 a[1]	6.7 a
C. liriodendri	6.6 h	17.2 i
C. macrodidymum	4.8 m	5.5 m
Cylindrocarpon spp. combined	12.0 y	27.0 z

[1]Within a row, values with different letters indicate values are significantly different (P≤0.05).

As previously, both wounding treatments were combined together as they showed similar trends. Disease incidences at 5 cm above stem bases were significantly affected by wound ages for all *Cylindrocarpon* spp. combined (*P*=0.009), and for *C. liriodendri* (*P*=0.016), both having a greater incidence than the no wound treatment only for days 1 and 5 (Figures 6.2 and 6.4) but not for *C. destructans* (*P*=0.823) and *C. macrodidymum* (*P*=0.425; A6.2.6). Overall, there were no clear trends of an ongoing effect due to the aging of wounds and the no wound treatments had similar levels of infection as the wounded treatments.

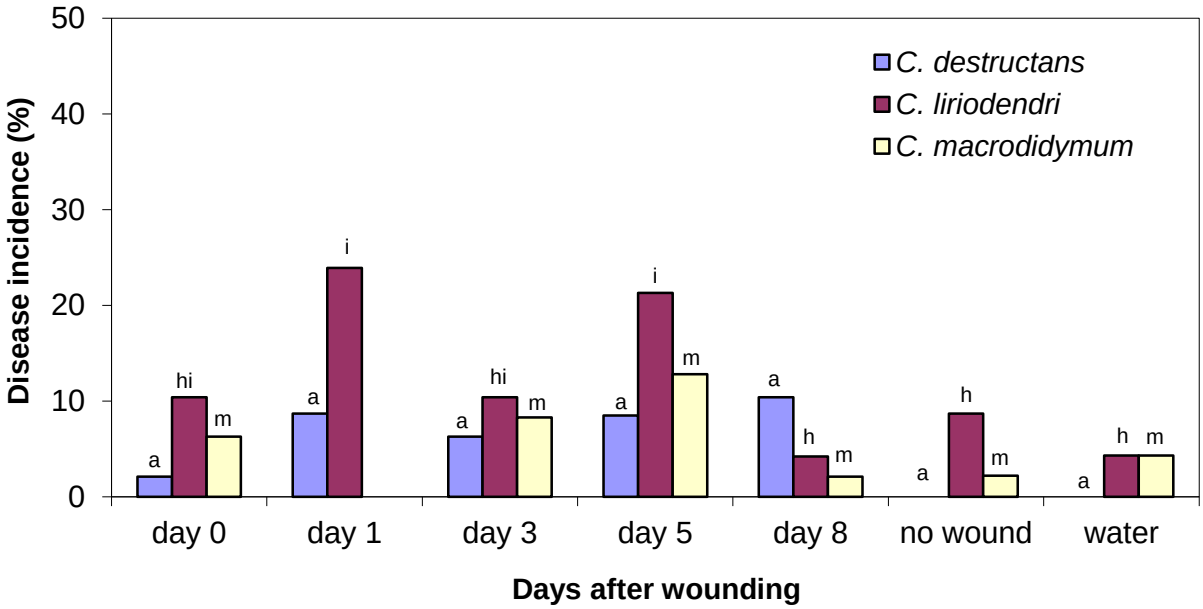

Figure 6.4. Mean disease incidences at 5 cm above stem bases of vines, 4 months after inoculation with *Cylindrocarpon* spp. at 0, 1, 3, 5 and 8 days after wounding, no wounding and water treatment. For each species, bars with different letters indicate values are significantly different (*P*≤0.05).

Overall, *C. liriodendri* caused significantly greater disease incidences at 5 cm above stem bases than *C. destructans* (*P*=0.001) and *C. macrodidymum* (*P*=0.003) which had similar disease incidences (*P*=1.000), with means of 11.8, 5.2 and 5.2% (A6.2.9), respectively.

Disease incidences for the different species at 5 cm above stem bases were not significantly different between the rootstock varieties (A6.2.6).

Disease severities at 1 cm above stem bases were significantly greater for stem than root wounds (*P*<0.001; A6.2.10), with means of 35.8 and 21.3%, respectively. The disease severities were significantly affected by wound ages for all *Cylindrocarpon* spp. combined (*P*<0.001; A6.2.10) however comparisons between treatments showed that only 5 day old wounds had a significantly greater mean disease severity than 8 day old wounds (Table 6.5). Disease severities were significantly greater for inoculated plants than water treated plants,

but did not differ between rootstock varieties or between the individual species with respect to wound ages.

Table 6.5. Disease severities at 1 cm above stem bases of vines, 4 months after inoculation with *Cylindrocarpon* spp. at 0, 1, 3, 5 and 8 days after wounding.

Days after wounding	Disease severity (%)
Day 0	31.2 bc[1]
Day 1	37.2 bc
Day 3	32.3 bc
Day 5	40.5 c
Day 8	26.6 b
No wounds	23.9 b
Control	8.7 a

[1]Values with different letters indicate values are significantly different ($P \leq 0.05$).

Root and shoot dry weights did not show any significant differences between stem wounded or root wounded plants. Root dry weights were significantly affected by wound age ($P<0.001$; A6.2.11), with plants inoculated 5 days after wounding having significantly lower root dry weights than plants inoculated 0, 1 and 8 days after wounding (Table 6.6). Shoot dry weights were also significantly affected by wound age ($P=0.002$; A6.2.12), with plants inoculated 5 days after wounding having significantly lower shoot dry weights than plants inoculated 0 days after wounding (Table 6.6).

Table 6.6. Mean root and shoot dry weights of rootstock varieties 3309 and 101-14, 4 months after inoculation with *Cylindrocarpon* spp. at 0, 1, 3, 5 and 8 days after stem and root wounding.

Treatment	Dry weights (g)	
	Roots	Shoots
Day 0	9.8 b[1]	34.5 b
Day 1	9.5 b	33.2 ab
Day 3	8.9 ab	32.5 ab
Day 5	7.4 a	28.0 a
Day 8	9.9 b	32.1 ab
No wounds	9.0 ab	29.7 ab
Control	8.6 ab	28.3 a

[1]Within a column, values with different letters indicate values are significantly different ($P \leq 0.05$).

Root dry weights were significantly different between rootstock varieties ($P<0.001$; A6.2.11), variety 101-14 having significantly greater root dry weights than variety 3309, with means of 9.7 and 8.3 g, respectively, but did not differ between root and stem wounded plants. Shoot dry weights were significantly different between rootstock varieties ($P<0.001$; A6.2.12), variety 101-14 having significantly higher root dry weights than variety 3309, with means of 35.9 and 26.5 g, respectively.

6.3.2.2. Graft unions

Cylindrocarpon species were not recovered from grafted plants inoculated at the graft-union.

6.3.2.3. Infection progression from trunk and root wounds

The statistical analyses for this section are shown in Section 6.3 of Appendix 6. For the root inoculated plants, their trunks exhibited black discoloration of the xylem vessels near the pith (Figure 6.5 A) while their roots were both asymptomatic (Figure 6.5 B) and symptomatic, with brown discoloration of the xylem vessels close to the pith (Figure 6.5 C).

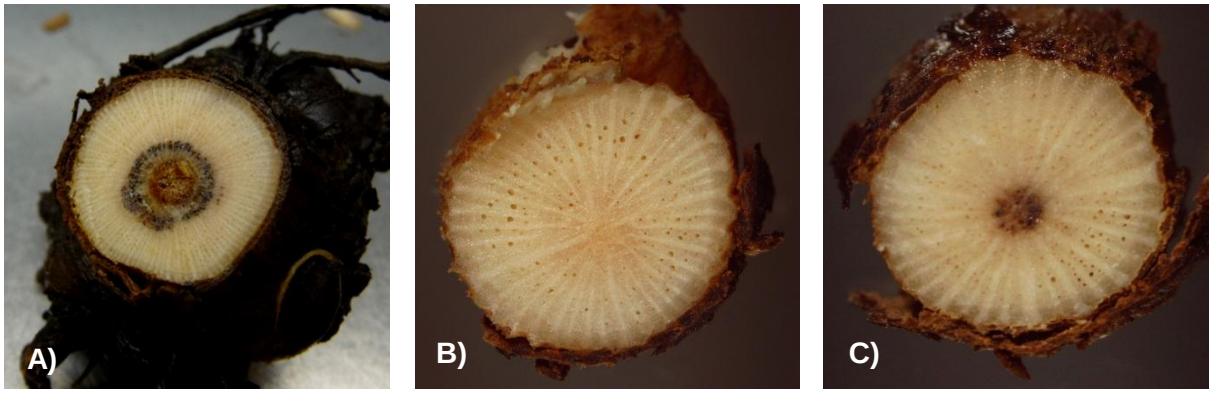

Figure 6.5. Cross-sections of trunk and roots from root wounded grapevines inoculated with *Cylindrocarpon* spp. A: symptomatic trunk; B: asymptomatic root; C: symptomatic root.

For root inoculated plants, disease incidences at 2.5, 5 and 7.5 cm above stem bases were not significantly different between inoculated root-wounded and unwounded vines, and root-wounded non-inoculated vines for the different *Cylindrocarpon* spp. (A6.3.1 to A6.3.4).

Pair-wise comparisons between disease incidences at 2.5, 5 and 7.5 cm above stem bases showed significant differences for *C. destructans* with incidences at 2.5 cm being significantly greater than those at 7.5 cm (P=0.016), with means of 6.8 and 0.8%, respectively, and for *C. macrodidymum*, with incidences at 5 cm being significantly greater than those at 7.5 cm (P=0.020), with means of 43.2 and 31.4%, respectively, but not for *C. liriodendri* (A6.3.5; Figure 6.6).

At 2.5, 5 and 7.5 cm above stem bases, *C. macrodidymum* caused significantly greater disease incidences than *C. liriodendri* and *C. destructans* which caused similar disease incidences (Figure 6.6 and A6.3.6). No significant differences were observed between rootstock varieties for the different species.

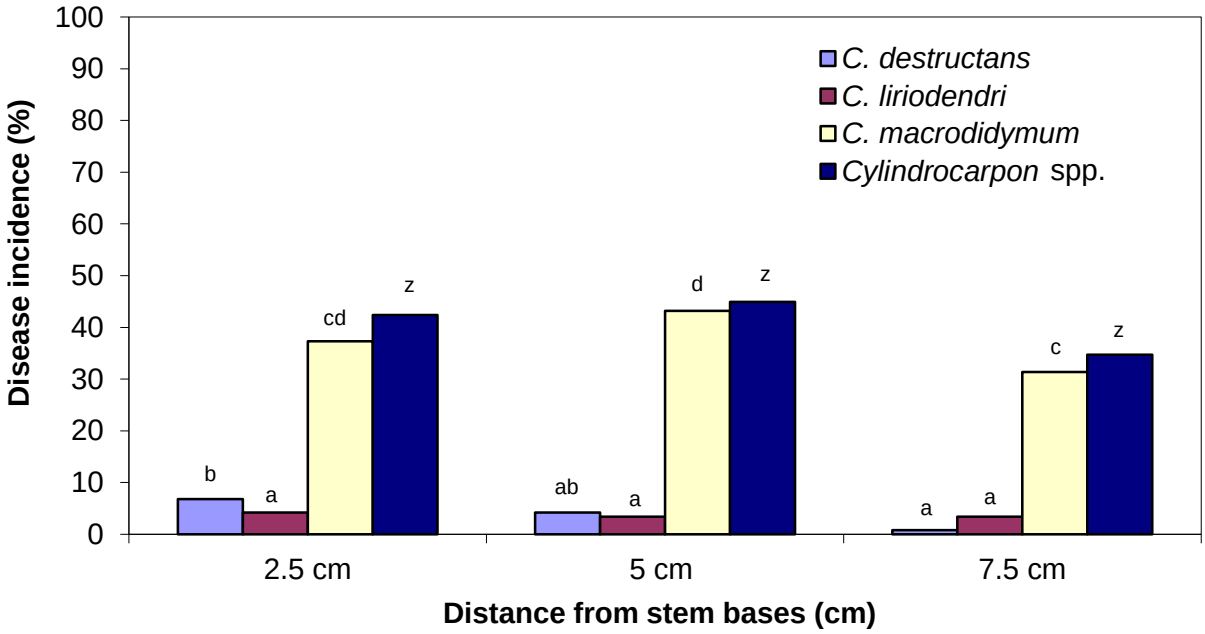

Figure 6.6. Mean disease incidences at 2.5, 5 and 7.5 cm above stem bases of grapevines, 6 months after inoculation with three *Cylindrocarpon* spp. Bars with different letters indicate values are significantly different ($P \leq 0.05$).

For root inoculated plants, disease severities at 2.5, 5 and 7.5 cm above stem bases were not significantly different between inoculated root-wounded, inoculated unwounded and root-wounded water treated vines for all *Cylindrocarpon* spp. combined, means being 22.2, 22.6 and 19.5% for 2.5, 5 and 7.5 cm above stem bases, and for *C. macrodidymum,* means being 18.3, 20.3 and 18.0%, respectively (A6.3.7 and A6.3.8). Results for *C. liriodendri* and *C. destructans* were too low to be analysed.

For root inoculated plants, proportions of infected roots were significantly different between inoculated root wounded, inoculated unwounded and root wounded non-inoculated vines for all *Cylindrocarpon* spp. combined ($P<0.001$), for *C. destructans* ($P<0.001$), for *C. liriodendri* ($P=0.052$) and for *C. macrodidymum* ($P=0.002$; A6.3.9). Proportions of infected roots were significantly greater for inoculated root wounded and unwounded plants than plants inoculated with water (Figure 6.7), except for unwounded vines inoculated with *C. liriodendri* which had similar proportions of infected roots to water treated plants.

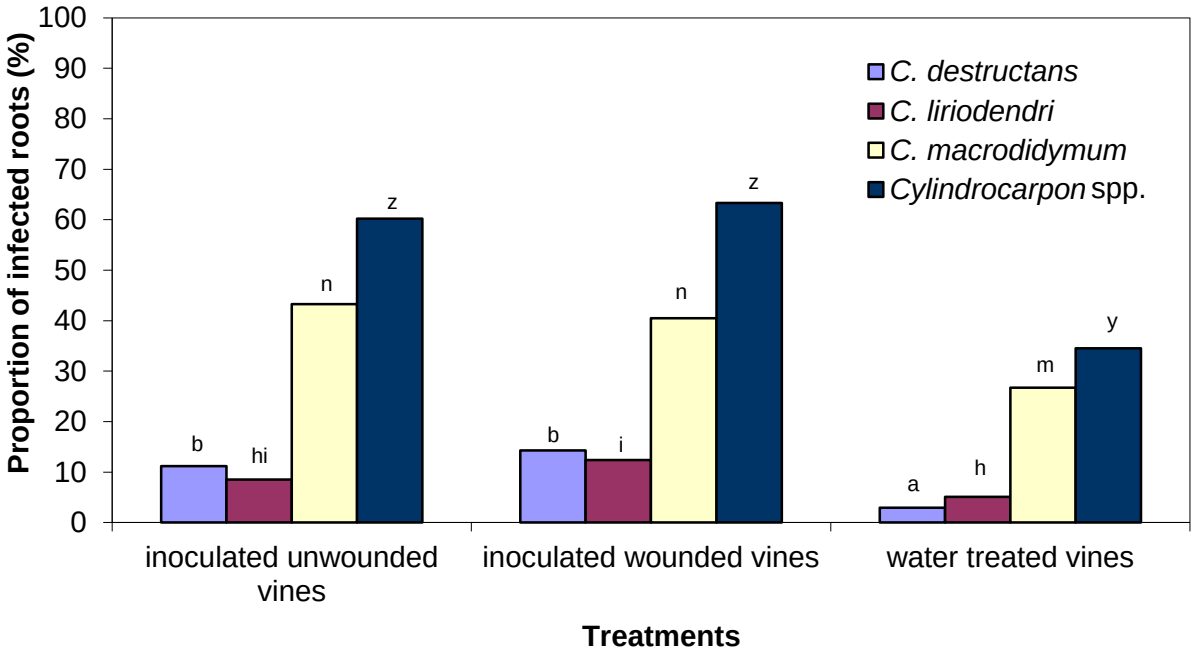

Figure 6.7. Mean proportion of infected roots of grafted vines, root wounded or unwounded, 6 months after inoculation with *Cylindrocarpon* spp. or water. For each species, bars with different letter indicate values are significantly different ($P \leq 0.05$).

Overall, *C. macrodidymum* infected significantly greater proportions of roots than *C. liriodendri* ($P<0.001$) and *C. destructans* ($P<0.001$) which both infected similar proportions of roots, with means of 36.9, 8.8 and 9.6%, respectively (A6.3.10). No significant differences between rootstock varieties were observed for the different species.

For cane and trunk inoculations, *Cylindrocarpon* spp. were not isolated from unwounded canes and trunks inoculated with *Cylindrocarpon* spp. Therefore, these data were omitted from the analyses as they interfered with them. The different species were isolated from 2.5 cm below the inoculation point, but not from 5 cm below it, and they were not isolated from above the inoculation point, at 2.5 cm or beyond it.

At the inoculation points, disease incidences showed significant differences between inoculated wounded canes and trunks, for all *Cylindrocarpon* spp. combined ($P=0.047$) and for *C. macrodidymum* ($P<0.001$) but not for *C. destructans* ($P=0.699$) and for *C. liriodendri* ($P=0.067$; A6.3.11 to A6.3.14).

At the inoculation points, disease incidences were significantly greater for canes than for trunks, for all *Cylindrocarpon* spp. combined, with means of 82.1 and 65.0%, respectively, and for *C. macrodidymum*, with means of 66.7 and 27.5%, respectively (Figure 6.8). However, the incidence for cane and trunk inoculations did not differ for *C. destructans*, with means of 33.3 and 30.0%, respectively or for *C. liriodendri*, with means of 69.2 and 52.5%,

respectively. The disease incidences at a distance of 2.5 cm below the inoculation point did not show any significant differences between cane and trunk inoculations for the different species.

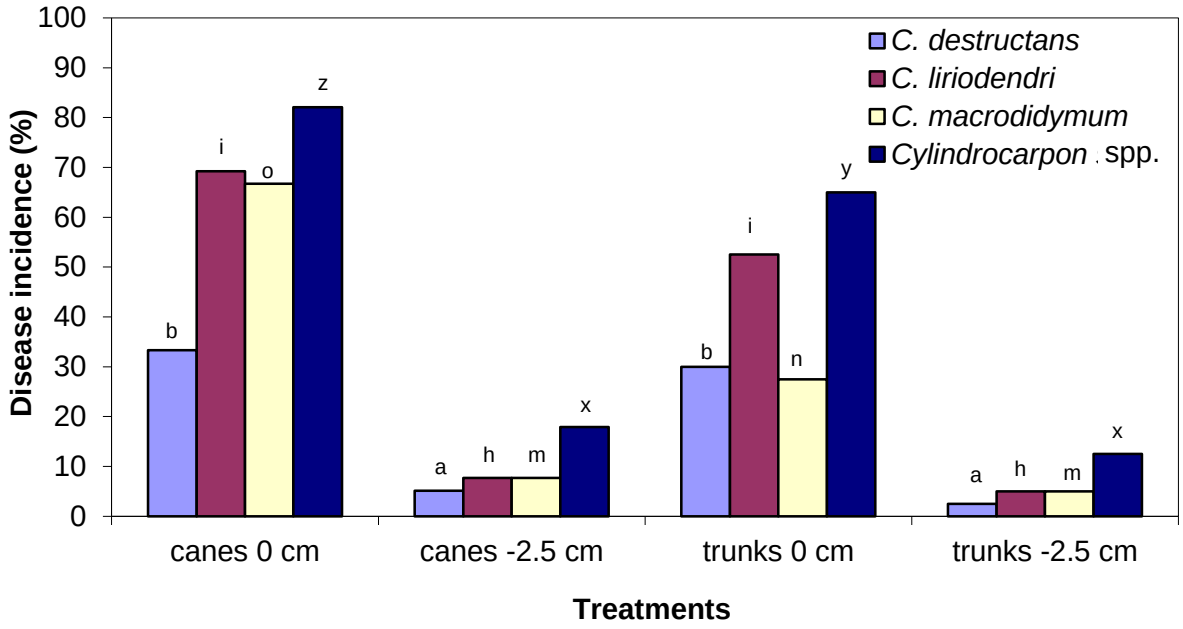

Figure 6.8. Mean disease incidences at inoculation point and at 2.5 cm below the inoculation point of grafted vines, 6 months after inoculation of canes and trunks with *Cylindrocarpon* spp. For each species, bars with different letters indicate values are significantly different ($P \leq 0.05$).

Overall, *C. liriodendri* caused significantly greater disease incidences at the inoculation point than *C. macrodidymum* and *C. destructans*, with means of 60.8, 46.8 and 31.6%, respectively (A6.3.15). At 2.5 cm below the inoculation point, all three species caused similar disease incidences, with means of 6.3, 6.3 and 3.8%, respectively.

For cane and trunk inoculations, disease severities at the inoculation point were significantly greater for canes than for trunks, for all *Cylindrocarpon* spp. combined ($P<0.001$), with means of 70.9 and 41.9%, respectively (Table 6.7), for *C. liriodendri* ($P=0.044$), with means of 50.0 and 31.9%, respectively, and for *C. macrodidymum* ($P=0.001$), with means of 35.2 and 12.5%, respectively, but not for *C. destructans* ($P=0.835$; A6.3.16). Overall, *C. liriodendri* caused significantly greater disease severities than *C. macrodidymum* and *C. destructans*, with means of 40.5, 23.7 and 14.2%, respectively (A6.3.17).

Table 6.7. Mean disease severity at the inoculation point of grafted vines, 6 months after inoculation of canes and trunks with *Cylindrocarpon* spp.

Species	Disease severity (%)	
	cane wounding	trunk wounding
C. destructans	14.8 a[1]	13.7 a
C. liriodendri	50.0 i	31.9 h
C. macrodidymum	35.2 n	12.5 m
Cylindrocarpon spp. combined	70.9 z	41.9 y

[1]Within a row, values with different letters are significantly different ($P \leq 0.05$).

6.3.3. Effect of cold storage

The statistical analyses for this section are shown in Section 6.4 of Appendix 6. Disease incidences at 1 cm above stem bases were significantly different between the inoculated vines and the controls (water treated plants) with $P<0.001$ for all *Cylindrocarpon* spp. combined, *C. destructans*, *C. macrodidymum* and *C. liriodendri* (A6.4.1) and the results are shown in Table 6.8.

Table 6.8. Mean disease incidences at 1 cm above stem bases of vines, 3 months after inoculation with three *Cylindrocarpon* spp.

Species	Disease incidence (%)	
	Inoculated vines	Non-inoculated vines
C. destructans	41.7 b[1]	11.1 a
C. liriodendri	75.0 c	0.0 a
C. macrodidymum	63.9 d	5.6 a
Cylindrocarpon spp. combined	96.5 z	16.7 y

[1]Values with different letters indicate values of inoculated vines are significantly different ($P \leq 0.05$). a-d for *Cylindrocarpon* spp. separately and y-z for the combined species.

The following analyses were conducted without data from the non-inoculated control (water treated) vines. Disease incidences at 1 cm above stem bases were significantly affected by cold storage for plants inoculated with *C. liriodendri* ($P=0.006$) but not all *Cylindrocarpon* spp. combined ($P=0.895$), *C. destructans* ($P=0.276$) or *C. macrodidymum* ($P=0.106$; A.6.4.2).

For all *Cylindrocarpon* spp. combined, disease incidences at 1 cm above stem bases ranged between 87.5% and 100% (Figure 6.9). Pair-wise comparisons between cold storage periods showed significantly greater disease incidences for *C. destructans* for plants inoculated after 6 months of cold storage than 1 and 2 months, with means of 58.3% for 6 months and 29.2% for 1 and 2 months (A6.4.3 and Figure 6.10). For *C. liriodendri*, there was significantly greater disease incidences for plants after 5 and 6 months of cold storage than 1 month, with means of 87.5, 95.8 and 58.3%, respectively (A6.4.4) and after 4, 5 and 6 months of cold storage than 3 months, with means of 83.3, 87.5, 95.8 and 50.0%, respectively. For *C.*

macrodidymum, there were significantly greater disease incidences for plants after 3 months of cold storage than 4 months, with means of 83.3 and 45.8%, respectively (A6.4.5).

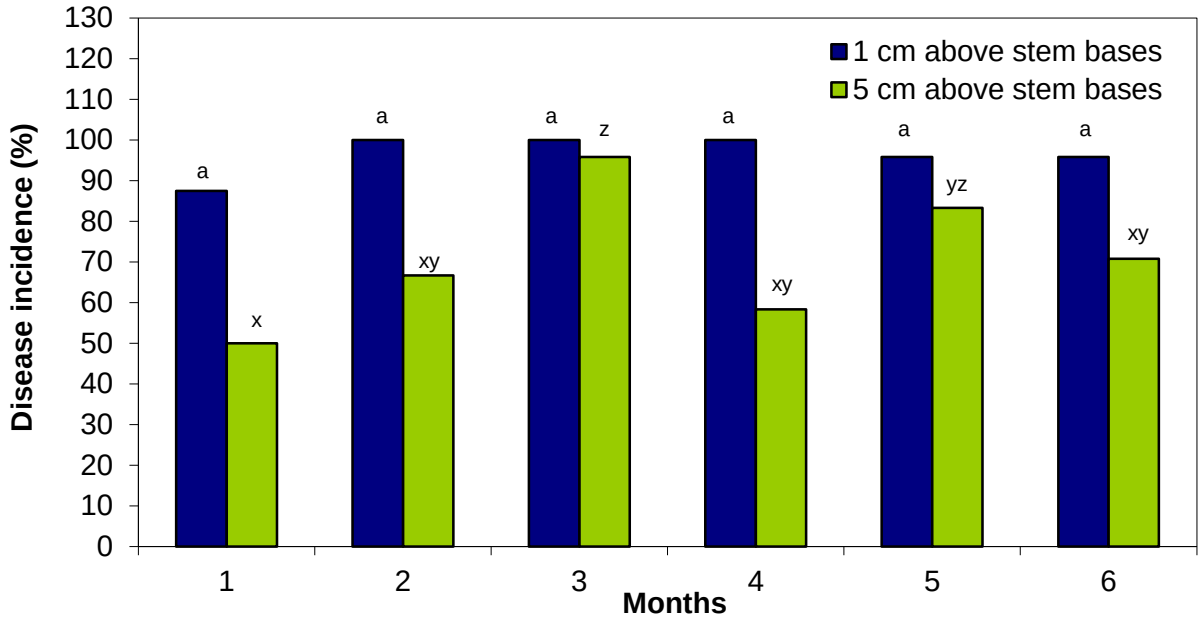

Figure 6.9. Mean disease incidences at 1 and 5 cm above stem bases of vines which were cold stored for 1 to 6 months and inoculated with *Cylindrocarpon* spp. For each distance, bars with different letters indicate values are significantly different ($P \leq 0.05$).

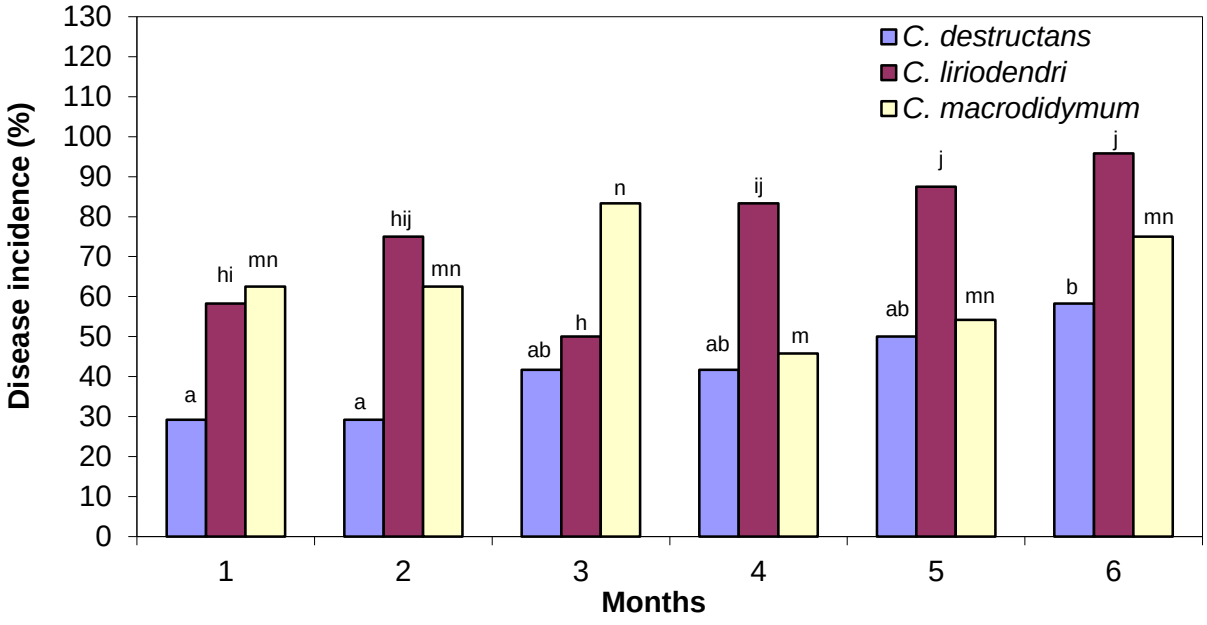

Figure 6.10. Mean disease incidences at 1 cm above stem bases of vines which were cold stored for 1 to 6 months and inoculated with *Cylindrocarpon* spp. For each species, bars with different letters indicate values are significantly different ($P \leq 0.05$).

Overall, *C. liriodendri* caused significantly greater disease incidences at 1 cm above stem bases than *C. macrodidymum* and *C. destructans*, with means of 75.0, 63.9 and 41.7%, respectively (A6.4.6 and Table 6.8). No significant differences were observed between varieties for the different species (A6.4.2).

Disease incidences at 5 cm above stem bases were significantly different between the inoculated vines and the water treated controls (A6.4.7), with $P<0.001$ for all *Cylindrocarpon* spp. combined, *C. destructans*, *C. macrodidymum* and *C. liriodendri* (Table 6.9).

Table 6.9. Mean disease incidences at 5 cm above stem bases of vines, 3 months after inoculation with three *Cylindrocarpon* spp.

Species	Disease incidence (%)	
	Inoculated vines	Non-inoculated vines
C. destructans	18.1 b[1]	2.1 a
C. liriodendri	38.9 c	0.0 a
C. macrodidymum	36.1 d	1.4 a
Cylindrocarpon spp. combined	70.8 z	3.5 y

[1]Values with different letters indicate values of inoculated vines are significantly different ($P \leq 0.05$). a-d for *Cylindrocarpon* spp. separately and y-z for the combined species.

The following analyses were conducted without data from the non-inoculated control water treated vines. Disease incidences at 5 cm above stem bases were significantly affected by cold storage (A.6.4.8) for plants inoculated with all *Cylindrocarpon* spp. combined ($P=0.027$), being significantly greater in plants cold-stored for 3 months than 1, 2, 4 and 6 months (A6.4.9), and for 5 months than 1 month (Figure 6.9). For *C. destructans* ($P=0.028$) the 5 cm disease incidences were significantly greater in plants cold-stored for 5 months than 1, 2 and 4 months (A6.4.10), and for 6 months than 1 month (Figure 6.11). However, the 5 cm disease incidences were not affected by cold storage in plants inoculated with *C. liriodendri* ($P=0.497$) or *C. macrodidymum* ($P=0.061$). For *C. liriodendri*, disease incidences ranged from 29.9 to 54.2%. For *C. macrodidymum*, pair-wise comparison showed significantly greater disease incidences for plants after 3 months of cold storage than 2, 4, 5 and 6 months. Overall, *C. liriodendri* caused significantly greater disease incidences at 5 cm above stem bases than *C. macrodidymum* and *C. destructans*, with means of 38.9, 36.1 and 18.1%, respectively (A6.4.12 and Table 6.9). No significant differences were observed between varieties for the different species (A6.4.8).

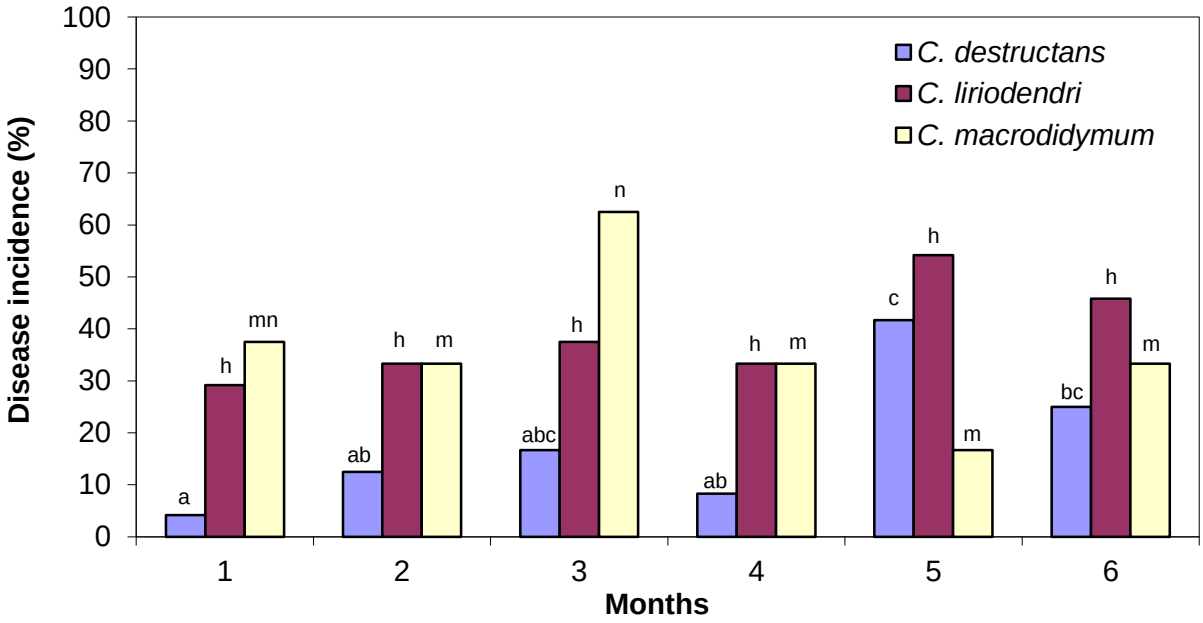

Figure 6.11. Mean disease incidences at 5 cm above stem bases of vines which were cold stored for 1 to 6 months and inoculated with *Cylindrocarpon* spp. For each species, bars with different letters indicate values are significantly different ($P \leq 0.05$).

Disease severities at 1 cm above stem bases of plants cold stored for 1 to 6 months showed significant differences ($P<0.001$; A6.4.13) between inoculated plants and their respective controls for *C. destructans*, *C. liriodendri*, *C. macrodidymum* and *Cylindrocarpon* spp. combined (Table 6.10).

Table 6.10. Mean disease severities at 1 cm above stem bases of vines, 3 months after inoculation with three *Cylindrocarpon* spp.

Species	Disease severity (%)	
	Inoculated vines	Non-inoculated vines
C. destructans	29.2 b[1]	8.1 a
C. liriodendri	58.3 c	0.0 a
C. macrodidymum	47.7 d	4.3 a
Cylindrocarpon spp. combined	84.2 z	12.5 y

Values with different letters indicate values of inoculated vines are significantly different ($P \leq 0.05$). a-d for *Cylindrocarpon* spp. separately and y-z for the combined species.

The following analyses were conducted without the non-inoculated (water treated) control vines. Disease severities at 1 cm above stem bases were significantly affected by cold storage for all *Cylindrocarpon* spp. combined ($P<0.001$), with significantly greater disease severities at 1 cm in plants cold-stored for 3, 5, and 6 months than 1 month (Table 6.11). For *C. destructans* ($P=0.038$), disease severities at 1 cm were significantly greater in plants cold-stored for 5 and 6 months than 1 and 2 months (Figure 6.12). For *C. liriodendri* ($P<0.001$), disease severities at 1 cm were significantly greater in plants cold-stored for 4, 5 and 6

months than 1 month, and for 5 and 6 months than 2 and 3 months. For *C. macrodidymum* (P<0.001), disease severities at 1 cm were significantly greater in plants cold-stored for 3 months than 1, 2, 4 and 5 months, and for 6 months than 1 and 4 months (A.6.4.14). Overall, *C. liriodendri* caused significantly greater disease severities at 1 cm above stem bases than *C. macrodidymum* and *C. destructans*, with means of 58.3, 47.7 and 29.2%, respectively (A6.4.15 and Table 6.10). No significant differences were observed between varieties for the different species (A6.4.20 to A6.4.23).

Table 6.11. Mean disease severities at 1 cm above stem bases of vines cold stored for 1 to 6 months and assessed 3 months after inoculation with *Cylindrocarpon* spp.

Month	Disease severity (%)
1	63.6 a[1]
2	75.0 ab
3	95.8 b
4	79.2 ab
5	95.8 b
6	95.8 b

[1]Values with different letters indicate values of inoculated vines are significantly different (P≤0.05).

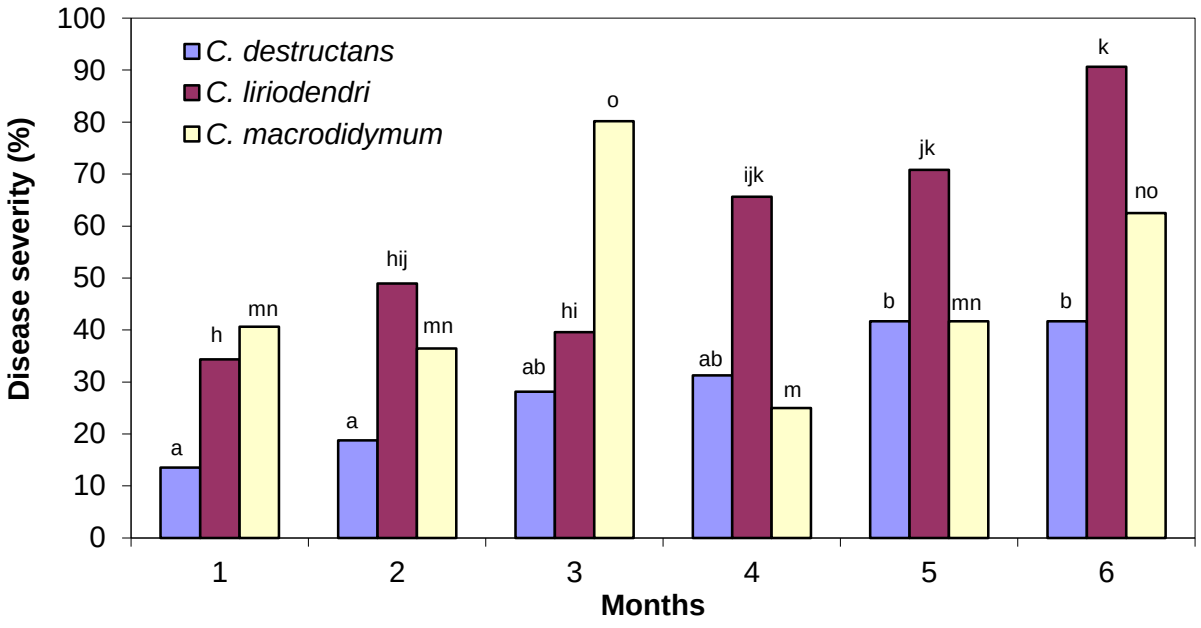

Figure 6.12. Mean disease severities at 1 cm above stem bases of vines which were cold stored for 1 to 6 months and inoculated with *Cylindrocarpon* spp. For each species, bars with different letters indicate values are significantly different (P≤0.05).

6.3.4. Prior infection with *Phaeomoniella chlamydospora*

The statistical analyses for this section are shown in Section 6.5 of Appendix 6. *Cylindrocarpon* spp. were not isolated from the controls (water treated) plants and the following analyses were conducted without these data.

Disease incidences at 1 cm above stem bases of inoculated plants showed significant differences between plants with or without *P. chlamydospora* for *C. liriodendri* (*P*=0.047), with means of 45.0 and 15.0%, respectively, but not for all *Cylindrocarpon* spp. combined (*P*=0.114), *C. destructans* (*P*=0.556) and *C. macrodidymum* (*P*=1.000; A6.5.1; Table 6.12). Overall, *C. liriodendri* and *C. macrodidymum* caused significantly greater disease incidences than *C. destructans* (A6.5.2), with means of 30.0, 30.0 and 7.5%, respectively.

Table 6.12. Mean disease incidences at 1 cm above stem bases of vines which were hot water treated or not and inoculated with *Cylindrocarpon* spp.

Species	Disease incidence (%)			
	no hot water treatment		hot water treatment	
C. destructans	10.0	a[1]	5.0	a
C. liriodendri	45.0	i	15.0	h
C. macrodidymum	30.0	m	30.0	m
Cylindrocarpon spp. combined	70.0	z	45.0	z

[1]Within a row, values with different letters indicate values of inoculated vines are significantly different (*P*≤0.05).

Disease incidences at 5 cm above stem bases of inoculated plants did not show any significant differences between plants with or without *P. chlamydospora* for all *Cylindrocarpon* spp. combined (*P*=0.678), for *C. destructans* (the pathogen was absent), for *C. liriodendri* (*P*=0.635) and *C. macrodidymum* (*P*=0.556; A6.5.3).

6.4. Discussion

In the vineyard, grapevines are subjected to different soil moisture levels throughout the year. The experiment investigating the effect of different water regimes showed that although disease incidences at 1 cm above stem bases were affected by the three water regimes, disease incidences were only significantly greater for inoculated water-logged plants than for plants watered with 75% FC, with means of 80.0 and 52.5%, respectively. This difference was mainly due to *C. macrodidymum* which caused greater disease incidences in water-logged plants than in plants from the lowest water regime (75% FC). The excess of water limits root growth because absorption of water and minerals is reduced, while oxygen levels gradually reduce and carbon dioxide levels increase (Childers and White, 1942; Kramer and

Boyer, 1995). In this environment, roots undergo changes to adapt to the stress (Burgess *et al*., 1999), which can be associated with greater susceptibility to disease. Colin-Belgrand *et al*. (1991) observed that 7 week flooding of three *Quercus* spp. caused root decay and production of adventitious roots. Such effects can be caused by a range of species, which are classified as weak pathogens, but become capable of infection under these conditions, as was illustrated by the work of Isaac (1956) who observed that very wet soil conditions were the only conditions under which *Verticillium nubilum* and *V. tricorpus* induced wilt symptoms in antirrhinum. Shivas *et al.* (1995) also reported that disease incidence of bananas caused by *Fusarium oxysporum* f. sp. *cubense* was increased with water-logging. Isaac (1957) studied the effect of three water regimes (dry, normal and water-logged soils) on the wilting of Brussel sprout plants caused by *Verticillium dahliae* and observed that water-logged plants were severely diseased after 6 weeks while plants in dry soil showed delayed symptoms and lower disease severity compared to water-logged plants.

In this study, the grapevines were subjected to the different water regimes before inoculation in order to only stress the plants, and not to directly affect the pathogen during the infection period. However, Sweetingham (1983) inoculated 6 month old rooted grapevines with 15 mL of 10^7 *C. destructans* conidia /mL and then maintained the water levels around the roots of the plants at 100% FC or water-logged for 2 weeks. All vines were then watered to 100% FC for 3 months, after which they were assessed. At the end of his experiment, 50% of the water-logged plants showed vascular discoloration and *C. destructans* was isolated from those tissues while the inoculated plants which were watered at 100% FC throughout were asymptomatic. *Cylindrocarpon* spp. are also able to survive in water-logged soils, as observed by Warcup (1951) who showed that the number of *C. destructans* colonies was greater from soil samples with high moisture content than low moisture content. Thornton (1965) also associated the high incidence of *C. destructans* with high soil moisture and cold temperature as *C. destructans* were not isolated from soils which had a moisture deficiency below wilting point for 2 to 3 months in summer. The high incidence of *C. destructans* in wet soils might be related to the mechanisms observed by Newcombe (1960) for *Fusarium oxysporum* f. c*ubense.* She reported that flooded soils contained abundant viable conidia of *Fusarium oxysporum* f. *cubense* up to 37 days after placing them in flooded soil and very few chlamydospores were produced. However, in moist soil, abundant chlamydospores were formed after 7 days and conidium numbers had decreased by 14 days. In flooded soil, the high germination of conidia contributed to the multiplication and spread of *Fusarium oxysporum* f. *cubense* in the presence of organic material. However, in the absence of the host or organic matter, soil isolation showed that the pathogen only survived for 3 months in soil under water whereas it was capable of surviving for at least 8 months in moist soil. Further investigations into the longevity of the various propagules of the common

Cylindrocarpon species and the changes in physiology of grapevine roots in soils subjected to different water regimes would provide additional information on the role of soil moisture in the disease cycle.

In the experiment investigating the effect of wound age prior to inoculation, stems inoculated 5 days after wounding had the greatest mean disease incidence at 1 cm above the wound site, while those inoculated 8 days after wounding showed the lowest mean disease incidence among inoculated plants, with 68.8 and 43.8%, respectively. However, there was no clear trend of reducing infection with increasing wound age. As plants were inoculated on the same day with the same conidium suspension, variations in the results are unlikely to result from the inoculum. When plants are wounded, they activate their defence responses to seal the wound site and to protect the plant from opportunistic pathogen invasion (De Bruxelles and Roberts, 2001). These defence responses consist of the formation of a physical barrier by reinforcing the existing cell walls and producing glycoproteins, lignin and suberin, as well as the synthesis of defence proteins and secondary metabolites, which are transported through the vascular system to other parts of the plants, and the production of reactive oxygen species (De Bruxelles and Roberts, 2001). As the plants put in place their defence systems following wounding, lower disease incidence and severity are expected in plants inoculated a few days following wounding.

Overall, the different wounding treatments in this experiment did not affect disease incidences caused by the different *Cylindrocarpon* species, being similar to the non-wounded controls. Munkvold and Marois (1995) inoculated 1 to 3 year old grapevine canes with *Eutypa lata* and observed a decrease in susceptibility with the increase of time after wounding, with a major decrease 28 days after wounding. They also observed that the time of pruning had an effect on the susceptibility of the plants, with a greater disease incidence when the plants were pruned early in the dormant season than plants pruned later in the dormant season. The same effects were reported by Munkvold and Marois (1994) on cherry trees infected at different times after wounding with *Eutypa lata*. Eskalen *et al.* (2007) inoculated *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora* at different times after wounding and observed that infection remained high in the first 2 months after wounding, was followed by a decrease in the following months, with no infection 5 months after wounding. Munkvold and Marois (1995) related the decrease in susceptibility with the increase of suberin and lignin, as also reported by Biggs (1989), who correlated a high suberin accumulation rate with the resistance of peach cultivars to *Leucostoma persoonii*. This experiment should be repeated with a longer wounding age to determine whether plants inoculated 8 days after wounding and beyond show a reduction in susceptibility.

Hawkins and Boudet (1996) detected lignin and suberin in the xylem wound zone 24 h after wounding 3 – 4 month old *Eucalyptus gunii* plants, in the bark wound zone 3 days after wounding, and they observed a wound periderm which was 2 – 3 cell layers thick in the bark wound zone. Cytological and biochemical studies such as the production of phytoalexins, lignin, suberin, oxidised phenolics and callose, and the activity of enzymes involved in the plant defence response could also be conducted to investigate the changes in wound sites over time.

In this study, no significant differences were observed between wounding treatments in the water stress experiment, which indicated that *Cylindrocarpon* spp. were capable of infecting wounded and non-wounded roots. Similarly, the experiments investigating the effect of wounding time prior to inoculation and the infection progression in grapevines in the vineyard showed that there was no significant differences in *Cylindrocarpon* disease incidences at 1 cm above stem bases between plants with wounded and unwounded roots. Although older stem and root tissues have lignified cell walls and bark, which provides some resistance to disease, pathogens are capable of penetrating young roots through the immature cortex of the apical region of the roots or through the wounds made when new lateral roots branch off the main roots (Garrett, 1970; Manners, 1993) which can explain greater disease incidences in water-logged soils. Rahman and Punja (2005) observed that *C. destructans* caused lesions mostly around the intersection between the primary and the lateral roots on ginseng. They also reported greater disease severity for wounded roots compared to unwounded roots. In the experiment investigating the effect of wounding time prior to inoculation, disease severities of wounded plants were non-significantly greater than for unwounded plants. A shorter growth period after inoculation may have provided greater differences in the disease incidences and severities between wounded and non-wounded plants. In nature, wounds in roots can also be caused by insects, other pathogenic microorganisms and nematodes (Wood, 1967), providing multiple entry points for *Cylindrocarpon* spp. but their role has not been investigated. However, Mundy *et al*. (2005) reported that the larvae of grass grub (*Costelytra zealandica),* which are known to feed on the roots of a range of plants, also damaged grapevines. In potted vine experiments, their feeding behaviours significantly reduced the mean length of roots and created grooves around the underground portions of the trunks. Whether this feeding damage by grassgrub larvae increases susceptibility to *Cylindrocarpon* warrants future investigation.

In the experiment investigating the infection progression, roots showed a brown discoloration of the pith and black streaks were observed in the wood of infected trunks (Figure 6.7). Black discoloration and gum inclusions in xylem vessels of grapevine stems infected with *Cylindrocarpon* spp. have been previously reported (Grasso and Magnano di San Lio, 1975; Sweetingham, 1983; Rego *et al*., 2000). Sweetingham (1983) described the presence of

hyphae in ray cells of the phloem and younger xylem which store starch, and the assocated the tissue discoloration with the presence of brown gum. Baayen *et al.* (1996) observed that carnation inoculated with *Fusarium oxysporum* f. sp. *dianthi* had their xylem vessels and intercellular spaces between the xylem and the cortex occluded with lignified gums. They concluded that the mechanisms were similar to the compartmentalisation of decay in trees, where colonisation of trees was inhibited vertically by obstructing the xylem vessels and laterally by reinforcing cell walls (Shigo, 1984).

Young grafted grapevines were inoculated at their graft-union to determine whether *Cylindrocarpon* spp. were capable of infecting vines through any incompletely healed sections of the grafting point. The usual nursery practice involves grafting the grapevines and then placing them into boxes of vermiculite in a warm room (27°C and 97% RH) for 3-4 weeks which allows the graft-union to heal and plant base to form a callus. The ‘graftlings‘ can then be planted into a field for one growing season, during which time they develop roots and shoots. If stem wounds were present and they were planted out into infested soil, infection could easily occur before the dormant grapevines were uprooted and sent to growers.The pathogen was not isolated from the graft-union which suggests an adequate healing of the graft-union at the nursery before sending the plants to growers. As unwounded canes and trunks were not infected, it seems that *Cylindrocarpon* spp. need a wounding site in above ground tissues to infect grapevines.

In wounded trunks and canes, *Cylindrocarpon* spp. were confined between 2.5 cm above and 5 cm below the inoculation point, since they were not isolated at or beyond these distances. Disease incidences were greater at the inoculation point than 2.5 cm below with 82.1 and 17.9%, respectively for canes and 65.0 and 12.5%, respectively for trunks. This indicated that the *Cylindrocarpon* spp. moved slowly in stems and mostly towards the base. This may be due to the fact that the phloem vessels are closer to the outer parts of grapevines, where wounding occurred, while xylem vessels are closer to the pith. In addition, Sweetingham (1983) observed the presence of hyphae in phloem vessels but never in xylem vessels indicating the preference of the pathogen for these tissues, which could also explain the movement of the pathogen towards the base. A possible explanation is that xylem vessels are thick-walled and lignify rapidly while phloem vessels are cellulosic (Galet, 2000). Sinclair *et al.* (1981) inoculated *Verticillium dahlia* in 4 to 7 year old *Acer rubrum* seedlings at 50 cm above ground and observed that in the first 10 months, *Verticillium dahlia* grew an average of 45 cm upward and 10 cm downward from the wound and no additional growth was observed in the following 14 months which they related to compartmentalisation. It is possible that the infection caused by *Cylindrocarpon* spp. was compartmentalised however cytological observations are needed to investigate changes at the inoculation point and in

surrounding tissues. Infection through wounded canes or trunks of grapevines constitute a potential threat to grapevine trunks as it is a common practice in vineyards to break off lateral shoots growing from the trunks, leaving a wound which can be used by *Cylindrocarpon* spp. as an entry point. As shown in the experiment investigating wounding age, the pathogen is still capable of infecting the plant 8 days after wounding. Although the pathogen is not reported to be airborne, it is likely to be splash-borne during heavy rainfall which may allow it to infect wounds in lower trunks. However, the occurrence of this event is likely to be masked by *Cylindrocarpon* infection through roots.

In plants inoculated with *Cylindrocarpon* spp. in the roots, the pathogens moved from the roots to the trunks reaching over 7.5 cm, 6 months after inoculation, with a disease incidence and severity of 34.7 and 19.5%, respectively at 7.5 cm above stem bases. The progression of *Cylindrocarpon* spp. from the roots to the trunk and the scion was observed by Halleen *et al.* (2003) who isolated increasing numbers of *Cylindrocarpon* spp. from roots, then rootstock and finally scion sections of callused grafted vines from 0 to 9 months after planting in nursery soil. Plant defences do not seem to be efficient to contain *Cylindrocarpon* spp. after root penetration.

Root wounded non-inoculated plants in the vineyard had a significantly lower proportion of infected roots than inoculated unwounded and wounded grapevines, with means of 34.5, 60.2 and 63.3%, respectively. However, some natural infection clearly occurred, either because the vineyard was sited in an infested area or because of cross-contamination between neighbouring vines, which were separated by only 75 cm.

In this experiment, the relative pathogenicities of the different species were different from those found in previous experiments. In almost all other experiments, *C. liriodendri* caused significantly greater disease incidences than *C. destructans* and *C. macrodidymum,* however in this experiment *C. macrodidymum* caused significantly greater disease incidences than the two other species, with means of 36.9, 8.8 and 9.6% for *C. macrodidymum*, *C. liriodendri* and *C. destructans*, respectively. The main difference between this and other experiments was the lack of frequent irrigation. Therefore, low soil moisture might have contributed to the low disease incidence of *C. liriodendri* and the higher disease incidence of *C. macrodidymum.* However, in a previous experiment using different soil types (Chapter 4) this pathogen species had not caused the greatest disease incidences in sandy loam, which was likely to present a drier root environment than the other soils. It is probable that the combination of low soil moisture and high temperature on the inoculation day have damaged *C. liriodendri* conidia as the same conidium suspension was used to inoculate canes and

trunks which exhibited significantly greater disease incidences for *C. liriodendri* than for *C. macrodidymum*, with means of 60.8 and 46.8%, respectively.

The experiment investigating the effect of cold storage time showed no significant effect on disease incidences at 1 cm above stem bases for any species except *C. liriodendri,* for which incidence increased significantly with cold storage time. Differences between cold storage periods were observed for the disease incidences at 5 cm above stem bases for all species combined and *C. destructans*, with incidence increasing after 1 month cold-storage. However, the disease severities did show a clear trend for increasing severity with storage time for all species. Plants cold stored for 1 month had the lowest disease severities and those for 6 months the highest severities, with means of 63.6 and 95.8%, respectively and the greatest disease severities were recorded for plants cold stored for 3, 5 and 6 months, with means of 95.8%. An increase in the disease incidences and severities of grapevines submitted to prolonged period of cold storage was expected as cuttings are weakened with extended cold storage as reported by Zencirkiran (2010) with rooted carnation cuttings. Zencirkiran (2010) observed a decline in survival rate with storage time, with a loss of 50% after 4 months. Snedden *et al.* (2010) found that the increase of cold storage time reduced the average root numbers and shoot height of *Populus tremuloides*. Eisenberg (1978) reported that the quality of stored ornamental cuttings decreased with time of storage. Alley and Koyama (1981) determined that grapevine cuttings stored for extended periods performed differently depending on the variety. They found that cuttings of grapevine cultivar Cabernet Sauvigon rooted as well as fresh cuttings after 2 years of cold storage whereas survival rates of cultivar Carignane decreased with storage time. However, in this experiment, there were no significant differences in cold storage effects on 101-14 and 3309.

The overall results from this experiment were not conclusive and it should be repeated with some additions. Plants were inoculated directly after being stored without the usual callusing period of 4 weeks before inoculation and this additional treatment is worth investigating. A period of acclimatisation after cold storage should be investigated to determine the effect on pathogenicity of *Cylindrocarpon* spp. and so should a shorter growth period before assessment, as there might have been some early effects of cold storage times on susceptibility, as indicated by the greater 5 cm incidences and severities after the longer storage times.

When grafted vines whose rootstock originated from mother-vines infected with *P. chlamydospora* were inoculated with *Cylindrocarpon* spp., only *C. liriodendri* caused significantly greater disease incidences at 1 cm above stem bases than in hot water treated, plants with 45.0 and 15.0%, respectively. The lack of significantly increased infection

incidence by all *Cylindrocarpon* spp. in inoculated vines already infected with *P. chlamydospora* was unexpected in this experiment as previous research had reported presence of multiple pathogens including *Cylindrocarpon* spp. in declining vines. Aroca *et al.* (2006) isolated more than one pathogen from 29.9% of young grapevines which were sampled before planting in vineyards, finding species which included *Phaeoacremonium* spp., *Botryosphaeria* spp., *Cylindrocarpon* spp., *Phomopsis* spp. and *P. chlamydospora*. Oliveira *et al.* (2004) reported finding *Cylindrocarpon* spp. in 87.5% of young plants sampled, of which 70% of plants were also infected with *Phaeomoniella chlamydospora.* This fungal pathogen causes grapevine decline and its main source is infected mother-vines (Whiteman *et al.*, 2007), as used this study. In other hosts it has been reported that the association of several pathogens enhanced disease severities. Santamaria *et al.* (2007) inoculated 1 year old *Pinus halepensis* seedlings with *Gremmeniella abietina* alone or with *Sclerophoma pythiophila*. The combination of the two pathogens resulted in an increase of disease severity compared with *G. abietina* alone. Mao *et al.* (1998) observed that the combination of *Phoma terrestris* and *Pythium irregular* resulted in a faster disease progression and being more severe symptoms in corn than with either pathogen alone, with 15 to 25% greater disease severities than *Phoma terrestris* and *Pythium irregular*, respectively alone. Gubler *et al.* (2004) also reported that in grapevines the association of *Phytophthora* spp. and *Cylindrocarpon* spp. caused a more rapid death than the pathogens alone. Since many symptomatic vines have been reported to contain several pathogens together, the prior infection by other pathogens may be a common factor in increasing the susceptibility of vines to black foot. While assessing PDA plates for the presence of *Cylindrocarpon* spp. from the different experiments the plates were frequently observed to also contain *Botryosphaeria* spp. and *Fusarium* spp. Further research should be conducted with a large number of hot-water treated vines that had been inoculated with *P. chlamydospora*. Furthermore, the presence of *P. chlamydospora* in the vines should be confirmed.

Results from this chapter confirm previous vineyard observations and converge to the general concept that stressed plants are more susceptible to black foot disease. An excess of water resulted in the increase of disease incidences and similar observations were made in heavy soils (Chapter 4), which are often linked to water-logging. In addition to root infection, *Cylindrocarpon* spp. are capable of infecting wounded canes and trunks which constitutes an additional threat to vines.

These studies have indicated that cultural practices could be modified in the nursery and vineyard to reduce infection. Soil pests such as grass grub should be controlled to prevent root damage. If stem trimming in nurseries and ‘bud-rubbing’ (removal of adventitious shoots from the trunk) were conducted during dry weather conditions, this might also avoid

infections that occur when conidia are splashed dispersed onto fresh wounds. Harvesting of young plants from nursery fields involves machine cutting of roots to allow lifting, which provides wounds for infection in conjunction with soil-borne inoculum. If the roots of the plants were thoroughly washed, followed by further root trimming this could remove the recently infected sections. To reduce storage time, nurseries could harvest cuttings and dormant plants in late winter. Additionally, they should discard cuttings not grafted in spring and grafted vines which were not used or sold to growers, to avoid long term cold storage which may enhance susceptibility to infection in the vineyard. In addition, vineyards and nursery fields should be well-drained to avoid the wet soil conditions that appear to enhance infection by *Cylindrocarpon* spp.

CHAPTER 7

General discussion

Grapevine losses in vineyards due to black foot disease became an increasing concern for New Zealand viticulture in the early 2000s, especially in areas where grapevines replaced orchards; it is estimated that black foot is responsible for 10% of losses of young vines in New Zealand. The same disease was reported in the 1990s in major viticultural areas including South Africa, California, Portugal and Australia where the causal pathogen was identified as *C. destructans* and *C. obtusisporum* (Scheck *et al.*, 1998; Rego *et al.*, 2001a). The replacement of own rooted *vinifera* grapevines by grafted vines was believed to have triggered the emergence of the disease. The pathogen is soilborne and has been linked to nursery sources and vineyard soils. With the improvement of molecular techniques, the identification of the pathogens responsible for this disease was clarified and continues to evolve with the addition of new species and one new genus to the list of causal agents. As the pathogens differ from one country to another, this study was focused on *Cylindrocarpon* spp. prevalent in New Zealand. A previous survey throughout New Zealand established that three *Cylindrocarpon* spp. were equally prevalent and so probably equally responsible for vine decline in vineyards: *C. liriodendri*, *C. destructans* and *C. macrodidymum.* These species were investigated in this study, the main goal of which was to improve understanding of the epidemiology of the pathogens so that appropriate control strategies could be developed to reduce frequencies of grapevine death due to *Cylindrocarpon* spp. in New Zealand.

7.1. Pathogenicity of different inoculum types

Throughout this study, *C. liriodendri* was consistently the most pathogenic species while *C. macrodidymum* and *C. destructans* had similar pathogenicities. However, with the introduction of molecular identification techniques, many of the "*C. destructans*" isolates which were believed to be the causal agent of black foot were reidentified and so this species was not considered to play a significant role in this disease and is no longer included in studies overseas (Halleen *et al*., 2007; Alaniz *et al*., 2009a). The particular situation in New Zealand where orchards are converted into vineyards seems to have allowed this pathogen to thrive in vineyards (Bonfiglioli, 2005) and the isolation of background levels of *C. destructans* in the different experiments here, using field soils is in agreement with this statement. Intra-specific variations in the virulence were observed for the three species in Chapter 2 and this observation is in agreement with previous reports (Rego *et al.*, 2001;

Rahman and Punja, 2005; Alaniz *et al.*, 2009). Further investigations should focus on the comparison of the pathogenicity of the different isolates in New Zealand and establish whether the degree of virulence is linked to the production of different enzymes, levels of enzyme present or toxins. Rahman and Punja (2005) observed that *C. destructans* virulence on ginseng was related to pectinase and polyphenoloxidase activities which were higher for highly virulent isolates than weakly virulent isolates, while Sweetingham (1983) and Evans and White (1966) demonstrated that brefeldin A, which is a toxin produced by *C. destructans*, was deleterious to *Eucalyptus pilularis* and *Pinus radiata*.

The pathogenicity of chlamydospores to grapevines was a novel finding as the pathogenicity of these propagules to grapevines had not been reported in the literature. A method to produce abundant chlamydospores was initiated from methods found in the literature. Additionally, a method to produced mycelium on wheat grains was used and the pathogenicities of conidia, chlamydospores and mycelium were studied simultaneously for the different species, in pots and in the field. All three propagules were capable of causing disease in grapevines. Conidia caused the greatest disease incidence among the three propagules in potting mix for all three species, whereas, no significant differences were observed between the different propagules when different inoculation concentrations were applied directly to plants. Increasing inoculum concentrations resulted in increased disease incidences and severities except for the highest concentration (10^6 conidia /mL) where it appeared that the maximum infectivity had been reached with an intermediate concentration (3.2×10^4 conidia /mL). An inoculum concentration of 10^2 spores /mL and 1 g of infested wheat grains were capable of inducing disease, which was a novel finding as all previous reports used concentrations between 10^4 and 10^6 conidia /mL when attempting to infect grapevines. However, additional investigations of the threshold level of propagules needed for infection should be conducted in potting mix and in soils since the lowest concentration used in this study was not low enough to demonstrate the minimum threshold concentration.

In the field, chlamydospores caused greatest disease incidences for *C. liriodendri* while conidia caused greatest disease incidences for *C. macrodidymum* and *C. destructans*. Generally, disease incidences of inoculated plants in soil were lower than those inoculated in the potting mix. This result could be attributed to the presence of other soil microorganisms as the soil was not sterilised. These outcomes are important to improve understanding of disease dynamics as previous studies on the pathogenicity of the *Cylindrocarpon* spp. only considered the presence of a single pathogen in sterilised soil or potting mix. However, in nursery and vineyard environments, *Cylindrocarpon* spp. are competing and interacting with a multitude of microorganisms and so these results need to be interpreted with caution. Arbuscular myccorhizal fungi have been demonstrated to increase plant uptake of minerals

and water and to reduce disease symptoms (Lioussanne, 2010). It is believed that these fungi influence the soil microorganism community, enhancing the development of beneficial bacteria and fungi which compete with soilborne pathogens (Lioussanne, 2010).

7.2. Development of a molecular identification method

The difficulties of using morphological characteristics to accurately identify *Cylindrocarpon* spp. led to the development of a molecular identification method for the different *Cylindrocarpon* spp. in Chapter 3. Methods available in the literature for fungal identification comprise conventional PCR, nested PCR and quantitative PCR. Species specific primers were not available for the different species in the literature at the beginning of this study. Since then, Alaniz *et al.* (2009) developed species specific primers for *C. macrodidymum* and *C. liriodendri* and Kernaghan *et al*. (2007) used primers specific for *C. destructans* f. sp. *panacis* which did not amplify other *C. destructans* isolates; all primer pairs target the rRNA ITS region sequences. In this study, the β-tubulin region was used as it has been shown to contain more polymorphisms than the ITS region. The different primers were tested for their specificity and sensitivity in a conventional PCR, followed by development of a nested PCR and a quantitative PCR. The optimised conventional PCR was capable of detecting 1 ng of pure DNA for *C. macrodidymum* and *C. destructans* and 0.1 ng of pure DNA for *C. liriodendri*. This method can be used when identifying *Cylindrocarpon* spp. from fungal cultures however it is not recommended for soil and plant tissue isolations as the sensitivity is low.

The nested PCR developed and optimised in this study used general β-tubulin primers in the primary PCR and species specific primers in the secondary PCR. Although the method was capable of detecting as little as 1 pg of pure DNA, false positives due to carry-over of contaminants delayed the development of the nested PCR method. A quantitative PCR was developed as an alternative to the nested PCR and had the advantage of also providing information on the quantity of DNA present in the sample whereas nested PCR only determined whether the pathogen was present or absent from the sample. The final detection limits for the different species using qPCR were 4.8, 66.0 and 11.5 pg for *C. liriodendri*, *C. destructans* and *C. macrodidymum*, respectively. Although Kernaghan *et al.* (2007) successfully developed a qPCR method for the detection of *C. destructans* f. sp. *panacis* using primers from the ITS region, this is the first report on the use of qPCR for the detection and quantification of the three different species using the β-tubulin gene.

The development of a multiplex system should be considered to determine the presence of *Cylindrocarpon* spp. in wood or soil samples. The primers used in this study amplified an approximately 200 bp sequence for *C. liriodendri* and *C. destructans* and a 300 bp sequence for *C. macrodidymum* which can therefore easily distinguish *C. macrodidymum* isolates from the two other species. Alaniz *et al.* (2009) have developed such a multiplex system to detect *C. liriodendri*, *C. macrodidymum* and *C. pauciseptatum* from grapevine wood with species specific primers from the ITS region and amplified a 253, 387 and 117 bp band for *C. liriodendri*, *C. macrodidymum* and *C. pauciseptatum*, respectively. In their study, species specific primers for *C. destructans* were not investigated as the pathogen was considered to not constitute a threat in Spain as it appears to do in New Zealand. Another approach for the detection of black foot disease on grapevines would be that of Dubrovsky and Fabritius (2007) who designed genus specific primers for *Cylindrocarpon* species and tested the presence of the pathogen in grapevine xylem. The primers would probably not be useful for detection in soil as they amplify ITS sequences from a wider range of *Cylindrocarpon* spp. than those known to infect grapevines.

A combination of PCR and restriction enzymes could be used to differentiate *C. liriodendri* from *C. destructans* and should be investigated further. Another approach for the detection of the different species would be the development of a dot blot hybridisation procedure where the PCR products are hybridised to a species specific oligonucleotide probe on a membrane. A chemiluminescent signal is generated when the probe binds to the PCR product. This method would allow testing many samples for a single pathogen (Koch and Utkhede, 2002; Mathur and Utkhede, 2004; Cao and Utkhede, 2005). Martos *et al.* (2011) successfully detected *P. chlamydospora* from infected grapevine wood using co-operational PCR combined with a dot blot hybridisation method. Co-operational PCR uses two primer pairs, one pair external to the other; it has a similar effect to nested PCR, but only a single PCR is needed (Olmos *et al.*, 2002). The primers used by Martos *et al.* (2011) targeted the ITS region and amplified a range of grapevine associated fungi however the probe used for the dot blot hybridisation was specific to *P. chlamydospora* and allowed the detection of the pathogen up to 0.1 pg of DNA. Similarly, this method could be used for the detection of the different *Cylindrocarpon* spp. using a genus specific PCR method and species specific probes. The method would have the advantage of requiring a single PCR and the hybridisation of the PCR products to different probes (Martos *et al*., 2011). Single-strand conformation polymorphism (SSCP) could also enable the differentiation of the *Cylindrocarpon* spp. as it is based on the folding of single nucleotide sequences and has been used to identify *Aspergillus* spp. section *Flavi* (Kumeda and Asao, 1996), *Siridium* spp. (Moricca *et al*., 2000) and *Phytophthora* spp. (Kong *et al*., 2003; Kong *et al*., 2004). The primers developed in this study could be used in a multiplex system and the PCR products

could be discriminated using SSCP. Recently, research has focused on the high resolution melting method which combines quantitative PCR using a dye such as SYBR Green with a gradual increase of temperature of the PCR products (Park *et al*., 2009; Adaszek and Winiarczyk, 2010). These products differentiate according to their nucleotide contents and their sequence length, showing different melting curve profiles. High resolution melting is capable of distinguishing single nucleotide polymorphism and has been used to map such differences in plant species (*Park et al.*, 2009; Wu *et al*., 2009). The species specific primers for *Cylindrocarpon* spp. could be used in a multiplex PCR and their melting curves should be capable of discriminating the different species.

7.3. Detection of *Cylindrocarpon* spp. in soil

Different baiting methods were investigated in Chapter 5 but none was successful. Although *C. destructans* infected different seedlings grown in infested potting mix, the pathogen was not isolated from seedlings in infested soil. Fast growing pathogens were able to infect seedlings and cause damping off. Similar observations were made by Sweetingham (1983) who attempted baiting with *Pinus radiata* seedlings.

As an alternative to the different baiting methods, a nested PCR and a qPCR were successfully optimised for the detection of *C. liriodendri* and *C. macrodidymum* in soil. Two soil DNA extraction methods were tested: the first was based on the principle of soil dilution plating while the second was based on that of Yeates *et al.* (1998). The second method was inhibited while the first one showed clear bands on a gel using general ITS primers and 0.2 µL of DNA. The advantage of the DNA extraction method developed in this study is that a soil sample of 10 g was used whereas the majority of the methods used in the literature tested soil samples of between 0.1 and 1 g of soil which is too small to provide a biologically useful information on the presence of microorganisms.

The nested PCR had a detection limit of 75.4 pg of DNA which was equivalent to 1.0×10^5 conidia /g for the three different species while the qPCR was capable of detecting as little as 4.8 pg for *C. liriodendri*, 11.5 pg for *C. macrodidymum* and 66 pg for *C. destructans* with pure DNA cultures. As the detection limit was very high for *C. destructans* and the time available was too short, qPCR was not optimised for this species. For the two remaining species, the detection limit was 1.0×10^3 conidia /g for *C. liriodendri* and 1.9×10^3 conidia /g for *C. macrodidymum*. Different factors such as the pathogenicity of isolates, spatial distribution of the inoculum and the physical and biological environment where the pathogen is present can influence disease incidence and severity and have to be considered when predicting a disease (Lees *et al*., 2002). A better understanding of the pathogen combined with quantitative PCR would provide tools to predict disease incidence and severity (Lees *et al.*,

2002). Quantitative PCR can also supply information on the survival of propagules over time and determine how long a plot should remain fallow before planting vines.

The ability of the qPCR to detect propagules in soil was tested using soil which was inoculated with 10^5 conidia /g of the different species. The method successfully detected *C. liriodendri* and *C. macrodidymum* when the conidia were mixed with the soil however C. *liriodendri* DNA quantities from soil samples after a week were very low but remained constantly low over the next 6 weeks, while the quantity of *C. macrodidymum* DNA was not detected during that period. The decrease of *Cylindrocarpon* propagules correlated well with the observations in Chapter 4 of the rapid conversion of conidia and mycelium into chlamydospores and the lysis of propagules. This is the first report on the behaviour of *C. liriodendri* and *C. macrodidymum* in soil. The results indicated that in an orchard, vineyard or nursery soil, however, resistant chlamydospores are likely to constitute the main type of *Cylindrocarpon* inoculum. This method could be used to test infestation levels of soil prior to planting.

Conidia and mycelium in the soil are transformed into chlamydospores when a suitable host is absent. The transformation occurs either at the terminal end or the side of a hypha while cells within a single or multiple conidia can combine their protoplasm to produce chlamydospores. These observations are comparable to those of Matturi and Stenton (1964) who reported the formation of chlamydospores from conidia and mycelium buried in soil on glass slides. The pathogens are likely to remain dormant as chlamydospores in the soil until the environmental conditions and the presence of a suitable host enable their germination. It would be interesting to determine the maximum distance needed between propagules and grapevine roots that allowed them to germinate and infect roots. Chlamydospores were also reported to need a period of maturation (Matturi and Stenton, 1964) and determining the time required for the chlamydospores of the different *Cylindrocarpon* spp. to mature would provide more information on the epidemiology of the *Cylindrocarpon* species. A single isolate of each species was used to investigate the conversion of conidia and mycelium to chlamydospores in soil, which is not sufficient to draw a general picture of the behaviour of each species in soil and repeating the experiment with a higher number of isolates would provide more robust information on the average rate of conversion for each species. A higher rate of conversion for a particular species would increase the probability of infection. It would also be valuable to determine the effect of both soil pH and soil moisture on survival of different propagules, allowing prediction of disease incidences and severities.

Further experiments are required to test the qPCR protocols developed in this study for *C. macrodidymum* and *C. liriodendri* using soil samples from infected nursery and vineyard soils

to determine whether the method is appropriate for their for detection in naturally infested soils. As only silt loam has been tested, the effect of heavier and lighter soils on the sensitivity of the detection should be investigated. The method was not optimised for the detection of *C. destructans* due to a lack of time and further investigations should explore the possibility of detecting *C. destructans* in soil and plant tissues. Quantification of *Cylindrocarpon* DNA in different plant tissues using qPCR and determining the progression of the pathogen in plant tissues using molecular tools were not investigated as time did not permit it, however, such studies could provide useful information about the epidemiology of the pathogen. The association of disease severity and incidence with quantities of *Cylindrocarpon* DNA in the soil should also be determined so that advice on whether the soil is suitable for planting grapevines or management measures are needed before planting can be provided to growers. Quantitative PCR shows great potential to be optimised as a routine test for detection of *Cylindrocarpon* spp. in soil.

7.4. Entry points for the pathogen

Two propagation methods were used to infect young grapevines in Chapter 2. These simulated infection in nurseries where callused cuttings are placed in the soil to root and in vineyards where vines with trimmed roots are planted. *Cylindrocarpon* spp. were isolated from the stems after 4 to 6 months, indicating that the pathogens can infect through the callus and wounded roots. Although disease incidences were lower for infected root-wounded plants than for callused plants, the different species were considered capable of moving from roots to the stems after a longer period. The results show the importance of early detection of the pathogen in nursery and in vineyard soils to enable appropriate control strategies to be implemented to prevent infection.

Wounding of root tissues was expected to enhance disease incidence as *Cylindrocarpon* spp. were reported to infect plants through wounds, however, disease incidences did not differ significantly between inoculated root wounded and unwounded grapevines in different experiments. Additionally, the numbers of infected roots were similar between inoculated root-wounded vines and non-wounded vines when grown in a vineyard. The different species were isolated at 7.5 cm above stem bases for both treatments. It is possible that the pathogen penetrated the roots through natural openings like the emergence of lateral roots or through wounds caused by nematodes, grass grubs or other fungal species. It would be interesting to infect roots at specific sites and follow the progression of the pathogen with time into and through the root and plant stem. Such studies have been carried out with different pathogens using transgenes encoding for the green fluorescent protein (Sprague *et al.*, 2007; Silva *et al*., 2009). Spague *et al.* (2007) successfully observed the colonisation of

Brassica napus roots by green fluorescent protein-expressing *Leptosphaeria maculans* isolates.

In trunks and 1 year old canes *Cylindrocarpon* spp. were unable to infect non-wounded tissues, which may been due to the presence of the secondary tissue layers, including bark and the periderm with suberised cell walls. Natural plant barriers are capable of preventing the entry of *Cylindrocarpon* spp. However, when canes and trunks of the plants were wounded, the different species were capable of infecting them, although they moved quite slowly, being isolated from only the wounding site and 2.5 cm below it at 6 months after inoculation. As it is common practice to cut lateral shoots from the trunk (a process called 'disbudding), *Cylindrocarpon* spp. that splash-dispersed from soil could infect grapevines through these wounds. Additionally, *Cylindrocarpon* spp. were capable of infecting grapevines 8 days after the wounds were made in stems. This is the first report of the movement of *Cylindrocarpon* spp. in trunks and in canes as all previous studies investigated the movement of the pathogens through roots towards the trunk. More research needs to be conducted to determine whether the pathogen is capable of spreading throughout the trunk bases when entering through a shoot or stem wound. These studies should be conducted over longer periods of time than the 6 months allowed for growth in this study, and if these wounds are found to be a significant source of infection and so vine deaths, the use of wound protectants should be examined.

In the different pot experiments, the presence of *Cylindrocarpon* spp. in the control plants was attributed to water splashing and was successfully eliminated by the use of a dripping system and by using wider plant spaces. This observation highlights the capacity of inoculum to spread in a vineyard by rainfall. As grapevines age, their root systems increase and can grow towards soil containing *Cylindrocarpon* propagules. This could have occurred in the experiment which investigated the progress of disease in the vineyard, when the non-inoculated vines showed similar disease incidences in trunks to the root-inoculated grapevines. When removing an infected and declining vine, growers should also consider removing neighbouring vines and try to eliminate the pathogen from the planting hole before replacing the diseased vine.

7.5. Stress factors affecting disease development

Different stress factors were investigated for their effects on disease development. Reports from vineyard observations suggested that excessive water and heavy soils were linked to high rates of the disease. The results from this study support those observations. Grapevines planted on heavy clay soils were more likely to develop black foot disease than plants grown

on light soils, probably because clay soils retain more water than sandy soils. Heavy soils can also contract when dry, thereby damaging roots, providing more entry sites for *Cylindrocarpon* spp. However, as the pathogen was capable of infecting grapevines in all soil types, selection of potential vineyards by soil type does not eliminate the threat, although light soil reduces its occurrence.

The investigation into the effect of water levels also demonstrated that vines with water-logged roots had greater disease incidences than those growing in soil with 75% field capacity. The viticultural regions of New Zealand have an annual rainfall which ranges between 400 mm in Central Otago and 1600 mm in Northland (Jackson and Schuster, 2001). A high rainfall level could favour the development of black foot, especially if they coincide with heavy soils. However, successive periods of drought and water logging are also likely to occur in vineyards and their effect on disease development should be investigated.

7.6. Conclusions

The results presented in this study have improved the understanding of the epidemiology of the *Cylindrocarpon* spp. as grapevines pathogens. The importance of conidia, mycelium and chlamydospores in disease development has been highlighted and the pathogen shown to be capable of infecting grapevines through wounded or intact roots, from which it invades the stem bases to ultimately the vines. The pathogen is also capable of infecting above ground parts of grapevines through wounds which are produced during vineyard management processes. In the absence of a suitable host, conidia and mycelium were shown to produce chlamydospores which are likely to remain dormant until activated by presence of a host in suitable soil conditions. These are likely to be the propagules responsible for longevity of the pathogen in vineyard and nursery soils.

The development of a molecular identification method for the different *Cylindrocarpon* spp. which is more accurate than traditional methods has enabled the correct identification of the different species. The further development of the molecular methods for quantifying *Cylindrocarpon* spp. in soils could enable scientists to determine the disease risk presented by a specific soil. It also has the potential for development into a diagnostic commercial tool.

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Appendix 1

List of isolates

Allocated number	Name	Species	Geographic origin
L1	LUPP1146	*Cylindrocarpon liriodendri*	Marlborough
L2	LUPP986	*Cylindrocarpon liriodendri*	Waipara
L3	LUPP959	*Cylindrocarpon liriodendri*	Hawke's Bay
L4	LUPP1031	*Cylindrocarpon liriodendri*	Gisborne
L5	LUPP953	*Cylindrocarpon liriodendri*	Hawke's Bay
L6	LUPP1000	*Cylindrocarpon liriodendri*	Central Otago
L7	LUPP1080	*Cylindrocarpon liriodendri*	Marlborough
L8	LUPP1014	*Cylindrocarpon liriodendri*	Auckland
L9	LUPP1102	*Cylindrocarpon liriodendri*	Marlborough
L10	LUPP993	*Cylindrocarpon liriodendri*	Central Otago
D1	LUPP1113	*Cylindrocarpon destructans*	Marlborough
D2	LUPP982	*Cylindrocarpon destructans*	Waipara
D3	LUPP994	*Cylindrocarpon destructans*	Central Otago
D4	LUPP1114	*Cylindrocarpon destructans*	Marlborough
D5	LUPP1071	*Cylindrocarpon destructans*	Marlborough
D6	LUPP989	*Cylindrocarpon destructans*	Waipara
D7	LUPP1002	*Cylindrocarpon destructans*	Central Otago
D8	LUPP967	*Cylindrocarpon destructans*	Hawke's Bay
D9	LUPP976	*Cylindrocarpon destructans*	Hawke's Bay
D10	LUPP950	*Cylindrocarpon destructans*	Martinborough
M1	LUPP1086	*Cylindrocarpon macrodidymum*	Marlborough
M2	LUPP1039	*Cylindrocarpon macrodidymum*	Gisborne
M3	LUPP1006	*Cylindrocarpon macrodidymum*	Central Otago
M4	LUPP1015	*Cylindrocarpon macrodidymum*	Auckland
M5	LUPP974	*Cylindrocarpon macrodidymum*	Hawke's Bay
M6	LUPP991	*Cylindrocarpon macrodidymum*	Waipara
M7	LUPP981	*Cylindrocarpon macrodidymum*	Nelson
M8	LUPP1120	*Cylindrocarpon macrodidymum*	Marlborough
M9	LUPP1145	*Cylindrocarpon macrodidymum*	Marlborough
M10	LUPP1147	*Cylindrocarpon macrodidymum*	Marlborough
N1	LUPP1045	Cylindrocarpon sp.nov.	Marlborough
N2	LUPP1048	Cylindrocarpon sp.nov.	Marlborough
N3	LUPP970	Cylindrocarpon sp.nov.	Hawke's Bay
N4	LUPP1044	Cylindrocarpon sp.nov.	Gisborne

Appendix 2

Media, solutions and potting mix recipes

2.1. Agars

Agarose
1 to 1.5 g of agarose (Progen Biosciences, Brisbane, Australia) per 100 mL of sterile nanopure water (SNW).

Potato dextrose agar (PDA)
39 g of PDA (Oxoid Ltd, Basingstoke, UK) was added to 1 L of reverse osmosis water (ROW) and autoclaved for 20 min at 121°C and 15 psi.

Potato dextrose agar (PDA) amended with chloramphenicol
39 g of potato dextrose agar (Oxoid Ltd, Basingstoke, UK) was added to 1 L of reverse osmosis water (ROW). Under a fume hood, 250 mg of chloramphenicol was added to the solution and autoclaved for 20 min at 121°C and 15 psi.

Spezieller Nährstoffarmer agar (SNA)
1.0 g KH2PO4
1.0 g KNO3
0.5 g MgSO4.7H20
0.5 g KCl
0.2 g glucose
0.2 g sucrose
20.0 g Oxoid agar no.3
All ingredient except agar were dissolved in 1L of SNW and the pH adjusted to 6 – 6.5. The agar was added and dissolved before the solution was autoclaved (15 min, 121°C, 15 psi) and allowed to cool to 50°C (Waller *et al.*, 2002)

2.2. Broths

Czapek Dox broth (CDB)
35 g of CDB (Sigma Chemicals, St. Louis, USA) was added to 1 L of ROW and autoclaved for 20 min at 121°C and 15 psi.

Potato dextrose broth (PDB)
24 g of PDB (Difco laboratories, Becton Dickinson, USA) was added to 1 L of ROW and autoclaved for 20 min at 121°C and 15 psi.

2.3. Solutions

Tris-acetate-EDTA (TAE)
242 g of Tris (Invitrogen life technologies, CA, USA)
57.1 mL of glacial acetic acid
100 mL of 0.5 M ethylenediaminetetra acetic acid (pH 8.0)
Reverse osmotic water to 1 L.

Tris-EDTA (TE)
10 mM of Tris HCl
1 mM of sodium EDTA (pH 8.0)

Extraction Buffer
100 mL of 100 mM Tris HCl (pH 8.0)
100 mM sodium EDTA (pH 8.0)
1.5 M NaCl

Loading dye
40% (w/v) of sucrose (Sigma Chemicals, USA)
0.25% of bromophenol blue
0.25% of xylene cyanol

Sybr®Green
1: 1000 dilution Sybr®Green I nucleic acid gel stain (×10,000 concentrate in DMSO)

2.4. Potting mix

3 – 4 month potting mix

80% horticultural bark (grade 2): 20% pumice (grade 3, 1-4 mm) amended with 2 kg of a 3-4 month fetiliser, Osmocote Exact (Scotts Australia Pty Ltd; (16:5:9.2) (N:P:K)),1 kg agricultural lime and 1 kg Hydraflo (Scotts Australia PTY Ltd) per 1 m^3.

8 – 9 month potting mix

80% horticultural bark (grade 2): 20% pumice (grade 3, 1-4 mm) amended with 5 kg of an 8-9 month fertiliser, Osmocote exact (Scotts Australia Pty Ltd; (15:4.0:7.5) (N:P:K)), 1 kg agricultural lime and 1 kg Hydraflo (Scotts Australia PTY Ltd) per 1 m^3.

Appendix 3

Statistical analysis for Chapter 2

3.1. Investigation of the pathogenicity of the three *Cylindrocarpon* spp.

3.1.1. Analysis by logistic regression of the effect of isolates, propagation methods and varieties on disease incidence at 1 cm above stem bases.

Source	B	S.E.	Wald	df	Probability
Isolates			38.435	9	0.000
Propagation methods	1.975	0.309	40.936	1	0.000
Varieties	-0.147	0.287	0.260	1	0.610
Constant	-2.491	0.562	19.658	1	0.000

3.1.2. Pair-wises comparisons among isolates for disease incidence at 1 cm above stem bases.

Control vs D1	*P*=0.017;	D1 vs D2	*P*=0.010;	D2 vs D3	*P*=0.000;
Control vs D2	*P*=0.000;	D1 vs D3	*P*=0.127;	D2 vs L1	*P*=0.481;
Control vs D3	*P*=0.330;	D1 vs L1	*P*=0.071;	D2 vs L2	*P*=0.077;
Control vs L1	*P*=0.000;	D1 vs L2	*P*=0.272;	D2 vs L3	*P*=0.011;
Control vs L2	*P*=0.003;	D1 vs L3	*P*=0.799;	D2 vs M1	*P*=0.008;
Control vs L3	*P*=0.008;	D1 vs M1	*P*=0.842;	D2 vs M2	*P*=0.003;
Control vs M1	*P*=0.006;	D1 vs M2	*P*=0.517;	D2 vs M3	*P*=0.500;
Control vs M2	*P*=0.076;	D1 vs M3	*P*=0.063;		
Control vs M3	*P*=0.000;				
D3 vs L1	*P*=0.002;	L1 vs L2	*P*=0.331;	L2 vs L3	*P*=0.326;
D3 vs L2	*P*=0.014;	L1 vs L3	*P*=0.084;	L2 vs M1	*P*=0.285;
D3 vs L3	*P*=0.067;	L1 vs M1	*P*=0.068;	L2 vs M2	*P*=0.097;
D3 vs M1	*P*=0.066;	L1 vs M2	*P*=0.018;	L2 vs M3	*P*=0.314;
D3 vs M2	*P*=0.379;	L1 vs M3	*P*=0.952;		
D3 vs M3	*P*=0.020;				
L3 vs M1	*P*=0.975;	M1 vs M2	*P*=0.384;	M2 vs M3	*P*=0.015.
L3 vs M2	*P*=0.364;	M1 vs M3	*P*=0.061;		
L3 vs M3	*P*=0.076;				

3.1.3. Pair-wise comparisons among *Cylindrocarpon* species for disease incidence at 1 cm above stem bases.

C. destructans vs *C. liriodendri*	*P*=0.216;
C. destructans vs *C. macrodidymum*	*P*=0.816;
C. liriodendri vs *C. macrodidymum*	*P*=0.324.

3.1.4. Analysis by logistic regression of the effect of isolates, propagation methods and varieties on disease incidence at 5 cm above stem bases.

Source	B	S.E.	Wald	df	Probability
Isolates			12.447	9	0.189
Propagation methods	1.281	0.314	16.661	1	0.000
Varieties	0.223	0.229	0.556	1	0.456
Constant	-3.033	0.684	19.683	1	0.000

3.1.5. Pair-wise comparisons among isolates for disease incidence at 5 cm above stem bases.

Control vs D1	*P*=0.043;	D1 vs D2	*P*=0.683;	D2 vs D3	*P*=0.037;
Control vs D2	*P*=0.077;	D1 vs D3	*P*=0.022;	D2 vs L1	*P*=0.442;
Control vs D3	*P*=0.622;	D1 vs L1	*P*=0.706;	D2 vs L2	*P*=0.758;
Control vs L1	*P*=0.016;	D1 vs L2	*P*=0.931;	D2 vs L3	*P*=0.746;
Control vs L2	*P*=0.052;	D1 vs L3	*P*=0.503;	D2 vs M1	*P*=0.619;
Control vs L3	*P*=0.140;	D1 vs M1	*P*=0.384;	D2 vs M2	*P*=0.323;
Control vs M1	*P*=0.222;	D1 vs M2	*P*=0.182;	D2 vs M3	*P*=1.000;
Control vs M2	*P*=0.437;	D1 vs M3	*P*=0.707;		
Control vs M3	*P*=0.101;				
D3 vs L1	*P*=0.008;	L1 vs L2	*P*=0.647;	L2 vs L3	*P*=0.532;
D3 vs L2	*P*=0.026;	L1 vs L3	*P*=0.236;	L2 vs M1	*P*=0.422;
D3 vs L3	*P*=0.630;	L1 vs M1	*P*=0.208;	L2 vs M2	*P*=0.215;
D3 vs M1	*P*=0.114;	L1 vs M2	*P*=0.094;	L2 vs M3	*P*=0.770;
D3 vs M2	*P*=0.233;	L1 vs M3	*P*=0.458;		
D3 vs M3	*P*=0.049;				
L3 vs M1	*P*=0.813;	M1 vs M2	*P*=0.660;	M2 vs M3	*P*=0.345.
L3 vs M2	*P*=0.500;	M1 vs M3	*P*=0.588;		
L3 vs M3	*P*=0.753;				

3.1.6. Pair-wise comparisons among species for disease incidence at 5 cm above stem bases.

C. destructans vs *C. liriodendri*	*P*=0.185;
C. destructans vs *C. macrodidymum*	*P*=0.976;
C. liriodendri vs *C. macrodidymum*	*P*=0.207.

3.1.7. Analysis by general linear model of the effect of isolates, propagation methods and varieties on disease severity at 1 cm above stem bases.

Source	Sum of squares	df	Mean square	F ratio	Probability
Isolates	125.483	9	13.943	7.641	0.000
Propagation methods	171.271	1	171.271	93.859	0.000
Varieties	3.375E-02	1	3.375E-02	0.18	0.892
Propagation methods*varieties	7.830	1	7.830	4.291	0.039
Isolates*propagation methods	42.274	9	4.697	2.574	0.008
Isolates*varieties	15.602	9	1.734	0.95	0.482
Error	445.242	244	1.825		

3.1.8. Comparisons between disease severities of plants inoculated with three *Cylindrocarpon* spp.

Source	Sum of squares	df	Mean square	F ratio	Probability
C. destructans vs *C. liriodendri*	7.669	1	7.669	3.423	0.066
Error	358.503	160	2.241		
C. destructans vs *C. macrodidymum*	0.071	1	0.071	0.030	0.863
Error	381.238	162	2.353		
C. liriodendri vs C*. macrodidymum*	6.116	1	6.116	2.665	0.105
Error	367.175	160	2.295		

3.1.9. Analysis by general linear model of the effect of species on disease severity at 1 cm above stem bases of callused grapevine cuttings.

Source	Sum of squares	df	Mean square	F	Probability
Species	17.114	2	8.557	3.208	0.044
error	314.767	118	2.668		

3.1.10. Pair-wise comparisons among species for disease severity at 1 cm above stem bases of callused plants.

C. destructans vs *C. liriodendri*	*P*=0.037;
C. destructans vs *C. macrodidymum*	*P*=0.843;
C. liriodendri vs *C. macrodidymum*	*P*=0.027.

3.1.11. Analysis by general linear model of the effects of isolates, propagation methods and varieties on root dry weights of callused and rooted grapevine cuttings.

Source	Sum of squares	df	Mean square	F ratio	Probability
Isolates	41.640	9	4.627	2.111	0.030
Propagation methods	163.911	1	163.911	74.770	0.000
Varieties	50.770	1	50.70	23.160	0.000
Propagation methods*varieties	11.466	1	11.466	5.230	0.023
Isolates*propagation methods	73.392	9	8.155	3.720	0.000
Isolates*varieties	30.237	9	3.360	1.533	0.137
Error	504.206	230	2.192		

3.1.12. Analysis by general linear model of the effect of species on root dry weights of grapevine cuttings, 4 months after inoculation with three *Cylindrocarpon* spp.

Source	Sum of squares	df	Mean square	F	Probability
Species	11.517	2	5.758	2.219	0.111
Error	589.102	227	2.595		

3.2. Investigation of the pathogenicity of the different pathogen propagules

3.2.1. Analysis by logistic regression of the effect of propagules and species on disease incidence at 1 cm above stem bases, 5 months after inoculation with three propagules of three *Cylindrocarpon* species (without the water and wheat grain controls for species).

Source	B	SE	Wald	df	Probability
Propagules			56.835	4	0.000
Constant	-1.853	0.488	14.415	1	0.000
Species			9.648	2	0.008
Constant	-1.802	0.521	11.95	1	0.001

3.2.2. Pair-wise comparisons among the propagule treatments and among species for disease incidence at 1 cm above stem bases.

Chlamydospores vs conidia	*P*=0.001;	Conidia vs mycelium	*P*=0.001;
Chlamydospores vs mycelium	*P*=0.280;	Conidia vs water	*P*=0.001;
Chlamydospores vs water	*P*=0.001;	Conidia vs wheat	*P*=0.001;
Chlamydospores vs wheat	*P*=0.001;		
Mycelium vs water	*P*=0.006;	Water vs wheat	*P*=0.827.
Mycelium vs wheat	*P*=0.003;		

C. destructans vs *C. liriodendri*	*P*=0.002;
C. destructans vs *C. macrodidymum*	*P*=0.374;
C. liriodendri vs *C. macrodidymum*	*P*=0.027.

3.2.3. Analysis by logistic regression of the effect of propagules from each species, propagation methods and varieties on disease incidence at 1 cm above stem bases.

Source	B	SE	Wald	df	Probability
Propagation methods	1.097	0.207	28.143	1	0.000
Varieties	0.348	0.203	2.938	1	0.087
Propagules * isolates			60.706	10	0.000
Constant	0.298	0.499	0.356	1	0.551

3.2.4. Pair-wise comparisons among propagule treatments of each species for disease incidence at 1 cm above stem bases (with L.: *C. liriodendri*; M.: *C. macrodidymum*; D.: *C. destructans*; Chl.: chlamydospores, Con.: conidia; Myc.: mycelium).

Control wheat vs water	*P*=0.800;	Control water vs L. Chl.	*P*=0.000;
Control wheat vs L. Chl.	*P*=0.000;	Control water vs L. Con.	*P*=0.000;
Control wheat vs L. Con.	*P*=0.000;	Control water vs L. Myc.	*P*=0.000;

Control wheat vs L. Myc.	*P*=0.000;	Control water vs M. Chl.	*P*=0.018;
Control wheat vs M. Chl.	*P*=0.007;	Control water vs M. Con.	*P*=0.000;
Control wheat vs M. Con.	*P*=0.000;	Control water vs M. Myc.	*P*=0.054;
Control wheat vs M. Myc.	*P*=0.031;	Control water vs D. Chl.	*P*=0.006;
Control wheat vs D. Chl.	*P*=0.000;	Control water vs D. Con.	*P*=0.254;
Control wheat vs D. Con.	*P*=0.003;	Control water vs D. Myc.	*P*=0.000;
Control wheat vs D. Myc.	*P*=0.137;		

L. Chl. vs L. Con.	*P*=0.002;	L. Con. vs L. Myc.	*P*=0.005;	L. Myc. vs M. Chl.	*P*=0.068;
L. Chl. vs L. Myc.	*P*=0.808;	L. Con. vs M. Chl.	*P*=0.000;	L. Myc. vs M. Con.	*P*=0.633;
L. Chl. vs M. Chl.	*P*=0.084;	L. Con. vs M. Con.	*P*=0.002;	L. Myc. vs M. Myc.	*P*=0.027;
L. Chl. vs M. Con.	*P*=0.800;	L. Con. vs M. Myc.	*P*=0.000;	L. Myc. vs D. Chl.	*P*=0.155;
L. Chl. vs M. Myc.	*P*=0.045;	L. Con. vs D. Chl.	*P*=0.001;	L. Myc. vs D. Con.	*P*=0.607;
L. Chl. vs D. Chl.	*P*=0.231;	L. Con. vs D. Con.	*P*=0.000;	L. Myc. vs D. Myc.	*P*=0.003;
L. Chl. vs D. Con.	*P*=0.402;	L. Con. vs D. Myc.	*P*=0.000;		
L. Chl. vs D. Myc.	*P*=0.004;				

M. Chl. vs M. Con.	*P*=0.145;	M. Con. vs M. Myc.	*P*=0.074;	M. Myc. vs D. Chl.	*P*=0.349;
M. Chl. vs M. Myc.	*P*=0.651;	M. Con. vs D. Chl.	*P*=0.331;	M. Myc. vs D. Con.	*P*=0.007;
M. Chl. vs D. Chl.	*P*=0.634;	M. Con. vs D. Con.	*P*=0.259;	M. Myc. vs D. Myc.	*P*=0.384;
M. Chl. vs D. Con.	*P*=0.012;	M. Con. vs D. Myc.	*P*=0.007;		
M. Chl. vs D. Myc.	*P*=0.158;				

D. Chl. vs D. Con.	*P*=0.037;	D. Con. vs D. Myc.	*P*=0.000.
D. Chl. vs D. Myc.	*P*=0.069;		

3.2.5. Analysis by logistic regression of the effects of propagules and species on disease incidence at 5 cm above stem bases, 5 months after inoculation with three propagules of three *Cylindrocarpon* species (without the water and wheat grain controls for species).

Source	B	SE	Wald	df	Probability
Propagules			58.030	4	0.000
Constant	-2.413	0.549	19.314	1	0.000
Species			9.096	2	0.011
Constant	-1.784	0.531	11.275	1	0.001

3.2.6. Pair-wise comparisons among propagule treatments and among species for disease incidence at 5 cm above stem bases.

Chlamydospores vs conidia	*P*=0.001;	Conidia vs mycelium	*P*=0.001;
Chlamydospores vs mycelium	*P*=0.375;	Conidia vs water	*P*=0.001;
Chlamydospores vs water	*P*=0.001;	Conidia vs wheat	*P*=0.001;
Chlamydospores vs wheat	*P*=0.023;		
Mycelium vs water	*P*=0.001;	Water vs wheat	*P*=0.109.
Mycelium vs wheat	*P*=0.087;		

C. destructans vs *C. liriodendri*	*P*=0.075;
C. destructans vs *C. macrodidymum*	*P*=0.199;
C. liriodendri vs *C. macrodidymum*	*P*=0.003.

3.2.7. Analysis by logistic regression of the effect of propagules from each species, propagation methods and varieties on disease incidence at 5 cm above stem base for plants grown from callused and rooted cuttings of rootstock varieties 101-14 and 5C, 5 months after inoculation with three propagules of three *Cylindrocarpon* species.

Source	B	SE	Wald	df	Probability
Propagation methods	1.230	0.216	32.376	1	0.000
Varieties	0.304	0.209	2.118	1	0.146
Propagules * isolates			78.610	10	0.000
Constant	-1.189	0.491	5.856	1	0.016

3.2.8. Pair-wise comparisons among propagules and species for disease incidence at 5 cm above stem bases (with L.: *C. liriodendri*; M.: *C. macrodidymum*; D.: *C. destructans*; Chl.: chlamydospores, Con.: conidia; Myc.: mycelium).

Control wheat vs control water	*P*=0.110;	Control water vs L. Chl.	*P*=0.000;
Control wheat vs L. Chl.	*P*=0.002;	Control water vs L. Con.	*P*=0.000;
Control wheat vs L. Con.	*P*=0.000;	Control water vs L. Myc.	*P*=0.000;
Control wheat vs L. Myc.	*P*=0.004;	Control water vs M. Chl.	*P*=0.002;
Control wheat vs M. Chl.	*P*=0.072;	Control water vs M. Con.	*P*=0.000;
Control wheat vs M. Con.	*P*=0.000;	Control water vs M. Myc.	*P*=0.092;
Control wheat vs M. Myc.	*P*=1.000;	Control water vs D. Chl.	*P*=0.011;
Control wheat vs D. Chl.	*P*=0.273;	Control water vs D. Con.	*P*=0.000;
Control wheat vs D. Con.	*P*=0.009;	Control water vs D. Myc.	*P*=0.006;
Control wheat vs D. Myc.	*P*=0.177;		

L. Chl. vs L. Con.	*P*=0.003;	L. Con. vs L. Myc.	*P*=0.002;	L. Myc. vs M. Chl.	*P*=0.170;
L. Chl. vs L. Myc.	*P*=0.823;	L. Con. vs M. Chl.	*P*=0.000;	L. Myc. vs M. Con.	*P*=0.015;
L. Chl. vs M. Chl.	*P*=0.109;	L. Con. vs M. Con.	*P*=0.386;	L. Myc. vs M. Myc.	*P*=0.003;
L. Chl. vs M. Con.	*P*=0.022;	L. Con. vs M. Myc.	*P*=0.000;	L. Myc. vs D. Chl.	*P*=0.046;
L. Chl. vs M. Myc.	*P*=0.002;	L. Con. vs D. Chl.	*P*=0.000;	L. Myc. vs D. Con.	*P*=0.662;
L. Chl. vs D. Chl.	*P*=0.027;	L. Con. vs D. Con.	*P*=0.000;	L. Myc. vs D. Myc.	*P*=0.069;
L. Chl. vs D. Con.	*P*=0.506;	L. Con. vs D. Myc.	*P*=0.000;		
L. Chl. vs D. Myc.	*P*=0.038;				

M. Chl. vs M. Con.	*P*=0.000;	M. Con. vs M. Myc.	*P*=0.000;	M. Myc. vs D. Chl.	*P*=0.274;
M. Chl. vs M. Myc.	*P*=0.065;	M. Con. vs D. Chl.	*P*=0.000;	M. Myc. vs D. Con.	*P*=0.009;
M. Chl. vs D. Chl.	*P*=0.442;	M. Con. vs D. Con.	*P*=0.004;	M. Myc. vs D. Myc.	*P*=0.177;
M. Chl. vs D. Con.	*P*=0.360;	M. Con. vs D. Myc.	*P*=0.000;		
M. Chl. vs D. Myc.	*P*=0.608;				

D. Chl. vs D. Con.	*P*=0.091;	D. Con. vs D. Myc.	*P*=0.160.
D. Chl. vs D. Myc.	*P*=0.806;		

3.2.9. Analysis by general linear model of the effect of propagules and species on disease severity at 1 cm above stem base, 5 months after inoculation with three propagules of three *Cylindrocarpon* species (without the water and wheat grain controls).

Source	Sum of square	df	Mean square	F	Probability
Propagules	97.366	2	48.683	25.453	0.000
Species	99.532	2	49.766	26.019	0.000
Error	784.199	410	1.913		

3.2.10. Pair-wise comparisons among propagule treatments and among species for disease severity at 1 cm above stem bases.

Chlamydospores vs conidia	*P*=0.000;
Chlamydospores vs mycelium	*P*=0.148;
Conidia vs mycelium	*P*=0.000.
C. destructans vs *C. liriodendri*	*P*=0.000;
C. destructans vs *C. macrodidymum*	*P*=0.766;
C. liriodendri vs *C. macrodidymum*	*P*=0.000.

3.2.11. Analysis by general linear model of the effect of propagules from each species, propagation methods and rootstock varieties on disease severity at 1 cm above stem bases, 5 months after inoculation with three propagules of three *Cylindrocarpon* species.

Source	Sum of squares	df	Mean square	F ratio	Probability
Propagules*species	316.129	10	31.613	18.363	0.000
Propagation methods	85.926	1	85.926	49.913	0.000
Varieties	4.547	1	4.547	2.641	0.105
Propagation methods* Propagules*species	44.720	10	4.472	2.598	0.004
Propagules*species*varieties	42.598	10	4.260	2.474	0.007
Propagation methods*varieties	1.381	1	1.381	0.802	0.371
Error	831.494	483	1.722		

3.2.12. Analysis by general linear model of the effect of propagules and species on root dry weights (without the water and wheat grain controls).

Source	Sum of square	df	Mean square	F	Probability
Propagules	159.131	2	79.565	23.681	0.000
Species	1.903	2	0.951	0.283	0.754
Error	1350.697	402	3.360		

3.2.13. Pair-wise comparisons among propagule treatments and among species for root dry weights.

Chlamydospores vs conidia	*P*=0.266;
Chlamydospores vs mycelium	*P*=0.000;
Conidia vs mycelium	*P*=0.000.
C. destructans vs *C. liriodendri*	*P*=0.963;
C. destructans vs *C. macrodidymum*	*P*=0.531;
C. liriodendri vs *C. macrodidymum*	*P*=0.502.

3.2.14. Analysis by general linear model of the effect of propagules from each species, propagation methods and rootstock varieties on root dry weights.

Source	Sum of squares	df	Mean square	F ratio	Probability
Propagules*species	274.509	10	27.451	10.154	0.000
Propagation methods	123.257	1	123.257	45.593	0.000
Varieties	5.507	1	5.507	2.037	0.154
Propagation methods* Propagules*species	230.079	10	23.008	8.511	0.000
Propagules*species *varieties	51.038	10	5.104	1.888	0.045
Propagation methods*varieties	0.122	1	0.122	0.045	0.832
Error	1278.713	473	2.703		

3.2.15. Analysis by general linear model of the effect of propagules and species on shoot dry weights (without the water and wheat grain controls).

Source	Sum of square	df	Mean square	F	Probability
Propagules	225.546	2	112.773	27.074	0.000
Species	151.287	2	73.643	18.160	0.000
Error	1674.456	402	4.165		

3.2.16. Pair-wise comparisons among propagule treatments and among the species for shoot dry weights.

Chlamydospores vs conidia	*P*=0.398;
Chlamydospores vs mycelium	*P*=0.000;
Conidia vs mycelium	*P*=0.000.
C. destructans vs *C. liriodendri*	*P*=0.710;
C. destructans vs *C. macrodidymum*	*P*=0.000;
C. liriodendri vs *C. macrodidymum*	*P*=0.000.

3.2.17. Analysis by general linear model of the effect of propagules from each species, propagation methods and rootstock varieties on shoot dry weights, 5 months after inoculation with three propagules of three *Cylindrocarpon* species.

Source	Sum of squares	df	Mean square	F ratio	Probability
Propagules*species	600.943	10	60.094	17.930	0.000
Propagation methods	172.659	1	172.659	51.514	0.000
Varieties	32.914	1	32.914	9.820	0.002
Propagation methods*propagules	269.186	10	26.919	8.031	0.000
Propagules*varieties	76.426	10	7.643	2.280	0.013
Propagation methods*varieties	4.297E-03	1	4.297E-03	0.001	0.971
Error	1585.333	473	3.352		

3.3. Investigation of the threshold number of pathogen propagules needed for infection

3.3.1. Analysis by logistic regression of the effect of propagule, concentration and species on disease incidence at 1 cm above stem bases, 5 months after inoculation with four concentrations of three propagules of two *Cylindrocarpon* species.

Source	B	SE	Wald	df	Probability
Species	1.196	0.294	16.548	1	0.000
Concentrations			28.973	2	0.000
Propagules			1.995	2	0.369
Constant	0.134	0.611	0.48	1	0.827
Species.propagules.concentration			13.621	4	0.009
Constant	0.960	0.670	2.054	1	0.152

3.3.2. Pair-wise comparisons among propagule treatments and among concentrations for disease incidence at 1 cm above stem bases with conc.: concentrations (concentrations: 0: 0 spores /mL or 0 g of infected wheat grains, 1: 10^2 spores /mL or 1 g of wheat grains, 2: 3.2×10^4 spores /mL or 3 g of wheat grains and 3: 10^6 spores /mL or 5g of wheat grains).

Chlamydospores vs conidia	*P*=0.155;
Chlamydospores vs mycelium	*P*=0.367;
Conidia vs mycelium	*P*=0.593.

Conc. 0 vs 1	*P*=0.806;	Conc.1 vs 2	*P*=0.000;	Conc. 2 vs 3	*P*=0.251.
Conc. 0 vs 2	*P*=0.660;	Conc. 1 vs 3	*P*=0.000;		
Conc. 0 vs 3	*P*=0.681;				

3.3.3. Analysis by logistic regression of the effect of interaction between propagules, concentrations and species on disease incidence at 1 cm above stem bases, 5 months after inoculation with four concentrations of three propagules of two *Cylindrocarpon* species using linear by linear association.

Propagules	Species	Value	df	Probability
Chlamydospores	*C. destructans*	8.371	1	0.004
	C. liriodendri	1.367	1	0.242
Conidia	*C. destructans*	6.493	1	0.011
	C. liriodendri	1.258	1	0.262
Mycelium	*C. destructans*	3.922	1	0.048
	C. liriodendri	11.322	1	0.001

3.3.4. Pair-wise comparisons among propagule concentrations of two *Cylindrocarpon* spp. for disease incidence at 1 cm above stem bases (concentrations: 0: 0 spores /mL or 0 g of infected wheat grains, 1: 10^2 spores /mL or 1 g of wheat grains, 2: 3.2×10^4 spores /mL or 3 g of wheat grains and 3: 10^6 spores /mL or 5g of wheat grains).

C. destructans:

- Chlamydospores:

Conc. 0 vs 1	*P*=0.000;	Conc.1 vs 2	*P*=0.596;	Conc. 2 vs 3	*P*=0.120.
Conc. 0 vs 2	*P*=0.000;	Conc. 1 vs 3	*P*=0.013;		
Conc. 0 vs 3	*P*=0.000;				

- Conidia:

Conc. 0 vs 1	*P*=0.000;	Conc.1 vs 2	*P*=0.596;	Conc. 2 vs 3	*P*=0.236.
Conc. 0 vs 2	*P*=0.000;	Conc. 1 vs 3	*P*=0.033;		
Conc. 0 vs 3	*P*=0.000;				

- Mycelium:

Conc. 0 vs 1	*P*=0.000;	Conc.1 vs 2	*P*=0.385;	Conc. 2 vs 3	*P*=0.740.
Conc. 0 vs 2	*P*=0.000;	Conc. 1 vs 3	*P*=0.103;		
Conc. 0 vs 3	*P*=0.000;				

C. liriodendri:

- Chlamydospores:

Conc. 0 vs 1	*P*=0.000;	Conc.1 vs 2	*P*=0.046;	Conc. 2 vs 3	*P*=0.389.
Conc. 0 vs 2	*P*=0.000;	Conc. 1 vs 3	*P*=0.449;		
Conc. 0 vs 3	*P*=0.000;				

- Conidia:

Conc. 0 vs 1	*P*=0.000;	Conc.1 vs 2	*P*=0.007;	Conc. 2 vs 3	*P*=0.120.
Conc. 0 vs 2	*P*=0.000;	Conc. 1 vs 3	*P*=0.420;		
Conc. 0 vs 3	*P*=0.000;				

- Mycelium:

Conc. 0 vs 1	*P*=0.000;	Conc.1 vs 2	*P*=0.103;	Conc. 2 vs 3	*P*=0.236.
Conc. 0 vs 2	*P*=0.000;	Conc. 1 vs 3	*P*=0.002;		
Conc. 0 vs 3	*P*=0.000;				

3.3.5. Analysis by general linear model of the effect of propagule, concentration and species on disease incidence at 5 cm above stem bases, 5 months after inoculation with four concentrations of three propagules of two *Cylindrocarpon* spp.

Source	B	SE	Wald	df	Probability
Species	1.750	0.405	18.695	1	0.000
Concentrations			1.674	2	0.433
Propagules			0.243	2	0.885
Constant	-2.049	0.746	7.545	1	0.006
Species.propagules.concentration			7.660	4	0.105
Constant	-1.141	0.785	2.112	1	0.146

3.3.6. Analysis by general linear model of the effect of propagule, concentration and species on disease severity at 1 cm above stem bases, 5 months after inoculation with four concentrations of three propagules of two *Cylindrocarpon* spp.

Source	Sum of squares	df	Mean square	F ratio	Probability
Cylindrocarpon species	24.766	1	24.766	12.365	0.001
Propagules	5.556E-02	2	2.778E-02	0.14	0.986
Concentrations	56.413	2	28.206	14.083	0.000
Species*propagules	2.056	2	1.028	0.513	0.599
Propagules*concentrations	4.825	4	1.206	0.602	0.661
Species*concentrations	7.746	2	3.873	1.934	0.147
Species*propagules*concentrations	25.452	12	2.121	1.066	0.390
Error	450.639	225	2.003		

3.3.7. Analysis by general linear model of the effect of propagule, concentration and species on root dry weights for plants grown from callused cuttings of rootstock variety 101-14, 5 months after inoculation with four concentrations of three propagules of two *Cylindrocarpon* spp.

Source	Sum of squares	df	Mean square	F ratio	Probability
Cylindrocarpon species	7.448E-03	1	7.448E-03	0.001	0.976
Propagules	7.400	2	3.700	0.461	0.631
Concentrations	20.274	2	10.137	1.263	0.285
Species*propagules	39.242	2	19.621	2.444	0.090
Propagules*concentrations	31.845	4	7.961	0.992	0.414
Species*concentrations	6.930	2	3.465	0.432	0.650
Error	1372.673	171	8.027		

Appendix 4

Additional information for Chapter 3

4.1. *C. destructans* primer development

4.1.1. Primer sequences

Four forward primers (Cyde F1, Cyde FA, Cyde F1 small and Cyde short) and two reverse primers (Cyde R1 and Cyde R2) were designed by Dr. Hayley Ridgway at Lincoln University and are listed in Table 4.1.1.

Table 4.1.1. Primer sequences tested for their specificity to *C. destructans*.

Primer name	Tm (°C)	sequence (5' to 3')
Cyde F1	63.6[1]	CGA CGT GCR GGS ATT CGC TAA CG
Cyde FA	52.7	CAG TGC GTA AGT GCT TCA
CydeF1 short	61.5	ACG TGC RGG SAT TCG CTA ACG
Cyde F1 small	57.0	TGC RGG SAT TCG CTA ACG
Cyde R1	52.7	GAG TTC RAC ASC TCY TGG
Cyde R2	52.3	CYT GGA TAK GGG CAG ATG

Tm: melting temperature.

4.1.2. Assessing the specificity of the primers

Methodology

The different primer combinations tested were: Cyde F1 /Cyde R1, Cyde FA /Cyde R1, Cyde F short /Cyde R1, Cyde F1 small /Cyde R1, Cyde short /Cyde R2, Cyde FA /Cyde R2 and Cyde F1 small /Cyde R2 for three *C. destructans* isolates (D1, D2, D3).

The PCR thermal cycle was as followed: 94°C for 3 min, 35 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C and a final extension at 72°C for 7 min. Each PCR contained: 2.5 µL of 10 × PCR buffer (Roche diagnostics GmbH, Germany), 200 µM of each dNTP (Fermentas Life Sciences, Ontario, Canada), 0.2 µM of each primer (Invitrogen Life Technologies, CA, USA), 1.25 U of FastStart Taq DNA polymerase (Roche diagnostics), 1 µL of 10 ng /µL DNA and SNW to a final volume of 25 µL. A negative control was used to detect the presence of

contamination by adding 1 µL of SNW to the PCR reaction mix instead of an aliquot of DNA. A positive control using general tubulin primers was amplified with the samples.

The best primer pair combinations were tested with all ten *C. destructans* isolates for their specificity.

Results

The general beta tubulin primers amplified a PCR product of 400 bp for the three *C. destructans* isolates tested (Figure 4.1.1). The negative control did not show any band. The primer combinations Cyde short /Cyde R1, Cyde F1 small /R1, Cyde FA /Cyde R2 failed to amplify a product. A 200 bp band was observed for all the three *C. destructans* DNA samples with the primers Cyde short /Cyde R2, Cyde F1 small /Cyde R2 and Cyde FA /Cyde R1.

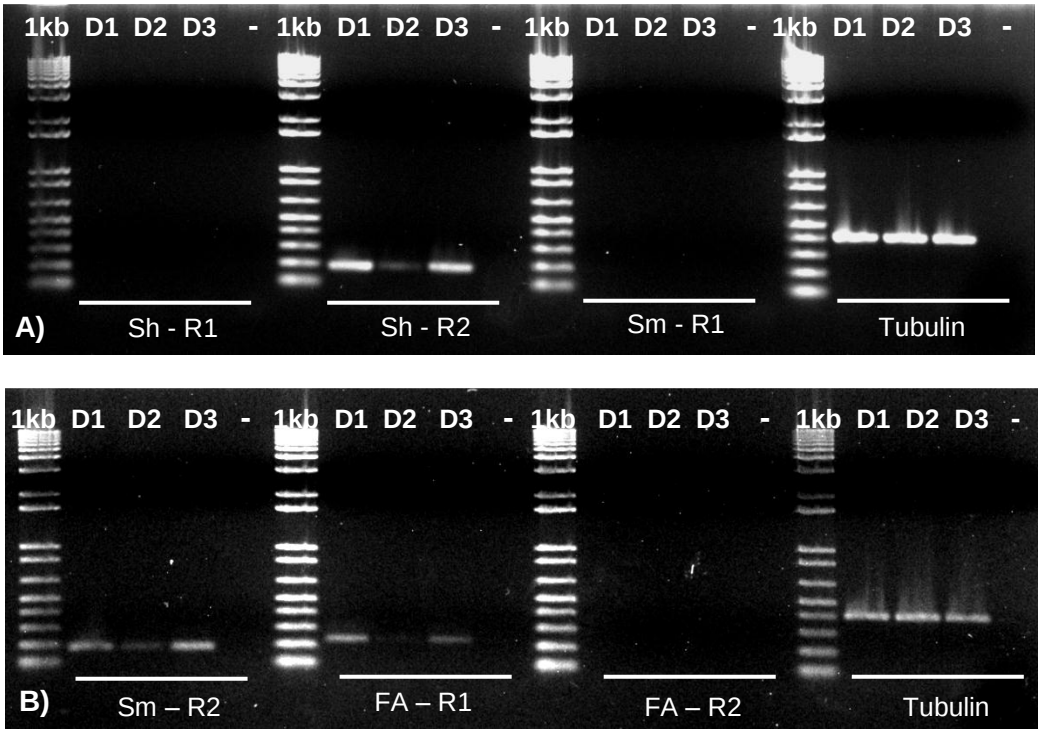


Figure 4.1.1. PCR products obtained with different primer pairs using three *C. destructans* genonomic DNA isolates. A: lane 2 to lane 5: primers Cyde short and Cyde R1; lane 7 to 10: Cyde short and Cyde R2; lane 12 to 15: Cyde F1 small and Cyde R1; lane 17 to 20: tubulin F and tubulin R. B: lane 2 to lane 5: primers Cyde F1 small and Cyde R2; lane 7 to 10: Cyde FA and Cyde R1; lane 12 to 15: Cyde FA and Cyde R2; lane 17 to 20: tubulin F and tubulin R (1kb: 1 Kb plus DNA ladder; -: negative control).

The primer pairs Cyde short /Cyde R2 and Cyde F1 small/ Cyde R2 were tested for their specificity to *C. destructans* using all ten isolates (Figure 4.1.2). No band was observed with the negative control. An expected 400 bp band was observed for the PCR products of the three isolates amplified with the general tubulin primers. All isolates were detected with the two primer pairs. As the primer pair Cyde short/ Cyde R2 showed differences between the brightness of the amplified bands on the gel, the primer pair Cyde F1 small/ Cyde R2 was selected for further experiments.

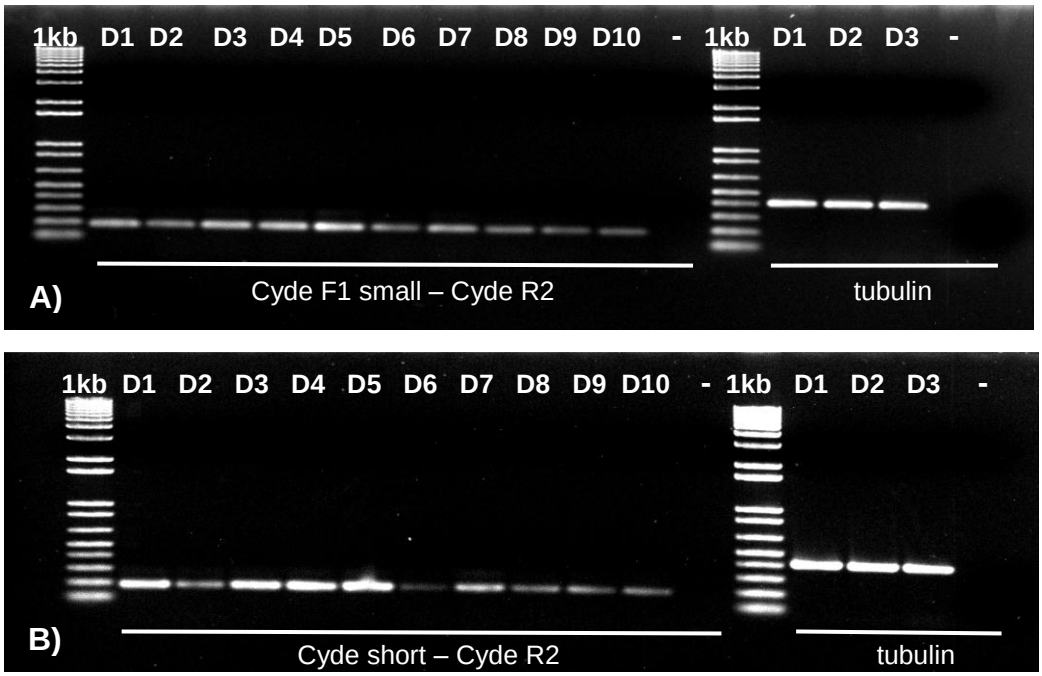
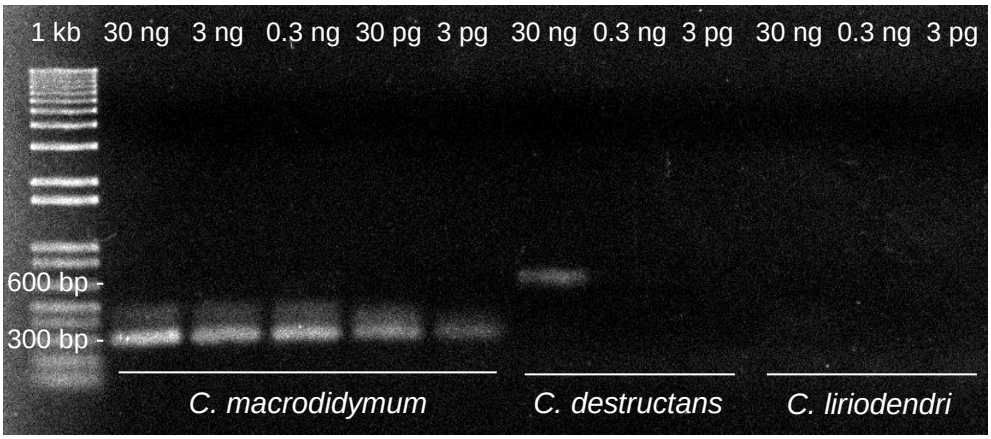


Figure 4.1.2. PCR products obtained with primer pairs Cyde F1 small /Cyde R2 (A), Cyde short /Cyde R2 (B) and general tubulin (A and B) of ten *C. destructans* isolates (D1 to 10: Mar 13 a, Wpa1 a, Co 1c, Mar 13b, Mar 7d, Wpa 2a, Co 5a, Hb 4d, Hb 6g and Mtb 1d; -: negative control, 1 kb: 1 Kb plus DNA ladder).

4.2. Cross reactivity using qPCR

4.2.1. Visualisation of products from qPCR using species specific primers Cyma F1 /Cyma R1 on a 1.5% agarose gel.



4.2.2. Sequences obtained after sequencing the PCR products obtained using 30 ng of *C. destructans* DNA.

Using Cyma F1

```
ORIGIN
1       CCGAGCTTGT GGGCGCCGCC GTTGGTACTA GAACTACCTG CATTGGAGGA
51      CGTTTCGTGA CCGGGACGAT GAGCAAGAGT CTCCTCCCCC CCTGGATCGT
101     TGGAGAATTT TGCAAGCTGT GAAGGTAGCC TCTGCTGCGT GGGAACACCT
151     CTTGGATTTC GTCAAGAATC CGGCGGGTGC CCGAGGTCGT TCTCCGTATT
201     CCCCTGGGCT CGTCAAACGA AATTGGCGTA ATCGGTTGCC ATACACCTGC
251     AAGAATTCTT CTTAGGCTAC TCACCTTTGT CGTCAGGTCT CCCGCCATAG
301     CTGTCGGAGT CGGTTGGTAT GAAGCCAACA TGCCTCCTGC ATTCAACCTC
351     CCATACGGCT CCTTCACGAC TGGCACGTCG GTAT //
```

Using Cyma R1

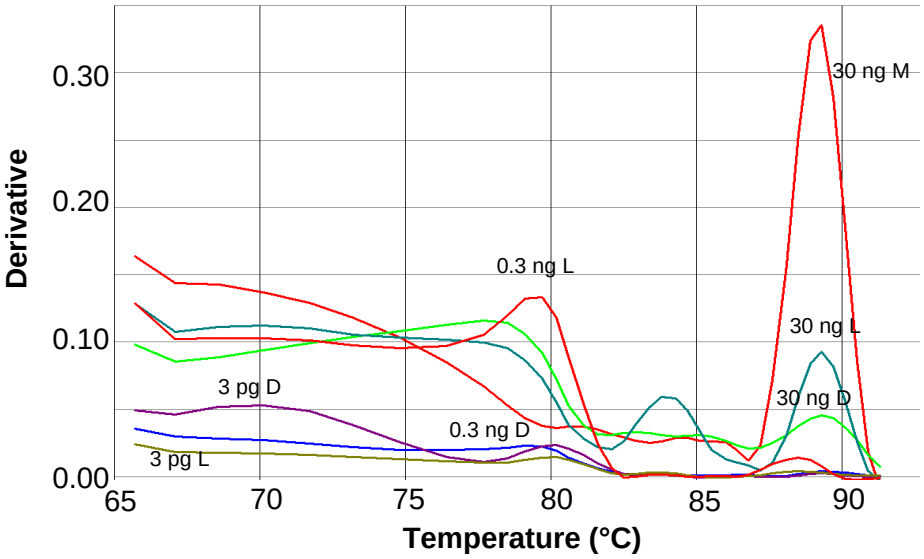
```
ORIGIN
1       GAGCTTGTGG GCGCCGCCGG TTGGTACTAG AACTACCTGC ATTGGCAGAC
51      GTTTCGTGAC CGGGACGATG AGCAAGAGTC TCCTCCCCCC CTGGATCGTT
101     GGAGAATTTT GCAAGCTGTG AAGGTAGCCT CTGCTGCGTG GGGCACCTCT
151     TGGATTTCGT CAAGAATCCG GCGGGTGCCC GAGGTCGTTC TCCGTATTCC
201     CCTGGGCTCG TCAAACGAAA TTGGCGTAAT CGGATGCCAT ACACCTGCAA
251     GAATTCTTCT TAGGCTACTC ACCTTTGTCG TCAGGTCTCC CGCAAAAGCT
301     GTCGGAGTCG GTTGG //
```

4.2.3. Sequence alignments using BLAST on Genbank.

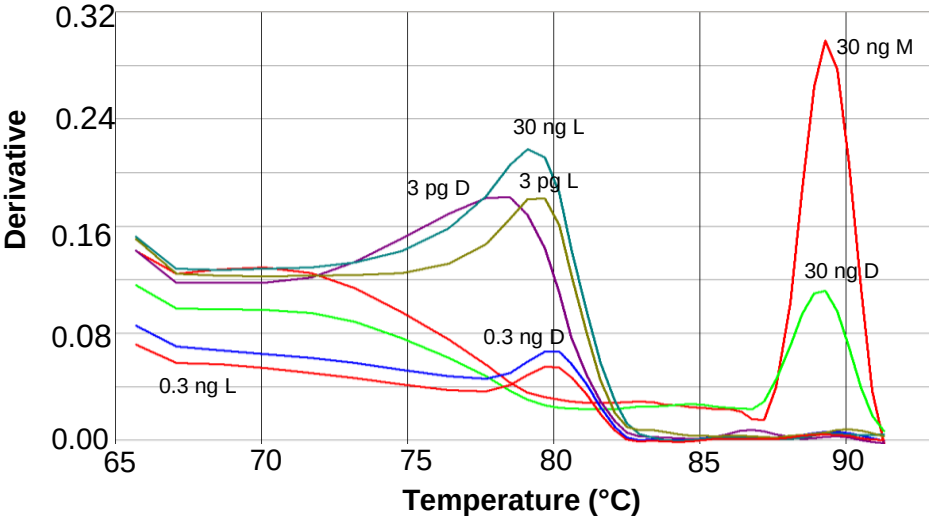
Description	Max. score	Total score	Query coverage	E value	Max. identity
Bradyrhizobium sp. nifD gene	44.6	44.6	9%	0.46	86%
Neosartorya fischeri ABC bile transporter, putative	42.8	42.8	14%	1.3	81%

4.2.4. Dissociation curves of qPCR products using 0.2 (A), 0.1 (B) and 0.05 µM (C) of primers Cyma F1 and Cyma R1 with 30, 0.3 and 0.003 ng of *C. destructans* and *C. liriodendri* DNA.

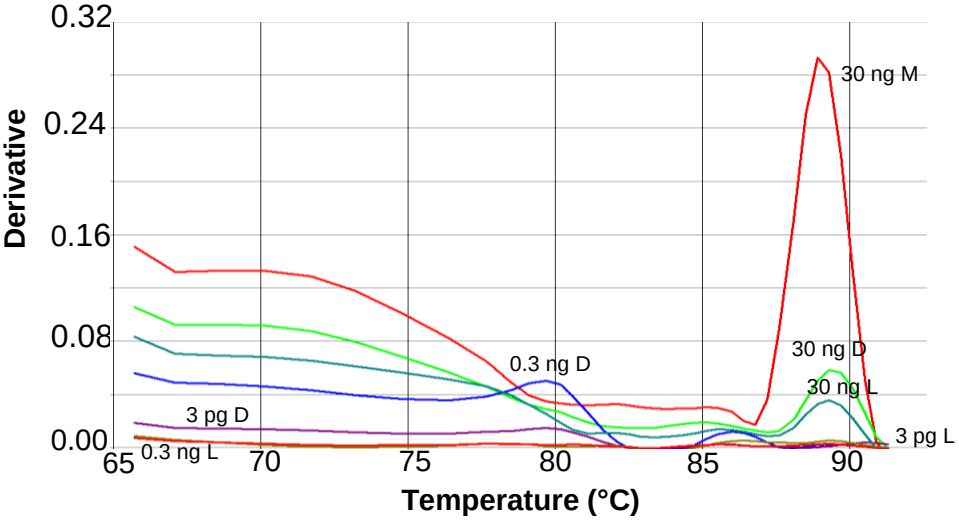
A)



B)



C)



4.3. Primers Mac1 and MaPa2

4.3.1. Results from primer sequence alignments on Genbank using BLAST.

Primers	Accession number	Species	Total score	Query coverage	E value	Max. identity
MaPa2	AJ875336.1	*Neonectria radicicola*	34.2	100%	1.00E-04	100%
	AJ875335.1	*Neonectria radicicola*	34.2	100%	1.00E-04	100%
	AJ875334.1	*Neonectria radicicola*	34.2	100%	1.00E-04	100%
	AJ875322.1	*Neonectria radicicola*	34.2	100%	1.00E-04	100%
	AJ875321.1	*Neonectria radicicola*	34.2	100%	1.00E-04	100%
Mac1	AJ875336.1	*Neonectria radicicola*	34.2	100%	1.00E-04	100%
	AJ875335.1	*Neonectria radicicola*	34.2	100%	1.00E-04	100%
	AJ875334.1	*Neonectria radicicola*	34.2	100%	1.00E-04	100%
	AJ875322.1	*Neonectria radicicola*	34.2	100%	1.00E-04	100%
	AJ875321.1	*Neonectria radicicola*	34.2	100%	1.00E-04	100%

Appendix 5

Statistical analyses for Chapter 4

5.1. Investigation of the pathogenicity of propagules in field conditions

5.1.1. Analysis by logistic regression of the effect of propagule treatments on disease incidence at 1 cm above stem bases, 6 months after inoculation with three propagules of three *Cylindrocarpon* species and planting in the field.

Species	Source	B	S.E.	Wald	df	Probability
Cylindrocarpon spp.	Treatments			102.967	4	0.000
	Constant	-0.423	0.319	1.753	1	0.186
C. destructans	Treatments			10.674	4	0.030
	Constant	-0.544	0.313	3.021	1	0.082
C. liriodendri	Treatments			65.323	4	0.000
	Constant	21.002	4066.667	0.000	1	0.996
C. macrodidymum	Treatments			41.894	4	0.000
	Constant	-3.089	0.602	26.288	1	0.046

5.1.2. Pair-wise comparisons among propagule treatments for disease incidence at 1 cm above stem bases of plants inoculated with *Cylindrocarpon* spp. (with chl.: chlamydospores).

Chl. vs conidia	*P*=0.699;	Conidia vs mycelium	*P*=0.009;
Chl. vs mycelium	*P*=0.003;	Conidia vs control water	*P*=0.000;
Chl. vs control water	*P*=0.000;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.000;		
Mycelium vs control water	*P*=0.000;	Control water vs control wheat	*P*=0.005.
Mycelium vs control wheat	*P*=0.000;		

5.1.3. Pair-wise comparisons among propagule treatments for disease incidence at 1 cm above stem bases of plants inoculated with *C. destructans* (with chl.: chlamydospores).

Chl. vs conidia	*P*=0.051;	Conidia vs mycelium	*P*=0.642;
Chl. vs mycelium	*P*=0.136;	Conidia vs control water	*P*=0.761;
Chl. vs control water	*P*=0.025;	Conidia vs control wheat	*P*=0.021;
Chl. vs control wheat	*P*=0.717;		
Mycelium vs control water	*P*=0.442;	Control water vs control wheat	*P*=0.009.
Mycelium vs control wheat	*P*=0.065;		

5.1.4. Pair-wise comparisons among propagule treatments for disease incidence at 1 cm above stem bases of plants inoculated with *C. liriodendri* (with chl.: chlamydospores).

Chl. vs conidia	*P*=0.000;	Conidia vs mycelium	*P*=0.059;
Chl. vs mycelium	*P*=0.000;	Conidia vs control water	*P*=0.000;
Chl. vs control water	*P*=0.000;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.000;		
Mycelium vs control water	*P*=0.000;	Control water vs control wheat	*P*=0.081.
Mycelium vs control wheat	*P*=0.000;		

5.1.5. Pair-wise comparisons among propagule treatments for disease incidence at 1 cm above stem bases of plants inoculated with *C. macrodidymum* (with chl.: chlamydospores).

Chl. vs conidia	*P*=0.000;	Conidia vs mycelium	*P*=0.005;
Chl. vs mycelium	*P*=0.003;	Conidia vs control water	*P*=0.000;
Chl. vs control water	*P*=0.516;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.013;		
Mycelium vs control water	*P*=0.000;	Control water vs control wheat	*P*=0.043.
Mycelium vs control wheat	*P*=0.000;		

5.1.6. Pair-wise comparisons among same propagule treatment of different species for disease incidence at 1 cm above stem bases.

Comparisons	Probability		
	Chlamydospores	Conidia	Mycelium
C. destructans vs *C. liriodendri*:	*P*=0.000	*P*=0.025	*P*=0.332
C. destructans vs *C. macrodidymum*:	*P*=0.009	*P*=0.305	*P*=0.233
C. liriodendri vs *C. macrodidymum*:	P=0.000	*P*=0.504	*P*=0.002

5.1.7. Pair-wise comparisons among species for disease incidence at 1 cm above stem bases, excluding the controls.

C. destructans vs *C. liriodendri*	*P*<0.001;
C. destructans vs *C. macrodidymum*	*P*=0.156;
C. liriodendri vs *C. macrodidymum*	*P*<0.001.

5.1.8. Analysis by logistic regression of the effect of propagule treatments on disease incidence at 5 cm above stem bases, 6 months after inoculation with three propagules of three *Cylindrocarpon* species and planting in the field.

Species	Source	B	S.E.	Wald	df	Probability
Cylindrocarpon spp.	Treatments			56.088	4	0.000
	Constant	-0.726	0.320	5.131	1	0.024
C. destructans	Treatments			8.564	4	0.073
	Constant	-1.081	0.368	8.604	1	0.003
C. liriodendri	Treatments			65.147	4	0.000
	Constant	-4.202	1.042	16.266	1	0.000
C. macrodidymum	Treatments			15.365	4	0.004
	Constant	-3.807	0.821	21.486	1	0.000

5.1.9. Pair-wise comparisons among propagule treatments for disease incidence at 5 cm above stem bases of plants inoculated with *Cylindrocarpon* spp. (with chl.: chlamydospores).

Chl. vs conidia	*P*=0.002;	Conidia vs mycelium	*P*=0.300;
Chl. vs mycelium	*P*=0.000;	Conidia vs control water	*P*=0.023;
Chl. vs control water	*P*=0.000;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.000;		
Mycelium vs control water	*P*=0.213;	Control water vs control wheat	*P*=0.011.
Mycelium vs control wheat	*P*=0.000;		

5.1.10. Pair-wise comparisons among propagule treatments for disease incidence at 5 cm above stem bases of plants inoculated with *C. destructans* (with chl.: chlamydospores).

Chl. vs conidia	*P*=0.220;	Conidia vs mycelium	*P*=0.824;
Chl. vs mycelium	*P*=0.314;	Conidia vs control water	*P*=0.023;
Chl. vs control water	*P*=0.286;	Conidia vs control wheat	*P*=1.000;
Chl. vs control wheat	*P*=0.220;		
Mycelium vs control water	*P*=0.040;	Control water vs control wheat	*P*=0.023.
Mycelium vs control wheat	*P*=0.824;		

5.1.11. Pair-wise comparisons among propagule treatments for disease incidence at 5 cm above stem bases of plants inoculated with *C. liriodendri* (with chl.: chlamydospores).

Chl. vs conidia	*P*=0.000;	Conidia vs mycelium	*P*=0.469;
Chl. vs mycelium	*P*=0.000;	Conidia vs control water	*P*=0.000;
Chl. vs control water	*P*=0.000;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.000;		
Mycelium vs control water	*P*=0.000;	Control water vs control wheat	*P*=1.000.
Mycelium vs control wheat	*P*=0.000;		

5.1.12. Pair-wise comparisons among propagule treatments for disease incidence at 5 cm above stem bases of plants inoculated with *C. macrodidymum* (with chl.: chlamydospores).

Chl. vs conidia	*P*=0.004;	Conidia vs mycelium	*P*=0.062;
Chl. vs mycelium	*P*=0.267;	Conidia vs control water	*P*=0.000;
Chl. vs control water	*P*=0.248;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.008;		
Mycelium vs control water	*P*=0.030;	Control water vs control wheat	*P*=0.155.
Mycelium vs control wheat	*P*=0.002;		

5.1.13. Pair-wise comparisons among same propagule treatment of different species for disease incidence at 5 cm above stem bases.

Comparisons	Probability		
	Chlamydospores	Conidia	Mycelium
C. destructans vs *C. liriodendri*:	*P*=0.000	*P*=0.087	*P*=0.458
C. destructans vs *C. macrodidymum*:	*P*=0.017	*P*=0.248	*P*=0.664
C. liriodendri vs *C. macrodidymum*:	*P*=0.000	*P*=0.701	*P*=0.096

5.1.14. Pair-wise comparisons among species for disease incidence at 5 cm above stem bases, excluding the controls.

C. destructans vs *C. liriodendri*	*P*<0.001;
C. destructans vs *C. macrodidymum*	*P*=0.403;
C. liriodendri vs *C. macrodidymum*	*P*<0.001.

5.1.15. Analysis by general linear model of the effect of propagule treatments on disease severity at 1 cm above stem bases, 6 months after inoculation with three propagules of three *Cylindrocarpon* species and planting in the field.

Variable	Source	Sum of squares	df	Mean square	F ratio	Probability
Cylindrocarpon spp.	Treatments	406.388	4	101.597	44.548	<0.001
	Error	1071.887	470	2.281		
C. destructans	Treatments	16.729	4	4.182	2.534	0.040
	Error	775.771	470	1.651		
C. liriodendri	Treatments	424.888	4	106.222	65.182	<0.001
	Error	765.925	470	1.630		
C. macrodidymum	Treatments	68.050	4	17.012	18.357	<0.001
	Error	435.575	470	0.927		

5.1.16. Pair-wise comparisons among species for disease severity at 1 cm above stem bases of plants inoculated with *Cylindrocarpon* spp. (with chl.: chlamydospores).

Chl. vs conidia	*P*=0.779;	Conidia vs mycelium	*P*=0.005;
Chl. vs mycelium	*P*=0.002;	Conidia vs control water	*P*=0.000;
Chl. vs control water	*P*=0.000;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.000;		
Mycelium vs control water	*P*=0.000;	Control water vs control wheat	*P*=0.007.
Mycelium vs control wheat	*P*=0.000;		

5.1.17. Pair-wise comparisons among species for disease severity at 1 cm above stem bases of plants inoculated with *C. destructans* (with chl.: chlamydospores).

Chl. vs conidia	*P*=0.082;	Conidia vs mycelium	*P*=0.670;
Chl. vs mycelium	*P*=0.194;	Conidia vs control water	*P*=0.795;
Chl. vs control water	*P*=0.049;	Conidia vs control wheat	*P*=0.017;
Chl. vs control wheat	*P*=0.526;		
Mycelium vs control water	*P*=0.499;	Control water vs control wheat	*P*=0.009.
Mycelium vs control wheat	*P*=0.052;		

5.1.18. Pair-wise comparisons among species for disease severity at 1 cm above stem bases of plants inoculated with *C. liriodendri* (with chl.: chlamydospores).

Chl. vs conidia	*P*=0.000;	Conidia vs mycelium	*P*=0.046;
Chl. vs mycelium	*P*=0.000;	Conidia vs control water	*P*=0.000;
Chl. vs control water	*P*=0.000;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.000;		
Mycelium vs control water	*P*=0.000;	Control water vs control wheat	*P*=0.093.
Mycelium vs control wheat	*P*=0.000;		

5.1.19. Pair-wise comparisons among species for disease severity at 1 cm above stem bases of plants inoculated with *C. macrodidymum* (with chl.: chlamydospores).

Chl. vs conidia	*P*=0.000;	Conidia vs mycelium	*P*=0.003;
Chl. vs mycelium	*P*=0.044;	Conidia vs control water	*P*=0.000;
Chl. vs control water	*P*=0.583;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.018;		
Mycelium vs control water	*P*=0.009;	Control water vs control wheat	*P*=0.046.
Mycelium vs control wheat	*P*=0.000;		

5.1.20. Pair-wise comparisons among same propagule treatment of different species for disease incidence at 5 cm above stem bases.

Comparisons	Probability		
	Chlamydospores	Conidia	Mycelium
C. destructans vs *C. liriodendri*:	*P*=0.001	*P*=0.006	*P*=0.113
C. destructans vs *C. macrodidymum*:	*P*=0.048	*P*=0.349	*P*=0.141
C. liriodendri vs *C. macrodidymum*:	*P*=0.001	*P*=0.010	*P*=0.003

5.1.21. Pair-wise comparison among species for disease severity at 1 cm above stem bases, excluding the controls.

C. destructans vs *C. liriodendri*	*P*<0.001;
C. destructans vs *C. macrodidymum*	*P*=0.255;
C. liriodendri vs *C. macrodidymum*	*P*<0.001.

5.1.22. Analysis by general linear model of the effect of treatments on root and shoot dry weights for plants grown from callused cuttings of rootstock varieties 101-14, 6 months after inoculation with three propagules of three *Cylindrocarpon* species and planting in the field.

Variable	Source	Sum of squares	df	Mean square	F ratio	Probability
Root dry weight	Treatments	23.879	4	5.970	1.147	0.334
	Error	2045.535	393	5.205		
Shoot dry weight	Treatments	24.245	4	6.061	2.350	0.054
	Error	941.604	365	2.580		

5.2. Investigation of the pathogenicity of propagules in different soil types

5.2.1. Analysis by logistic regression of the effect of propagule treatments on disease incidence at 1 cm above stem base for plants grown from callused cuttings of rootstock varieties 101-14, 6 months after inoculation with three propagules of *Cylindrocarpon* spp. and planting in three different soil types.

Species	Source	B	S.E.	Wald	df	Probability
Cylindrocarpon spp.	Propagules			78.802	4	0.000
	Soil types			20.181	2	0.000
	Constant	2.542	0.804	9.997	1	0.002
C. destructans	Propagules			17.470	4	0.002
	Soil types			36.311	2	0.000
	Constant	0.363	0.695	0.273	1	0.601
C. liriodendri	Propagules			1.425	4	0.840
	Soil types			6.719	2	0.035
	Constant	-0.088	0.789	0.012	1	0.912
C. macrodidymum	Propagules			26.578	4	0.000
	Soil types			3.773	2	0.152
	Constant	-2.431	1.074	5.121	1	0.024

5.2.2. Pair-wise comparisons among soil types for disease incidence at 1 cm above stem bases of plants inoculated with *Cylindrocarpon* spp, without controls.

Comparisons	Probability		
	Cylindrocarpon spp.	*C. destructans*	*C. liriodendri*
Light soil vs medium soil:	*P*=0.334	*P*=0.154	*P*=0.581
Light soil vs soil heavy soil:	*P*=0.000	*P*=0.000	*P*=0.016
Medium soil vs heavy soil:	*P*=0.001	*P*=0.000	*P*=0.064

5.2.3. Pair-wise comparisons among same soil type of different species for disease incidence at 1 cm above stem bases.

Comparisons	Probability		
	Light soil	Medium soil	Heavy soil
C. destructans vs *C. liriodendri*:	*P*=0.065	*P*=0.360	*P*=0.458
C. destructans vs *C. macrodidymum*:	*P*=0.383	*P*=0.016	*P*=0.001
C. liriodendri vs *C. macrodidymum*:	*P*=0.003	*P*<0.001	*P*<0.001

5.2.4. Analysis by logistic regression of the interaction between propagule treatments and soil types on disease incidence at 1 cm above stem bases.

Species	Wald	df	Probability
Cylindrocarpon spp.	2.531	4	0.639
C. destructans	8.552	4	0.073
C. liriodendri	7.167	4	0.127
C. macrodidymum	1.545	4	0.819

5.2.5. Chi-square test for disease incidence at 1 cm above stem bases for different propagule types.

Species	Value	df	Probability
Cylindrocarpon spp.	98.566	4	<0.001
C. destructans	15.458	4	0.004
C. liriodendri	96.988	4	<0.001
C. macrodidymum	53.369	4	<0.001

5.2.6. Pair-wise comparisons among propagule treatments for disease incidence at 1 cm above stem bases of plants inoculated with *Cylindrocarpon* spp. (with Chl.: chlamydospores).

Chl. vs conidia	*P*=0.098;	Conidia vs mycelium	*P*=0.299;
Chl. vs mycelium	*P*=0.478;	Conidia vs control water	*P*=0.000;
Chl. vs control water	*P*=0.000;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.000;		
Mycelium vs control water	*P*=0.000;	Control water vs control wheat	*P*=0.071.
Mycelium vs control wheat	*P*=0.000;		

5.2.7. Pair-wise comparisons among propagule treatments for disease incidence at 1 cm above stem bases of plants inoculated with *C. destructans* (with Chl.: chlamydospores).

Chl. vs conidia	*P*=0.027;	Conidia vs mycelium	*P*=0.060;
Chl. vs mycelium	*P*=0.627;	Conidia vs control water	*P*=0.004;
Chl. vs control water	*P*=0.416;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.038;		
Mycelium vs control water	*P*=0.194;	Control water vs control wheat	*P*=0.132.
Mycelium vs control wheat	*P*=0.011;		

5.2.8. Pair-wise comparisons among propagule treatments for disease incidence at 1 cm above stem bases of plants inoculated with *C. liriodendri* (with Chl.: chlamydospores).

Chl. vs conidia	*P*=0.418;	Conidia vs mycelium	*P*=0.223;
Chl. vs mycelium	*P*=0.686;	Conidia vs control water	*P*=0.000;
Chl. vs control water	*P*=0.000;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.000;		
Mycelium vs control water	*P*=0.000;	Control water vs control wheat	*P*=1.000.
Mycelium vs control wheat	*P*=0.000;		

5.2.9. Pair-wise comparisons among propagule treatments for disease incidence at 1 cm above stem bases of plants inoculated with *C. macrodidymum* (with Chl.: chlamydospores).

Chl. vs conidia	*P*=0.000;	Conidia vs mycelium	*P*=0.003;
Chl. vs mycelium	*P*=0.063;	Conidia vs control water	*P*=0.000;
Chl. vs control water	*P*=0.229;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.922;		
Mycelium vs control water	*P*=0.016;	Control water vs control wheat	*P*=0.994.
Mycelium vs control wheat	*P*=0.000;		

5.2.10. Pair-wise comparisons among same propagule treatment of different species for disease incidence at 1 cm above stem bases.

Comparisons	Probability		
	Chlamydospores	Conidia	Mycelium
C. destructans vs *C. liriodendri*:	*P*=0.014	*P*=1.000	*P*=0.017
C. destructans vs *C. macrodidymum*:	*P*=0.001	*P*=0.311	*P*=0.035
C. liriodendri vs *C. macrodidymum*:	*P*<0.001	*P*=0.230	*P*<0.001

5.2.11. Pair-wise comparisons among species for disease incidence at 1 cm above stem bases, excluding controls.

C. destructans vs *C. liriodendri*	*P*=0.004;
C. destructans vs *C. macrodidymum*	*P*<0.001;
C. liriodendri vs *C. macrodidymum*	*P*<0.001.

5.2.12. Analysis by logistic regression of the effect of propagule treatments and soil types on disease incidence at 5 cm above stem bases, 6 months after inoculation with three propagules of *Cylindrocarpon* spp. and planting in three different soil types.

Species	Source	B	S.E.	Wald	df	Probability
Cylindrocarpon spp.	Treatments			54.043	4	0.000
	Soil types			16.524	2	0.000
	Constant	0.587	0.710	0.685	1	0.408
C. destructans	Treatments			14.560	4	0.006
	Soil types			20.574	2	0.000
	Constant	-0.356	0.772	0.213	1	0.645
C. liriodendri	Treatments			0.772	4	0.942
	Soil types			9.168	2	0.010
	Constant	-1.396	1.119	1.557	1	0.212
C. macrodidymum	Treatments			13.968	4	0.007
	Soil types			0.216	2	0.898
	Constant	-3.894	1.586	6.0299	1	0.014

5.2.13. Pair-wise comparisons among soil types for disease incidence at 5 cm above stem bases of plants inoculated with *Cylindrocarpon* spp., without controls.

Comparisons	Probability		
	Cylindrocarpon spp.	*C. destructans*	*C. liriodendri*
Light soil vs medium soil:	*P*=0.175	*P*=0.058	*P*=1.000
Light soil vs soil heavy soil:	*P*=0.000	*P*=0.000	*P*=0.008
Medium soil vs heavy soil:	*P*=0.006	*P*=0.004	*P*=0.007

5.2.14. Pair-wise comparisons among same soil type of different species for disease incidence at 5 cm above stem bases.

Comparisons	Probability		
	Light soil	Medium soil	Heavy soil
C. destructans vs *C. liriodendri*:	*P*=0.076	*P*=0.345	*P*=0.052
C. destructans vs *C. macrodidymum*:	*P*=0.267	*P*=0.049	*P*=0.002
C. liriodendri vs *C. macrodidymum*:	*P*=0.001	*P*=0.001	*P*<0.001

5.2.15. Analysis by logistic regression of the interaction between propagule treatments and soil types on disease incidence at 5 cm above stem bases for plants grown from callused cuttings of rootstock varieties 101-14, 6 months after inoculation.

Species	Wald	df	Probability
Cylindrocarpon spp.	2.478	4	0.649
C. destructans	6.970	4	0.137
C. liriodendri	2.960	4	0.565
C. macrodidymum	0.043	4	1.000

5.2.16. Chi-square test for disease incidence at 5 cm above stem base for different propagule treatments.

Species	Value	df	Probability
Cylindrocarpon spp.	58.645	4	<0.001
C. destructans	13.542	4	0.009
C. liriodendri	61.677	4	<0.001
C. macrodidymum	25.025	4	<0.001

5.2.17. Pair-wise comparisons among propagule treatments for disease incidence at 5 cm above stem bases of plants inoculated with *Cylindrocarpon* spp.

Chl. vs conidia	*P*=0.047;	Conidia vs mycelium	*P*=0.015;
Chl. vs mycelium	*P*=0.671;	Conidia vs control water	*P*=0.000;
Chl. vs control water	*P*=0.000;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.000;		
Mycelium vs control water	*P*=0.000;	Control water vs control wheat	*P*=0.191.
Mycelium vs control wheat	*P*=0.000;		

5.2.18. Pair-wise comparisons among propagule treatments for disease incidence at 5 cm above stem bases of plants inoculated with *C. destructans*.

Chl. vs conidia	*P*=0.129;	Conidia vs mycelium	*P*=0.003;
Chl. vs mycelium	*P*=0.105;	Conidia vs control water	*P*=0.013;
Chl. vs control water	*P*=0.258;	Conidia vs control wheat	*P*=0.002;
Chl. vs control wheat	*P*=0.031;		
Mycelium vs control water	*P*=0.542;	Control water vs control wheat	*P*=0.308.
Mycelium vs control wheat	*P*=0.749;		

5.2.19. Pair-wise comparisons among propagule treatments for disease incidence at 5 cm above stem bases of plants inoculated with *C. liriodendri.*

Chl. vs conidia	*P*=0.518;	Conidia vs mycelium	*P*=0.374;
Chl. vs mycelium	*P*=0.828;	Conidia vs control water	*P*=0.000;
Chl. vs control water	*P*=0.000;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.000;		
Mycelium vs control water	*P*=0.000;	Control water vs control wheat	*P*=1.000.
Mycelium vs control wheat	*P*=0.000;		

5.2.20. Pair-wise comparisons among propagule treatments for disease incidence at 5 cm above stem bases of plants inoculated with *C. macrodidymum*.

Chl. vs conidia	*P*=0.011;	Conidia vs mycelium	*P*=0.014;
Chl. vs mycelium	*P*=0.504;	Conidia vs control water	*P*=0.011;
Chl. vs control water	*P*=1.000;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.994;		
Mycelium vs control water	*P*=0.444;	Control water vs control wheat	*P*=0.994.
Mycelium vs control wheat	*P*=0.994;		

5.2.21. Pair-wise comparisons among same propagule treatments of different species for disease incidence at 5 cm above stem bases.

Comparisons	Probability		
	Chlamydospores	Conidia	Mycelium
C. destructans vs *C. liriodendri*:	*P*=0.076	*P*=1.000	*P*=0.002
C. destructans vs *C. macrodidymum*:	*P*=0.001	*P*=0.071	*P*=0.109
C. liriodendri vs *C. macrodidymum*:	*P*<0.001	*P*=0.035	*P*<0.001

5.2.22. Pair-wise comparisons among species for disease incidence at 5 cm above stem bases, excluding the controls.

C. destructans vs *C. liriodendri*	*P*=0.004;
C. destructans vs *C. macrodidymum*	*P*<0.001;
C. liriodendri vs *C. macrodidymum*	*P*<0.001.

5.2.23. Analysis by general linear model of the effect of propagule treatments and soil types on disease severity at 1 cm above stem bases, 6 months after inoculation with three propagules of three *Cylindrocarpon* species and planting in three different soil types.

Species	Source	Sum of squares	df	Mean square	F ratio	Probability
Cylindrocarpon spp.	Treatments	285.387	4	71.347	36.381	<0.001
	Soil types	77.607	2	38.803	19.787	<0.001
	Error	521.647	266	1.961		
C. destructans	Treatments	32.433	4	8.108	4.772	0.001
	Soil types	92.207	2	46.103	27.136	<0.001
	Error	451.933	266	1.699		
C. liriodendri	Treatments	199.153	4	49.788	28.962	<0.001
	Soil types	18.027	2	9.013	5.243	0.006
	Error	457.280	266	1.719		
C. macrodidymum	Treatments	36.913	4	9.228	14.495	<0.001
	Soil types	2.287	2	1.143	1.796	0.168
	Error	169.353	266	0.637		

5.2.24. Pair-wise comparisons among soil types for disease severity at 1 cm above stem bases of plants inoculated with *Cylindrocarpon* spp.

Comparisons	Probability		
	Cylindrocarpon spp.	*C. destructans*	*C. liriodendri*
Light soil vs medium soil:	*P*=0.007	*P*=0.041	*P*=1.000
Light soil vs soil heavy soil:	*P*=0.000	*P*=0.000	*P*=0.008
Medium soil vs heavy soil:	*P*=0.000	*P*=0.000	*P*=0.005

5.2.25. Pair-wise comparisons among same soil type of different species for disease severity at 1 cm above stem bases.

Comparisons	Probability		
	Light soil	Medium soil	Heavy soil
C. destructans vs *C. liriodendri*:	*P*=0.004	*P*=0.070	*P*=0.001
C. destructans vs *C. macrodidymum*:	*P*=0.420	*P*=0.068	*P*=0.266
C. liriodendri vs *C. macrodidymum*:	*P*=0.001	*P*=0.001	*P*=0.001

5.2.26. Pair-wise comparisons among species for disease severity at 1 cm above stem bases of plants inoculated with *Cylindrocarpon* spp.

Chl. vs conidia	*P*=0.024;	Conidia vs mycelium	*P*=0.088;
Chl. vs mycelium	*P*=0.939;	Conidia vs control water	*P*=0.000;
Chl. vs control water	*P*=0.000;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.000;		
Mycelium vs control water	*P*=0.000;	Control water vs control wheat	*P*=0.223.
Mycelium vs control wheat	*P*=0.000;		

5.2.27. Pair-wise comparisons among species for disease severity at 1 cm above stem bases of plants inoculated with *C. destructans*.

Chl. vs conidia	*P*=0.046;	Conidia vs mycelium	*P*=0.044;
Chl. vs mycelium	*P*=0.874;	Conidia vs control water	*P*=0.004;
Chl. vs control water	*P*=0.354;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.061;		
Mycelium vs control water	*P*=0.304;	Control water vs control wheat	*P*=0.302.
Mycelium vs control wheat	*P*=0.051;		

5.2.28. Pair-wise comparisons among species for disease severity at 1 cm above stem bases of plants inoculated with *C. liriodendri*.

Chl. vs conidia	*P*=0.569;	Conidia vs mycelium	*P*=0.435;
Chl. vs mycelium	*P*=0.250;	Conidia vs control water	*P*=0.000;
Chl. vs control water	*P*=0.000;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.000;		
Mycelium vs control water	*P*=0.000;	Control water vs control wheat	*P*=1.000.
Mycelium vs control wheat	*P*=0.000;		

5.2.29. Pair-wise comparisons among species for disease severity at 1 cm above stem bases of plants inoculated with *C. macrodidymum*.

Chl. vs conidia	*P*=0.000;	Conidia vs mycelium	*P*=0.013;
Chl. vs mycelium	*P*=0.036;	Conidia vs control water	*P*=0.000;
Chl. vs control water	*P*=0.397;	Conidia vs control wheat	*P*=0.000;
Chl. vs control wheat	*P*=0.131;		
Mycelium vs control water	*P*=0.010;	Control water vs control wheat	*P*=1.000.
Mycelium vs control wheat	*P*=0.003;		

5.2.30. Pair-wise comparisons among same propagule treatment of different species for disease severity at 1 cm above stem bases.

Comparisons	Probability		
	Chlamydospores	Conidia	Mycelium
C. destructans vs *C. liriodendri*:	*P*=0.005	*P*=0.814	*P*=0.005
C. destructans vs *C. macrodidymum*:	*P*=0.001	*P*=0.098	*P*=0.001
C. liriodendri vs *C. macrodidymum*:	*P*=0.001	*P*=0.032	*P*=0.001

5.2.31. Pair-wise comparisons among species for disease severity at 1 cm above stem bases, excluding the controls.

C. destructans vs *C. liriodendri*	*P*=0.001;
C. destructans vs *C. macrodidymum*	*P*=0.001;
C. liriodendri vs *C. macrodidymum*	*P*=0.001.

5.2.32. Analysis by general linear model of the effect of propagule treatments and soil types on root dry weights, 6 months after inoculation with three propagules of *C. macrodidymum* and planting in three different soil types.

Source	Sum of squares	df	Mean square	F ratio	Probability
Treatments	112.971	4	28.243	2.473	0.045
Soil types	1834.721	2	917.360	80.331	<0.001
Soil types. Treatments	446.887	8	55.861	4.892	<0.001
Error	2443.835	214	11.420		

5.2.33. Analysis by general linear model of the effect of propagule treatments and soil types on shoot dry weights, 6 months after inoculation with three propagules of *C. macrodidymum* and planting in three different soil types.

Source	Sum of squares	df	Mean square	F ratio	Probability
Treatments	5.310	4	1.328	1.970	0.100
Soil types	98.673	2	49.337	73.211	<0.001
Soil types. Treatments	14.189	8	1.774	2.632	0.009
Error	144.213	214	0.674		

5.3. Fate of mycelium and conidia in the soil environment

5.3.1. Analysis of variance of the effect of three isolates and two inocula on the number of conidia in the nylon bags after 3 weeks using GenStat version 12.1.

Source of variation	Sum of squares	d.f.	Mean square	F ratio	F probability
Isolate	1.421e+13	2	7.103e+12	79.38	<0.001
Inoculum	1.736e+13	1	1.736e+13	193.96	<0.001
Isolate.Inoculum	1.522e+13	2	7.611e+12	85.05	<0.001
Residual	1.611e+12	18	8.949e+10		
Total	4.840e+13	23			

5.3.2. Analysis of variance of the effect of three isolates and two inocula on the number of chlamydospores in the nylon bags after 3 weeks using GenStat version 12.1.

Source of variation	Sum of squares	df	Mean square	F ratio	F probability
Isolate	1.759e+11	2	8.794e+10	2.19	0.141
Inoculum	1.117e+11	1	1.117e+11	2.78	0.113
Isolate.Inoculum	4.558e+11	2	2.279e+11	5.67	0.012
Residual	7.231e+11	18	4.017e+10		
Total	1.467e+12	23			

Appendix 6

Statistical analyses for Chapter 6

6.1. Investigation of the effects of water-logging stress

6.1.1. Analysis by logistic regression of the effect of plant inoculation on disease incidence at 1 cm above stem bases, 6 months after inoculation with *Cylindrocarpon* spp.

Species	B	SE	Wald	df	Probability
Cylindrocarpon spp.	3.764	0.478	61.984	1	<0.001
C. liriodendri	4.331	0.777	31.080	1	<0.001
C. macrodidymum	3.119	0.637	23.963	1	<0.001
C. destructans	2.998	0.646	21.507	1	<0.001

6.1.2. Pair-wise comparisons among species for disease incidences at 1 cm above stem bases of plants inoculated with *Cylindrocarpon* spp.

C. destructans vs *C. liriodendri*: *P*=0.010;
C. destructans vs *C. macrodidymum*: *P*=0.683;
C. liriodendri vs *C. macrodidymum*: *P*=0.065.

6.1.3. Analysis by logistic regression of the effect of water stress, rootstock varieties and wounding on disease incidence at 1 cm above stem bases, 6 months after inoculation with *Cylindrocarpon* spp.

Species	Source	B	SE	Wald	df	Probability
Cylindrocarpon spp.	Water regimes			7.615	2	0.022
	Wounding	-0.375	0.435	0.774	1	0.388
	Variety	1.758	0.458	14.719	1	<0.001
	Constant	1.551	0.865	3.218	1	0.073
C. destructans	Water regimes			0.397	2	0.820
	Wounding	-0.597	0.452	1.746	1	0.186
	Variety	0.794	0.456	3.036	1	0.081
	Constant	0.740	0.771	0.920	1	0.337
C. liriodendri	Water regimes			2.147	2	0.342
	Wounding	-0.355	0.423	0.704	1	0.402
	Variety	1.798	0.441	16.594	1	<0.001
	Constant	-0.914	0.775	1.389	1	0.239
C. macrodidymum	Water regimes			6.187	2	0.045
	Wounding	0.000	0.425	0.000	1	1.000
	Variety	0.539	0.428	1.487	1	0.208
	Constant	-0.773	0.795	0.946	1	0.331

6.1.4. Pair-wise comparisons among water regimes for disease incidences at 1 cm above stem bases of plants inoculated with *Cylindrocarpon* spp.

75% field capacity vs water-logging:	*P*=0.006;
100% field capacity vs water-logging:	*P*=0.064;
75% field capacity vs 100% field capacity:	*P*=0.317.

6.1.5. Pair-wise comparisons among water regimes for disease incidences at 1 cm above stem bases of plants inoculated with *C. macrodidymum*.

75% field capacity vs water-logging:	*P*=0.012;
100% field capacity vs water-logging:	*P*=0.143;
75% field capacity vs 100% field capacity:	*P*=0.287.

6.1.6. Mean disease incidences at 1 cm above stem bases of wounded and unwounded plants, 6 months after inoculation with *Cylindrocarpon* spp.

Wounding	Disease incidence at 1 cm (%)			
	Cylindrocarpon spp.	*C. destructans*	*C. liriodendri*	*C. macrodidymum*
No wound	39.2	18.3	25.0	17.5
Wound	32.5	12.5	20.0	16.7

6.1.7. Analysis by logistic regression of the effect of plant inoculation on disease incidence in the water stress experiment at 5 cm above stem bases, 6 months after inoculation with *Cylindrocarpon* spp. or water.

Species	B	SE	Wald	df	Probability
C. destructans	2.096	0.790	7.040	1	0.008
C. liriodendri	3.399	0.768	19.565	1	<0.001
C. macrodidymum	1.568	0.885	3.137	1	0.697
Cylindrocarpon spp.	2.841	0.522	29.558	1	<0.001

6.1.8. Pair-wise comparison among species for disease incidences at 1 cm above stem bases of plants inoculated with *Cylindrocarpon* spp.

C. destructans vs *C. liriodendri*:	*P*=0.003;
C. destructans vs *C. macrodidymum*:	*P*=0.263;
C. liriodendri vs *C. macrodidymum*:	*P*<0.001.

6.1.9. Analysis by logistic regression of the effect of water stress, rootstock varieties and wounding on disease incidence at 5 cm above stem bases, 6 months after inoculation with *Cylindrocarpon* spp.

Species	Source	B	SE	Wald	df	Probability
Cylindrocarpon spp.	Water regimes			4.241	2	0.120
	Wounding	0.093	0.431	0.046	1	0.829
	Variety	1.946	0.456	18.214	1	<0.001
	Constant	-1.398	0.816	2.934	1	0.087
C. destructans	Water regimes			0.2	2	0.905
	Wounding	-0.605	0.645	0.878	1	0.349
	Variety	1.453	0.713	4.153	1	0.042
	Constant	-2.337	1.095	4.553	1	0.033
C. liriodendri	Water regimes			3.170	2	0.205
	Wounding	0.000	0.468	0.000	1	1.000
	Variety	2.092	0.530	15.563	1	0.000
	Constant	-2.671	0.997	7.177	1	0.007
C. macrodidymum	Water regimes			0.235	2	0.889
	Wounding	0.422	0.925	0.208	1	0.648
	Variety	1.259	0.974	1.672	1	0.196
	Constant	-12.484	118.162	0.011	1	0.916

6.1.10. Mean disease incidences at 5 cm above stem bases of plants watered with three different water regimes for a month, 6 months after inoculation with *Cylindrocarpon* spp.

Species	Water regimes		
	75% field capacity	100% field capacity	Water-logging
C. destructans	12.5	10.0	10.0
C. liriodendri	17.5	30.0	32.5
C. macrodidymum	0.0	7.5	10.0
Cylindrocarpon spp.	25.0	37.5	45.5

6.1.11. Mean disease incidences at 5 cm above stem bases of wounded and unwounded plants, 6 months after inoculation with *Cylindrocarpon* spp.

Wounding	Disease incidence at 5 cm (%)			
	Cylindrocarpon spp.	*C. destructans*	*C. liriodendri*	*C. macrodidymum*
No wounds	20.8	7.5	15.0	4.2
Wounds	19.2	5.0	13.3	3.3

6.1.12. Analysis by general linear model of the effect of water stress, rootstock varieties and wounding on disease severity at 1 cm above stem bases, 6 months after inoculation with *Cylindrocarpon* spp.

Species	Source	B	SE	Wald	df	Probability
Cylindrocarpon spp.	Variety	49.408	1	49.408	17.597	0.000
	Water levels	14.717	2	7.358	2.621	0.077
	Wounding	1.008	1	1.008	0.359	0.550
	Error	297.617	106	2.808		
C. destructans	Variety	8.533	1	8.533	4.492	0.036
	Water levels	0.650	2	0.325	0.171	0.843
	Wounding	4.800	1	4.800	2.527	0.115
	Error	201.350	106	1.900		
C. liriodendri	Variety	39.675	1	39.675	15.272	0.000
	Water levels	1.517	2	0.758	0.292	0.747
	Wounding	0.675	1	0.675	0.260	0.611
	Error	275.383	106	2.598		
C. macrodidymum	Variety	4.408	1	4.408	2.066	0.154
	Water levels	6.617	2	3.308	1.550	0.217
	Wounding	8.33E-03	1	8.33E-03	0.004	0.950
	Error	226.217	106	2.134		

6.1.13. Mean disease severity at 1 cm above stem bases of plants watered with three different water regimes for a month, 6 months after inoculation with *Cylindrocarpon* spp.

Species	Water regimes		
	75% field capacity	100% field capacity	Water-logging
C. destructans	17.5	21.9	20.6
C. liriodendri	30.0	36.9	33.7
C. macrodidymum	15.0	22.5	29.3
Cylindrocarpon spp.	43.7	56.9	65.0

6.1.14. Mean disease severity at 1 cm above stem bases of wounded and unwounded plants, 6 months after inoculation with *Cylindrocarpon* spp.

Wounding	Disease incidence at 1 cm (%)			
	Cylindrocarpon spp.	*C. destructans*	*C. liriodendri*	*C. macrodidymum*
No wound	57.5	25.0	35.4	22.5
Wound	52.9	15.0	31.7	22.1

6.1.15. Analysis by general linear model of the effect of water stress, varieties and wounding on root dry weights, 6 months after inoculation with *Cylindrocarpon* spp. or water.

Source	Sum of squares	df	Mean square	F ratio	Probability
Variety	0.533	1	0.533	0.389	0.535
Water regimes	3.299	2	1.65	1.204	0.308
Inoculation	1.163	1	1.163	0.849	0.361
Wounding	0.305	1	0.305	0.222	0.639
Error	68.488	50	1.370		

6.1.16. Analysis by general linear model of the effect of water stress, varieties and wounding on shoot dry weights, 6 months after inoculation with *Cylindrocarpon* spp. or water.

Source	Sum of squares	df	Mean square	F ratio	Probability
Variety	0.374	1	0.374	0.482	0.491
Water regimes	9.790E-02	2	4.895E-02	0.063	0.939
Inoculation	0.848	1	0.848	1.094	0.301
Wounding	1.370	1	1.370	1.768	0.190
Error	38.756	50	0.775		

6.2. Investigation of the effect of wound ages

6.2.1. Analysis by logistic regression of the effect of wound ages, rootstock varieties and wound types on disease incidence at 1 cm above stem bases, 4 months after inoculation with *Cylindrocarpon* spp.

Species	Treatments	B	S.E.	Wald	df	Probability
Cylindrocarpon spp.	Wound types	-1.005	0.239	17.65	1	<0.001
	Wound ages			26.907	6	<0.001
	Variety	0.084	0.237	0.126	1	0.723
	Constant	0.537	0.520	1.065	1	0.302
C. destructans	Wound type	-0.052	0.323	0.026	1	0.872
	Wound ages			9.749	6	0.136
	Variety	-0.469	0.326	2.071	1	0.150
	Constant	-0.923	0.654	1.988	1	0.159
C. liriodendri	Wound type	-1.182	0.257	21.226	1	<0.001
	Wound ages			28.404	6	<0.001
	Variety	0.343	0.251	1.874	1	0.171
	Constant	0.287	0.540	0.282	1	0.595
C. macrodidymum	Wound type	-0.06	0.346	0.030	1	0.863
	Wound ages			6.444	6	0.375
	Variety	0.788	0.360	4.806	1	0.028
	Constant	-1.925	0.726	7.023	1	0.008

6.2.2. Pair-wise comparisons among wound ages for disease incidences at 1 cm above stem base of plants inoculated with *Cylindrocarpon* spp.

Day 0 vs day 1:	*P*=0.159;	Day 1 vs day 3:	*P*=0.095;	Day 3 vs day 5:	*P*=0.032;
Day 0 vs day 3:	*P*=0.814;	Day 1 vs day 5:	*P*=0.635;	Day 3 vs day 8:	*P*=0.512;
Day 0 vs day 5:	*P*=0.067;	Day 1 vs day 8:	*P*=0.028;	Day 3 vs no wound:	*P*=0.821;
Day 0 vs day 8:	*P*=0.392;	Day 1 vs no wound:	*P*=0.189;	Day 3 vs control:	*P*=0.002;
Day 0 vs no wound:	*P*=1.000;	Day 1 vs control:	*P*=0.000;		
Day 0 vs control:	*P*=0.001;				
Day 5 vs day 8:	*P*=0.009;	Day 8 vs no wound:	*P*=0.385;	Day 5 vs control:	*P*=0.000;
Day 5 vs no wound:	*P*=0.064;	Day 8 vs control:	*P*=0.014;	No wound vs control:	*P*=0.001.

6.2.3. Pair-wise comparisons among wound ages for disease incidences at 1 cm above stem bases of plants inoculated with *C. destructans.*

Day 0 vs day 1:	*P*=0.753;	Day 1 vs day 3:	*P*=0.372;	Day 3 vs day 5:	*P*=0.777;
Day 0 vs day 3:	*P*=0.242;	Day 1 vs day 5:	*P*=0.265;	Day 3 vs day 8:	*P*=1.000;
Day 0 vs day 5:	*P*=0.157;	Day 1 vs day 8:	*P*=0.370;	Day 3 vs no wound:	*P*=0.105;
Day 0 vs day 8:	*P*=0.242;	Day 1 vs no wound:	*P*=0.484;	Day 3 vs control:	*P*=0.013;
Day 0 vs no wound:	*P*=0.717;	Day 1 vs control:	*P*=0.069;		
Day 0 vs control:	*P*=0.136;				
Day 5 vs day 8:	*P*=0.772;	Day 8 vs no wound:	*P*=0.130;	Day 5 vs control:	*P*=0.010;
Day 5 vs no wound:	*P*=0.082;	Day 8 vs control:	*P*=0.015;	No wound vs control:	*P*=0.165.

6.2.4. Pair-wise comparisons among wound ages for disease incidences at 1 cm above stem bases of plants inoculated with *C. liriodendri.*

Day 0 vs day 1:	*P*=0.014;	Day 1 vs day 3:	*P*=0.001;	Day 3 vs day 5:	*P*=0.021;
Day 0 vs day 3:	*P*=0.306;	Day 1 vs day 5:	*P*=0.275;	Day 3 vs day 8:	*P*=0.789;
Day 0 vs day 5:	*P*=0.139;	Day 1 vs day 8:	*P*=0.001;	Day 3 vs no wound:	*P*=0.021;
Day 0 vs day 8:	*P*=0.198;	Day 1 vs no wound:	*P*=0.284;	Day 3 vs control:	*P*=0.147;
Day 0 vs no wound:	*P*=0.167;	Day 1 vs control:	*P*=0.000;		
Day 0 vs control:	*P*=0.007;				
Day 5 vs day 8:	*P*=0.013;	Day 8 vs no wound:	*P*=0.014;	Day 5 vs control:	*P*=0.000;
Day 5 vs no wound:	*P*=1.000;	Day 8 vs control:	*P*=0.247;	No wound vs control:	*P*=0.001.

6.2.5. Pair-wise comparisons among species for disease incidences at 1 cm above stem bases of plants inoculated with three *Cylindrocarpon* spp.

C. destructans vs *C. liriodendri*:	*P*<0.001;
C. destructans vs *C. macrodidymum*:	*P*=0.295;
C. liriodendri vs *C. macrodidymum*:	*P*<0.001.

6.2.6. Analysis by logistic regression of the effect of wound ages, wound type and rootstock varieties on disease incidences at 5 cm above stem bases, 4 months after inoculation with *Cylindrocarpon* spp.

Species	Treatments	B	S.E.	Wald	df	Probability
Cylindrocarpon spp.	Wound types	-1.163	0.321	13.130	1	<0.001
	Wound ages			16.982	6	0.009
	Variety	0.163	0.307	0.280	1	0.596
	Constant	-2.014	0.776	6.728	1	0.009
C. destructans	Wound type	-0.741	0.547	1.835	1	0.175
	Wound ages			2.883	6	0.823
	Variety	0.459	0.535	0.734	1	0.392
	Constant	-10.566	37.115	0.081	1	0.776
C. liriodendri	Wound type	-1.213	0.396	13.130	1	<0.001
	Wound ages			16.982	6	0.009
	Variety	0.142	0.368	0.28	1	0.596
	Constant	-2.383	0.955	6.227	1	0.013
C. macrodidymum	Wound type	-0.152	0.524	0.084	1	0.772
	Wound ages			5.980	6	0.425
	Variety	0.167	0.525	0.101	1	0.751
	Constant	-2.869	1.249	5.274	1	0.022

6.2.7. Pair-wise comparison among wound ages for disease incidences at 5 cm above stem bases of plants inoculated with *Cylindrocarpon* spp.

Day 0 vs day 1:	*P*=0.192;	Day 1 vs day 3:	*P*=0.084;	Day 3 vs day 5:	*P*=0.030;
Day 0 vs day 3:	*P*=0.753;	Day 1 vs day 5:	*P*=0.667;	Day 3 vs day 8:	*P*=1.000;
Day 0 vs day 5:	*P*=0.060;	Day 1 vs day 8:	*P*=0.098;	Day 3 vs no wound:	*P*=0.445;
Day 0 vs day 8:	*P*=0.756;	Day 1 vs no wound:	*P*=0.014;	Day 3 vs control:	*P*=0.232;
Day 0 vs no wound:	*P*=0.176;	Day 1 vs control:	*P*=0.007;		
Day 0 vs control:	*P*=0.109;				
Day 5 vs day 8:	*P*=0.030;	Day 8 vs no wound:	*P*=0.276;	Day 5 vs control:	*P*=0.002;
Day 5 vs no wound:	*P*=0.002;	Day 8 vs control:	*P*=0.168;	No wound vs control:	*P*=0.644.

6.2.8. Pair-wise comparison among wound ages for disease incidences at 5 cm above stem bases of plants inoculated with *C. liriodendri.*

Day 0 vs day 1:	*P*=0.080;	Day 1 vs day 3:	*P*=0.075;	Day 3 vs day 5:	*P*=0.119;
Day 0 vs day 3:	*P*=1.000;	Day 1 vs day 5:	*P*=0.756;	Day 3 vs day 8:	*P*=0.199;
Day 0 vs day 5:	*P*=0.111;	Day 1 vs day 8:	*P*=0.008;	Day 3 vs no wound:	*P*=0.994;
Day 0 vs day 8:	*P*=0.992;	Day 1 vs no wound:	*P*=0.039;	Day 3 vs control:	*P*=0.288;
Day 0 vs no wound:	*P*=0.668;	Day 1 vs control:	*P*=0.010;		
Day 0 vs control:	*P*=0.135;				
Day 5 vs day 8:	*P*=0.011;	Day 8 vs no wound:	*P*=0.295;	Day 5 vs control:	*P*=0.009;
Day 5 vs no wound:	*P*=0.034;	Day 8 vs control:	*P*=1.000;	No wound vs control:	*P*=0.204.

6.2.9. Pair-wise comparison among species for disease incidences at 5 cm above stem bases of plants inoculated with three *Cylindrocarpon* spp.

C. destructans vs *C. liriodendri*:	*P*=0.001;
C. destructans vs *C. macrodidymum*:	*P*=1.000;
C. liriodendri vs *C. macrodidymum*:	*P*=0.003.

6.2.10. Analysis by general linear model of the effect of wounding ages, wound type and rootstock varieties on disease severity at 1 cm above stem bases, 4 months after inoculation with *Cylindrocarpon* spp.

Source	Sum of squares	df	Mean square	F ratio	Probability
Variety	1.520	1	1.520	0.853	0.357
Wound type	27.749	1	27.749	15.564	0.000
Days	49.513	6	8.252	4.629	0.000
Error	552.697	310	1.783		

6.2.11. Analysis by general linear model of the effect of wounding ages, wound type and rootstock varieties on root dry, 4 months after inoculation with *Cylindrocarpon* spp.

Source	Sum of squares	df	Mean square	F ratio	Probability
Variety	150.903	1	150.903	23.996	<0.001
Wound type	2.933	1	2.933	0.466	0.495
Days	179.203	6	29.867	4.749	<0.001
Error	1653	263	6.289		

6.2.12. Analysis by general linear model of the effect of wounding ages, wound type and rootstock varieties on shoot dry weights, 4 months after inoculation with *Cylindrocarpon* spp.

Source	Sum of squares	df	Mean square	F ratio	Probability
Variety	6573.260	1	6573.26	87.231	<0.001
Wound type	44.830	1	44.830	0.595	0.441
Days	1631.648	6	271.941	3.609	0.002
Error	20195.028	268	75.355		

6.3. Investigation of the infection progression from trunk and root wounds

6.3.1. Analysis by logistic regression of the effect of inoculated root wounded, inoculated unwounded and root wounded water treated vines (Treatment) on disease incidences at 2.5, 5 and 7.5 cm above stem bases, 6 months after inoculation with *Cylindrocarpon* spp.

Distance	Source	B	S.E.	Wald	df	Probability
2.5 cm	Variety	-0.263	0.423	0.387	1	0.534
	Treatment			4.176	2	0.124
	Constant	1.693	1.172	20.89	1	0.148
5 cm	Variety	0.126	0.42	0.09	1	0.764
	Treatment			3.893	2	0.143
	Constant	0.941	0.96	0.961	1	0.327
7.5 cm	Variety	-0.277	0.472	0.344	1	0.557
	Treatment			2.003	2	0.367
	Constant	0.171	0.924	0.034	1	0.853

6.3.2. Analysis by logistic regression of the effect of of inoculated root wounded, inoculated unwounded and root wounded water treated vines (Treatment) on disease incidences at 2.5, 5 and 7.5 cm above stem bases, 6 months after inoculation with *C. destructans*.

Distance	Source	B	S.E.	Wald	df	Probability
2.5 cm	Variety	-1.237	0.874	2.002	1	0.157
	Treatment			3.945	2	0.139
	Constant	-3.218	1.037	9.626	1	0.002
5 cm	Variety	-0.399	0.954	0.175	1	0.676
	Treatment			1.746	2	0.418
	Constant	-3.483	1.085	10.309	1	0.031
7.5 cm	Variety	9.95	137.304	0.005	1	0.942
	Treatment			0.007	2	0.997
	Constant	-23.28	222.74	0.011	1	0.917

6.3.3. Analysis by logistic regression of the effect of inoculated root wounded, inoculated unwounded and root wounded water treated vines (Treatment) on disease incidences at 2.5, 5 and 7.5 cm above stem bases, 6 months after inoculation with *C. liriodendri*.

Distance	Source	B	S.E.	Wald	df	Probability
2.5 cm	Variety	-1.415	1.140	1.541	1	0.214
	Treatment			1.559	2	0.459
	Constant	-3.179	1.044	9.27	1	0.002
5 cm	Variety	0.041	1.034	0.002	1	0.968
	Treatment			1.04	2	0.594
	Constant	-3.684	1.142	10.415	1	0.001
7.5 cm	Variety	-1.112	1.172	0.9	1	0.343
	Treatment			0.484	2	0.785
	Constant	-2.523	0.794	10.087	1	0.001

6.3.4. Analysis by logistic regression of the effect of of inoculated root wounded, inoculated unwounded and root wounded water treated vines (Treatment) on disease incidences at 2.5, 5 and 7.5 cm above stem bases, 6 months after inoculation with *C. macrodidymum*.

Distance	Treaments	B	S.E.	Wald	df	Probability
2.5 cm	Variety	-0.087	0.417	0.044	1	0.835
	Treatment			1.586	2	0.452
	Constant	-0.654	0.944	0.481	1	0.488
5 cm	Variety	-0.051	0.424	0.014	1	0.905
	Treatment			1.70904	2	0.426
	Constant	0.269	0.903	0.089	1	0.765
7.5 cm	Variety	-0.279	0.477	0.343	1	0.558
	Treatment			2.083	2	0.353
	Constant	-0.648	0.969	0.447	1	0.504

6.3.5. Pair-wise comparisons among disease incidences at 2.5, 5 and 7.5 cm above stem bases of plants inoculated with three *Cylindrocarpon* spp. For each species:

Cylindrocarpon spp.*:*	2.5 cm vs 5 cm	*P*=0.728;
	2.5 cm vs 7.5 cm	*P*=0.211;
	5 cm vs 7.5 cm	*P*=0.058.
C. destructans:	2.5 cm vs 5 cm	*P*=0.375;
	2.5 cm vs 7.5 cm	*P*=0.016;
	5 cm vs 7.5 cm	*P*=0.324.
C. liriodendri:	2.5 cm vs 5 cm	*P*=1.000;
	2.5 cm vs 7.5 cm	*P*=1.000;
	5 cm vs 7.5 cm	*P*=1.000.
C. macrodidymum:	2.5 cm vs 5 cm	*P*=0.265;
	2.5 cm vs 7.5 cm	*P*=0.324;
	5 cm vs 7.5 cm	*P*=0.020.

6.3.6. Pair-wise comparisons among species for disease incidences at 2.5, 5 and 7.5 cm above stem bases of plants inoculated with three *Cylindrocarpon* spp.

2.5 cm:	*C. destructans* vs *C. liriodendri*:	*P*=0.549;
	C. destructans vs *C. macrodidymum*:	*P*<0.001;
	C. liriodendri vs *C. macrodidymum*:	*P*<0.001.
5 cm:	*C. destructans vs C. liriodendri:*	*P*=1.000;
	C. destructans vs C. macrodidymum:	*P*<0.001;
	C. liriodendri vs C. macrodidymum:	*P*<0.001.
7.5 cm:	*C. destructans vs C. liriodendri:*	*P*=0.375;
	C. destructans vs C.macrodidymum:	*P*<0.001;
	C. liriodendri vs C. macrodidymum:	*P*<0.001.

6.3.7. Analysis by general linear model of the effect of of inoculated root wounded, inoculated unwounded and root wounded water treated vines (Treatment) on disease severities at 2.5, 5 and 7.5 cm above stem bases, 6 months after inoculation with *Cylindrocarpon* spp.

Source	Sum of squares	df	Mean square	F ratio	Probability
Varieties	0.192	1	0.192	1.074	0.303
Treatments	0.520	2	0.260	1.453	0.239
Varieties. Treatments	0.342	2	0.171	0.956	0.388
Error	16.650	93	0.179		
Distance	6.697E-02	2	2.249E-02	0.641	0.528
Distance. Variety	1.617E-02	2	8.086E-03	0.155	0.857
Distance. Treatment	7.430E-02	4	1.857E-02	0.356	0.840
Error	9.710	186	5.220E-02		

6.3.8. Analysis by general linear model of the effect of inoculated root wounded, inoculated unwounded and root wounded water treated vines (Treatment) on disease severities at 2.5, 5 and 7.5 cm above stem bases, 6 months after inoculation with *C.macrodidymum*.

Source	Sum of squares	df	Mean square	F	Probability
Varieties	0.118	1	0.118	0.707	0.402
Treatments	0.232	2	0.116	0.699	0.500
Varieties. Treatments	0.410	2	0.205	1.233	0.296
Error	15.461	93	0.166		
Distance	3.631E-02	2	1.816E-02	0.399	0.672
Distance. Variety	2.641E-03	2	1.320E-03	0.029	0.971
Distance. Treatment	6.954E-02	4	1.739E-02	0.382	0.821
Error	8.470	93	9.107E-02		

6.3.9. Analysis by general linear model of the effect of of inoculated root wounded, inoculated unwounded and root wounded water treated vines (Treatment) on the proportion of infected roots of grafted vines, 6 months after inoculation with *Cylindrocarpon* spp.

Species	Source	Sum of squares	df	Mean square	F ratio	Probability
Cylindrocarpon spp.	Varieties	1.210E-03	1	1.210E-03	0.028	0.867
	Treatments	1.960	2	0.980	22.75	<0.001
	Error	4.005	93	4.307E-02		
C. destructans	Varieties	3.743E-03	1	3.743E-03	0.332	0.566
	Treatments	0.267	2	0.133	11.826	<0.001
	Error	1.049	93	1.128E-02		
C. liriodendri	Varieties	7.073E-03	1	7.073E-03	0.417	0.520
	Treatments	0.103	2	5.161E-02	3.043	0.052
	Error	4.203	93	4.519E-02		
C. macrodidymum	Varieties	4.263E-03	1	4.263E-03	0.094	0.759
	Treatments	0.621	2	0.310	6.869	0.002
	Error	4.203	93	4.519E-02		

6.3.10. Pair-wise comparisons among species for the proportion of infected roots of grafted vines, 6 months after inoculation with *Cylindrocarpon* spp.

C. destructans vs *C. liriodendri*: P=0.630;
C. destructans vs *C. macrodidymum*: P<0.001;
C. liriodendri vs *C. macrodidymum*: P<0.001.

6.3.11. Analysis by logistic regression of the effect of trunk and shoot wounding treatments on the disease incidence at inoculation point and 2.5 cm below the inoculation point of grafted vines, 6 months after inoculation with *Cylindrocarpon* spp.

Source	Treatments	B	SE	Wald	df	Probability
0 cm	Varieties	-0.899	0.615	2.133	1	0.144
	Treatments	-1.242	0.626	3.941	1	0.047
	Constant	2.310	1.343	2.957	1	0.085
-2.5 cm	Varieties	0.102	0.699	0.021	1	0.884
	Treatments	-0.568	0.703	0.653	1	0.419
	Constant	-10.000	81.687	0.015	1	0.903

6.3.12. Analysis by logistic regression of the effect of trunk and shoot wounding treatments on the disease incidence at inoculation point and 2.5 cm below the inoculation point of grafted vines, 6 months after inoculation with *C. destructans*.

Source	Treatments	B	SE	Wald	df	Probability
0 cm	Varieties	-0.110	0.589	0.035	1	0.852
	Treatments	-0.228	0.589	0.149	1	0.699
	Constant	0.169	1.083	0.024	1	0876
-2.5 cm	Varieties	12.075	152.940	0.006	1	0.937
	Treatments	-1.386	1.732	0.641	1	0.423
	Constant	-24.863	556.653	0.002	1	0.964

6.3.13. Analysis by logistic regression of the effect of trunk and shoot wounding treatments on the disease incidence at inoculation point and 2.5 cm below the inoculation point of grafted vines, 6 months after inoculation with *C. liriodendri*.

Source	Treatments	B	SE	Wald	df	Probability
0 cm	Varieties	-0.447	0.549	0.662	1	0.416
	Treatments	-1.023	0.558	3.362	1	0.067
	Constant	0.735	1.113	0.436	1	0.509
-2.5 cm	Varieties	-0.546	1.057	0.267	1	0.605
	Treatments	-0.546	1.057	0.267	1	0.605
	Constant	-11.714	221.419	0.003	1	0.958

6.3.14. Analysis by logistic regression of the effect of trunk and shoot wounding treatments on the disease incidence at inoculation point and 2.5 cm below the inoculation point of grafted vines, 6 months after inoculation with *C. macrodidymum*.

Source	Treatments	B	SE	Wald	df	Probability
0 cm	Varieties	-0.961	0.579	2.761	1	0.097
	Treatments	-2.267	0.626	13.133	1	0.000
	Constant	0.156	1.326	0.014	1	0.906
-2.5 cm	Varieties	-0.322	1.085	0.088	1	0.766
	Treatments	-0.727	1.083	0.451	1	0.502
	Constant	-11.738	221.320	0.003	1	0.958

6.3.15. Pair-wise wise comparisons among species for the disease incidences at the inoculation point and 2.5 cm below of grafted vines, 6 months after inoculation with *Cylindrocarpon* spp.

0 cm :	*C. destructans* vs *C. liriodendri*:	*P*<0.001;
	C. destructans vs *C. macrodidymum*:	*P*=0.036;
	C. liriodendri vs *C. macrodidymum*:	*P*=0.043.
-2.5 cm :	*C. destructans vs C. liriodendri:*	*P*=0.687*;*
	C. destructans vs C. macrodidymum:	*P*=0.727*;*
	C. liriodendri vs C. macrodidymum:	*P*=1.000*.*

6.3.16. Analysis by general linear model of the effect of trunk and shoot wounding treatments on the disease severity at inoculation point of grafted vines, 6 months after inoculation with *Cylindrocarpon* spp.

Species	Source	Sum of squares	df	Mean square	F ratio	Probability
Cylindrocarpon spp.	Varieties	7.082E-03	1	7.082E-03	0.096	0.757
	Treatments	6.096	2	3.048	41.450	<0.001
	Error	6.912	94	7.353E-02		
C. destructans	Varieties	0.174	1	0.174	0.199	0.657
	Treatments	0.038	1	0.038	0.044	0.835
	Error	49.708	57	0.872		
C. liriodendri	Varieties	0.995	1	0.995	0.410	0.524
	Treatments	10.334	1	10.334	4.262	0.044
	Error	138.225	57	2.425		
C. macrodidymum	Varieties	2.280	1	2.280	1.570	0.215
	Treatments	16.263	1	16.263	11.203	0.001
	Error	82.746	57	1.452		

6.3.17. Pair-wise wise comparisons among species for the disease severities at the inoculation of grafted vines, 6 months after inoculation with *Cylindrocarpon* spp.

C. destructans vs *C. liriodendri*:	*P*<0.001;
C. destructans vs *C. macrodidymum*:	*P*=0.006;
C. liriodendri vs *C. macrodidymum*:	*P*<0.001.

6.4. Investigation of the effect of cold storage

6.4.1. Analysis by Chi-square of the effect of cold storage on disease incidences at 1 cm above stem bases of vines, 3 months after inoculation with *Cylindrocarpon* spp. or water treatment.

Chi-square	Value	df	Probability
C. destructans	34.606	1	0.000
C. liriodendri	172.800	1	0.000
C. macrodidymum	108.092	1	0.000
Cylindrocarpon spp.	186.935	1	0.000

6.4.2. Analysis by logistic regression of the effect of 1 to 6 months of cold storage and rootstock varieties on disease incidences at 1 cm above stem bases, 3 months after inoculation with *Cylindrocarpon* spp.

Species	Treatments	B	SE	Wald	df	Probability
Cylindrocarpon spp.	Months			1.649	5	0.895
	Variety	1.509	1.155	1.707	1	0.191
	Constant	2.625	1.055	6.187	1	0.013
C. destructans	Months			6.323	5	0.276
	Variety	0.000	0.346	0.000	1	1.000
	Constant	0.336	0.449	0.562	1	0.453
C. liriodendri	Months			16.273	5	0.006
	Variety	0.345	0.417	0.685	1	0.408
	Constant	2.972	1.035	8.243	1	0.004
C. macrodidymum	Months			9.081	5	0.106
	Variety	0.389	0.36	1.156	1	0.282
	Constant	0.914	0.500	3.334	1	0.068

6.4.3. Pair-wise wise comparisons among cold storage months for the disease incidences 1 cm above stem bases of vines cold stored for 1 to 6 months and inoculated with *C. destructans*.

Month 1 vs month 2	*P*=1.000;	Month 2 vs month 3	*P*=0.363;
Month 1 vs month 3	*P*=0.367;	Month 2 vs month 4	*P*=0.367;
Month 1 vs month 4	*P*=0.063;	Month 2 vs month 5	*P*=0.127;
Month 1 vs month 5	*P*=0.135;	Month 2 vs month 6	*P*=0.045;
Month 1 vs month 6	*P*=0.044;		
Month 3 vs month 4	*P*=1.000;	Month 4 vs month 5	*P*=0.563;
Month 3 vs month 5	*P*=0.557;	Month 4 vs month 6	*P*=0.224;
Month 3 vs month 6	*P*=0.244;	Month 5 vs month 6	*P*=0.563.

6.4.4. Pair-wise wise comparisons among cold storage months for the disease incidences 1 cm above stem bases of vines cold stored for 1 to 6 months and inoculated with *C. liriodendri*.

Month 1 vs month 2	*P*=0.196;	Month 2 vs month 3	*P*=0.077;
Month 1 vs month 3	*P*=0.550;	Month 2 vs month 4	*P*=0.477;
Month 1 vs month 4	*P*=0.234;	Month 2 vs month 5	*P*=0.269;
Month 1 vs month 5	*P*=0.023;	Month 2 vs month 6	*P*=0.070;
Month 1 vs month 6	*P*=0.008;		
Month 3 vs month 4	*P*=0.017;	Month 4 vs month 5	*P*=0.679;
Month 3 vs month 5	*P*=0.008;	Month 4 vs month 6	*P*=0.159;
Month 3 vs month 6	*P*=0.004;	Month 5 vs month 6	*P*=0.319.

6.4.5. Pair-wise wise comparisons among cold storage months for the disease incidences 1 cm above stem bases of vines cold stored for 1 to 6 months and inoculated with *C. macrodidymum*.

Month 1 vs month 2	*P*=1.000;	Month 2 vs month 3	*P*=0.094;
Month 1 vs month 3	*P*=0.103;	Month 2 vs month 4	*P*=0.223;
Month 1 vs month 4	*P*=0.901;	Month 2 vs month 5	*P*=0.546;
Month 1 vs month 5	*P*=0.553;	Month 2 vs month 6	*P*=0.352;
Month 1 vs month 6	*P*=0.352;		
Month 3 vs month 4	*P*=0.009;	Month 4 vs month 5	*P*=0.564;
Month 3 vs month 5	*P*=0.035;	Month 4 vs month 6	*P*=039;
Month 3 vs month 6	*P*=0.458;	Month 5 vs month 6	*P*=0.119.

6.4.6. Pair-wise wise comparisons among species for the disease incidences 1 cm above stem bases of vines cold stored for 1 to 6 months and inoculated with *Cylindrocarpon* spp.

C. destructans vs *C. liriodendri*:	*P*<0.001;
C. destructans vs *C. macrodidymum*:	*P*=0.001;
C. liriodendri vs *C. macrodidymum*:	*P*=0.048.

6.4.7. Analysis by Chi-square tests of the effect of inoculation on disease incidences at 5 cm above stem bases of vines, 3 months after inoculation with *Cylindrocarpon* spp.

Chi-square	Value	df	Probability
C. destructans	20.284	1	0.000
C. liriodendri	69.517	1	0.000
C. macrodidymum	56.980	1	0.000
Cylindrocarpon spp.	139.918	1	0.000

6.4.8. Analysis by logistic regression of the effect of 1 to 6 months of cold storage and rootstock varieties on disease incidences at 5 cm above stem bases, 3 months after inoculation with *Cylindrocarpon* spp.

Species	Treatments	B	SE	Wald	df	Probability
Cylindrocarpon spp.	Months			12.617	5	0.027
	Variety	0.771	0.400	3.719	1	0.054
	Constant	0.533	0.486	1.205	1	0.270
C. destructans	Months			12.576	5	0.028
	Variety	-0.639	0.470	1.855	1	0.173
	Constant	-0.804	0.515	2.440	1	0.118
C. liriodendri	Months			4.374	5	0.497
	Variety	0.363	0.349	1.082	1	0.298
	Constant	-0.350	0.448	0.610	1	0.435
C. macrodidymum	Months			10.532	5	0.061
	Variety	0.527	0.366	2.076	1	0.150
	Constant	-0.968	0.480	4.076	1	0.043

6.4.9. Pair-wise wise comparisons among cold storage months for the disease incidences 5 cm above stem bases of vines cold stored for 1 to 6 months and inoculated with *Cylindrocarpon* spp.

Month 1 vs month 2	*P*=0.242;	Month 2 vs month 3	*P*=0.010;
Month 1 vs month 3	*P*<0.001;	Month 2 vs month 4	*P*=0.551;
Month 1 vs month 4	*P*=0.562;	Month 2 vs month 5	*P*=0.182;
Month 1 vs month 5	*P*=0.014;	Month 2 vs month 6	*P*=0.755;
Month 1 vs month 6	*P*=0.140;		
Month 3 vs month 4	*P*=0.002;	Month 4 vs month 5	*P*=0.057;
Month 3 vs month 5	*P*=0.156;	Month 4 vs month 6	*P*=0.365;
Month 3 vs month 6	*P*=0.020;	Month 5 vs month 6	*P*=0.303.

6.4.10. Pair-wise wise comparisons among cold storage months for the disease incidences 5 cm above stem bases of vines cold stored for 1 to 6 months and inoculated with *C. destructans*.

Month 1 vs month 2	*P*=0.296;	Month 2 vs month 3	*P*=0.683;
Month 1 vs month 3	*P*=0.156;	Month 2 vs month 4	*P*=0.637;
Month 1 vs month 4	*P*=0.551;	Month 2 vs month 5	*P*=0.023;
Month 1 vs month 5	*P*=0.002;	Month 2 vs month 6	*P*=0.267;
Month 1 vs month 6	*P*=0.041;		
Month 3 vs month 4	*P*=0.383;	Month 4 vs month 5	*P*=0.121;
Month 3 vs month 5	*P*=0.057;	Month 4 vs month 6	*P*=0.221;
Month 3 vs month 6	*P*=0.447;	Month 5 vs month 6	*P*=0.008.

6.4.11. Pair-wise wise comparisons among cold storage months for the disease incidences 5 cm above stem bases of vines cold stored for 1 to 6 months and inoculated with *C. macrodidymum*.

Month 1 vs month 2	*P*=0.763;	Month 2 vs month 3	*P*=0.043;
Month 1 vs month 3	*P*=0.083;	Month 2 vs month 4	*P*=1.000;
Month 1 vs month 4	*P*=0.763;	Month 2 vs month 5	*P*=0.182;
Month 1 vs month 5	*P*=0.104;	Month 2 vs month 6	*P*=1.000;
Month 1 vs month 6	*P*=0.763;		
Month 3 vs month 4	*P*=0.043;	Month 4 vs month 5	*P*=1.000;
Month 3 vs month 5	*P*=0.001;	Month 4 vs month 6	*P*=0.182;
Month 3 vs month 6	*P*=0.043;	Month 5 vs month 6	*P*=0.182.

6.4.12. Pair-wise wise comparisons among species for the disease incidences 5 cm above stem bases of vines inoculated with *Cylindrocarpon* spp.

C. destructans vs *C. liriodendri*:	*P*<0.001;
C. destructans vs *C. macrodidymum*:	*P*=0.003;
C. liriodendri vs *C. macrodidymum*:	*P*<0.001.

6.4.13. Analysis by general linear model of the effect of inoculation after 1 to 6 months of storage on disease severities at 1 cm above stem bases, 3 months after inoculation with *Cylindrocarpon* spp. or water treatment.

Species	Source	Sum of squares	df	Mean square	F ratio	Probability
Cylindrocarpon spp.	Treatments	592.253	1	592.253	467.590	<0.001
	error	257.122	203	1.267		
C. destructans	Treatments	50.837	1	50.837	30.887	<0.001
	error	334.122	203	1.646		
C. liriodendri	Treatments	392.000	1	392.000	360.299	<0.001
	error	220.861	203	1.088		
C. macrodidymum	Treatments	217.014	1	217.014	137.186	<0.001
	error	321.125	203	1.582		

6.4.14. Analysis by general linear model of the effect of cold storage after 1 to 6 months of storage and rootstock varieties on disease severities at 1 cm above stem bases, 3 months after inoculation with *Cylindrocarpon* spp.

Species	Source	Sum of squares	df	Mean square	F ratio	Probability
Cylindrocarpon spp.	Month	36.201	5	7.240	8.128	<0.001
	Error	58.792	66	0.891		
	Variety	2.507	1	2.507	2.202	0.143
	Error	75.125	66	1.138		
C. destructans	Month	25.750	5	5.150	2.513	0.038
	Error	135.250	66	2.049		
	Variety	2.778E-02	1	2.778E-02	0.011	0.917
	Error	168.417	66	2.552		
C. liriodendri	Month	87.000	5	17.400	7.178	<0.001
	Error	160.00	66	2.424		
	Variety	0.111	1	0.111	0.054	0.816
	Error	134.833	66	2.043		
C. macrodidymum	Month	76.951	5	15.390	9.460	<0.001
	Error	107.375	66	1.627		
	Variety	0.562	1	0.562	0.242	0.625
	Error	153.708	66	2.329		

6.4.15. Pair-wise wise comparisons among species for the disease severities 1 cm above stem bases of vines cold stored for 1 to 6 months and inoculated with *Cylindrocarpon* spp.

C. destructans vs *C. liriodendri*: $P<0.001$;
C. destructans vs *C. macrodidymum*: $P=0.001$;
C. liriodendri vs *C. macrodidymum*: $P=0.025$.

6.5. Prior infection with *Phaeomoniella chlamydospora*

6.5.1. Analysis by logistic regression of the effect of the presence of *Phaeomoniella chlamydospora* on disease incidences at 1 cm above stem bases, 6 months after inoculation with *Cylindrocarpon* spp.

Species	Treatment	B	SE	Wald	df	Probablility
Cylindrocarpon spp.	Presence of *P. chlamydospora*	-1.048	0.663	2.495	1	0.114
	Constant	0.847	0.488	3.015	1	0.082
C. destructans	Presence of *P. chlamydospora*	-0.747	1.268	0.347	1	0.556
	Constant	-2.197	0.745	8.69	1	0.003
C. liriodendri	Presence of *P. chlamydospora*	-1.534	0.771	3.959	1	0.047
	Constant	-0.201	0.449	0.199	1	0.655
C. macrodidymum	Presence of *P. chlamydospora*	0.00	0.69	0.000	1	1.000
	Constant	-0.847	0.488	3.015	1	0.082

6.5.2. Pair-wise wise comparisons among species for the disease incidences 1 cm above stem bases of vines inoculated with *Cylindrocarpon* spp.

C. destructans vs *C. liriodendri*: *P*=0.035;
C. destructans vs *C. macrodidymum*: *P*=0.035;
C. liriodendri vs *C. macrodidymum*: *P*=1.000.

6.5.3. Analysis by logistic regression of the effect of the presence of *Phaeomoniella chlamydospora* on disease incidences at 5 cm above stem bases, 6 months after inoculation with *Cylindrocarpon* spp.

Species	Treatment	B	SE	Wald	df	Probablility
Cylindrocarpon spp.	Presence of *P. chlamydospora*	-0.348	0.839	0.172	1	0.678
	Constant	-1.386	0.559	6.15	1	0.013
C. liriodendri	Presence of *P. chlamydospora*	-0.463	0.974	0.226	1	0.635
	Constant	-1.735	0.626	7.673	1	0.006
C. macrodidymum	Presence of *P. chlamydospora*	0.747	1.268	0.347	1	0.556
	Constant	-2.944	1.026	8.236	1	0.004