GRAPEVINE RHIZOSPHERE BACTERIA: INFLUENCE OF DIVERSITY AND FUNCTION ON TWO ROOT DISEASES

A thesis submitted in fulfilment

of the requirements for the degree

of

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by

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DEDICATION

This thesis is dedicated to my late grandfather,
Dennis James Joyce "Pa-Pa"
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ABSTRACT

Abstract of a thesis submitted in fulfilment of the Degree of Master of Science

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The overall goal of this research was to determine what, if any, role grapevine rhizosphere bacteria play in the differing susceptibilities of New Zealand grown rootstocks to Cylindrocarpon black foot disease. The size and diversity of bacterial populations associated with the rhizospheres of grapevine rootstocks: 101-14, 5C, Schwarzmann and Riparia Gloire were evaluated. Dilution plating showed that total bacterial (P=0.012, P=0.005 for NA and KB, respectively) and fluorescent Pseudomonad (P=0.035) rhizosphere counts differed between rhizosphere and bulk soils but did not correlate with the differing susceptibilities of the rootstock varieties to black foot. No varietal differences were found for spore forming bacteria (P=0.201). SSCP banding patterns showed that species diversity was similar for most rootstocks, but that there were some differences in the composition of bacterial populations, probably attributable to vigour.

Some functional characteristics of the bacteria isolated from the rhizospheres of the most and least susceptible rootstock varieties were assessed to investigate their potential to suppress the pathogen. In dual culture, bacteria from Riparia Gloire, 101-14 and the control soil all had little ability to antagonise *Cylindrocarpon destructans*. However, they differed in their degrees of activity for glucanase (P=0.000), protease (P=0.001) and siderophores (P=0.000). In all tests, bacterial isolates from the rhizosphere of 101-14 had the largest number of active isolates ($P \leq 0.002$); however, those from Riparia Gloire had the greatest degree of positive responses for the glucanase and siderophore assays. Bacterial isolates from the control soil produced few glucanases and no siderophores, but had the highest degree of protease activity. Bands excised and sequenced from SSCP gels frequently matched to other 'uncultured bacteria' in GenBank, as well as to other bacterial phyla, classes and genera commonly isolated from soil and sediment samples. These included members of the Firmicutes, Proteobacteria (α, δ, γ), Verrucomicrobia, Acidobacteria and Chromatiales.

The pathogenicity of *C. destructans* and *Fusarium oxysporum* was investigated by inoculating soil containing wounded ungrafted rootstocks of 101-14, 5C, Schwarzmann and Riparia Gloire. Results indicated that *F. oxysporum* might be a more aggressive pathogen than *C. destructans*. Inoculation with *F. oxysporum* or *C. destructans* increased disease severity, P=0.018 and P=0.056, respectively at 0 cm. Rootstock variety influenced disease severity caused by *C. destructans* (P<0.001) and *F. oxysporum* (P=0.090), with rootstocks 101-14 and 5C being most susceptible to *C. destructans*, and Riparia Gloire and Schwarzmann most susceptible to *F. oxysporum*. There was also an indication that inoculation with one pathogen increased plant susceptibility to the other, with increased *F. oxysporum* infection in the *C. destructans* inoculated treatments of Riparia Gloire and Schwarzmann (P<0.05).

The effect of carbohydrate stress (leaf trimming) and inoculation on *C. destructans* disease severity, incidence, and rootstock rhizosphere bacterial populations was evaluated by inoculating the soil containing one year old plants of Sauvignon Blanc scion wood grafted to rootstocks 101-14 and Schwarzmann. Disease severity and incidence was similar for both Schwarzmann (8.4% and 29.3%, respectively) and 101-14 (14.9% and 31.0%, respectively). When data for the moderate and no stress treatments were combined, because their effects were similar, the disease severity was significantly higher for the highly stressed plants (P=0.043). Stress did not influence disease incidence (P=0.551). Infection occurred in the non-inoculated plants, but disease severity was higher in the plants inoculated with *C. destructans* than those that were not. Root dry weight of highly stressed plants was lower than in both the moderately stressed (P=0.000) and unstressed plants (P=0.003). An interaction between inoculation and stress (P=0.031) showed that inoculated and highly stressed plants had the lowest root dry weight but there was no effect of rootstocks (P=0.062). There was no significant effect of carbohydrate stress (P=0.259) or inoculation (P=0.885) on shoot dry weight. SSCP banding patterns showed that bacterial diversity was generally similar between rootstocks, but stress and inoculation altered rhizosphere bacterial communities.

This study has demonstrated that functionality of grapevine rhizosphere bacteria do differ between grapevine rootstock varieties that have different susceptibilities to black foot disease, but that this role needs to be further investigated if more accurate and practically relevant conclusions are to be drawn.

Keywords: *Cylindrocarpon destructans*, *Fusarium oxysporum*, grapevine, rootstock, bacteria, rhizosphere, SSCP, pathogen, soil, carbohydrate, stress, biocontrol

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LIST OF ABBREVIATIONS

SDW	sterile distilled water
CFU	colony forming units
PDA	potato dextrose agar
NA	nutrient agar
KB	King's Medium B agar
SSCP	single strand conformation polymorphism
DGGE	denaturing gradient gel electrophoresis
BLAST	Basic Local Alignment Search Tool
bp	base pair (s)
d	day (s)
h	hour (s)
min	minutes
Schw.	Schwarzmann
R.Gl.	Riparia Gloire
Inoc	inoculation treatment
r.p.m	revolutions per minute
mL	millilitre
µL	microlitre
LSD	least significant difference
GWC	gravimetric water content
ISR	induced systemic resistance
PCR	polymerase chain reaction

*SI units not included

CHAPTER 1
INTRODUCTION

1.1 GRAPEVINES

1.1.1 New Zealand grapevine industry

The last ten to twenty years has seen the New Zealand wine industry grow enormously (Figure 1.1). In 2008 there were almost 30,000 hectares of grapevines planted in New Zealand which produced 205.2 million litres of wine. A total of 88.6 million litres of wine was exported, bringing $NZ 797.8 million into the country. Domestic sales in 2008 were equivalent to about half this, with 46.5 million litres of wine reported to be sold within the country (Smith & Fistonch, 2008). Grapevines and their products, particularly with respect to wine, are clearly of great importance to the New Zealand economy.

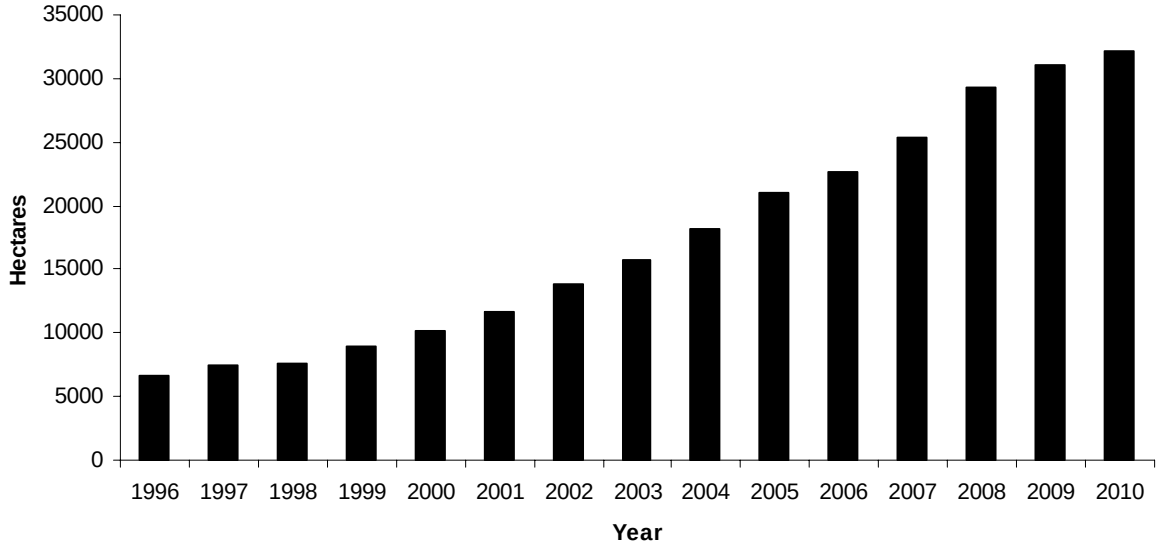


Figure 1.1: Total New Zealand producing vineyard area 1996-2008 (actual) and 2009-2010 (forecast). Data from the New Zealand Winegrower's Statistical Annual 2008 (www.nzwine.com/statistics).

1.1.2 Grapevine rootstocks

In the last 20-30 years, the world-wide losses caused by grape phylloxera (*Daktulosphaira vitifoliae*), a root-feeding insect, has led to the introduction of phylloxera resistant grapevine rootstock varieties into most of the wine producing regions of the world. To minimise losses caused by grape phylloxera, new vineyards used grafted plants, *Vitis vinifera* (with its desired fruit quality) grafted onto phylloxera resistant rootstocks (North American *Vitis* species or hybrids of these species and the European *V. vinifera*) (Powell, 2008). North American *Vitis* species are largely resistant to grape phylloxera, while the European *V. vinifera* is extremely

susceptible (Mullins et al., 1992). The insect that causes phylloxera feeds only on the leaves and non-lignified roots of American species or hybrids, however, on European species, it feeds on leaves and all root tissues, even the lignified roots. The main damage caused to the plants is through reduction of nutrient and water uptake, causing reduced yields and even death of plants (Powell, 2008). Additionally, the feeding sites provide for entry of fungal pathogens including *Cylindrocarpon destructans* and *Fusarium oxysporum* which then cause root necrosis (Omer et al., 1995; Granett et al., 1998).

The rootstock varieties onto which the fruiting scion wood is grafted can have very different characteristics, and so are selected according to the characteristics of the intended vineyard soil. They may confer more or less vigour and tolerance to drought, water-logging, lime content, acidity and salinity. They may also affect fruit composition and wine type as some rootstock varieties have a greater uptake of potassium which can alter the pH of the wine (Whiting & Buchanan, 1992). They also differ in scion compatibility and resistance to particular pests and diseases. However, the need for resistance to phylloxera has been the primary driver of rootstock breeding and selection (Granett et al., 2001).

The introduction of the phylloxera resistant rootstocks has had some adverse effects; it has been accompanied by widespread vine losses in many regions, due to infection of trunks and roots by fungal pathogens. Vines planted on American and hybrid rootstocks seem to be more susceptible to some diseases than *V. vinifera* cultivars (Wallace et al., 2003). For example, Eskalen et al (2001) reported that Petri disease was not a serious threat to Californian vineyards prior to the 1990s when grafted vines were introduced, even though the fungi responsible (*Phaeomoniella chlamydospora* and *Phaeoacremonium* species) were first recorded in the 1900s. Petri disease, which causes a young vine decline, became prevalent only after the introduction of the grafted rootstock varieties currently in use (e.g. 101-14, 5C, 3309 and 110R). Above ground symptoms of Petri disease are similar to those of Cylindrocarpon black foot (Eskalen et al., 2001) whose prevalence in young vines has also been anecdotally noted to increase with the use of phylloxera resistant rootstocks. The same may be true for other root-infecting diseases, for example, wilt caused by *Fusarium oxysporum*.

1.1.3 Grapevine root diseases

Cylindrocarpon destructans and *F. oxysporum* are two common grapevine root pathogens (Edwards et al., 2007). When investigating decline of young vines in Sicily, Grasso (1984)

isolated both species from rotting roots. Other fungal grapevine root rot pathogens have included *Pythium* spp., *Phytophthora* spp. (Marais, 1980) and *Armillaria mellea* (Pertot et al., 2008).

1.1.3.1 *Cylindrocarpon destructans* and black foot disease

Black foot disease is a serious and relatively new threat to vineyards around the world, including those in South Africa (Halleen et al., 2003; Fourie & Halleen, 2004), Australia (Edwards & Pascoe, 2004; Whitelaw-Weckert et al., 2007), the Mediterranean region (Rego et al., 2000; Aroca et al., 2006; Rumbos et al., 2008), North and South America (Scheck et al., 1998a; Scheck et al., 1998b; Gubler et al., 2004; Halleen et al., 2006a), and New Zealand (Bleach et al., 2007). *Cylindrocarpon* species are commonly found in soil, and are typically associated with the roots of herbaceous woody plants where they may exist as saprophytes or weak pathogens (Brayford, 1992; Halleen et al., 2006a). Black foot disease of grapevines is caused by several *Cylindrocarpon* species including *C. destructans*, *C. macrodidymum*, and *C. liriodendri* (Halleen et al., 2007) which were almost uniformly isolated in a survey of symptomatic grapevines in New Zealand (Bleach et al., 2007). In North America, the disease is mainly caused by *C. destructans*, which was reported by Gubler et al (2004) to be "by far the most serious pathogen of those recently discovered". The disease will often cause decline of the grapevines in the year following their planting and is known to kill both young and mature vines; primarily it is the young vines, between the ages of 2 and 8 years, that are killed (Larignon, 1998; Halleen et al., 2004).

Symptoms of Cylindrocarpon black foot disease may be different for nursery and vineyard infections. In nurseries, and newly established vineyards, the young vines may be chlorotic and stunted, often dying during the hot dry conditions of their first summer. In established vineyards (> 2 years) however, the infection causes a more gradual decline as plants age, and death may take up to a year or more (Gubler et al., 2004; Halleen et al., 2006), indicating that plant vigour may play a role in tolerance to the disease (Gubler et al., 2004).

Plants with black foot disease typically have "reduced vigour, poor growth, small trunks, abnormally shortened internodes, uneven wood maturity, sparse foliage, and small leaves, and interveinal chlorosis" (Halleen et al., 2006a) (Figure 1.2, A), which precedes necrosis and premature defoliation (Scheck et al., 1998a). Budding and shoot formation are typically absent or delayed (Halleen et al., 2007) after winter dormancy, and shoots may dry out and die during summer (Halleen et al., 2004; Halleen et al., 2006). Those that remain may perish the following winter (Halleen et al., 2006a), or be noticeably stunted (Petit & Gubler, 2005).

Feeder roots and root hairs may have sunken necrotic lesions (Scheck et al., 1998a; Halleen et al., 2006a), and total root biomass may be reduced due to fewer feeder roots or root hairs. To compensate for the loss of functional roots at the basal crown, a second crown of roots may develop, with roots emerging from the upper level of the rootstock and running parallel to the soil surface (Fourie et al., 2000; Halleen et al., 2007) (Figure 1.2, B). Root damage results in reduced water and mineral acquisition and eventually plant dieback (Petit & Gubler, 2006).

Internal symptoms of black foot disease include dark brown to black vascular streaking caused by the blockage of most of the xylem vessels, and sometimes functional phloem, with thick-walled tyloses or gum, as well as internal necrosis often extending from the bark to the pith which is compacted and discoloured (Larignon, 1998; Halleen et al., 2006a). Trunks of infected vines are often discoloured below ground level, sometimes at ground level and up to 15 cm above ground level (Figure 1.2, C). However, hyphae are not usually located in the xylem vessels or functional phloem, often being found in the ray cells (a convenient supply of metabolisable carbon for the pathogen) of the phloem and in the younger xylem (Halleen et al., 2006a).

Figure 1.2: Symptoms of black foot disease: stunting and chlorosis in vines (A), development of a second root crown (B) and dark staining of the stem base (C).

Preliminary work has indicated that the different grapevine rootstocks used in New Zealand exhibit different susceptibilities/tolerances to Cylindrocarpon black foot disease (Harvey & Jaspers, 2006). Harvey and Jaspers (2006) performed two pathogenicity trials (2005/2006) from which they reported some trends in resistance (Table 1.1). Overall rankings were based on the recovery of *Cylindrocarpon* species from plant tissue, with a ranking of 1 being the most tolerant and 14 being the most susceptible. Prior to this very little information was available regarding rootstock susceptibility (Gubler et al., 2004). Eskalen et al (2001)

suggested that differences in susceptibilities may be due to the differing ability of *Cylindrocarpon* species to move in the vascular tissue of rootstocks.

Table 1.1: Results table from the PLANTwise Services Ltd report to the New Zealand Winegrowers research council (Harvey & Jaspers, 2006). Stem base isolation incidence scores from the root-soak inoculated grapevine treatments, with lowest ranks being the most tolerant.

	Trial 1: 2005	**Trial 2: 2006**
Variety	**Ranking**	**Ranking**
Rupestris St. George	1	-
Riparia Gloire	2	4
R 140	3	1
3309	4	3
3306	5=	7
420 A	5=	6
Schwarzmann	5=	11
SO4 (5C)	8=	2
Paulsen 1103	8=	9
Gravsac	8=	-
T 5BB	8=	10
101-14	12	8
R 110	13	-
Fercal	14	5

1.1.3.2 *Fusarium oxysporum* and root rot /wilt

Fusarium oxysporum is a fungal species common in agricultural soils around the world and closely associated with plant roots (Gordon & Martyn, 1997). Granett et al (1998) reported that *F. oxysporum* is relatively common in all root systems. Non-pathogenic strains are effective colonisers of the root cortex and it is thought that pathogenic strains evolved from these originally non-pathogenic forms. Gordon and Martyn (1997) postulated that over time, endophytic associations may have become latent infections and that in wilt diseases, these pathogens resulted from the selection of forms with shorter latent periods. These *F. oxysporum* infections can reduce the water conducting capacity of the plant, causing wilting (Beckman & Roberts, 1995).

Fusarium oxysporum is frequently isolated from grapevine trunks when surface sterilised tissue fragments are plated out onto agar. These *F. oxysporum* isolates and other *Fusarium* species are some of the most common plate contaminants in studies looking at the pathogenicity of other fungi in grapevine trunk bases (pers. comm. C. Bleach, 2007).

Fusarium oxysporum is also frequently isolated from necrotic grapevine roots in phylloxera infested soils, and was reported by Edwards et al (2007) to be the most virulent fungal pathogen associated with such root necrosis. It appears to enter the roots via wounds associated with the feeding sites of phylloxera and the subsequent root rot can be severe enough to cause portions of the root system to become detached from the vine, resulting in vine death (Omer et al., 1999). However, although a known cause of root rots and wilts in other plants (de Andrade, 1993), and present in the rotting roots of declining grapevines (Grasso, 1984), *F. oxysporum* was not regarded as being the causal agent in the past. In California, Granett et al., (1998) reported that while *Fusarium* species were common in vineyard soils, they were rarely invasive unless roots were damaged or phylloxera galled. However, Highet and Nair (1995) confirmed the pathogenicity of *F. oxysporum* with isolates taken from the roots of declining grapevines that were used to infect grapevines in glasshouse trials. Fungal hyphae could also be seen in sections of roots under a transmission electron microscope within and between root cortex cells. There was limited disintegration of cortical cells and loss of cell contents, however, the fungus did not pierce the endodermis. van Coller et al (2005) also confirmed the pathogenicity of *Fusarium* species including *F. oxysporum* on grapevines grafted onto 99 Richter and 101-14 Mgt.

In Brazil, *F. oxysporum* f.sp. *herbemontis* was considered by de Andrade et al (1993; 1995) to be the most important pathogen of grapevine cultivars since it caused significant plant death. Their infection experiments showed that the wilt caused by this pathogen was largely responsible for decline and mortality of young grapevines, since inoculated cuttings failed to establish and decline occurred in older inoculated plants. Root symptoms included root decay with brown vascular discoloration (de Andrade et al., 1995), pink discolouration in the cortical and vascular tissues, tyloses in the xylem and rotting of epidermal tissues of the roots of infected plants (Highet & Nair, 1995). Highly infected roots had a tendency to die, and this loss of roots may be partly responsible for vine decline (Granett et al., 1998). Foliar symptoms have also been reported. They included yellow spots which expanded into streaks before leaves died and dropped and were reported by de Andrade et al (1995) and also by Grasso (1984) in Sicily. In Australia, Highet and Nair (1995) also found above ground symptoms included delayed, weak shoot growth and low fruit yields.

While the resistances, of rootstocks commonly used in New Zealand, to *F. oxysporum* are not well established, de Andrade et al (1993) observed differences in the resistance of grapevine rootstocks to *F. oxysporum* f.sp. *herbemontis* infection in Brazil. Hybrids of *Vitis riparia* x *Vitis berlandieri* were highly susceptible, but some degree of resistance was noted in *Vitis*

berlandieri x *Vitis rupestris* hybrids. Rankings (1-4) were based on disease symptoms: absence of symptoms on roots (1), vascular discolouration of roots 10 cm from the shoot base (2), vascular discolouration of the roots 5 cm from the shoot base (3), and vascular discoloration of roots at the shoot base (4) (Table 1.2).

Table 1.2: Results table adapted from de Andrade et al (1995). The reported resistance of grapevine rootstock varieties to *F. oxysporum* f.sp. *herbemontis* in Brazil, in inoculated pots and naturally infected soils in field conditions. Ranking 1-4, with 1 being most resistant. Data from the 1989-1991 period.

1.1.4 Economic implications of grapevine root diseases

Soil-borne diseases are a major concern for nurseries that supply grafted grapevine plants. In New Zealand, 30-40% of grafted grapevine plants fail every year (about 5,000,000 plants worth about NZ$25 M), and some of this is thought to be caused by grapevine root disease, especially black foot. Since mature mother-vines which are used to produce the cuttings for grafting are also affected, there may be additional production losses of NZ$3 M per year, due to re-establishment costs (pers. comm. B. Corban, 2006). When vineyards are affected by these root diseases, there are additional losses from reduced yield and inferior fruit quality (Gugino & Travis, 2003). Diseased plants must be removed and it is the subsequent replanting costs that cause most of the high economic losses (Petit & Gubler, 2005). The economic impact that comes from needing to replant infected young vineyards is felt most in major production regions. For example, California has experienced severe economic losses associated with such replantings and the associated downtime. Sometimes only the weakest vines are replaced but growers have been known to replant substantial areas where the young vines have been growing very slowly (Scheck et al., 1998a).

1.1.5 Control of grapevine root diseases

Very little is known about managing *F. oxysporum* infections of grapevines, particularly in New Zealand. However, since *Fusarium* spp. like *F. oxysporum* are soil-borne and able to multiply rapidly, leading to high levels of inoculum and root disease, management strategies need to focus on reducing *Fusarium* populations in both plant and soil (van Coller et al., 2005). Since both *F. oxysporum* and *C. destructans* are (primarily) soil-borne root pathogens of grapevines, one would expect control measures to be similar. Control of Cylindrocarpon black foot currently focuses on eliminating inoculum from the soil and on preventing infection of young plants, either through chemical or biological treatments or with hot water treatment. Management processes for young vines, which primarily focus on avoiding plant stress have also been successful in the control of other vine decline diseases (Gubler et al., 2004).

Inoculum of soil-borne diseases may build up over time if grapevine nurseries and vineyards use the same soil repeatedly, as has been reported with black foot disease increase (Halleen et al., 2003; Halleen et al., 2004). Many soil-borne pathogens can survive as saprophytes in soil. The ability of *Cylindrocarpon* species and *F. oxysporum* to produce chlamydospores also allows them to survive in the soil environment without a host for significant lengths of time. In light of this, the standard two year rotation system employed by some propagation nurseries may need to be reviewed (Toussoun & Nelson, 1968; Scheck et al., 1998b). Fumigation of the soil with methyl bromide prior to planting has been successful in controlling black foot disease but the use of such fumigants is being phased out (Petit & Gubler, 2006).

Using plant material sourced from reputable nurseries is a good pre-emptive disease management strategy (Stamp, 2001). In some nurseries, only 5% of vines carry the pathogen and have symptoms, while in others it may be as high as 40% (Gubler et al., 2004). Additionally, the basal ends of cuttings provide one of the highest risk areas in terms of infection and so the focus should be on protecting them. Various treatments can be used prior to planting, but, whether chemical or biological in nature, their effectiveness has not been convincing. However, it may still be worth examining the effectiveness of dipping the basal end of cuttings in a fungicide or biocontrol agent (Halleen et al., 2003). *In vitro* studies indicated that benomyl, flusilazole and prochloraz manganese chloride fungicides, used alone or with a wax formulation, had the most promise (pers. comm. F. Halleen, 2006). Due to there being several fungi involved in causing Cylindrocarpon black foot disease, control is more complicated, on account of different fungi having significantly different epidemiology and

susceptibilities to fungicides (Halleen et al., 2004). Hot water treatment of dormant nursery grapevines has also shown strong potential (Halleen et al., 2006a), with 30 minutes at 50°C seemingly sufficient (pers. comm. F. Halleen, 2006), but when grown in cool climates, 47°C and 48°C were equally effective (pers. comm. C. Bleach, 2007).

There is no curative strategy for declining grapevines in vineyards and currently no fungicides are registered for the control of Cylindrocarpon black foot in vineyards (Halleen et al., 2006a). However, recently, the efficacy of several fungicides was tested *in vitro* and in the field to protect young vines preplanting, with some success (pers. comm. C. Bleach, 2008). *Trichoderma* treatments were also shown to reduce the incidence of *Cylindrocarpon* species and greatly improve root development in young vines (Fourie & Halleen, 2001). The mycorrhizal fungus *Glomus intraradices* has also been reported to protect against infection by *C. destructans* if applied early (Gubler et al., 2004; Halleen et al., 2006). Arbuscular mycorrhizae (AM) are able to increase plant resistance to abiotic and biotic stresses and consequently, colonised plants are less susceptible to disease, due to an improved nutritional status (especially an enhanced uptake of phosphorous), or because the AM occupies potential infection sites. Alternatively, the fungus may suppress the pathogen by stimulating the growth of antagonistic microorganisms in the rhizosphere (Petit & Gubler, 2006).

Soil health is also an important factor in suppression of root diseases because soil can contain significant populations of bacteria and other microorganisms that are capable of killing or suppressing plant pathogenic fungi (Broadbent et al., 1971; Sturz et al., 1997). Changes in the soil organic matter content, due to composted soil amendments can alter the populations of bacteria and actinomycetes (Hoitink & Boehm, 1999). Some of these microorganisms could be antagonistic towards pathogens like *C. destructans* or *F. oxysporum*. For example, it has been established that some bacteria isolated from vineyard soil are able to antagonise both these grapevine root pathogens (*in vitro*) possibly through the production of diffusible antibiotics (Whitelaw-Weckert, 2004). Soils with higher organic matter content and higher microbial activity, with significant populations of soil bacteria tend to display high fungistatic activity (Sturz et al., 1997). Composts have been demonstrated to reduce *C. destructans* populations in soils due to the antagonism of microorganisms associated with these soils (Gugino & Travis, 2003). High levels of microbial activity in soils can also result in competition effects that could reduce pathogen activity and survival, helping to reduce the pressure of a high pathogen inoculum and even resulting in disease suppression (Sturz et al., 1997). Therefore, managing soil microbial communities could provide a means of

diminishing the activity of soil-borne plant pathogens and, by doing so, improve plant health (Mazzola, 2004).

1.1.6 Abiotic factors influencing disease development

Abiotic stresses have been demonstrated to influence plant disease development in several pathogen-host systems (Schoeneweiss, 1981). With respect to Cylindrocarpon black foot, growers have focussed on eliminating or preventing the stresses that predispose the plants to disease (Halleen et al., 2006a). Malnutrition or an excess of nutrients, poor water drainage, soil compaction, heavy crop loads on young plants, planting of vines in poorly prepared soil and improper plant holes are stresses that can impair root development, favour disease development and worsen disease severity (Larignon, 1998; Fourie et al., 2000; Fourie & Halleen, 2001; Halleen et al., 2004). Planting in heavy and wet soils or on susceptible rootstocks may also increase losses to this disease (Gubler et al., 2004). During summer, the high temperatures also mean that infected vines (with their compromised root systems) are not able to obtain the water needed to compensate for the increased transpiration rate (Halleen et al., 2006a).

A potential cause of stress is the use of canopy thinning which is a standard practice in cool climate viticultural regions. It involves the removal of leaves from around fruit clusters and/or shoot trimming several times over the growing season thereby reducing grapevine carbohydrate stores and causing stress (Chanishvili et al., 2005). However, controlled and properly timed partial defoliation of grapevines has been demonstrated to confer benefits such as increased yields, improved bud fertility (Hunter et al., 1995), higher numbers of fine and extension roots that improve the ability of the plants to penetrate deeper soil (Hunter et al., 1994; Hunter et al., 1995). Grapevine carbohydrate reserves are also significantly influenced by environmental and management factors, including seasonal weather (Bains et al., 1981), crop load (Balasubrahmanyam et al., 1978), pests, diseases (Ryan et al., 2000) and methods of vine pruning and training (Koblet et al., 1993; Schultz et al., 2000).

The dominant forms of carbohydrate in grapevines are starch and soluble sugars (sucrose, glucose, fructose and myo-inositol) (Hamman et al., 1996), although others including raffinose and stachyose are present in less significant quantities (Loescher et al., 1990). In grapevines, both the trunk and roots are storage sites for carbohydrate during dormancy (Wood, 2000), although roots are the primary storage organs (Loescher et al., 1990). They provide the carbohydrates for new growth in spring, including shoots and inflorescences (Scholefield et al., 1978).

With defoliation, carbohydrate production and transport by the leaves is reduced, decreasing its availability to sinks. “The number, severity and timing (within growing season and number of consecutive years) of defoliations and the physiological condition of the tree when defoliation occurs contribute to the outcome” (Horsley et al., 2000). Defoliation alters the carbohydrate physiology of mature grapevines (Bennett, 2002) and if too severe can cause stress. For example, in grapevines, incorrect timing or excessive leaf removal can result in unwanted outcomes, including greatly reduced yields (the consequence of fewer flowers per inflorescence and fewer inflorescences per grapevine) (Bennett, 2002), reduced bud fertility and delayed ripening (Hunter et al., 1995), reduced berry weight (Kliewer, 1970), as well as significantly reduced cane, trunk, root and total vine dry weights.

1.2 THE RHIZOSPHERE

1.2.1 Definitions

The rhizosphere is the zone of soil close enough to living plant roots to be directly influenced by the root activity and exudates (Hinsinger et al., 2005). Pinton et al. (2000) defined it as “the field of action or influence of a root”. Similarly Darrah (1993) defined it as a “zone of soil surrounding the root which is affected by it”.

1.2.2 Rhizodeposits

The interface between plant roots and the immediate soil layer is affected by the compounds (rhizodeposits) released into that area by the plant. These include exudates, sloughed off cells and decaying root material (Gregory, 2006). Of the carbon that plants assimilate via photosynthesis, between 10% and 30% is transferred to the rhizosphere as rhizodeposits (Morgan et al., 2005). Marschner (1995) put this figure at between 5 and 21% of all photosynthetically fixed carbon. These deposits into the rhizosphere alter its chemical and physical characteristics and stimulate the growth of soil microorganisms which are able to utilise them (Gregory, 2006).

Root exudates are generally grouped into low molecular weight compounds such as simple polysaccharides, amino acids, amides, organic acids, phenolics and some secondary metabolites, and high molecular weight compounds such as flavonoids, peptides, proteins (e.g. enzymes), fatty acids, growth regulators, nucleotides, tannins, carbohydrates, steroids, terpenoids, alkaloids, polyacetylenes, vitamins and mucilage (Curl & Truelove, 1986; Bertin et al., 2003; Walker et al., 2003; Gregory, 2006). Root exudates also contain lesser amounts of ions (e.g. H^+ and inorganic ions), oxygen and water (Bertin et al., 2003). Some of these compounds are water soluble and leak from the root, some are secretions that depend on

metabolic processes for their release, and some are released when cells autolyse and still others are released as gases (Lynch & Whipps, 1990).

The organic materials released from the roots are a key source of carbon and energy for soil microorganisms (Whipps, 2001), with some exudates even serving as specific signals to soil microorganisms (Grayston et al., 1995). All root exudates in the rhizosphere are believed to "stimulate or inhibit microbial populations and their activities" (Morgan et al., 2005). Generally, saprotrophic and biotrophic organisms in the rhizosphere grow in response to the presence of plant derived rhizodeposits, but it is also possible for soil-borne plant pathogens to do so. This rhizodeposition can encourage pathogen growth towards the roots, resulting in infection (Whipps, 2001). Consequently, the rhizosphere community structure is influenced by the quality and quantity of organic substances released from plant roots (Bazin et al., 1990). Biocidal compounds released by roots may also significantly influence the composition of rhizosphere microbial communities (Rumberger & Marschner, 2003). The composition, size and activity of soil microorganism populations can influence plant development and growth, alter nutrient dynamics and modify plant susceptibility to disease and abiotic stress (Morgan et al., 2005).

Root exudates are released by living root hairs, as well as by actively growing primary and secondary roots (Bertin et al., 2003). However, the pattern of exudation is not homogenous along the root axis (Walker et al., 2003). Along the root axis there are qualitative and quantitative differences in root exudation, meaning that different root zones can have distinct bacterial communities (Yang & Crowley, 2000). Additionally, different types of exudates are released from different parts of the root system. Near the root tip recently assimilated carbon is secreted as relatively simple molecules, while more complex compounds are released from the more mature root zones, mostly in the form of sloughed off root cells (Jaeger et al., 1999; Gregory, 2006).

Plant developmental phase can also influence the quantitative and qualitative characteristics of root exudates (Marschner et al., 2002; Morgan et al., 2005), with root exudation typically decreasing as plants age. However, abiotic stresses frequently result in increased root exudation rates (Bertin et al., 2003), likely due to biotic and abiotic plant stresses which compromise cell membranes, causing increased permeability, and so diffusional loss of carbon compounds into the rhizosphere, thereby increasing microbial growth (Farrar et al., 2003). Soil type can also influence root exudation because it affects carbon availability and root-soil adhesion (Gregory, 2006). The rhizosphere bacteria themselves can produce metabolites that induce root exudation responses (Sturz & Christie, 2003). For example, in

one study of perennial ryegrass, when the plants were not inoculated with a selection of bacteria or fungi (including *Penicillium* spp., *Aspergillus* spp., *Fusarium* spp., *Mucor* sp., *Cladosporium* sp., and *Pseudomonas* sp.), only 1% of an assimilated label was detected in root exudates, but when plants were inoculated the percentage exuded increased to 3-34% (Meharg & Killham, 1995).

Vigour can also influence the quantity and quality of root exudation, and therefore soil microbial populations, since soil microbial biomass increases with rhizodeposition (Merckx et al., 1985; 1987). More vigorous plants are likely to have greater root exudation than less vigorous plants because shoot development and root growth rate significantly influence rhizodeposition (Kuzyakov, 2002). Farrar et al (2003) stated that the flow of carbon into the rhizosphere is dependent upon the amount of root tissue, as well as how much exudate is released from individual root apices and root lengths. Root exudation can also differ between plant species or even cultivars of the same species (Grayston et al., 1998), with differences being both qualitative and quantitative (Badri & Vivanco, 2009).

1.2.3 Plant selection of microorganisms

It is still not known conclusively if plants can "selectively favour microbial communities most beneficial to themselves" (Frey-Klett et al., 2005), but it has often been assumed that plant communities control the below-ground soil microorganism diversity. Although little is known about how plant species composition and diversity actually influence the rhizosphere community composition (Sharma et al., 2005), some results indicate that the composition of the most dominant bacterial populations in the rhizosphere is strongly driven by plant species and their processes (Kowalchuk et al., 2002). The release of particular root exudates and leachates influences which specific bacterial communities are activated and sustained in a plant's rhizosphere (Sturz & Christie, 2003). Jaeger et al (1999) also concluded that the structural and functional diversity of rhizosphere populations is in part a result of plant species and the associated differences in root exudation.

Ibekwe and Kennedy (1999) reported that rhizosphere microbial communities varied with the host plant species (wheat, barley, pea, jointed goat-grass and downy brome). Smalla et al (2001) used DGGE to show plant specific shifts in the relative abundance of bacterial populations in the rhizosphere of potato, strawberry and oilseed rape. Some *Nocardia* populations for example, were specific to strawberry. Germida et al (1998) concluded that canola and wheat plants must have different effects on the composition and diversity of their root associated bacteria because bacterial communities of canola and wheat differed when plants were grown at the same site, with there being significantly more bacilli in the wheat

rhizoplane than the canola. Rhizosphere bacterial communities can also differ between cultivars of the same species. For example, Fromin et al (2001) reported that the genetic structure of *Pseudomonas brassicacearum* rhizosphere populations was influenced by *Arabidopsis thaliana* genotype. The *P. brassicacearum* bacterial populations were isolated from the rhizospheres of *A. thaliana* Wassilewskija (WS) and *A. thaliana* Columbia (COL), as well as an *A. thaliana* mutant and a genetically distinct plant species (wheat). However, the genetic structure of the *P. brassicacearum* populations varied between plant species and between *A. thaliana* ecotypes.

Allelochemicals released by plant roots can inhibit the growth of only one microorganism, or several species of microorganisms (Bertin et al., 2003). Some of the root exudates, or compounds produced by other soil flora, may act as "messengers", causing the initiation of root-root, root-microbe and root-faunal interactions (Walker et al., 2003). Bauer and Teplitski (2001) noted that exudates have the potential to repress or simulate microbial signalling in the rhizosphere, and suggested that plants can actively stimulate specific microbial populations in the rhizosphere. These compounds include signal mimics, signal blockers and signal-degrading enzymes (Gregory, 2006).

1.2.4 Microbial diversity in the rhizosphere

The microbial composition of the rhizosphere is significantly different from that of the bulk soil, being "a hot spot for microbial colonization and activity" (Sharma et al., 2005). The biological, biochemical, chemical and physical processes in the rhizosphere are responsible for the differences between rhizosphere and bulk soils. Root growth, respiration, rhizodeposition, and water and nutrient uptake are some of the driving forces for the processes above. Differences between the bulk and rhizosphere soils are also due to the activity of microorganisms stimulated in the rhizosphere by root exudates, microorganisms which can harm or benefit the plant (Hinsinger et al., 2005). The rhizosphere will thus contain populations of bacteria, actinomycetes, fungi, protozoa, viruses and nematodes significantly different to those of the bulk soil (Gregory, 2006), with the rhizosphere typically having a lower diversity of soil microorganisms than the bulk soil (Morgan et al., 2005). Bacterial and fungal communities can also be significantly different within the rhizosphere; varying between the root tip and the more mature parts of the root system (Gregory, 2006).

1.2.5 Disease suppression

In some soils, disease development is minimal, even when a pathogen and its susceptible host are present. These soils are termed 'suppressive', and the suppression can be general or

specific. General suppression of a soil-borne pathogen is related to the total amount of microbial activity in a soil, and cannot be attributed to a specific microorganism, or group of microorganisms while specific suppression can be attributed to a specific microorganism, or group of microorganisms that are inhibitory or antagonistic to specific pathogens (Mazzola, 2002).

Pathogen or disease suppressive soils are able to limit the survival or growth of soil-borne bacterial or fungal pathogens. Biotic and /or abiotic factors may also influence a soil's suppressiveness to a particular plant pathogen, although soil microorganisms are thought to be the most influential factor (Mazzola, 2002; Garbeva et al., 2004). For example, van Os and van Ginkel (2001), concluded that high microbial biomass and activity suppressed *Pythium* root rot in bulbous Iris. Workneh and van Bruggen (1994) also observed a link between soil microbial diversity and the suppression of root disease. They reported that the total numbers and diversity of actinomycetes and numbers of fluorescent Pseudomonads, as well as the proportions of cellulolytic actinomycetes and chitinolytic fungi, were all positively correlated with the suppression of corky root of tomato caused by *Pyrenochaeta lycopersici*. In a study on suppression of watermelon root rot caused by *F. oxysporum* f. sp. *niveum*, suppressive soils had larger populations of actinomycetes, fluorescent Pseudomonads and total bacteria than conducive soils (Larkin et al., 1993a).

Disease suppression can also be specific. For example, several *Pseudomonas* species have been shown to have effective biological control capabilities against the soil-borne fungus, *Gaeumannomyces graminis* var. *tritici* (Ggt), the causal agent of take-all disease of wheat. With successive wheat monoculture, the proportion of pathogen antagonistic bacteria in the rhizosphere has been shown to increase (Cook & Rovira, 1976), resulting in take-all decline. This is a natural phenomenon observed in the field whereby the disease severity of Ggt decreases over time. It is attributed to the selection of populations of fluorescent Pseudomonads in these soils and on wheat roots (Sanguin et al., 2008). For example, Chapon et al (2002) isolated a bacterial strain *P. fluorescens* Pf29Arp, from a suppressive soil which was capable of antagonising this pathogen and reducing lesion size. Take-all decline is believed to be attributed to the ability of these bacteria to produce metabolites like 2,4-diacetylphloroglucinol (2,4-DAPG) that are able to suppress the pathogen (Raaijmakers & Weller, 1998).

1.2.6 Biocontrol of plant pathogens by rhizosphere bacteria

Rhizosphere bacteria are able to inhibit plant pathogenic fungi or bacteria through: the production of anti-fungal metabolites, niche exclusion, competition for nutrients, and/or

induced systemic resistance (ISR) (Bloemberg & Lugtenberg, 2001). However, the rhizosphere bacteria involved in biological control may exhibit several of these characteristics (Whipps, 2001). The following sections will outline how bacteria can potentially interact with plant pathogenic fungi to restrict their development.

1.2.6.1 Competition

Competition for colonisation sites on the host, and for nutrients and minerals can result in the biological suppression of pathogenic fungi in the rhizosphere (Morgan et al., 2005). For example, some rhizosphere bacteria produce siderophores, which are iron chelating compounds with a high affinity for ferric iron (Fe^{3+}) (Whipps, 2001), that allow the bacteria to compete for iron in the soil environment under iron-limiting conditions. Siderophores are produced by nearly all bacteria and fungi when iron is scarce (Buyer & Sikora, 1990), but fungal siderophores are thought to have a lower affinity for iron (Compant et al., 2005) than those of bacteria. Some siderophores can only be utilized by the bacteria that produce them while others can be used by a range of bacteria (Frey-Klett et al., 2005). Bacterial siderophores which can scavenge and sequester the iron in the rhizosphere, then transport it into the bacterial cells, make it unavailable to pathogenic fungi, and so restrict their development (Chet et al., 1990; Whipps, 2001). This competition associated with the production of siderophores can result in the displacement of the phytopathogen (Chet et al., 1990). For example, siderophores, most commonly from *Pseudomonas* spp., have been shown to have a suppressive effect on several plant pathogens including: *Rhizoctonia solani*, *Fusarium oxysporum*, *Pythium aphanidermatum* and *Phytophthora parasitica* (Whipps, 2001; Dianez et al., 2006).

1.2.6.2 Antibiosis

Antibiosis is the inhibition of the fungal pathogen through the production of antimicrobial metabolites (excluding metal chelators and enzymes) by some soil bacteria. These compounds can inhibit or slow fungal growth and have been implicated in disease suppression (Whipps, 2001). Fluorescent Pseudomonads in particular have been found to produce many antifungal compounds that have a broad spectrum of activity against phytopathogens (Dwivedi & Johri, 2003; Compant et al., 2005). The main classes of antibiosis-related compounds produced by Pseudomonads are: phloroglucinols including DAPG, phenazines, pyoluteorin, pyrrolnitrin, lipopeptides and hydrogen cyanide (HCN) (Haas & Keel, 2003). *Bacillus*, *Streptomyces* and *Stenotrophomonas* species are also key players in soil antibiosis, producing compounds such as kanosamine, oligomycin A, zwittermicin A and xanthobaccin (Compant et al., 2005).

Pseudomonads and other rhizosphere bacteria can produce HCN which inhibits the development of some root diseases (Schippers et al., 1990). For example, HCN produced by *P. fluorescens* CHAO can suppress black root rot of tobacco caused by the fungal pathogen *Thielaviopsis basicola* (Haas & Keel, 2003). Production of HCN however, depends largely on the amino acid composition of the substrate, e.g. root exudates. Glycine and proline are key stimulators of HCN production, but light intensity, soil water potential and the availability of ferric iron and inorganic phosphate are also important. HCN directly inhibits the activities of root pathogens but may also be involved in the induction of plant resistance responses (Schippers et al., 1990).

1.2.6.3 Parasitism

Some rhizosphere bacteria are able to parasitize plant pathogenic fungi (Whipps, 2001). Bacteria, especially actinomycetes, are capable of parasitizing and degrading fungal spores and some bacteria can disrupt the attachment of hyphae to plant root cells through to the complete lysis and degradation of the fungal hyphae. A range of enzymes, including cell-wall degrading hydrolytic enzymes may be responsible, including cellulases, glucanases (β-1,3 β-1,4 and β-1,6), chitinases, laminarinases, pectinases and proteases (Whipps, 2001; Krechel et al., 2002).

1.2.6.4 Induced resistance

Some microorganisms, particularly non-pathogenic rhizosphere bacteria, may also be able to induce systemic plant resistance (ISR). ISR is a state of heightened defence-related preparedness in plants, which can be expressed locally or systemically and provides protection against a broad spectrum of phytopathogens including fungi, bacteria and viruses. It has been reported in plant species including *Arabidopsis*, bean, carnation, cucumber, radish, tomato and tobacco (van Loon et al., 1998). The rhizosphere bacteria most commonly involved in ISR are *Pseudomonas* species (Bloemberg & Lugtenberg, 2001). Rhizosphere bacteria could induce ISR via membrane lipopolysaccharides, siderophores, volatile compounds, salicylic acid and/or flagellation factors. Some of these elements can enhance the production of plant defence chemicals when the plant is challenged by biotic or abiotic stresses (Compant et al., 2005).

Plant roots displaying ISR may show strengthening of epidermal and cortical cell walls, deposition of barriers made from compounds such as lignin and phenolics, elevated enzyme levels (e.g. chitinases, peroxidase, polyphenol oxidase and or chalcone synthase) (Compant et al., 2005), enhanced phytoalexin production and increased expression of stress-associated genes (Whipps, 2001). Defence related genes may also be activated, including those

responsible for the production of jasmonate, peroxidase and the synthesis of phytoalexins (Sturz & Christie, 2003). Throughout the process the inducing rhizosphere bacteria and the challenging pathogen remain spatially separated. For example, a strain of *Pseudomonas fluorescens* and *F. oxysporum* f.sp. *dianthi* were applied to different parts of a carnation plant; the roots were treated with *Pseudomonas fluorescens*, and the fungal pathogen was introduced a week later via a stem wound. ISR caused the *Fusarium* wilt to be suppressed (van Loon et al., 1998).

1.2.6.5 Plant growth promotion

Plant growth-promoting rhizobacteria (PGPR) often have flagella, and may be directed to root surfaces by chemical attractants in the root exudates (Compant et al., 2005). Their growth-promoting activity may minimise the negative effects of minor pathogens (Whipps, 2001; Dwivedi & Johri, 2003). These rhizosphere bacteria stimulate plant growth through the secretion of phytohormones like auxins, cytokinins and gibberellins (Bloemberg & Lugtenberg, 2001), for example, many produce indole-3-acetic acid (IAA). Others promote plant growth by making nutrients more readily available, for example, by solubilising inorganic phosphate or by N_2 fixation (Frey-Klett et al., 2005; Morgan et al., 2005). Still others may regulate ethylene production in roots, decrease heavy metal toxicity (Whipps, 2001) or stimulate the growth of beneficial ectomycorrhizal fungi. These mycorrhizal associations can enhance plant resistance to soil-borne pathogens by producing antibiotic substances or, due to their hyphal mantle surrounding the plant roots mechanically exclude the pathogens from the plant roots (Frey-Klett et al., 2005).

1.2.7 Abiotic factors influencing rhizosphere microorganisms

Many physical factors influence the survival and activity of microorganisms in the soil. These include temperature, pH, nutrient availability, light intensity, soil moisture content, carbon dioxide concentration and oxygen availability. The physical structure of the soil, root growth, water dynamics and microbial dynamics all interact, and changes in soil structure directly affect moisture levels and gas diffusion to the microbial components of the rhizosphere (Young, 1998). Soil structure and water potential also influence the spatial exploration of the rhizosphere by root pathogens (Hinsinger et al., 2005).

1.2.8 Methods used to study rhizosphere bacteria

The spatial and temporal dimensions of the rhizosphere change when different processes are taken into account, and as such, its definition is not exact. For example, when only microorganism populations and immobile nutrients (including carboxylates and extracellular

enzymes) are considered, the width of the rhizosphere could be less than a millimetre. However, its width could be tens of millimetres with respect to mobile nutrients and water, and its width could be much larger still with respect to volatile compounds and gases (e.g. carbon dioxide). Therefore, it seems that the spatial extent of the rhizosphere is dependent on the mobility of the rhizodeposits which makes distinguishing the boundary between the rhizosphere and bulk soil virtually impossible. The rhizosphere is not a small cylinder of soil around the roots with a set radius. The architecture of the rhizosphere is closely tied to that of the root system, and as such will vary between plant species, plant genotype and in response to the environment (Hinsinger et al., 2005).

Gregory (2006) stated that for much of a growing season, most of the upper 0.1 m of the soil profile can be considered as rhizosphere due to the presence of covering plants, while Bertin et al (2003) defined the rhizosphere as the zone of soil 0 – 2 mm away from the root surface, being influenced by the living roots. In many field and pot trials, rhizosphere soil has been considered to be the soil left firmly adhering to the roots after shaking (Gregory & Hinsinger, 1999; Jaillard et al., 2002; Kowalchuk et al., 2002; Sharma et al., 2005) and this will be used to define the rhizosphere soil used in this project, "soil both loosely adhering to the roots as well as soil that could be brushed or scraped off the root surface" (Kowalchuk et al., 2002).

A range of culture-dependent and culture-independent methods can be used to determine the abundance and diversity of rhizosphere bacteria. Some rhizosphere studies have used only culture-dependent methods, but this would have underestimated soil microbial diversity since only 1-10 % of all soil microorganisms are culturable (Torsvik et al., 1998). To improve estimates of the numbers and diversity of soil bacteria, culture-independent methods can be used in conjunction with culture-dependent methods (Garbeva et al., 2008). Dilution plating can determine the size of culturable bacteria populations associated with plant roots, providing insights into reasons for differences in susceptibilities between plant cultivars (Neal et al., 1973; Gilbert et al., 1994). Groups commonly cultured include the fluorescent Pseudomonads, which have high nutrient demands and can grow rapidly in this environment (Marilley & Aragno, 1999). These bacteria often have biocontrol capabilities, and have been implicated in the suppression of several soil-borne diseases (Weller, 1988). For example, Berg et al (2006) reported that *Pseudomonas* spp. was the most dominant (77%) antagonistic genus against *V. dahliae*. *Pseudomonas fluorescens* has been shown to be a potential antagonist of root-rot pathogens (including *F. oxysporum*, *Macrophomina phaseolina* and *Rhizoctonia solani*) isolated from the grapevine rhizosphere, reducing disease severity and incidence as

well as reducing root colonisation by the pathogens (Ziedan et al., 2005; Ziedan & El-Mohamedy, 2008).

Culturable *Bacillus* species have also been found to be dominant within the rhizosphere populations of several plants including chrysanthemum, barley and grassland species including *Lolium perenne* (Felske et al., 1998; Smalla et al., 2001). These and other spore forming bacteria have also been implicated in the biological control of plant pathogens (Jeon et al., 2003; J. Yang et al., 2004). For example, Yang et al (2004) observed that cucumber seedling blight and root rot caused by *Pythium* species can potentially be controlled by a strain of *Paenibacillus polymyxa* (PKB1) which reduced disease severity and increased plant yield. Spore forming genera include: *Bacillus*, *Clostridium*, *Sporolactobacillus*, *Desulfotomaculum*, *Sporosarcina* (Stolp, 1988) and *Paenibacillu*s (Bent & Chanway, 2002). These spore forming bacteria can form endospores which are resistant to heat and dry conditions, which makes them an easy group to differentiate from other bacteria at the isolation stage.

Appropriate culture-independent methods for studying the rhizosphere bacterial populations include community level physiological profiling (CLPP), phospholipid fatty acid analysis (PLFA), terminal restriction fragment polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP). As these methods rely on discriminating between DNA sequences directly amplified from soil they are not influenced by whether the organism can be cultured. SSCP and DGGE have the advantage of allowing the identification of the soil microorganism species by DNA sequencing of bands excised from the gels. They can be used to characterise bacterial communities from rhizosphere soil samples, allowing identification of dominant bacteria to the genus or species level (Kerr & Curran, 1996). SSCP is the simpler of the two techniques and can be done without specialised equipment. It is deemed to be a simple, sensitive, time efficient and inexpensive tool (Hayashi, 1991; Sunnucks et al., 2000). Another possible advantage of SSCP over DGGE is that it does not appear to be affected by heteroduplex induced ‘pseudobands’, which occur in DGGE (Ferris & Ward, 1997), and requires little or no specialised equipment (Liu et al., 1999; Sunnucks et al., 2000). Other researchers have shown that when investigating bacterial communities, by amplifying their V3-4 region of 16S rDNA, SSCP gels produced sharper, more easily differentiated bands, detecting more subtle differences than DGGE gels. Resolution was also comparable or greater in SSCP gels than in DGGE gels (Hori et al., 2006).

1.3 EXPERIMENTAL OBJECTIVES

This project aims to investigate some characteristics of grapevine rootstock rhizospheres, in which bacteria are usually the dominant flora (Anderson & Domsch, 1978). It will investigate the diversity of rhizosphere bacteria associated with selected rootstock varieties which reportedly have different levels of resistance to *C. destructans*. By assessing the biocontrol activity, of the rhizosphere bacteria isolated from the rhizospheres of the different rootstocks, *in vitro*, it will try to elucidate some of the mechanisms behind the differing susceptibilities of grapevine rootstock varieties. The experimental objectives are to determine:

1. Whether the grapevine rootstock varieties with different levels of greenhouse resistance to *C. destructans* develop different populations of rhizosphere bacteria when grown in soil, using both isolation and molecular techniques for assessment.

2. Whether bacterial isolates, representative of the rhizosphere bacterial populations of the different grapevine rootstock varieties, have physiological attributes commonly associated with biocontrol of fungal pathogens.

3. The resistance of these same grapevine rootstock varieties (grown in soil) to *C. destructans* and *F. oxysporum* infection, and assessing the importance of the latter as a pathogen of rootstocks commonly grown in New Zealand.

4. Whether partial defoliation, by leaf trimming, affects the resistance of grapevines to *C. destructans* and/or alters rhizosphere bacterial community structure.

CHAPTER 2

DIVERSITY AND FUNCTIONALITY OF GRAPEVINE RHIZOSPHERE BACTERIA

2.1 INTRODUCTION

Plant species or cultivar specific differences in rhizosphere bacterial populations are frequently attributed to differences in root exudates (Haichar et al., 2008), particularly the quantity and quality of the exudates (Jaeger et al., 1999; Marschner et al., 2004; Badri & Vivanco, 2009). The variation in exudation patterns between genotypes or cultivars of the same plant species were concluded to cause differences in the extent of bacterial root colonisation (Miller et al., 1998; Rengel, 1997; Grayston et al., 1998; Rengel et al., 1998; Badri & Vivanco, 2009). For example, wheat genotypes more tolerant of Zn deficiency released larger amounts of phytosiderophore and 2'-deoxymugineic acid than the more susceptible wheat genotypes. In Zn deficient situations, these more tolerant wheat genotypes also showed greater rhizosphere populations of fluorescent Pseudomonads than the more susceptible wheat genotypes (Rengel, 1997). Similarly, Rengel et al (1998) reported that root populations of Pseudomonads differed between wheat genotypes. In addition, wheat genotypes that were efficient in Mn uptake had greater root colonisation by Pseudomonads than those that were less efficient in Mn uptake. They concluded "it remains to be elucidated whether these crop genotypes can modify the quantitative and/or the qualitative composition of their root exudates and secretions under nutrient deficiency conditions in order to stimulate beneficial interactions with microorganisms".

The ability of soil microorganisms, like bacteria, to colonise plant roots appears to be associated with their capacity to make use of plant root exudates (Parke, 1991; Lemanceau et al., 1995), which is why different root exudates can be differentially selective in their stimulation of rhizosphere microbial communities (Chiarini et al., 1998; Garbeva et al., 2004). However, it is important to acknowledge that environmental factors can also influence the composition of rhizosphere communities (Smalla et al., 2001), but that plant and soil type are the most significant determining factors (Garbeva et al., 2004; Berg & Smalla, 2009).

The rhizosphere bacteria selected for by specific plant species may be able to suppress plant diseases caused by fungi or bacteria. However, Berg et al (2002) observed that different plant species have rhizosphere conditions that are not equally supportive of bacterial isolates capable of disease suppression. They determined that the abundance and diversity (phenotypic and genotypic) of bacterial isolates antagonistic to the soil-borne pathogen, *Verticillium*

dahliae, was plant species specific. Antagonists from the strawberry rhizosphere were predominantly *Pseudomonas putida* B (69%), while those from oilseed rape had a much greater diversity and included more Enterobacteriaceae (*Serratia*, *Pantoea*, *Enterobacter* and *Weeksella* species). *Pseudomonas putida* A and B, which displayed diversity at the subspecies level, had plant species specific clusters, indicating that they were being selected for by a particular rhizosphere. Additionally, Rengel (1997) observed that ratios of certain bacterial groups in the rhizosphere can influence the tolerance of wheat and barley plants to take-all disease caused by *Gaeumannomyces graminis*. Neal et al (1970; 1973) noted cultivar specific selection of bacterial species, whereby the rhizospheres of wheat lines more resistant to a root rot caused by *Cochliobus sativus* had smaller populations of bacteria than the susceptible lines (with fewer cellulolytic, pectinolytic and amylolytic bacteria in particular). However, there are not always obvious differences in the composition of bacterial communities between susceptible and more tolerant plants. For example, Chiarini et al (1998) found no effect of maize cultivar type on the rhizosphere microbial densities in relation to their susceptibilities to *Fusarium* species.

Rhizosphere bacteria are able to suppress plant disease by a range of different mechanisms including: the production of anti-fungal metabolites, parasitism, niche exclusion, competition for nutrients, plant growth promotion and induced systemic resistance (ISR) (Bloemberg & Lugtenberg, 2001). The most well known bacterial antagonists of soil-borne plant pathogens are species of *Pseudomonas*, *Burkholderia*, *Bacillus*, *Serratia* and Actinomycetes (Thomashow, 1996; Garbeva et al., 2004). The rhizosphere is a rich source of pathogen antagonistic bacteria, as reported by Berg et al (2005); after comparing bacterial communities from the rhizosphere, phyllosphere, endorhiza and endosphere of field grown potatoes they concluded that the rhizosphere and endorhiza were the main sources of *V. dahliae* antagonists. As Chet et al (1990) aptly phrased it, “the rhizosphere is the first-line defence for roots against attack by pathogenic fungi”.

In New Zealand, the most widely planted rootstock between 1997 and 2002 was 101-14, which accounted for 35% of vine plantings, however this decreased to 20% between 2003 and 2006, with a current favourite being Schwarzmann (Hoskins, 2008). In these experiments, rootstock varieties Riparia Gloire (*V. riparia* x *V. riparia)*, 5C (*V. riparia* x *V. berlandieri*), Schwarzmann and 101-14 (both *V. riparia* x *V. rupestris*) were used (Jackson & Schuster, 2001). As hybrids of a range of *Vitis* species and different parent vines, they encompass a substantial proportion of the significant heterogeneity within the genus (May, 1994).

In this chapter, isolation and molecular techniques (SSCP) were used to determine whether four grapevine rootstock varieties (101-14, Riparia Gloire, 5C and Schwarzmann), with different levels of greenhouse resistance to *C. destructans*, select for different populations of rhizosphere bacteria when grown in soil. Harvey and Jaspers (2006) have reported that Riparia Gloire has low susceptibility to *C. destructans*, and 101-14 moderate to high susceptibility. The susceptibilities of 5C and Schwarzmann were more variable (Table 1.1). The ability of a random selection of bacterial strains isolated from the rhizospheres of the most and least *C. destructans* susceptible rootstocks (101-14 and Riparia Gloire, respectively), to produce specific hydrolytic enzymes (protease, β-glucanase), and siderophores was assessed. In addition, the ability of the bacterial isolates to inhibit *C. destructans* in dual plate culture was also assessed.

2.2 METHODS

2.2.1 Pot experiment with different grapevine rootstocks

For this experiment, rootstock varieties Riparia Gloire, 5C, Schwarzmann, 3309 and 101-14 were used. Ten rooted cuttings of each rootstock variety were grown in individual 2.5 L pots containing a 50/50 mix of soil (Wakanui silt loam classified as a mottled immature pallic soil), sourced from the Lincoln University vineyard, and potting mix [(80% horticultural bark (grade 2): 20% pumice (grade 3, 1-4 mm)]. The potting mix was 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. Ten control pots, consisting of the same 50/50 mix of soil and potting mix, but without a plant were also incorporated. All pots were laid out in a completely randomised design on a mesh table in the greenhouse. For the duration of the experiment, high pressure sodium lamps (Son-T Agro 400, Philips) were suspended above the table to ensure light levels were sufficient for good plant growth. Each day plants had light from 4 am to 12 pm then 4 pm to 8 pm, giving them a 16 h day. Temperatures ranged from 14ºC (minimum) to 30ºC (maximum) and plants were kept moist by daily watering. While in the greenhouse, weeds from all pots were removed by hand.

Plants were grown for 3 months over autumn (March 2006–May 2006), but experienced a hypersensitive response to a light powdery mildew infection (on the underside of their leaves) at this time (pers. comm. I. Harvey, 2006). Efforts made to restore plant health included two sprays of each of Chlorotek (3-5 mL/L) and Sulphur (Super Six, 720 g/L, diluted at 16.5 mL/10 L), and the application of Hoagland's plant nutrient solutions (100 mL per pot)

(Hoagland & Arnon, 1950). Plant health did not improve; leaves became brittle and dry, and it was decided that dormancy should be induced. Lights were switched off and watering discontinued. After the leaves had fallen the plants were placed in a cold room (4ºC) for the remaining winter months (July 2006–August 2006). The plants were then pruned back to two buds and returned to the greenhouse. After bud break (end of August) the apparently healthy plants were grown for a further 6-7 weeks to allow rhizosphere bacterial populations to re-establish. No plants of 3309 recovered so this rootstock treatment was omitted from the experiment.

2.2.2 Collection and assessment of rhizosphere soil

Prior to harvest the plant and control pots were not watered for 24 h as high soil moisture levels would have interfered with the process of rhizosphere soil collection. From the ten replicate plants set up for each rootstock variety, six of the most uniform plants were selected for sampling. Six of the ten control ('minus plant') replicates were sampled at the start of the experiment in March 2006 (initial), and again when the plants were harvested in October 2006 (final). Rhizosphere soil was collected by shaking loose soil from the roots and then collecting the soil that remained attached to the roots (Kowalchuk et al., 2002). To do this, plants were carefully lifted out of their pots, rolled ten times on a plastic sheet (to loosen the soil) and firmly shaken three times to dislodge non-rhizosphere soil. The soil remaining on the roots was shaken onto a tray where it was thoroughly mixed and then roots and large pumice pieces were picked out by hand. For the controls ('minus plant') an equivalent volume of soil was removed from the central core of each pot using a hand trowel. To prevent cross contamination between soil samples, new plastic sheets and gloves were used for separate samples and the trowel was wiped with cotton wool soaked in 70% ethanol. For each pot (control or plant) approximately 40 g of this rhizosphere soil was collected, of which about one third was set aside for bacterial isolation onto agar (soil dilutions), a third for the molecular characterisation of the bacterial populations [single-stranded conformation polymorphism (SSCP)], and the final third for drying so that the colony forming units (CFU) could be calculated per gram of dried soil. While soil samples to be used for the molecular characterisation of bacteria were immediately stored in plastic tubes at -80˚C until use, the remainder of each rhizosphere sample was stored in an individual snap-lock bag with the plant roots and refrigerated at 4˚C until they were processed the following day.

The soil gravimetric water content (GWC) was calculated by taking 10 g replicates of fresh rhizosphere soil from each of six rootstock plants or control treatment. The weight of these was recorded before and after they were oven dried at 105˚C for 24 h.

The GWC was calculated using the following formula: $m = (m_w - m_{od})/m_{od}$. Where m is the gravimetric water content, mw is the weight of fresh soil and mod is the weight of oven dried soil (Klute, 1986).

Figure 2.1: Rhizosphere soil adhering to grapevine roots (A), rhizosphere soil collected for assessment (B) and grapevine roots with the rhizosphere soil removed (C).

2.2.3 Isolation of culturable bacteria

2.2.3.1 Dilution plating and agar types

Soil solutions were made of the rhizosphere soil from each plant, or control (bulk soil), by placing 10 g of soil into individual 200 mL bottles containing 90 mL of sterile distilled water (SDW) and mixing with a wrist-action shaker for 10 min. After standing the solutions for 10 min, a 1 mL supernatant sample (10^{-1}) was used to produce serial dilutions in SDW of 10^{-1} to 10^{-6}/mL. For total bacterial counts, concentrations of 10^{-4}, 10^{-5} and 10^{-6}/mL were plated out in triplicate on each of the agars: Nutrient agar (NA, Oxoid) and King's medium B (KB). KB plates (Appendix 1.1) were simultaneously used to determine the numbers of fluorescent Pseudomonads, which appeared as bright luminous green-yellow colonies when viewed under ultra violet (UV) light, and were easily distinguishable from the colour of the medium (King et al., 1954; Johnsen & Nielsen, 1999).

To isolate only the spore forming bacteria, the above dilutions were heated to 80˚C in a water bath for 10 min to kill all but spore forming bacteria (Grasso et al., 1996). These heat- treated samples were cooled to room temperature before being plated out in triplicate on NA. Concentrations of 10^{-3}, 10^{-4} and 10^{-5}/mL were plated out since the assessment of the initial

controls (at the start of the experiment) showed that counts were too low at the 10^{-6} concentration (Table 2.5-1, Appendix 2.5).

The NA plates were incubated for 3 d, while the KB plates were incubated for 6 d (King et al., 1954), both at 20˚C in the dark. After incubation, individual colonies were counted using a SUNTEX Colony Counter 570. To calculate the CFUs per gram of oven dried soil, plate count data (from plates that yielded 30-300 colonies per plate) were used in conjunction with the GWC values. Values were obtained for each individual plate, averaged over the replicates and then the mean value for each plant log transformed. For the total NA and total KB counts the 10^{-4} dilution plates were used, and for the spore former counts the 10^{-3} dilution plates were used.

2.2.3.2 Storage of bacterial isolates

From each of the total (NA and KB) and spore forming counts, per rootstock variety and control treatment, 20 bacteria were selected, resulting in a sample size of 60 bacterial isolates per treatment. The selection of the bacterial colonies reflected the range of types on the dilution plates which were uniformly considered for selection. After selection, each isolate was streaked onto NA and then subcultured (twice) to ensure pure culture. These plates were incubated for 2-3 days (5 for slower growing isolates) at 20°C in the dark. Single bacterial colonies were transferred to sterile 1.5 mL plastic microtubes containing a 30% (v/v) glycerol: water solution, gently vortexed and then stored at -80°C for later testing. Two tubes were made for each bacterial isolate.

2.2.3.3 Functionality testing

Stored bacterial isolates from the rhizospheres of the most (101-14) and least (Riparia Gloire) susceptible rootstock varieties, as well as those from the final controls, underwent functionality testing. Of the 60 bacteria available per treatment, 40 were randomly selected using the Excel (Microsoft Corporation, USA) random number generator, cultured from the -80°C stocks onto NA and incubated at 20˚C in the dark for 3-4 days. For each bacterial isolate, three single colonies were each inoculated into 10 mL of sterile nutrient broth (NB, Oxoid). The universal tubes containing the broth cultures were incubated at 20˚C for 24 h on an orbital shaker (133 r.p.m) in the dark before being used to inoculate all the indicator agar plates (Sections 2.2.3.3.2-2.2.3.3.5) and the dual plates. For the indicator agar plates, control bacterial isolates EJ28 and EJ50 were used as their response to these assays was previously established. These bacteria had been isolated from the rhizosphere of *Pinus radiata* seedlings (grown in forest soil) by B. Pottinger in 2005.

2.2.3.3.1 Dual plating to test for biocontrol activity

A dual plating assay was used to detect any inhibition of a *C. destructans* isolate by adjacent bacterial colonies. The *C. destructans* strain used was isolated from a diseased grapevine from Marlborough region by C. Bleach in 2005. The isolate was stored on Spezieller-Nährstoffarmer agar (SNA, Appendix 1.1) slopes at 4°C and routinely cultured onto PDA prior to this test. For each bacterial isolate, six PDA (Oxoid) plates were set up (two plates for each of the three replicate overnight broth cultures). Each plate was inoculated centrally with a mycelium plug (6 mm diameter) cut from the leading edge of a *C. destructans* culture grown on PDA at 20°C with 12 h light: 12 h dark for a period of 5 d. This plug was surrounded by four 10 µL drops of bacterial broth placed equidistantly around and 1 cm from the perimeter of the agar plate. Control plates were set up in the same way except that SDW rather than broth cultures were placed around each fungal plug. After incubation at 20°C for 7-10 days in the dark, the inhibitory effect of the bacteria was assessed by the size, if any, of the inhibition zones (Berg et al., 2002; 2005; Frey-Klett et al., 2005) according to the criteria in Appendix 2.3 (Table 2.3-1).

2.2.3.3.2 Glucanase activity

Production of β -glucanase by the rhizosphere bacteria was detected using carboxymethylcellulose (CMC) agar amended with the chromogenic 4-methylumbelliferyl (MUF)-β-D-lactoside substrate (Sigma-Aldrich, Germany). Agar preparation is detailed in Appendix 2.1. This medium is relatively expensive and so multiwell square Petri dishes (10 cm x 10 cm, divided into 25 wells; Bibby Sterilin Ltd, U.K.) were used instead of standard agar plates. Six individual wells were used per bacterial isolate (two wells for each of three replicate overnight NB broth cultures). Each well was centrally inoculated with 10 µL of an overnight NB culture, before plates were incubated at 21˚C for 8 days in the dark. During incubation, plates were viewed daily on a UV light-box (366 nm) where colonies were assessed according to the criteria in Appendix 2.3 (Table 2.3-2). β-glucanase production could be seen as fluorescence of and around bacterial colonies (Miller et al., 1998). To ensure detection was consistent EJ50 and EJ28, bacteria known to be positive or negative for β-glucanase production, respectively, were used.

2.2.3.3.3 Protease activity

The production of protease by bacteria was detected using skimmed milk agar (SMA) (Opelt & Berg, 2004), the preparation of which is detailed in Appendix 2.1. As with the above assay, six plates per bacterial isolate were set up, two plates per overnight NB broth culture. The SMA plates were centrally inoculated with 10 µL of an overnight NB culture of each

bacterium and incubated at 20˚C for 3 days in the dark. At this time, any visible casein degradation (clearing) was noted and graded according to the criteria in Appendix 2.3 (Table 2.3-3). To ensure detection was consistent EJ28 and EJ50, bacteria known to be positive or negative for protease production, respectively, were used.

2.2.3.3.4 Siderophore production

The chrome azural S (CAS) method (modified) was used to test for the production and secretion of siderophores by rhizosphere bacteria. Agar preparation is detailed in Appendix 2.1. As with the above assays, six plates per bacterial isolate were set up, two plates per overnight NB broth culture. These indicator plates were centrally inoculated with 10 µL of an overnight NB culture of the bacteria. Plates were assessed according to the criteria in Appendix 2.3 (Table 2.3-4), after incubation at 25˚C for 3 days in the dark. Siderophore production on this medium results in orange halos around bacterial colonies, due to the transfer of iron (Fe) from the Fe-CAS dye complex (blue) to the siderophores (Alexander & Zuberer, 1991; Neilands, 1995; Frey-Klett et al., 2005). To ensure detection was consistent EJ50 and EJ28, bacteria known to be positive or negative for siderophore production, respectively, were used.

2.2.3.3.5 Chitinase activity

An attempt was made to test for chitinase production by bacteria using CM-Chitin-RBV (remazol brilliant violet-linked chitin) solution (Loewe Biochemica GmbH, Germany), a chromogenic chitin substrate, within a basal medium that contains no carbon compounds. Agar preparation is detailed in Appendix 2.1. Six wells on a multiwell Petri dish were used per bacterial isolate (two wells for each of the three replicate overnight NB broth cultures). Each well was inoculated centrally with 10 µL of an overnight NB culture of the specific bacterium, before plates were incubated at 23˚C for 14 days in the dark. During this time plates were checked every day for chitinase activity (Wakelin, 2001). Any degradation of chitin would be seen as a clearing zone around the colonies due to the release of the violet dye from the chitin substrate (manufacturer's instructions). A *Serratia entomophila* isolate from the Agricultural and Life Sciences Division (Lincoln University) was used as a positive control, since this bacterium is known to produce chitinases (Upadhyaya et al., 1992). Some difficulties were experienced while undertaking the assay and no satisfactory data could be obtained. The chitin solution experienced accidental freezing while being stored in the 4˚C fridge, something the manufacturers warned should be prevented due to it causing precipitation. However, several attempts were made to improve experimental design and the trouble shooting is outlined in Appendix 2.2.

2.2.4 Molecular assessment

Single-strand conformation polymorphism (SSCP) was used to detect differences in the diversity of bacterial communities present in the soil samples of the different control (initial and final) and rootstock variety treatments: Riparia Gloire, 5C, Schwarzmann and 101-14.

2.2.4.1 DNA extraction

From the six randomly selected replicates for each of the six treatments, 0.25 g subsamples were used for DNA extraction using the PowerSoil™ DNA Kit (MO BIO Laboratories, Inc, CA, USA) as per the manufacturer's instructions. The quality of the DNA was confirmed by separating 5 µL of the extracted DNA by electrophoresis at 10V/cm for 45 min in a 1% agarose gel (Progen Biosciences, Brisbane, Australia). Gels were stained with ethidium bromide [0.5 µg per mL 1xTAE (Appendix 1.1)] for 30 min, rinsed in sterile distilled water (SDW) for 10 min and then photographed under UV light using the VersaDoc™ Imaging System (Model 3000, Bio-Rad, CA, USA). DNA was stored at -20 °C.

2.2.4.2 PCR

Each 25 µL PCR reaction contained 1 U *Taq* DNA polymerase, 2 mM $MgCl_2$, 0.2 mM of each of dATP, dCTP, dGTP, dTTP (Master Mix, Fermentas, Vilnius, Lithuania) and 5 pmole of each of the universal bacterial primers (Invitrogen, Auckland, New Zealand; Life Technologies Corporation, California, USA) B342If (5'- CTACGGGIGGCIGCAGT - 3' and U806Ir-Ph (5'- GGACTACCIGGGTITCTAA – 3') (Hori et al., 2006). To each tube, 1 µL of the extracted DNA was added. A negative control was included which contained all ingredients except the DNA. The tubes containing the reaction mix were placed in a thermal cycler (iCycler, Bio-Rad, Auckland, New Zealand; California, USA) and amplified using the following protocol: 94°C for 2 min (denaturation), then 32 cycles of: 1 min at 94°C (denaturation), 1 min at 55°C (annealing) and 1 min at 72°C (elongation), and a final cycle of 5 min at 72°C. The resulting products were frozen at -20°C.

To determine if a PCR fragment of the correct size (464 bp) had been produced, 5 µL of each amplification product was mixed with 2 µL of 6x loading dye (0.025% bromophenol blue, 0.025% xylene cyanol, 40% w/v sucrose) and separated by electrophoresis in a 1% agarose gel (Progen Biosciences) at 10 V/cm for 45 min. The gel was stained and photographed as described previously. Samples were stored at -20°C until SSCP analyses.

2.2.4.3 SSCP

All PCR products were analysed by SSCP using polyacrylamide gels. Each polyacrylamide gel contained 10% acrylamide (37.5:1, Bio-Rad Laboratories, CA, USA) and 10% Urea (w/v)

in 1xTBE (445 mM Tris Base, 445 mM Borate, 10 mM EDTA) buffer (Appendix 1.1). The catalysts, N,N,N',N'-tetra-methyl-ethylenediamine (30 µL) (Bio-Rad Laboratories, CA, USA) and 10% w/v ammonium persulfate (280 µL) (Bio-Rad Laboratories, CA, USA), were added to this mixture immediately prior to pouring into the assembled Protean ® II xi cell (Bio-Rad Laboratories, CA, USA) casting apparatus. The acrylamide was left to polymerize for 2 h.

Prior to loading onto the polyacrylamide gel, 2 µL of PCR product was mixed with 30 µL loading dye (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, 10 mM NaOH, 0.8 mM EDTA pH 8). These samples were then heat denatured for 7 min at 99°C, plunged into wet ice and loaded into wells on the gel within 5-10 min. The gel was run at 15°C for 19-20 h at 250 V in a Protean ® II xi cell (Bio-Rad Laboratories) in 1x TBE buffer. A cooling system (Grant LVF6, Grant Instruments Ltd, Cambridge, England) was used to keep the temperature constant.

Gels were silver stained by placing in a glass tray of fixer (10% ethanol, 0.5% acetic acid) for 3 min, then stained (10% ethanol, 0.5% acetic acid, 0.2% $AgNO_3$) for 5 min. They were then rinsed briefly in SDW and washed in SDW for 2 min, prior to placing in a developer (3.0% NaOH, and 0.1% formaldehyde) for 40 min and rinsed again in SDW (Bassam et al., 1991; Benbouza et al., 2006). Polyacrylamide gels were dried for storage on filter paper on a gel dryer (SGD210D Speed Gel™ System, Thermo Savant, NY, USA) at 65°C for 2 h.

After staining, the gels were placed between acetate sheets and scanned on a Canon iR2270 photocopier/scanner. Bands were excised from each of the gels using a scalpel (cleaned and sterilized between bands by wiping with fresh cotton wool soaked in 70% ethanol). A fragment of each excised band (~2 mm^2) was crushed using a sterile new pipette tip and added to a PCR reaction mix, and then reamplified as described previously except that the number of cycles was increased to 35. Reamplified DNA was separated in a 1% agarose gel as described previously.

2.2.4.4 Sequence

A selection of PCR products, generated from excised bands, were sequenced using primer U806Ir-Ph at the Lincoln University Sequencing Facility. Sequences were manually checked and trimmed of any ambiguous sequence on either end. For most products, a clear sequence of at least 250 bp was obtained. The GenBank (http://www.ncbi.nlm.nih.gov/) nucleotide BLAST tool (Altschul et al., 1990) was used to find the closest match for each sequence based on the maximum identity and E values returned.

2.2.4.5 Sequence Editing

Several sequences contained small regions of ambiguity and this was likely due to parallel sequencing of very similar products. Thus, each sequence was proof read and edited to ensure that the correct base had been assigned. Chromas Lite (Technelysium Pty Ltd, Helensvale, Australia) was used to view the electropherograms and DNAMAN version 4 (Lynnon Corporation, Quebec, Canada) was used to edit the sequences. In regions where the sequence differed from the returned GenBank match and two bases were apparent in the returned sequence, the base that corresponded with the GenBank match was used. Edited sequences were re-submitted into GenBank and analysed by BLAST.

2.2.4.6 Confirmation

To confirm that they were the desired product, PCR products derived from excised bands were run on an SSCP gel alongside the original sample from which they were excised.

2.2.5 Statistical methodology

Dilution plating data (CFUs) were $\log_{10}$ transformed and analysed using a general analysis of variance (ANOVA). To cope with the large number of zero counts recorded for fluorescent Pseudomonads, the value of one was added to all counts before the data was logged. When the overall effect was significant ($P<0.05$) according to the ANOVAs, pairwise comparisons were carried out with Fisher's protected least significant difference test (LSD). Data were analysed using the statistical software GenStat® version 9.0 (USN International Ltd.).

Non parametric tests were used to analyse the functionality assay data (raw data counts). The Kruskal-Wallis test was used to determine overall significance. If this was significant ($P<0.05$), then pairwise comparisons, by way of the Mann-Whitney test, could proceed. Analysis done on the number of plates (6 per isolate, 40 isolates) placed within each grade of each functionality assay allowed comparison across rootstock variety and control treatments. The analysis was then repeated using presence (1) and absence (0) variables for the different assays. Here, data were regrouped and analysed by Chi-Square tests, with significance determined by Pearson's Chi-Square ($P<0.05$). Data were analysed using SPSS® for Windows® V13.0 (SPSS Inc., Chicago, IL, USA, 2007).

2.3 RESULTS

2.3.1 Dilution plating

The dilution plating gave CFUs of total culturable (NB and KB) and groups of certain bacteria (spore forming and fluorescent Pseudomonad) in the bulk control soil and in the rhizospheres of the grapevine rootstocks tested.

2.3.1.1 Total bacteria (NA)

The rhizospheres of the rootstock varieties tested and the control treatment soil (final) differed in the mean number of total bacteria they supported on NA (P=0.012) (ANOVA Table 2.4-1 in Appendix 2.4). As shown in Table 2.1, the rhizospheres of rootstock varieties 5C ($7.48x10^5$ CFU/g dry soil) and Schwarzmann ($5.39x10^5$ CFU/g dry soil) supported more culturable bacteria than the control soil ($1.97x10^5$ CFU/g dry soil), but 101-14 ($3.46x10^5$ CFU/g dry soil) and Riparia Gloire ($2.96x10^5$ CFU/g dry soil) did not. Rootstock variety 5C had significantly greater total bacterial counts than either 101-14 or Riparia Gloire, but did not differ significantly from Schwarzmann, which had similar numbers to 101-14 and Riparia Gloire (P>0.05).

Table 2.1: CFU counts $\log_{10}$ per gram of oven dried (OD) soil for the rhizospheres of different grapevine rootstocks and a control treatment. Means for total culturable bacteria (NA and KB), spore forming bacteria and fluorescent Pseudomonads are given. Values within the same column followed by different letters were significantly different at P=0.05 according to Fisher's Protected LSD test

	Log_{10} CFU/g OD soil			
Rootstock	**Total (NA)**	**Total (KB)**	**Spore formers**	**Fluorescent Pseudomonads**
Control	5.295 c	4.826 c	4.789	N/A
5C	5.874 a	5.738 a	4.703	4.40 a
101-14	5.539 bc	5.113 bc	4.630	3.03 ab
Riparia Gloire	5.472 bc	5.223 bc	4.738	1.20 b
Schwarzmann	5.732 ab	5.598 ab	4.618	3.79 a
LSD	0.3297	0.4865	0.1659	2.194
***P* value**	0.012 *	0.005 *	0.201	0.035 *

* Denotes significance $P \leq 0.05$

N/A = not applicable.

2.3.1.2 Total bacteria (KB)

As with total bacterial counts on NA, the rhizospheres of the rootstock varieties tested and control treatment soil differed in the mean number of total bacteria they supported on KB (P=0.005) (ANOVA Table 2.4-2, Appendix 2.4). Pairwise comparisons showed that rootstock varieties 5C ($5.47x10^5$ CFU/g dry soil) and Schwarzmann ($3.96x10^5$ CFU/g dry soil)

supported significantly more culturable bacteria than the control soil ($6.7x10^4$ CFU/g dry soil). The total bacterial counts for 101-14 ($1.29x10^5$ CFU/g dry soil) and Riparia Gloire ($1.67x10^5$ CFU/g dry soil) did not differ significantly from the control. Rootstock variety 5C had a significantly larger population of bacteria than Riparia Gloire and 101-14, but did not differ significantly from Schwarzmann. Rootstock variety Schwarzmann was similar to Riparia Gloire and 101-14 (P>0.05) (Table 2.1).

2.3.1.3 Spore forming bacteria

The rootstock varieties tested did not differ from each other or the control treatment in the mean number of culturable spore forming bacteria they supported in their rhizospheres (P=0.201). Pairwise testing could not proceed (ANOVA Table 2.4-4, Appendix 2.4). The log values are shown in Table 2.1.

2.3.1.4 Fluorescent Pseudomonads

The rhizospheres of the rootstock varieties tested and the control treatment soil differed in the mean number of culturable fluorescent Pseudomonads they supported (P=0.035) (ANOVA Table 2.4-3, Appendix 2.4). Pairwise comparisons showed that rootstock 5C ($2.51x10^4$ CFU/g dry soil) had a significantly larger population of fluorescent Pseudomonads than Riparia Gloire ($1.58x10^1$ CFU/g dry soil). Rootstocks 5C, 101-14 ($1.07x10^3$ CFU/g dry soil) and Schwarzmann ($6.16x10^3$ CFU/g dry soil) all supported very similar numbers of fluorescent Pseudomonads (Table 2.1). No fluorescent Pseudomonads were isolated from the soil controls and so this treatment was omitted from the analysis.

2.3.2 Bacterial functionality testing

A list of all the bacterial isolates selected and stored for functionality testing can be found in Appendix 2.6. For all 40 isolates, there was a high level of consistency for the six replicate plates, except that with the glucanase assay, some bacteria did not grow (two from 101-14, three from the control and four from Riparia Gloire). To compensate for this, results for each response category are presented as a percentage of the total number of plates in each treatment. However, the analysis (numbers of plates per grade) did take this into account. Overall, between treatments, fewer differences were found in the spectrum of bacterial functionality when dual plating and protease assays were considered than when glucanase and siderophore assays were considered.

2.3.2.1 Dual plating to test for biocontrol activity

The range of inhibitory responses are shown in Figure 2.2. The bacterial isolates from Riparia Gloire, 101-14 and the control treatment did not differ in presence or absence variables of inhibition (P=0.545) or their degree of inhibition towards *C. destructans* in dual culture (P=0.104) and so pairwise comparisons could not be made. The similarities in dual plating responses can be seen in Figure 2.3. For the control, 101-14 and Riparia Gloire there was no fungal inhibition (-) for 57.5%, 52.5% and 55.0% of isolates and only slight fungal inhibition (+/-) for 40%, 32.5% and 35% of isolates, respectively. Moderate fungal inhibition (+) was shown by a few isolates from 101-14 (12.5%) and Riparia Gloire (10%), while strong fungal inhibition (++) was shown for only one isolate from 101-14 and the control soil (2.5%). Overall, it seemed that rhizosphere soils had more antagonistic isolates that the control soils.

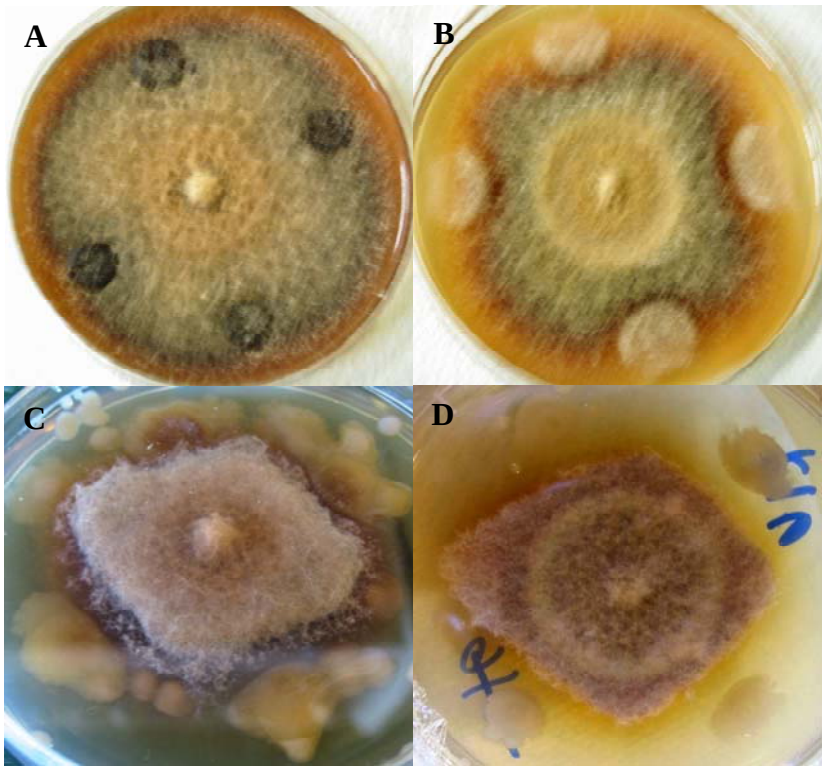


Figure 2.2: Representatives of the different *C. destructans* inhibition classes: A) – no fungal inhibition, B) +/- slight fungal inhibition, C) + moderate fungal inhibition and D) ++ strong fungal inhibition.

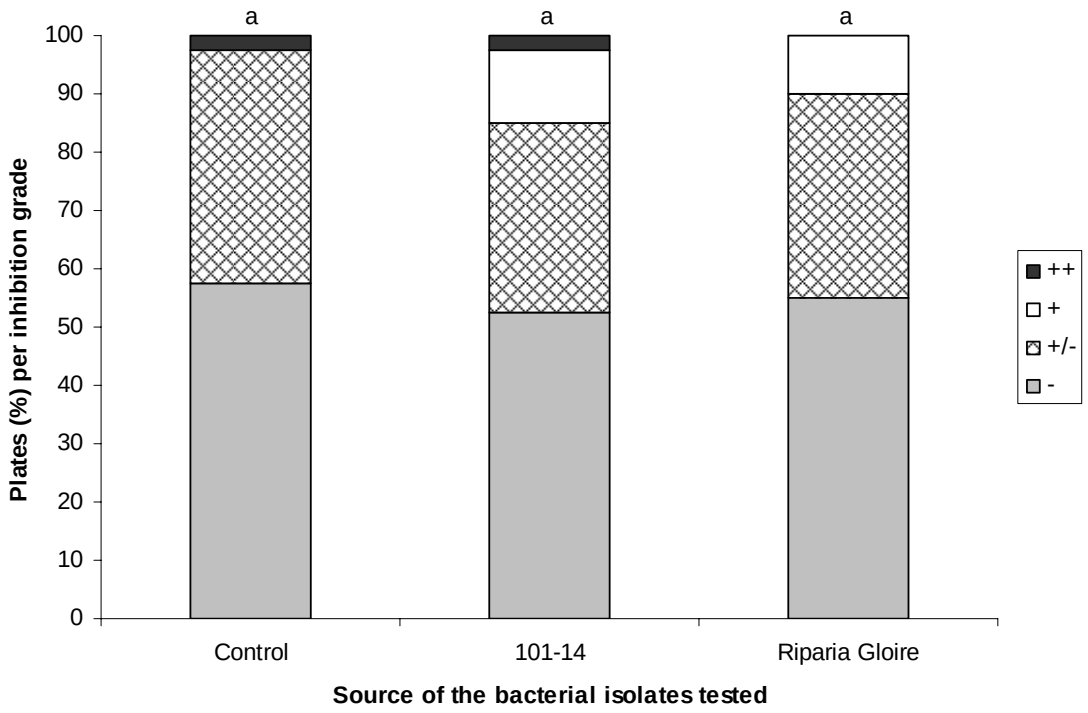


Figure 2.3: The percentage of isolates from each treatment that corresponded to a particular grade of *C. destructans* inhibition (++, +, +/- and -) on dual plate assays, by bacteria isolated from the rhizospheres of grapevine rootstocks 101-14 and Riparia Gloire, and from control soils. The same letters above the treatment bars indicates a similar response at *P*=0.05 (according to the Mann-Whitney test).

2.3.2.2 Glucanase activity

The glucanase activity responses observed were graded and are shown in Figure 2.4. Riparia Gloire, 101-14 and the control treatment differed by the number of isolates in the degrees of glucanase activity on CMC agar (*P*=0.000). Pairwise comparisons showed that the bacterial isolates from 101-14 were significantly different from those of Riparia Gloire (*P*=0.003) and the control (*P*=0.000), which also differed from each other (*P*=0.022). The following trends were observed. Compared to 101-14 (55.3%), a greater number of bacterial isolates from Riparia Gloire (75.0%) and the control (81.1%) had no glucanase activity (–) (Figure 2.5). However, more bacterial isolates from 101-14 had the +/- response (15.8%) than from either Riparia Gloire (2.8%) or the control treatment (5.4%). Bacterial isolates from the control (13.5%) and 101-14 (21%) had more + responses than Riparia Gloire (5.6%). However, more isolates from Riparia Gloire had ++ responses (16.6%) than those of 101-14 (7.9%) and the control (0%). When the data was analysed using presence and absence variables, there was a treatment difference (*P*=0.000). Pairwise comparisons showed that 101-14 (44.7%) had more glucanase active isolates that either the control or Riparia Gloire (*P*=0.000), and that Riparia Gloire isolates (25.0%) did not differ significantly from the control (18.9%, *P*=0.124).

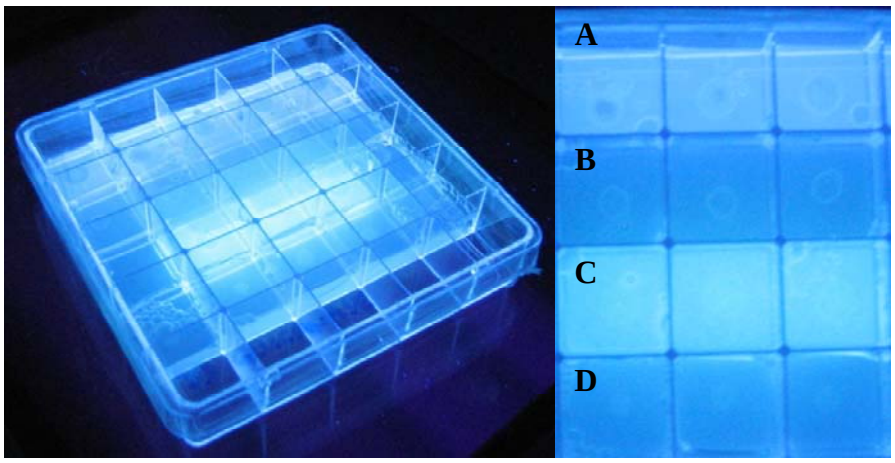


Figure 2.4: A glucanase assay plate viewed on a light box (366 nm) showing the differing degrees of fluorescence (left). Representatives of the different glucanase production classes: A) + moderate fluorescence, B) – no fluorescence, C) ++ high fluorescence and D) +/- slight fluorescence (right).

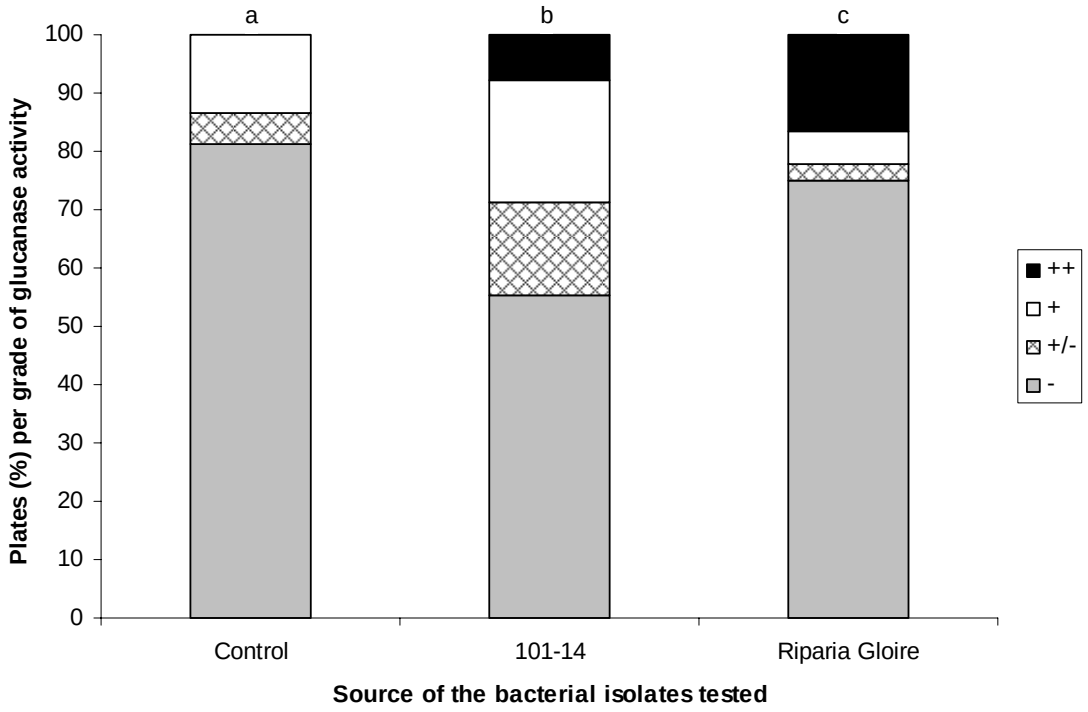


Figure 2.5: The percentage of isolates from each treatment that corresponded to a grade of glucanase activity (++, +, +/- and -) on CMC agar. Bacteria isolated from the rhizospheres of grapevine rootstocks 101-14 and Riparia Gloire, and control soils. Different lettering above treatment bars indicates significantly different glucanase activity responses at P=0.05 (according to the Mann-Whitney test).

2.3.2.3 Protease activity

The protease production responses observed were graded and are shown in Figure 2.6. Riparia Gloire, 101-14 and the control treatment differed by the number of isolates in each degree of protease production (*P*=0.001) (Figure 2.7). Pairwise comparisons showed that the bacterial isolates from the 101-14 treatment were significantly different from those of the control (*P*=0.001) and Riparia Gloire (*P*=0.001). The response of the Riparia Gloire isolates was not significantly different from the control (*P*=0.703). The following trends were observed. Fewer isolates from rootstock variety 101-14 (20.0%) had no protease activity (-) than those from Riparia Gloire (32.5%) and the control (37.5%) treatment. Additionally, while 101-14 had fewer + (5%) and +++ (2.5%) responses than Riparia Gloire (12.5% and 5%, respectively) or the control (10 and 12.5%, respectively), it did have more ++ (55%) responses than either. Riparia Gloire had 35% of its isolates in this category, and the control treatment, 25%. All treatments had very similar numbers of +/- responses being 15%, 17.5% and 15% for the bacterial isolates from the control, 101-14 and Riparia Gloire, respectively (Figure 2.4). When the data was analysed using presence and absence variables, there was a treatment difference (*P*=0.000). Pairwise comparisons showed that 101-14 (80.0%) had more protease producing bacterial isolates than the control (*P*=0.000) or Riparia Gloire (*P*=0.002), and that Riparia Gloire (67.5%) did not differ from the control (62.5%) in its proportion of protease production (*P*=0.251).

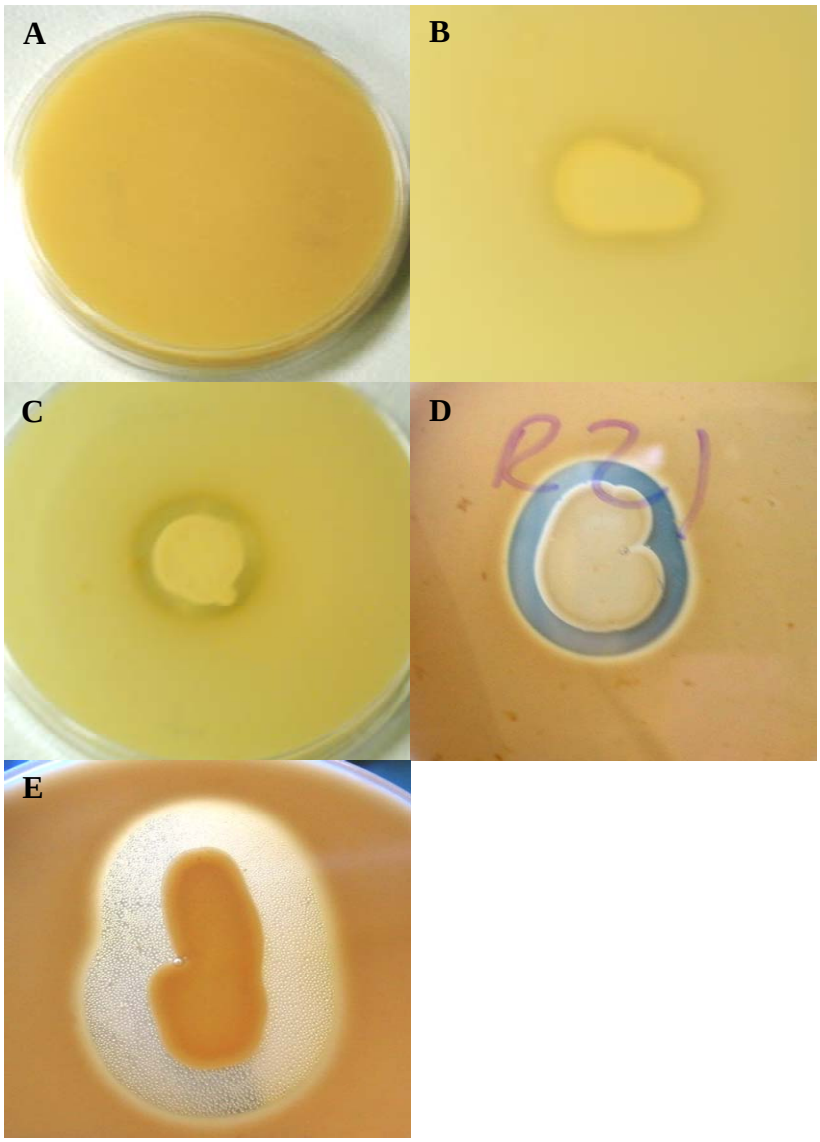


Figure 2.6: Representatives of the different protease activity classes: A) – no protease activity, B) +- inconclusive protease activity, C) + a halo of <5 mm, D) ++ a halo of 5-10 mm and E) +++ indicating the most protease activity with a halo of ≥ 1 cm.

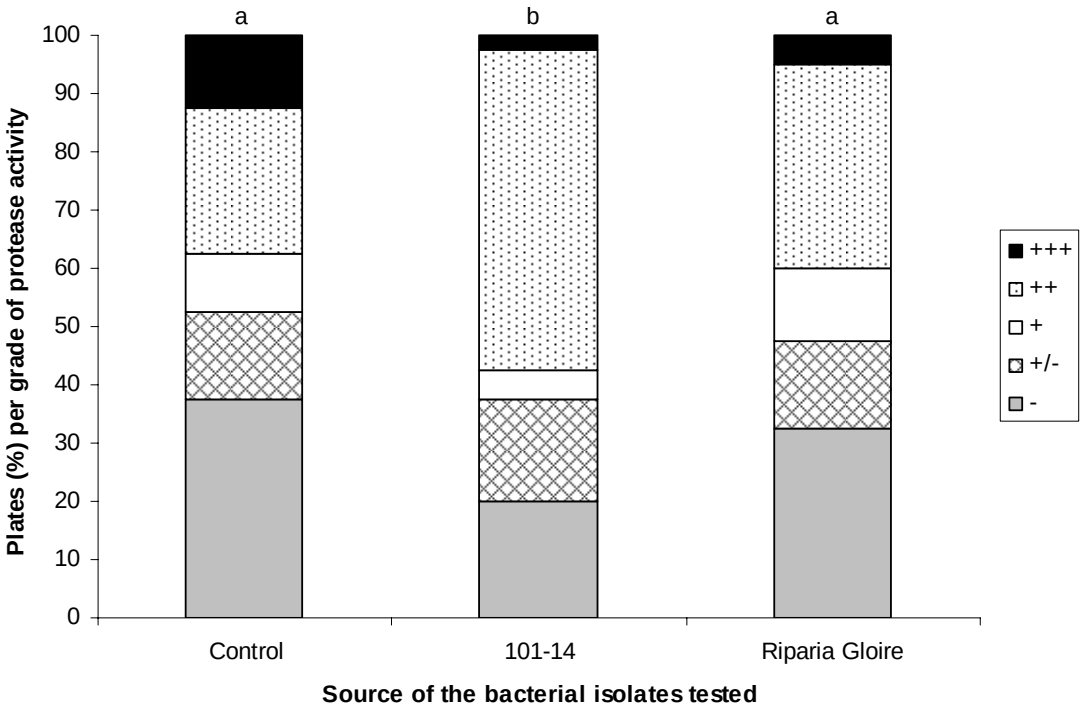


Figure 2.7: The percentage of isolates from each treatment that corresponded to a particular grade of protease activity (+++, ++, +, +/- and -) on skim milk agar by bacteria isolated from the rhizospheres of grapevine rootstocks 101-14 and Riparia Gloire and from control soils. Different lettering above treatment bars indicates significantly different protease activity responses at *P*=0.05 (according to the Mann-Whitney test).

2.3.2.4 Siderophore production

The siderophore production responses observed were graded and are shown in Figure 2.8. Riparia Gloire, 101-14 and the control treatment differed by the number of isolates in the degrees of siderophore activity on CAS agar (*P*=0.000). Pairwise comparisons showed that the bacterial isolates in the control treatment were significantly different from those of 101-14 (*P*=0.000) and Riparia Gloire (*P*=0.000), which also differed in their degree of siderophore activity (*P*=0.000). The following trends were observed. The control isolates yielded only - - (97.5%) and +/- (2.5%) responses, indicating that most control bacteria would not grow on the CAS agar and the few that did, did not produce siderophores. Bacterial isolates from 101-14 (25%) produced more siderophores by the +/- and ++ criteria (25% and 35%, respectively) than those from the Riparia Gloire (5% and 10%, respectively). However, in terms of the greatest level of siderophore production (+++), more isolates from Riparia Gloire (17.5%) than from 101-14 (12.5%), were found (Figure 2.9).

When the data was analysed using presence and absence variables, there was a treatment difference (*P*=0.000). Pairwise comparisons showed that compared to the control (0%), and Riparia Gloire treatments (27.5%), 101-14 had the most siderophore active isolates (47.5%) (*P*=0.000 and *P*=0.001, respectively).

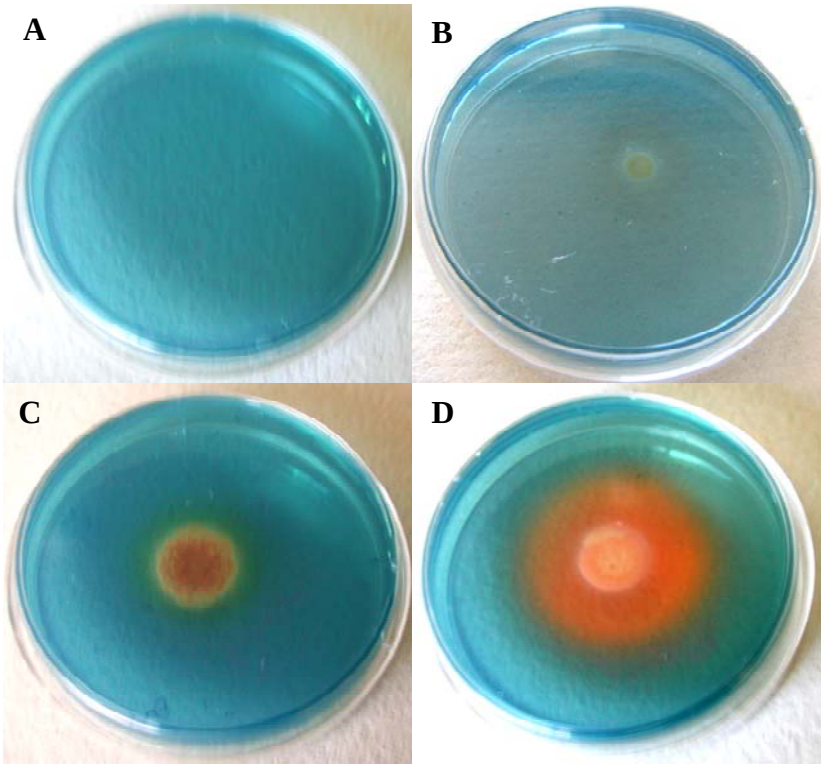


Figure 2.8: Representatives of the different siderophore activity classes. A) - - no bacterial growth and no siderophore activity, B) +/- bacterial growth but no siderophore activity, C) ++ bacterial growth with some siderophore activity (orange halo of <1 cm) and D) +++ bacterial growth with strong siderophore activity (orange halo of >1 cm).

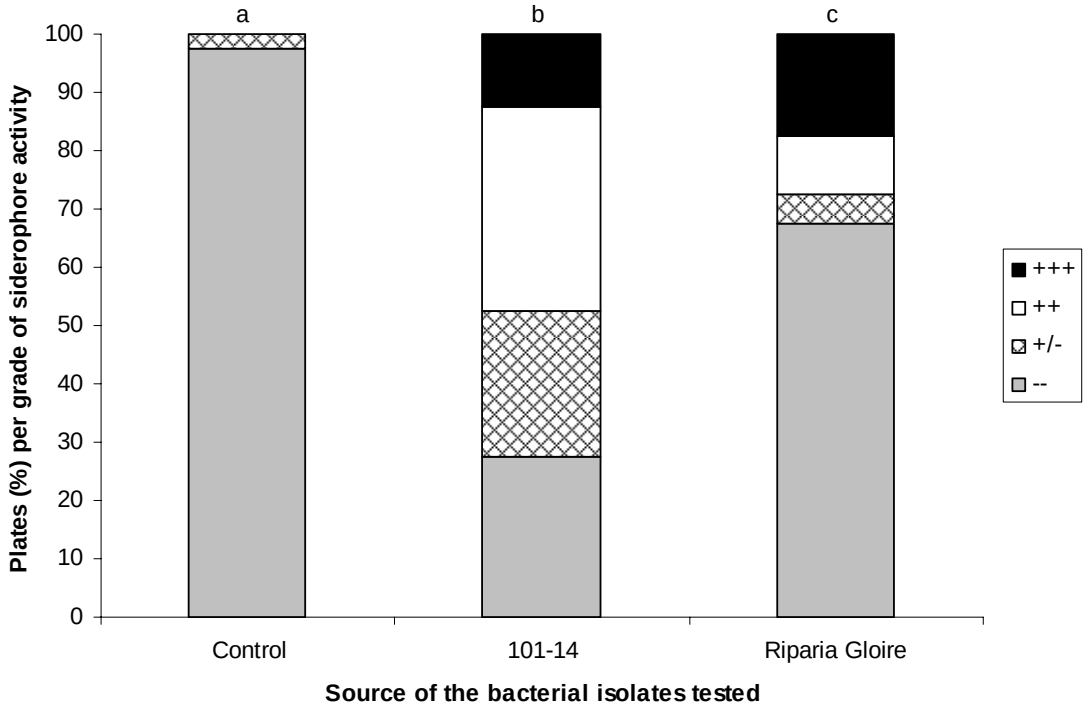


Figure 2.9: The percentage of isolates from each treatment that corresponded to a grade of siderophore activity (+++, ++, +/- and - -) on CAS agar. Bacteria isolated from the rhizospheres of grapevine rootstocks 101-14 and Riparia Gloire, and control soils. Different lettering above treatment bars indicates significantly different siderophore activity responses at P=0.05 (according to the Mann-Whitney test).

2.3.3 Molecular analysis of bacterial rhizosphere populations

2.3.3.1 SSCP banding patterns

Six replicates of soil were assessed for the rhizospheres of each grapevine rootstock variety and the control treatment (initial and final). The SSCP analysis produced gels whose banding patterns were very consistent (Figure 2.10) within each control treatment, indicating that the sampling system used provided good consistency. The gels showed clear differences in the banding patterns between initial and final controls (minus plant), with noticeably more bands in the initial soil controls than in the final soil controls. For example, there were several more bands in the initial control gels between bands 12 and 14 than in the final control gels. The presence of more bands reflects greater species and/or strain diversity in the initial controls. In addition, intensity of bands was typically more intense in the initial controls than in the final controls. For example, bands 8, 9, 11 and 12 were noticeably darker in the initial controls than in the final controls. However, band 1 was stronger in the final controls than in the initial controls. Although not quantitative it is possible that any shift in the relative intensity of a band between rootstock varieties or control treatments may reflect changes in the abundance of that bacterial species in the grapevine rhizosphere. Bands 7 and 13 did not differ significantly in intensity between control treatments.

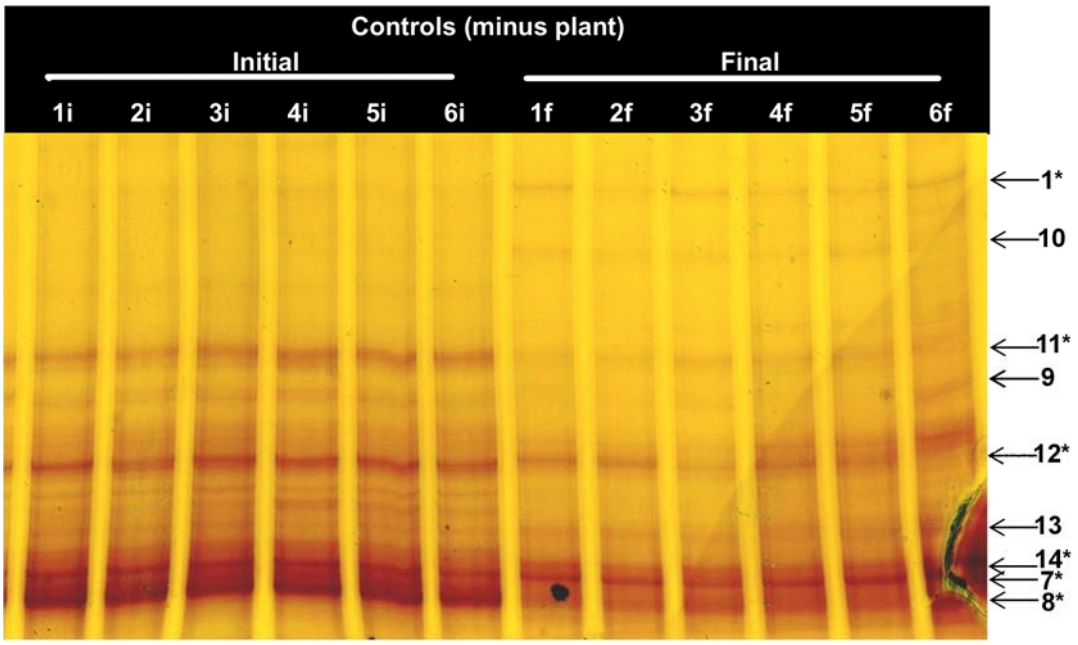


Figure 2.10: SSCP banding patterns for bacteria from bulk soil, showing six replicates (1-6) for the initial (i) and final (f) control treatments ('minus plant'). Numbers and arrows identify bands that were successfully reamplified and their excise position. Those followed by an asterisk were successfully sequenced.

The numbers of bands in the gels (Figures 2.11 and 2.12) indicated that the rootstock variety that had the most variability in its rhizosphere bacterial populations was 5C. For 5C there was wide variation in banding patterns between replicate plants, particularly in lanes 2 and 3 which seemed to have greater species diversity than lanes 1 and 4 to 6. Within the rhizospheres of 101-14 replicate plants, banding patterns were quite consistent, but there was some variation, particularly in lanes 1 and 6. The rootstock varieties that had the least variation between replicate plants were Riparia Gloire and Schwarzmann (Figure 2.11 and Figure 2.12).

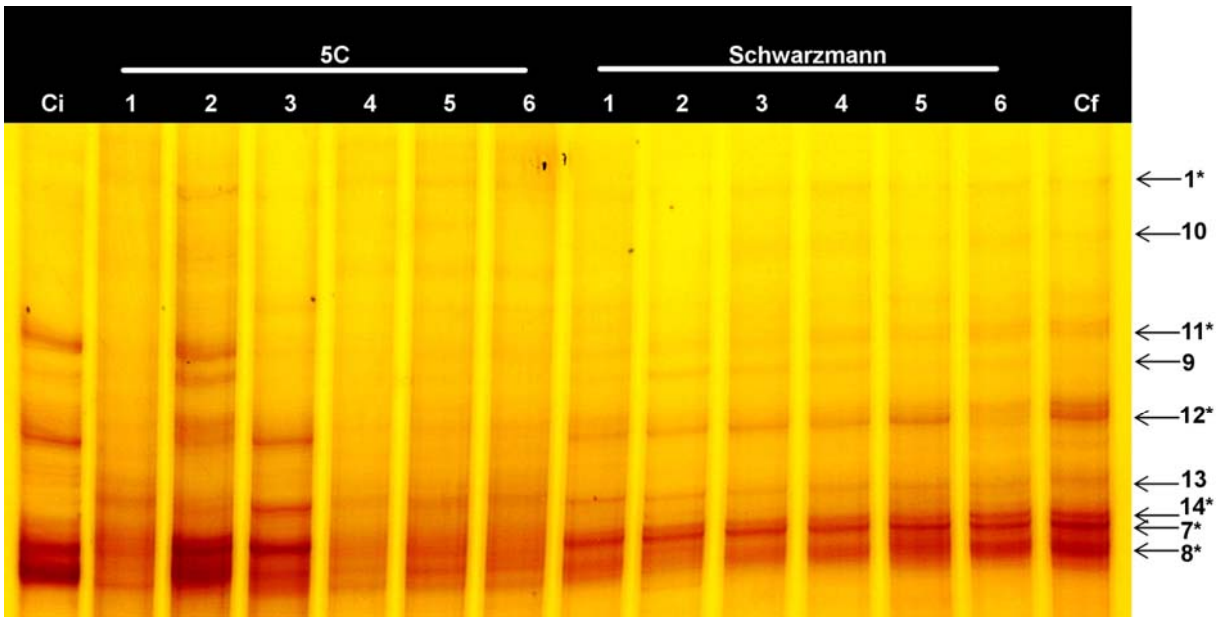


Figure 2.11: SSCP banding pattern for bacteria from grapevine rhizosphere soil, showing six replicates (1-6) for the 5C and Schwarzmann rootstock treatments. Also shown on the gel is a typical initial (Ci) and final (Cf) control replicate. Numbers and arrows identify bands that were successfully reamplified and their excise position. Those followed by an asterisk were successfully sequenced.

When the banding patterns of the rootstock varieties are compared to those of the controls (as seen in the Ci and Cf lanes on each treatment gel), they do not differ greatly. There were more bands in the gels of the initial controls than in most of the rootstock treatments, and rootstock banding patterns were most similar to those of the final controls. All bands successfully sequenced (1, 7, 8, 11, 12, 14) that were found in the rootstock samples were also present in the final controls.

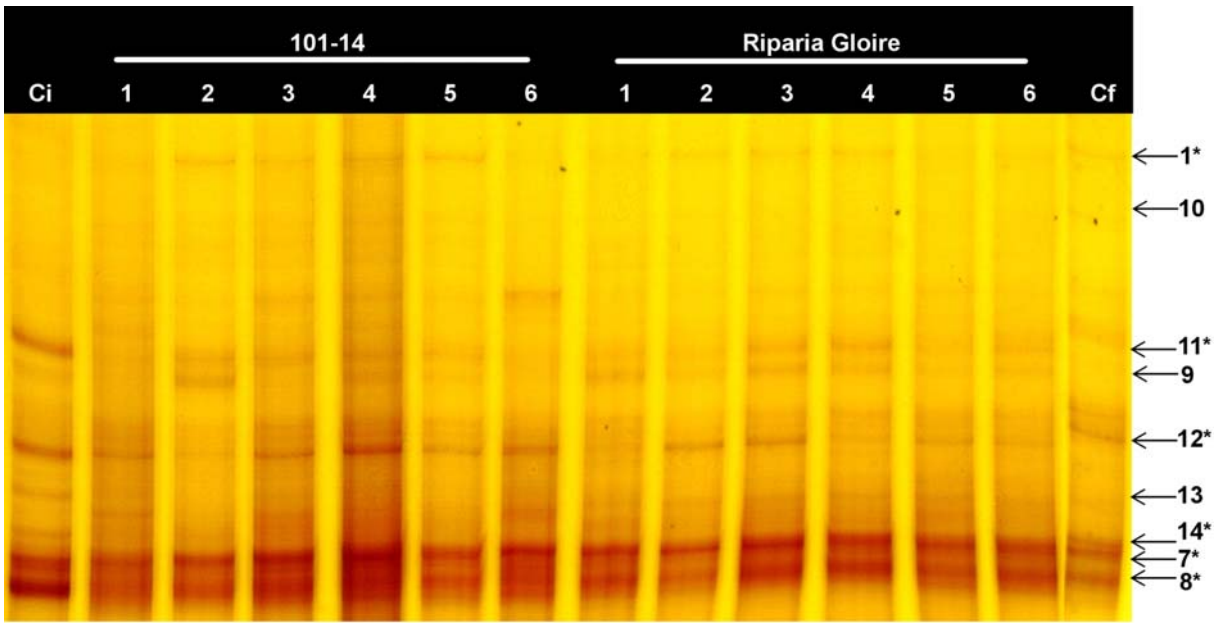


Figure 2.12: SSCP banding pattern for bacteria from grapevine rhizosphere soil, showing six replicates (1-6) for the 101-14 and Riparia Gloire rootstock treatments. Also shown on the gel is a typical initial (Ci) and final (Cf) control replicate. Numbers and arrows identify bands that were successfully reamplified and their excise position. Those followed by an asterisk were successfully sequenced.

2.3.3.2 Sequenced bands

A sample of eleven distinct bands were excised from the SSCP gels and reamplified for sequencing. However, only six of these ultimately produced readable sequences (Table 2.2). Several other bands were excised and successfully reamplified from the gel matrix but sequence quality was not adequate for similarity matching. For example, although a sequence was obtained for band 13, the BLAST search was unsuccessful. DNA sequences used for similarity matching within the GenBank database ranged in size from 265 bp to 304 bp. For each sequence, the closest BLAST matches were to uncultured bacteria whose bands were from denaturing gradient gel electrophoresis (DGGE) gels, to which they had greater than 90% similarity and 95-100% sequence coverage. It was not always possible to get a bacterial group or species level identification from the BLAST, and so the closest match and a match that contained a genus name were included in the table. All matches were to bacteria that had been isolated from soil and sediments. Three of the sequences appeared to be from γ-Proteobacteria (bands 8, 12 and 14), which include the Chromatiales (Imhoff, 2005), while two sequences were likely to be from α-Proteobacteria (bands 1 and 7) including a *Rhodopseudomonas* sp., and one band (band 11) was likely to be from a δ-Proteobacteria (www. ncbi.nlm.nih.gov/).

Due to the similarity in GenBank matches between some of the bands, DNAMAN 4 (Lynnon Corporation, Canada) for Windows® was used to run sequence alignments. Flanking regions of sequence that were present in one sample, but for which there were no corresponding regions in the other sequences were removed. It was determined that sequences for bands 7 and 8 were 94% similar to each other. The sequence for band 1 was 91% similar to both bands 7 and 8. Additionally, the sequences for bands 12 and 14 were 96% similar to each other. The sequence for band 1 was 91% similar to both bands 12 and 14.

2.3.3.3 Sequence gels

SSCP gels that were run to confirm the correct bands had been excised showed very weak bands for the re-amplified PCR products compared to the original source lanes (Figure 2.13). Due to the difficulty in discretely excising a single band from the original SSCP gels, sometimes more than one band was seen in a gel lane for such a product. Bands 1, 11 and 12 were relatively distinct from those clustered at the base of the gel and easily excised. However, these still produced patterns similar to those of bands 7, 8 and 14. This may have also been due to a single band having more than one stable conformation.

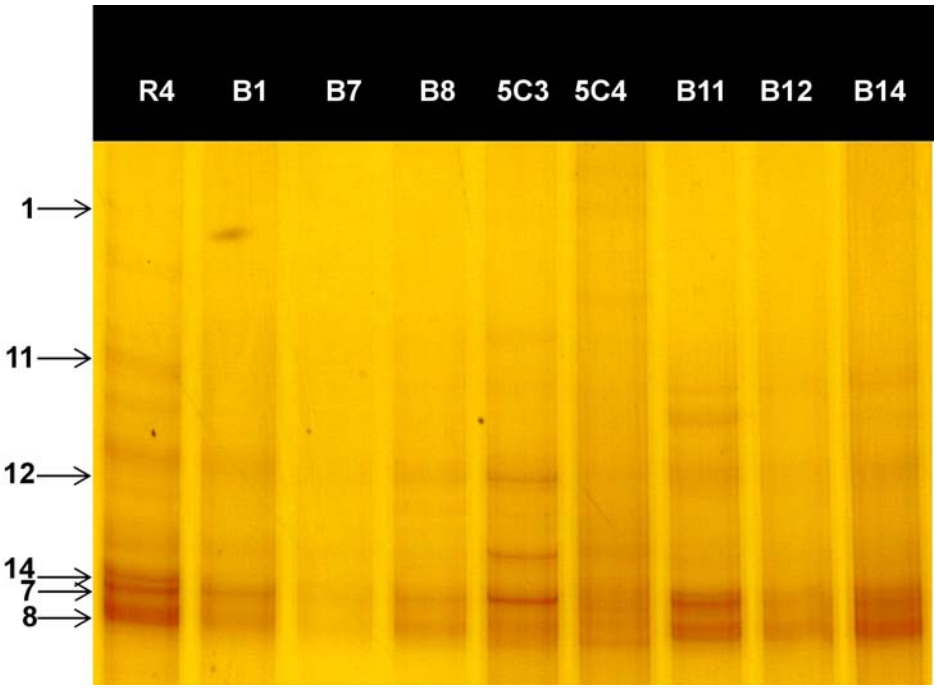


Figure 2.13: An SSCP gel of the sequenced bands (B1, B7, B8, B11, B12, B14) together with the original source samples from Riparia Gloire (R4) and 5C (5C3, 5C4).

Table 2.2: Sequenced bands excised from SSCP gel of amplified grapevine rhizosphere bacteria (16S DNA) with their highest matches from GenBank.

Band	Name	Source	Highest Accession #	Coverage	Max ident	Size
1	Uncultured bacterium DGGE band	heavy metal and PAH-contaminated soil	AY649345.1	99%	93%	265 bp
	Rhodopseudomonas sp.	environmental sample	D14426.2	100%	90%	
7	Uncultured bacterium isolate DGGE band	endophytic and rhizoplane	EU635967.1	100%	93%	280 bp
	Uncultured alpha Proteobacterium clone	alpine tundra soil	FJ569895.1	100%	91%	
8	Uncultured bacterium clone	marine sediment	AY171368.1	100%	93%	270 bp
	Uncultured Chromatiales bacterium	lagoon sediment	AM501811.1	100%	93%	
11	Uncultured bacterium DGGE band	heavy metal and PAH-contaminated soil	AY649340.1	65%	92%	290 bp
	Uncultured delta Proteobacterium clone	marsh sediment	AY374777.1	65%	88%	
12	Uncultured bacterium DGGE band	heavy metal and PAH-contaminated soil	AY649345.1	97%	94%	272 bp
	Uncultured Chromatiales bacterium	lagoon sediment	AM501645.1	98%	91%	
14	Uncultured bacterium DGGE band	heavy metal and PAH-contaminated soil	AY649345.1	95%	93%	304 bp
	Uncultured gamma Proteobacterium clone	alpine tundra soils	FJ568431.1	99%	90%	

2.4 DISCUSSION

The rhizosphere bacterial populations of grapevine rootstock varieties 101-14, 5C, Riparia Gloire and Schwarzmann were studied to identify potential mechanisms for their different susceptibilities to black foot disease (as caused by the pathogen *C. destructans*). This was achieved through plate counts, functionality assays and molecular characterisation. These functionality assays were performed because the ability of rhizosphere bacteria to produce hydrolytic enzymes (like protease, chitinase and β-glucanase), secondary metabolites (including siderophores), or the ability to inhibit pathogen growth in dual culture are characteristics frequently associated with the suppression of soil borne plant diseases (Berg et al., 2005). Information obtained from the dilution plate counts and SSCP characterisation of bacterial populations did not directly correlate with the reported differences in susceptibilities. In contrast, the functionality assays indicated that although bacteria isolated from the rhizosphere of the most susceptible rootstock, 101-14, had more isolates with some biocontrol potential than those isolated from the rhizosphere of the least susceptible rootstock, Riparia Gloire, or the bulk soil, isolates from the rhizosphere of Riparia Gloire had more of the intense responses. These results, like those of Berg et al (2002), showed that different plant varieties have rhizosphere conditions that do not equally select for bacterial isolates with biocontrol capabilities and this may partially explain the differing susceptibilities to black foot.

In this study, the grapevine rootstock varieties supported different total NA bacterial counts ranging between 3.0 - 7.5x10^5 CFU per gram of dry soil in the rhizosphere. Total KB bacterial counts also differed between varieties, ranging from 1.7 - 5.5x10^5 CFU per gram of dry soil, while fluorescent Pseudomonad counts ranged from 1.6x10^1 to 2.5x10^4 CFU per gram of dry soil. Spore forming bacterial counts, which did not differ significantly between treatments, ranged from 4.2 - 6.2x10^4 CFU per gram of dry soil. These findings are in accordance with what others have reported for grapevine soils (Song et al., 2004).

Both Pseudomonad and spore forming bacteria are known for their potential as biological control agents. This study showed that total (NA and KB) and fluorescent Pseudomonad rhizosphere populations of the rootstock varieties tested did not correlate with their different susceptibilities to Cylindrocarpon black foot rot, as both the most (101-14) and least (Riparia Gloire) susceptible varieties supported similar rhizosphere populations, with similar counts recorded for the other rootstocks. However, no fluorescent Pseudomonads were isolated from the final control soil, suggesting that the plant roots supported this bacterial group. There were however, fluorescent Pseudomonads in the initial control soil (Appendix 2.5, Table 2.5-1),

with counts of $5.0x10^4$ CFU per gram of dried soil (similar to those of 5C at the same dilution). This is probably due to the previous growth of weeds in this vineyard soil, further supporting the idea that plant roots and/or exudates support this bacterial group. The number of spore forming bacteria did not differ between rootstock varieties, or the control, making it unlikely that these bacteria were responsible for the differences in black foot susceptibility either. However, it is possible that while counts of these bacteria did not differ significantly between rootstocks, their functionality and bacterial species composition, or strains, within the groups might have differed.

One possible explanation for the observed differences in the different bacterial plate counts is grapevine rootstock vigour. Plants of varieties 5C and Schwarzmann, which had the highest numbers of total bacteria and fluorescent Pseudomonads, typically have moderate vigour, while 101-14 is said to be of low-moderate vigour, but more vigorous than Riparia Gloire which has low vigour (Hoskins et al., 2003; pers. comm. G. Creasy, 2008). More vigorous grapevine rootstocks are likely to have greater root exudation than the less vigorous grapevine rootstocks since shoot development and root growth rate influence rhizodeposition (Kuzyakov, 2002). To confirm that the reported vigorousness of the rootstocks matched growth in this experiment, it would have been useful to record the root and shoot dry weights of the plants used in the experiment. However, since grapevine rootstock vigour is dependent on site (Kennison, 2008), what was apparent in the greenhouse may not apply to the vineyard environment.

Given that the observed differences in the bacterial plate counts do not appear to correlate with grapevine rootstock susceptibility to black foot, it is possible that the bacterial groups assessed here were too broad for differences in rhizosphere populations of specific bacteria to be detected. It is also possible that other kinds of functionality tests could have been more appropriate. In their study looking at the effect of plant species on the abundance and diversity of *V. dahliae* antagonistic bacteria, Berg et al (2002) used phenotypic characterisation to determine the biological control potential of these bacterial isolates. Their diverse analysis included dual plating assays, as well as the detection of lytic enzymes (glucanases, chitinases, proteases), siderophores, cyanide (HCN), indole-3-acetic acid (IAA) and N-acylhomoserine lactones (AHLs), compounds involved in quorum sensing.

Berg et al (2005) used these same functionality assays and employed a point system whereby bacterial isolates received points for antagonistic traits: 0-3 points for antagonism of *V. dahliae* and *Rhizoctonia solani* and 1 point for each other positive response (up to a total of 13 points). A similar system could have been employed here to detect more subtle differences

in the biocontrol potential of bacteria from different rootstock rhizospheres. While Berg et al (2005) found that no particular assay was best at identifying bacterial isolates with high antagonistic potential, the 20 bacterial isolates that scored the highest with the point system had the following traits: 20 were antagonistic to *R. solani*, 17 to *V. dahliae*, 18 had proteolytic activity, 17 had cellulytic activity and siderophore production and just 3 had chitinolytic activity. From this, one might conclude that the most important functionality tests when looking for bacteria with biocontrol potential are the dual plate, protease, cellulase and siderophore assays, however this is probably dependent on the pathogen and microenvironment.

The differences in susceptibility of the rootstocks might also be due to an unculturable bacterial component, or a non-bacterial component, of the grapevine rhizosphere. It has been established that mycorrhizal associations can be responsible for differences in plant disease resistance, with arbuscular mycorrhizae in particular having been shown to increase plant resistance to some soil-borne fungal and bacterial pathogens (Waschkies et al., 1994). For example, *Glomus fasciculatus* increased resistance of citrus plants to *Phytophthora parasitica* (Davis & Menge, 1980), *Glomus mosseae* increased resistance of tomato plants to *Fusarium oxysporum* (Dehne & Schonbeck, 1979) and a *Glomus* species increased resistance of cucumber plants to *Pythium ultimatum* (Rosendahl & Rosendahl, 1990).

The SSCP gel for the soil controls showed that there were noticeably more bands for the initial controls than for the final controls, probably reflecting a loss in species diversity. The soil used in this study was sourced from a vineyard where it was supporting a range of weed and grass species. This meant that at the time of sampling, the bacterial populations in the initial control soil had been influenced by this prior plant growth and associated root exudates. It is well known that the density and functional diversity of culturable soil bacteria increases linearly with the number of plant species, which is typically attributed to the increased range and volume of rhizodeposits, or the development of more diverse soil microhabitats (Stephan et al., 2000). In contrast, the final control soil had not experienced the addition of any fresh root exudates over the course of the study, and showed a concomitant decrease in bacterial diversity. This correlates well with the literature as Berg et al (2002) reported that bacterial counts for fallow soils were typically lower than those from rhizosphere soils, due to root exudates being a major carbon source for soil bacteria. In this study, bacteria were still present in the final control soils, but populations were probably dominated by more tolerant, saprophytic or spore-forming bacterial species.

There were some correlations between the SSCP findings and those of the dilution plating, regarding rootstock plant variability. The SSCP gels showed more bacterial diversity in the rhizosphere soil of 101-14 than for Riparia Gloire. However, when considering the dilution plating data, 101-14 and Riparia Gloire had very similar CFU counts for the different bacterial groups. This would seem to indicate that while species differed between plant varieties, the total and broad groups of rhizosphere bacteria were very similar. Perhaps of interest, is that as with the dilution plating, the rootstock with the most obvious result was 5C. Variety 5C had both the greatest banding variability between rhizosphere samples with SSCP, and the highest bacterial plate counts. This is somewhat unexpected because it is often the most susceptible plant cultivars that have the largest bacterial populations (Gilbert et al., 1994). However, 5C and Schwarzmann are reportedly more vigorous that the other rootstocks, and so this, in relation to the greater root exudation, probably accounts for them having the largest rhizosphere bacterial populations. However, 5C and Schwarzmann differed in the diversity of their rhizosphere bacteria since bacteria from the rhizosphere of Schwarzmann were not found to be highly diverse by SSCP.

The SSCP results were qualitative but not quantitative. So, while species diversity (as indicated by the banding patterns) was very similar for most plants (including those from the most and least susceptible rootstock varieties), the abundance or activity of these dominant species could have varied. It is possible that the very broad amplification of a relatively conserved ribosomal region missed potential species differences in rootstock rhizosphere composition. There might also have been less similarity in banding patterns between rootstock varieties if the plants had been left to grow for longer. For example, Smalla et al (2001) noted that the similarity between the rhizosphere DGGE patterns and those of the bulk soil decreased in the second year of an experiment. The plant dependent shifts in the relative abundance of bacterial populations between crops also became more distinct for strawberry, potato and oilseed rape. The primers used in this experiment, B342If and U806Ir, were also employed by Hori et al (2006) to investigate bacterial community structure and succession within a methanogenic bioreactor. B342If and U806Ir are universal bacterial primers which amplify the V3-4 region of 16S rDNA. The selection of the 16S rDNA region for amplification reflected an attempt to encompass the total soil bacterial community (Garbeva et al., 2008), although it is acknowledged that universal bacterial primers may not amplify all dominant members of the microbial community (Marschner et al., 2002). The primers may preferentially amplify some species over others, due to the secondary structure of the DNA flanking the priming site (Cullings et al., 2005). Further, DNA extraction efficiency varies for members of the microbial community (Marschner et al., 2002). Gel banding complexity could

have been reduced through the use of genus specific primers, however, this would have required prior choice of target genera. Such primers may have shown greater variation in the soil microbial community structure.

It is also possible that the size of the amplimer used for SSCP was too large. However, the size of the fragments used for SSCP here (464 bp) were similar to those of Hori et al (2006) (450 bp). The sensitivity of SSCP tends to be inversely proportional to the size of the fragments used, and Zinger et al (2007) stated that a 150-250 bp fragment is ideal. Differences of 1 bp can be resolved 99% of the time for fragments of 100-300 bp (Sunnucks et al., 2000; Zinger et al., 2007), 80% of the time for fragments of 400 bp (Girman, 1996), and only adequate resolution can be achieved for fragments up to 775 bp (Orti et al., 1997). Primers yielding smaller products could be used if this experiment were to be repeated. However when the universal bacterial primers Com1 and Com2 (Schwieger & Tebbe, 2000), which produced a 407 bp fragment, were trialled in a preliminary study, they were not successful.

DNA sequencing was used to identify the bacterial species that generated the SSCP banding patterns. Band excision was technically and spatially very difficult due to the closeness and high density of the bands, particularly in the lower regions of the gels. Using a longer gel might have reduced this by allowing the gels to run for longer, with greater separation on the lower portion of the gel, but such facilities were unavailable. This density of bands produced some ambiguity, with multiple products evident in several of the returned sequences, and some re-amplified bands producing multiple bands in the confirming SSCP gels. However, although some of this may have been due to imprecise excision it is also likely that some of the multiple bands were due to less favourable conformations of the same single stranded DNA sequence (Hayashi, 1991).

All bands sequenced from the SSCP gels were found to match most closely to uncultured bacteria, typically from DGGE bands of soil or sediment samples. The findings are in agreement with those of Dohrmann and Tebbe (2005) who reported that the majority of their bacterial sequences from the rhizosphere of herbaceous plants were most similar to those of uncultured microorganisms from soil and sediment. In the current study, secondary GenBank matches included *Rhodopseudomonas* species, Chromatiales, α-Proteobacterium, δ-Proteobacterium and γ-Proteobacterium representatives. *Rhodopseudomonas* is a genus in the α-Proteobacteria that includes culturable species (Okubo & Hiraishi, 2007) so it is possible that the species excised from the SSCP gels were represented in the culture plates. The order Chromatiales also includes culturable members and belongs to the γ-Proteobacteria class (Lee et al., 2007). Given the large size of these three Proteobacteria classes, they are

likely to include both culturable and unculturable members. Another explanation for why the sequences were most closely matched to uncultured microorganisms could in part be due to the relatively small size (464 bp) of the products (smaller when trimmed; 265-304 bp). Opelt et al (2007) also found in their study that *Burkholderia* bacterial sequences (412 bp) were too short to be unambiguously identified to species level. Their sequences showed matches of 88-100% to the NCBI database, which were similar to the 88-94% in our study. Alternatively, the lack of a genus or species match could indicate that novel groups of bacteria were present in the grapevine rhizosphere (Ahmad et al., 2009). It would have been useful to get sequences for the representative bacteria that were cultured (40-60 per rootstock variety), as well as for all the SSCP bands. This would have allowed correlations with functionality to be made.

One of the obvious difficulties in interpreting the SSCP gels was due to the tightly clustered bands at the bases of the gels. Although several attempts were made to improve this distribution, the outcome was still not ideal. Two different acrylamide: bis-acrylamide ratios were tested, and the superior band resolution was achieved with 37.5:1 rather than 29:1. Varying the concentration of acrylamide was also explored. The 6% acrylamide gels which are commonly used (Hayashi, 1991) resulted in smear-like bands, but 10% (wt/vol) gels gave better resolution, and were similar to those used by Opelt et al (2007) who used 8% and 9% gels. Although complementary single strands of DNA tend to separate more efficiently in gels with a low cross linking ratio (Hayashi, 1991), bands can be sharpened by the use of higher concentrations of acrylamide and crosslinker (Yip et al., 1999). Results might have been improved through the use of the specialist gel matrix MDE® (FMC Bioproducts) which others have used to get clearer separation of DNA fragments (Schwieger & Tebbe, 2000; Hori et al., 2006). Several different running times (10 h, 14 h, 18 h, 20 h, 21 h and 24 h), voltages (250 V, 300 V and 350 V) and running temperatures (15°C, 18°C and 20°C) were trialled, and 21 h at 250V and 15°C gave the best spread of bands without overheating the gel. Temperature choice is critical, as a particular temperature can either sharpen bands or reduce separation (Yip et al., 1999).

During the initial optimisation studies, the SSCP bands were very faint. Sunnucks et al (2000) recommended optimising PCR conditions until strong, clear products could be seen on agarose gels, since the faint bands may have been due to a low concentration of the PCR product. An ammonium acetate DNA precipitation was carried out to concentrate the DNA as outlined by Sambrook and Russel (2001). However, this did not have the intended effect as bands appeared even fainter, with increased streaking along the sides of the lanes. Hongyo et al (1993) have reported that reducing the amount of PCR product used in SSCP is a way of

combating incomplete denaturation which causes such streaking (provided the product is sufficiently concentrated). However, highly concentrated PCR products appear to be not ideal either.

Incomplete denaturation was thought to be the reason for the bands remaining incompletely resolved (with poor sharpness) and is frequently the reason behind unclear banding (Sunnucks et al., 2000). There is known to be a high rate of reannealing after denaturation (Selvakumar et al., 1997), and this can be a problem when high concentrations of DNA are used, as is typically the case in community analysis (Schwieger & Tebbe, 1998). In an attempt to overcome this, different gel additives were tested. The incorporation of 10% urea (a denaturant) gave significantly crisper bands than 5% urea, 5% glycerol, or combinations of both. Yip et al (1999) hypothesised that band smearing was the result of multiple conformations of single stranded DNA having very similar mobilities, but that these conformations could be made to move more uniformly by a denaturing environment. Alternatively, the strands can be in a transitional state between two or more conformations, or at equilibrium between two or more conformations (Hayashi, 1991).

Loading dye was made fresh and performed best when less than a week old. Different additives were incorporated in the dye solution, both individually, and in combination. Additives included 0.1% SDS, 20 μM EDTA and 100 μM NaOH, but a combination of EDTA and NaOH yielded the clearest result. The ratio of loading dye to PCR product was also varied in an attempt to increase denaturation, with 2 μL of PCR product to every 30 μL of loading dye giving the sharpest bands. Large volumes of denaturant and loading dye probably reduced the likelihood of complementary DNA strands reannealing. Other concentrations of loading dye to PCR product tested included: 17:5, 25:7, or 30 parts to 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, or 8. The best ratio of loading dye to product (30:2) was used in conjunction with a longer (7 min) heat denaturation protocol than initially trialled (5 min), yielding crisper bands with less streaking.

In an attempt to simplify the dense gel banding patterns, the primers B342If and U806Ir-(Ph) were used so that one of each of the complementary DNA strands might be phosphorylated. Lambda exonuclease (Fermentas, Vilnius, Lithuania) was used to digest the phosphorylated strand of each PCR generated DNA molecule, resulting in completely single stranded product. While this was used successfully by others including Schweiger and Tebbe (1998) and Hori et al (2006) to get a less complex gel, it was not successful in this instance, even when a purification kit (QIAGEN QiaQuick® spin columns, microcentrifuge and vacuum protocol, Germany) was used prior to or after the digestion.

After extensive optimisation, an attempt to decrease PCR product concentration revealed that reducing the number of PCR cycles from 32 to 30, significantly increased the resolution of the bands. There was not sufficient time to re-amplify all the samples, but if the experiment were repeated, it might be advisable to use fewer PCR cycles. Finally, different gel stains were tested in an attempt to gain clearer visualisation of the bands without excessive background staining. The silver stain with the NaOH buffer (Zhou et al., 2005) gave the strongest bands, although background staining was significant. Other stains tested include SYBR® Safe (Invitrogen), Syber Gold (Invitrogen), Ethidium Bromide and three modifications of the silver stain (Bassam & Gresshoff, 2007; Ji et al., 2007; Han et al., 2008). It is possible that the pale SSCP banding, despite strong PCR products (as seen on agarose gels), could be due to most of the sequences having very high mobility conformations, which were lost off the SSCP gels. However, this is normally only the case for some sequences (Sunnucks et al., 2000). A time staggered experiment could determine if the main bands were running off the gel. Overall, given that SSCP is meant to be simpler and require little optimisation (Sunnucks et al., 2000), and taking into account how much time was spent on optimisation, perhaps DGGE, although more involved, would have been a better choice.

Ideally, given the findings of dilution plating and molecular characterisation, it would have been desirable to use rootstock 5C in the functionality assays, but experimental constraints did not allow for this. Instead, Riparia Gloire and 101-14, with their different susceptibilities to black foot were used, meaning that comparisons across all experiments could only be made for these rootstocks. More bacteria with potential biocontrol activity were isolated from the rhizosphere of 101-14 than Riparia Gloire or the control. However, perhaps differences in susceptibility are due to the activity of a few dominant and highly active rhizosphere bacteria (as seen with Riparia Gloire's glucanase and siderophore activity), rather than the less intense activity of a larger number of bacterial isolates (as seen with 101-14). With the observed differences in the functionality profiles of the different treatments, it is apparent that the rootstocks are showing some degree of influence in terms of their rhizosphere bacterial community. This is not unexpected given that different plant species and even cultivars of the same species have been shown to select different rhizosphere bacterial communities, probably due to differences in root exudation, as outlined previously.

The colony morphologies of the bacterial isolates used for functionality testing were very similar between treatments (Appendix 2.6). However, given the differences observed in rootstock activity, with bacteria isolated from the rhizosphere of 101-14 having the largest proportion of protease, glucanase and siderophore producing bacterial isolates, one might expect them to be from a more morphologically diverse selection. Isolates in this trial were selected based by their apparent frequency, and some may have been of the same bacterial species or strain. In other trials like those by Berg et al (Berg et al., 2002; 2005), selection of isolates was based on differences in colony morphology, thereby potentially increasing isolate diversity. Perhaps this is part of the reason why they found a higher number of functionally active isolates. Furthermore, Berg et al (2002) used the nutrient poor medium, R2A for dilution plating, sometimes in combination with high molecular weight substrate enrichment plates. They noted that the enrichment plates favoured the fast growing γ-Proteobacteria. Perhaps the nutrient rich NA used here also favoured the faster growing groups at the expense of the slower growing ones, resulting in a bias in the bacteria selected as representatives for the different rootstock varieties, and this could have a follow on effect for the functionality assays. However, since the Proteobacteria were common in the molecular characterisation of the grapevine rhizosphere, this does not appear to have been a major concern here.

Dual cultures with *C. destructans* were used to test for antagonistic activity. Bacterial isolates from the rhizospheres of Riparia Gloire and 101-14 as well as the bulk soil of the control treatment did not differ in their degree of antagonism towards *C. destructans* in dual culture. Thus, there was not a correlation between the dual plating results and the increased number of bacteria that produced high levels (degree) of metabolites (although at times a lower percentage of active isolates), associated with biocontrol activity, from the rhizosphere of Riparia Gloire. Although the bacteria from the rhizosphere of grapevine plants were not antagonistic towards *C. destructans* in dual culture, it does not mean that they were incapable of doing this *in vivo*. For the dual plating assays, the nutrient rich fungal medium, PDA was used, rather than the Waksman agar used by Berg et al (Berg et al., 2002; 2005). As PDA is a general fungal growth media it may not be conducive for antibiotic production and may have limited bacterial growth. False negatives were possible since the dual plating environment is artificial compared to the soil environment, and antibiotic synthesis is known to be reliant on the metabolic status of the cell, nutrient availability and environmental stimuli (Thomashow, 1996). Plant development also influences antibiotic production, something not replicated in the dual plate assay. For example, DAPG production is induced by the root exudation of older plants, but not those of young plants (Picard et al., 2000). Barka et al (2002) also reported that dual plates are not always an accurate measure of bacterial antagonism of fungi.

The percentage of rhizosphere bacterial isolates antagonistic to fungal pathogens in other dual plating trials was similar to that found here. For example, Berg et al (2002) reported that the proportion of bacteria isolated from the soil that were antagonistic varied between the rhizospheres of strawberry (9.5%), oil seed rape (6.3%), potato (3.7%) and bulk soil (3.3%). Berg et al (ref 2005) observed that 3-13% of rhizosphere isolates were antagonistic to the soil-borne pathogens *V. dahliae* and *Rhizoctonia solani*. In another study, Berg et al (2006) found that the proportion of antagonistic bacterial isolates was 9.6% in rhizospheres, and that there were a higher proportion of antagonists from the strawberry rhizosphere (10.2%) than from the rhizosphere of oilseed rape (8.9%). Here, few isolates from the rhizosphere of 101-14 (12.5%) and Riparia Gloire (10%) showed moderate inhibition of *C. destructans*, and just one from both the control and 101-14 treatments showed strong inhibition (2.5%).

Considering the functionality assays performed, it is possible that false negatives occurred as a function of the bacterial concentration in the nutrient broths used to inoculate the plates. Certain numbers of bacteria may be required before they can produce certain secondary metabolites or extracellular hydrolytic enzymes. Berg et al (2002) noted that for a large number of fungal antagonistic bacteria, the genes involved in disease suppression are regulated in response to bacterial population densities, a phenomenon known as quorum sensing (Pierson et al., 1994; 1998; Eberl, 1999). If time had been sufficient, it would have been useful to have determined the number of CFUs used to inoculate the assay plates. For the purpose of this experiment, this issue was dealt with by consistently using one bacterial colony to inoculate broths which were grown for similar periods and conditions.

While large differences between grapevine rootstocks susceptible to and more tolerant of black foot were not detected, rhizosphere bacteria may still be responsible for such differences in susceptibility to black foot. The rhizosphere bacteria of Riparia Gloire may not directly antagonise *C. destructans* through the production of secondary metabolites or hydrolytic enzymes, but they may have an indirect effect in the complex grapevine rhizosphere system. For example, plant growth promoting bacteria have been implicated in the biological control of plant pathogens through competition for ecological niches (root surface and rhizosphere), thereby possibly reducing infection by colonising infection sites, producing inhibitory secondary metabolites or by inducing systemic resistance in the plant. Competition for nutrients may also play a part (Compant et al., 2005).

In conclusion, the role of grapevine rhizosphere bacteria in influencing grapevine rootstock susceptibility to black foot is inconclusive. Dilution plating showed that total (NA and KB) and fluorescent Pseudomonad rhizosphere populations of the rootstock varieties tested did not correlate with their different susceptibilities to Cylindrocarpon black foot rot. SSCP reinforced this, showing genus/species diversity to be very similar for most rootstocks. However, the functionality tests indicated that Riparia Gloire, the least susceptible rootstock to Cylindrocarpon black foot rot, had more rhizosphere bacteria with strong responses than did the more susceptible 101-14. These rhizosphere bacteria with the increased ability to produce hydrolytic enzymes or siderophores may play a role in suppressing Cylindrocarpon black foot disease.

CHAPTER 3

CYLINDROCARPON DESTRUCTANS AND *FUSARIUM OXYSPORUM* PATHOGENICITY TRIAL

3.1 INTRODUCTION

Cylindrocarpon destructans and *Fusarium oxysporum* are two root rot pathogens of grapevines. *Cylindrocarpon destructans* is a causal agent of black foot, a disease that is a serious threat to grapevines around the world (Halleen et al., 2006a). *Fusarium oxysporum* has been isolated from the rotting roots of declining grapevines (Grasso, 1984) but has not always been regarded as a causal agent of the rot. Granett et al (1998) reported that it was rarely invasive unless roots were damaged or phylloxera-galled. However, in glasshouse trials, Highet and Nair (1995) confirmed the potential of *F. oxysporum* as a grapevine pathogen. Further, *F. oxysporum* f.sp. *herbemontis* was considered by de Andrade (1993) to be the most important pathogen of grapevine cultivars in Brazil, where it causes significant plant death.

As established earlier (Table 1.1), grapevine rootstocks differ in their susceptibility to black foot disease, as demonstrated by Harvey and Jaspers (2006) and Jaspers et al (2007). However, little is known about the significance of *F. oxysporum* in New Zealand vineyards since the susceptibilities of commonly used New Zealand rootstocks to this pathogen are unknown. Differences in susceptibility have been investigated in Brazil, where de Andrade et al (1995) reported that the resistance of rootstock varieties to *F. oxysporum* f.sp. *herbemontis* was variable. In Australia, Edwards et al (2007) observed that the extent of root necrosis caused by *F. oxysporum* was influenced by plant vigour, with low vigour vines having greater disease severity than more vigorous vines.

The research trial reported here investigated the resistance of grapevine rootstock varieties Riparia Gloire, 5C, Schwarzmann and 101-14 to *C. destructans* and *F. oxysporum*. It also aimed to confirm the previously recorded susceptibilities of grapevine rootstocks to *C. destructans*. With soil being used as the growth medium, the trial could make some comparisons between previous glasshouse trials done in potting mix (Harvey & Jaspers, 2006), and field trials in vineyards (Bleach, 2007).

3.2 METHODS

3.2.1 Plant preparation

For this experiment, four ungrafted rootstock varieties were used. They were: 101-14, Schwarzmann, 5C and Riparia Gloire. Callused cuttings of 5C and 101-14 were obtained from a commercial nursery, Corbans Viticulture (Auckland, New Zealand). Cuttings of Schwarzmann and Riparia Gloire were obtained from the Lincoln University vineyard and callused in pots of vermiculite set on heat pads at 25°C for two months (April and May 2006). The cuttings of all four rootstock varieties were grown in 2.5 L pots containing a 50/50 mix of soil (Wakanui silt loam classified as a mottled immature pallic soil), which was sourced from the Lincoln University vineyard, and a potting mix [(80% horticultural bark (grade 2): 20% pumice (grade 3, 1-4 mm)]. The potting mix was amended with 2 kg of a 3-4 month fertilizer, 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. Soil was introduced into the potting mix in order to ensure that key soil fungi and bacteria were available for the development of the complex interactions typical of a natural soil environment, and to more closely reflect natural infection of grapevines in a vineyard. After three months growth, the plants were repotted into 4 L pots of the same soil/potting mix amended with 5 kg of an 8-9 month fertilizer, 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. Any dead vines were replaced with rooted, callused cuttings (September 2006) from the same source which had been grown to the same developmental stage in potting mix (for numbers of replacements, see Table 3.6-1 in Appendix 3.6). These were also grown in 4 L pots.

Pots were laid out in a completely randomised design on corrugated metal tables for 1 month, and on mesh tables for the remaining period. Vines were kept moist by daily watering and grown in a greenhouse for a total of 11 months spanning winter (June 2006) – winter (May 2007). During this time, temperatures ranged from 14ºC (minimum) to 30ºC (maximum). Vines were placed under high pressure sodium lamps (Son-T Agro 400, Philips) from the start of autumn (March 2007) until harvest (May 2007) to ensure light levels were sufficient for good growth. All weeds were removed by hand.

3.2.2 Pathogen inoculation

Plants of each rootstock variety were inoculated with *C. destructans*, *F. oxysporum*, or SDW (controls), in spring (November 2006). The *C. destructans* inoculum was a mixture of three isolates that had been isolated from vineyards and were reported as pathogenic to grapevines

in other trials, being Col c (Central Otago), Mar 13a (Marlborough), WPA 1a (Waipara) (pers. comm. C. Probst, 2006) and B1N3P4 (Lincoln) (pers. comm. C. Bleach, 2006). Cultures of these isolates were grown on PDA for 7-14 d at 20ºC.

The *F. oxysporum* isolates used were originally isolated from grapevine material, morphologically identified by Ian Harvey (Plantwise, Lincoln) and made available as several mixed culture plates. From these, six isolates (80B, 81B, 105A, 39A, 127B and 24B) were selected, grown in pure culture and their identities confirmed by sequencing. The ribosomal DNA (rDNA) was amplified from fungal mycelia using REDExtract-N-Amp™ (Sigma-Aldrich, USA) as per the manufacturer's instructions. Fragments of the internal transcribed spacer (ITS) regions of fungal rDNA were amplified using primers ITS4 and ITS5 (5′-TCCTCCGCTTATTGATATGC-3′ and 5′-GGAAGTAAAAGTCGTAACAAGG-3′, respectively) using the following protocol: 94°C for 2 min (denaturation), then 35 cycles of: 30 s at 94°C (denaturation), 30 s at 55°C (annealing) and 1 min at 72°C (elongation), with a final cycle of 7 min at 72°C.

Sequencing of PCR products was done in one direction at the Lincoln University Sequencing Facility using primer ITS5. Sequences of about 520 bp were obtained and analysed using the GenBank (http://www.ncbi.nlm.nih.gov/) BLAST (Altschul et al., 1990) function, confirming all isolates were *F. oxysporum* (99% maximum identity and 100% coverage) (Table 3.2-1, Appendix 3.2). For this trial, *F. oxysporum* isolates that differed in colour and colony morphology were selected in an effort to maximise the genetic diversity of the isolates used to inoculate plants (Figure 3.3-2, Appendix 3.3). However, a sequence comparison using DNAMAN version 4 (Lynnon Corporation, Quebec, Canada) revealed isolates did not significantly differ from each other and shared great sequence homology (Table 3.2-2, Appendix 3.2). Cultures of these six isolates were grown on oatmeal agar (30 g oatmeal, 20 g agar per 1L SDW), with plates left unwrapped and exposed to natural light so as to increase sporulation (Yamoah, 2007) for 14 d at 18-20ºC.

Cultures of *C. destructans* and *F. oxysporum* were washed with SDW containing 0.001% Tween 80 (polyoxyethylene 20 sorbitan mono-oleate; BDH Chemicals Ltd), and gently stroked with sterile glass slides to loosen conidia. These suspensions were sieved (150 µm pore size) to exclude any bulk mycelium. A haemocytometer was used to count conidia and concentrations adjusted to 10^6/mL with SDW and Tween 80 (0.001%). For each pathogen, inoculum was prepared by mixing equal volumes of conidial suspensions from all isolates. A diluted sample (100 µL of 10^3/mL) of this mixed-isolate suspension was plated on PDA,

incubated at 20ºC for 2 d and colony forming units (CFU) counted. An average of 97 colonies grew on each of the three replicate plates, indicating 97% conidial viability.

On the day of inoculation, immediately prior to the addition of the conidial suspensions, the root systems of all the vines were wounded using an asparagus knife (which had a sharp, square tip) that was driven vertically down into the soil at four equidistant locations approximately 8 cm from the trunk. All plants were inoculated with 50 mL (per plant) of the appropriate mixed 10^6 conidial suspension, or 50 mL SDW (controls). The conidial suspension was gently poured over the soil surface close to the wounding sites. All pots were given a further 50 mL of tap water to help carry conidia into the soil, and then left undisturbed for 24 h. After inoculation, the plants were grown for a further 6 months to allow symptoms to develop. Fifteen replicates were set up per treatment to allow for loss of plants. Subsequent assessment of pathogen infection was conducted on all living replicates, 8-10 plants per treatment. The different treatments are summarised in Table 3.1.

Table 3.1: The 12 treatment combinations in this experiment, showing rootstock variety, inoculum type and the number of replicates assessed.

Rootstock	Treatment	Description	Replicates
101-14	O	not inoculated	10
	F	inoculated with *F. oxysporum*	9
	C	inoculated with *C. destructans*	10
5C	O	not inoculated	10
	F	inoculated with *F. oxysporum*	9
	C	inoculated with *C. destructans*	8
Schwarzmann	O	not inoculated	10
	F	inoculated with *F. oxysporum*	8
	C	inoculated with *C. destructans*	9
Riparia Gloire	O	not inoculated	10
	F	inoculated with *F. oxysporum*	10
	C	inoculated with *C. destructans*	8

3.2.3 Plant assessment

3.2.3.1 Root and shoot dry weights

After 11 months growth, grapevine plants were uprooted, washed to remove all soil, air dried for 2 h at room temperature and the roots cut off at the trunk base. Roots from each plant were placed in a separate brown paper bag which was closed with a single staple. The shoots of each plant, minus the trunk, leaves and petioles, were placed in a brown paper bag and closed

with a single staple. The bags of roots and shoots were dried in a 50˚C oven to constant weight at the Field Service Centre, Lincoln University, and after 14 d were weighed (Scheck et al., 1998b). At this time the recorded dry weights were found to be stable, since weights of random samples did not differ between two consecutive days. Due to the dark colour of the roots it was not possible to examine them for the presence of disease related necrosis.

3.2.3.2 Infection status

3.2.3.2.1 Surface sterilisation

The vine trunks were sterilised in batches of the same treatment. They were washed thoroughly with tap water, air dried and the lower 20 cm cut off to enable this section to be completely submerged in the tanks of sterilising solutions. These trunks were soaked in 70% ethanol for 30 s, in a 0.35% sodium hypochlorite solution for 5 min and in 70% ethanol for 30 s. Each trunk was then wrapped in a new paper towel to absorb the excess ethanol, then placed in a new plastic bag, and stored at 4ºC until tissue isolation (which was done within 24 h of surface sterilisation).

3.2.3.2.2 Tissue isolation

The root crown, comprising the lowest 1-2 cm of the trunk base was discarded. A 1-2 mm slice of the basal end of the trunk (0 cm) was then cut into four uniform tissue pieces (~3 mm^2) and placed equidistantly near the outer edge of a PDA plate amended with the antibiotic chloramphenicol (Sigma; 0.25 g/L). Another sliver of trunk tissue was taken 5 cm above the basal section and placed in the centre of the same PDA plate (Figure 3.1). Plates were incubated at 20˚C with 12 h of dark and 12 h of light, for a period of 7 d, after which the tissue sections that gave rise to characteristic *C. destructans* and *F. oxysporum* colonies were counted (Figures 3.2 and 3.3).

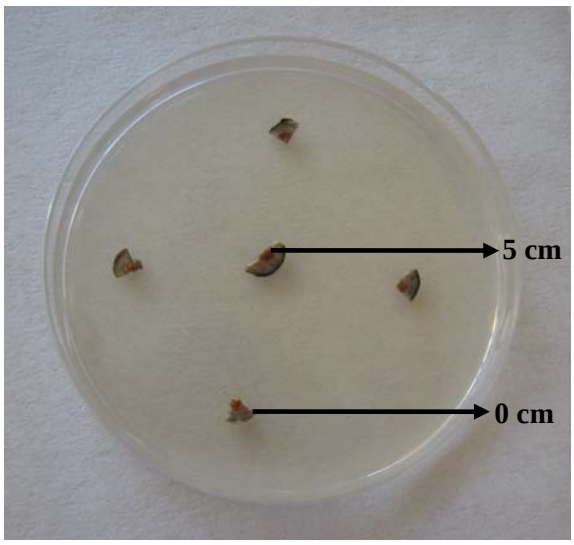


Figure 3.1: The orientation on PDA plates of tissue fragments, four taken from the basal end (0 cm) and one taken 5 cm from the basal end (5 cm), of a grapevine trunk.

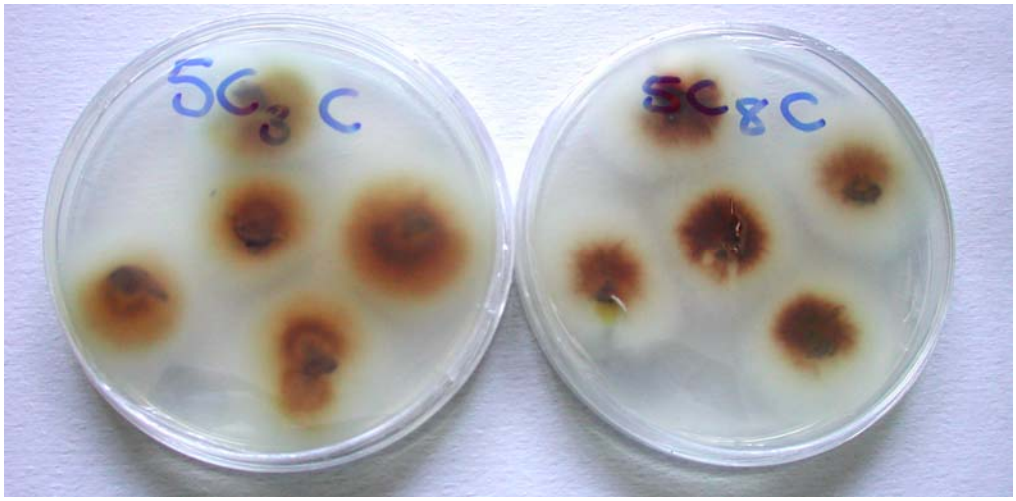


Figure 3.2: Examples of stem pieces infected with *Cylindrocarpon destructans* isolates after incubation on agar.

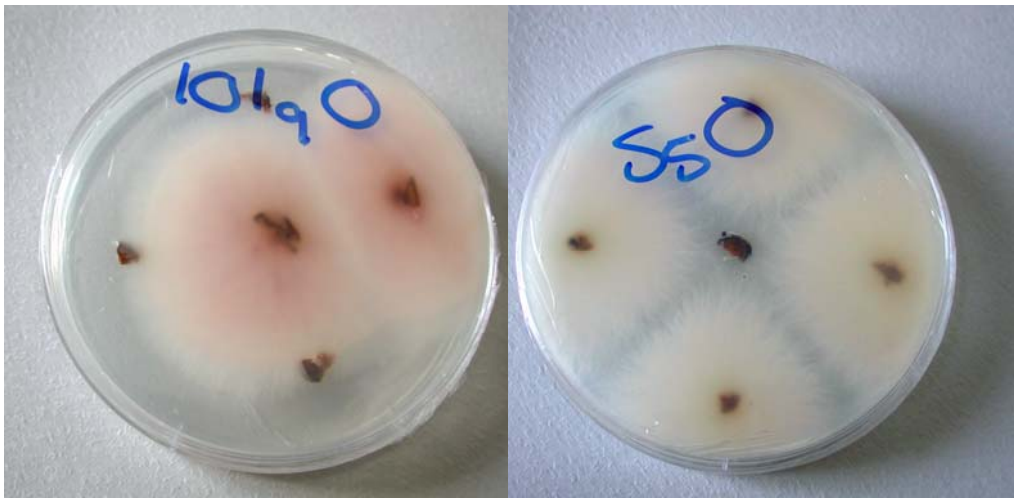


Figure 3.3: Examples of stem pieces infected with *Fusarium oxysporum* isolates after incubation on agar.

3.2.3.2.3 Identification

Fungal isolates from the experimental plants were identified as *C. destructans* and *F. oxysporum* by comparison with culture plates and conidia of the pathogens used for inoculation. All isolates were identified by colony morphology first, and at least 30% of all suspected positives (for both pathogens) were randomly selected for subculture. From these subcultures, slide mounts of conidia were made and examined by light microscope. The criteria used in the identification of *C. destructans* and *F. oxysporum* isolates are described in Appendix 3.3 and Appendix 4.4. Accuracy of identification was calculated by dividing the number of correctly identified isolates from the tissue pieces (based on spore morphology) by the number of isolates subcultured, and multiplying by 100.

3.2.4 Statistical analysis

Experimental data was analysed by Univariate Analysis of Variance (ANOVA) using SPSS® for Windows® V13.0 (SPSS Inc., Chicago, IL, USA, 2007). The normality of the data set was confirmed through visual assessment of the residual plots and considered adequate. ANOVA was considered the most appropriate analysis as it allowed for the experimental design. Non-parametric tests would not have accurately represented the data (pers. comm. C. Frampton, 2008). When ANOVA indicated overall significance for disease severity data (proportion of infected tissue pieces), and dry weight data, multiple comparison tests (pair-wise) were conducted with Fisher's Protected Least Significant Difference (LSD) tests. ANOVA was also used to determine the effect of overall (plant) *C. destructans* and *F. oxysporum* disease incidence on root and shoot dry weights (Tables 3.5-9 and 3.5-10, Appendix 3.5). Logistic regression was conducted on disease incidence data, (proportion of infected plants) to account for experimental design, and then treatments compared using Chi-square and Pearson Chi-square, to determine significance of the differences. Overall incidence was determined from samples taken at 0 cm and 5 cm from the stem base. The mean values given in the text are those predicted by the ANOVA (raw data means shown in Appendix 3.5). As there was no indication of variation between them, replacement and original plants were analysed together.

3.3 RESULTS

3.3.1 Infection status: tissue isolation

3.3.1.1 Identification of isolates

Accuracy of identification from colonies grown on PDA was deemed to be approximately 70% for *C. destructans* and 90% for *F. oxysporum*. Isolates of *F. oxysporum* and *C. destructans* cultured from the experimental plants had spore and colony morphologies that

matched those of the isolates used to inoculate the plants. It was considered acceptable to include the other similar *Cylindrocarpon* or *Fusarium* species in the assessment because they reflected the natural situation being investigated in this experiment. The original isolation plates contained other fungi, mainly *Fusarium* sp., *Alternaria* sp., *Botryosphaeria* sp., *Pythium* sp., *Penicillium* sp. and *Paecilomyces* sp. (Table 3.1-1, Appendix 3.1).

3.3.1.2 Disease severity (0 cm) and incidence (5 cm)

The significances and sources of variation for disease severity at 0 cm differed for *F. oxysporum* and *C. destructans*. Inoculation with *F. oxysporum* or *C. destructans* increased disease severity, *P*=0.018 and *P*=0.056, respectively (Table 3.2). Non-inoculated controls had the lowest disease severity for both pathogens, but *C. destructans* (10.0%) background infection was slightly lower than that of *F. oxysporum* (13.1%). Background infection was present because the soil provided inoculum. The effect of rootstock variety on disease severity by the two pathogens at 0 cm was large (*P*=0.000) for *C. destructans* and (*P*=0.090) for *F. oxysporum*. There was an interaction between inoculation treatment and rootstock variety for *F. oxysporum* (*P*=0.062) but not *C. destructans* (*P*=0.300).

Analysis of both *F. oxysporum* and *C. destructans* disease incidence at 5 cm revealed that neither inoculation treatment (*P*=0.165 and *P*=0.116, respectively) nor the interaction between rootstock variety and inoculation treatment was significant (*P*=0.904 and *P*=0.894, respectively). *Cylindrocarpon destructans* disease incidence was influenced by rootstock variety (*P*=0.002), mimicking the significance at 0 cm. However, *F. oxysporum* disease incidence at 5 cm was not influenced by rootstock variety (*P*=0.385).

Table 3.2: The effect of inoculation treatment (*C. destructans*, *F. oxysporum* or none) on *F. oxysporum* and *C. destructans* disease severity (% infected wood pieces) at 0 cm (*P*=0.018). Within a column, values followed by different letters were significantly different at *P*=0.05 according to Fisher's Protected LSD.

Inoculation treatment	***F. oxysporum* disease severity (%)**	***C. destructans* disease severity (%)**
C (*C. destructans*)	26.9 ab	23.0 a
F (*F. oxysporum*)	34.1 a	9.45 b
O (control, none, SDW)	13.1 b	10.0 b

3.3.1.2.1 Fusarium oxysporum disease severity

Analysis of *F. oxysporum* disease severity data at 0 cm from the stem base (ANOVA Table 3.4-3, Appendix 3.4) revealed significant differences in severity, depending on whether a plant was inoculated with *F. oxysporum*, *C. destructans* or left non-inoculated (*P*=0.018)

(Table 3.2). There was greater disease severity in the *F. oxysporum* inoculated plants than in the non-inoculated controls (P=0.005) but it was not greater than in the *C. destructans* inoculated plants (P=0.340). The difference between plants inoculated with *C. destructans* or left non-inoculated was almost significant, with a trend for greater disease severity in the *C. destructans* inoculated plants than in the non-inoculated controls (P=0.065). Rootstock variety alone caused some effect on *F. oxysporum* disease severity (P=0.090) at 0 cm. Riparia Gloire (32.7%) had the greatest *F. oxysporum* disease severity, while 101-14 (26.8%) and Schwarzmann (27.4%) had similar, moderate *F. oxysporum* disease severity, and 5C (11.8%) had the least (Figure 3.4).

There was some interaction between grapevine rootstock variety and inoculation treatment (P=0.062) which affected *F. oxysporum* disease severity at 0 cm. The susceptibilities of the rootstocks were not consistent for the different inoculation treatments. Figure 3.4 shows the effect of *F. oxysporum* inoculation on the disease severity of both *F. oxysporum* and *C. destructans*. Inoculation with *F. oxysporum* resulted in 101-14 having the greatest *F. oxysporum* disease severity (52.9%), Riparia Gloire (32.5%) and Schwarzmann (36.9%) having moderate *F. oxysporum* disease severity, and 5C having the lowest *F. oxysporum* disease severity (14.0%). Inoculation with *F. oxysporum* did not increase *C. destructans* disease severity at 0 cm. For *F. oxysporum* inoculated and non-inoculated plants *C. destructans* disease severity was 22.2% and 25.0% (101-14), 16.6% and 15.0% (5C), 0% and 0% (Riparia Gloire) and 1.0% and 0% (Schwarzmann), respectively.

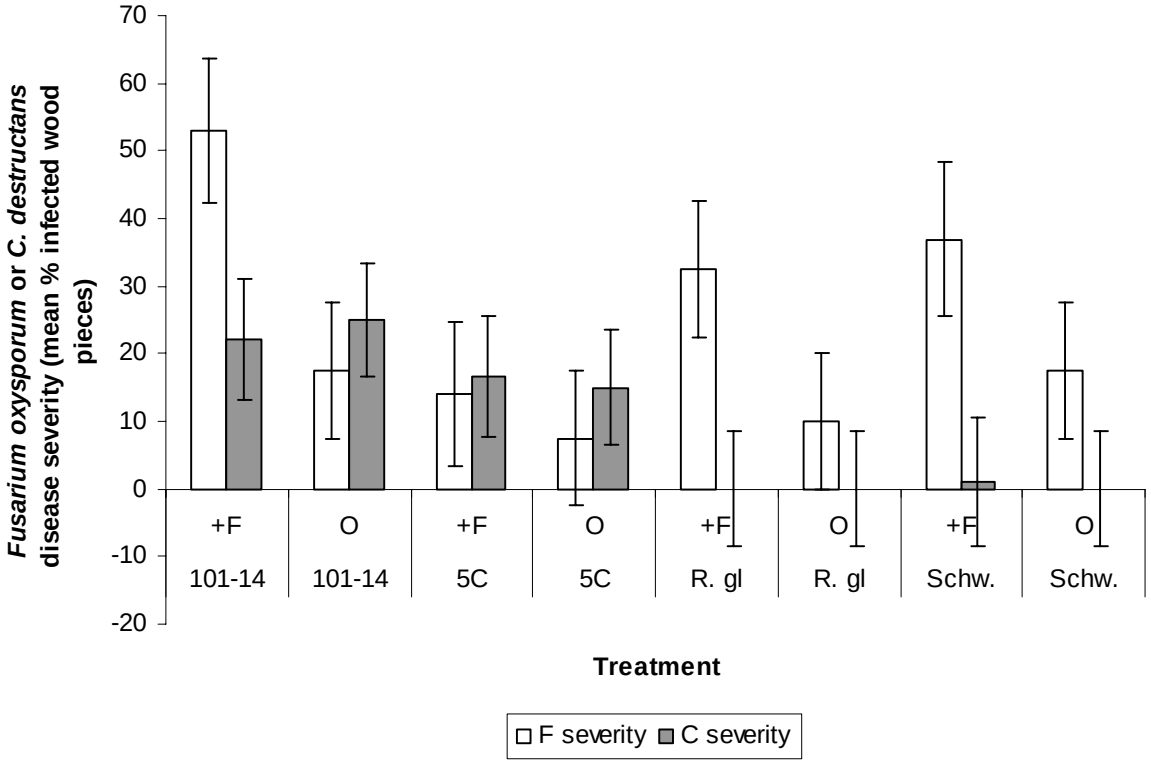


Figure 3.4: The effect of *F. oxysporum* inoculation (+F) compared to no inoculation (O) on both *F. oxysporum* (F) and *C. destructans* (C) disease severity for the different grapevine rootstocks, at 0 cm from stem base. Error bars are SE of the means and show overall differences between treatment combinations.

3.3.1.2.2 Fusarium oxysporum disease incidence

Analysis of *F. oxysporum* disease incidence at 5 cm from the stem bases (ANOVA Table 3.4-4, Appendix 3.4) showed that it was not affected by *F. oxysporum* inoculation (P=0.165), by rootstock variety (P=0.385), or by the interaction between rootstock variety and inoculation treatment (P=0.904). When overall *F. oxysporum* disease incidence was considered, taking into account infection at both 0 cm and 5 cm, the effect of rootstock variety was not significant (P=0.123), and pairwise comparisons could not be undertaken. Overall *F. oxysporum* disease incidence for the rootstock varieties was 55.6% (Schwarzmann), 48.3% (101-14), 42.9% (Riparia Gloire) and 25.0% (5C).

3.3.1.2.3 Cylindrocarpon destructans disease severity

Inoculation treatment influenced *C. destructans* disease severity at 0 cm (P=0.056). As expected, there was greater *C. destructans* disease severity when plants were inoculated with *C. destructans* (23.0%), than when they were inoculated with *F. oxysporum* (9.5%) or left non-inoculated (10.0%). Pairwise comparisons showed that plants inoculated with *C. destructans* had greater *C. destructans* disease severity than those inoculated with

F. oxysporum (*P*=0.034) or non-inoculated (*P*=0.039), the latter being similar (*P*=0.931) (Table 3.2).

Analysis of *C. destructans* disease severity data at 0 cm from the stem base, (ANOVA Table 3.4-1, Appendix 3.4) revealed significant differences in severity depending on the grapevine rootstock variety involved (*P*=0.000). Rootstock varieties 101-14 and 5C did not differ in their disease severities (*P*=0.988) (28.2% and 28.1%, respectively), and both had significantly higher disease severity than either Riparia Gloire (0.3%, *P*=0.000) or Schwarzmann (0.6%, *P*=0.000). The latter two were similar in their disease severities (*P*=0.900) (Table 3.3).

Table 3.3: *Cylindrocarpon destructans* disease incidence data (5 cm and overall per plant) and disease severity data (% infected wood pieces at 0 cm) for different grapevine rootstock varieties. Within a column, values followed by different letters were significantly different at *P*=0.05 (according to Fisher's Protected LSD).

Rootstock variety	***C. destructans* disease incidence (%)**		***C. destructans* disease severity (%)**
	5 cm	**Overall***	
101-14	33.9 a	44.8 a	28.2 a
5C	16.7 ab	50.0 a	28.1 a
Riparia Gloire	4.1 b	3.6 b	0.3 b
Schwarzmann	3.1 b	7.4 b	0.6 b
***P* value**	0.002	0.000	0.000

* Overall disease incidence being the combined plant mean for 0 cm and 5 cm incidence data.

The interaction between rootstock variety and inoculation treatment on *C. destructans* disease severity was not significant (*P*=0.300). Figure 3.5 shows the effect of *C. destructans* inoculation on the disease severity of both *F. oxysporum* and *C. destructans*. Inoculation with *C. destructans* resulted in 5C (52.7%) having the greatest *C. destructans* disease severity, followed closely by 101-14 (37.5%), while Riparia Gloire (1.0%) and Schwarzmann (2.7%) had low *C. destructans* disease severity.

The effect of *C. destructans* inoculation on *F. oxysporum* disease severity differed between rootstocks (*P*=0.062). There was no significant increase caused in 101-14 (10%) and 5C (14%) (Figure 3.5), however, in Riparia Gloire, *C. destructans* inoculation caused a significant (*P*<0.05) increase in *F. oxysporum* disease severity (55.7%) compared to non-inoculated (10%) and *F. oxysporum* inoculated (32.5%). For Schwarzmann, the *F. oxysporum* disease severities for these treatments were 27.9%, 17.5% and 36.9%, respectively. For Riparia Gloire and Schwarzmann, inoculation with *C. destructans* increased *F. oxysporum* disease severity at 0 cm.

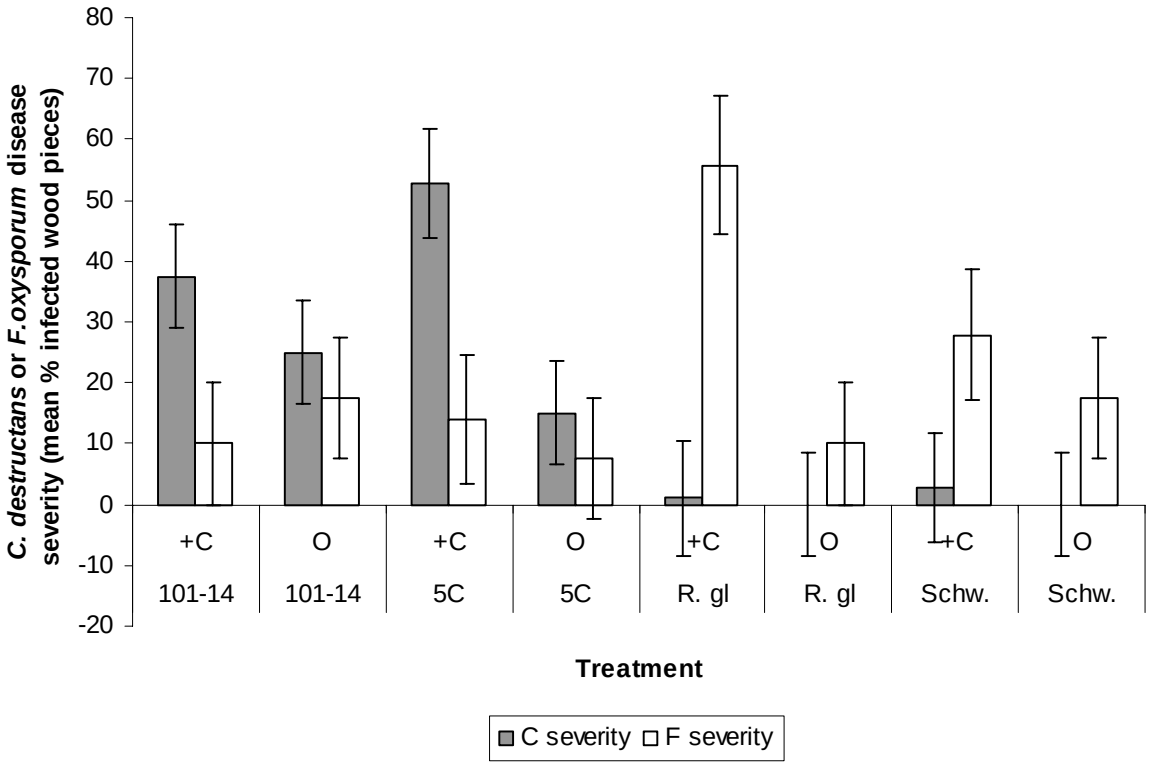


Figure 3.5: The effect of *C. destructans* inoculation (+C) compared to no inoculation (O) on both *C. destructans* (C) and *F. oxysporum* (F) disease severity for the different grapevine rootstocks, at 0 cm from stem base. Error bars are SE of the means and show overall differences between treatment combinations.

3.3.1.2.4 Cylindrocarpon destructans disease incidence

Overall, *C. destructans* disease incidence at 5 cm from the stem base (ANOVA Table 3.4-2, Appendix 3.4) was not affected by *C. destructans* inoculation (P=0.116), nor was it affected by the interaction between rootstock variety and inoculation treatment (P=0.894). Only rootstock variety significantly influenced *C. destructans* disease incidence (P=0.002). Plants of 101-14 had greater *C. destructans* disease incidence than all other varieties ($P \leq 0.05$) which were similar ($P \geq 0.05$) (Table 3.3). Similar trends in disease incidence were observed at 0 and 5 cm. When overall *C. destructans* disease incidence was considered, taking into account both 0 cm and 5 cm infection, there was a significant effect of rootstock variety (P=0.000). Pairwise comparisons revealed that 101-14 and 5C did not differ significantly in the level of disease incidence (P=0.696) and both had greater disease incidence than Riparia Gloire (P=0.000) and Schwarzmann (P=0.002) which were similar (P=0.531) (Table 3.3).

3.3.1.3 Root Dry Weights

ANOVA analysis (ANOVA Table 3.4-5, Appendix 3.4) showed that root dry weight was not significantly influenced by inoculation treatment (P=0.628) or the interaction between rootstock variety and inoculation treatment (P=0.586). However, rootstock variety alone did

influence root dry weight (P=0.000). Plants of 5C had greater mean root dry weights than all other varieties ($P \leq 0.002$) which were similar (Table 3.4).

Table 3.4: Mean root dry weights (g) of grapevine rootstocks when data for non-inoculated and inoculated plants were combined. Values followed by different letters were significantly different at P=0.05 (according to Fisher's Protected LSD).

Rootstock variety	Root dry weight (g)
101-14	26.9 a
5C	34.7 b
Riparia Gloire	24.7 a
Schwarzmann	24.3 a

ANOVA analysis (ANOVA Tables 3.4-7–3.4-14, Appendix 3.4) of individual rootstock varieties showed that overall disease incidence of *C. destructans* and *F. oxysporum* affected the root dry weight of some varieties (P=0.023 and P=0.019, respectively). There was no correlation between *F. oxysporum* incidences and the root dry weights of 101-14 (P=0.127), Riparia Gloire (P=0.590) or Schwarzmann plants (P=0.127), but there was for 5C whose root dry weights were significantly lower in plants infected with *F. oxysporum* than those that were not (P=0.019) (Figure 3.6). The incidence of *C. destructans* had no effect on the root dry weights of Riparia Gloire (P=0.929) or Schwarzmann plants (P=0.498). However, plants of 101-14 had significantly higher root dry weights when infected with *C. destructans*, than when not (P=0.023), a trend also reflected by 5C (P=0.051) (Figure 3.7).

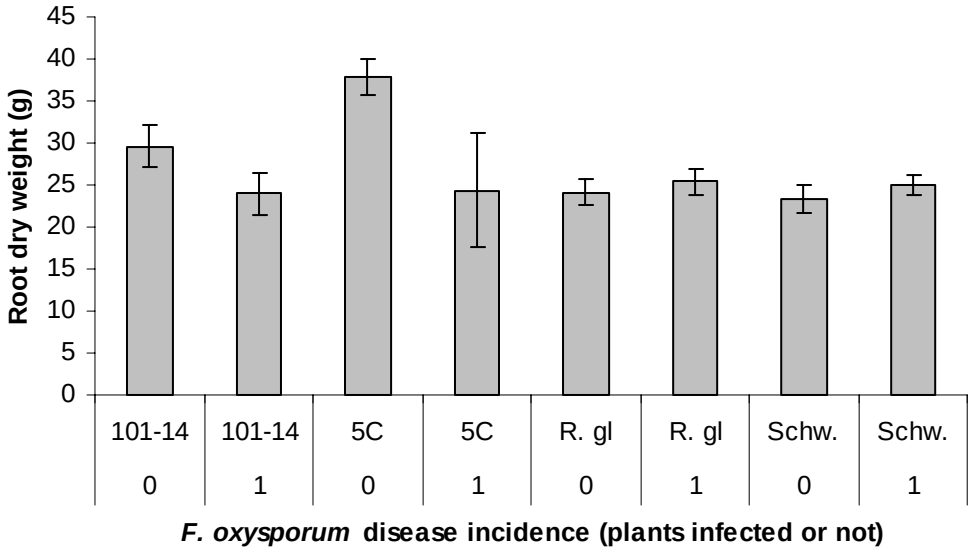


Figure 3.6: Mean root dry weights (g) of different grapevine rootstock varieties infected with *F. oxysporum* (1) or not infected (0). Error bars are SE of the means and show differences in mean root dry weights between infected and uninfected plants for a particular rootstock.

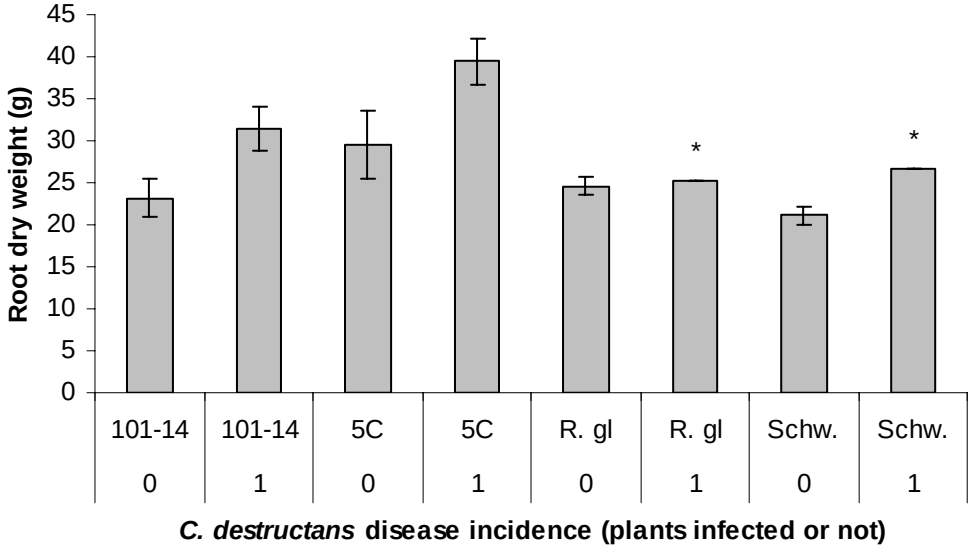


Figure 3.7: Mean root dry weights (g) of different grapevine rootstock varieties infected with *C. destructans* (1) or not infected (0). Error bars are SE of the means and show differences in mean root dry weights between infected and uninfected plants for a particular rootstock (* indicates data from one plant only for Riparia Gloire and two plants for Schwarzmann).

3.3.1.4 Shoot Dry Weights

ANOVA analysis (ANOVA Table 3.4-6, Appendix 3.4) showed that shoot dry weight was not significantly influenced by inoculation treatment (P=0.198) or the interaction between rootstock variety and inoculation treatment (P=0.313). However, rootstock variety alone did

influence shoot dry weight (P=0.000). Plants of rootstock 5C had a mean shoot dry weight that was greater than all other varieties ($P \leq 0.05$) which were similar (Table 3.5).

Table 3.5: Mean shoot dry weights (g) of grapevine rootstocks when data for non-inoculated and inoculated plants were combined. Values followed by different letters were significantly different at P=0.05 (according to Fisher's Protected LSD).

Rootstock variety	Shoot dry weight (g)
101-14	5.3 a
5C	9.0 b
Riparia Gloire	4.2 a
Schwarzmann	5.5 a

ANOVA analysis (ANOVA Tables 3.4-7–3.4-14, Appendix 3.4) of individual rootstock varieties showed that *F. oxysporum* overall disease incidence affected shoot dry weight of some varieties (P=0.006). There was no correlation between *F. oxysporum* incidences and the shoot dry weights of 101-14 (P=0.276), Riparia Gloire (P=0.390) or Schwarzmann plants (P=0.317), but there was for 5C, which had significantly lower shoot weights in plants infected with *F. oxysporum* than those that were not (P=0.006) (Figure 3.8). The incidence of *C. destructans* had no effect on the shoot dry weight of 101-14 (P=0.276), 5C (P=0.560), Riparia Gloire (P=0.929) or Schwarzmann (P=0.498) (Figure 3.9).

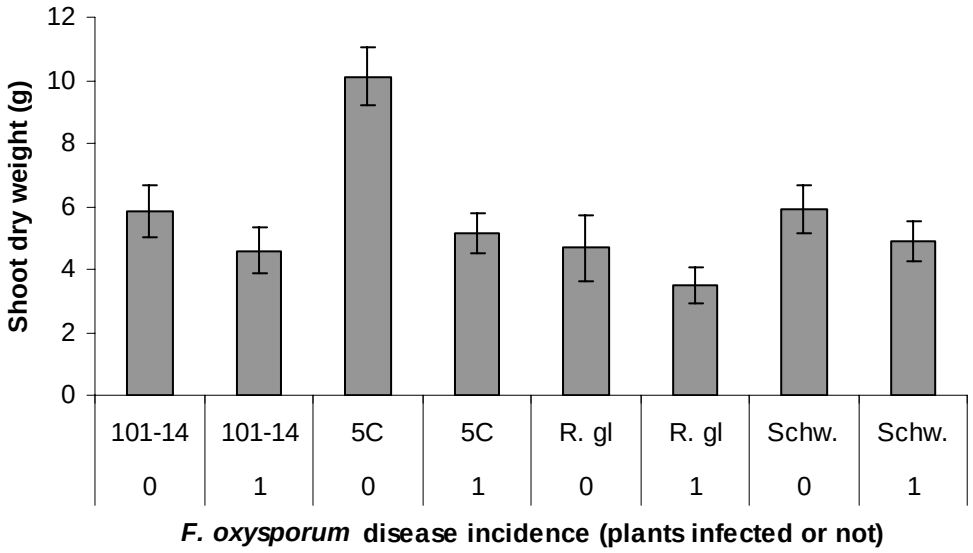


Figure 3.8: Mean shoot dry weights (g) of different grapevine rootstock varieties infected with *F. oxysporum* (1) or not infected (0). Error bars are SE of the means and show differences in mean shoot dry weights between infected and uninfected plants for a particular rootstock.

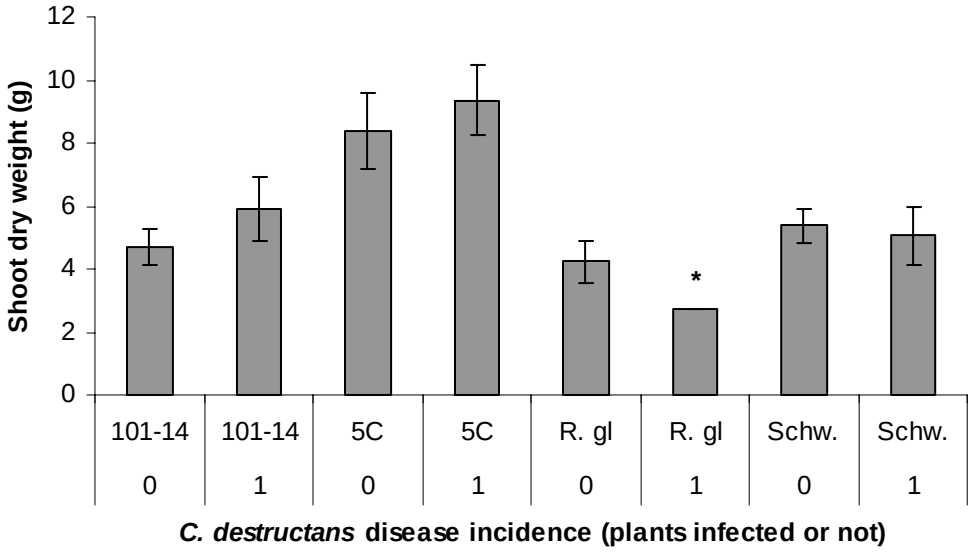


Figure 3.9: Mean shoot dry weights (g) of different grapevine rootstock varieties infected with *C. destructans* (1) or not infected (0). Error bars are SE of the means and show differences in mean shoot dry weights between infected and uninfected plants for a particular rootstock (* indicates data from one plant only).

3.3.1.5 Dual infection

The number of plants infected concurrently by both *C. destructans* and *F. oxysporum* were too small to analyse and so raw data are presented. Overall, only six out of 112 vines had both *C. destructans* and *F. oxysporum* incidence. Of these, two belonged to 101-14, two to 5C and one to each of Riparia Gloire and Schwarzmann. Four had been inoculated with *F. oxysporum* and two had been inoculated with *C. destructans*. No dual infections occurred in the controls. This level of dual infection was low considering that the severity data (Figures 3.4 and 3.5 and Table 3.6) and the incidence data (Table 3.7) indicated the potential for dual infection in *C. destructans* inoculated plants.

Table 3.6: *Fusarium oxysporum* and *C. destructans* disease severity (% pieced of wood infected) for control plants (O), plants inoculated with *C. destructans* (C) or those inoculated with *F. oxysporum* (F).

Inoculation treatment	***F. oxysporum* disease severity (%)**	***C. destructans* disease severity (%)**
O	13.1	10.0
C	25.7	25.0
F	34.0	9.7

Table 3.7: Overall *F. oxysporum* and *C. destructans* disease incidence (% plants infected) for control plants (O), plants inoculated with *C. destructans* (C) or those inoculated with *F. oxysporum* (F).

Inoculation treatment	Overall *F. oxysporum* disease incidence (%)	Overall *C destructans* disease incidence (%)
O	22.5	15.0
C	44.4	41.7
F	63.9	25.0

3.4 DISCUSSION

This greenhouse experiment was designed to test the susceptibilities of four of the most commonly grown New Zealand grapevine rootstocks (5C, 101-14, Schwarzmann and Riparia Gloire) to two common root pathogens, *F. oxysporum* and *C. destructans*. Assessments included the disease severities and disease incidences of both pathogens as well as the root and shoot dry weights. Both *C. destructans* and *F. oxysporum* were able to infect grapevine roots when inoculated into the soil, the high levels of natural infection was unexpected since the vineyard from which the soil was taken had not been reported to have a problem with black foot. Results indicated that *F. oxysporum* was more pathogenic than *C. destructans*. *Fusarium oxysporum* severity was high when plants were inoculated with either *F. oxysporum* or *C. destructans*, while *C. destructans* severity was only high when plants were inoculated with *C. destructans*. The rootstock varieties also displayed different susceptibilities to the pathogens *F. oxysporum* and *C. destructans*. Results showed that 101-14 and 5C were most susceptible to *C. destructans*, whereas susceptibility to *F. oxysporum* was greatest in 101-14 and moderate in Riparia Gloire and Schwarzmann (Figures 3.4 and 3.5).

Rootstock varieties differed in their mean levels of *F. oxysporum* disease severity, and in the interaction between rootstock variety and inoculation treatment. Differences in *F. oxysporum* disease severity between *F. oxysporum* inoculated and non-inoculated treatments were not significant for any varieties except 101-14 which had the highest *F. oxysporum* disease severity (Figure 3.4). The rootstock varieties also differed in their mean levels of *C. destructans* disease severity since inoculation increased *C. destructans* infection in variety 5C and 101-14 only (Figure 3.5). Inoculation with *C. destructans* increased *F. oxysporum* infection in Riparia Gloire (significant) and Schwarzmann (not significant) and inoculation with *F. oxysporum* resulted in slightly increased *C. destructans* infection only in 5C. The infections not due to inoculation were caused by the low natural populations and partially reflected the susceptibility of the rootstocks to the two pathogens.

The absence of dual infections in individual plants seems to indicate that these two pathogens compete with each other in infection sites. If both fungi occupied the same niche within the plant or rhizosphere one might out-compete the other. Since most of the "*F. oxysporum* susceptible" rootstock varieties were not infected with *C. destructans*, but many of the tissue fragments for the "most *F. oxysporum* resistant" rootstock varieties were infected with *C. destructans*, this indicated that *F. oxysporum* could out-compete *C. destructans*. Tables 3.6 and 3.7 clearly show that *C. destructans* inoculation treatments had approximately equal incidence and severity of the two pathogens, while the *F. oxysporum* inoculation caused high incidence and severity of *F. oxysporum* infection, but *C. destructans* infection was low. Given the results, one would have expected more dual infections.

It is possible that *C. destructans* moved ahead of *F. oxysporum* in the plant to an extent where the section sampled was no longer dominated by *C. destructans*, but by *F. oxysporum*. Alternatively, growth of one pathogen on the agar may have prevented the growth of the other. Sequential analysis of trunks, with more pieces than just at 0 cm and 5 cm and smaller isolation fragments could determine if this was occurring. A better understanding of dual infection would also require a trial with a *C. destructans* treatment, a *F. oxysporum* treatment, a combined (*C. destructans* and *F. oxysporum*) treatment, and a non-inoculated control. Accurate conclusions cannot be drawn here because no plants in this trial were inoculated with both pathogens. Another example of infection by one root pathogen preventing infection by another was reported by Macia-Vicente et al (2008) who observed that barley roots colonised by a number of endophytic fungi, in the rhizosphere and sometimes the root cortex, reduced symptoms caused by the root pathogen *Gaeumannomyces graminis* var. *tritici*. These endophytic fungi included *Aspergillus*, *Phoma*, *Acremonium* and *Fusarium* species as well as *C. destructans*.

The finding that grapevine rootstocks differ in their susceptibilities to *C. destructans* and *F. oxysporum* infection is not unexpected since this has been demonstrated previously. de Andrade et al (1995) used potted cuttings (rooted) grown in methyl bromide treated soil inoculated with fragments of woody shoots infected with *F. oxysporum* f.sp. *herbemontis*. Results showed that hybrids of *Vitis riparia* x *Vitis berlandieri* (including 5C) and *Vitis riparia* x *Vitis rupestris* (including 101-14 and Schwarzmann) were the most susceptible, and hybrids of *Vitis berlandieri* x *Vitis rupestris* (including 420 A and Paulsen 1103) had moderate resistance (Table 1.2). There is some similarity between their findings and those here, for example, *F. oxysporum* disease severity was greatest in 101-14 and Schwarzmann in

both. However, the isolates used were quite different and so the results cannot be expected to be the same.

In contrast to de Andrade et al (1995), Omer et al (1999), reported that a *F. oxysporum* isolate from rotten grapevine roots was unable to cause infection in certain rootstocks including 5C, when their roots were wounded and inoculated with a conidial suspension of 10^6 conidia per mL. This study correlates with the finding here that 5C is not particularly susceptible to *F. oxysporum*. The fact that rootstock varieties differ in their susceptibility to this pathogen is not unexpected as infection rate, damage severity, and spread of *F. oxysporum* within the plants is determined at least in part by their genetic and physiological characteristics (Granett et al., 1998).

Here, the rootstock susceptibilities to *C. destructans* were in general agreement with the results Harvey and Jaspers (2006) reported for their pot trials (Table 1.1). However, they found that the susceptibility of grapevine rootstock varieties to *C. destructans* was variable between seasons, with results differing for the two trials. In agreement with the findings here, Riparia Gloire was consistently reported to have low susceptibility to *C. destructans*, and 101-14 moderate to high susceptibility, however the reported susceptibilities of 5C and Schwarzmann were inconsistent, with 5C being a lot more resistant in their trials than this one. These differences might be due to the different inoculation methods used as they soaked the plant roots in a conidial suspension ($1x10^6$), rather than inoculating the soil as was done here. Their plants were also grown in a semi sterile peat and pumice mix, rather than the blend of potting mix and soil used here. Due to the presence of soil and associated microorganisms, the *C. destructans* in this trial could have been influenced by potentially pathogen-antagonistic or plant beneficial bacteria. For example, Schwarzmann, which was one of the most susceptible rootstocks tested in their trial, was one of the least susceptible in this experiment.

Differences in the susceptibility of rootstock varieties to *C. destructans* and *F. oxysporum* infection could in part be due to differences in rootstock vigour, root exudates and other factors discussed in Chapter 2. For example, the most vigorous rootstocks (101-14 and 5C) were found to be the most susceptible to *C. destructans*. It is possible that their higher rate of root tip advancement and root exudation are in part responsible. Different plant species, or even cultivars of the same species are able to exude different quantities and qualities of organic compounds from their roots (Lynch & Whipps, 1990), altering the diversity and structure of rhizosphere microbial populations (Grayston et al., 1998; Garbeva et al., 2008). Information on the composition of grapevine root exudates from different rootstock varieties

is lacking. However, in a study looking at the sugar composition of grapevine rootstock cuttings (stem and root zone), Reed et al (2004) proposed that varietal differences might be the reason for discrepancies between their work and that of others. Additionally, while the grapevine rootstocks tested by Nikolaou et al (2000) were not the same as those used here, differences in cytokinin content of the xylem exudates, and the volume of exudates were detected between rootstock varieties.

Variation in root exudation between susceptible and more resistant rootstocks has been demonstrated in other systems. For example, apple rootstocks, M.26 and M.7, which are susceptible to apple replant disease (ARD) support greater numbers of culturable fungi and bacteria in their rhizosphere than some of the more tolerant rootstocks, G.16, G.30 and CG.6210. It was suggested that the reason for this might be that the susceptible rootstocks were releasing more root exudates, but this was not quantified (Rumberger et al., 2007). It was also postulated that rhizosphere Pseudomonads were responsible for the greater susceptibility since Yao et al (2006) found lower numbers and different species of *Pseudomonas* in the rhizospheres of the ARD-susceptible rootstocks than the ARD-tolerant rootstocks.

Competition from the naturally occurring non-pathogenic strains of *C. destructans* and *F. oxysporum* with the pathogenic strains, for nutrients and colonisation sites may also reduce the success of pathogenic strains. For example, non pathogenic strains of *F. oxysporum* reduced stem colonisation by pathogenic strains in carnation, reducing disease severity (Postma & Luttikholt, 1996). It is likely that rhizosphere *F. oxysporum* populations differ between grapevine rootstock varieties since plant cultivar has been shown to influence *Fusarium* populations in other plants (Fravel et al., 2003). For example, differences in root exudates of the watermelon cultivar 'Crimson Sweet', which also had increased populations of beneficial *F. oxysporum* in its rhizosphere, were considered to be the cause of its reduced susceptibility to *Fusarium* wilt compared to other cultivars (Larkin et al., 1993b).

Another possible explanation for the differences in rootstock susceptibility to *C. destructans* and *F. oxysporum* could be related the ability of each rootstock's rhizosphere bacterial populations to colonise root wounds. Both *C. destructans* and *F. oxysporum* are wound pathogens, and so if beneficial rhizosphere bacteria were able to reach and colonise grapevine root wounds before these pathogens, this could affect their susceptibility. Roots colonised by beneficial bacteria would be less likely to become infected (as discussed in Chapter 1). This is something that was not looked at in these experiments, but it is recommended that future work consider the implications of this aspect of rhizosphere bacteria.

Identification of the isolates as *C. destructans* and *F. oxysporum* in the assessment reported here was not 100% accurate. However, identification was considered satisfactory as 10-15 % of all isolates considered to be positive for a given pathogen were subcultured onto PDA and grown to view both colony and spore morphologies. Accuracy was deemed to be approximately 70% for *C. destructans* (total 30 subcultured) and, approximately 90% for *F. oxysporum* (total 30 subcultured). The number of *C. destructans* isolates may have been underestimated, since the diverse and overlapping morphology of the isolates from *Cylindrocarpon* species was not fully appreciated. However, this was acknowledged and assessment criteria were adjusted for the experiment reported in Chapter 4. While *F. oxysporum* identification appeared to be more accurate, the presence of "microconidia borne in false heads on short monophialides" was not determined. This character was used to distinguish *F. oxysporum* from other closely related species by Burgess et al (1989). Consequently, molecular identification of all suspected positive isolates, for both fungi, would have been beneficial. Alternatively, the use of selective agars might have been useful as they allow fungi recovered at a low rate on PDA (due to poor growth) to be more successfully recovered (Bandyopadhyay et al., 2006). For a trial like this, these could have included *Cylindrocarpon* selective agar (CSA) (Sweetingham, 1983), and selective *Fusarium* agar (SFA) (Leslie & Summerell, 2006), although the selectiveness of CSA has not proven to be sufficient in other work (pers. comm. C. Probst, 2008).

Root dry weight was not influenced by inoculation treatment, or associated interactions, but was influenced by rootstock variety. Varieties 5C and Schwarzmann were reported to have greater, similar vigour but only 5C had a higher mean root dry weight than the other rootstocks, which were comparable. It therefore seems likely that the higher root dry weight of variety 5C was caused by another factor, possibly the presence of root galls which were found on many of its plants (Figure 3.6-1, Appendix 3.6). These galls might have been the result of crown gall caused by *Agrobacterium tumefaciens* (Kawaguchi et al., 2007), especially since 5C is known to be particularly sensitive to this disease (Goodman et al., 1993). In hindsight, it would have been useful to weigh these galls and deduct their weight from the root weights.

Overall disease incidence (i.e. from both sampling points) affected root dry weight only in 5C, which had significantly lower root dry weights in plants infected with *F. oxysporum* than uninfected plants. A very different response was observed for overall *C. destructans* incidence which increased the root dry weight of 101-14 and 5C. These different effects could be attributed to the different responses of the rootstock varieties to *F. oxysporum* and

C. destructans. Highet and Nair (1995) also observed that artificially infecting grapevines with *F. oxysporum* severely stunted the root systems. Conversely, the loss of roots in black foot disease is sometimes compensated for by the development of a second crown of horizontally growing roots close to and parallel to the soil surface (Halleen et al., 2007). The development of another root axis was not observed, possibly because they were confined in a pot, but the tendency to grow more roots might have accounted for the increase in the root dry weight of 5C and 101-14, the rootstocks most susceptible to *C. destructans*. In addition, the roots of *C. destructans* infected plants could have taken longer to die and fall off than those of *F. oxysporum* infected plants, which were therefore not included in the weighing.

Like root dry weights, shoot dry weights were not influenced by inoculation or associated interactions, but were affected by rootstock variety. Rootstock variety 5C had the greatest mean shoot dry weight, reflecting the root dry weight findings, which was not unexpected as there is a well established relationship between above ground and below ground growth (May, 1994). The higher vigour of 5C than Schwarzmann found in this experiment could be due to the higher greenhouse temperatures favouring its growth more than in the vineyard. Support for this can be found in vigour ratings of the different rootstocks in the warmer grape-growing regions of Australia (Nicholas, 1996), where S04 (sometimes wrongly identified as 5C in New Zealand) was considered a moderate to high vigour rootstock, whereas Schwarzmann was only reported to have moderate vigour. Vigour ratings also tend to refer to the vigour the rootstock induces in the scion, but the plants here were not grafted. Some rootstocks that are very vigorous on their own roots actually seem to inhibit vigour on the scion grafted to it, for example, Riparia Gloire (*Vitis riparia*) (pers. comm. G. Creasy, 2008).

Overall, *F. oxysporum* disease incidence significantly reduced mean shoot dry weight in 5C. This was not unexpected as Highet and Nair (1995) found *F. oxysporum* infected vines to experience delayed, weak shoot growth. However, there was no effect of overall *C. destructans* incidence on shoot dry weight. This is surprising given that black foot affected vines often experience reduced vigour, sparse foliage and smaller leaves (Halleen et al., 2006a). In this experiment, the omission of leaf weight from the shoot dry weights was due to variable leaf fall at the time of harvest; inclusion of leaf weight might have detected such effects.

In this trial the presence of the *F. oxysporum* in the stem was not correlated with any symptoms. The work of de Andrade et al (1995) evaluated *F. oxysporum* disease severity based on observations of internal symptoms such as the vascular discolouration of roots 5 and 10 cm from the shoot base, and this would have been useful additional data for the current

experiment. However, given that *F. oxysporum* was isolated from above the roots, and non pathogenic strains are usually restricted to the roots (Fravel et al., 2003), *F. oxysporum* cannot be discounted as a pathogen of grapevines, even if it is only present when roots are wounded. In the nursery and vineyard environments it is unlikely that grapevine roots are ever without injury. For example, Halleen et al (2003) noted that the basal ends of nursery cuttings are semi or fully exposed and that callus roots can break when vines are planted out. Both of these, and potentially the feeding of grass grub larvae (Mundy et al., 2005), create wounds that leave the grapevines open to infection.

Highet and Nair (1995) confirmed the pathogenicity, or at least the potential of *F. oxysporum* to be a pathogen of grapevines in Australia in a glasshouse trial where grapevines were infected with *F. oxysporum* obtained from roots of declining field grown vines. They tested two types of inoculum; a suspension of conidia (2×10^6 conidia per mL) of which 10 mL was poured over the upper 4 cm of exposed root system, and pieces of grapevine roots infected with *F. oxysporum*, placed 5 mm from the exposed roots. Plants were assessed after only eight weeks, much less time than was allowed here. However, they still observed obvious symptoms. Surface sterilised roots, rather than the stems used here, were plated on PDA. Ultra thin sections of root tissue were also studied with a light microscope and a transmission electron microscope (TEM) which revealed *F. oxysporum* hyphae growing within and between cells of the root cortex. Cortical cells were partly degraded and infected cells had lost their contents. However, the endodermis was not perforated. Similar studies could be performed for an experiment such as this one.

In this trial, 101-14 and 5C cuttings were sourced from a commercial nursery, whereas Schwarzmann and Riparia Gloire were from the Lincoln University vineyard. This was unavoidable as no one source could supply plant material of the four varieties. However, all callused cuttings were introduced to the potting mix and soil blend at the same time and grown in the same way. Although results showed greater incidence of *C. destructans* in 101-14 and 5C than Schwarzmann and Riparia Gloire, this reflected previous trends in susceptibility. It therefore seemed unlikely that the plant sources had affected the outcomes of the experiment.

The vines that died three months after planting were all from 101-14 and 5C but since they were replaced with vines of the same batch and treatment, this potential source of variation was considered to be minor and so the plants were assessed together. Removing them from the trial or analysing them separately would have reduced the sample size, compromising the analysis. In hindsight, it could have been beneficial to assess some plants from the

replacement batch to determine if either *C. destructans* or *F. oxysporum* was present. However, it seemed likely that the more susceptible 5C and 101-14 had succumbed to pathogens present in the field soil. The natural levels of infection in the soil was evident since *C. destructans* and *F. oxysporum* were both detected in the control plants, to similar levels, being 10.0% for *C. destructans* and 13.1% for *F. oxysporum*. The similar trends for the control treatments are seen in Figures 3.4 and 3.5.

Contamination of control soils could also have occurred via the splash dispersal of spores, during watering, as plants were laid out in a completely randomised design. The potential for splash dispersal of spores in drainage water was recognised but only a corrugated metal table was initially available in the greenhouse; the pots were moved onto a mesh table as soon as one was available, within a month, so as to reduce the likelihood of contamination. Although disease incidence varied between control pots, the vineyard soil was considered the most likely source of external *C. destructans* inoculum, especially since other researchers have subsequently found similar *C. destructans* incidence while using the same soil (pers. comm. C. Probst, 2008) It is unlikely that the plants were the source of natural inoculum since natural infection by *F. oxysporum* and/or *C. destructans* occurred in all rootstock varieties, even those where canes (rooted in pumice and not exposed to pathogens prior to planting) and not grafted/rooted vines were used.

Natural contamination by *Cylindrocarpon* and *Fusarium* species could have been avoided by using gamma sterilised soil, with the necessary rhizosphere bacteria introduced as a mixed bacterial filtrate. Shishido and Chanway (1998) used a forest soil suspension (in sterile phosphate buffer) and sterile (oven dried and autoclaved) sand microcosms to study the effect of plant-growth-promoting bacteria on spruce seedlings and the composition of soil microbial communities. A short-coming of that experiment was that the soil communities in the microcosms came from a small 0.5 g soil sample (per 37 g microcosm), thereby introducing too few microorganisms to adequately represent the normal numbers and diversity of the soil microbial community. However in this trial, a much larger volume of soil was used, perhaps better representing the microbial community.

Although they differed in colour and colony morphology, the *F. oxysporum* isolates used to inoculate the grapevines were shown to share great sequence homology. This indicated a large degree of morphological plasticity within *F. oxysporum*. Alternatively, the primers used here (ITS4, ITS5) might have been partly responsible for this apparent similarity, as reported by O'Donnell and Cigelnik (1997) who concluded that while ITS regions are effective for distinguishing many fungal species, they are not the best suited to *Fusarium* species due to

homology in the ITS region. Sequences of the translation elongation factor EF-1α and the mitochondrial small subunit ribosomal RNA genes have proven more useful (Fravel et al., 2003).

In conclusion, this greenhouse trial showed that both *C. destructans* and *F. oxysporum* were able to infect grapevine rootstocks commonly grown in New Zealand (101-14, 5C, Schwarzmann and Riparia Gloire), but that *F. oxysporum* appeared to be a more aggressive pathogen than *C. destructans*. Disease incidence by *F. oxysporum* was significantly related to reduced mean shoot and root dry weight of rootstock 5C, while *C. destructans* incidence increased the root dry weight of 101-14 and 5C. While reinforcing that grapevine rootstocks differ in their susceptibilities to *C. destructans*, this trial also showed that rootstock varieties differ in their susceptibilities to *F. oxysporum*. Rootstocks 101-14 and 5C were most susceptible to *C. destructans*, whereas 101-14, Riparia Gloire and Schwarzmann were more susceptible to *F. oxysporum*. These findings need to be confirmed with field trial data, but would seem to suggest that grape growers in New Zealand should consider *F. oxysporum* a considerable threat.

CHAPTER 4

THE EFFECT OF CARBOHYDRATE STRESS ON GRAPEVINE RHIZOSPHERE BACTERIAL POPULATIONS AND *CYLINDROCARPON DESTRUCTANS* DISEASE SEVERITY AND INCIDENCE

4.1 INTRODUCTION

Plant carbohydrates provide energy for plant metabolism and growth (Shoresh & Harman, 2008). In grapevines, this is reflected by the extent of canopy growth and the level of carbohydrate reserves accumulated, which is in part determined by the grapevine variety and climate (Bennett et al., 2005). Environmental factors and host stress have been shown to play a role in the development of Cylindrocarpon black foot disease. Host stresses include malnutrition, poor soil drainage or compaction, heavy crop loads on young vines, poorly prepared soil and poor planting technique (Fourie & Halleen, 2001). The loss of leaf photosynthetic area through defoliation also has the potential to cause carbohydrate stress to grapevines (Kliewer & Fuller, 1973).

A standard practice in cool climate viticultural regions is for leaves to be removed from around fruit clusters and/or by shoot trimming several times over the growing season (Chanishvili et al., 2005). Canopy thinning allows for better exposure of the fruit to sunlight, while also increasing the photosynthetic activity in the older leaves. Additionally, the practice alters canopy structure and microclimate, making foliar pest and disease control easier (Hunter et al., 1994). Vineyard canopy thinning protocols are believed to have no adverse effects on grapevines, as they have been shown to leave sugar and organic acid levels unchanged (Hunter et al., 1995). However, defoliation is known to stress plants by decreasing the availability and concentrations of photosynthate, compromising their resistance to other biotic and abiotic stresses (Marcais & Breda, 2006). Multiple or severe defoliation events lower root storage carbohydrate levels and eventually result in carbohydrate starvation (Horsley et al., 2000). Early and harsh defoliation in one season was also reported to result in the premature mobilization of carbohydrate stored in the roots, trunks and canes, decreasing the next season's bud fertility and thereby having an undesirable flow through effect on yield (Hunter et al., 1995).

Stress factors play a part in predisposing woody plants to pathogen infection, increasing both disease incidence and severity (Schoeneweiss, 1981). It is thought that stress factors, working individually or together, aid pathogen invasion by compromising host resistance (Rayachhetry

et al., 1996), however, the stress intensity needs to reach a minimum threshold to predispose a plant to disease. This predisposition can be reversible, unless defoliation is prolonged (Schoeneweiss, 1981). In woody plants, stem and crown cankers and dieback diseases typically increase in prevalence when plants are stressed, and high intensity defoliation can make plants more susceptible to root rots (Schoeneweiss, 1981).

Defoliation alters both the quantity and quality of carbon flow from plant roots (Guitian & Bardgett, 2000). However, the reported effects of defoliation on plant root exudation differ; it may be increased (Paterson & Sim, 2000), decreased (Miller & Rose, 1992), or left unchanged (Bazot et al., 2005). Root exudation can also be altered by a plant's response to biotic and abiotic stresses and by plant species or even cultivars (Grayston et al., 1998). The carbon substrates deposited in the rhizosphere by plant roots are of great importance to the soil microbial community (Paterson, 2003), as a carbon and energy source for rhizosphere bacteria and fungi (Lynch & Whipps, 1990), determining microbial activity, biomass and community structure (Grayston et al., 1995). Consequently, any such changes in exudation could increase the growth of some soil microorganisms (Albareda et al., 2006), which may be pathogenic or competitive to pathogens (Mazzola, 2002). Bertin et al (2003) concluded that root exudation typically increases when plants are stressed. However, Guitian and Bardgett (2000) found that microbial activity was typically at its highest in non-defoliated plants. Since these changes in soil microbial communities, due to defoliation events, are independent of changes in root biomass, it seemed likely that such community changes resulted from modified root exudation (Guitian & Bardgett, 2000).

The aim of this experiment was to determine the effect of partial defoliation, a common practice in viticulture, on the species and populations of rhizosphere bacteria, and on the susceptibility of grafted grapevines to infection by *Cylindrocarpon destructans*. Two rootstock varieties were used, both being commonly grown in New Zealand vineyards (Hoskins, 2008) where *Cylindrocarpon* species have caused high losses due to black foot disease (Bleach et al., 2006). Both rootstocks used in this experiment are hybrids of the same American *Vitis* species: *Vitis riparia* x *Vitis rupestris* (Jackson & Schuster, 2001), making them genetically quite similar, however, Schwarzmann was shown to be less susceptible to black foot than 101-14 in a recent trial, with susceptibility rankings of 5 and 12, respectively, in a 14 variety trial (Harvey & Jaspers, 2006).

4.2 METHODS

4.2.1 Plant preparation

For this experiment, plants of Sauvignon Blanc scion wood grafted to two rootstock varieties, 101-14 or Schwarzmann, were used. The one year old dormant grafted plants were grown in 2.5 L pots containing a 50/50 mix of soil (Wakanui silt loam classified as a mottled immature pallic soil), which was sourced from the Lincoln University vineyard, and a potting mix [(80% horticultural bark (grade 2): 20% pumice (grade 3, 1-4 mm)]. The potting mix was 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. The 'soil only' controls, included in the randomised design, were kept with the other pots and consisted of the same 50/50 mix of soil and potting mix in 2.5 L pots, but did not contain vine plants.

Vines were kept moist by daily watering and grown in a greenhouse for a total of 10 months spanning spring (September 2006) – winter (July 2007). During this time, temperatures ranged from 15ºC (minimum) to 30ºC (maximum). Vines were placed under high pressure sodium lamps (Son-T Agro 400, Philips) from the start of autumn (March 2007) until harvest (July 2007), to ensure light levels were sufficient for good growth. All weeds were removed by hand to prevent the root exudates of weeds altering the rhizosphere communities in the pots. All flower buds were removed when visible on the vines as flower development could also reduce carbohydrate stores within the vines.

4.2.2 Carbohydrate stress

In summer (November 2006, 2 months after planting), leaves were trimmed from all scion shoots above the fourth node to induce three different levels of carbohydrate stress, being none (level 0), moderate (level 1) and high (level 2). For stress level 0, none of the leaves were removed, for stress level 1 every third leaf was removed, and for stress level 2 every third leaf was left on the vine, the rest being removed (Bennett, 2002). Vines were then left undisturbed for a period of three weeks, after which the trimming treatment was repeated on the new sections of the shoots. This was done a total of three times at three weeks apart.

4.2.3 Pathogen inoculation

The *C. destructans* isolates used were the same isolates listed in Section 3.2, with mixed conidial suspensions (10^6/mL) prepared in the same way. Conidial viability of this mixed suspension was checked after plating onto PDA, with the average germination rate being 98%. Three weeks after the third trimming treatment, the root systems of all the vines were

wounded and inoculated as in Section 3.2.2 with 50 mL of conidial suspension. The non-inoculated plants ('Cyl –' treatments) were inoculated with 50 mL SDW, and the 'soil only' controls had inoculated and non-inoculated treatments, with either 50 mL of SDW or *C. destructans* conidial suspension. All pots were given a further 50 mL of tap water to help carry conidia into the soil, and then left undisturbed for 24 h. The different treatments are summarised in Table 4.1.

Table 4.1: The eight treatments in this experiment based on *C. destructans* (Cyl) inoculation status and stress (trimming) level.

Treatment abbreviation	Inoculation status	Trimming level	Description
Cyl +	Inoculated	N/A*	Inoculated 'soil only' control
Cyl + 0		0: unstressed	Inoculated, un-trimmed
Cyl + 1		1: moderate stress	Inoculated, 1/3 leaves removed
Cyl + 2		2: high stress	Inoculated, 2/3 leaves removed
Cyl -	Not inoculated	N/A	Non-inoculated 'soil only' control
Cyl – 0		0: unstressed	Non-inoculated, un-trimmed
Cyl – 1		1: moderate stress	Non-inoculated, 1/3 leaves removed
Cyl – 2		2: high stress	Non-inoculated, 2/3 leaves removed

* N/A = not applicable

After inoculation, all pots in this 10 replicate (per treatment) trial were laid out in a completely randomised block design, and the plants grown for a further 6 months in a greenhouse to allow symptoms to develop. Assessment of pathogen infection was conducted on seven of the most consistent looking replicates of each treatment, except for Schwarzmann 'Cyl – 1', for which only six live plants were available.

4.2.4 Plant assessment

4.2.4.1 Collection of rhizosphere soil

The rhizosphere soil for each plant was dislodged and mixed on a tray as described in Section 2.2 and one 15 g sample collected per plant (or pot in the case of the 'soil only' controls). There were 96 samples in total, six replicates per treatment per variety. The six replicates chosen from the seven plants selected for infection assessment were the plants numbered 1-6 in the design. Soil samples were placed in sterile plastic vials and stored at -80˚C until they were needed for molecular characterisation of the bacterial communities.

4.2.4.2 Root and shoot dry weights

The root and shoot dry weights of the grapevine plants was determined as described in Section 3.2.3.1.

4.2.4.3 Infection status

Surface sterilisation, isolation and identification of trunk isolates was carried out as described in Section 3.2.3.2. The slide mounts were examined under the microscope and isolates identified according to the criteria stated in Appendix 4.4.

4.2.5 Molecular assessment

Single-strand conformation polymorphism (SSCP) was used to detect differences in the diversity of bacterial communities present in the soil samples of the different treatments. Three randomly selected replicates for each of the eight treatments (per rootstock variety) were assessed. DNA extraction, amplification, SSCP and sequencing were performed as described in Chapter 2.

4.2.6 Statistical analysis

Experimental data for *C. destructans* disease severity and incidence, as well as plant root and shoot dry weight, were analysed as described in Section 3.2.4. The mean values given in the text are those predicted by the ANOVA (raw data means shown in Appendix 4.2).

4.3 RESULTS

4.3.1 Infection status: tissue isolation

4.3.1.1 Identification of isolates

Isolates of *C. destructans* cultured from the experimental plants displayed some intra-specific variation (Figure 4.1) that corresponded with the morphology of the isolates used to inoculate the plants. All *Cylindrocarpon* isolates were identified as *C. destructans* and the few filamentous fungi or yeasts that contaminated the plates were identified as *Fusarium oxysporum*, *Fusarium* sp., *Alternaria* sp., *Botryosphaeria* sp., *Pythium* sp., *Penicillium* sp., *Phomopsis* sp. and *Paecilomyces* sp. (Table 4.3-1, Appendix 4.3).

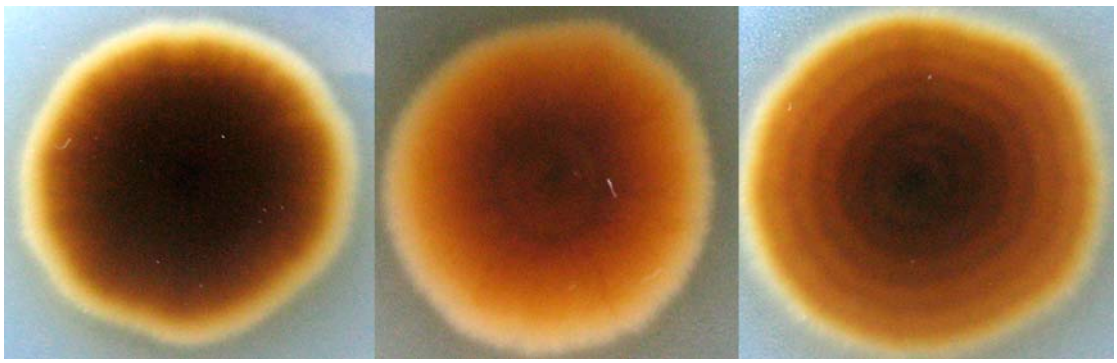


Figure 4.1: Examples of the colony morphology variation seen in the re-isolated *C. destructans* isolates.

4.3.1.2 Grapevine trunk base infection at 0 cm

Overall, *C. destructans* disease severity data provided a more significant treatment effect (ANOVA Tables 4.1-1 and 4.1-2, Appendix 4.1) than disease incidence data. When disease severity data (number of infected wood pieces) was analysed, carbohydrate stress, rootstock variety, and the interactions were not significant. However, there was a trend for greater disease severity in 101-14 than in Schwarzmann, with the average number of *C. destructans* infected wood pieces being 14.9% and 8.4%, respectively. Although stress was not a significant factor (P=0.138) when the stress treatments were analysed as three separate treatments, there was a trend for increasing disease severity with severity of trimming (for both rootstock varieties combined) (Table 4.2). Highly stressed plants had notably greater disease severity than both unstressed and moderately stressed plant, while the disease severity levels of unstressed and moderately stressed plants were similar. Only inoculation affected disease severity (P=0.031). Plants inoculated with *C. destructans* had 17.9% infected wood pieces while non-inoculated plants had 5.4%.

Analysis of overall (0 cm and 5 cm) disease incidence data showed little significance. *Cylindrocarpon destructans* inoculation was the only significant factor affecting disease incidence (P=0.037), with 40.5% disease incidence observed in inoculated plants and 19.5% in non-inoculated plants. There was also a trend, although not significant, for slightly greater disease incidence in 101-14 plants (31.0%) than in Schwarzmann plants (29.3%) (P=0.867). When combining inoculated and non-inoculated treatments and rootstock varieties, there was a non-significant trend, for greater *C. destructans* incidence in highly stressed plants (32.1%) and moderately stressed plants (40.7%) than in unstressed plants (17.9%) (P=0.174).

When data of disease severity in unstressed and moderately stressed treatments were combined in a single treatment (being not significantly different from each other) and the analysis repeated with only two stress treatments, carbohydrate stress was found to significantly influence disease severity (*P*=0.043), with highly stressed plants having the greatest disease severity (Table 4.2). In this second analysis, inoculation also influenced disease severity (*P*=0.009) with inoculated plants having a disease severity of 21.4% and non-inoculated plants 5.8%. Replicate, rootstock variety and all interactions remained non-significant.

Table 4.2: Effects of three carbohydrate stress treatments, alone and after combining the two lower (similar) stress categories, on *C. destructans* disease severity (% infected wood pieces) of grapevine rootstocks at 0 cm from stem base. Data are combined averages for inoculated and non-inoculated plants. Values followed by different letters were significantly different at *P*=0.05 (according to Fisher's Protected LSD).

Stress Level	**Infected wood pieces (%)**	**Stress Level**	**Infected wood pieces (%)**
0: unstressed	7.15	0 + 1	7.60 a
1: moderate	8.15		
2: high	19.65	2	19.65 b
***P* value**	0.138		0.043

4.3.1.3 Grapevine trunk infection at 5 cm

Analysis of the three stress treatments showed that neither stress, inoculation, nor variety treatments, nor interactions between them, significantly influenced *C. destructans* disease incidence at 5 cm (ANOVA Table 4.1-3, Appendix 4.1). When pruning treatments 0 and 1 were combined as a single treatment and the analysis repeated as before, effects remained non-significant. However, in this second analysis, there was a non-significant trend, for the plants exposed to the highest stress to have a greater *C. destructans* incidence (17.9%) than those of the combined (unstressed and moderately stressed) group (12.6%) (*P*=0.551) (ANOVA Table 4.1-4, Appendix 4.1).

4.3.2 Root dry weights

ANOVA analysis conducted with the three stress treatments showed that plant root dry weight was significantly influenced by rootstock variety (*P*=0.003), carbohydrate stress (*P*=0.000) and the interaction between inoculation and carbohydrate stress (*P*=0.031). Inoculation and other interactions were not significant. Fisher's Protected LSD tests showed that the mean root dry weights of unstressed plants were not significantly different from moderately stressed plants (*P*=0.162). However, the root dry weight of highly stressed plants

was significantly lower than both unstressed (P=0.003) and moderately stressed plants (P=0.000) (Table 4.3).

Table 4.3: Root dry weights (g) of grapevine rootstocks under different carbohydrate stress. Data for non-inoculated and inoculated plants and rootstock variety were combined. Values followed by different letters were significantly different at P=0.05 (according to Fisher's Protected LSD).

Stress Level	Root dry weight (g)
0: unstressed	18.5 a
1: moderate	19.9 a
2: high	15.6 b

101-14 plants had significantly higher root dry weights than Schwarzmann plants, with the raw data means being 19.2 g and 16.8 g, respectively (P=0.001). Inoculation did not have a significant effect (P=0.986) on root dry weight, with both non-inoculated and inoculated plants having a mean dry weight of 18.0 g. However there was a significant interaction between inoculation and stress as shown in Figure 4.2. Inoculation caused reductions in root weights of the two stressed treatments, but an increase (P=0.031) in the unstressed treatment with mean weights of 20.1 g for inoculated plants and 17.0 g for non-inoculated plants. In the non-inoculated treatments, the moderately stressed plants had higher root dry weights than in the other two stress treatments, which were similar. In the inoculated treatments, the highly stressed plants had lower root dry weights than the unstressed and moderately stressed plants which had similar root dry weights.

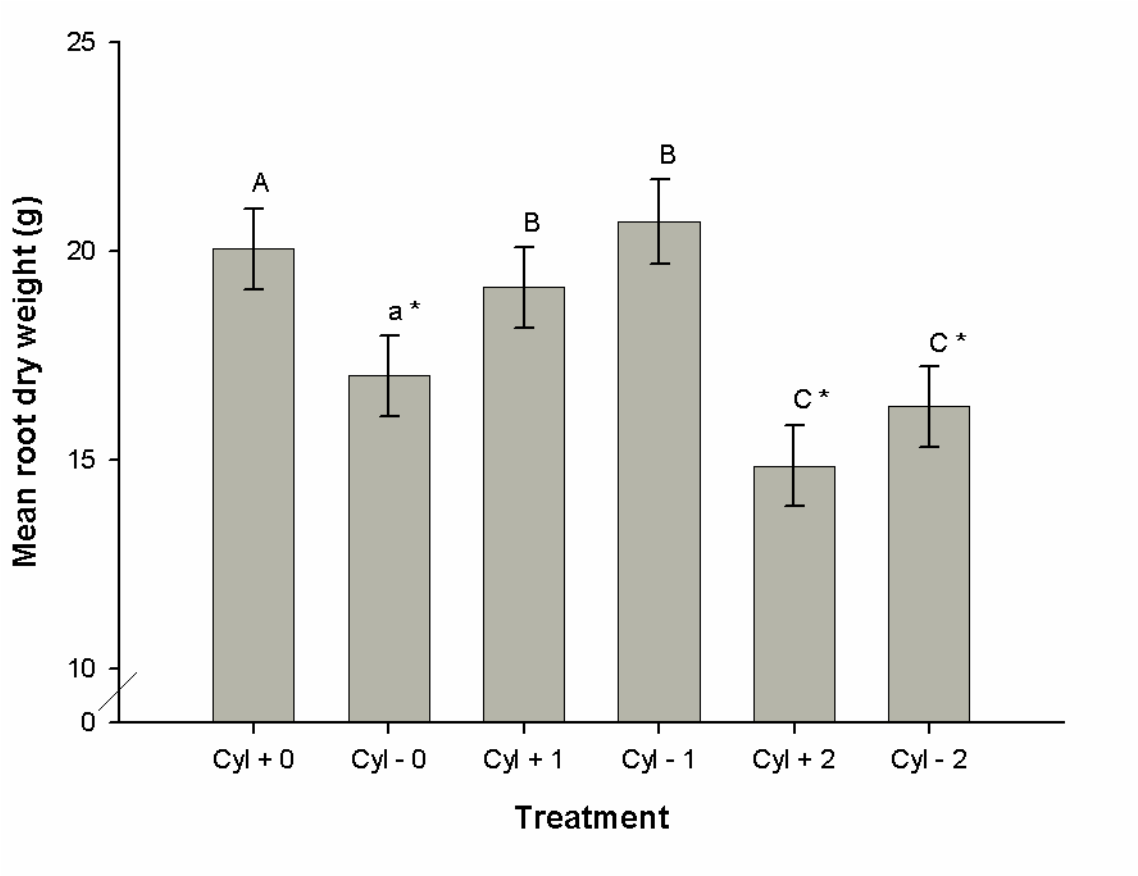


Figure 4.2: Root dry weight (g) of *Cylindrocarpon destructans* inoculated and non-inoculated (Cyl +/-) grapevine plants under different carbohydrate stress (0=unstressed, 1=moderate, 2=high stress) treatments. Error bars are SE of the means and show overall differences between treatment combinations. Capital and lower case lettering denotes a significant difference between inoculated and non-inoculated pairs, while an asterisk indicates the treatment is significantly different from the *C. destructans* inoculated unstressed (Cyl + 0) control.

When ANOVA analysis was conducted with root dry weight data of only two stress treatments (treatments 0 and 1 combined), rootstock variety (P=0.001) and carbohydrate stress (P=0.000) remained significant factors. Although differences were seen in the response of the two rootstocks to stress, with the root dry weight of Schwarzmann being more affected by carbohydrate stress than 101-14, the interaction between variety and stress was not significant (P=0.062) (Figure 4.3). As expected, highly stressed plants had a significantly lower mean root dry weight than did the combined unstressed and moderately stressed plants, being 15.6 g and 19.2 g, respectively.

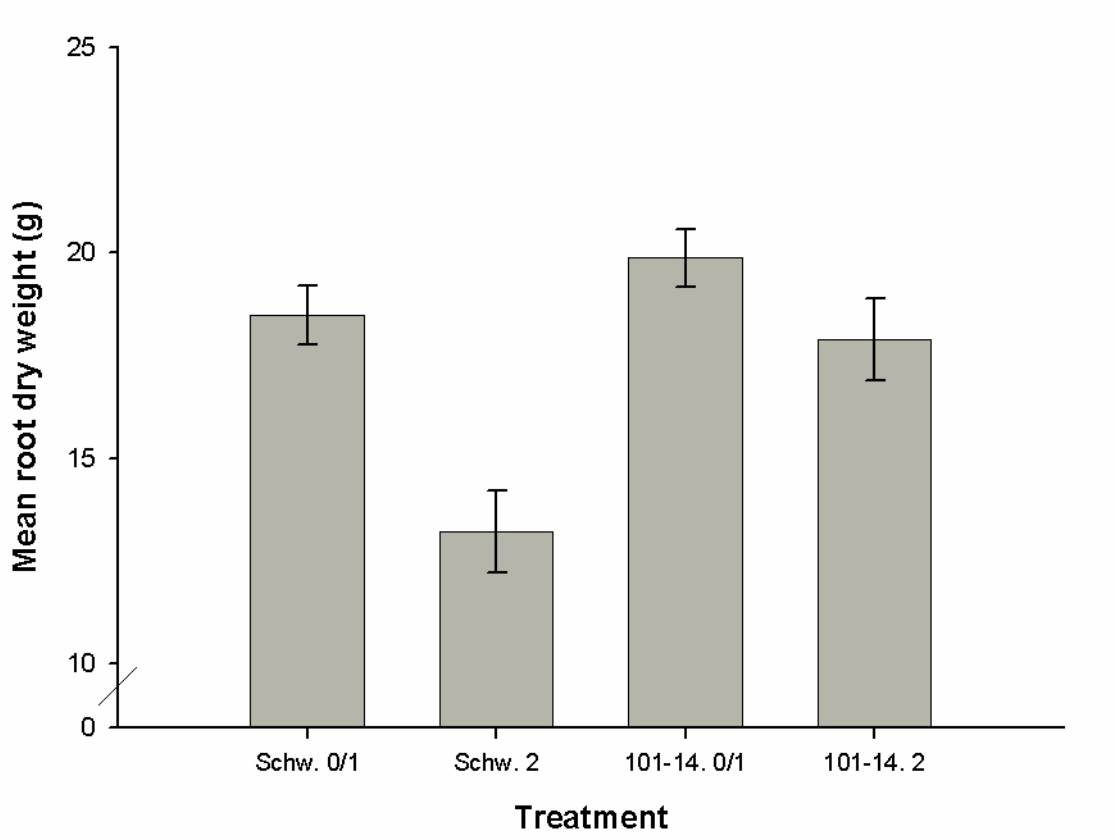


Figure 4.3: Mean root dry weight of Schwarzmann (Schw) and 101-14 grapevine rootstocks under high (2) or no/moderate (0/1) carbohydrate stress. Error bars are SE of the means and show differences (*P*=0.062) in the means.

4.3.3 Shoot dry weights

The shoot dry weight of plants was significantly influenced by rootstock variety (*P*=0.000), with 101-14 having a higher mean shoot dry weight (4.3 g) than Schwarzmann (2.4 g). Inoculation, carbohydrate stress, and all related interactions were not significant. When the analysis used the combined stress treatments (treatments 0 and 1) in comparison with stress treatment 2, the overall outcome was similar to when shoot dry weights were analysed using the three separate stress treatments. Again only rootstock variety was a significant factor (*P*=0.000).

ANOVA analysis revealed no significant effect of *C. destructans* incidence on root (*P*=0.892) or shoot (*P*=0.467) dry weights. For Schwarzmann, the *P*-values were 0.693 and 0.140, respectively. For 101-14, the *P*-values were 0.467 and 0.985, respectively.

4.3.4 Molecular assessment of grapevine rhizosphere bacterial communities

4.3.4.1 SSCP banding patterns

The SSCP analysis produced a gel that had bands tightly clustered at the base. The three replicates used showed that the results were generally consistent, so no further replicates were assessed (Figure 4.5-1, Appendix 4.5). In analysis of band patterns, only bands that appeared consistently for all the replicates were considered. Across the different treatments, the specific bands were often consistently present but at different intensities (Table 4.5-1, Appendix 4.5).

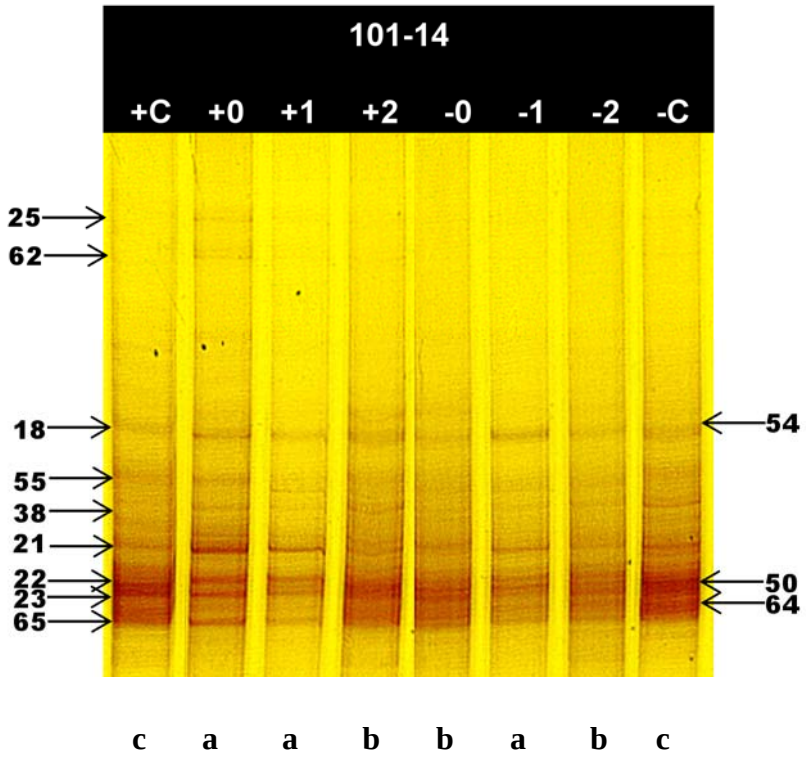


Figure 4.4: SSCP banding pattern for bacterial rhizosphere soil representing one replicate for all treatments with the 101-14 rootstock, inoculated (+) or not inoculated (-) with *C. destructans* and exposed to no (0), moderate (1) or high stress (2). Numbers and arrows identify bands and their excise position. The +C abbreviation denotes a soil only control (no plant) that was inoculated with *C. destructans*, while –C indicates a soil only control that was not inoculated. Lettering along the bottom of the figure (a, b, c) denotes similarity.

For grafted plants with 101-14 rootstock, there was a noticeable difference in the pattern of bands between the different treatments (Figure 4.4). In the inoculated plants (+), those that were unstressed (+0) or slightly stressed (+1) gave a different result to those that were highly stressed (+2). The pattern of four distinct clear bands (21, 22, 23 and 65) in the former was replaced by several closely grouped bands in the +2 treatment, which was similar to non-inoculated -C, -0, -2 and +C treatments. In the non-inoculated plants (-) the unstressed (-0) and highly stressed (-2) plants were more similar to each other than to the moderately stressed (-1) plants. The intensity of bands 21, 22 and 23 changed substantially in the inoculated

101-14 treatments where band 21 became less intense as the stress increases and band 22 faded as a new band was observed immediately above it.

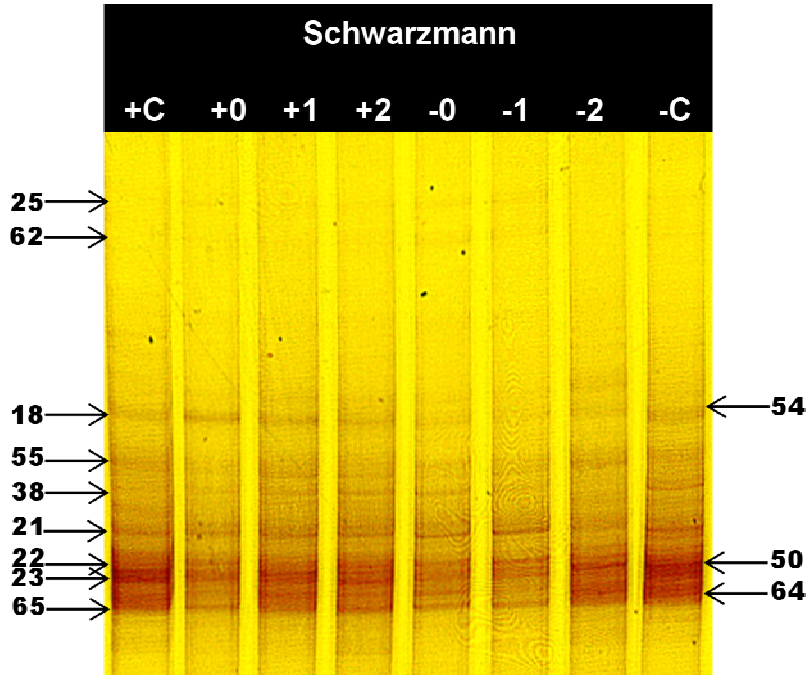


Figure 4.5: SSCP banding pattern for bacterial rhizosphere soil representing one replicate for all treatments with the Schwarzmann rootstock, inoculated (+) or not inoculated (-) with *C. destructans* and exposed to no (0), moderate (1) or high stress (2). Numbers and arrows identify bands and their excise positions. The +C abbreviation denotes a plant only control that was inoculated with *C. destructans*, while –C indicates a plant only control that was not inoculated.

In general, changes in band patterns due to the treatments were less obvious in Schwarzmann than in 101-14. For grafted plants with Schwarzmann rootstock, there was also a minor but noticeable change in the pattern of bands when plants were subjected to different treatments (Figure 4.5). Several bands from the non-inoculated plants (-) showed different intensities across the stress treatments. Bands 21 and 65 were sharper and more distinct in the unstressed (-0) or moderately stressed (-1) treatment than the highly stressed (-2) treatment. The band pattern for this treatment (-2) was indistinct and more similar to the inoculated treatments (+) and the controls. Thus, the Schwarzmann bacterial rhizosphere communities (as shown by band patterns) were most similar in all inoculated plants and those with high carbohydrate stress. Overall, in Schwarzmann these bands were more consistent and less changeable than those of 101-14.

4.3.4.2 Sequenced bands

Several bands were excised from the SSCP gels and sequenced (Table 4.4). A broad range of DNA sequence quality was obtained from these PCR products. Useful DNA sequences for similarity matching ranged in size from 220 to 331 bp. Most BLAST matches were to

uncultured bacterial bands isolated from DGGE gels and had greater than 90% similarity. Band 18 was common to all treatments and rootstock varieties, with only minor changes in relative intensity. In Schwarzmann, band 18 was stronger in the *C. destructans* inoculated treatments (+0, +1, +2) than in the non-inoculated treatments (-0, -1, -2), but the same did not hold true for 101-14. This band had 91% similarity to an uncultured Verrucomicrobia isolate from agricultural soil (http://www.ncbi.nlm.nih.gov/). Bands 21, 22 and 23 were also common to all treatments and rootstock varieties. Band 38 was found in most samples and there were no obvious trends in the distribution and intensity of this band. Bands 25 and 62 were typically paired, faint and intermittent in all treatments and replicates. Several other bands with inconsistent intensities, namely 50, 54, 55, 64 and 65 were also excised, reamplified and sequenced.

4.3.4.3 Sequence gels

SSCP gels that were run to confirm the correct bands had been excised showed very weak bands for the re-amplified PCR products (Figure 4.5-2, Appendix 4.5). Sometimes more than one band was seen in a lane for such a product (Figure 4.6).

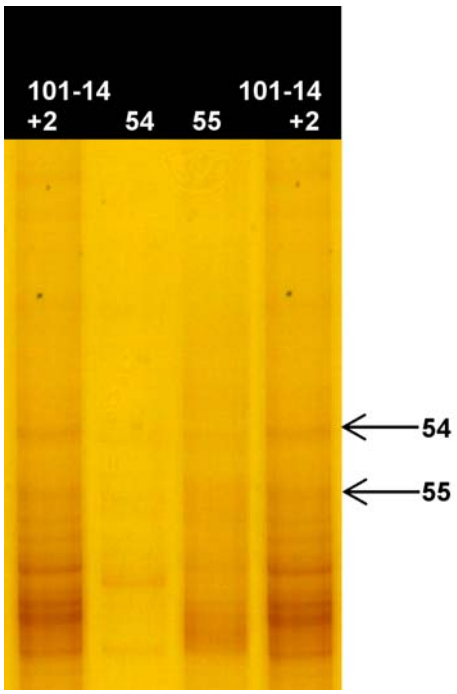


Figure 4.6: An example of band excision: column 54 and column 55. Both bands were originally excised from a 101-14 'Cyl + 2' sample.

Table 4.4: Sequenced bands excised from SSCP gel of amplified grapevine rhizosphere bacteria (16S DNA) with their highest matches from GenBank.

Band	Name	Source	Accession #	Coverage	Max ident	Size
18	Uncultured Verrucomicrobia bacterium	agricultural soil	EU297643.1	94%	91%	290 bp
21	Uncultured bacterium	arctic tundra soil	AM945475.1	97%	91%	220 bp
	Uncultured Verrucomicrobia bacterium	agricultural soil	EU297643.1	97%	91%	
22	Uncultured Acidobacteria bacterium	agricultural soil	EF662429.1	91%	94%	270 bp
23	Uncultured bacterium	rice field	AM909906.1	95%	92%	280 bp
	Uncultured gamma Proteobacterium	environmental sample	AY580826.1	97%	90%	
25	Uncultured bacterium isolate DGGE band	alpine soil	DQ525812.1	85%	95%	282 bp
	Delta subdivision Proteobacterium	Fjord	L40767.1	96%	89%	
38	Uncultured bacterium DGGE band	phytoremediation site	AY649351.1	88%	93%	331 bp
	Prosthecomicrobium pneumaticum	*Prosthecomicrobium pneumaticum*	AB017203.1	89%	90%	
50	Uncultured bacterium	anaerobic sludge	AB267019.1	98%	92%	270 bp
	Desulforegula conservatrix	freshwater sediments	AF243334.1	97%	91%	
54	*Rhodovulum* sp.	deep sea sediments	AY214344.1	95%	88%	260 bp
55	Uncultured Acidobacteria bacterium	agricultural soil	EF662429.1	99%	91%	281 bp
62	Uncultured Proteobacterium	Soil	EF018977.1	96%	91%	280 bp
64	Uncultured bacterium	prairie soil	EU135421.1	100%	92%	280 bp
	Uncultured Firmicutes bacterium	agricultural soil	EU299385.1	100%	89%	
65	Uncultured bacterium	prairie soil	EU133286.1	97%	91%	286 bp
	Uncultured Verrucomicrobia bacterium	agricultural soil	EU299074.1	94%	91%	

4.4 DISCUSSION

This greenhouse trial investigated the effect of carbohydrate stress, as induced by leaf trimming, on the disease severity and disease incidence of Cylindrocarpon black foot disease in Sauvignon Blanc scion wood grafted to grapevine rootstocks 101-14 and Schwarzmann. Disease severity was significantly higher in highly carbohydrate stressed plants than in the unstressed or moderately stressed plant group. There was also a trend for increasing disease incidence with severity of trimming, with highly and moderately stressed plants having the greatest disease incidence. These findings were reinforced by the mean root dry weight data which was lower in highly stressed plants than unstressed and moderately stressed plants. Inoculation also reduced the root dry weights of the stressed treatments, with Schwarzmann seemingly more affected by carbohydrate stress than 101-14.

Problems with natural infection and death of some replicates contributed to confounding the statistical analysis, and it is possible that potential significance of data for some treatments was obscured by these factors. Although the ANOVA estimated means were not significant, the raw data shows promising trends for differences in incidence and severity as well as shoot dry weights for the stress treatments (Appendix 4.2). Since other research reported significantly greater susceptibility for 101-14 than Schwarzmann, the lack of strong significance in this study was probably caused by low replication, natural infection from the soil and the low levels of infection achieved through inoculation. In this pot trial, about 40% infection was achieved, while others have had as much as 50% in the field or 70% incidence with *C. destructans* inoculation in similar pot trials, and up to 100% with *C. liriodendri* (pers comm. C. Probst & C. Bleach, 2008). If the experiment was repeated, with more replicates and these confounding factors removed, then the trends presented and discussed could have been significant.

Only *C. destructans* inoculum was used in this trial, and identified in isolations, although other *Cylindrocarpon* species may have been present in the soil. Although colony morphology and even conidium morphology are not sufficient for the correct identification of isolates to species level, (Petit & Gubler, 2005) all species have been implicated in causing black foot (Probst et al., 2007) so even if there had been any misidentification, black foot disease incidence and severity was still able to be analysed. If there had been sufficient time and resources, the identity of isolates could have been confirmed using PCR with species specific primers (pers. comm. C. Probst, 2007). Although some native *C. destructans* isolates present in the soil may have caused infection, the introduced isolates were considered more

abundant as seen in the infection data with disease incidence being 19.5% in non-inoculated plants and 40.5% in *C. destructans* inoculated plants.

The trend found here for increasing disease severity with increasing severity of trimming agrees with other reports of stress causing increased disease severity and incidence. For example, Schoeneweiss (1981) concluded from a review of his and others' investigations that environmental stresses, including drought and freezing, greatly influence the incidence and severity of plant diseases. In this study, the significantly greater disease severity found in plants with the greatest carbohydrate deficiency (highly stressed plants) versus unstressed or moderately stressed plants indicates that *C. destructans* is better able to exploit stressed plants than unstressed plants. Halleen et al (2007) also considered that abiotic stresses such as malnutrition, inadequate drainage, improper planting and soil compaction play a role in the development of black foot disease. A relationship between defoliation (carbohydrate stress) and disease development was also shown with cottonwood seedlings which developed stem cankers after inoculation with *Cytospora* species, only when manually defoliated (Schoeneweiss, 1981). In *Eucalyptus* species, drought and defoliation increased their susceptibility to stem cankers caused by *Endothia gyrosa* and *Botryosphaeria ribis*. In unstressed trees, cankers were limited in extent and often healed, while stressed trees had much longer lesions and more canker development (Old et al., 1990). The authors concluded that the greater symptom development was due to poorer compartmentalisation responses and callus formation which were inhibited by the low carbohydrate levels in the stems of the stressed trees.

Wargo (1996) concluded that plants can be predisposed to infection when severely stressed, possibly because of the reduced energy available for defence. Under conditions of carbohydrate stress, the available carbohydrates could be preferentially allocated to maintain metabolism rather than for use in creating physical and chemical barriers around wounds (Rayachhetry et al., 1996). The quantity and quality of plant sugars play a crucial part in disease resistance (Daniele et al., 2003) with higher sugar levels in leaf tissues conferring increased resistance to some plant pathogens (Daniele et al., 2003; Murillo et al., 2003). This is not unexpected given that soluble sugars are involved in signalling and the regulation of gene expression (Shoresh & Harman, 2008). Carbohydrate stress in trees can also result in modification of amino acids and phenolic defensive compounds including phytoalexins and lignin (Wargo & Houston, 1974; Horsley et al., 2000), which use plant carbohydrates in their synthesis (Vidhyasekaran, 1974; Daniele et al., 2003). Measuring the quantity and composition of carbohydrates in the roots and shoots of the different stress treatments, before

and after the infection period, would have added an interesting dimension to this study, as was reported for oak trees, in which defoliation significantly decreased starch and sucrose levels in the bark and outer wood of their roots. However, in that example, defoliation increased the levels of reducing sugars, such as glucose and fructose, which can stimulate the growth of some pathogens (Wargo, 1996).

Defoliation may also negatively affect the activity of the naturally occurring glucanase and chitinase enzymes in the bark and wood of some plants. Since these enzymes disrupt fungal growth, this provides another mechanism for increasing plant susceptibility (Wargo, 1996). Fungal cell walls typically contain significant levels of chitins and glucans and these are hydrolysed by the glucanase and chitinase enzymes produced by the plant (Collinge et al., 1993) in response to wounding and pathogen attack (Giannakis et al., 1998). In grapevines, varieties that have genes for endochitinases and glucanases have been shown to have greater tolerance or resistance to the grapevine dieback fungus, *Eutypa lata* (pers. comm. S. Delrot, 2008). Giannakis et al (1998) also reported a positive correlation between resistance to powdery mildew, caused by *Uncinula necator*, and the activity of chitinase and β-1,3-glucanase in different grapevine genotypes. Their studies used agars containing leaf extracts with these enzymes, which inhibited fungal growth and resulted in hyphal tip rupture. The plants' responses to *C. destructans* infection and different defoliation treatments should also be investigated in relation to pathogenesis related proteins in the roots or stems of different grapevine rootstocks. This may be worthwhile since stimulation of chitinase and glucanase activity has been reported in grapevine tissues, including roots (Derckel et al., 1996), in response to abiotic stress such as cold stress and drought (de los Reyes et al., 2001; Castro et al., 2008).

The differences in rootstock susceptibility to *C. destructans* could be due to differences in the carbohydrate concentrations of 101-14 and Schwarzmann roots. Higher tissue carbohydrate concentrations, often associated with more resistant hybrids of some crops, can sometimes inhibit pathogen growth, as shown for resistant and susceptible potato hybrids by Daniele et al (2003). The same could be true for grapevine rootstocks, since grape variety is known to influence carbohydrate reserves (J. Bennett et al., 2005).

Disease incidence data for infection at 5 cm up the stem was not significant for stress, variety, or even inoculation treatments. There was a trend for highly carbohydrate stressed plants to have higher disease incidence (19.65%) than the combined unstressed and moderately stressed group (7.60%), further confirming that carbohydrate stress could increase black foot development. For most treatments, incidence at 5 cm (16.1% with inoculation) was less than

at 0 cm (21.4% with inoculation) but reflected it. The most likely reason for the lower incidence at 5 cm was that the progression of the disease was too slow for the infections to reach the 5 cm region by the time of assessment. However, the time allowed for infection to develop (10 months) should have been sufficient as others have had up to 35% death rate due to Cylindrocarpon black foot in grapevines after inoculation with the same concentration of a *C. destructans* isolate, when only 4.5 months was allowed before destructive assessment. Their different method of root inoculation, by dipping them in the inoculum rather than inoculating the soil, could have accelerated infection (Halleen et al., 2004). In addition, their pots did not contain soil and so lacked competition by soil microorganisms. Inoculation of field soil caused a similar trend, with more infection at 0 cm than at 5 cm, when 8 months was allowed for disease development (pers. comm. C. Bleach, 2009).

Perhaps disease incidence was not significantly influenced by carbohydrate stress because the plants were left to grow for a significant period of time. The infection might have occurred earlier in 101-14 than Schwarzmann, resulting in higher disease severity because the infection had longer to infect the trunk. If the plants had been assessed earlier, a similar incidence at 0 cm might have been observed. Alternatively, perhaps the rootstocks had equal susceptibility to *C. destructans* infection (incidence), but the rate of disease progression within the rootstock tissues differed. If 101-14 had a greater degree of tissue invasion or break-down, then this could account for its higher disease severity.

In this study, root dry weights were reduced in relation to the level of carbohydrate stress. This is in line with other research which reported that roots were particularly sensitive to defoliation, as it reduced root starch concentrations dramatically (Bennett et al., 2005) and decreased root dry weight (Kliewer & Fuller, 1973). The defoliation treatments used in this trial could have been more frequent or intense if larger plants had been used, potentially causing even greater disease severity or incidence. However, Bennett (2002) used similar defoliation treatments as employed in this trial; with mature Chardonnay vines (>5 years old), he conducted the experiment in a vineyard and plucked new leaves according to the same ratio, but more frequently during the growing season.

Kliewer (1970) and Kleiwer and Fuller (1973) used defoliation treatments on grapevines of 0, 25, 50, 75 and 100% in rooted cutting pot trials similar to this one. They performed defoliations at different grapevine developmental stages, including fruit-set, veraison and fruit maturity. Defoliation at fruitset caused lower dry weights for canes, trunks and roots, at veraison it caused lower dry weights for trunks and roots, and at harvest it only reduced the root dry weights. In their study, the root dry weights of 50 and 75% defoliated vines were

significantly less than those of the 25 or 0% defoliated vines ($P<0.05$). For example, roots from 0% defoliated vines weighed 106.2 g, those from 25% defoliated vines weighed 97.2 g, those from 50% defoliated vines weighed 83.6 g, and those from 75% defoliated vines weighed 78.5 g, indicating a reduction of about 26% between the control and the most intense defoliation treatment. Kliewer and Fuller (1973) allowed a much longer timeframe for their study (2 years) than was available for this masters' research program, which allowed them to carry out the defoliation treatments a total of six times, three times a year. In addition, the plants used here were smaller than theirs, and so fewer differences between treatments might have been observed because larger plants lose proportionally more leaves for any given percentage based trimming treatment, making a greater difference to their carbohydrate reserves. They also used rooted cuttings, not grafted vines, and Sultana vines rather than the wine varieties used here.

The variety by stress interaction ($P=0.062$) was associated with the greater reduction in the root dry weight of Schwarzmann than 101-14, especially with the highly stressed plants. Schwarzmann was more affected by the carbohydrate stress than 101-14, probably due to 101-14 being more vigorous and therefore having a greater ability to quickly regenerate the lost leaves and replenish carbohydrate stores. Despite its apparent better tolerance of carbohydrate stress, 101-14 was found to be more susceptible to Cylindrocarpon black foot than Schwarzmann, in this trial and in others (Harvey & Jaspers, 2006). Regardless, 101-14 is currently the most commonly used rootstock in New Zealand vineyards (Verstappen, 2008), possibly for its agronomic qualities.

The significant interaction between inoculation and carbohydrate stress was evident with inoculated plants that had reduced root dry weight in moderate and high stress treatments, but increased root dry weight in unstressed treatments (when compared for the non-inoculated plants). *Cylindrocarpon destructans* infection was more likely when the plants were carbohydrate stressed, and the infected vines probably lost their necrotic diseased roots, and had poor root development from necrotic crowns. Halleen et al (2004) also reported that inoculation with *Cylindrocarpon* species, including *C. destructans*, significantly reduced both root and shoot mass in potted grapevines. The increased root mass seen in unstressed inoculated vines, which have the carbohydrate reserves, could perhaps be attributed to the loss of root material stimulating the plant to produce more roots, as was seen with citrus rootstock cultivars infected with *Phytophthora nicotianae* (Graham, 1995).

In terms of the shoot dry weight data, only rootstock variety was a significant factor, with 101-14 again having higher shoot dry weights than Schwarzmann, indicating that 101-14 was

more vigorous than Schwarzmann. Shoot dry weight was not influenced by carbohydrate stress, possibly because the study period and defoliation intensities used in this study were not sufficient for such changes to be detected. Kliewer and Fuller (1973) reported that defoliated plants typically experienced a decrease in shoot growth and internode length, a useful measurement that could have been taken in this study.

Single stranded conformational polymorphism (SSCP), a culture independent methodology, was used to monitor changes in the bacterial populations of the rhizosphere, by using universal bacterial primers. Although the bands on the SSCP gels clustered at the bottom of the gel and this often made it difficult to clearly see individual bands, three main results were still apparent. Firstly, there were different banding patterns, indicating changes in the diversity of bacteria in the rhizosphere, caused by some of the treatments. Secondly, the banding patterns on gels were different between the two rootstock varieties, indicating that rootstock variety also affected bacterial rhizosphere communities. Thirdly, the observed changes in banding patterns were more often an alteration in band intensity rather than a complete change in presence or absence of bands. These differences were consistent between gel replicates.

The greater vigour of 101-14 than Schwarzmann was shown by it producing higher root dry weight and greater shoot dry weight in the scion. Shoot development and root growth rate significantly influence rhizodeposition and therefore the size and composition of the soil microbial community (Kuzyakov, 2002). Due to its observed greater vigour, rootstock variety 101-14 probably had greater root exudation, and supported a greater soil bacterial population than Schwarzmann, mechanisms already discussed in Chapter 2. Schwarzmann appeared to be more susceptible to defoliation induced carbohydrate stress than 101-14, with Schwarzmann's root dry weights suffering a greater reduction when plants were highly stressed (compared to the unstressed and moderately stressed treatment) than those of 101-14 (Figure 4.3). It is possible that the differences between the stress treatments were caused by the effects on exudates released from the roots. Vestergard et al (2008) suggested that recently assimilated carbon was released to the rhizosphere more slowly in defoliated plants than in non defoliated plants. If less carbon was allocated to the rhizosphere in the highly stressed plant samples, this could have resulted in a loss of bacterial density from key taxonomic groups, which depend on the exudates as a carbon source, causing SSCP bands to fade.

The changes in bacterial communities due to carbohydrate stress differed with grapevine rootstock variety. For rootstock 101-14 the banding patterns of the no stress and high stress treatments were more similar to each other than to the moderate stress treatment (Figure 4.4).

For rootstock Schwarzmann, the no stress and moderate stress banding patterns were more similar to each other than for the high stress treatment (Figure 4.5). These results reflect the trends in disease severity at 0 cm shown in the raw data (Appendix Table 4.2-1). They suggest that the bacterial rhizosphere communities associated with the rootstocks were affected by the amount of disease at the stem base. The exact identity of all of the bands in each pattern is unknown but a representative group was sequenced (Table 4.4). Rootstock variety differences in SSCP bands were also apparent when the differentially stressed plants had been inoculated with *C. destructans*. For rootstock 101-14, the banding pattern of the bacterial populations changed between the moderate and high stress in the inoculated treatments, unlike the banding patterns of rootstock Schwarzmann which remained relatively similar with only small shifts in band intensities due to defoliation treatments. This indicated that in 101-14, the combination of pathogen and defoliation was able to alter the bacterial rhizosphere population only when defoliation was severe.

In this experiment, the trend was for 101-14 to have greater infection than Schwarzmann, so perhaps this difference in rootstock susceptibility was partially responsible for the differing response of bacterial communities to carbohydrate stress. Having higher vigour, 101-14 was able to tolerate moderate carbohydrate stress, but being more susceptible to *C. destructans*, when the carbohydrate stress passed a certain threshold (high intensity), disease severity increased. The different banding profile of 101-14 for the high stress treatment might be due to both decreased root exudation due to defoliation, and also infection causing the loss of necrotic root material, thereby reducing the root area available for exudation. This would mean a loss of habitat and nutrients for groups of bacteria on or in this root tissue. Schwarzmann bacterial populations not responding significantly differently when inoculated than when non-inoculated perhaps reflect the lack of susceptibility to the pathogen.

Defoliation induced stress can increase the release of soluble organic compounds from the roots (Paterson & Sim, 2000), or compromise plant physical barriers (as discussed earlier). This combination might encourage pathogen growth and increase disease incidence, resulting in leakage of carbon and electrolytes from pathogen entry wounds. Naseby et al (2000) reported that inoculating pea plants with *Pythium ultimum* increased the root and rhizosphere bacterial (including fluorescent *Pseudomonas* species) and fungal populations. These population increases were attributed to *Pythium* causing root damage and or nutrient leakage from the roots. In this study, such leakage could also have stimulated microbial biomass and activity, including pathogen growth or the growth of other fungal and bacterial groups in the rhizosphere. Additionally, *C. destructans* might be able produce toxins or enzymes which

increase leakage from the roots. For example, when cocoyam roots are infected with *Pythium myriotylum*, loss of electrolytes is due mainly to the disruption of cell membranes caused by enzymes and toxins (Nyochembeng et al., 2007). In one study by Meharg and Killham (1995), root exudation of perennial ryegrass increased as a result of inoculation by a range of soil fungi and bacteria. Modification of exudation was induced by microbial metabolites which increased plasmalemma permeability or compromised its protein function.

Bacterial populations could vary between the rhizospheres of infected and non infected plants due to the presence of decaying or lost roots in the former. This is because soil microorganisms are dramatically affected by the quality of plant litter and root turnover (Paterson, 2003). The shifts in the SSCP banding patterns could also reflect the loss or gain of bacteria affected by *C. destructans* antagonistic bacteria. The effect of inoculation on banding patterns could be partly explained by the fact that diseased plants can have different fungal and bacterial rhizosphere communities than those of healthy plants. For example, healthy black spruce seedlings had a higher proportion of Acidobacteria and γ-Proteobacteria than the diseased seedlings which sustained a higher proportion of Actinobacteria (Filion et al., 2004). In the same study, it was also reported that beneficial microorganisms and those with biocontrol potential against soil-borne pathogens, for example *Pseudomonas*, *Bacillus* and *Paenibacillus*, were detected in the rhizosphere of only healthy seedlings. However, there was no evidence about whether the communities associated with healthy roots promoted disease suppression, or if they were present because the pathogen was not.

Marschner et al (2002) analysed bacterial (16S rDNA) and eukaryotic (18S rDNA) community structure of white lupin using PCR-DGGE. They reported that soil microbial communities were modified by organic acid exudation and that the changes in community structure were reflected in the DGGE banding patterns, with several bands being present for all organic acid concentrations, but others for low or high concentrations. By sequencing of bands, Schwieger and Tebbe (2000) found that inoculation of alfalfa with the pathogen *Sinorhizobium meliloti* L33 modified the rhizosphere bacterial community, decreasing the proportion of γ-Proteobacteria while increasing the proportion of α-Proteobacteria. In this study, the shift seen in the SSCP banding pattern when defoliation stress was severe could perhaps be attributed to similar pathogen induced leakage.

In several gels, the banding pattern of the inoculated and/or stressed treatment was quite different from the non-inoculated and unstressed treatments, with particular groups of bands most affected. It is possible that the absent bands represent bacteria that may have been inhibited by the pathogen itself, or the induced change in root exudates was not conducive to

their growth. Specific bacterial groups could require large amounts of carbon from their host, often being dependent on specific root exudate components, as was found to be the case with soil fungi from defoliated trees (Cullings et al., 2005). Bacteria differ in their ability to utilise particular carbon substrates in the rhizosphere, for example, organic acids. When the relative availability of these change/fluctuate, the competitive ability of some species may increase or decrease accordingly, changing the microbial community structure (Marschner et al., 2002). Defoliation treatments may also have altered the grapevine root exudate quantity and quality in such a way.

The soil only controls give an indication of how much the grapevine rootstocks contributed to the soil bacterial populations, as opposed to what was already present in bulk soil. These controls were similar to several of the stress/inoculation treatments, such as in 101-14 for the low stress non-inoculated and high stress non-inoculated /inoculated treatments. For rootstock Schwarzmann they were most similar to the high stress non-inoculated and all of the inoculated treatments. This suggests that the bacterial communities in all of these treatments are similar to those in bulk soil, where there is likely to be less carbon and organic matter than around plants, due to the lack of root exudation and decaying plant tissues. Maize plants for example, are estimated to deposit 100 mg C kg^{-1} soil per day (Iijima et al., 2000). Looking at Schwarzmann in particular, it seems likely that the similarity between rhizospheres of highly stressed plants and the control soils is the result of low root exudation by this low vigour rootstock.

The great similarity in types and numbers of bands for both rootstock varieties and all treatments and the differences in band intensities suggested that the response to pathogen and /or defoliation stress is not related to the presence or absence of a specific bacterial group, but rather to the abundance or functionalities of bacteria. Overall, the findings of this study were in line with those of Costa et al (2006a), who found no significant differences in the *Pseudomonas* species diversity (DGGE fingerprints) for different maize cultivars, but rather, shifts in the relative abundance of dominant populations related to plant development. However, as discussed in Chapter 2, one of the obvious difficulties in interpreting the SSCP gels was the tightly clustered bands at the base of the gel. Optimisation methods are also outlined in that section.

Again, as with Chapter 2, DNA sequencing was used to identify the bacterial species generating the banding patterns. Matches to GenBank sequences were not 100%, and ranged from 88-95%. The maximum identification percentage given for the sequenced bands are not high enough for an absolute assignment of bacterium species identity. However, the

percentage identity was considered adequate to give some indication of phylum, class or genus level match as similar results were obtained by Opelt et al (2007). Sequences corresponding to SSCP bands showed that the grapevine rhizosphere included members of the bacterial phylum Firmicutes (Table 4.4), a group that includes genera implicated in the biological control of fungal pathogens, including but not limited to, *Bacillus*, *Clostridium* and *Paenibacillus* (de Vos et al., 1984). For example, a *Bacillus* isolate (UYBC38) was demonstrated to be antagonistic towards *Botrytis cinerea* (Rabosto et al., 2006), a *Clostridium* species, isolated from rice field soil was antagonistic to the pathogen *Rhizoctonia solani* (Niza et al., 2007), and a strain of *Paenibacillus polymyxa* (BRF-1) suppressed the growth of *Phialophora gregata*, the cause of soybean brown stem rot (Zhou et al., 2008). Members of the Proteobacterium phylum were also detected in the grapevine rhizosphere soil. This phylum includes such genera as the *Pseudomonas* (γ-Proteobacteria) and *Burkholderia* (β-Proteobacteria) (Brenner et al., 2005) which have also been implicated in the biocontrol of fungal pathogens (Costa et al., 2006a; Opelt et al., 2007). Additionally, the bacterial groups found here were similar to those detected on roots of black spruce seedlings grown in a nursery (Filion et al., 2004), including bacterial groups such as Acidobacteria, Actinobacteria, Firmicutes, Proteobacteria (alpha, beta, delta, gamma), and Verrucomicrobia. A large number of the bands sequenced in their experiment also matched up with an "uncultured bacterium".

In the SSCP analysis of grapevine rhizosphere bacterial communities, the bands that changed the most in response to treatment were 21, 22, 23 and 65. In the *C. destructans* inoculated rhizosphere, of 101-14 in particular, high intensity defoliation resulted in fading of the bands, indicating a decrease in the populations of these bacteria. Bands 21 and 65 represented members of the Verrucomicrobia, while band 22 was a member of the Acidobacteria. Cardenas and Tiedje (2008) found that Verrucomicrobia and Acidobacteria were largely unculturable but ubiquitous soil bacteria, which would not have been identified as dominant members of the rhizosphere had only culture dependent methods been employed. The role of these bacteria is uncertain but some members of the Verrucomicrobia are known cellulose degrading organisms (Schmalenberger & Tebbe, 2002), and methane oxidisers (Dunfield et al., 2007). Band 23 was probably a member of the γ-Proteobacteria. Some members of the Proteobacteria are known to be physiologically flexible and able to make use of several root exudate compounds (Zul et al., 2007). Decreasing intensity of this band occurred when 101-14 was highly carbohydrate stressed, providing further evidence to the hypothesis that this treatment could cause reduction in quantity of root exudates.

With so many similarities in the sequences between grapevine rootstock varieties, it is likely that many of the bacterial groups were common to both rootstocks. However, strains of antagonistic bacteria have been shown to have different genotypic and phenotypic profiles, but the same mobility on a DGGE gel (Costa et al., 2006a), and perhaps the same could occur with SSCP.

In summary, this trial shows that carbohydrate stress increases *C. destructans* black foot disease incidence and severity. Of the two rootstock varieties grafted to the scion wood, 101-14 appears more susceptible to infection than Schwarzmann. However, given the large effect of high levels of defoliation on Schwarzmann root dry weight, it would seem that this rootstock is more susceptible to carbohydrate stress. The SSCP analysis indicates that stress and pathogen presence can alter bacterial communities in the rhizosphere. These changes may either contribute to, or be a product of, the increased susceptibility of 101-14 to black foot infection and the low root dry weight of Schwarzmann caused by carbohydrate stress.

CHAPTER 5
CONCLUDING DISCUSSION AND FUTURE WORK

This research set out to determine whether grapevine rhizosphere bacteria play a role in the reportedly different susceptibilities of grapevine rootstock varieties to Cylindrocarpon black foot disease. When this study began, it was considered that *C. destructans* was mainly responsible for black foot disease (Gubler et al., 2004), but it was also acknowledged that other species might be involved (Bonfiglioli, 2005). In South Africa, another *Cylindrocarpon* species, *C. liriodendri* is now believed to be the dominant pathogen; some isolates were re-identified from *C. destructans* to *C. liriodendri* using DNA sequence data (Halleen et al., 2006b). Recent research in New Zealand by Probst et al (2007) also found *C. liriodendri* isolates to be more pathogenic than those of *C. destructans* and *C. macrodidymum*.

5.1 CHAPTER 2: POPULATION, DIVERSITY AND FUNCTIONALITY OF RHIZOSPHERE BACTERIA

The size and diversity of bacterial populations associated with the rhizospheres of the different grapevine rootstocks was evaluated by dilution plate counts and molecular characterisation with SSCP. The results indicated that the size and diversity of bacterial populations did not directly correlate with the reported differences in susceptibilities of the rootstocks to Cylindrocarpon black foot disease. Rootstock 5C had bacterial populations that were larger and more varied between plants than those from the rhizospheres of 101-14, Riparia Gloire and Schwarzmann. The larger bacterial populations of 5C could be attributed to its greater vigour, since Merckx et al (1985; 1987) showed that rhizosphere soil microbial biomass increases with rhizodeposition. Root exudates may differentially inhibit or stimulate *C. destructans* growth, since they can include compounds toxic to some microorganisms (Fu & Cheng, 2002). For example, those of the common bean were shown to inhibit the growth of *Rhizobium* species, probably through the action of phenolic compounds (Kato et al., 1997). Conversely, allelochemicals exuded from some rice cultivars, but not others, were shown to stimulate soil bacterial communities, but inhibit fungi (Kong et al., 2008).

To establish whether root exudates can influence the growth of *Cylindrocarpon* species, exudates could be collected from the different rootstocks and plate assays used to determine whether they stimulate or inhibit fungal growth or spore germination. Buxton (1962) impregnated filter paper with root exudates from banana varieties with different susceptibilities to Panama wilt. Strips of this filter paper were placed on agar plates inoculated with spores of *F. oxysporum* f.sp. *cubense* and its effect on germination determined. Alternatively, percent germination could be calculated by adding spores to root exudates, in microtiter plates or samples spotted onto glass slides (Gunawardena et al., 2005). However, influencing factors such as plant age, pathogen strain or root surface area and volume should be considered when undertaking such an experiment.

Functionality assays indicated that bacterial isolates from the rhizosphere of Riparia Gloire had more strongly positive responses than those from the more susceptible 101-14. More bacteria from Riparia Gloire produced highly active hydrolytic enzymes and siderophores than bacteria from other rootstocks, and these bacteria may play a role in suppressing black foot disease. However, the results are inconclusive and it is recommended that further research be carried out. More comprehensive functionality tests employing a larger number of assays, such as those employed by Berg et al (2002; 2005), could be used in combination with a larger number of bacterial isolates (from all rootstock varieties). This might provide a more complete picture of the role of grapevine rhizosphere bacteria in rootstock susceptibility to black foot disease as was found by Berg et al (2002; 2005) from their studies on the pathogen *V. dahliae*. A total of 120 bacterial isolates were screened in the current experiment, but others have tested a significantly larger sample, for example, Berg et al (2002) assessed 5854 bacterial isolates and Berg et al (2005) assessed 2648. However, for such a large scale experiment high through-put and partly automated methods would be necessary. Identifying all the bacterial isolates, at the dilution plating stage, would also have been advantageous as a range of isolates with known diversity could have then been assessed for functionality. The effect of reinoculating promising beneficial bacterial isolates back into the grapevine rhizosphere should also be investigated. Bacterial isolates that were demonstrated to have strongly positive functionality assay responses could be made into a filtrate and introduced to the soil of plants prior to inoculation with *Cylindrocarpon* species.

5.2 CHAPTER 3: SUSCEPTIBILITY OF GRAPEVINE ROOTSTOCKS TO *C. DESTRUCTANS* AND *F. OXYSPORUM*

A pathogenicity trial involving both *C. destructans* and *F. oxysporum* determined their pathogenicity to four rootstock varieties. These pathogens were able to infect the roots of the grapevine rootstocks tested to different extents. Rootstocks 101-14 and 5C were most susceptible to *C. destructans*, while Riparia Gloire and Schwarzmann were more susceptible to *F. oxysporum*. Results suggested that *F. oxysporum* might be the more aggressive pathogen because it caused greater disease severity overall. There was also an indication that inoculation with one pathogen increased plant susceptibility to another, with increased *F. oxysporum* infection in the *C. destructans* inoculated treatments of Riparia Gloire and Schwarzmann. In the report by Harvey and Jaspers (2006) and the current experiment, Riparia Gloire demonstrated low susceptibility to *C. destructans*, and in the latter was shown to have a relatively small bacterial population. In the current study 5C had high susceptibility and a relatively large bacterial population. Together, these findings appear to support the work of Gilbert et al (1994), Neal et al (1973) and Rumberger et al (2007) where more susceptible plant cultivars had larger bacterial populations than the less susceptible plant cultivars. No other significant differences were noted between the rhizosphere bacterial populations of the rootstocks that could be correlated with their susceptibilities.

Any differences in the susceptibilities of grapevine rootstocks to *C. destructans* and *F. oxysporum* could in part be due to differences in physical barriers to fungal penetration. The production of secondary wall material increases disease resistance by providing a barrier to fungal penetration, or by some juvenile tissue being unavailable to the invading fungi (Taher & Cooke, 1975). Future research could investigate whether different grapevine rootstocks differ in how much carbohydrate they invest in these barriers; it is possible that the more resistant ones may have greater internal differentiation and lignification, limiting the movement of invading fungi within the plant. Plants could be inoculated with the pathogens, and then the number and thickness of these barriers examined under the light microscope. Investigations should also be conducted to determine whether the more susceptible varieties differ in exudates that attract either *C. destructans* or *F. oxysporum* to their roots. For example, Botha et al (1990) showed that root exudates of avocado varieties with high resistance to *Phytophthora cinnamomi* attracted fewer of the pathogen's zoospores than the more susceptible varieties. Botha and Kotze (1989) found that particular amino acids (glutamic acid, aspartic acid and arginine) responsible for attracting the pathogen were most

abundant in the susceptible plants. Marais and Hattingh (1985) found similar interactions between grapevines and *P. cinnamomi*.

5.3 CHAPTER 4: CARBOHYDRATE STRESS EFFECTS ON *C. DESTRUCTANS* DISEASE SEVERITY AND RHIZOSPHERE POPULATIONS

Carbohydrate stress significantly increased *C. destructans* black foot disease severity in grafted grapevine plants with rootstocks 101-14 and Schwarzmann. As before, rootstock variety 101-14 appeared more susceptible to infection than Schwarzmann, but Schwarzmann root dry weight was more depleted by the carbohydrate stress. Stress and inoculation also altered grapevine rhizosphere bacterial communities, and these changes appeared to be related to the differences in rootstock susceptibility to Cylindrocarpon black foot disease. Further investigations should evaluate the effects of leaf trimming treatments and rootstock variety on grapevine root exudation volume and composition to provide a more complete picture of how these factors affect the grapevine rhizosphere.

No conclusive results could be reported for the role of grapevine rhizosphere bacteria in the susceptibilities of rootstocks to Cylindrocarpon black foot disease, when stressed or unstressed, but further research, using additional molecular tools and functionality assays involving more bacterial isolates (from all the rootstocks) might offer some clarification. It is likely that the mechanisms of action might be more subtle than could be determined by the methods used in this study.

Several other experiments have incorporated the use of both universal and genus-specific bacterial primers. For example, Opelt et al (2007) used SSCP to analyse the endophytic and ectophytic bacterial populations associated with Sphagnum mosses. They used both universal bacterial primers (16S rDNA), and *Burkholderia*-specific primers because *Burkholderia* species were known to antagonise *V. dahliae*. In the current study, bacterial groups containing genera implicated in biocontrol were identified by sequencing, and so further studies could repeat this experiment using both universal bacterial primers and genus specific primers. Bacterial genera known to have biocontrol capabilities could be targeted, for example, *Pseudomonas*, *Burkholderia*, *Paenibacillus*, *Bacillus*, *Clostridia* and Streptomycetes (family Streptomycetaceae, class Actinobacteria) (Inbar et al., 2005). The latter are known to produce a large array of secondary metabolites including antibiotics and extracellular enzymes, which could be investigated with primers specific to the Actinobacteria as reported by Inbar et al (2005). Challis and Hopwood (2003) used Streptomycetaceae specific primers to study the distribution of these bacteria in response to root proximity and compost amendment.

Actinomycetes have been shown to inhibit *C. destructans in vitro* (Whitelaw-Weckert, 2004) and so they would be an ideal group to monitor in a defoliation study as root exudates are their primary carbon source (Crawford et al., 1993).

The sequencing results obtained here could be used as a guide to choose group specific primers for species present in the grapevine rhizosphere, particularly species known to inhibit the growth of *C. destructans in vitro*. This would be a useful addition, as species of biological importance may be at levels too low to be detected by the use of universal bacterial primers, particularly with the very small size of the samples analysed. Future investigations could also determine the diversity of fungi (including the pathogens) in the rhizosphere of grapevines since Costa et al (2006b) found that plant species affected rhizosphere community structure of both bacteria and fungi. Arbuscular mycorrhizal fungi would be a useful group to monitor because they are known to colonise grapevine roots (Schreiner, 2003) and are a dominant beneficial influence in the rhizosphere (Bending et al., 2006).

Primers that target specific functionalities could also be investigated, for example, genes implicated in fungal antagonism, such as those coding for hydrolytic enzymes or secondary metabolites. Genes involved in siderophore production in particular, might be good targets as reported by Opelt and Berg (2004) who found that nearly all of the *V. dahliae* antagonistic *Pseudomonas* and *Burkholderia* species isolated from bryophytes produced siderophores. To gain a better functional understanding of the mechanisms behind bacterial antagonism of fungal pathogens, Berg et al (2002) also used a PCR screening approach to target the *phlD* gene in *Pseudomonas putida* isolates. This gene codes for DAPG, one of the antibiotics frequently implicated in the biocontrol of fungal pathogens. A similar study was undertaken by Frey-Klett et al (2005) who used primers to amplify regions of genes involved in the synthesis of the antibiotics phenazine-1-carboxylic acid, pyrrolnitrin and pyoluteorin. Such primers directed to genes might have been informative in this experiment, targeting plant-beneficial bacterial functionalities or genes that encode antifungal compounds.

To determine which genes were being actively expressed in the grapevine rhizospheres, or which bacterial groups were most active, it might have been beneficial to use primers that target RNA rather than DNA, as DNA based analyses do not differentiate between dormant and active populations. The total rhizosphere microbial DNA extracted could be from dormant or dead cells (Sharma et al., 2005). Vestergard et al (2008), used DGGE for studying both 16SrRNA and 16SrDNA genes to determine the effect of arbuscular mycorrhizal fungi on the bacterial communities of pea plants. Sharma et al (2005) also amplified both 16S rRNA and rDNA in their DGGE analysis of bacterial community structure in the rhizosphere

of grain legumes. Use of RNA is crucial because changes in microbial activity are not always reflected in changes in bacterial community structure (Vestergard et al., 2008). However, DNA and RNA based analyses can yield very similar results, perhaps indicating that in the rhizosphere, the metabolically active bacteria are dominant (Poulsen et al., 1993). It is therefore possible that the bacteria dominating the SSCP gels in this study were the metabolically active ones. However, the use of quantitative PCR with genus specific, RNA specific, or function specific primers would have allowed more conclusive proof of the abundance and activities of bacterial groups within rhizosphere communities. For example, Kandeler et al (2009) used quantitative PCR to determine the abundance of total bacteria, nitrate reducers and denitrifiers in the different soil layers of a forest, using the 16S rRNA gene as well as nitrate reduction and denitrification genes, respectively.

The amount of soil used for analysis limited the overall effectiveness of the molecular methodology used in the current study, and a method using more soil would have been ideal. In this study, samples of 0.25 g were used whereas up to 1 g has been used in other studies (Costa et al., 2006a). Rhizosphere soil was chosen over the rhizoplane or bulk soil because it is the microbial habitat most strongly influenced by plants and commonly used in studies that investigated the composition of root microbial communities (Kowalchuk et al., 2002; Dohrmann & Tebbe, 2005), however, useful information might have been gained from the rhizoplane soil as well. Using soil from different depths, at different locations along the root axis, or from older/younger plants may also have affected the outcome since root exudation and its associated soil bacterial communities, are influenced by these factors (Yang & Crowley, 2000; Walker et al., 2003; Morgan et al., 2005).

Better sequence matches at the genus or species level would have provided a better picture of the grapevine rhizosphere communities, and could have allowed the detection of more subtle differences between rootstock varieties. As discussed in previous sections, matches were considered adequate for the broader levels of identification, although the accuracy of any genus or species level matches should not be relied upon. However, it appears that multiple methods are sometimes necessary to successfully identify rhizosphere bacteria to genus or species level. For example, Berg et al (2005) identified all *V. dahliae* antagonistic bacterial isolates from their study on potato rhizosphere, phyllosphere, endorhiza and endosphere communities. They used fatty acid methyl ester gas chromatography (FAME-GC), sequencing of the 16S rRNA gene covering variable regions v1-v4, with additional characterisation by rep-PCR with BOX primers and gas chromatography using the MIDI system (Microbial Identification System, Inc., USA). This allowed them to successfully identify 316 of their 349

isolates to the genus or species level. Using multiple methods might have improved identification in the present study.

If given further time and resources, it could have been worthwhile to carry out a field trial with a greater number of replicates, rootstock varieties and scion combinations. This would allow one to determine whether the trends observed here, in terms of carbohydrate stress increasing disease severity, were consistent in the vineyard situation. Plants grown in pots in a greenhouse, with the associated physical constraints and controlled watering and lighting, may react differently to those grown in the field when exposed to artificially induced stress. This is because natural environmental stresses are highly variable in amount and time (Schoeneweiss, 1981). Pathogen inoculum levels could also vary between the field and the greenhouse, with inoculum potentially being more localised in the former due to soil characteristics and water flow. Future investigations could also determine the effect of carbohydrate stress on a selection of the implicated *Cylindrocarpon* species, or genotypes, since subspecies variation in pathogenicity has been shown to occur (Probst et al., 2007). Different inoculum types could also be trialled, although conidia and mycelia of *C. destructans*, *C. liriodendri* and *C. macrodidymum* have recently been shown to convert to chlamydospores when placed in the soil environment (pers. comm. M. Jaspers, 2009).

It is also important to note that soil, site, plant developmental stage and season are capable of altering exudation, root morphology and, therefore, structure of rhizosphere communities, including the composition, relative abundance and diversity of antagonistic bacteria (Berg et al., 2005; 2006). The molecular component of this trial could be further expanded to examine rhizosphere bacterial populations over a longer period of time, but this was not possible due to time constraints. For example, sampling rhizosphere soil across seasons could have revealed seasonal fluctuations in soil microbial populations not seen in the single end of trial sampling done here. For example, temperature gradient gel electrophoresis profiles from maize grown in tropical soils revealed strong seasonal shifts in bacterial diversity, in the eubacterial bacterial profiles as well as in the group specific profiles of Proteobacteria and Actinomycetes (Gomes et al., 2001).

5.4 RELEVANCE OF THIS STUDY TO THE MANAGEMENT OF CYLINDROCARPON BLACK FOOT DISEASE

The results of the current study are relevant to the New Zealand grape growing industry. They have confirmed that commonly used grapevine rootstocks are susceptible to both *C. destructans* and the less studied *F. oxysporum*, indicating that greater efforts should be made to monitor both of these in New Zealand vineyards. Since the removal of leaves was shown to increase *C. destructans* disease severity, growers should take every precaution to protect their plants from abiotic and biotic stresses that result in loss of foliage. Managing environmental factors (as well as insects and diseases that cause defoliation) that could stress grapevines should be an important part of integrated pest management strategies (Schoeneweiss, 1981). This is of particular importance in cool climate environments like New Zealand, because there is little or no postharvest carbohydrate reserve accumulation when leaf senescence is soon after, or coincides with, fruit harvest (Bennett et al., 2005).

As part of normal vineyard management, canopy thinning is considered to confer benefits such as improved yield, rooting and bud fertility. It also improves canopy air flow and humidity, making pest and disease control easier (Smart et al., 1990; Stapleton & Grant, 1992; Hunter et al., 1995). However, these results indicate that care should be taken by grape growers when deciding the intensity of canopy thinning in areas at risk to *Cylindrocarpon* infection. In fact, it should probably be considered with respect to other grapevine pathogens as well. This study showed that removal of 33% of leaves from above the fourth node was not detrimental to the rootstocks tested, but this percentage could vary with rootstock and scion combinations depending on their vigour.

Treatments used in this experiment were more severe than used in normal trimming practices, but are also relevant in that they show how vines are likely to respond to more severe defoliation stress events such as early frost (Bennett, 2002). In vineyards, the mature vines have developing fruit clusters that are very strong sinks for photosynthate (Motomura, 1990) which could exacerbate the stress caused by normal trimming. Drought can also create carbohydrate stress (Horsley et al., 2000), and it is thought that the decrease in summer rains and an increase in the evapotranspiration potential due to climate change may increase this effect (pers. comm. S. Delrot, 2008). Additionally, since black foot disease primarily affects young vines, growers should be cautious with trimming young vines and perhaps not harvest from vines in their second year, which is currently a common practice in New Zealand. Further research is necessary for clarification, but it seems likely that any factors that cause severe carbohydrate stress may increase black foot disease severity.

5.5 CONCLUSIONS

This study has demonstrated that diversity and abundance of grapevine rhizosphere bacteria vary between grapevine rootstock varieties with different susceptibilities to Cylindrocarpon black foot disease. These bacteria may therefore play a role in the differing susceptibilities, but this needs to be further investigated in field trials and with more comprehensive methodology. If the role of grapevine rhizosphere bacteria is better understood, they could potentially be managed to allow New Zealand grape growers to minimise the damaging effects of this serious and economically important disease. In the future it might possible to manage, by cultural practices or introduction of species, natural rhizosphere bacterial populations in such a way that they promote optimal plant growth and health. To do this the factors influencing the abundance and diversity of bacteria with antagonistic potential need to be better understood. Berg et al (2006) concluded that this knowledge "could be the key for a successful biological control by bacterial antagonists".

APPENDIX 1

1.1 GENERAL MEDIA AND RECIPES

King's Medium B (KB): for fluorescent Pseudomonads

20.0 g Protease peptone No.3 (Difco)

10 mL glycerol

1.5 g K_2HPO_4

1.5 g $MgSO_4.7H_2O$

15.0 g Technical Agar No.3 (Oxoid)

H_2O to make 1 litre

The pH adjusted to 7.2 with 1M NaOH at 25ºC prior to autoclaving.

Fluorescent Pseudomonads were observed under UV light as fluorescent colonies (King et al., 1954; Atlas, 2004).

Nährstoffarmer agar

1.0 g KH_2PO_4

1.0 g KNO_3

0.5 g $MgSO_4.7H_2O$

0.5 g KCl

0.2 g glucose

0.2 g sucrose

20.0 g Oxoid agar no. 3

All ingredients except the agar were dissolved in 1 L of SDW and the pH adjusted to 6-6.5. The agar was added and dissolved before the solution was autoclaved (15 min, 121ºC, 15 Psi) and allowed to cool to 50ºC (Waller et al., 2002).

50x TAE (Tris-acetate) Stock

Dissolve 242 g Tris in 500 mL SDW.

Add 100 mL 0.5 M Na_2EDTA (pH 8.0) and 57.1 mL glacial acetic acid before adjusting the volume to 1 Litre with SDW.

Store at room temperature and then dilute to 1x as required.

10x TBE (Tris-borate) Stock

Dissolve 108 g Tris and 55 g of boric acid in 900 mL SDW.

Add 40 ml 0.5 M Na_2EDTA (pH 8.0) and then make up to 1 Litre with SDW.

Store at room temperature and then dilute to 1x as required.

APPENDIX 2

2.1 ADDITIONAL MATERIAL FOR CHAPTER 2

Chrome azural S (CAS) agar: for siderophores

The following solutions were prepared separately. In total this made 1 Litre of CAS agar.

Solution 1: Fe-CAS indicator solution

10 mL of 1 mM $FeCl_3.6H_2O$ in 10 mM HCL

50 mL aqueous solution of CAS (1.21 mg mL^{-1}).

This dark purple mixture was gradually added, with constant stirring, to 40 mL of an aqueous solution of hexadecyltrimethylammonium bromide (HDTMA; 1.82 mg mL^{-1}) causing the colour to change to dark blue. The solution was then autoclaved (15 min, 121ºC, 15 Psi) and allowed to cool to 50ºC.

Solution 2: the buffer solution

Dissolve 30.24 g of piperazine-N,N′-bis (2-ethanesulfonic acid) (PIPES) in 750 mL of a salt solution containing:

0.3 g KH_2PO_4

0.5 g NaCl

1.0 g NH_4Cl

The pH was adjusted to 6.8 using 50 % (w:v) KOH, then water was added to make up to a total volume of 800 mL. 15 g of agar was added to this solution before it was autoclaved and allowed to cool to 50ºC.

Solution 3

The following were added to 70 mL of water:

2 g glucose

2 g mannitol

493 mg $MgSO_4.7H_2O$

11 mg $CaCl_2$

1.17 mg $MnSO_4.H_2O$

1.4 mg H_3BO_3

0.04 mg $CuSO_4.5H_2O$

1.2 mg $ZnSO_4.7H_2O$

1.0 mg $Na_2MoO_4.2H_20$.

The solution was then autoclaved and allowed to cool to 50ºC.

Solution 4

3 g casamino acids was dissolved into 30 mL of water (10% w:v).

The casamino acid solution was filter sterilised using a sterile syringe and 0.22 μm Millipore filter unit. The solution was not autoclaved.

Finally, under aseptic conditions, solution 3 was added to solution 2, solution 4 was then added to this, and lastly, solution 1 was added with enough stirring to thoroughly mix the solutions without producing bubbles. The poured agar was cyan blue to sea-green in colour (Alexander & Zuberer, 1991; pers. comm. B. Pottinger, 2006).

Skimmed milk agar (SMA): for protease

Solution 1

In a 500 mL Schott bottle, 50 g of skim milk powder (Anchor) was dissolved in SDW, and made up to 500 mL with SDW.

Solution 2

In a 1 L Schott bottle, 2.8 g Nutrient Agar (Oxoid) and 20 g agar (Davis) were dissolved and made up to 500 mL with SDW.

Solution 1 and 2 were autoclaved separately and cooled to 55ºC before adding solution 1 to solution 2 under aseptic conditions. The plates were immediately poured and the set agar was a peachy-cream colour (Opelt & Berg, 2004; pers. comm. B. Pottinger, 2006).

Carboxymethylcellulose (CMC) agar: for glucanase

10 g CMC (Preswollen, microgranular, Sigma-Aldrich, Germany)

0.5 g asparagine

0.5 g yeast extract (Difco, USA)

0.5 g $MgSO_4.7H_2O$

0.5 g $(NH_4)_2SO_4$

1.0 g KH_2PO_4

0.5 g KCl

1.0 g $CaCl_2$

15 g agar (Davis)

SDW to make the solution to 1 Litre.

After autoclaving and cooling to 50 ºC, 2 mL of filter sterilised (0.22 µm Millipore filter unit) 200 µM MUF β-D-lactoside (Sigma-Aldrich, Germany) was added to the CMC agar and the plates poured (Miller et al., 1998).

Chitinase assay

- ### Basal agar

15 g agar (Davis)

2.0 g $(NH_4)_2SO_4$

1.1 g Na_2HPO_4

0.7 g KH_2PO_4

0.2 g $MgSO_4.7H_2O$

1.0 mg $FeSO_4$

1.0 mg $MnSO_4$

SDW to make the solution to 1 Litre (Atlas, 2004).

- **Chitin agar**

Half (50 mL) of the CM-Chitin-RBV (remazol brilliant violet-linked chitin) solution (Loewe, Germany) was dialysed at 4ºC overnight in a length of dialysis tubing (MWCO 10,000 Da), presoaked in hot water and secured with clips. Dialysis was carried out in a 10 L bucket of SDW with a magnetic stirrer to remove the sodium azide.

The basal agar and the chitin were autoclaved separately, cooled to 50ºC and then 50 mL of each mixed in a 1:1 ratio as per the manufacturer's instruction. The pH was found to be within the recommended 5-7.5 range. Plates were poured immediately. The agar set a deep violet colour (Johansen & Binnerup, 2002).

2.2 CHITIN AGAR TROUBLESHOOTING

Testing basal agar medium

Ten randomly selected bacterial isolates taken from the rhizosphere soils and the *S. entomophila* positive control were grown on the basal medium. Plates were incubated at 23ºC in the dark for 14 d. All bacteria grew slowly and so the agar was considered an adequate nutrient agar medium.

Chitin concentration

To determine the best chitin concentration, dialysed chitin was mixed into the agar at 1:1 and 1:4 concentrations, but clearing was not observed in the sample or control bacterial plates after incubation at 23ºC in the dark for 14 d, with plates being assessed every day for 14 d. The same was procedure was carried out for the non dialysed chitin with the same result. Twenty randomly selected bacteria were used, inoculating with overnight Nutrient Broth cultures. Assessment of clearing was attempted with the naked eye, against a dark or white background, and by holding up to both natural and artificial lights sources, but reactions were not conclusively strong enough for this assay to be used.

Growth trial

Ten randomly selected bacteria, and *Serratia entomophila,* a known chitinase producer, were used for this trial. The following agars were used: NA, basal medium – chitin, basal medium + non-dialysed chitin (1:1) and basal medium + dialysed chitin (1:1). All agar was visually confirmed to be at the same colour intensity. Wells of multiwell plates which contained these agars were inoculated with 10 μL of an overnight Nutrient Broth culture, of which there were

ten replicates. Incubation was for 14 d at 23ºC in the dark, with plates checked at 7 d and 14 d for growth of bacteria and any visible clearing.

All the bacteria grew well on the NA, while growth was slow on the basal medium alone, and very slow on the agar containing the dialysed and non-dialysed chitin. Since clearing was not observed, even on the different concentrations of chitin, the aim was to use differential growth rates to rank chitinase production. However, it was concluded that the difference in bacterial growth between the plates that included chitin and those that did not was not sufficient for growth to be used as an indicator of chitin utilisation. The chitin in the agars was the main carbon source, and as such was expected to enhance the growth of bacteria. This not working meant that the chitinase assay could not be included in this study.

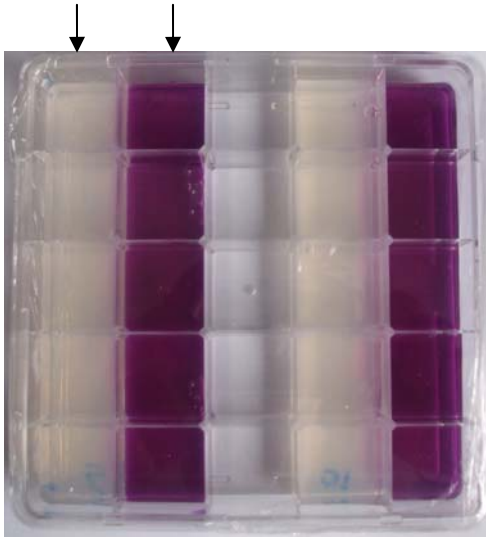


Figure 2.2-1: Set up of the multiwell plates showing basal media (clear) and basal media plus either dialysed chitin (left) or non-dialysed chitin (right).

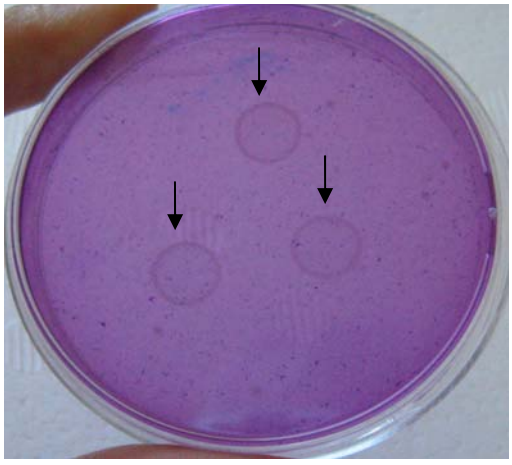


Figure 2.2-2: Bacteria growing (arrow) on plate where chitin (1:1 ratio) was the primary carbon source. No clearing was visible.

2.3 ASSESSMENT CRITERIA FOR PLATE BIOASSAYS

2.3.1 Dual plating assay

Table 2.3-1: The criteria used in dual plating to grade the inhibition of a *C. destructans* isolate by grapevine rhizosphere bacteria.

Criteria used for dual plating assessment	Grade
No fungal inhibition, mycelium growth to plate edge, through bacterial colony	-
Slight fungal inhibition with small indent seen in fungal colony around the bacterial colony, but mycelial growth still to the plate edge with no zone of inhibition visible	+/-
Moderate fungal inhibition, fungal growth up to bacterial colonies but restricted, colony taking on a diamond shape	+
Strong fungal inhibition, with zone of inhibition (>1mm) where bacteria and fungus do not touch, fungal colony taking on a diamond shape	++

2.3.2 Glucanase assay

Table 2.3-2: The criteria used to grade the fluorescence resulting from glucanase activity in grapevine rhizosphere bacteria on modified CMC agar.

Criteria used for the assessment of glucanases production	Grade
No fluorescence, dark	-
Slight fluorescence, very little light emitted by colony, hard to determine	+/-
Moderate fluorescence, some white-blue light emitted by bacterial colony	+
High fluorescence, bright white-blue light emitted by bacterial colony	++

2.3.3 Protease assay

Table 2.3-3: The criteria used to grade the clearing resulting from protease activity in grapevine rhizosphere bacteria on SMA agar.

Criteria used for assessment of protease production	Grade
No clearing of agar around bacterial colony	-
Faint or incomplete clearing of agar around bacterial colony	+/-
Small clearing zone around bacterial colony <5 mm wide	+
Moderate clearing zone around bacterial colony 5-10 mm wide	++
Large clearing zone around bacterial colony >1 cm wide	+++

2.3.4 Siderophore assay

Table 2.3-4: The criteria used to grade siderophore secretion by grapevine rhizosphere bacteria grown on CAS agar.

Criteria used for the assessment of siderophore production	Grade
No bacterial growth, no orange colony pigmentation or halo	--
Bacterial growth, no orange colony pigmentation or halo	+/-
Bacterial growth, orange pigmentation of colony, orange halo 0-1 cm	++
Bacterial growth, orange pigmentation of colony, orange halo >1 cm	+++

2.4 ANOVA TABLES AND ADDITIONAL STATISTICAL MATERIAL FOR CHAPTER 2

Table 2.4-1: Analysis of variance (ANOVA) table showing the effect of grapevine rootstock variety (treatment) on the mean total bacterial count (NA).

Variate	**Log_{10} (total bacterial count on NA)**				
Source of variation	d.f.*	s.s.*	m.s.*	v.r.*	F pr.*
Treatment	4	1.22572	0.30643	3.99	0.012
Residual	25	1.92235	0.07689		
Total	29	3.14807			

* d.f. = degrees of freedom, s.s. = sums of squares, m.s. = mean square, v.r. = variance ratio, F pr. = F probability

Table 2.4-2: Analysis of variance (ANOVA) table showing the effect of grapevine rootstock variety (treatment) on the mean total bacterial count (KB).

Variate	**Log_{10} (total bacterial count on KB)**				
Source of variation	d.f.*	s.s.*	m.s.*	v.r.*	F pr.*
Treatment	4	3.2760	0.8190	4.89	0.005
Residual	25	4.1845	0.1674		
Total	29	7.4604			

* d.f. = degrees of freedom, s.s. = sums of squares, m.s. = mean square, v.r. = variance ratio, F pr. = F probability

Table 2.4-3: Analysis of variance (ANOVA) table showing the effect of grapevine rootstock variety (treatment) on the mean fluorescent Pseudomonad count

Variate	**Log_{10} (total fluorescent Pseudomonad count +1)**				
Source of variation	d.f.*	s.s.*	m.s.*	v.r.*	F pr.*
Treatment	3	34.712	11.571	3.49	0.035
Residual	20	66.364	3.318		
Total	23	101.076			

* d.f. = degrees of freedom, s.s. = sums of squares, m.s. = mean square, v.r. = variance ratio, F pr. = F probability

Table 2.4-4: Analysis of variance (ANOVA) table showing the effect of grapevine rootstock variety (treatment) on the mean spore forming bacterial count.

Variate	**Log_{10} (spore forming bacterial count)**				
Source of variation	d.f.*	s.s.*	m.s.*	v.r.*	F pr.*
Treatment	4	0.12579	0.03145	1.62	0.201
Residual	25	0.48658	0.01946		
Total	29	0.61237			

* d.f. = degrees of freedom, s.s. = sums of squares, m.s. = mean square, v.r. = variance ratio, F pr. = F probability

2.5 DILUTION PLATING DATA FOR THE INITIAL SOIL CONTROLS

Table 2.5-1: Raw data means from the dilution plate assessment of the ten soil only controls (initial) at the start of the experiment. The data includes the three replicate plates used for each of the ten controls. For each of the bacterial count types: total (NA and KB), fluorescent Pseudomonad and spore forming, the mean number of CFU per agar plate, as well as the $\log_{10}$ CFU per gram of oven dried soil are shown.

Dilution	Count	Agar type	CFU (plate)	Log_{10} CFU
10^{-4}	Total bacteria	NA	51.77	6.48
10^{-5}			7.23	6.84
10^{-6}			1.53	7.15
10^{-4}	Total bacteria	KB	57.93	6.73
10^{-5}			8.40	6.87
10^{-6}			1.20	6.95
10^{-4}	Florescent Pseudomonads		0.50	4.71
10^{-5}			0.25	5.91
10^{-6}			0.03	6.52
10^{-3}	Spore forming bacteria	NA	52.00	5.71
10^{-4}			10.77	6.02
10^{-5}			1.37	6.05

2.6 THE BACTERIA ISOLATED FROM THE RHIZOSPHERE OF GRAPEVINE CULTIVARS AND BULK SOIL IN CHAPTER 2

Table 2.6-1: The origin and colony morphology of the bacterial isolates selected from the control soils and used in the functionality tests.

Isolate	Replicate	Media type	Plate*	Dilution	Colony morphology (size, form, opacity, colour, elevation, surface, margin)
C1	1	KB	P	10^{-4}	large, circular, opaque, raised, smooth, shiny, entire margin
C2	1	KB	P	10^{-4}	small, irregular, opaque, cream, raised, smooth, shiny, entire margin
C3	1	KB	P	10^{-4}	very small, circular, opaque, cream, raised, smooth, shiny, entire margin
C4	1	NA	S	10^{-3}	medium, circular, opaque, cream, crateriform, smooth, dull, entire margin
C5	1	NA	S	10^{-5}	small, circular, opaque, cream, flat, smooth, shiny, entire margin
C6	1	NA	S	10^{-3}	medium, circular, opaque, cream, flat, thick, smooth, dull, entire margin
C7	1	NA	T	10^{-5}	medium, circular, opaque, cream, flat, smooth, shiny, entire margin
C8	1	NA	T	10^{-5}	medium, circular, opaque, peach, raised, smooth, shiny, entire margin
C9	1	NA	T	10^{-4}	small, circular, opaque, salmon-brown, raised, smooth, shiny, entire margin
C10	1	NA	T	10^{-4}	medium, circular, opaque, cream, flat, thick, smooth, shiny, entire margin
C11	2	KB	P	10^{-5}	medium, circular, opaque, cream-white, raised, smooth, shiny, entire margin
C12	2	KB	P	10^{-5}	small, circular, opaque, raised, smooth, shiny, entire margin
C13	2	KB	P	10^{-4}	medium, circular, opaque, cream, raised, smooth, shiny, entire margin
C14	2	NA	S	10^{-4}	large, circular, opaque, cream, flat, concentric rings, thick, dull, entire margin
C15	2	NA	S	10^{-3}	medium, circular, opaque, cream, flat, smooth, dull, entire margin
C16	2	NA	S	10^{-3}	medium, circular, opaque, cream, crateriform, smooth, dull, entire margin
C17	2	NA	T	10^{-4}	small, circular, opaque, yellow, raised, smooth, shiny, entire margin
C18	2	NA	T	10^{-4}	small, circular, opaque, yellow, raised, smooth, shiny, entire margin
C19	2	NA	T	10^{-4}	very small, circular, opaque, cream-white, raised, smooth, shiny, entire margin

C20	2	NA	T	10^{-4}	large, irregular, opaque, cream-white, flat, smooth, dull, entire margin
C21	3	KB	P	10^{-4}	medium, circular, opaque, yellow, raised, smooth, shiny, entire margin
C22	3	KB	P	10^{-4}	medium, circular, opaque, cream-white, flat, smooth, shiny, entire margin
C23	3	KB	P	10^{-4}	very small, circular, opaque, cream-white, raised, smooth, shiny, entire margin
C24	3	NA	S	10^{-4}	large, circular, opaque, cream-white, flat, thick, smooth, shiny, entire margin
C25	3	NA	S	10^{-5}	medium, irregular, opaque, cream-brown, thick, crateriform, shiny, entire margin
C26	3	NA	S	10^{-5}	medium, circular, opaque, cream, flat, thick, smooth, shiny, entire margin
C27	3	NA	T	10^{-4}	large, irregular, opaque, raised with convoluted ridges, thick, dull, entire margin
C28	3	NA	T	10^{-4}	large, circular, opaque, cream, thick, crateriform, dull, entire margin
C29	3	NA	T	10^{-4}	very small, circular, opaque, orange-brown, raised, smooth, shiny, entire margin
C30	3	NA	T	10^{-4}	very small, circular, opaque, cream, raised, smooth, shiny, entire margin
C31	4	KB	P	10^{-4}	small, circular, opaque, cream, raised, smooth, shiny, entire margin
C32	4	KB	P	10^{-4}	medium, circular, opaque, cream-white, raised, smooth, shiny, entire margin
C33	4	KB	P	10^{-4}	small, circular, opaque, yellow, raised, smooth, shiny, entire margin
C34	4	NA	S	10^{-4}	small, circular, opaque, cream-white, flat, smooth, shiny, entire margin
C35	4	NA	S	10^{-3}	medium, irregular, translucent, cream-white, flat, dull, entire margin
C36	4	NA	S	10^{-3}	very small, circular, translucent, cream-white, flat, smooth, dull, entire margin
C37	4	NA	T	10^{-4}	medium, circular, opaque, yellow, raised, smooth, shiny, entire margin
C38	4	NA	T	10^{-4}	medium, circular, opaque, cream, raised, smooth, shiny, entire margin
C39	4	NA	T	10^{-4}	very small, circular, opaque, yellow, raised, shiny, smooth, entire margin
C40	4	NA	T	10^{-4}	medium, irregular, opaque, cream-white, flat, smooth, dull, entire margin

*Plate types: T (total bacteria), P (total bacteria KB) and S (spore forming bacteria).

Table 2.6-2: The origin and colony morphology of the bacterial isolates selected from the rhizosphere of 101-14 and used in the functionality tests.

Isolate	Replicate	Media type	Plate*	Dilution	Colony morphology (size, form, opacity, colour, elevation, surface, margin)
1.1	1	KB	P	10^{-5}	medium, circular, opaque, cream, convex, smooth, shiny, entire margin
1.2	1	KB	P	10^{-5}	small, circular, opaque, cream, flat, dry, entire margin
1.3	1	KB	P	10^{-4}	fluorescent, large, opaque, cream, convex, smooth, shiny, entire margin
1.4	1	NA	S	10^{-3}	small, circular, opaque, cream, flat, smooth, shiny, entire margin
1.5	1	NA	S	10^{-4}	large, circular, opaque, cream, crateriform, thick, dull, entire margin
1.6	1	NA	S	10^{-3}	small, circular, opaque, cream, flat, shiny, smooth, entire margin
1.7	1	NA	T	10^{-5}	small, circular, opaque, cream, convex, shiny, smooth, entire margin
1.8	1	NA	T	10^{-4}	small, circular, opaque, salmon, convex, shiny, smooth, entire margin
1.9	1	NA	T	10^{-4}	medium, circular, opaque, cream, flat, shiny, smooth, entire margin
1.10	1	NA	T	10^{-4}	small, circular, opaque, cream, raised, dry, entire margin
1.11	2	KB	P	10^{-5}	very small, circular, opaque, cream, raised, dull, entire margin
1.12	2	KB	P	10^{-4}	medium, circular, opaque, yellow, raised, smooth, shiny, entire margin
1.13	2	KB	P	10^{-4}	very small, circular, opaque, cream, raised, smooth, shiny, entire margin
1.14	2	NA	S	10^{-5}	medium, circular, opaque, salmon, flat, smooth, shiny, entire margin
1.15	2	NA	S	10^{-4}	small, circular, opaque, white-cream, flat, dull, entire margin
1.16	2	NA	S	10^{-3}	medium, circular, opaque, cream, flat, smooth, dull, entire margin
1.17	2	NA	T	10^{-4}	large, circular, opaque, cream, thick, crateriform, dull, entire margin
1.18	2	NA	T	10^{-4}	large, circular, opaque, cream, thick, crateriform, dull, entire margin
1.19	2	NA	T	10^{-4}	small, circular, opaque, cream-white, raised, smooth, shiny, entire margin
1.20	2	NA	T	10^{-5}	medium, circular, opaque, cream-white, convex, smooth, shiny, entire margin
1.21	3	KB	P	10^{-4}	fluorescent, large, irregular, opaque, cream, convex, smooth, shiny, entire margin

1.22	3	KB	P	10^{-5}	medium, circular, opaque, cream-apricot, convex, smooth, shiny, entire margin
1.23	3	KB	P	10^{-4}	fluorescent, circular, opaque, cream, raised, smooth, shiny, entire margin
1.24	3	NA	S	10^{-4}	large, circular, opaque, cream, thick, crateriform, dull, entire margin
1.25	3	NA	S	10^{-4}	small, circular, opaque, cream-white, flat, dull, entire margin
1.26	3	NA	S	10^{-3}	medium, circular, opaque, cream, raised, smooth, dull, entire margin
1.27	3	NA	T	10^{-5}	medium, circular, opaque, cream, raised, smooth, dull, entire margin
1.28	3	NA	T	10^{-5}	medium, circular, opaque, cream, raised, smooth, shiny, entire margin
1.29	3	NA	T	10^{-4}	small, circular, opaque, cream, flat, smooth, dull, entire margin
1.30	3	NA	T	10^{-6}	small, circular, opaque, cream, raised, smooth, shiny, entire margin
1.31	4	KB	P	10^{-4}	large, circular, opaque, cream, umbonate, smooth, shiny, entire margin
1.32	4	KB	P	10^{-4}	small, circular, opaque, cream-white, raised, smooth, shiny, entire margin
1.33	4	KB	P	10^{-4}	small, circular, opaque, yellow, raised, smooth, shiny, entire margin
1.34	4	NA	S	10^{-4}	large, circular, opaque, cream, thick, crateriform, dull, entire margin
1.35	4	NA	S	10^{-3}	medium, circular, opaque, cream, raised circular ridge, dull, entire margin
1.36	4	NA	S	10^{-3}	small, circular, white, flat, dull, entire margin
1.37	4	NA	T	10^{-4}	medium, circular, opaque, apricot-cream, flat, smooth, shiny, entire margin
1.38	4	NA	T	10^{-6}	small, circular, opaque, convex, smooth, shiny, entire margin
1.39	4	NA	T	10^{-4}	*Bacillus mycoides*, large, filamentous, cream, flat, dull, filiform margin
1.40	4	NA	T	10^{-4}	small, circular, opaque, cream-white, convex, smooth, shiny, entire margin

*Plate types: T (total bacteria), P (total bacteria KB) and S (spore forming bacteria).

Table 2.6-3: The origin and colony morphology of the bacterial isolates selected from the rhizosphere of Riparia Gloire and used in the functionality tests.

Isolate	Replicate	Media type	Plate*	Dilution	Colony morphology (size, form, opacity, colour, elevation, surface, margin)
R1	1	KB	P	10^{-5}	medium, circular, opaque, orange-yellow, raised, smooth, shiny
R2	1	KB	P	10^{-4}	large, circular, opaque, cream, raised, smooth, shiny
R3	1	KB	P	10^{-4}	medium, circular, opaque, cream, umbonate (slight), smooth, dull
R4	1	NA	S	10^{-5}	large, circular, opaque, cream, thick, crateriform, creased, dull, entire margin
R5	1	NA	S	10^{-4}	small, circular, slightly translucent, cream-white, flat, smooth, shiny
R6	1	NA	S	10^{-4}	Bacillus mycoides, large, filamentous, cream, flat, dull, filiform margin
R7	1	NA	T	10^{-4}	very small, circular, opaque, orange-yellow, raised, shiny, smooth
R8	1	NA	T	10^{-4}	medium, circular, opaque, cream, raised, smooth, shiny
R9	1	NA	T	10^{-5}	medium, circular, opaque, orange-yellow, raised, smooth, shiny
R10	1	NA	T	10^{-4}	small, irregular, slightly translucent, cream-white, flat, smooth, shiny
R11	2	KB	P	10^{-6}	medium, circular, opaque, cream, raised, smooth, shiny
R12	2	KB	P	10^{-4}	large, circular, opaque, apricot, surrounded by a thinner ring, raised, smooth, shiny
R13	2	KB	P	10^{-5}	medium, circular, opaque, cream-white, raised, smooth, shiny
R14	2	NA	S	10^{-5}	large, circular, opaque, cream, thick, crateriform, creased, dull, entire margin
R15	2	NA	S	10^{-3}	medium, circular, opaque, cream-white, flat, smooth, dull
R16	2	NA	S	10^{-3}	very small, circular, translucent, cream, flat, thin, smooth, shiny
R17	2	NA	T	10^{-6}	medium, circular, opaque, cream, raised, smooth, shiny
R18	2	NA	T	10^{-5}	medium, circular, opaque, cream-peach, raised, smooth, shiny
R19	2	NA	T	10^{-4}	medium, circular, cream, opaque, filliform margin
R20	2	NA	T	10^{-4}	very small, circular, opaque, cream-white, raised, smooth, shiny
R21	3	KB	P	10^{-5}	medium, circular, opaque, cream-orange, raised, smooth, shiny

R22	3	KB	P	10^{-4}	small, circular, opaque, orange, raised, smooth, shiny
R23	3	KB	P	10^{-4}	small, circular, opaque, cream-white, raised, smooth, shiny
R24	3	NA	S	10^{-3}	medium, circular, opaque, cream, thick, crateriform, creased, dull, entire margin
R25	3	NA	S	10^{-4}	small, circular, opaque, cream-white, flat, smooth, shiny
R26	3	NA	S	10^{-4}	medium, circular, opaque, brown-apricot, flat, smooth, dull
R27	3	NA	T	10^{-5}	medium, circular, opaque, cream, flat, smooth, dull
R28	3	NA	T	10^{-5}	very small, circular, opaque, cream-white, raised, smooth, shiny
R29	3	NA	T	10^{-4}	very small, circular, opaque, cream , raised, convoluted surface, dull
R30	3	NA	T	10^{-4}	very small, circular, opaque, cream-white, raised, smooth, shiny
R31	4	KB	P	10^{-4}	medium, irregular, opaque, reddish-apricot, flat, smooth, shiny
R32	4	KB	P	10^{-4}	medium, circular, opaque, cream-white, raised, smooth, shiny
R33	4	KB	P	10^{-4}	large, circular, opaque, cream, raised, smooth, shiny
R34	4	NA	S	10^{-4}	medium, circular, opaque, cream, thick, crateriform, dull
R35	4	NA	S	10^{-4}	small, circular, opaque, cream-white, flat, smooth, shiny
R36	4	NA	S	10^{-3}	medium, irregular, opaque, cream, flat, smooth, dull
R37	4	NA	T	10^{-5}	large, circular, opaque, cream, thick, crateriform, creased, dull, entire margin
R38	4	NA	T	10^{-5}	small, circular, opaque, orange-yellow, raised, smooth, shiny
R39	4	NA	T	10^{-4}	medium, circular, opaque, cream, thick, crateriform, creased, dull, entire margin
R40	4	NA	T	10^{-4}	large, circular, opaque, cream, flat, smooth, shiny

*Plate types: T (total bacteria), P (total bacteria KB) and S (spore forming bacteria)

.

APPENDIX 3

3.1 "OTHER" FUNGAL INFECTION DATA FOR CHAPTER 3

Table 3.1-1: Infection data for all non *F. oxysporum* and *C. destructans* fungi isolated from four rootstock varieties inoculated with *F. oxysporum* (F), *C. destructans* (C) or no inoculation (O) at 0 cm and 5 cm from the stem base. Values are total colonies and may include more than one isolate per tissue fragment.

Treatment		**0 cm**					**5 cm**				
Variety	**Inoc**	**F**	**B**	**A**	**Pi**	**Pe**	**F**	**B**	**A**	**Pi**	**Pe**
101-14	O	9	0	0	0	14	3	0	0	0	1
	F	5	4	0	6	7	3	0	0	2	1
	C	14	2	3	0	8	2	1	0	1	0
5C	O	11	0	2	0	2	4	0	0	3	0
	F	9	1	1	6	5	3	1	0	2	0
	C	8	0	3	0	8	1	0	1	1	0
Schw.	O	17	2	2	1	0	3	0	1	1	1
	F	13	0	0	0	5	3	0	0	0	1
	C	21	1	0	5	4	5	0	0	0	1
R.Gl	O	27	1	1	1	10	6	0	0	0	3
	F	28	3	2	0	7	3	1	1	0	1
	C	14	0	0	4	10	2	0	0	0	3

Key

Inoc: inoculation status (N=not inoculated, Y=inoculated)

Schw: Schwarzmann

R.Gl: Riparia Gloire

F: *Fusarium* sp.

B: *Botryosphaeria* sp.

A: *Alternaria* sp.

Pi: *Pythium* sp.

Pe: *Penicillium* sp.

NB: No *Phomopsis* or *Paecilomyces* spp. were isolated in this experiment.

3.2 *FUSARIUM OXYSPORUM* SEQUENCE DATA FOR CHAPTER 3

Table 3.2-1: *Fusarium oxysporum* isolates used in this experiment were sequenced using the primer ITS5. The following sequence data was obtained from GenBank.

Isolate	Fragment size (bp)	Best match	Accession number	Coverage	Maximum identity
24B	520	*Fusarium oxysporum* isolate	EU839400.1	100%	99%
39A	518		EU839400.1	100%	99%
80B	519		EU839400.1	100%	99%
81B	514		EU839400.1	100%	99%
105A	520		EU839400.1	100%	99%
127B	519		EU839400.1	100%	99%
			EU839400.1	100%	99%

Table 3.2-2: Sequence alignment data for the *Fusarium oxysporum* isolates used to inoculate the grapevines in Chapter 3. Both homology and distance matrices are given.

Homology matrix of the 6 sequences:

24B 100%
39A 100.0% 100%
80B 100.0% 100.0% 100%
105A 100.0% 100.0% 100.0% 100%
81B 100.0% 100.0% 100.0% 100.0% 100%
127B 100.0% 100.0% 100.0% 100.0% 100.0% 100%

Distance matrix of the 6 sequences:

24B 0
39A 0.000 0
80B 0.000 0.000 0
105A 0.000 0.000 0.000 0
81B 0.000 0.000 0.000 0.000 0
127B 0.000 0.000 0.000 0.000 0.000 0

3.3 IDENTIFICATION OF *FUSARIUM OXYSPORUM*

Table 3.3-1: Characters used in the identification of *Fusarium oxysporum* cultures grown on PDA and Oatmeal Agar.

Item	Description	References
Mycelia	Fluccose (woolly). Sparse, abundant, spreading. Colour: white-pale violet, vinaceous, grey-purple, salmon, peach.	Booth, 1977 Leslie & Summerell, 2006 Pio et al., 2008
Microconidia	Oval, elliptical or kidney shaped. Straight or curved. Typically aseptate. Size: 5-12 x 2.2-3.5 µm.	Booth, 1977 Leslie & Summerell, 2006
Macroconidia	Slightly sickle-shaped. Typically 3 septate (can be 2-5). Size: 27-60 x 3-5 µm. Thin walled. Typically produce abundant sporodochia. Apical cell: tapered/curved with or without slight hook. Basal cell: foot-shaped to pointed.	Booth, 1977 Nelson et al., 1983 Burgess et al., 1989 Leslie & Summerell, 2006
Chlamydospores	May be formed. Often abundant. Singly, pairs, clusters, or short chains. Smooth or rough walled. Terminal or intercalary.	Leslie & Summerell, 2006 Toussoun & Nelson, 1968
Sclerotia	Small. May be abundant. Light brown, blue to blue-black or violet.	Leslie & Summerell, 2006 Toussoun & Nelson, 1968
Agar pigmentation	Pale to dark violet or dark magenta. Some isolates.	Leslie & Summerell, 2006

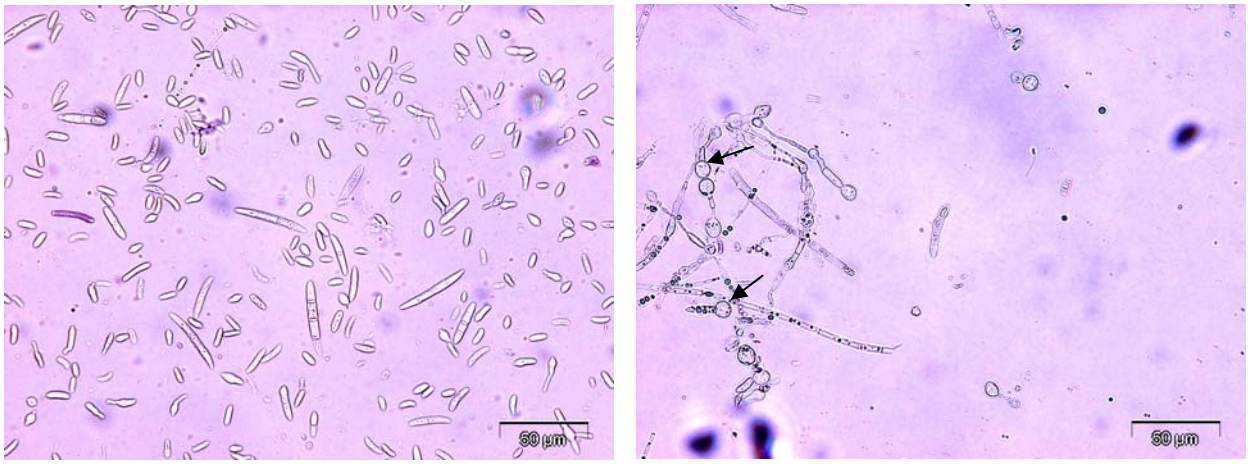


Figure 3.3-1: *Fusarium oxysporum* spores (left) and chlamydospores (right, arrow) when grown on OA. Incubated 20ºC, 12 h dark: 12 h light for 14 days.

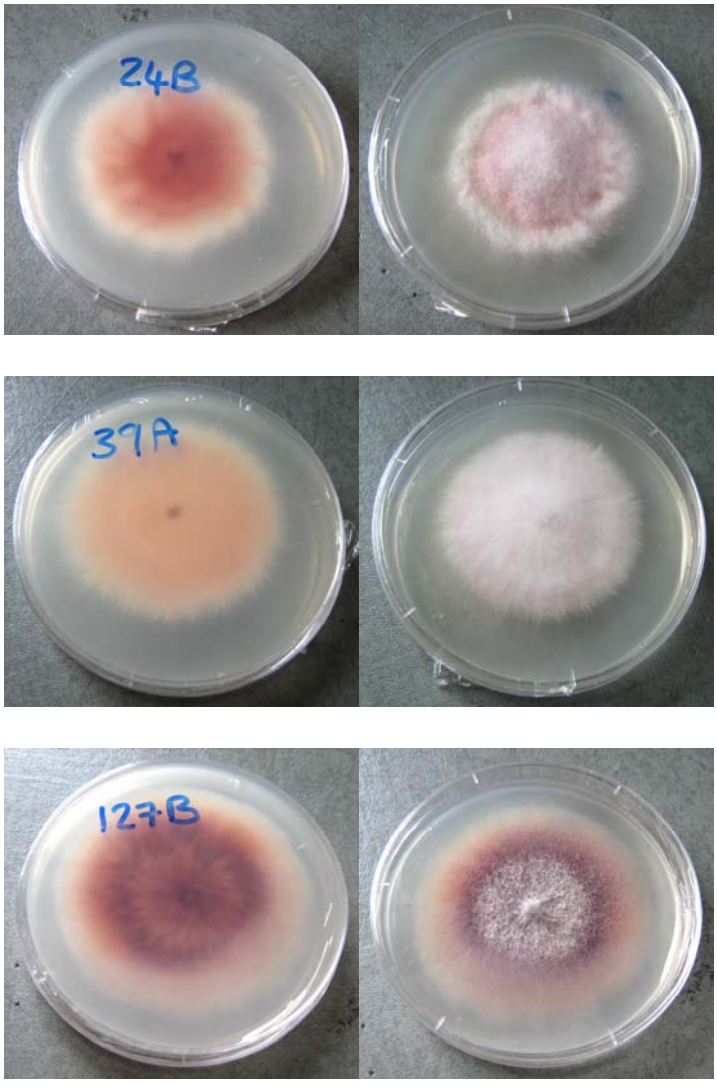


Figure 3.3-2: An example of different *Fusarium oxysporum* colony morphologies on PDA. Top and reverse of colonies shown. Incubated 20ºC, 12 h dark: 12 h light for 14 days.

3.4 ANOVA TABLES FOR CHAPTER 3

Table 3.4-1: Analysis of variance (ANOVA) for *C. destructans* disease severity of grapevine rootstocks at 0 cm from the stem base.

Dependent Variable: 0cm_severity_C

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	67.609[a]	20	3.380	2.967	.000
Intercept	33.779	1	33.779	29.651	.000
REP	17.899	9	1.989	1.746	.090
VAR	34.868	3	11.623	10.202	.000
TRT	6.798	2	3.399	2.984	.056
VAR * TRT	8.383	6	1.397	1.226	.300
Error	103.668	91	1.139		
Total	209.000	112			
Corrected Total	171.277	111			

Table 3.4-2: Analysis of variance (ANOVA) for *C. destructans* disease incidence of grapevine rootstocks at 5 cm from the stem base.

Dependent Variable: 5cm_severity_C

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4.665[a]	20	.233	2.176	.007
Intercept	2.198	1	2.198	20.509	.000
REP	2.110	9	.234	2.187	.030
VAR	1.739	3	.580	5.408	.002
TRT	.474	2	.237	2.209	.116
VAR * TRT	.241	6	.040	.374	.894
Error	9.754	91	.107		
Total	17.000	112			
Corrected Total	14.420	111			

Table 3.4-3: Analysis of variance (ANOVA) for *F. oxysporum* disease severity of grapevine rootstocks at 0 cm from the stem base.

Dependent Variable: 0cm_severity_ F

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	62.770[a]	20	3.138	1.956	.017
Intercept	102.796	1	102.796	64.069	.000
REP	18.915	9	2.102	1.310	.243
VAR	10.742	3	3.581	2.232	.090
TRT	13.492	2	6.746	4.204	.018
VAR * TRT	20.102	6	3.350	2.088	.062
Error	146.007	91	1.604		
Total	311.000	112			
Corrected Total	208.777	111			

Table 3.4-4: Analysis of variance (ANOVA) for *F. oxysporum* disease incidence of grapevine rootstocks at 5 cm from the stem base.

Dependent Variable: 5cm_severity_F

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.108[a]	20	.155	.867	.627
Intercept	5.389	1	5.389	30.066	.000
REP	1.586	9	.176	.983	.459
VAR	.552	3	.184	1.026	.385
TRT	.659	2	.329	1.837	.165
VAR * TRT	.384	6	.064	.357	.904
Error	16.311	91	.179		
Total	25.000	112			
Corrected Total	19.420	111			

Table 3.4-5: Analysis of variance (ANOVA) for root dry weight data.

Dependent Variable: ROOT_DW

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3083.237[a]	20	154.162	1.742	.040
Intercept	80445.082	1	80445.082	908.787	.000
REP	683.198	9	75.911	.858	.566
VAR	1929.377	3	643.126	7.265	.000
TRT	82.767	2	41.383	.468	.628
VAR * TRT	415.227	6	69.204	.782	.586
Error	8055.241	91	88.519		
Total	96477.680	112			
Corrected Total	11138.479	111			

Table 3.4-6: Analysis of variance (ANOVA) for shoot dry weight data.

Dependent Variable: SHOOT_DW

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	576.732[a]	20	28.837	2.562	.001
Intercept	3803.363	1	3803.363	337.911	.000
REP	101.894	9	11.322	1.006	.441
VAR	366.876	3	122.292	10.865	.000
TRT	37.075	2	18.537	1.647	.198
VAR * TRT	81.115	6	13.519	1.201	.313
Error	1024.253	91	11.256		
Total	5507.970	112			
Corrected Total	1600.986	111			

Table 3.4-7: Analysis of variance (ANOVA) showing the difference in root and shoot dry weights between *F. oxysporum* infected and non-infected 101-14 plants.

			Sum of Squares	df	Mean Square	F	Sig.
ROOT_DW * INCID_F	Between Groups	(Combined)	228.443	1	228.443	2.473	.127
	Within Groups		2493.708	27	92.360		
	Total		2722.152	28			
SHOOT_DW * INCID_F	Between Groups	(Combined)	11.514	1	11.514	1.292	.266
	Within Groups		240.634	27	8.912		
	Total		252.148	28			

Table 3.4-8: Analysis of variance (ANOVA) showing the difference in root and shoot dry weights between *C. destructans* infected and non-infected 101-14 plants.

			Sum of Squares	df	Mean Square	F	Sig.
ROOT_DW * INCID_C	Between Groups	(Combined)	485.048	1	485.048	5.854	.023
	Within Groups		2237.104	27	82.856		
	Total		2722.152	28			
SHOOT_DW * INCID_C	Between Groups	(Combined)	11.061	1	11.061	1.239	.276
	Within Groups		241.087	27	8.929		
	Total		252.148	28			

Table 3.4-9: Analysis of variance (ANOVA) showing the difference in root and shoot dry weights between *F. oxysporum* infected and non-infected 5C plants.

			Sum of Squares	df	Mean Square	F	Sig.
ROOT_DW * INCID_F	Between Groups	(Combined)	955.463	1	955.463	6.282	.019
	Within Groups		3954.478	26	152.095		
	Total		4909.941	27			
SHOOT_DW * INCID_F	Between Groups	(Combined)	130.501	1	130.501	9.020	.006
	Within Groups		376.180	26	14.468		
	Total		506.681	27			

Table 3.4-10: Analysis of variance (ANOVA) showing the difference in root and shoot dry weights between *C. destructans* infected and non-infected 5C plants.

			Sum of Squares	df	Mean Square	F	Sig.
ROOT_DW * INCID_C	Between Groups	(Combined)	679.158	1	679.158	4.174	.051
	Within Groups		4230.784	26	162.722		
	Total		4909.941	27			
SHOOT_DW * INCID_C	Between Groups	(Combined)	6.703	1	6.703	.349	.560
	Within Groups		499.978	26	19.230		
	Total		506.681	27			

Table 3.4-11: Analysis of variance (ANOVA) showing the difference in root and shoot dry weights between *F. oxysporum* infected and non-infected Riparia Gloire plants.

			Sum of Squares	df	Mean Square	F	Sig.
ROOT_DW * INCID_F	Between Groups	(Combined)	10.858	1	10.858	.297	.590
	Within Groups		950.007	26	36.539		
	Total		960.864	27			
SHOOT_DW * INCID_F	Between Groups	(Combined)	9.333	1	9.333	.764	.390
	Within Groups		317.419	26	12.208		
	Total		326.752	27			

Table 3.4-12: Analysis of variance (ANOVA) showing the difference in root and shoot dry weights between *C. destructans* infected and non-infected Riparia Gloire plants.

			Sum of Squares	df	Mean Square	F	Sig.
ROOT_DW * INCID_C	Between Groups	(Combined)	.298	1	.298	.008	.929
	Within Groups		960.567	26	36.945		
	Total		960.864	27			
SHOOT_DW * INCID_C	Between Groups	(Combined)	2.256	1	2.256	.181	.674
	Within Groups		324.496	26	12.481		
	Total		326.752	27			

Table 3.4-13: Analysis of variance (ANOVA) showing the difference in root and shoot dry weights between *F. oxysporum* infected and non-infected Schwarzmann plants.

			Sum of Squares	df	Mean Square	F	Sig.
ROOT_DW * INCID_F	Between Groups	(Combined)	18.891	1	18.891	.747	.396
	Within Groups		632.563	25	25.303		
	Total		651.454	26			
SHOOT_DW * INCID_F	Between Groups	(Combined)	6.578	1	6.578	1.041	.317
	Within Groups		157.949	25	6.318		
	Total		164.527	26			

Table 3.4-14: Analysis of variance (ANOVA) showing the difference in root and shoot dry weights between *C. destructans* infected and non-infected Schwarzmann plants.

			Sum of Squares	df	Mean Square	F	Sig.
ROOT_DW * INCID_C	Between Groups	(Combined)	12.079	1	12.079	.472	.498
	Within Groups		639.375	25	25.575		
	Total		651.454	26			
SHOOT_DW * INCID_C	Between Groups	(Combined)	.192	1	.192	.029	.866
	Within Groups		164.335	25	6.573		
	Total		164.527	26			

3.5 RAW DATA MEANS FOR CHAPTER 3

Table 3.5-1: Raw data means for *F. oxysporum* disease severity at 0 cm. Values are shown for the three inoculation treatments: *C. destructans* (C), *F. oxysporum* (F) and none (O).

Inoc	Rootstock variety	Mean (%)
C	101-14	10.00
	5C	13.89
	Riparia Gloire	56.25
	Schwarzmann	27.78
F	101-14	52.78
	5C	13.89
	Riparia Gloire	32.50
	Schwarzmann	37.50
O	101-14	17.50
	5C	7.50
	Riparia Gloire	10.00
	Schwarzmann	17.50

Table 3.5-2: Raw data means for *C. destructans* disease severity at 0 cm. Values are shown for the three inoculation treatments: *C. destructans* (C), *F. oxysporum* (F) and none (O).

Inoc	Rootstock variety	Mean (%)
C	101-14	37.50
	5C	52.78
	Riparia Gloire	0.00
	Schwarzmann	2.78
F	101-14	22.23
	5C	16.67
	Riparia Gloire	0.00
	Schwarzmann	0.00
O	101-14	27.78
	5C	15.00
	Riparia Gloire	0.00
	Schwarzmann	0.00

Table 3.5-3: Raw data means for *F. oxysporum* disease incidence 0 cm and 5 cm from trunk base. Values are shown for the three inoculation treatments: *C. destructans* (C), *F. oxysporum* (F) and none (O).

Inoc	Rootstock variety	0 cm Mean (%)	5 cm Mean (%)
C	101-14	20.00	30.00
	5C	22.22	22.22
	Riparia Gloire	62.50	50.00
	Schwarzmann	66.67	22.22
F	101-14	77.78	22.22
	5C	44.44	22.22
	Riparia Gloire	50.00	40.00
	Schwarzmann	75.00	12.50
O	101-14	40.00	20.00
	5C	10.00	10.00
	Riparia Gloire	10.00	10.00
	Schwarzmann	30.00	10.00

Table 3.5-4: Raw data means for *C. destructans* disease incidence 0 cm and 5 cm from stem base. Values are shown for the three inoculation treatments: *C. destructans* (C), *F. oxysporum* (F) and none (O).

Inoc	Rootstock variety	0 cm Mean (%)	5 cm Mean (%)
C	101-14	50.00	40.00
	5C	66.67	33.33
	Riparia Gloire	0.00	12.50
	Schwarzmann	11.11	11.11
F	101-14	33.33	33.33
	5C	33.33	0.00
	Riparia Gloire	0.00	0.00
	Schwarzmann	0.00	0.00
O	101-14	30.00	30.00
	5C	20.00	20.00
	Riparia Gloire	0.00	0.00
	Schwarzmann	0.00	0.00

Table 3.5-5: Raw data means for overall (0 cm and 5 cm) *F. oxysporum* disease incidence. Values are shown for the three inoculation treatments: *C. destructans* (C), *F. oxysporum* (F) and none (O).

Inoc	Rootstock variety	Mean (%)
C	101-14	30.00
	5C	22.22
	Riparia Gloire	62.50
	Schwarzmann	66.67
F	101-14	77.78
	5C	44.44
	Riparia Gloire	60.00
	Schwarzmann	75.00
O	101-14	40.00
	5C	10.00
	Riparia Gloire	10.00
	Schwarzmann	30.00

Table 3.5-6: Raw data means for overall (0 cm and 5 cm) *C. destructans* disease incidence. Values are shown for the three inoculation treatments: *C. destructans* (C), *F. oxysporum* (F) and none (O).

Inoc	Rootstock variety	Mean (%)
C	101-14	60.00
	5C	66.67
	Riparia Gloire	12.50
	Schwarzmann	13.89
F	101-14	44.44
	5C	55.56
	Riparia Gloire	0.00
	Schwarzmann	0.00
O	101-14	30.00
	5C	30.00
	Riparia Gloire	0.00
	Schwarzmann	0.00

Table 3.5-7: Raw data means for the root dry weights of grapevine rootstocks when exposed to different inoculation treatments: *C. destructans* (C), *F. oxysporum* (F) and none (O).

Inoc	Rootstock variety	Mean (g)
C	101-14	24.92
	5C	32.96
	Riparia Gloire	25.55
	Schwarzmann	26.04
F	101-14	25.11
	5C	36.84
	Riparia Gloire	21.21
	Schwarzmann	23.31
O	101-14	30.36
	5C	33.83
	Riparia Gloire	27.41
	Schwarzmann	23.48

Table 3.5-8: Raw data means for the shoot dry weights of grapevine rootstocks when exposed to different inoculation treatments: *C. destructans* (C), *F. oxysporum* (F) and none (O).

Inoc	Rootstock variety	Mean (g)
C	101-14	4.49
	5C	8.08
	Riparia Gloire	3.20
	Schwarzmann	6.90
F	101-14	5.09
	5C	8.28
	Riparia Gloire	3.13
	Schwarzmann	4.21
O	101-14	6.12
	5C	10.11
	Riparia Gloire	6.00
	Schwarzmann	4.86

Table 3.5-9: Raw data showing the effect of *C. destructans* disease incidence (0=absent, 1=present) on the mean root and shoot dry weights of grapevine rootstocks.

	Root dry weight		**Shoot dry weight**	
	Incidence		**Incidence**	
Variety	**0**	**1**	**0**	**1**
101-14	23.17 (n=16)	31.39 (n=13)	4.68 (n=16)	5.92 (n=13)
5C	29.59 (n=14)	39.44 (n=14)	8.38 (n=14)	9.36 (n=14)
Riparia Gloire	26.64 (n=27)	25.20 (n=1)	4.23 (n=27)	2.70 (n=1)
Schwarzmann	24.10 (n=25)	26.65 (n=2)	5.37 (n=25)	5.05 (n=2)

Table 3.5-10: Raw data showing the effect of *F. oxysporum* incidence (0=absent, 1=present) on the mean root and shoot dry weights of grapevine rootstocks.

	Root dry weight		**Shoot dry weight**	
	Incidence		**Incidence**	
Variety	**0**	**1**	**0**	**1**
101-14	29.69 (n=15)	23.74 (n=14)	5.85 (n=15)	4.59 (n=14)
5C	37.89 (n=21)	24.40 (n=7)	10.11 (n=21)	5.13 (n=7)
Riparia Gloire	24.13 (n=16)	25.38 (n=12)	4.68 (n=16)	3.51 (n=12)
Schwarzmann	23.35 (n=12)	25.03 (n=15)	5.90 (n=12)	4.91 (n=15)

Table 3.5-11: Raw data showing the effect of grapevine rootstock on the mean shoot and root dry weights of vines.

Variety	**Root dry weight (g)**	**Shoot dry weight (g)**
101-14	26.86	5.24
5C	34.52	8.87
Riparia Gloire	24.31	4.57
Schwarzmann	24.29	5.35

3.6 PLANT INFORMATION FOR CHAPTER 3

Table 3.6-1: The different grapevine rootstock variety and treatment combinations, showing how many plants of each were replaced and how many were the original plants.

Rootstock	Trt	Description	Original	Replacement
101-14	O	not inoculated	4	6
	F	inoculated with *F. oxysporum*	6	3
	C	inoculated with *C. destructans*	5	5
5C	O	not inoculated	0	10
	F	inoculated with *F. oxysporum*	2	7
	C	inoculated with *C. destructans*	3	5
Schwarzmann	O	not inoculated	10	0
	F	inoculated with *F. oxysporum*	8	0
	C	inoculated with *C. destructans*	9	0
Riparia Gloire	O	not inoculated	10	0
	F	inoculated with *F. oxysporum*	10	0
	C	inoculated with *C. destructans*	8	0

Figure 3.6-1: An example of the root galls observed on plants belonging to grapevine rootstock 5C.

APPENDIX 4

4.1 ANOVA TABLES FOR CHAPTER 4

Table 4.1-1: Analysis of variance (ANOVA) for *C. destructans* disease severity of grapevine rootstocks at 0 cm from the trunk base. Stress treatments 0, 1 and 2 are treated separately.

Dependent Variable: 0cm_severity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	17.953	1	17.953	18.578	.005
	Error	5.811	6.013	.966[a]		
REP	Hypothesis	5.798	6	.966	.918	.488
	Error	70.536	67	1.053[b]		
VAR	Hypothesis	1.389	1	1.389	1.320	.255
	Error	70.536	67	1.053[b]		
INOC	Hypothesis	5.116	1	5.116	4.860	.031
	Error	70.536	67	1.053[b]		
STRESS	Hypothesis	4.300	2	2.150	2.042	.138
	Error	70.536	67	1.053[b]		
INOC * STRESS	Hypothesis	3.066	2	1.533	1.456	.240
	Error	70.536	67	1.053[b]		
VAR * STRESS	Hypothesis	.315	2	.157	.149	.861
	Error	70.536	67	1.053[b]		
VAR * INOC	Hypothesis	.097	1	.097	.093	.762
	Error	70.536	67	1.053[b]		

a. .999 MS(REP) + .001 MS(Error)

b. MS(Error)

Table 4.1-2: Analysis of variance (ANOVA) for *C. destructans* disease severity of grapevine rootstocks at 0 cm from the trunk base. Stress treatments 0 and 1 are combined.

Dependent Variable: 0cm_severity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	22.002	1	22.002	22.619	.002
	Error	7.365	7.571	.973[a]		
REP	Hypothesis	5.806	6	.968	.954	.463
	Error	71.030	70	1.015[b]		
VARIETY	Hypothesis	1.707	1	1.707	1.682	.199
	Error	71.030	70	1.015[b]		
INOC	Hypothesis	7.239	1	7.239	7.134	.009
	Error	71.030	70	1.015[b]		
VARIETY * INOC	Hypothesis	.105	1	.105	.104	.748
	Error	71.030	70	1.015[b]		
STRESS2	Hypothesis	4.307	1	4.307	4.245	.043
	Error	71.030	70	1.015[b]		
INOC * STRESS2	Hypothesis	2.609	1	2.609	2.571	.113
	Error	71.030	70	1.015[b]		
VARIETY * STRESS2	Hypothesis	.290	1	.290	.286	.594
	Error	71.030	70	1.015[b]		

a. .894 MS(REP) + .106 MS(Error)

b. MS(Error)

Table 4.1-3: Analysis of variance (ANOVA) for *C. destructans* disease incidence of grapevine rootstocks at 5 cm from the trunk base. Stress treatments 0, 1 and 2 are treated separately.

Dependent Variable: 5cm_severity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	1.731	1	1.731	38.812	.001
	Error	.269	6.039	.045[a]		
REP	Hypothesis	.267	6	.045	.310	.930
	Error	9.625	67	.144[b]		
VAR	Hypothesis	.000	1	.000	.002	.967
	Error	9.625	67	.144[b]		
INOC	Hypothesis	.000	1	.000	.002	.967
	Error	9.625	67	.144[b]		
STRESS	Hypothesis	.223	2	.112	.778	.464
	Error	9.625	67	.144[b]		
INOC * STRESS	Hypothesis	.081	2	.040	.281	.756
	Error	9.625	67	.144[b]		
VAR * STRESS	Hypothesis	.060	2	.030	.209	.812
	Error	9.625	67	.144[b]		
VAR * INOC	Hypothesis	.000	1	.000	.002	.967
	Error	9.625	67	.144[b]		

a. .999 MS(REP) + .001 MS(Error)

b. MS(Error)

Table 4.1-4: Analysis of variance (ANOVA) for *C. destructans* disease incidence of grapevine rootstocks at 5 cm from the trunk base. Stress treatments 0 and 1 are combined.

Dependent Variable: 5cm_severity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	1.724	1	1.724	30.950	.000
	Error	.615	11.039	.056[a]		
REP	Hypothesis	.274	6	.046	.325	.922
	Error	9.834	70	.140[b]		
VARIETY	Hypothesis	.007	1	.007	.049	.826
	Error	9.834	70	.140[b]		
INOC	Hypothesis	.005	1	.005	.036	.850
	Error	9.834	70	.140[b]		
VARIETY * INOC	Hypothesis	6.758E-05	1	6.758E-05	.000	.983
	Error	9.834	70	.140[b]		
STRESS2	Hypothesis	.051	1	.051	.360	.551
	Error	9.834	70	.140[b]		
INOC * STRESS2	Hypothesis	.056	1	.056	.398	.530
	Error	9.834	70	.140[b]		
VARIETY * STRESS2	Hypothesis	.051	1	.051	.360	.551
	Error	9.834	70	.140[b]		

a. .894 MS(REP) + .106 MS(Error)

b. MS(Error)

Table 4.1-5: Analysis of variance (ANOVA) for root dry weight data. Stress treatments 0, 1 and 2 are treated separately.

Dependent Variable: ROOT_DW

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	26842.700	1	26842.700	4359.268	.000
	Error	37.102	6.025	6.158[a]		
REP	Hypothesis	36.904	6	6.151	.471	.828
	Error	875.564	67	13.068[b]		
VAR	Hypothesis	122.073	1	122.073	9.341	.003
	Error	875.564	67	13.068[b]		
INOC	Hypothesis	.004	1	.004	.000	.986
	Error	875.564	67	13.068[b]		
STRESS	Hypothesis	272.252	2	136.126	10.417	.000
	Error	875.564	67	13.068[b]		
INOC * STRESS	Hypothesis	95.627	2	47.814	3.659	.031
	Error	875.564	67	13.068[b]		
VAR * STRESS	Hypothesis	56.594	2	28.297	2.165	.123
	Error	875.564	67	13.068[b]		
VAR * INOC	Hypothesis	7.218	1	7.218	.552	.460
	Error	875.564	67	13.068[b]		

a. .999 MS(REP) + .001 MS(Error)

b. MS(Error)

Table 4.1-6: Analysis of variance (ANOVA) for root dry weight data. Stress treatments 0 and 1 are combined.

Dependent Variable: ROOT_DW

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	22387.600	1	22387.600	3501.184	.000
	Error	64.338	10.062	6.394[a]		
REP	Hypothesis	33.005	6	5.501	.394	.880
	Error	976.110	70	13.944[b]		
VARIETY	Hypothesis	170.773	1	170.773	12.247	.001
	Error	976.110	70	13.944[b]		
INOC	Hypothesis	1.676	1	1.676	.120	.730
	Error	976.110	70	13.944[b]		
VARIETY * INOC	Hypothesis	8.729	1	8.729	.626	.432
	Error	976.110	70	13.944[b]		
STRESS2	Hypothesis	242.259	1	242.259	17.373	.000
	Error	976.110	70	13.944[b]		
INOC * STRESS2	Hypothesis	23.288	1	23.288	1.670	.201
	Error	976.110	70	13.944[b]		
VARIETY * STRESS2	Hypothesis	50.075	1	50.075	3.591	.062
	Error	976.110	70	13.944[b]		

a. .894 MS(REP) + .106 MS(Error)

b. MS(Error)

Table 4.1-7: Analysis of variance (ANOVA) for shoot dry weight data. Stress treatments 0, 1 and 2 are treated separately.

Dependent Variable: SHOOT_DW

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	920.715	1	920.715	442.790	.000
	Error	12.510	6.016	2.079[a]		
REP	Hypothesis	12.472	6	2.079	.731	.626
	Error	190.502	67	2.843[b]		
VAR	Hypothesis	72.227	1	72.227	25.402	.000
	Error	190.502	67	2.843[b]		
INOC	Hypothesis	.060	1	.060	.021	.885
	Error	190.502	67	2.843[b]		
STRESS	Hypothesis	7.847	2	3.923	1.380	.259
	Error	190.502	67	2.843[b]		
INOC * STRESS	Hypothesis	4.862	2	2.431	.855	.430
	Error	190.502	67	2.843[b]		
VAR * STRESS	Hypothesis	3.312	2	1.656	.582	.561
	Error	190.502	67	2.843[b]		
VAR * INOC	Hypothesis	.028	1	.028	.010	.922
	Error	190.502	67	2.843[b]		

a. .999 MS(REP) + .001 MS(Error)

b. MS(Error)

Table 4.1-8: Analysis of variance (ANOVA) for shoot dry weight data. Stress treatments 0 and 1 are combined.

Dependent Variable: SHOOT_DW

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	858.825	1	858.825	397.380	.000
	Error	17.567	8.128	2.161[a]		
REP	Hypothesis	12.445	6	2.074	.716	.638
	Error	202.800	70	2.897[b]		
VARIETY	Hypothesis	63.017	1	63.017	21.752	.000
	Error	202.800	70	2.897[b]		
INOC	Hypothesis	.175	1	.175	.061	.806
	Error	202.800	70	2.897[b]		
VARIETY * INOC	Hypothesis	.016	1	.016	.005	.942
	Error	202.800	70	2.897[b]		
STRESS2	Hypothesis	3.533	1	3.533	1.220	.273
	Error	202.800	70	2.897[b]		
INOC * STRESS2	Hypothesis	.196	1	.196	.068	.796
	Error	202.800	70	2.897[b]		
VARIETY * STRESS2	Hypothesis	.189	1	.189	.065	.799
	Error	202.800	70	2.897[b]		

a. .894 MS(REP) + .106 MS(Error)

b. MS(Error)

4.2 RAW DATA MEANS FOR CHAPTER 4

Table 4.2-1: Raw data means for *C. destructans* disease severity 0 cm from trunk base.

	0 cm disease severity (%)					
	101-14			**Schw.**		
Inoc	**0**	**1**	**2**	**0**	**1**	**2**
N	0	14.29	14.29	3.57	0	0
Y	17.86	7.14	35.72	7.14	10.72	28.57

Table 4.2-2: Raw data means for *C. destructans* disease incidence 5 cm from trunk base.

	5 cm disease incidence (%)					
	101-14			**Schw.**		
Inoc	**0**	**1**	**2**	**0**	**1**	**2**
N	0	28.57	14.29	14.29	16.67	14.29
Y	14.29	14.29	14.29	0	14.29	28.57

Table 4.2-3: Raw data means for overall plant *C. destructans* disease incidence.

	Overall disease incidence data (% plants)					
	101-14			**Schw.**		
Inoc	**0**	**1**	**2**	**0**	**1**	**2**
N	0	42.86	14.29	28.57	16.67	14.29
Y	28.57	42.86	57.14	14.29	57.14	42.86

* All out of a total of 7 plants, except Schw. N. 1 where only 6 plants were available.

Table 4.2-4: Raw data means for the root dry weights of grapevine rootstocks 101-14 and Schwarzmann, when inoculated (Y) and not inoculated (N) with *C. destructans*.

	Root dry weight: Raw data means (g)					
	101-14			**Schw.**		
Inoc	**0**	**1**	**2**	**0**	**1**	**2**
N	18.89	21.51	18.13	15.14	19.72	14.43
Y	20.04	19.07	17.69	20.07	19.19	12.03

Table 4.2-5: Raw data means for the shoot dry weights of grapevine rootstocks 101-14 and Schwarzmann, when inoculated (Y) and not inoculated (N) with *C. destructans*.

	Shoot dry weight: Raw data means (g)					
	101-14			**Schw.**		
Inoc	**0**	**1**	**2**	**0**	**1**	**2**
N	3.414	5.343	3.914	1.843	2.133	3.129
Y	3.857	4.014	5.071	2.529	2.329	2.371

Table 4.2-6: Raw data showing the effect of *C. destructans* incidence on grapevine shoot and root dry weights for varieties 101-14 and Schwarzmann.

	Root dry weight		**Shoot dry weight**	
	Incidence		**Incidence**	
Variety	**0**	**1**	**0**	**1**
101-14	18.98	19.77	4.27	4.28
Schw.	16.88	16.23	2.62	1.85

* The number of plants in each category vary: 101-14 and Schwarzmann both have 29 plants with 0 incidence. 101-14 has 13 plants with an incidence of 1, while Schwarzmann has 12.

Table 4.2-7: *Cylindrocarpon destructans* disease severity 0 cm from the trunk base. Means provided for variety, inoculation and stress effects.

Variety	**Mean**	**%**
101-14	0.595	14.9
Schw.	0.341	8.5

Inoc	**Mean**	**%**
N	0.220	5.5
Y	0.714	17.9

Stress	**Mean**	**%**
0	0.286	7.2
1	0.333	8.3
2	0.786	19.7

Table 4.2-8: *Cylindrocarpon destructans* disease incidence 5 cm from the trunk base. Means provided for variety, inoculation and stress effects.

Variety	**Mean**	**%**
101-14	0.143	14.3
Schw.	0.146	14.6

Inoc	**Mean**	**%**
N	0.15	15
Y	0.143	14.3

Stress	**Mean**	**%**
0	0.071	7.1
1	0.185	18.5
2	0.179	17.9

Table 4.2-9: *Cylindrocarpon destructans* disease incidence combined for 0 cm and 5 cm from the trunk base. Means provided for variety, inoculation and stress effects.

Variety	**Mean**	**%**
101-14	0.31	31
Schw.	0.29	29

Inoc	**Mean**	**%**
N	0.20	20
Y	0.40	40

Stress	**Mean**	**%**
0	0.179	17.9
1	0.407	40.7
2	0.343	34.3

Table 4.2-10: Root dry weight (g) data showing the influence of variety, inoculation and stress.

Variety	Mean
101-14	16.690
Schw.	19.22

Inoc	Mean
N	17.93
Y	18.01

Stress	Mean
0	18.54
1	19.88
2	15.57

Table 4.2-11: Shoot dry weight (g) data showing the influence of variety, inoculation and stress.

Variety	Mean
101-14	4.27
Schw.	2.4

Inoc	Mean
N	3.32
Y	3.36

Stress	Mean
0	2.91
1	3.50
2	3.62

4.3 FUNGAL INFECTION DATA FOR CHAPTER 4

Table 4.3-1: Total numbers of the most common fungi isolated from plants in each treatment combination. Values are total colonies may include more than one isolate per tissue fragment. If a column is not present there were no isolates for that pathogen at that location.

			0 cm						**5 cm**						
Variety	**Inoc**	**Stress**	**Cd**	**F**	**B**	**A**	**Py**	**Pe**	**Cd**	**F**	**B**	**A**	**Py**	**Pe**	**Ph**
Schw.	N	0	3	8	0	1	2	12	1	2	0	0	2	1	1
Schw.	N	1	1	16	4	0	3	1	1	3	1	0	0	0	0
Schw.	N	2	3	11	2	0	4	8	1	2	3	1	1	1	2
Schw.	Y	0	6	13	0	0	1	3	0	4	1	0	0	0	0
Schw.	Y	1	4	13	0	1	1	7	2	0	0	1	0	3	3
Schw.	Y	2	9	1	12	0	8	0	2	0	2	0	4	0	0
101-14	N	0	3	10	0	0	9	1	0	1	2	1	1	1	0
101-14	N	1	4	7	1	0	5	1	2	3	2	0	0	0	0
101-14	N	2	5	9	4	0	4	2	1	2	1	1	1	2	0
101-14	Y	0	5	6	4	0	1	2	1	2	1	1	0	1	0
101-14	Y	1	3	7	5	0	7	2	1	2	0	3	3	0	0
101-14	Y	2	10	4	1	1	2	2	1	3	0	0	4	0	0

Key

Inoc: inoculation status (N=not inoculated, Y=inoculated)

Stress: stress level (0=unstressed, 1=moderate, 2=high stress)

Cd: *Cylindrocarpon destructans*

F: *Fusarium* sp.

B: *Botryosphaeria* sp.

A: *Alternaria* sp.

Pi: *Pythium* sp.

Pe: *Penicillium* sp.

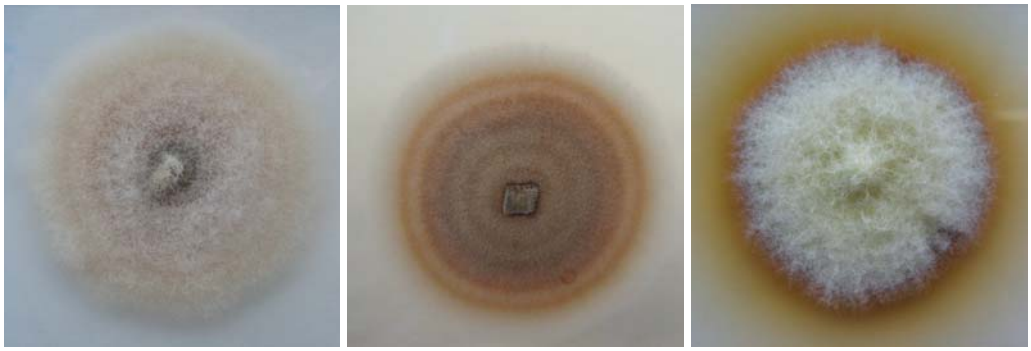
Ph: *Phomopsis* sp.

4.4 IDENTIFICATION OF *CYLINDROCARPON* SPP. ISOLATES

C. destructans colonies are dark brown, cinnamon or hazel; the dense aerial mycelium has a felt appearance and an even concentric growth pattern (Samuels & Brayford, 1990) (pers. comm. C. Bleach, 2006). The reverse colour of colonies is similar to the surface and ranges from buff to hazel. *C. destructans* produces macroconidia (15-25 µm x 4-8 µm), predominantly 1-3 septate (depending on colony age), straight and cylindrical, with apical cells broadly rounded. Chlamydospores are brown and readily produced (Samuels & Brayford, 1990; Brayford, 1992; Halleen et al., 2004).

C. liriodendri colonies are typically cream but at times slightly sepia, with sparse aerial mycelium giving colonies a felt-like appearance (Halleen, 2005; Whitelaw-Weckert et al., 2007). Colonies have a concentric growth pattern (pers. comm. C. Bleach, 2006). The reverse colour of colonies is similar to the surface and ranges from cream to slightly cinnamon or sepia. *C. liriodendri* produces numerous macroconidia (24-55 µm x 4.5-6.5 µm), which are 1-3 septate, straight or slightly curved and cylindrical. Chlamydospores are brown and commonly produced (Halleen et al., 2004; Whitelaw-Weckert et al., 2007).

C. macrodidymum colonies have no concentric growth pattern and the aerial mycelium is predominant, yellowish and covering the whole colony with a felt-like or woolly appearance. The reverse colour of colonies is dark brown to burnt umber (Halleen et al., 2004). *C. macrodidymum* produces numerous macro-conidia (26-45 µm x 4-8 µm) which are 1-4 septate (typically 3), straight, cylindrical or sometimes slightly clavate, and with slightly bent apical cells. It rarely forms chlamydospores (Halleen et al., 2004; Halleen et al., 2006a).



C. liriodendri *C. destructans* *C. macrodidymum*

Figure 4.4-1: Colony characteristics of *Cylindrocarpon* species (associated with black foot) on PDA.

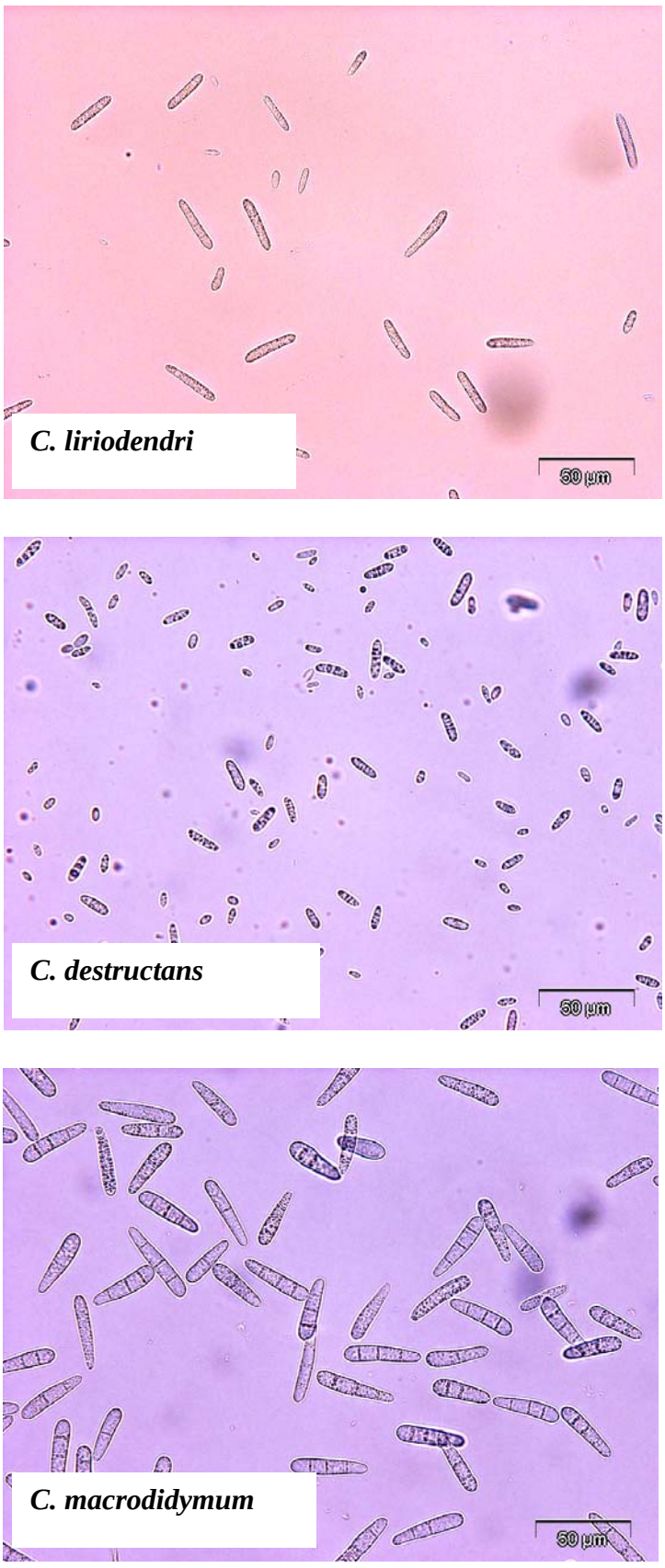


Figure 4.4-2: Spore characteristics (macroconidia) of *Cylindrocarpon* species (associated with black foot) grown on PDA.

4.5 SUPPLEMENTARY MOLECULAR DATA FOR CHAPTER 4

Table 4.5-1: Band intensity data for bands excised and sequenced from SSCP gels of DNA extracted from bacteria from the rhizosphere of grapevine rootstocks Schwarzmann and 101-14, inoculated (+) or not inoculated (-) with *Cylindrocarpon destructans* and exposed to no (0) moderate (1) or high (2) stress. C denotes soil only controls.

	Controls		**101-14**						**Schwarzmann**					
Band	**+C**	**-C**	**+0**	**+1**	**+2**	**-0**	**-1**	**-2**	**+0**	**+1**	**+2**	**-0**	**-1**	**-2**
18	S	S	S	S	S	S	S	S	S	S	S	M	M	M
21	S	S	S!	S!	M	M	S	M	S	S	S	S	S	M
22	M	M	S!	S!	W	M	M	M	M	M	W	S	S	M
23	S	M	S!	S!	M	M	M	M	M	M	S	S	S	S
25	W	M	S	M	W	W	W	W	M	M	M	M	M	W
38	W	S!	S	M	S!	W	W	W	W	M	S	S	M	W
50	S	S	M	S	S	S	S	M	W	S	S	W	S	S
54	M	M	S	W	M	W	W	W	S	M	W	W	W	M
55	S	M	S!	W	M	W	W	W	W	W	W	M	W	W
62	W	M	S	M	M	W	W	W	M	M	M	M	M	W
64	S	S	M	M	W	W	S	M	S	S	S	S!	M	W
65	S	M	S!	S	M	M	M	M	S	M	S	S	S	M

Key

S strong

M moderate

W weak

! very strong

Ratings of M and W can collectively be called weak. Bands rated W could not always be seen in the digital reproduction.

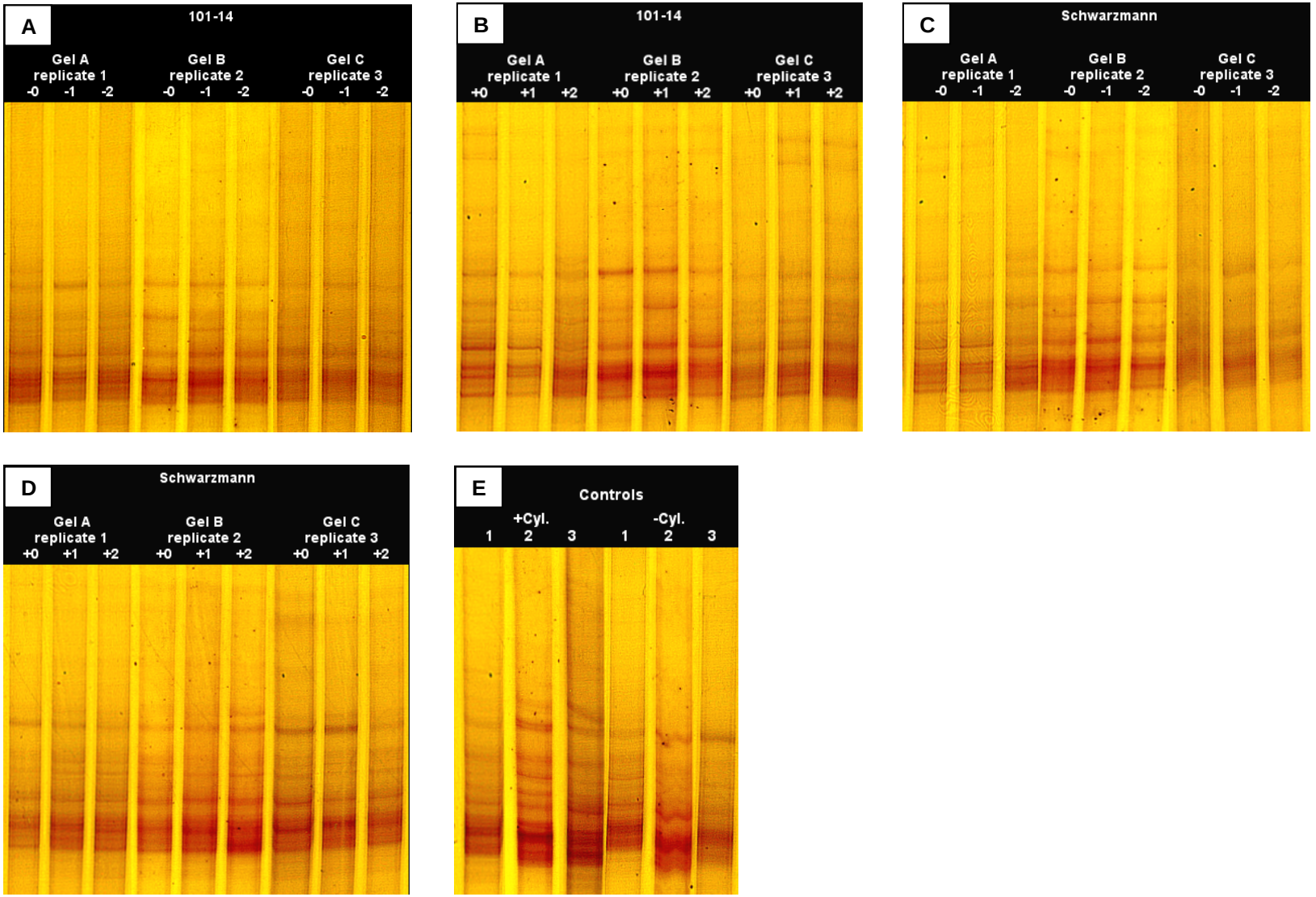


Figure 4.5-1: Composite SSCP gels of the three replicates per treatment for grapevine rootstocks 101-14 (A, B) and Schwarzmann (C,D), inoculated (+) or not inoculated (-) with *Cylindrocarpon destructans* and exposed to no (0), moderate (1) and high (2) stress. Soil only controls (E). Control gels show results for three replicates.

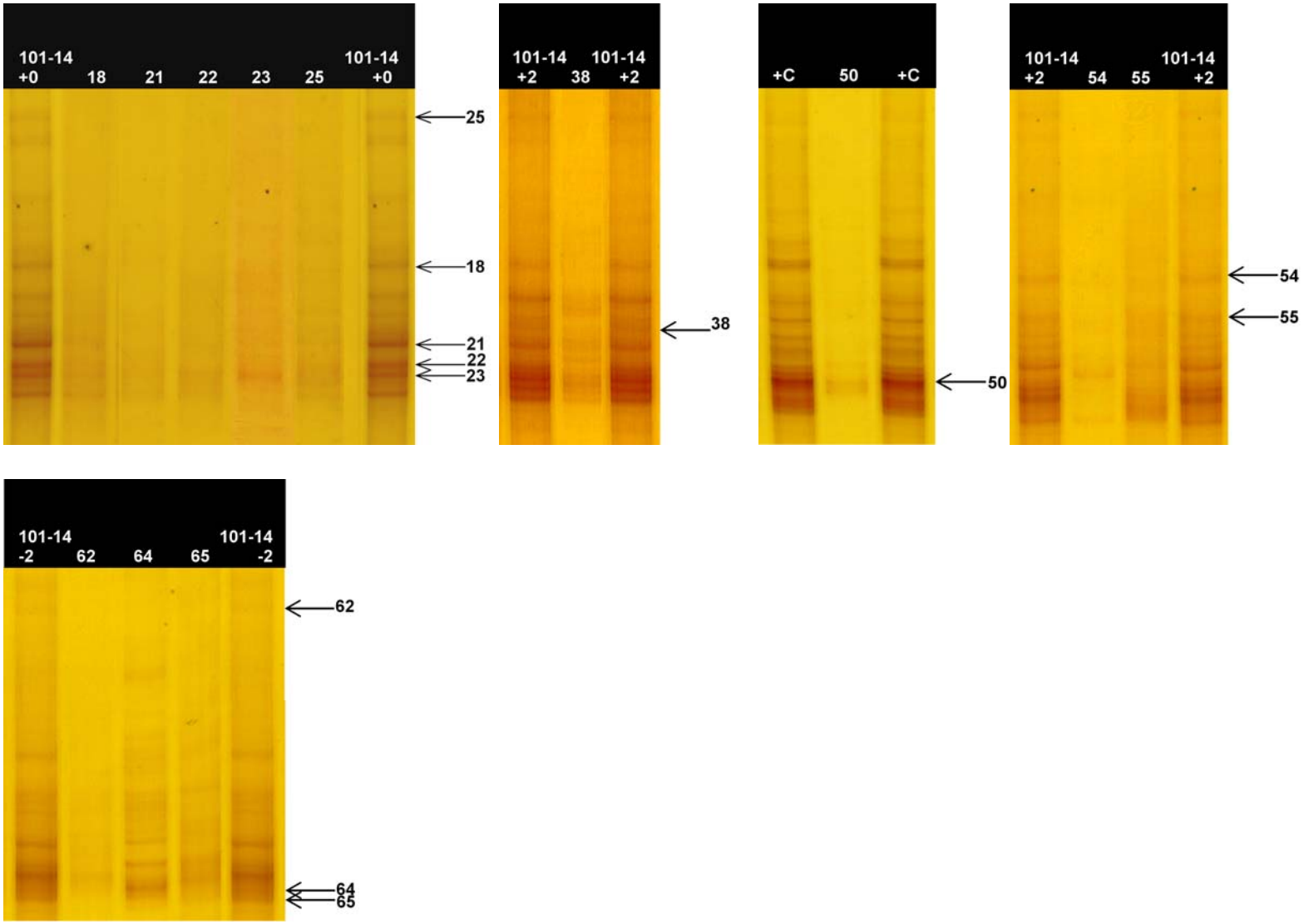


Figure 4.5-2: Composite SSCP gel scans of the bands excised, amplified, and run again after re-amplification. Band numbers are shown above the columns.

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