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Metarhizium strains associated with grass grub (*Costelytra giveni*) in New Zealand, and their potential as biocontrol agents

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A thesis
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of the requirements for the Degree of
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Nghia Thi Nguyen

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Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

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by

Nghia Thi Nguyen

Grass grub (*Costelytra giveni*) (Coleoptera: Scarabaeidae), an endemic pest of improved pasture in New Zealand, is a damaging root herbivore which lives in soils for most of its life cycle. The pest is resistant to most microbes, but larvae are susceptible to some strains of the fungi belonging to the genus *Metarhizium*. However, fungal diseases are rarely sufficient to control the pest naturally. It has been shown that other microbes can influence infection by the fungal propagules, but it is not known if this is due to specific species of microbes, and the mode of action of microbial interactions is yet to be elucidated. Plant and soil type can also influence the effectiveness of fungal entomopathogens, as they release compounds which can directly and indirectly affect soil microbial communities and potentially the infection process. Also, the abundance of *Metarhizium* in pasture soils and the host range of *Metarhizium* to New Zealand insect pests is unknown for most strains.

In this project, the goal was to find *Metarhizium* strains with the potential to be used as a biocontrol agent of grass grub in New Zealand and to assess the effect of microbial interactions with *Metarhizium* on the control achieved. *Metarhizium* spp. were isolated from the environment, identified through molecular approaches, and bioassays conducted against second and third instar grass grub larvae to determine relative virulence.

A number of *Metarhizium* strains were isolated from infected grass grub larvae, soil and obtained from existing collections. At one field site, *Metarhizium* spp. were infecting around 5% of field-collected larvae. These isolates were shown to belong to the species, *M. anisopliae, M. novozealandicum, M. robertsii. M. guizhouense, M. pemphigi, M. brunneum, M. frigidum* and *M. pinghaense*. Among these isolates *M. novozealandicum* (C14), *M. novozealandicum* (F99), *M. anisopliae* (M2), *M. anisopliae* (F672) and *M. pinghaense* (JK) caused high grass grub larval mortality after 35 days in bioassays (Chapter 2). *M. novozealandicum* (C14) caused the highest larval mortality in second and third instar grass grub in both semi-sterilised and non-sterilised soil. The soil type did

not affect the pathogenicity of *Metarhizium*. The LT_{50} and LC_{50} values of *M. novozealandicum* (C14) were lower than for other *Metarhizium* isolates. *M. novozealandicum* (C14) at 10^7 conidia/ml ($LC_{50} <$ 2.5×10^7 conidia/ 10 g soil after 28 days) was effective against both second and third instar grass grub (nearly 100% larval mortality after 35 days) (Chapter 3).

Interactions between *M. novozealandicum* (C14) and another potential deterrent of grass grub, the grass endophyte *Epichloë,* were investigated. *M. novozealandicum* (C14) applied at a rate of 10^5 conidia/ml had no interaction with meadow fescue endophyte (*Epichloë uncinatum*) and could not be detected as an endophyte inside grass tissues. However, *M. novozealandicum* (C14) established as an endophyte inside meadow fescue when soil was inoculated with 10^7 conidia/ml. After surface sterilisation and culturing *M. novozealandicum* (C14) was isolated from inside colonised plants, and inhibition of other endophytic fungi was found when isolating on MSM medium. Fungal hyphae were seen inside plant tissue using fluorescent microscopy, but could not specifically be confirmed as *M. novozealandicum* (C14) (Chapter 4).

A range of bacteria were isolated from soil and dead field collected grass grub larvae to examine the effect on *M. novozealandicum* (C14) infection and larval mortality, as a proxy for bacteria- fungal interactions. Most selected bacteria had a synergistic interaction with *M. novozealandicum* (C14) against the second instar larvae but there was no synergism against the third instar larvae. Isolate 6-1 (*Yersinia enterocolitica*) when combined with *M. novozealandicum* (C14) was more effective than the other bacterial isolates. This bacterial isolate also produced more chitinase than other bacteria tested but not proteinase. Isolate *Yersinia enterocolitica* 6-1 produced volatiles that inhibited the growth of *M. novozealandicum* (C14) when not in direct contact, but did not show direct antagonism against *M. novozealandicum* (C14) on PDA medium. There is potential to combine isolate *Yersinia enterocolitica* 6-1 with *M. novozealandicum* (C14) for the biocontrol of grass grub (Chapter 5).

The host range of *Metarhizium* was assessed for some New Zealand insect pests in bioassays. *M. novozealandicum* (C14) had high pathogenicity against two insect pests, *Helicoverpa armigera* (Lepidoptera) and *Tenebrio molitor* (Coleoptera) while there was less effective against three insect pests, *Plutella xylostella, Wiseana* sp. and *Myzus persicae* (Chapter 6).

The distribution of *Metarhizium* in pasture soil from three sites (Lincoln, Oxford and West Coast) was also investigated. Both *M. novozealandicum* and *M. anisopliae* were isolated, but *M. novozealandicum* was dominant at all three sites. At the Lincoln site there were high *Metarhizium* CFU counts and relatively low grass grub larval counts while the Oxford site had low CFU counts and high grass grub larval counts, but the West Coast site produced low counts of both. Although a correlation between high *Metarhizium* abundance and low grass grub abundance at Lincoln and the

opposite at Oxford site was indicated, the number of samples was low, and this study needs to be repeated with further replicates to provide more robust data (Chapter 7).

Overall, the results obtained in this thesis have provided essential biological evidence to help understand the potential of *M. novozealandicum* (C14) as a biocontrol agent against grass grub in New Zealand. Combining *M. novozealandicum* (C14) with bacteria, such as *Y. enterocolitica*, may achieve even better control than with *M. novozealandicum* (C14) alone. *M. novozealandicum* (C14) can also colonise meadow fescue endophytically, which may have implications for other modes of action, although no evidence of additional effects were found in this study. *M. novozealandicum* (C14) was also able to infect other insect pest species. A wide host range could be beneficial depending on impacts on natural enemies. The correlation between the presence of *Metarhizium* and decreasing populations of grass grub at two sites was shown, but this result needs to be replicated over more areas. In conclusion, *M. novozealandicum* (C14) has the potential to become a biocontrol agent against grass grub in New Zealand.

Keywords: Grass grub, *Costelytra giveni*, *Metarhizium* spp., CFUs, *Yersinia enterocolitica*, Fluorescent microscopy, Lepidoptera, Hemiptera, Coleoptera, *M. anisopliae, M. novozealandicum, M. robertsii. M. guizhouense, M. pemphigi, M. brunneum, M. frigidum, M. pinghaense*, *M. novozealandicum* (C14), *M. novozealandicum* (F99), *M. anisopliae* (M2), *M. anisopliae* (F672) and *M. pinghaense* (JK)

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List of acronyms

MSM	*Metarhizium* Selective Media
C14	15-T2-P-C14
F672	AgR F672
JK	JB (K1-4)
CFU	Colony forming unit
LSD	Least significant differences
TX-100	Triton X-100
ITS	Internal transcribed spacer
EF1α	Elongation factor 1-alpha
RCBD	Randomized complete block design
CRD	Completely randomised design
PBS	Phosphate buffered solution
LB	Luria-Bertani Miller
PDA	Potato Dextrose Agar
E-M-	without *E. uncinatum* endophytes in meadow fescue grass without *M. novozealandicum* C14
E-M+	without *E. uncinatum* endophytes in meadow fescue grass with *M. novozealandicum* C14
E+M-	with *E. uncinatum* endophytes in meadow fescue grass without *M. novozealandicum* C14
E+M+	with *E. uncinatum* endophytes in meadow fescue grass with *M. novozealandicum* C14

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Chapter 1
Introduction

1.1 Background

Pastures, consisting mainly of grasses and clovers, play a very important role in the New Zealand economy because New Zealand exported dairy products worth around $NZ 19.7 billion in 2020, accounting for 42% of GDP (Anon, 2020). Insect pest infestation and plant diseases are a constant threat facing pastoral farmers, particularly in improved grasslands. One such pest is the New Zealand grass grub, *Costelytra giveni* White (previously called *C. zealandica*)(Coleoptera: Scarabaeidae) which is one of the only few native insects that have become a major pest in New Zealand pastures (Scott, 1984; Grimont *et al*., 1988; Glare, 1994; Richards *et al*., 1997; Johnson *et al*., 2001; Townsend *et al*., 2004; O'Callaghan and Gerard, 2005; Wright *et al*., 2005; Marshall *et al*., 2008; Coca-Abia & Romero-Samper, 2016). This insect has been observed feeding on the roots of native tussocks which are believed to have been their main food source prior to European settlement (Kelsey, 1957). Post European settlement, converted pastures provided new habitats for *C. giveni* (Yeates, 1991). This has lead to major infestations in livestock pastures across wide geographic ranges with significant economic damage to the pasture industry (Osborne & Boyd, 1974; Glare, 1994; O'Callaghan & Gerard, 2005; Marshall *et al*., 2008; Lefort, 2013).

Safe and effective control of *C. giveni* in New Zealand pastures is a significant goal of the pasture industry. Control options for grass grub include insecticides, management practices such as tillage and crop rotation, and biological control. The insecticide diazinon coated onto ryegrass seed has been used to establish new dairy pastures (Zydenbos *et al*., 2016), and diazinon, fensulfothion and lindane have provided very cost effective control (Pfeffer and Heath, 2010). However, those insecticides have potential negative effects on human health over time (Anon, 2011; Jackson, 1990). Phenol has been used as an aggregation attractant for the adult males of grass grubs (Henzell & Lowe, 1970; Henzell *et al*., 1970; Chapman, 1975; Lauren, 1979). Starlings are predators of grass grub in pasture (East & Pottinger, 1975). A heavy roller was used on pasture to control grass grub, and a 61% larval mortality was achieved (Stewart & Toor, 1983). The use of microorganisms such as viruses (Dearing *et al*., 1980; Glare, 1992a), bacteria (Fowler, 1974; East & Willoughby, 1983; Trought *et al*., 1982; Klein, 1992) protozoa (Miln, 1978; Hanula & Andreadis, 1992), fungi (Glare, 1992b) and nematodes (Jackson & Trought, 1982) to control grass grub have all been studied. Biological control agents, such as parasitic nematodes, bacteria, viruses, fungi and microsporidia (Villalobos-

Hernandez, 1994; Lacey *et al*., 2001; Hajek, 2004) are generally safe to mammals and natural enemies of the target pests and are ecologically non-disruptive (Roberts & St Leger, 2004). Not all biocontrol agents have been successful in controlling grass grub. In particular, the use of parasitic nematodes as a biocontrol agent against grass grub was difficult because of the high production cost involved in formulation. On the other hand, the bacterium *Serratia entomophila* has been successfully used as a biopesticide to control grass grub (Jackson, 1990). Among the potential biocontrol agents, fungi are known pathogens of grass grub (Glare *et al*., 1993b; Bourner *et al*., 1996) and have previously been developed into biopesticides for other pest insects around the world (Mazid *et al*., 2011). Other promising entomopathogens can be found in the Fungi kingdom. The fungi *Beauveria* and *Metarhizium* spp. are mostly distributed in the soil environment and infect hosts through the cuticle (Hajek & St Leger, 1994; Inyang *et al*., 1998; Lacey *et al*., 2001; Meyling & Eilenberg, 2007; Cory & Ericsson, 2010; Safavi, 2010). They have been reported as pathogens of *C. giveni* (Latch, 1965; Bourner *et al*., 1996). Unlike bacteria or viruses, fungi penetrate directly and do not require ingestion for infection. Insect pest species are targeted by epidermal contact with the fungal pathogen which means all life stages are potentially infected (Zimmermann, 1993; Roberts & St Leger, 2004; Meyling & Eilenberg, 2007; St Leger, 2008; Behie *et al*., 2015; Keyser, 2015; Steinwender *et al*., 2015).

The aim of this study was to investigate: (I) the biocontrol potential of *Metarhizium* against *C. giveni*; (II) the interaction of *Metarhizium* and the *Epichloë* endophyte of meadow fescue; and (III) any interactions between *Metarhizium spp.* and common soil bacteria, and the effect of these interactions on the ability of *Metarhizium* spp. to control *C. giveni* infestations in New Zealand pastures.

1.2 Grass grub

1.2.1 Life cycle

The life cycle of grass grub has four stages (egg-larvae-pupae-adult) (Cottier, 1962; Villalobos-Hernandez, 1994; Lefort, 2013) (Figure 1.1). Adults are dark brown in colour and present in October and November (Cottier, 1962). They feed on grasses and clovers, on the foliage of stone fruit trees, and a wide range of other plants and trees. The female lays eggs below the soil surface with 3 to 40 eggs in each cluster (Cotter, 1962). Eggs are light-coloured, oval in shape, and coated with a clear sticky fluid to make them adhere closely until they hatch. The young larvae are crescent-shaped, white and begin to feed on roots (Cottier, 1962). The larvae go through three larval instars before pupation, emerging from January to September (Cottier, 1962; Villalobos-Hernandez, 1994; Lefort,

2013) (Figure 1.1) when they cause damage to pasture (Cottier, 1962). The pupal stage occurs in October and takes about 4-6 weeks (Cottier, 1962).

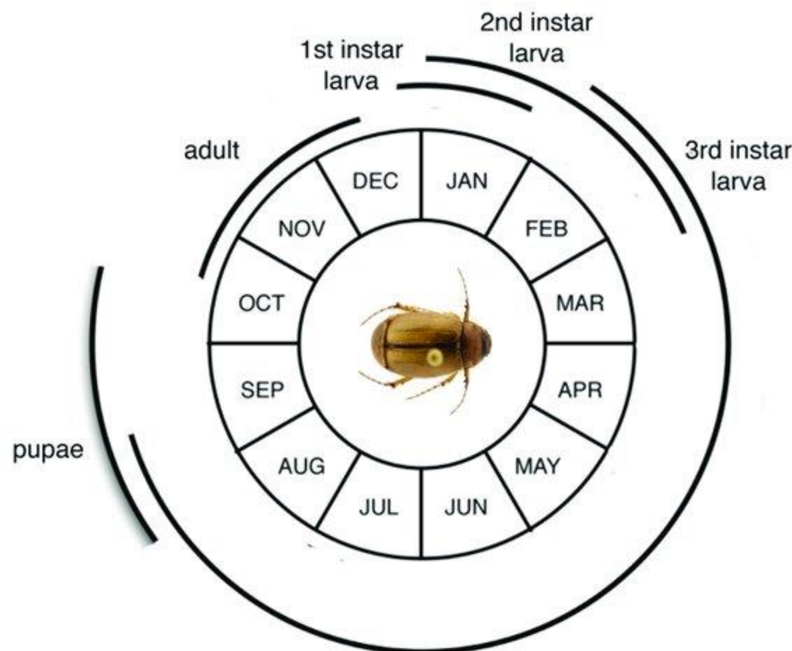


Figure 1.1. Life cycle of *Costelytra giveni* with permission from Lefort (2013).

1.2.2 Distribution and damage of *Costelytra giveni*

Abundance of *C. giveni* larvae and damage varies among pastures and years. Density of *C. giveni* larvae is generally low in new pastures while it is high in 3-6 year old pastures (Villalobos-Hernandez, 1994). Ferguson *et al*. (2019) have estimated the economic damage by grass grub to be between NZ$215 - $585M/year across New Zealand. Economic loss occurs when larval densities increase above 150 larvae/m^2 for drought-prone pastures and 200 larvae/m^2 in irrigated pastures in Canterbury, New Zealand (Townsend & Jackson, 1997), reaching 50% of pasture area damaged at a density of about 300-400 larvae/m^2 (Villalobos-Hernandez, 1994). This can change in other places depending on regional conditions; for example, massive damage of pasture with 430 larvae/m^2 and maximum number over 2200 larvae/m^2 was found in the Amuri irrigation scheme in Canterbury, while the number was about 700 larvae/m^2 on the North Island volcanic plateau (Jackson *et al*., 2012) (Figures 1.2 and 1.3) .

Figure 1.2 Pasture damaged by *Costelytra giveni* larvae at the Lincoln University Dairy Research Farm (May, 2019)

Figure 1.3 *Costelytra giveni* larvae dug up at the Lincoln University Dairy Research Farm (May, 2019)

1.3 Potential approaches to control of grass grub

1.3.1 Meadow fescue (*Festuca pratensis*)

In New Zealand, meadow fescue is a minor pasture species. Most of the meadow fescue grown in New Zealand contains a natural endophyte (*Epichloë uncinata*). Loline alkaloids produced by *E. uncinata* were effective against insect pests such as argentine stem weevil (Patchett *et al*., 2008a, Popay *et al.*, 2009; Popay & Hume, 2011), Japanese beetle (Patterson *et al*., 1991), and grass grub (Patchett *et al*., 2008b). In meadow fescue infected with *Epichloë uncinata*, the concentration of loline alkaloids increased in younger plant tissue after insect damage (Gonthier *et al.,* 2008, Zhang *et al*., 2009). Loline concentrations in the root of meadow fescue of more than 450 µg/g affected the larval weight of grass grub (Patchett *et al*., 2011).

1.3.2 Entomopathogens

Several microorganisms could be used for biocontrol of insect pests (Bourner *et al.,* 1996; Inglis *et al*., 2001; Lacey *et al*., 2001). Flock house virus (FHV), protozoan parasites (*Nosema* spp.), bacteria (*Paenibacillus popilliae, Serratia entomophila* and *Serratia proteamaculans*) and nematodes (e.g. *Steinernema* spp.) were found to attack grass grub larvae (Miln, 1978; Wigley & Miln, 1982; Jackson, 1990). The fungi *M. anisopliae sensu lato* and *Beauveria* spp. are known entomopathogenic fungi with the potential to control grass grub (Latch & Kain, 1983; Glare, 1994; Bourner *et al*., 1996).

1.3.2.1 *Metarhizium* spp.

The fungi *Metarhizium*, which belong to the order Hypocreales, and the family Clavicipitaceae, are globally distributed. There are between 750-1000 insect species known to be attacked by *Metarhizium* (Lacey *et al*., 2001; Meyling & Eilenberg, 2007; Schneider *et al.*, 2011; Vega *et al*., 2012; Keyser, 2015). *Metarhizium* has been isolated directly from soil and from infected insects. The fungus was given the common name “green muscardine” based on the appearance of dead insects carrying green fungal conidia (Roberts & St Leger, 2004). *Metarhizium* spp. have been found largely in U.S. pasture soils at a depth of 2-6 cm, at a concentration of up to 10^6 conidia/g (St Leger, 2008). *M. anisopliae sensu lato* has been used for over 100 years for controlling insect pests (Roberts & St Leger, 2004). It was an original broadly defined species with a large host range. Subsequently, *M. anisopliae* has been divided into a number of species. However, the taxonomy has been modified and it is likely that it was *M. brunneum* and *M. robertsii* infecting more than seven insect orders rather than the originally described *M. anisopliae*, which is now restricted to a few hosts (Veen, 1968; Zimmermann, 1993). Many *Metarhizium* species are morphologically similar, and identification is difficult using morphological attributes alone. There are currently ten species within

the *M*. *anisopliae* complex (viz., *M*. *anisopliae*, *M*. *acridum*, *M*. *brunneum*, *M*. *globosum*, *M.guizhouense*, *M*. *lepidiotae*, *M*. *majus*, *M*. *pingshaense*, *M*. *robertsii* and *M. indigotica*). The other major species complex, *M*. *flavoviride* was resolved into five species based on sequencing (viz., *M*. *flavoviride*, *M*. *koreanum*, *M*. *minus*, *M*. *pemphigi,* and *M. frigidum*) (Kepler *et al*., 2014). The species complex *M. anisopliae* have been used to control species of Acari (Ixodidae, Tetranychidae), Blattodea (Blattidae, Blattellidae), Coleoptera (Curculionidae, Nitidulidae, Scarabaeidae), Diptera (Ephydridae, Mycetophilidae, Sciaridae, Tipulidae), Hemiptera (Aphididae, Cercopidae, Cicadellidae, Delphacidae, Miridae, Pentatomidae), Isoptera (Kalotermitidae, Rhinotermitidae, Termopsidae), Hymenoptera (Formicidae), Lepidoptera (Crambidae, Noctuidae), Orthoptera, Siphonaptera (Pulicidae), Thysanoptera (Thripidae) in many countries all over the world (Faria & Wraight, 2007). Some strains of *Metarhizium* have been isolated for use as biological control agents to manage insect pests like locusts, termites, spittlebugs and white grubs (Nishi *et al.,* 2013).

1.3.2.2 *Metarhizium* in New Zealand

Metarhizium species have been recorded as pathogens of insect pests in New Zealand (Glare *et al*., 1993a). *M. anisopliae* has been isolated from Coleoptera and *M. novozealandicum* from Lepidoptera and Coleoptera (Driver *et al*., 2000). *M*. *novozealandicum* have been isolated from pinhole borer (*Platypus* sp.) in New Zealand (Reay *et al*., 2007). Studies have reported that *M. anisopliae*, *M. brunneum*, *M. frigidum*, *M. guizhouense, M. novozealandicum*, *M. pemphigi*, *M. rileyi* and *M. robertsii* were endemic in New Zealand while *M. acridum, M. majus, M. pingshaense* and *M. lepidiotae* are present only in recognised collections (Glare, pers. comm., 2018).

1.3.2.3 *Metarhizium* on scarabs

M. anisopliae has been isolated from insects belonging to the order Coleoptera and was used to successfully control the scarab *Adoryphorus couloni* in pasture in Tasmania (Rath, 1992; Hajek & St Leger, 1994)*. M. anisopliae* strain CLO 53 has been used successfully to control white grub (*Hoplia philanthus)* (Ansari *et al*., 2004b). There was also a synergistic interaction between *M. anisopliae* strain CLO 53 and the nematodes *Heterorhabditis megidis* and *Steinernema glaseri* at a high fungus spore concentration (2 x 10^{12} and 2 x 10^{13} conidia/ha) (Ansari *et al*., 2004a). *M. anisopliae* has also been used to control black vine weevil (Ansari *et al*., 2008, 2010), red palm weevil (Wakil *et al*., 2017) and sugarcane whitegrubs (*Dermolepida albohirtum*) in Australia (Samson *et al.,* 2001). However, these fungi are not always particularly effective against scarabs as the larvae might have developed resistance because they are constantly exposed to conidia in the soil. For example, in a laboratory experiment using seventeen *Beauveria* spp. and two *Metarhizium* spp. strains isolated from different sites in Mexico from white grub, *Phyllophaga polyphylla* (Scarabaeidae), mortality was

less than 50% (Carrillo-Benitez *et al*., 2013). Similarly, there was no significant difference between *Beauveria* spp. and *Metarhizium* spp. strains when *P. polyphylla* larvae were infected and larval mortality did not exceed 20% (Guzman-Franco *et al*., 2012).

1.3.2.4 *Metarhizium*, soil microbial communities, soil insects and plant interactions

Metarhizium spp. have been isolated from roots of different plants species. *M. robertsii* is mostly associated with grass roots, whereas *M. guizhouense* is found in roots of trees and *M. brunneum* has been isolated from the roots of woody plants and strawberry and blueberry plants. Switchgrass and haricot bean plants treated with *M. robertsii* had increased root hair growth (Keyser *et al.*, 2015). Additionally, *M. brunneum*, *M. anisopliae* and *M. robertsii* populations have been shown to promote the growth of some plants apart from their role as entomopathogens. Some strains of *M. robertsii* (strain 2575), *M. brunneum* (strain 3738), and *M. anisopliae* (strain 8248) have the ability to boost seedling growth based on their rhizosphere competence in maize roots (Liao *et al*., 2014). Moreover, increased plant growth has been observed on some crops such as soybean, tomato and maize (Keyser, 2015), as the yield of maize was increased after seed was treated with *M. brunneum* (Keyser *et al*., 2014). Additionally, nitrogen transported directly from soil insects to the plant through the interaction between the plant endophyte and insect pathogen (*Metarhizium robertsii*) promoted plant growth (Behie *et al*., 2012; Sasan & Bidochka, 2013).

Soil microbial species have key roles in maintaining the broad potential of the soil ecosystem function (Alvarez-Martin *et al*., 2016) through the processes of soil structure formation, decomposition of organic matter, toxin removal and the cycling of carbon, nitrogen, phosphorus, and sulphur (Kong *et al*., 2006; Zhong *et al*., 2010). Moreover, these microorganisms also contribute to controlling some soil borne diseases of plants and promoting plant growth (Garbeva *et al*., 2004; Batten *et al.,* 2006). In the rhizosphere, plants, fungi, protozoa, bacteria, nematodes and invertebrates interact in numerous ways (Liao *et al*., 2014), such as in transporting some nutrients (N, P and Fe) and auxins for plant growth (Batten *et al*., 2006). There are multitrophic interactions among plants, insect pests and entomopathogens that could help or hinder the efficacy of entomopathogens (Shikano, 2017).

Metarhizium spp. have provided a model system for the study of such interactions, with a lot of potential benefits of interaction between fungus, insect and plant in agriculture. Maize yield was increased significantly with treatment using *M. anisopliae* (Liao *et al.,* 2014). Some studies suggest that *B. bassiana* and *M. anisopliae* interact directly with the plant (Meyling & Eilenberg, 2007) through the absorption of nitrogen from insects by the plant (Behie *et al*., 2012; Behie & Bidochka, 2014). The relationships between the soil microbial community with *Metarhizium*, grass grub and

the plant are likely to involve multi-trophic interactions with a lot of potential benefits for studying entomopathogenic fungi for controlling grass grub.

1.3.2.5 Bacterial pathogens of *Costelytra giveni* larvae

A common disease of grass grub, called amber disease, is caused by the bacteria *Serratia entomophila* (Jackson *et al.,* 1983; Grimont *et al*., 1988; Allardyce *et al*., 1991; Jackson *et al*., 1999) and *Serratia proteamaculans* (Glare *et al*., 1993b; Grkovic *et al*., 1995; Glare *et al*., 1996; Jackson *et al*., 1997; Hurst *et al*., 2007). A survey indicated that up to 86% of grass grub larvae populations had symptoms of amber disease in some paddocks in Canterbury (Trought *et al*., 1982). Hurst *et al*. (2004) reported that amber disease symptoms were associated with a 155-kb plasmid, pADAP which carries the genes sepA, sepB and sepC. Milky disease caused by *Paenibacillus popilliae* is a less common disease of grass grub in New Zealand (Jackson, 1990; Glare *et al.*, 1993a). The bacterium *Yersinia entomophaga* has been isolated from infected larvae and is a pathogen of grass grub (Hurst *et al*., 2011; Hurst *et al*., 2014).

1.3.2.6 Combination of bacteria and *Metarhizium* to control insect pests

Studies have shown potential interactions between bacteria and *Metarhizium* for control of insect pests. A combination of *M. anisopliae* and *S. entomophila* had a synergistic effect on control of early-instar grass grub but not in older instar grass grub (Glare, 1994). Jackson & Chinn (1993) also reported that the combination of *M. guizhouense* and *S. entomophila* could produce a synergistic effect to control the grass grub. Mantzoukas *et al.* (2019) showed that a significantly higher larval mortality was observed in both 2nd and 4th instar larvae of tomato leafminer due to the synergy between *M. anisopliae* and *Bacillus thuringiensis* compared to each single pathogen alone. This synergy was reported against the larval stage of Colorado potato beetle (Kriukov *et al.,* 2009). In addition, the synergistic effects of *M. robertsii* and *B. thuringiensis* were noted in both 2nd and 4th instar larvae of *H. armigera* (Mantzoukas, 2019); 4th instar larvae of the Colorado potato beetle *Leptinotarsa decemlineata* (Yaroslavtseva *et al.*, 2017); on larvae of *Sesamia nonagrioides* (Mantzoukas *et al.,* 2012) and in insect pests of aubergine such as the jassid, *Amrasca bigutulla*, and the aphid, *A. gossypii* (Jugno *et al.,* 2018).

1.4 Aims and objectives of the present study

This study aims to better understand the interactions between entomopathogenic fungal species of the genus *Metarhizium*, the host (*C. giveni)*, members of the soil microbial community, and the grass plant. This knowledge could be applied to improve the biocontrol potential of *Metarhizium* spp. species against grass grub in pasture soils in New Zealand.

I hypothesised that:

1) Some isolated and identified *Metarhizium* strains will have the potential to control *C. giveni*

2) Soil type influences the pathogenicity of *Metarhizium* to *C. giveni*

3) Soil microbial community alters the infectivity and pathogenicity of *Metarhizium* spp. against soil dwelling insects

4) Interactions between common soil bacteria and *Metarhizium* influence the biological control of *C. giveni*.

The objectives of this study were to

Objective 1: Isolate and identify *Metarhizium* spp. pathogenic to *C. giveni*

Objective 2: Establish the pathogenicity of *Metarhizium* against grass grub in different soil types

Objective 3: Determine the effect of the presence of the meadow fescue (*Festuca pratensis*) endophyte (*Epichloë uncinatum*) on the pathogenicity of *Metarhizium novozealandicum* C14 to grass grub (*C. giveni*)

Objective 4: Investigate the effect of selected soil bacteria on the virulence of *M. novozealandicum* C14 to grass grub larvae

Objective 5: Explore the host range of the grass grub-active *M. novozealandicum* C14

Objective 6: Determine the field distribution of *Metarhizium* in several regions of the South Island of New Zealand

Chapter 2

Isolation and identification of *Metarhizium* spp. pathogenic to *Costelytra giveni*

2.1 Introduction

Metarhizium spp. are entomopathogenic fungi with broad host ranges (Trizelia *et al.,* 2017) and have been isolated from infected insects and agricultural and non-agricultural soils across every continent except Antarctica (Vanninen, 1996; Bidochka *et al.*, 1998; Keller *et al.*, 2003; Bruck, 2004; Silva *et al.*, 2004; Becerra-Velásquez *et al.*, 2007; Quesada-Moraga *et al.*, 2007; Derakhshan, 2009; Sahayaraj and Borgio 2009; Goble *et al.*, 2010; Hussein *et al.*, 2010; Meyling *et al.*, 2011; Weisi *et al*, 2012; Shin *et al.*, 2013; Sowmya, 2016; Kryukov *et al.*, 2017; Nishi and Sato, 2017; Brunner-Mendoza *et al.*, 2018; Islam 2018; Kilic, 2019; Tkaczuk, 2019; Mongkolsamrit *et al.*, 2020). Within this genus, *M. anisopliae sensu lato* has been found worldwide (see above references) and is one of several entomopathogenic fungi used extensively in commercial biocontrol products that are available worldwide (Brunner-Mendoza *et al.*, 2018). Complicating this however, Bischoff *et al.* (2009) recognized *M. anisopliae sensu lato* as a species complex and split it into nine different species: *M. anisopliae*, *M. acridum*, *M. brunneum*, *M. globosum*, *M. guizhouense*, *M. lepidiotae*, *M. majus*, *M. pingshaense* and *M. robertsii*. Mongkolsamrit *et al.* (2020) recently added two more: *M. clavatum* and *M. sulphureum*. Because of this, species identifications in this group can be ambiguous in the literature from before 2009, and/or that uses morphological features for identification.

In New Zealand, *Metarhizium* has been collected widely (Glare *et al.*, 1993a and references therein; Barker & Barker, 1998; Brownbridge *et al.*, 2010). Glare *et al.* (1993a) complied a checklist of entomopathogenic species in New Zealand, which lists *M. anisopliae sensu lato* isolated from Coleoptera and Lepidoptera and *M. flavoviride* isolated from Coleoptera. *Metarhizium* species have been recorded as pathogens of insects considered serious agriculture pests in New Zealand (Glare *et al.*, 1993a). Recently, several *Metarhizium* species, including *M. anisopliae sensu stricto*, *M. brunneum*, *M. frigidum*, *M. guizhouense, M. novozealandicum*, *M. pemphigi*, *M. rileyi* (syn: *Nomuraea rileyi*) and *M. robertsii,* were found occurring naturally in New Zealand, while *M. acridum, M. majus, M. pingshaense* and *M. lepidiotae* were identified only from recognised collections (Glare, pers. comm., 2018). *M. rileyi* appears to be rare. *Metarhizium novozealandicum* and *M. pemphigi* were previously considered variants of *M. flavoviride. Metarhizium guizhouense* strain AgRF16 (previously identified as *M. anisopliae*) was isolated from dead larvae of the grass grub and this

fungus was used to attempt control of this pest in New Zealand pasture, but had little impact (Latch, 1965; Glare *et al.,* 1994). Using AgRF16, Glare *et al.* (1994) found that the strain was not effective due to lack of activity at a soil temperature less than 16°C.

This chapter aims to report on:

1) Isolation and identification of *Metarhizium* spp. from infected *C. giveni* larvae and from three different soil types, Wakanui, Templeton and Temuka, in the Canterbury region of New Zealand.

2) The occurrence of *Metarhizium* from infected larvae collected in pasture soil.

3) The pathogenicity of selected *Metarhizium* isolates against *C. giveni* in bioassays.

2.2 Material and methods

2.2.1 *Metarhizium* Selective Media (MSM)

Potato Dextrose Agar (PDA-Merck) with antibiotics (Streptomycin sulphate and Chlortetracycline, concentrations listed in Appendix A.2.1.1) was used for isolating *Metarhizium* from soil and infected larvae.

2.2.2 Isolation from infected larvae

Spores were harvested from infected larvae of *C. giveni* with green fungal conidia (Figure 2.1C), collected from a field at Lincoln University, and transferred to a tube with 10 ml of sterile 0.01% Triton X-100 (TX-100) solution. Spore suspensions were mixed well, and serial dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were prepared. One hundred µl of each dilution was spread over the surface of an MSM plate using disposable hockey sticks. Plates were incubated at 25°C with a light regime of 16h light/8h dark and observed daily for 6-7 days after which *Metarhizium* colonies were identified and transferred to fresh PDA medium (Oxoid) for further development. Conidia were observed using a Leica DM2500 microscope with an Olympus SC100 camera.

2.2.3 Isolation from field soils

Metarhizium were isolated from each of the three collected soil types (Templeton, Temuka and Wakanui; see Chapter 3 for a description of soil characteristics). Four replicates of 10 g soil (for a total of 40 g per soil type) were used. The samples were taken from established dairy pastures at 0-15 cm soil depth. Ninety ml of sterile 0.01% TX-100 was added to each 10 g soil sample. These suspensions were then homogenised using a Stuart (Staffordshire, UK) flask shaker at 300-400 rpm for 30 min. The initial soil suspension was considered 10^{0}, from which further dilutions were

prepared. Serial dilutions from 10^{-1} to 10^{-4} were made by taking 1 ml of each dilution and adding to 9 ml of sterile 0.01% TX-100. Each dilution was plated on MSM with two plates per dilution. One hundred µl of each dilution was spread over the surface of a MSM plate and incubated at 25˚C with a 16h light/8h dark photoperiod. The inoculated plates were observed daily for 6-7 days after which *Metarhizium* colonies were identified based on their morphology and transferred to fresh PDA medium for development. Conidia were observed using a Leica DM2500 microscope with an Olympus SC100 camera.

2.2.4 Molecular identification of strains

All putative *Metarhizium* isolates were identified (including those from existing collections) using polymerase chain reaction (PCR) followed by Sanger sequencing (Kepler *et al.*, 2014). Elongation factor 1 -alpha (EF1α), and internal transcribed spacer (ITS) regions (Bischoff *et al.*, 2009; Kepler *et al.,* 2014) were used as barcodes for fungal identification. For DNA extraction, a small sample was cut from the growing edge of a culture and transferred to a micro centrifuge tube with 500 µl of a 5% Chelex suspension following the method of Alizadeh *et al*. (2017). These sample tubes were mixed thoroughly and incubated for 12 minutes at 100°C. After cooling to room temperature, samples were centrifuged for 20 minutes at 16,000 × g to separate the aqueous and particulate phase. The clear top layer (up to 200 µl) was transferred to a new tube and stored in a - 20°C freezer until further analysis.

Prior to PCR, DNA concentration (ng/µl) was estimated for each sample using spectrophotometry (Nanodrop 3.0.0 spectrophotometer; Nanodrop Technologies Inc., Delaware, USA). PCR reactions were carried out using 5 µl of 5× MyTaq Reaction Buffer (5 mM dNTPs, 15 mM $MgCl_2$, stabilizers and enhancers; Bioline), 1 µl of each primer (10 µM), 0.25 µl MyTaq HS DNA Polymerase (Bioline), approximately 100-300 ng genomic DNA and ddH_2O up to a final volume of 25 µl. The same reaction mixture with no template DNA added was included in each PCR reaction as a negative template control. Primers used are listed in Table 2.1. For amplification, a Kyratec thermal cycler was used, starting with an initial denaturation of 5 min at 95°C, followed by 40 cycles of 45 sec at 95°C, 45 sec at 55°C and 2 min at 72°C and concluding with a final extension of 7 minutes at 72°C. For problematic samples the annealing temperature was reduced to 52°C. The quality and size of the PCR products were assessed by agarose gel electrophoresis, using a 1% agarose gel in 1× TAE buffer (40 mM Tris-OH, 20 mM Acetic Acid, pH 7.8, 1 mM EDTA). Five microlitres of each PCR product along with loading dye were loaded in each lane of an agarose gel containing a DNA gel stain (RedSafe™). A 1 kb plus DNA ladder (Hyperladder II, Bioline, USA) was used to estimate the lengths of PCR products. PCR products were separated by electrophoresis in 1×TAE buffer at 100 V for 45 minutes

and then visualised following exposure to UV light using the UVITEC Imaging Systems Model 3000 (Bio-Rad, USA).

All amplified products were sequenced at the Lincoln University Sequencing Unit (Lincoln, NZ) and the sequences generated were edited and assembled using ChromasPro software (http://www.technelysium.com.au/ChromasPro.html) before being compared to the nucleotide GenBank database (Alizadeh *et al*., 2017).

Table 2.1 Primers used for identification

Gene region	Est. fragment size	Primers	Sequence 5'- 3'	Reference
EF1α (3' exon region)	1000	983F	GCYCCYGGHCAYCGTGAYTTYAT	Rehner and Buckley 2005
		2218R	ATGACACCRACRGCRACRGTYTG	
ITS	500	ITS1F	TCCGTAGGTGAACCTGCGG	White *et al.* 1990
		ITS4R	TCCTCCGCTTATTGATATGC	

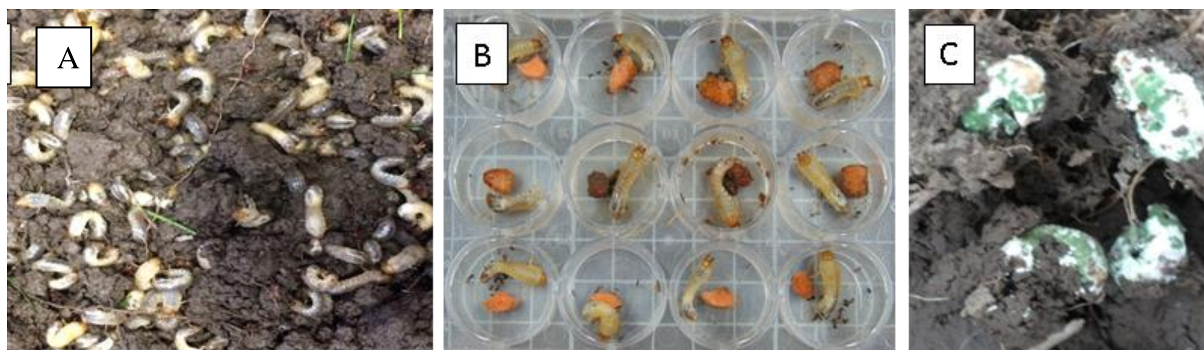


Figure 2.1 A) A heavy *Costelytra giveni* infestation in a Canterbury pasture soil. B) A cell culture plate used for maintaining individual larvae in the laboratory at 15°C with carrot as food. C*) C. giveni* larvae cadavers showing green spore mats typical of *Metarhizium* infection.

2.2.5 Collection of larvae for bioassays and mortality levels in field collected *Costelytra giveni*

Larvae were collected from the Research Dairy Farm at Lincoln University (Figure 2.1A). Second and third instar larvae were present in soil from January mid-March, and from February to mid-September, respectively. Third instar larvae have a larger head capsule than the second instar larvae (Cottier, 1962; Lefort, 2013). Based on morphology and collection time, second and third instar larvae were collected from the field and pretested for feeding activity as well as health (*i.e.*, ability to feed) upon return to the laboratory. Each larva was placed in a separate compartment of a 12-well cell culture plate with a carrot cube as food (figure 2.1B). Plates were incubated at 20°C for 14 days from the 14th to 28th of March, 2018. Mortality and incidence of *Metarhizium* in the natural population, identified by the presence of green fungal spores growing from the cadaver (Figure

2.1C), was then recorded. After this, actively feeding second and third instar larvae were selected for the bioassay experiment on the 14th day after setting up the carrot feeding plates. Only larvae from compartments with clear evidence of feeding, *i.e.*, bite marks on the carrot cube, were selected for use in bioassays.

2.2.6 Comparing the virulence of nine *Metarhizium* strains against second and third instar *Costelytra giveni* larvae

2.2.6.1 Preparation of inoculum

The virulence of eight *Metarhizium* strains from existing collections (Table 2.3), along with one strain collected for this study, in total representing six species, was tested against *C. giveni* larvae. All strains were grown on PDA plates at room temperature (20°C). After 15 days, fungal conidia of each strain were harvested directly from the plates and placed into 9 ml of sterile 0.01% v/v TX-100 solution (Autoclaved Milli-Q water plus Triton X-100). Conidial density was estimated using the original solution in a haemocytometer counting chamber and a Leica DM2500 microscope. Serial dilutions from 10^{-2} to 10^{-4} were made by adding 1 ml of each dilution to 9 ml of sterile 0.01% Triton X -100. Two hundred µl from the 10^{-4} dilution was used to estimate conidial density in a counting chamber, and the original solutions were adjusted to a concentration of 10^{8} conidia/ml by the formula presented in Appendix A.2.1.2. and A.2.1.3.

2.2.6.2 Bioassays

Metarhizium virulence was tested in 10 g subsamples of dry Wakanui soil (oven dried, 90° C) placed in universal vials with screw-on lids. For the inoculated treatment, 10^{8} conidia, suspended in 2 ml of sterile 0.01% Triton X-100 solution (1 ml 10^{8} conidia/ml suspension plus 1 ml 0.01% TX-100), was added to each vial for a final concentration of 10^{7} spores/g dry soil. For the non-inoculated controls, 2 ml of 0.01% TX-100 was added. All vials were shaken to mix soil and suspensions thoroughly before the larvae were placed in the vial. One *C. giveni* larva was added per vial, and a carrot cube was placed in each vial as food. The experiment was laid out in a randomized complete block design (RCBD) with 10 treatments (9 strains plus a control) with either four vials each for second instar larvae, or twenty vials each for third instar larvae which were more abundant in the larva collections. Vials were incubated at 22°C in environmental cabinets (16-h photoperiod). Data on larval mortality were taken 1, 3, 5, 7, 10, 14, 21, 28 and 35 days after inoculation. This experiment had 10 treatments, nine *Metarhizium* strains and one control, with five replicates per treatment and with 4 larvae in each replicate (total =20 larvae per treatment).

2.2.6.3 Data analyses

Mortality of larvae was compared as a binary state variable: each larva was either dead (0) or live (1) at the time of data recording. The data were corrected for control mortality by using Abbott's formula (Abbott, 1925). The results were analysed by a general ANOVA in GenStat®, 19th edition by comparison of mean values of the treatments using Fisher's unprotected least significance difference.

2.3 Results

2.3.1 Isolation of *Metarhizium* strains

Eleven *Metarhizium* strains were isolated from *C. giveni* larvae, and one each from a *Wiseana* sp. larva, *Wiseana* sp. pupa and Wakanui soil, after 6-7 days of observation. Species determinations of all the samples used in this study are presented in Table 2.2. Individual gene alignments were used for species identification. Seven *M. anisopliae* isolates and seven *M. novozealandicum* isolates were identified using the internal transcribed spacer (ITS) region of the rDNA. Seven of eleven fungal strains isolated from infected *C. giveni* larvae were identified as *M. anisopliae* while the four remaining strains isolated from infected *Wiseana* sp. larva and pupa, and Wakanui soil were identified as *M. novozealandicum*.

Table 2.2 Species identification of the new *Metarhizium* strains isolated in this study.

<table>
<tr><th>Isolate code</th><th>Metarhizium Species</th><th>Gene region</th><th>Isolation source</th><th>Origin</th></tr>
<tr><td>M1</td><td rowspan="8">anisopliae</td><td rowspan="14">ITS</td><td rowspan="8">Costelytra giveni larva (Coleoptera)</td><td rowspan="8">Field Research Centre, Lincoln University, Canterbury</td></tr>
<tr><td>M2</td></tr>
<tr><td>M3</td></tr>
<tr><td>M3</td></tr>
<tr><td>M4</td></tr>
<tr><td>M5</td></tr>
<tr><td>M6</td></tr>
<tr><td>M7</td></tr>
<tr><td>M8</td><td rowspan="6">novozealandicum</td><td>Wiseana sp. pupa (Lepidoptera)</td><td rowspan="6">Research Dairy Farm, Lincoln University, Canterbury</td></tr>
<tr><td>M9</td><td>Wiseana sp. larva (Lepidoptera)</td></tr>
<tr><td>M10</td><td rowspan="4">Costelytra giveni larva (Coleoptera)</td></tr>
<tr><td>M11</td></tr>
<tr><td>M12</td></tr>
<tr><td>M13</td></tr>
</table>

M14			Wakanui soil	Field Research Centre, Lincoln University, Canterbury

Table 2.3 *Metarhizium* strains obtained from existing collections and used in the bioassays.

Isolate code	*Metarhizium* Species	Gene region	Isolation source	Origin	Isolated by
15-T2-P-C14	*novozealandicum*	ITS	Kiwifruit soil	Nelson	MC Lefort
AgR F672	*anisopliae*		*Pinus radiata* roots	NZ	AgResearch
FCC 447	*robertsii*		Kiwifruit roots	NZ	AgResearch
JB (K4-1)	*guizhouense*		Kiwifruit leaves	Nelson	Jenny Bookes
E1037	*pemphigi*	EF1α	Coleoptera	Nelson	Nic Cummings
WetaMet #2	*frigidum*		Weta (Orthoptera)	West coast	Travis Glare
E1035	*novozealandicum*		*Plecoptera*	Canterbury	Nic Cummings
F99	*novozealandicum*		*Costelytra giveni* (Coleoptera)	Methven, Canterbury	AgResearch

Species identities and relevant information on *Metarhizium* species from existing collections used this study are presented in Table 2.3. Individual gene alignments were used for species identification. Four (one *M. novozealandicum*, one *M. anisopliae*, one *M. robertsii* and one *M. pingshaense*) were identified using the ITS region because those isolates produced a clear band in gels following PCR. Four more species were identified (one *M. pemphigi*, one *M. brunneum*, one *M. frigidum* and two *M. novozealandicum*) using the EF1α gene region as ITS did not amplify cleanly.

2.3.2 Mortality levels in field collected grass grub

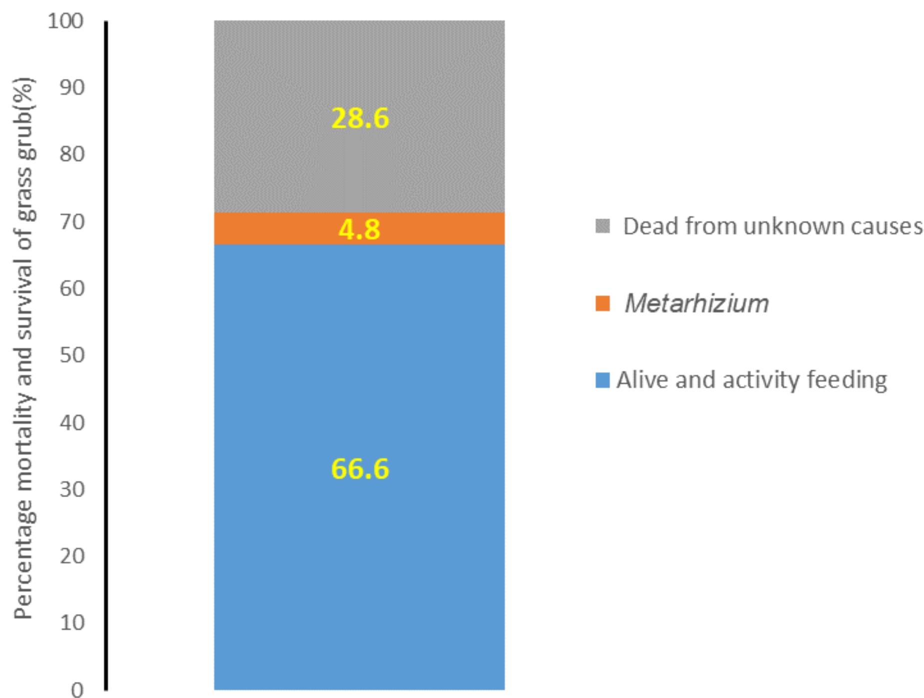


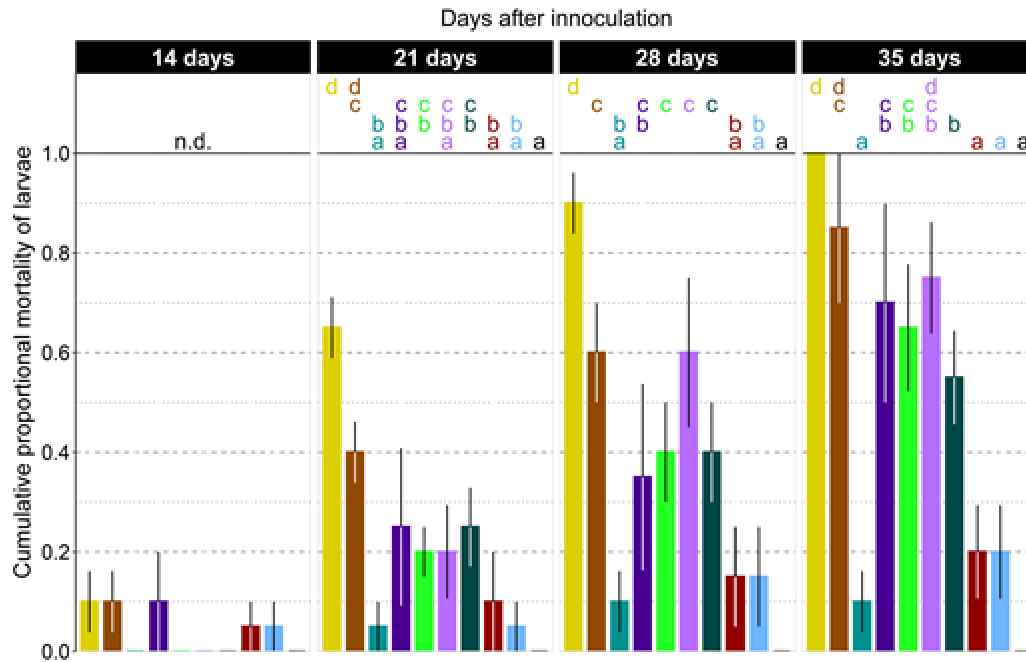
Figure 2.2 Percentage mortality, including incidence of *Metarhizium* infection, and survival in field-collected *Costelytra giveni* from Lincoln University, Canterbury.

In total, 755 larvae were collected and observed to assess mortality levels (Figure 2.2). After 14 days, 503 (66.6%) remained healthy and actively feeding, 36 (4.8%) *Metarhizium* infected larvae were identified by the presence of green fungal spores growing from the cadaver and 216 (28.6%) were observed without any evidence of *Metarhizium* infection, dead from unknown causes. These may have died for a number of reasons including handling damage, bacteria and/or other microbial attack.

2.3.3 Comparison of the virulence of nine *Metarhizium* strains against second and third instar *Costelytra giveni* larvae

Mortality in the *Metarhizium* treatments was first seen on day 14 of the trial in both instars. *Metarhizium* strain 15-T2-P-C14 (*M. novozealandicum*) consistently produced the highest mean larval mortality across the duration of the study for both second and third instar larvae (Figure 2.3). However, when compared to other high mortality-causing strains, it was significantly higher in only two instances: day 28 against 2nd instar larvae and day 14 against 3rd instar larvae. Mortality in strain F99 (*M. novozealandicum*) was second to 15-T2-P-C14, except on day 14 against 2nd instars, but in no instance was it significantly different from at least two other strains. On the other hand, mean mortality when inoculated with strains WetaMet #2 (*M. frigidum*), E1035 (*M. novozealandicum*) and

E1037 (*M. pemphigi*) was consistently lower than other inoculated treatments against both instars. Against 2nd instar larvae, none of these strains produced significantly higher mortality than the control (which had no mortality), though this may have been due to a lower statistical power in those bioassays. By day 28, mortality in these treatments were significantly lower than the other *Metarhizium* treatments (except for strain AgR F672 (*M. anisopliae*) at day 28 against 2nd instar). Strains AgR F672, M2 (*M. anisopliae*), JB (K1-4) (*M. pinghaense*), FCC 447 (*M. robertsii*) all produced substantial mortality, at least greater than 50%. Although there were differences between these strains, they were not consistent across days or experiments. Overall, the general relative patterns between strains described above were consistent in both larval instars.



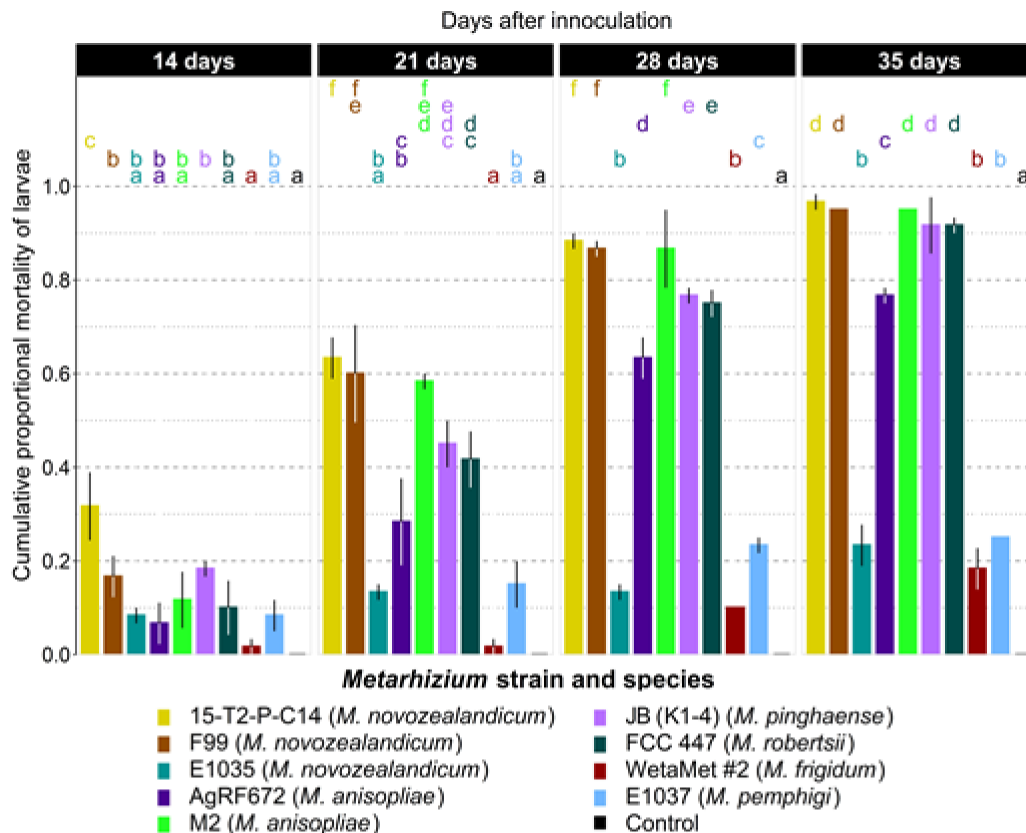


Figure 2.3 Mean proportional mortality and standard error of 2nd (above) and 3rd (below) instar larvae inoculated with one of nine *Metarhizium* strains at spore concentration of 10^7spores/g dry soil. Letters at the top of each graph indicate significance groupings based on Fisher's unprotected LSD test statistic: means with the same letters were not significantly different.

2.4 Discussion

This research aimed to collect and identify *Metarhizium* strains occurring under pasture in three soil types, assess the abundance of *Metarhizium* naturally occurring therein at one site and identify strains with the potential to control *C. giveni* grass grub infestations in pasture soils for subsequent studies. Some *Metarhizium* strains collected from soil, infected larvae and from existing collections were further identified using sequencing of either the the ITS or EF1α gene regions, to determine of the species of *Metarhizium*. *Metarhizium* strains in this study were recovered from field collected grass grubs, a larva and a pupa of porina moth, (*Wiseana* sp., Lepidoptera, Hepialidae) and soil. All strains belonged to two species, *M. novozealandicum* and *M. anisopliae*, similar to previous reports which also found these species to be abundant (Glare *et al*., 1993; Glare, pers. comm., 2018). Glare *et al.* (1993a) and Liu *et al.* (2020) also reported isolation of *M. novozealandicum* from larvae of *Wiseana* sp.; *M. anisopliae* was previously also isolated from *Crambus* sp. (Lepidoptera*), Mythimna separata, Persectania aversa, Scellodes cordalis, Heteronychus arator, Pericoptus truncatus, Listronotus bonariensis* (Coleoptera) and unidentified wireworm larvae (Coleoptera).

Five percent of field-collected larvae which died showed signs of *Metarhizium* infection, indicating that *Metarhizium* was at least partly responsible for their death. A further 29% of the deaths recorded were due to undetermined causes, indicating that *Metarhizium* spp. were unlikely to be the main cause of mortality in this field. Hussein *et al*. (2010) reported that 14% of mortality in field-collected wax moth larvae was caused by *M. anisopliae*.

One *Metarhizium* isolate was isolated from the Wakanui soil but not from the two remaining soil types (Templeton and Temuka). This may have been by chance because of the small sample size taken, so using larger samples may have increased the opportunity for detection. Molloy (1993) reported that Temuka, Templeton and Wakanui soils were important agricultural soils in Canterbury, but no information regarding pests status provided.

Patterns of relative virulence between the *Metarhizium* strains remained similar in both 2nd and 3rd instar larvae, i.e., a strain that produced relatively high or low mortality in one instar did so in both instars. Although mortality appeared to be generally lower in the younger, 2nd instar larvae, instars could not be statistically compared to each other. For 2nd instar larvae, results showed that 15-T2-P-C14 (shortened to C14) (*M. novozealandicum*) in particular generally produced greater virulence relative to the other strains. Both strains F99 (*M. novozealandicum*) and JB (K1-4) (*M. pinghaense*) were also effective, and after 35 days, mortality caused by these strains was not significantly different. For 3rd instar larvae, strain C14 again produced the highest mortality. In this case however,

F99 and M2 (*M. anisopliae*) performed equally early in the experiment, and by the end of the experiment JB (K1-4) and FCC 447 (*M. robertsii*) had also caught up to C14. Based on the growth morphology on PDA plates and virulence in both bioassays, strains C14, F99, M2, JB (K1-4) and AgR F672 (*M. anisopliae*) were considered to have potential as biocontrol agents against grass grub infection by *C. giveni* and were selected for the further study presented in Chapter 3.

Chapter 3

Pathogenicity of *Metarhizium* against grass grub in different soil types

3.1 Introduction

Grass grub is susceptible to some soil-borne entomopathogenic fungi strains belonging to the genera *Beauveria* and *Metarhizium* (Jackson, 1990; Glare *et al.*, 1993a). *Metarhizium* spp. have previously been considered as potential biological control agents against grass grub in New Zealand (Jackson, 1990; Glare, 1994; Rivas-Franco *et al*., 2019) but no commercial products based on these fungi are yet available.

Different soil factors such as soil texture, pH, organic matter and soil microbial community could influence the pathogenicity of *Metarhizium* to grass grub. Soil is a complex system and has an important role in providing microhabitats for the survival of organisms including fungi, bacteria, protozoa, nematodes and viruses (Nannipieri *et al.*, 2003). Clifton (2013) has shown that soil type affected the presence of soil-borne entomopathogenic fungi, as organic soil was more suitable than non-organic soil for fungal growth. In addition, soil is an important source of diversity of microbial organisms, particularly bacteria and fungi, and interactions between fungi and bacteria occur in the soil ecosystem (Deveau *et al*., 2018). For example, Glare (1994) reported that the combination of *M. guizhouense* with the bacterial entomopathogen *Serratia entomophila* was synergistic against second instar grass grubs in laboratory conditions. Ansari *et al.* (2004) also demonstrated that combining the fungus *M. anisopliae* CLO 53 and entomopathogenic nematodes *Heterorhabditis megidis* and *Steinernema glaseri* caused additive and synergistic effects against third instar *Hoplia philanthus* larvae in both the laboratory and greenhouse. However, Medina *et al.* (2020) reported that there was antagonism between the fungus *Trichoderma atroviride* and *Metarhizium robertsii* on PDA medium and *Trichoderma* decreased the germination of *M. robertsii* conidia.

Soil properties (soil texture, pH, organic matter, cation exchange capacity (CEC)) also have an important effect on the survival of *Metarhizium*. Generally, fungi can survive in soils across a wide pH range, with fungal growth increasing with decreasing pH between the ranges from 4.5 to 8.3, with pH 4.5 supporting optimal fungal growth (Rousk *et al*., 2009). Medo and Cagan (2011) reported that *M. anisopliae* was less common in fine-textured and high organic matter Slovakia soils than *Beauveria bassiana*. In addition, Jabbour and Barbercheck (2009) found that the presence of

Metarhizium was not related to soil mineral content (phosphorus, potassium, magnesium, calcium and also cation exchange capacity). There is little information about soil minerals and the occurrence of *Metarhizium* in pasture soils.

The aim for this chapter was to use three soil types (Wakanui, Temuka and Templeton) to investigate the effect of soil types and their characteristics on *Metarhizium*, as well as to examine the pathogenicity of *Metarhizium* on grass grub in the three soils in the presence and absence of their natural soil-inhabiting microbes, using different spore concentrations of the *Metarhizium* isolates.

3.2 Material and methods

The semi-sterilised soil types experiment used second instar larvae while the non-sterilised soil experiment used third instar larvae because of time constraints which limited the experiments to one year of larval availability.

3.2.1 The pathogenicity of *Metarhizium* against second instar grass grub in semi-sterilised soils

3.2.1.1 Hypothesis

Soil type influences the pathogenicity of *Metarhizium* to grass grub.

3.2.1.2 Soil preparation

Three soil types (Templeton, Temuka and Wakanui) were collected from Lincoln University and Selwyn District, Canterbury for this project. Wakanui soil (silt loam) and Temuka soil (clay loam) were collected at Lincoln University from two sites (43.647872^{o} S, 172.467853^{o} E for Wakanui) and (43.648924^{o} S, 172.468711^{o} E for Temuka). Templeton soil (silt loam) was collected from a farm in the Selwyn District, Canterbury (43.647586^{o} S, 172.458100^{o} E) (Figure 3.1). Samples to a depth of about 15 cm were taken from the three sites with ten replicates of each soil type in the same paddock. For each soil type, 50 kg soil was collected from 10 different positions and mixed before they were transferred to the laboratory at the Bio-Protection Research Centre in February 2019 and kept in plastic bags in boxes at 4°C. A 500 g sample from each soil type was sent to Hill Laboratories (Hamilton, New Zealand) for determination of general chemical characteristics, including pH, contents of phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sodium (Na); cation exchange capacity (CEC), total base saturation, organic matter and total carbon contents (Table 3.1).

Figure 3.1 Templeton soil collected from a farm in the Selwyn District, Canterbury in 2019

Table 3.1 Characteristic of the three Canterbury soil types (Hill Laboratories, 2018)

Analysis	Temuka	Templeton	Wakanui	Sterilized Wakanui
pH	6.0	5.7	6.3	5.9
Olsen Phosphorus (mg/L)	5	23	14	31
Potassium (me/100g)	0.43	0.56	1.36	0.96
Calcium (me/100g)	7.8	5.6	6.5	7.2
Magnesium (me/100g)	1.91	0.87	1.51	1.33
Sodium (me/100g)	0.16	0.15	0.14	0.13
CEC (me/100g)	15	11	13	14
Total Base Saturation (%)	70	63	72	67
Volume Weight (g/mL)	0.95	1.03	0.93	1.04
Sulphate Sulphur (mg/kg)	2	7	1	6
Potentially Available Nitrogen (15cm Depth) (kg/ha)	73	43	82	131
Anaerobically Mineralisable N (µg/g)	51	28	58	84
Organic Matter (%)	5.4	3.7	5.5	5.5
Total Carbon (%)	3.1	2.2	3.2	3.2
Total Nitrogen (%)	0.30	0.17	0.31	0.27
C/N Ratio	10.4	12.5	10.1	11.6
Anaerobically Mineralisable N/Total N Ratio (%)	1.7	1.6	1.9	3.1
Base Saturation % K	2.9	4.9	10.3	6.6
Ca	53	49	50	50
Mg	13	7.7	11.5	9.2
Na	1.1	1.3	1	0.9
MAF Units K	8	12	26	20
Ca	9	7	8	9
Mg	41	20	32	31
Na	7	7	6	6

3.2.1.3 Semi-sterilised soils

A 10 kg sample from each soil type (Temuka, Templeton, and Wakanui) was sieved through a 2 mm mesh to remove plant debris and stones (Figure 3.2), homogenised by hand and placed in an oven at 90°C for three days to semi-sterilise. The alternate soil sterilisation methods of autoclaving or gamma irradiation were not suitable or available for these experiments. The soil was semi-sterile, as not all microbes were eliminated by this method, but in general, few species remained and only in low numbers. All soils were checked for culturable microbes (including *Metarhizium* and bacteria) after sterilising by plating soil extracts on MSM and LB media (see recipes in Chapter 2 and Chapter 5).

3.2.1.4 Soil moisture content determination

The soil moisture content (MC) was determined using the method of Rex *et al.* (2015) with some modifications. Briefly, 50 g soil (3 replicates) was placed in a Petri plate with a lid and oven-dried at 90°C then weighed. The MC of the soil was determined as follows:

MC (%) = [(Wet soil – Dry soil)/Dry soil] × 100

Where: - MC (%): Soil moisture content of the air-dried or field soil (%)

- *Wet soil: air-dried soil (g) or field soil (g)*

- *Dry soil: Oven-dried soil (g) at 90°C*

Figure 3.2 Collected field soil samples were sieved through a 2 mm mesh.

3.2.1.5 Pre-screening of *Costelytra giveni* larvae

This experiment used 2nd instar larvae that were pre-screened for feeding activity as an indicator of health as described in Chapter 2.

3.2.1.6 Bioassays

The experiment was set up to compare the effect of the three semi-sterilised soil types on the pathogenicity of *Metarhizium* against 2nd instar *C. giveni* larvae. For each soil type, 10 g sterilised soil was placed in each universal vial with a screw-on lid. Five *Metarhizium* isolates were tested: *M. anisopliae* (M2), *M. novozealandicum* (C14), *M. anisopliae* (F672), *M. guizhouense* (JK) and *M. novozealandicum* (F99), selected from the results in Chapter 2, with each *Metarhizium* isolate used at four spore concentrations. The preparation of the spore solution was described in Chapter 2. The spore solution were quantified using an improved Neubauer chamber, and adjusted to 10^6, 10^7, 10^8 and 10^9 conidia/10 g sterilised soil, and used immediately. Each inoculated treatment received 1 ml of the *Metarhizium* solution added directly to the soil and 1 ml of 0.01% TX-100. For the non-inoculated controls of the three soil types, 2 ml of 0.01% TX-100 was added. All vials were shaken to mix soil and solutions thoroughly before the larvae were added. Field collected larvae (see Chapter 2) were placed individually in the soil with a carrot cube for food and incubated at 22°C (Glare, 1994) in environmental cabinets using a 16 light/8 h dark photoperiod from 30 January to 1 May 2019. Data on larval mortality were taken 1, 3, 5, 7, 10, 14, 21, 28 and 35 days after inoculation.

3.2.1.7 Experimental design

The experiment used a randomized complete block design (RCBD) with three soil types and with four spore concentrations of each of five *Metarhizium* strains, and with three extra non-inoculated controls for each *Metarhizium* isolate as the experiments were set up separately. For each *Metarhizium* isolate, there was 15 treatments, each treatment with five replicates, and each replicate with four larvae (one larva/vial) for a total of 300 larvae.

3.2.2 The pathogenicity of *Metarhizium* against third instar grass grub in non-sterilised soils

3.2.2.1 Soil preparation

All 3rd instar larval experiments used non-sterilised soils. Soil (6 kg) of each type (Temuka, Templeton, and Wakanui) was sieved through a 2 mm mesh to remove plant debris and stones (Figure 3.2), and air-dried at room temperature (20°C) for 14 days. The soil moisture content was determined (see 3.2.14) and based on the results (see Appendix 3.1), the spore suspension in 0.01% TX-100 was adjusted to 2 ml for each vial in bioassay 2.

3.2.2.2 Pre-screening and bioassays

Larvae were pre-screened as described in Chapter 2. The same bioassay method as for the 2nd instar larvae was followed in this experiment. The experiment was designed to compare the effect of non-sterilised soil on the pathogenicity of *Metarhizium* against 3rd instar *C. giveni* larvae. For each soil type, 10 g air-dried soil was placed in a universal vial with a screw-on lid. The best three *Metarhizium* isolates: *M. anisopliae* (M2)*, M. novozealandicum* (C14) and *M. novozealandicum* (F99), were used, selected from the results of the experiments against 2nd instar larvae. The spore suspension used for the treatments and 0.01% TX-100 for the control was calculated based on the water needed to achieve the required soil moisture using the formula in 3.2.1.3 and adjusted to 2 ml for each vial. Data on larval mortality were taken 1, 3, 5, 7, 10, 14, 21, 28 and 35 days after inoculation from 25th May to 8th July 2019. The experimental design was described in 3.2.1.5

3.2.3 Data analyses

In all the experiments in this chapter, the pathogenicity of the *Metarhizium* isolates was tested with reference to the grass grub mortality data as the variable of interest. Mortality was compared as a binary state variable: each larva was either dead (0) or alive (1) at the time of data recording. The data were corrected for control mortality by using Abbott's formula (Abbott, 1925). Probit analysis was performed to determine median lethal concentration (LC_{50}) of the *Metarhizium* isolates. The results of larval mortality and LT_{50} value were analysed by a general ANOVA in GenStat®, 19th edition by comparison of mean values of the treatments using Fisher's unprotected least significance difference.

3.3 Results

3.3.1 The pathogenicity of five *Metarhizium* isolates against second instar grass grub in semi-sterilised soils

Some soil properties may change after semi-sterilising. Using the Wakanui (silt loam) soil type as an example, pH decreased, Olsen Phosphorus increased and Potassium decreased following sterilisation (Table 3.1). On MSM, there was no evidence of *Metarhizium* surviving soil sterilisation, however, some bacteria grew on LB medium suggesting that a small number of bacteria survived, most likely sporeformers such as *Bacillus*.

Mean cumulative percent mortality of larvae increased from day 1 to day 35 in all treatments in the three semi-sterilised soil types (Figure 3.3). Larval mortality was 20% or less from day 1 to day 7 for all treatments. In the untreated controls larval death began at day 14 in some treatments/soil types and by day 35 all the treatment/soil types had dead larvae (Figure 3.3). These did not exceed 20%

mortality. At day 35, there were no significant differences among soil types for C14, M2, F672 and JK, but for F99 larval mortality was significantly greater in the Templeton soil than in the Wakanui soil (Figure 3.3.).

For each *Metarhizium* strain larval mortality increased rapidly between days 14 and 35. Mortality at day 35 was significantly greater than the untreated control for each spore concentration with the exception of the 10^6 conidia/g soil for M2, F672 and JK (Figure 3.3). Soil type had no effect on mortality for any of the five *Metarhizium* strains.

At the 10^6 spore concentration, larval mortality at day 35 did not differ from that of the untreated control for F99, M2, F672 and JK, but did so for C14. Mortality increased with increasing spore concentration reaching 100% for the two highest spore concentrations (10^8 and 10^9) in all the *Metarhizium* strains except F672 (Figure 3.3). Mortality did not differ between 10^8 and 10^9 spore concentrations and was significantly higher for these two concentrations than the 10^7 concentration (Figure 3.3).

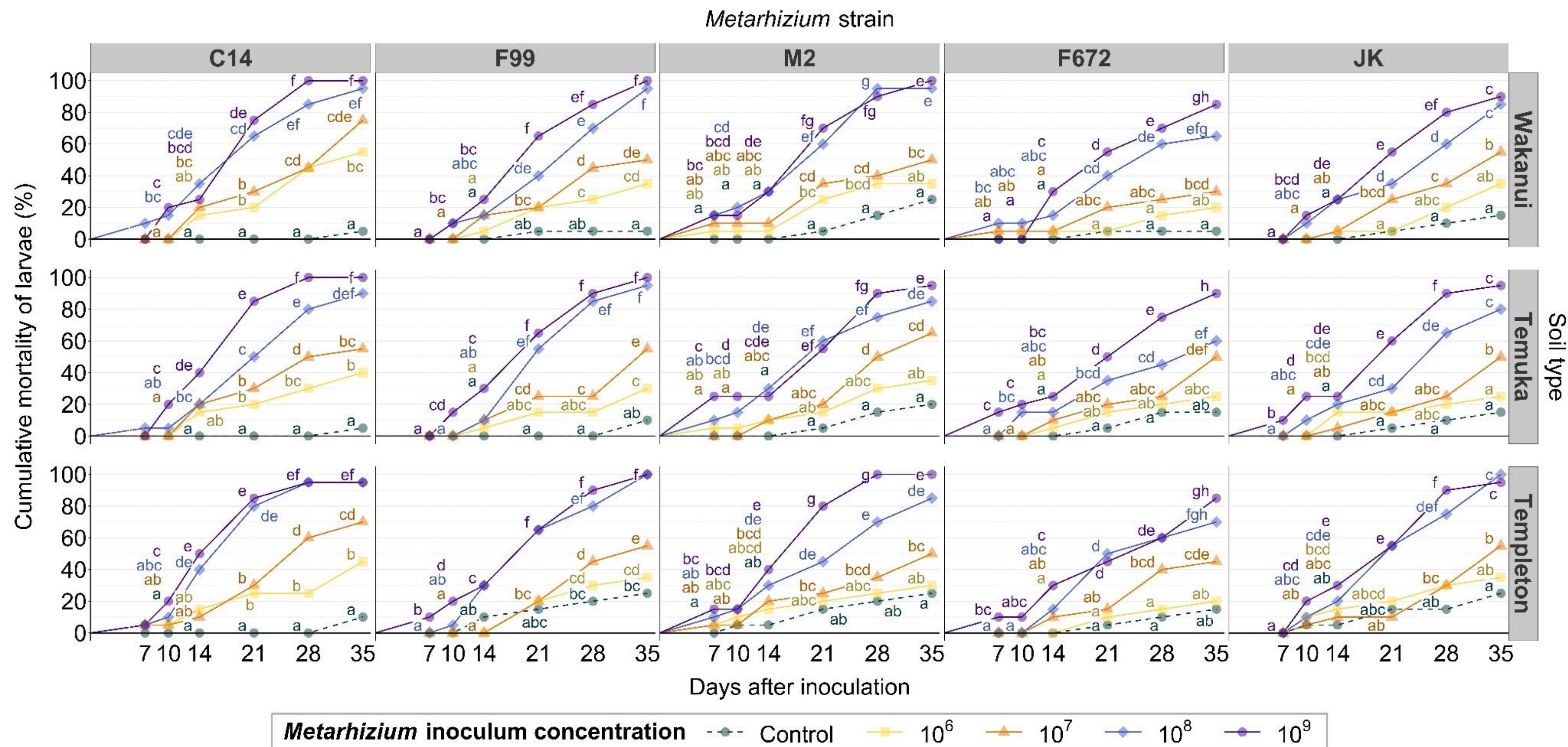


Figure 3.3 Mean cumulative mortality over time of 2nd instar *Costelytra giveni* larvae inoculated with four concentrations each of five *Metarhizium* isolates (columns). All were tested in three semi-sterilised soil types (rows). Letters indicate significance groupings based on Unprotected LSD test statistic: means with no letters in common are significantly different (P<0.05). Different days after inoculation and different strains were tested separately but different soils were compared within these groups, i.e., all vertically aligned means across all three sterilised soils can be compared.

Overall, *M. novozealandicum* (C14), *M. novozealandicum* (F99), *M. anisopliae* (M2) and *M. guizhouense* (JK) were more virulent than *M. anisopliae* (F672), producing high larval mortality by the end of the experiment consistently across all the soil types and inoculation intensities studied. In addition, soil type did not significantly affect the pathogenicity of *Metarhizium* isolates against second instar grass grub. The 10^8 conidia/ 10 g dry soil treatment (nearly 100% larval mortality) was able to control 2nd instar grass grub in all three soils in bioassays.

The time taken for 50% of larvae to die (LT_{50}) calculated over 35 days for each spore concentration in each soil type against 2nd instar grass grub is shown in Figure 3.4. LT_{50} for *M. novozealandicum* (C14), *M. novozealandicum* (F99), *M. anisopliae* (M2), *M. guizhouense* (JK) and *M. anisopliae* (F672) did not differ significantly at each spore concentration in each soil type. The mean LT_{50} for *M. novozealandicum* (C14) and (F99), *M. anisopliae* (M2) and (F672), and *M. guizhouense* (JK) did not differ significantly at the 10^8 and 10^9 spore concentrations, but was significantly lower at the 10^7 spore concentration in all these soils (Figure 3.4).

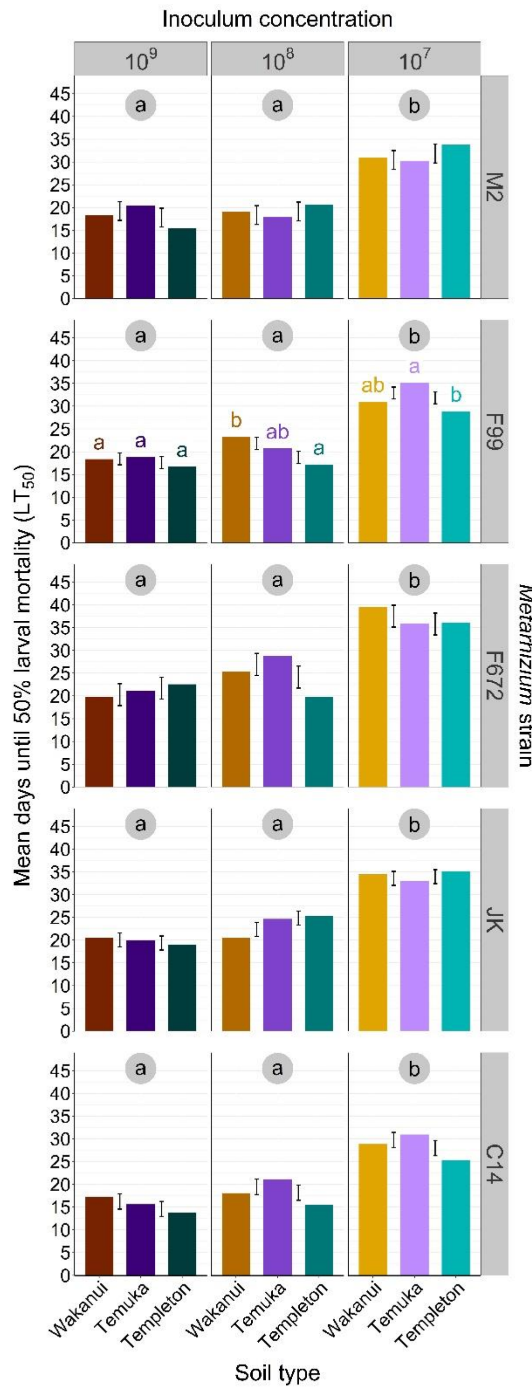


Figure 3.4 The mean calculated length of time, in days, until 50% *Costelytra giveni* larval mortality occurred (LT_{50}) after inoculation with five *Metarhizium* strains in three sterilised soils. Several conidial concentrations were tested. Letters indicate significance groupings based on Unprotected LSD test statistic: means with no letters in common are significantly different ($P<0.05$) using F99 as an example and are there for information only as soil type was never a significant factor in ANOVA tests. Bars indicated in the figure are LSD.

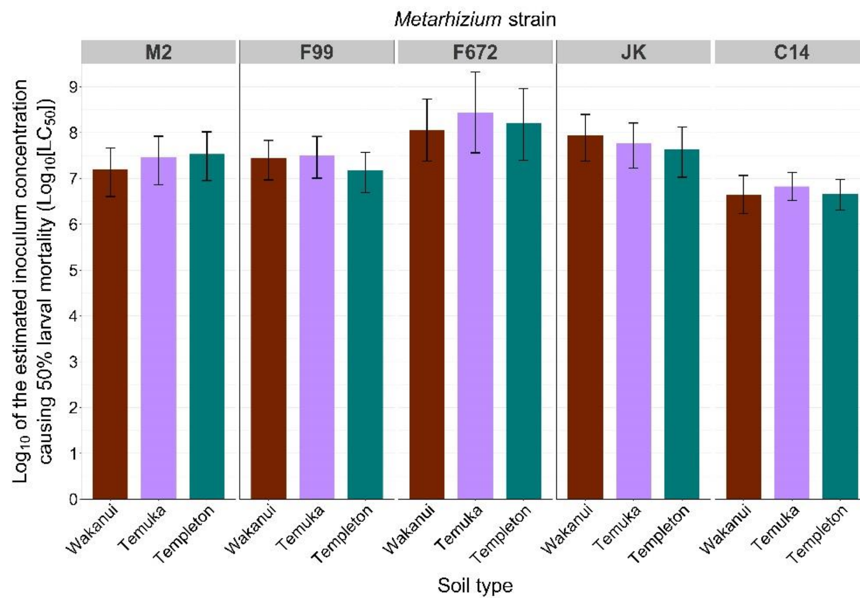


Figure 3.5 The mean Log_{10} of the calculated conidial-inoculum concentration of five *Metarhizium* isolates required to cause 50% mortality ($Log_{10}[LC_{50}]$) of *Costelytra giveni* 2nd instar larvae after 28 days. Each *Metarhizium* isolate was tested in three sterilised soils. Error bars on each mean show the 95% confidence interval derived from logistic regressions.

The number of conidia required to kill 50% (LC_{50}) of 2nd instar larvae of *C. giveni* after 28 days for the five *Metarhizium* isolates in the three soils was calculated (Figure 3.5). *M. novozealandicum* (C14) had a lower LC_{50} value (< 6.5×10^6 conidia/10 g dry soil) than other *Metarhizium* isolates in the experiment. *M. novozealandicum* (F99) and *M. anisopliae* (M2) had lower LC_{50} values (< 3.3×10^7 conidia/10 g dry soil) than *M. guizhouense* (JK) and *M. anisopliae* (F672) which had LC_{50} values of > 4.2×10^7 conidia/10 g dry soil.

3.3.2 The pathogenicity of *Metarhizium* against third instar grass grub larvae in non-sterilised soils

For these experiments, three isolates, *M. novozealandicum* (C14), *M. novozealandicum* (F99) and *M. anisopliae* (M2), were used based on having the lowest LC_{50} results of the first experiment.

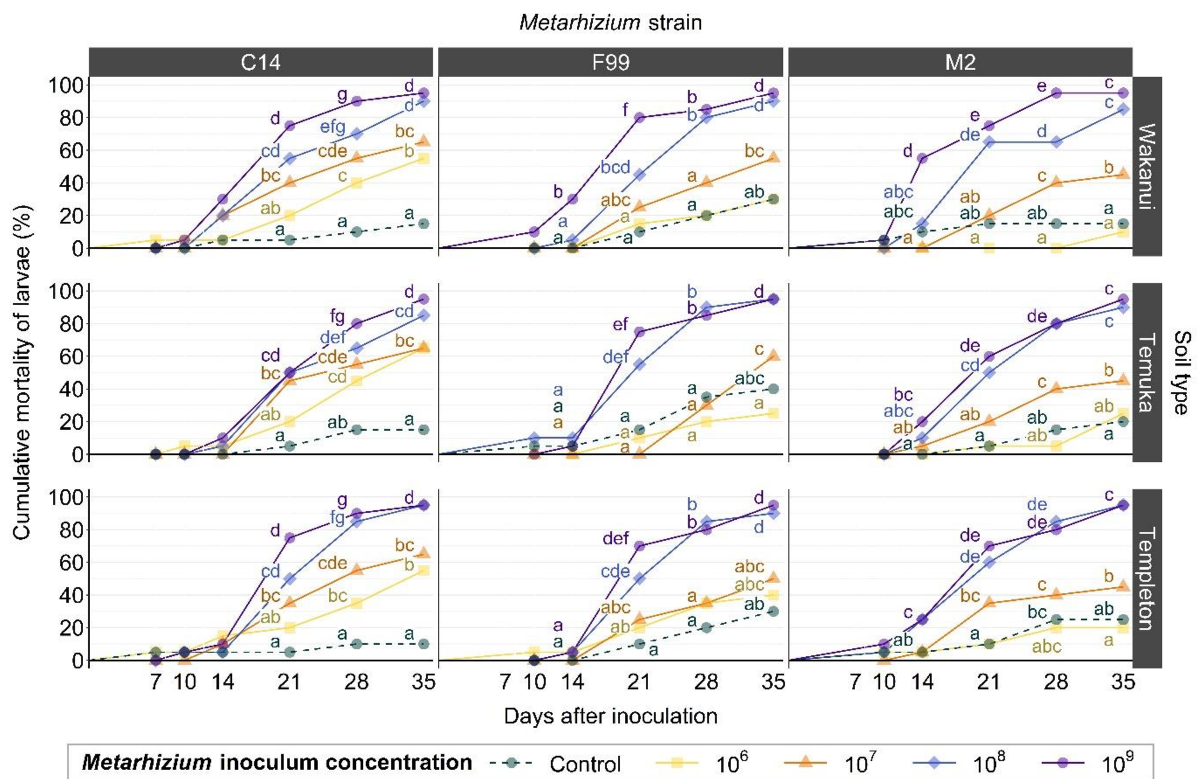


Figure 3.6 Mean mortality over time of 3rd instar *Costelytra giveni* larvae inoculated with four concentrations each of three *Metarhizium* strains (columns). All were tested on three unsterilised soils (rows). Letters indicate significance groupings based on Unprotected LSD test statistic; means with no letters in common are significantly different (P<0.05). Different days after inoculation and different strains were tested separately but different soils were compared within these groups, i.e., all vertically aligned means across all three soils can be compared.

In all three non-sterilised soils, mean cumulative percent mortality of larvae increased across the duration of the experiment beginning at around day 14 (Figure 3.6). For C14, the untreated control was always lower than other treatments over all time points. For F99, concentration of 10^6 and 10^7 did not differ from the control at 21, 28 and 35 days for the three soil types. For M2, these spore concentrations did not differ from the control at 28 and 35 days, except in the Wakanui soil at 10^7 spore concentration. There was a significant difference between the 10^8 and 10^9 conidial concentrations and the untreated controls (P<0.05). No significant differences among the three strains were found at any point during the experiment. Soil type did not affect the pathogenicity of *M. novozealandicum* (C14) and (F99) and *M. anisopliae* (M2) against 3rd instar grass grub larvae.

Among the inoculation concentrations, larval mortality was low when inoculated with the spore solution concentration of 10^6 spores. At this concentration, larval mortality was significantly lower than that of both high spore concentrations (10^8 and 10^9) on the 14th to the 35th day but there was no significant difference between 10^6 and 10^7 spore concentration in each non-sterilised soil. This was similar to the pattern found when inoculating the same three sterilised soil types with *M. novozealandicum* (C14), *M. novozealandicum* (F99), and *M. anisopliae* (M2) at the same concentration. Mortality from *M. novozealandicum* (C14) and (F99), and *M. anisopliae* (M2)

treatments reached nearly 100% at the highest spore concertation, but no significant difference between both high spore concentrations was found. Larval mortality at the 10^7 spore concentration, when inoculated with *M. novozealandicum* (C14), was higher than that of *M. novozealandicum* (F99) and *M. anisopliae* (M2) in the three sterilised soils.

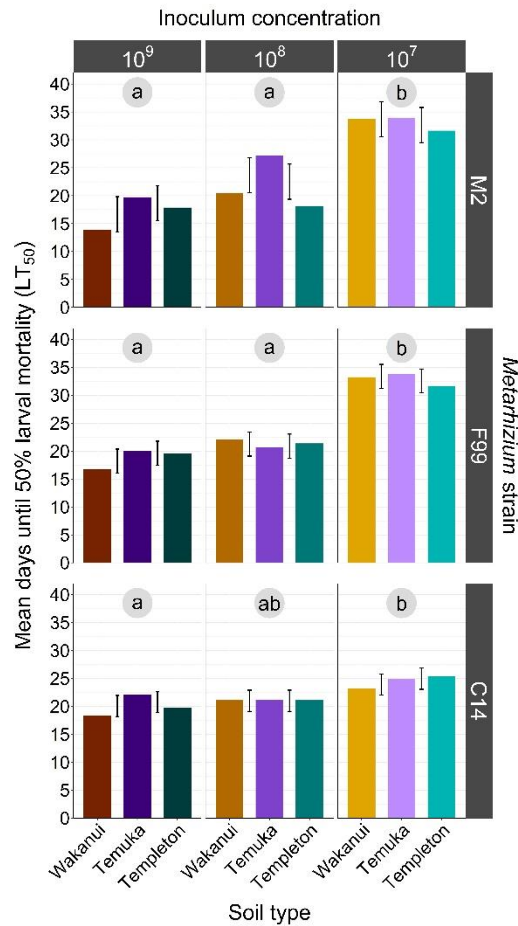


Figure 3.7 The mean calculated length of time, in days, until 50% *Costelytra giveni* larval mortality occurred (LT_{50}) after inoculation with three *Metarhizium* strains in three non-sterilised soils. Several conidial concentrations were tested. Letters indicate significance groupings based on Unprotected LSD test statistic: means with no letters in common are significantly different ($P<0.05$) and are there for information only as soil type was never a significant factor in ANOVA tests. Bars indicated in the figure are LSD.

The time taken for 50% of larvae to die (LT_{50}), calculated over 35 days for each spore concentration in all three non-sterilised soils against 3rd instar grass grub, is shown in Figure 3.7. The LT_{50} values of *M. novozealandicum* (C14), *M. novozealandicum* (F99) and *M. anisopliae* (M2) were significantly lower at both the 10^8 and 10^9 spore concentrations compared to the 10^7 spore concentration, but *M. novozealandicum* (C14) didn't significantly differ between the 10^8 and 10^7 spore concentrations. Also, the LT_{50} value for *M. novozealandicum* (C14) was lower than that of *M. anisopliae* (M2) and *M. novozealandicum* (F99) at the 10^7 spore level, indicating that *M. novozealandicum* (C14) was more virulent than *M. novozealandicum* (F99) and *M. anisopliae* (M2) against 3rd instar grass grub.

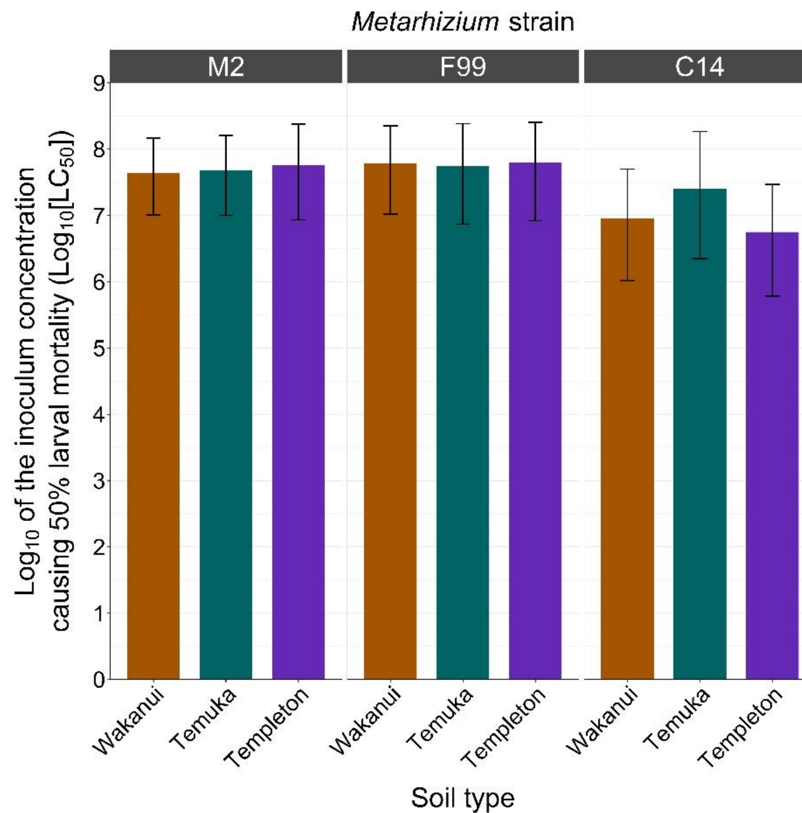


Figure 3.8 The mean Log_{10} of the calculated conidial-inoculum concentration of three *Metarhizium* strains required to cause 50% mortality ($Log_{10}[LC_{50}]$) of *Costelytra giveni* 3rd instar larvae after 28 days. Each *Metarhizium* strain was tested in three non-sterilised soils. Error bars on each mean show the 95% confidence interval derived from logistic regressions.

The number of conidia required to kill 50% (LC_{50}) of 3rd instar larvae of *C. giveni* after 28 days was calculated for the three *Metarhizium* isolates using the mortality from inoculation with 10^6, 10^7, 10^8 and 10^9 conidia per 10 g air-dried soil in the three non-sterilised soil types (Figure 3.8). *M. novozealandicum* (C14) had a lower LC_{50} value (2.5 ×10^7 conidia per 10 g dry soil) than the other two isolates while *M. novozealandicum* (F99) and *M. anisopliae* (M2) showed the same LC_{50} values (around 5.3 × 10^7 conidia per 10 g dry soil) in all three soils.

3.4 Discussion

In this chapter, the relationship between soil type and the pathogenicity of five *Metarhizium* isolates (*M. novozealandicum* (C14), *M. novozealandicum* (F99), *M. anisopliae* (M2), *M. guizhouense* (JK) and *M. anisopliae* (F672)) towards 2nd instar grass grub was evaluated. Three isolates (*M. novozealandicum* (C14), *M. novozealandicum* (F99), *M. anisopliae* (M2)) were selected for further testing against 3rd instar larvae in non-sterilised soil. Of the three isolates tested against third instar larvae, *M. novozealandicum* C14 had the highest pathogenicity at four spore concentrations and lower LT_{50} value (23.1- 25.2 days at 10^7 conidia/10 g air-dried soil) and LC_{50} value (0.9 × 10^7 – 2.5 ×

10^7 conidia/10 g air-dried soil after 28 days). A concentration of 10^8 conidia/10 g dry soil against 2nd and 3rd instar grass grub produced almost 100% mortality. High amounts of inoculum appear to increase the onset of the disease, perhaps by increasing the number of conidia-initiated penetrations of larva and or wearing down larval defences more quickly.

Soil type did not affect the pathogenicity of *Metarhizium* isolates against grass grub. Based on the information in Table 3.1, there was a slight decrease of some factors such as pH, potassium, magnesium and sodium while there was an increase in Olsen phosphorus, calcium, CEC and sulphate in semi-sterilised Wakanui soil comparing to non-sterilised Wakanui soil. This information showed that physical and biological properties of soil did not greatly affect the pathogenicity of *Metarhizium* and soils with more sand and clay soils were not tested. This result aligns with the report of Garrido-Jurado *et al.* (2011) which showed that soil types or $CaCl_2$ concentration did not affect the pathogenicity of *M. anisopliae* against medfly puparia. Randhawa (2017) have reported that *M. robertsii* pathogenicity was not impacted by the presence of phosphorus, potassium, magnesium and calcium in the soil. Moreover, Vanninen (1995) showed that soil types did not affect the occurrence of *M. anisopliae*. The current results and these previous reports suggest *Metarhizium* infections are not greatly impacted by soil characteristics, so that the original hypothesis was not supported.

The larval mortality caused by *Metarhizium* isolates did not differ between sterilised and non-sterilised soils. This result indicated that the natural soil microbial community of these soil types also did not affect the pathogenicity of *Metarhizium* isolates against grass grub. However, Parsa *et al*. (2018) found that there was diminished endophyte colonization (by *B. bassiana* and *M. anisopliae*) in non-sterilise soil compared with sterilised soil. One main reason for this result could be that the microbial community level might be low in these soils, especially after drying. The pathogenicity of *Metarhizium* was not affected by physical properties and soil microbial community of three soil types (Wakanui, Temuka and Templeton) against 2nd and 3rd instar larvae. The effect of specific soil bacteria at higher concentrations is the subject of Chapter 5.

Overall, these results suggests that *M. novozealandicum* (C14) may be a potential candidate for controlling 2nd and 3rd instar grass grub, and this strain will be the focus of subsequent chapters.

Chapter 4

The effect of the presence of the meadow fescue (*Festuca pratensis*) endophyte (*Epichloë uncinatum*) on the pathogenicity of *Metarhizium novozealandicum* C14 to grass grub (*Costelytra giveni*)

4.1 Introduction

Grass endophytes have important roles in protecting their host grasses against biotic (such as pathogenic microbes and insect pests) and abiotic (such as drought and salt) stresses (Malinowski *et al.*, 1997; Leuchtmann, 2000; Vega *et al.,* 2008; Crawford *et al*., 2010; Shoji *et al.*, 2015; Xia *et al*., 2018; Zhang *et al*., 2019; Carvalho *et al.,* 2020; Hume *et al*., 2020; Mantzoukas & Eliopoulos, 2020), and can also contribute to preventing weed incursions (Saikkonen *et al*., 2013). One genus of grass endophytes, *Epichloë* spp., can produce several groups of active alkaloids that have activity against chewing insects (Scott, 2001; Bastias *et al*., 2017). These endophytes have become very important in New Zealand, with almost all ryegrass now sold containing an *Epichloë* endophyte. In New Zealand, there are at least 12 grasses species including native and naturalised species that have been recorded as being infected with *Epichloë,* also called *Neotyphodium* (asexual genus of the sexual *Epichloë*) endophytes (Hume *et al*., 2020) with *Epichloë festucae* var*. lolii* in perennial ryegrass (*Lolium perenne*), *E. coenophiala* in tall fescue (*Schedonorus phoenix* syn. *Festuca arundinacea*), and *E. uncinatum* in meadow fescue (*S. pratensis* syn. *F. pratensis*) (Hume *et al*., 2020).

There are three alkaloid groups (lolines, ergopeptides and peramine) produced by *Epichloë* endophytes which protect grasses against insect pests (Leuchtmann *et al.,* 2000; Blankenship *et al.,* 2001). Loline distribution in endophyte-infected *Festuca pratensis* (meadow fescue) varied during the season (high loline levels in the leaves and roots during summer and in the crowns during early autumn) (Den-wen *et al*., 2006), but loline concentrations were similar in the roots and shoots of meadow fescue in autumn (Patchett *et al.*, 2008). Loline alkaloids in *F. pratensis* infected with the endophyte *E. uncinatum* provide control of several pasture insects such as adult argentine stem weevil (Jensen *et al*., 2009), black beetle and red-headed pasture cockchafer larvae (Bryant *et al*., 2010) and root aphids (Schmidt & Guy, 1997). In addition, some plant pathogens were inhibited by *E. uncinatum* endophyte in *F. pratensis,* as shown by the low mycelium growth *in vitro* of *Gaeumannomyces graminis, Fusarium equiseti, F. culmorum* and *F. graminearum* (Panka, 2006).

M. anisopliae is known to be capable of endophytic colonisation in maize (Akello, 2012) and soybean (Khan *et al*., 2012), and has also been reported to establish as an endophyte after foliar application

and artificial inoculation through seed and soil treatment in crops such as oilseed rape (Batta, 2013), tomato (Elena *et al*., 2011; Garcia *et al*., 2011; Dutta *et al*., 2015), tea (Kaushik & Datta, 2016), cabbage (Razinger *et al*., 2014), and sorghum (Mantzoukas *et al*., 2015). Moreover, coating maize seed with *M. anisopliae* improved plant growth and reduced infection by *F. graminearum* and root feeding by *C. giveni* larvae (Rivas-Franco *et al.*, 2019).

As both fungi could be present in and around plants at the same time, the aim of the research reported in this chapter was to characterise the interaction of the endophyte *E. uncinatum* of *F. pratensis* and *M. novozealandicum* (C14) at a low spore concentration (10^5 spores/g) against the New Zealand grass grub (*C. giveni*). In a second experiment, the presence of *Metarhizium* in the plant tissues after soil inoculation and in the absence of grass grub was investigated.

4.2 Materials and methods

4.2.1 Meadow fescue grass preparation

Seeds of *F. pratensis* (with endophyte E+ and without E-) were obtained from Cropmark Seeds Ltd. Eight seeds were placed in a small tube (50 ml) containing 20 g of air-dried Wakanui soil and 1 ml of tap water. The tubes were then placed in an incubator at 22^0C, using a 16 light /8h dark photoperiod. The three smallest plants (out of eight plants) were removed from each tube after one week to leave reassembly five uniform plants in each tube at the end. Meadow fescue plants were grown for about 20 days before use in bioassays. In total, there were 40 tubes including 20 tubes for *F. pratensis* with endophyte (E+) and 20 tubes for *F. pratensis* without endophyte (E-).

4.2.2 Pre-screening of *Costelytra giveni* larvae

This experiment used field collected 3rd instar larvae that were pre-screened for feeding activity as described in Chapter 2.

4.2.3 Preparation of inoculum

M. novozealandicum (C14) was grown on PDA plates at room temperature (20°C). After 15 days, fungal conidia were harvested directly from the plates as described in Chapter 2. Conidial density was estimated as described in Chapter 2 and the original solutions adjusted to a concentration of 10^5 conidia/ml.

4.2.4 Experimental design

The experiments were conducted in an incubator and laid out in a completely randomised design (CRD) with four treatments and ten replicates. Each inoculated tube (M+) received one ml of *M. novozealandicum* (C14) with 10^5 conidia/g of air-dried soil (20 g soil/tube). Non-inoculated controls

(M-) received 1 ml of 0.01% TX-100. A single third instar larva was added to each tube and the larval movement inside the soil was observed prior to incubation at 22°C in environmental cabinets using a 16/8-h light/dark photoperiod. This experiment was set up on 2nd July 2019 and ran to 29th July 2019, a total of 28 days. *Costelytra giveni* mortality and infection (as determined by outgrowth from the cadaver) was recorded on the 14th and 28th day, and plant weight was measured at the end of the experiment (Figure 4.1).

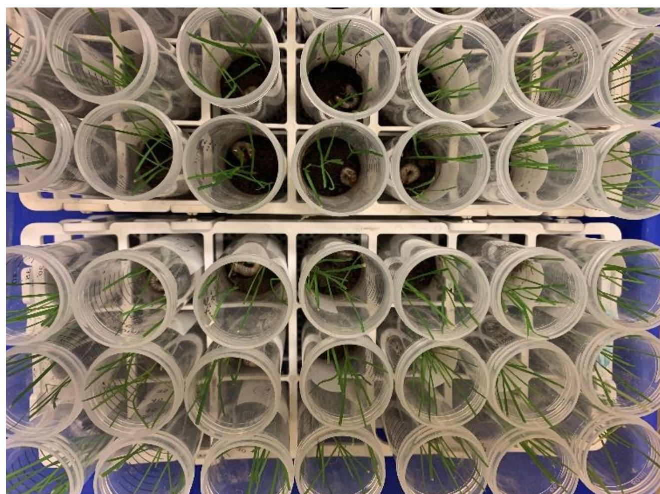


Figure 4.1 The layout of the experiment in the incubator at 22°C

4.2.5 Testing for the presence of *Metarhizium novozealandicum* (C14) inside *Festuca pratensis* tissues

One plant selected at random from the five plants in each tube was completely washed with running tap water and air-dried before sterilisation in class 1 laminar flow chamber. Each plant was divided into roots, stem and leaves and each plant part was processed separately using the following protocol: 0.01% TX-100 for 3 min, 2% bleach solution for 5 mins, 70% ETOH for 1 min and then washed in sterilised H_2O for 1 min (repeated 3 times). Solutions were changed after every 3-4 samples. Each part of the plant was completely submerged and gently shaken in solution. Utensils were sterilised between every sample and the bench area was wiped with 70% ETOH. Each plant part (root, stem or leaves) was cut into pieces small enough to be plated on MSM selective medium (Chapter 2). There were ten replicate plants per treatment. Plates were incubated at 25ºC for 3-4 days or until visible fungal growth.

4.2.6 Dry weight of *Festuca pratensis*

The four remaining plants were removed from the tube, and washed with tap water to remove soil. Plants were then placed in a paper bag. These were oven-dried at 65ºC for three days before dry weight was recorded.

4.2.7 Data analyses

Dry weight data as well as the mortality of insects and the number of cadavers supporting sporulation were analysed using ANOVA and Probit in Genstat-19th edition. Fisher's exact test, which is the standard method for comparing two treatments, was used for larval mortality.

4.2.8 Presence of *Metarhizium novozealandicum* (C14) in *Festuca pratensis* tissue in the absence of grass grub

The same procedures, conditions and experimental design as was used in the insect bioassay (see section 4.2.1 to 4.2.5) were used in this experiment except that no larvae were added to tubes and a higher concentration of inocula, 10^7 conidia/g of non-sterilised soil (20 g soil/tube), five plants per one tube, were used.

4.2.9 Molecular identification of *Metarhizium* isolates

Fungi growing on the MSM selective medium and tentatively identified as *Metarhizium* based on fungal morphology were identified using polymerase chain reaction (PCR) followed by Sanger sequencing. DNA was extracted using the method of Alizadeh *et al.* (2017). Elongation factor 1-alpha (EF1α) (Bischoff *et al*., 2009; Kepler *et al*., 2014; Stielow *et al*., 2015; Meyer *et al*., 2019) gene region was targeted as specific primers (Chapter 2), for fungal identification. Identification was confirmed by comparing the sequences to the nearest matches in the GenBank database and previous sequences obtained in this study (Alizadeh *et al*., 2017).

PCR was run as described in Chapter 2 with the primer pair used to target the EF1α gene region being 983F (GCY CCY GGH CAY CGT GAY TTY AT) and 2218R (ATG ACA CCR ACR GCR ACR GTY TG (Rehner & Buckley 2005).

All amplified products were sequenced at the Lincoln University Sequencing Unit (Lincoln, NZ) and the sequences generated were edited and assembled using ChromasPro before being compared to the nucleotide GenBank database (Alizadeh *et al*., 2017).

4.2.10 Determination of the presence of fungi by fluorescent microscopy

After 30 days, whole meadow fescue plants collected from the experiment in section 4.2.2.1 were stained with fluorescent dyes. Each plant was divided into roots, stems and leaves and stained separately prior to microscopic observation. Samples were analysed for the presence of fungal structures on the surface of the grass tissues, or internally as endophytes, using fluorescent microscopy.

4.2.10.1 Meadow fescue sample preparation

Roots, stems and leaves of meadow fescue plants were first dehydrated by soaking samples individually in EtOH (96%) in 50 ml Falcon tubes and incubating overnight at 4°C. Subsequently, EtOH was carefully discarded and replaced with a solution of KOH (10%) and the samples incubated at room temperature overnight. After incubation, KOH was discarded, and samples were washed once in 1x phosphate-buffered saline (PBS; pH7.4, 0.137M NaCl; 0.0027M KCl; 0.01M Na2HPO4 and 0.0018M KH2PO4).

4.2.10.2 Staining of fungi in grass tissues

For roots, shoots and leaves, wheat germ agglutinin conjugated to Alexa Fluor (WGA-AF488; Molecular Probes, Eugene, OR, USA) was used to stain chitin present in the fungal hyphae (Ramonell *et al*. 2005). ConA is generally used to visualize glycoproteins since it binds to sugar residues like α-mannopyranosyl and α-glucopyranosyl found in glycoproteins and glycolipids (Zuccaro *et al*., 2011). Grass cells were visualized using Pontamine (Direct Red 80) as a counterstain (Thomas *et al.* 2018). Samples were incubated at room temperature for 30 min in 0.1% TX-100 in 1 x PBS (pH 7.4) staining solution containing 10 µg/ml WGA-AF488 and 0.025% Pontamine and during this time vacuum-infiltration was done three times at 2 min intervals. Finally, grass samples were washed in the PBS buffer for 3 h and in fresh buffer overnight. Samples were stored in the dark at 4°C until analysis.

4.2.10.3 Fluorescent microscope image acquisition

Small root segments were mounted on glass slides for microscopic observation. Roots were chosen because *M. novozealandicum* (C14) moves first to the roots of the plant after being inoculated into the soil. The visualization of the different fluorophores/chrome and dyes in hyphae and grass cells was achieved using an excitation of 495 nm for WGA-AF488 and 561 nm for Pontamine. Fluorescence images were recorded on a multichannel Olympus BX51 fluorescent microscope (Olympus, Germany) using the program Cell (Olympus).

4.3 Results

4.3.1 Effect of the presence of *Epichloë* inside *Festuca pratensis* on pathogenicity of *Metarhizium novozealandicum* C14 against grass grub larvae

4.3.1.1 Mortality of larvae

The number of dead and live larvae in each treatment after 28 days is presented in Table 4.1. Larval mortality in three treatments, E+M+, E+M- and E-M+, was 30% after 28 days while meadow fescue with neither endophyte nor Metarhizium (E-M-) had only 10% larval mortality. However, there was no significant difference among any of the treatments ($P>0.05$).

Table 4.1 Effect of the presence of *Epichloë uncinatum* endophytes in meadow fescue grass (*Festuca pratensis*) on percentage mortality of *Costelytra giveni* larvae after 28 days: with endophyte (E+), without endophyte (E-), with *Metarhizium novozealandicum* (C14) (M+) and without C14 (M-).

Treatment	Larval mortality (%)
E-M-	10
E-M+	30
E+M-	30
E+M+	30

4.3.1.2 Determination of *Metarhizium novozealandicum* (C14) presence inside *Festuca pratensis* tissues

In the first experiment, *M. novozealandicum* (C14), inoculated at 10^5 conidia/ml, was not detected in grass samples collected from any treatment. No surface sterilised grass supported *Metarhizium* outgrowth, but other fungi were present.

4.3.1.3 Plant dry weight

Mean dry weight in each treatment was recorded after 28 days (Table 4.2). It was lowest (0.086 g) in the E-M- treatment, but this was not significantly different from the E-M+ treatment. The two *E. uncinatum* treatments did not differ significantly but their dry weight was significantly greater than the E-M- treatment. The E-M+ treatment was not significantly different from any other treatment.

Table 4.2 Mean dry weight of meadow fescue grass in treatments with (E+) and without endophyte (E-) inoculated with *Metarhizium novozealandicum* (C14) and uninoculated control after 28 days

Treatment	Mean dry weight of plant (g)
E-M-	0.086a
E-M+	0.127ab
E+M-	0.139b
E+M+	0.146b
LSD (5%)	0.049

4.3.2 Presence of *Metarhizium novozealandicum* (C14) inside grasses

4.3.2.1 Detection of C14 after surface sterilisation

In the second experiment, *M. novozealandicum* (C14) was observed in grass roots, stems and leaves when inoculated at a concentration of 10^7 conidia/g soil. Control plates of meadow fescue without *M. novozealandicum* (C14) (with endophyte E+ and without endophyte E-) did not show the presence of *M. novozealandicum* (C14), but other fungi were observed (Figure 4.2). This result indicates that the presence of *M. novozealandicum* (C14) may inhibit the growth of other fungi.

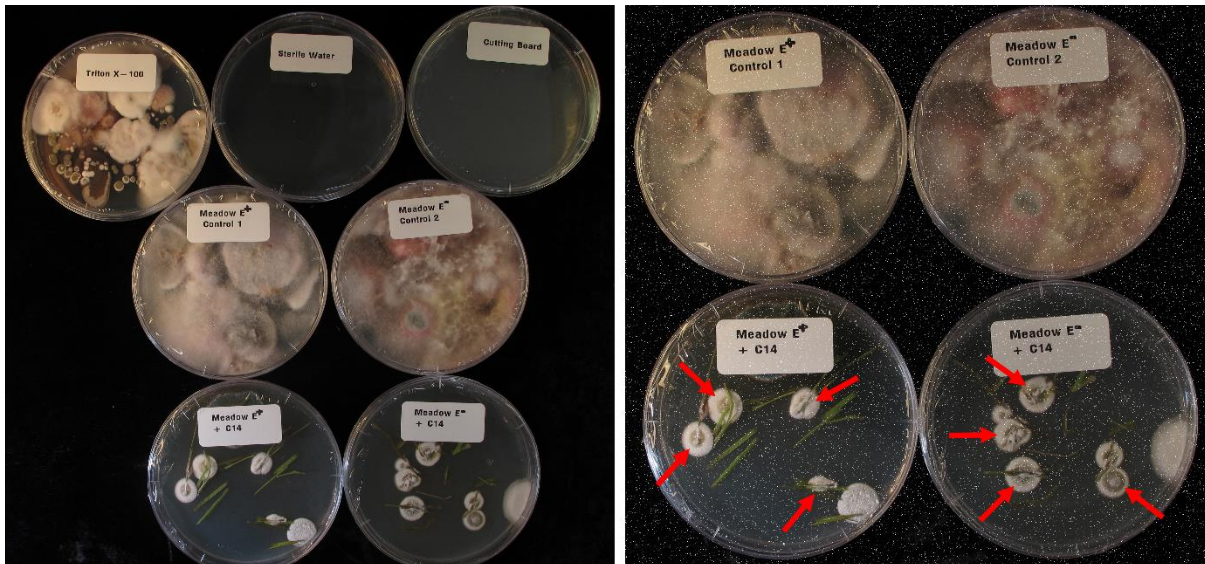


Figure 4.2 Examples of MSM semi-selective medium used to test for the presence of *Metarhizium* inside roots, stems and leaves of meadow fescue grasses with and without endophyte. Red arrows indicate growing *Metarhizium* colonies.

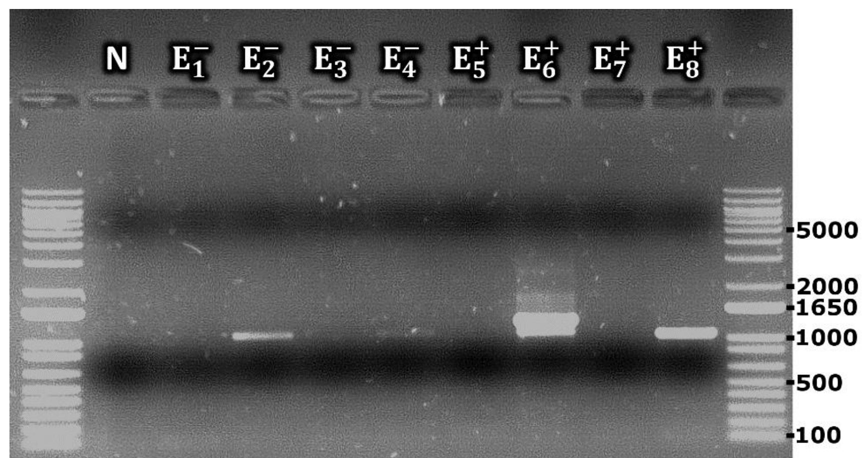


Figure 4.3 PCR amplification with elongation factor 1α of genomic DNA extracted from fungal isolates in surface sterilised meadow fescue grass as shown in Figure 2: Control (N); Meadow fescue grass treated with C14 without endophyte (E^{-}_{1} - E^{-}_{4}); Meadow fescue grass treated with C14 with endophyte (E^{+}_{5} - E^{+}_{8}).

All *Metarhizium* isolates were selected from colonies growing from surface sterilised grasses (Figure 4.2). Molecular identification of the selected isolates was done by elongation factor partial sequence and aligned with the partial sequence of C14 (Chapter 2). Complete sequence alignment was noted between the selected isolates and C14, confirming the identity of the isolates to be C14.

4.3.2.2 Fluorescent microscopy

Fungal hyphae growing inside the roots were observed in all treatments. It was not possible to determine if the fungus was *Metarhizium* using this method, and it was clear from plate culturing that other fungi were present inside meadow fescue. The hyphae were observed anywhere inside the roots, indicating the ability of the fungi to colonize the roots where important nutrient reserves for the development of meadow fescue grasses are stored (Figure 4.4).

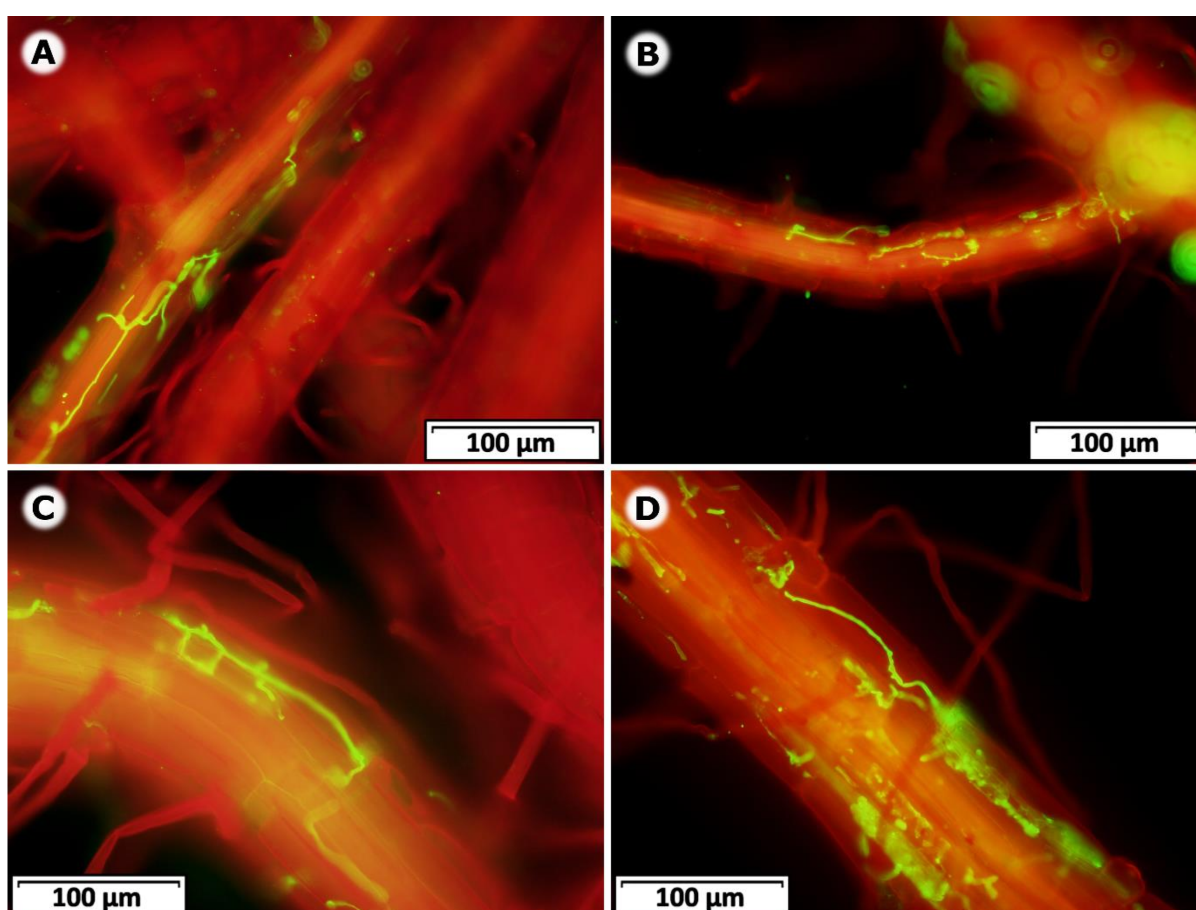


Figure 4.4 Fungal hyphae (green) observed by fluorescent microscopy inside the root of *Festuca pratensis* grown either from *Epichloë uncinatum* infected seeds (E+) or from non-infected seeds (E-) and either inoculated with *Metarhizium* (M+) or not inoculated (M-): A) E-M-, B) E+M-, C) E-M+, D) E+M+.

4.4 Discussion

The aim of this study was to determine the effect of the presence of *Epichloë* endophyte in meadow fescue on *M. novozealandicum* (C14) pathogenicity at a low spore concentration (10^5 spores/g soil) on grass grub, with the number of dead and live larvae and mean dry weight of the grass as

parameters. Likewise, the endophytic ability of *M. novozealandicum* (C14) inside meadow fescue grasses at both high and low spore concentrations was determined using a surface sterilization and plating method, and non-specific fluorescent microscopy. No difference in larval mortality was found between any of the treatments with *M. novozealandicum* (C14) inoculated at low conidial concentrations and without C14 in either endophyte (E+) or without endophyte (E-) meadow fescue. The result indicates that the presence of *M. novozealandicum* (C14) at 10^5 conidia/g soil was relatively ineffective. The presence of *M. novozealandicum* (C14) inside the meadow fescue grasses at a low conidial concentration was not detected after surface sterilisation, but was at the higher inoculum level of 10^7 conidia/g soil, suggesting inoculum concentration influences the establishment of *Metarhizium* as an endophyte.

Mean dry weight of meadow fescue showed that there was significantly higher growth with *Epichloë* (E+) and *Metarhizium* than without endophyte (E-) + no *Metarhizium.* However, there were no significant differences among meadow fescue without endophyte (E-) + *Metarhizium*, meadow fescue with endophyte (E+) + *Metarhizium* and meadow fescue with endophyte (E+) without *Metarhizium* after 28 days. This suggests that *E. uncinatum* in meadow fescue could produce a toxin or poison which reduced the feeding activity of grass grubs on the root, increased *F. pratensis* growth, or both. Patchett *et al*. (2008) have shown that grass grub attack of roots led to the production of a high loline concentration inside the root, decreasing the feeding activity of larvae. In addition, Popay *et al.* (2003) recorded that root consumption of grass grub was significantly lower in meadow fescue with endophyte (E+) than meadow fescue without endophyte (E-). In this current study, it was shown that there was no evidence of a synergistic effect between *Epichloë* endophyte and *Metarhizium*. Further study is required to determine effects on grass grub larval mortality and meadow fescue root dry weight when there was a combination between C14 at a high dose and endophyte.

M. novozealandicum C14 was detected after surface sterilisation of plants 28 days after inoculation at a high spore concentration (10^7 conidia/g soil). The presence of other fast-growing fungi was observed in uninoculated controls with and without endophyte in both experiments but not in C14-inoculated treatments, indicating that the presence of *Metarhizium* could inhibit the growth of other fungi because these fungi likely came from non-sterilised soil. There is a need to isolate fungi from non-sterilised soil and check for any antagonism between these fungi and *Metarhizium*. Previous studies found *M. robertsii* and *M. brunneum* inhibited the growth of *Fusarium solani in vitro* (Sasan & Bidochka, 2013; Jaber & Alananbeh, 2018). Using PCR-targeted sequencing for identification of some *Metarhizium* isolates from the surface sterilisation method showed that the isolates were confirmed to be *M. novozealandicum* (C14) as described in Chapter 2. Behie *et al*. (2015) isolated *M. robertsii* from inside the root of haricot bean plants but not the stem or leaves. On the other hand, *M.*

brunneum has been isolated from the stem of the soybean plant (Clifton *et al.,* 2018). Ahmad *et al.* (2020) reported that *M. robertsii* was an endophyte of maize roots and leaves. It can also establish as an endophyte in the roots of switchgrass and haricot beans (Sasan & Bidochka, 2012). In addition *M. robertsii* has been established as beneficial endophyte of root and leaf after inoculating maize seeds (Ahmad *et al*., 2020) while *M. anisopliae* was endophytically established inside roots of maize plants by coating seed (Cai *et al*., 2019; Rivas-Franco *et al*., 2019; Ramos *et al.,* 2020) and shown to be an endophyte in stem and leaf of maize and sorghum (Ramanujam & Poornesha, 2018).

The results of fluorescent microscopy to determine the presence of *M. novozealandicum* (C14) inside the roots of meadow fescue grasses were not conclusive because the non-sterilised soil used might have been contaminated with other fungi. *M. novozealandicum* (C14) at the low spore concentration did not have any interaction with endophyte (E+) of meadow fescue against third instar grass grubs. *M. novozealandicum* (C14) was recovered after surface sterilisation in treatments with and without endophyte (E+ and E-) when inoculated with high spore concentration, indicating its endophytic ability. Further experiments are required to determine the endophytic ability of *M. novozealandicum* (C14) in meadow fescue by using green fluorescent protein tags (Stretton *et al*., 1998). This method can be transferred to *Metarhizium* to tag them to confirm presence endophytic in grass.

Chapter 5

The effect of selected soil bacteria on the virulence of *Metarhizium novozealandicum* (C14) to grass grub larvae

5.1 Introduction

Soil microorganisms are involved in complex interactions which can affect soil structure, the breakdown of organic compounds and the cycling of nutrients important for plant growth. Some studies have predicted that one gram of soil may have up to 10^{10} bacterial and 10^{6} fungal cells, and consist of thousands of bacterial and fungal species (Torsvik *et al*., 1990; Bridge & Spooner, 2001; Roesch *et al*., 2007; Trevors, 2009). Soil contains a diversity of entomopathogens from across the groups of bacteria, viruses, fungi, protozoa and nematodes (Mayerhofer, 2017), some of which can act as biological control agents of insects, plant pathogens and weeds (Kennedy, 1999). Such diverse microbial communities can be isolated from different soils and insect samples (Fuxa & Kunimi, 1997).

In addition to abiotic factors influencing *Metarhizium* infections, biotic factors can have an effect. Some bacteria isolated from New Zealand soils have been shown to infect grass grub larvae. These bacteria could be promising as synergists with the fungus against grass grub in pasture. Amber disease, a disease of grass grub larvae, is caused by two species of bacteria, *Serratia entomophila* and *S. proteamaculans*, originally isolated from diseased grass grub (Glare *et al*., 1996; Jackson *at al*., 2001; Jackson *et al*., 2004). The disease is associated with a 153-kb mega plasmid determined by pADAP (Glare *et al*., 1993; Grkovic *et al*., 1995; Hurst *et al*., 2002, 2003, 2011). Seed coating with *S. entomophila* resulted in protection of seedlings against grass grub larvae (Wright *et al.,* 2005; Young *et al*, 2010). In addition, *Yersinia entomophaga* was isolated from a diseased grass grub larva and is showing promise as an insect biocontrol agent (Hurst *et al*., 2011, 2014). Milky disease of grass grub is caused by *Paenibacillus popillae* (Radcliffe, 1971; Warren & Potter, 1983; East & Wigley, 1985), and a strain of *Bacillus thuringiensis* has been used against grass grub in a previous study (Chilcott & Wigley, 1990).

Interactions between microorganisms have been reported to affect biocontrol. There can be interactions between nematodes and fungi affecting their virulence towards insect hosts (Davies, 2005). For example, there was an interaction between *M. anisopliae* and the nematodes *Heterorhabditis megidis* and *Steinernema glaseri* which produced synergistic control of the beetle *Hoplia philanthus* (Ansari *et al*., 2004). Similarly, the pathogen combination of *Metarhizium brunneum* and *Paranosema locustae* (Microsporidia) to control the migratory grasshopper produced synergism under laboratory and greenhouse conditions (Dakhel *et al*., 2019). There was a synergistic

response between *M. anisopliae* and *Heterorhabditis bacteriophora* when applied to control the weevil *Rhynchophorus ferrugineus*, as there was higher virulence when the fungus and nematode were paired than when alone. This was evidenced by a decrease in the number of pupae, adults and hatching eggs in the treatments where entomopathogen combinations were applied (Wakil *et al.,* 2017). Moreover, Glare (1994) reported that there was a synergistic interaction between *M. guizhouense* and *S. entomophila* against second instar grass grub larvae, but not third instar larvae.

This study extended the approach to examine the effect of bacteria isolated from soil and dead grass grub larvae that are not known to be entomopathogens or induce *Metarhizium* mortality. The hypothesis was that one or more of these bacteria, when combined with *M. novozealandicum* (C14), would increase the mortality of grass grub larvae over that obtained from the application of *M. novozealandicum* (C14) alone. Also, whether components of the bacteria microflora of soil (including *Serratia* spp. and *Yersinia* spp.) influence the virulence of *M. novozealandicum* (C14) by acting as antagonists or synergists through producing chitinase and volatile compounds was investigated. Potential enzymes such as chitinase, which can affect an insect's cuticle, could be produced by the bacteria and result in increased virulence of the *M. novozealandicum* (C14). Chitinase production was assessed for each bacterium used in these experiments to determine correlations with virulence.

5.2 Material and methods

5.2.1 Culturing of bacteria

Bacteria were cultured in Luria-Bertani Miller broth (LB) with 1.5% agar (Appendix D 5.1.1) and grown at 30ºC for one day.

5.2.2 Isolation of bacteria from dead *Costelytra giveni* larvae

Field collected larvae were stored at 4°C in the original field soil. Many larvae, mainly third instar, died during the storage. Each dead larva was transferred into a 2 ml tube using sterile forceps, and 1 ml of sterile PBS (Appendix D 5.1.2) was added to each tube. Dead larvae were then ground using a sterile 1 ml pipette tip. Serial dilutions up to 10^4 were made from the homogenised larvae in the PBS. For the 10:1 solution, 100 µl of the homogenised larvae solution was added to 900 µl PBS and mixed thoroughly. The same steps were followed until dilutions of 10^4 were reached. One hundred µl of diluted solution was plated onto LB agar and spread with a hockey stick. Inoculated plates were sealed and incubated at 30°C with a 16 h photoperiod. Two plates per dilution were used. The plates were checked for any bacterial growth at three days after inoculation. Bacterial colonies were initially characterised based on colony morphology such as colour, shape and size.

5.2.3 Isolation of bacteria from soil

Four lots of 10 g soil samples were taken from each of three collected soil types: Templeton (silt loam), Temuka (clay loam), and Wakanui (silt loam) as described in Chapter 3. Nine ml of sterile peptone water (0.1%, Appendix D 5.1.3) was added to each 1 g soil sample. The peptone-soil solutions were mixed using a Stuart (Staffordshire, UK) flask shaker at 300-400 rpm for 30 min. This constituted a 10:1 peptone water-soil solution. From this, serial dilutions of 10^2, 10^3 and 10^4 were prepared in sterile PBS. The 10^2, 10^3 and 10^4 dilution solutions were plated in LB agar as described in the above section with two plates per dilution. One hundred µl of each dilution was spread over the surface of a fresh LB agar plate using disposable sterile hockey sticks. The inoculated plates were placed in an incubator at 30°C with a 16 h photoperiod. Bacterial growth was observed three days after the inoculation. Morphology of each colony was recorded as described in the above section.

5.2.4 Molecular identification of bacterial isolates

All putative bacteria isolates were identified by amplifying (using polymerase chain reaction (PCR)) part of the 16s RNA gene for sequencing. Identification was confirmed by comparing the sequences to the nearest matches in the GenBank database (Alizadeh *et al*., 2017). DNA was extracted using the method of Alizadeh *et al.* (2017). All bacterial strains were grown overnight in sterile LB broth at 30°C and shaken at 250 rpm.

Prior to PCR, DNA concentration (ng/µl) was determined for each sample using spectrophotometry (Nanodrop 3.0.0 spectrophotometer; Nanodrop Technologies Inc., Delaware, USA). PCR reaction mixes were prepared using 5 µl of 5× MyTaq Reaction Buffer (5 mM dNTPs, 15 mM MgCl2, stabilizers and enhancers; Bioline), 1 µl of each primer (10 µM), 0.25 µl of MyTaq HS DNA Polymerase (Bioline), 1 µl of genomic DNA (100-300 ng/µl) and 12.75 µl of sterile PCR water to make up a final volume of 25 µl. The same reaction mixture with sterile PCR water was used as the negative control. Primers used were f8–27 and r1510 (Table 5.1). Amplification was performed in a Kyratec thermal cycler starting with an initial denaturation at 95°C for 5 minutes, followed by 40 cycles at 95°C for 45 sec, 60°C for 45 sec and 2 min at 72°C and a final extension of 7 minutes at 72°C. The quality and size of the PCR products were assessed by agarose gel electrophoresis, using a 1% gel in 1× TAE (40 mM Tris-OH, 20 mM Acetic Acid, pH 7.8, 1 mM ethylenediaminetetraacetic acid [EDTA]). Five µl of each PCR product along with loading dye were loaded in each lane of an agarose gel with RedSafeTM. One kb plus DNA ladder (Hyperladder II, Bioline, USA) was used to estimate the size of PCR products. Electrophoresis was performed at 100 V for 45 minutes and then visualised following exposure to UV light using the UVIDOC HD6 Touch (UVITEC Cambridge).

All amplified products were sequenced at the Lincoln University Sequencing Unit (Lincoln, NZ) and the sequences generated were edited and assembled using ChromasPro before being compared to the nucleotide database (Alizadeh *et al.*, 2017).

Table 5.1 Primers used

Target gene region	Primer	Sequence	Reference
16S rRNA	f8–27	AGAGTTTGATCCTGGCTCAG	Lipson & Schmidt, 2004
	r1510	GGTTACCTTGTTACGACTT	Lipson & Schmidt, 2004
Putative Afp18 toxin	Afp18F	GAACGTGCAGAATCTCAGCCTGAG	Hurst *et al.,* 2018
	Afp18R	AAGCGTAGCGTTCATCGAAGCCAG	Hurst *et al.,* 2018
S. entomophila pADAP derived SepC TC-A component	SepCF	CAAGAAGTTCAGCATGCCGAGGAG	Hurst *et al.,* 2018
	SepCR	TCAATGAGCGTAAGGGAAGCTGGC	Hurst *et al.,* 2018

5.2.5 Visualisation of the megaplasmid pADAP in *Serratia* spp. and *Yersinia* spp.

A megaplasmid pADAP is known to encode amber disease and can serve as an indicator of disease-causing ability in *Serratia* towards grass grub larvae. Variations of the plasmid have also been found in other species, including *Yersinia* sp. (Sitter, 2020). To test for the presence of pADAP in both the *Serratia* spp. and *Yersinia* spp. isolated from *C. giveni* larvae, visualization of pADAP plasmids using gel electrophoresis was performed following the protocol of Kado & Liu (1981). Bacterial colonies from each isolate were grown overnight in 7 ml of sterile LB broth using a shaker at 250 rpm and 30°C. Two hundred to five hundred µl of each overnight culture was pelleted and resuspended in 100 µl E buffer (40 mM Tris-acetate, 2mM Na2EDTA, pH 7.9). Two hundred to three hundred µl of lysis solution including 50 mM Tris, 3% SDS, pH 12.6 was added and the mixture was incubated for 30-90 min at 55°C. Following the incubation, most of the protein was removed by mixing with 300 µl of phenol/chloroform and the aqueous phase was directly used for electrophoresis following the methods of Sambrook et al. (1989). The presence of plasmid DNA was then assessed by agarose gel electrophoresis using 70 µl of each aqueous phase with six µl of loading dye in each lane of a 0.8% agarose gel in 0.5-1x TBE buffer. The DNA was run on a gel at 120 V for 60 minutes until the dye reached the bottom end of the lane. Gel photos were taken using the UVIDOC HD6 Touch (UVITEC Cambridge). Lambda DNA/Hind III Marker (Thermo Fisher Scientific Inc.) was used to estimate the size of plasmid DNA.

5.2.6 Detection of the pADAP megaplasmid in the bacterial isolates by amplification of the *sep* and *afp* disease encoding clusters.

The amber disease encoding plasmid pADAP can have both a *sep* and *afp* disease encoding cluster (Hurst *et al*., 2011). Therefore, primer pairs specific to these regions were used as a second means to

confirm the presence of pADAP. On pADAP, there are two potential virulence encoding clusters, Sep (*Serratia entomophila* pathogenicity) and Afp (antifeeding prophage). The SepCF and SepCR primer pair (Table 5.1) was used to test the presence of the *Serratia* pADAP derived SepC TC-A component gene region. The Afp18F and Afp18R primer pair (Table 5.1) was used to test the presence of the putative Afp18 toxin encoding gene region. All 18 bacteria strains isolated in this study were tested using this method even though only *Serratia* species are known to cause amber disease. *Yersinia entomophaga* is toxic to grass grub using a different mechanism. For DNA extraction, the same protocol as described above, using the method of Alizadeh *et al*. (2017) for DNA extraction, and PCRs as described in section 5.1.4 except that different primer pairs were used.

5.2.7 Bioassay against grass grub larvae using bacteria and *Metarhizium novozealandicum* alone and in combination

The strain of *M. novozealandicum* used in this study, C14, was originally isolated from soil in a Kiwifruit orchard in Nelson, New Zealand. Seven bacterial isolates coded 2-2, 3-1, 3-2, 3-3, 5-1, 6-1 and S3 were isolated from either soil or dead *C. giveni* larvae as described in sections 5.1.2 and 5.1.3. Two additional disease causing bacteria strains identified as A1MO2 (*S. entomophila*) and 145WT (*S. proteamaculans*) obtained from AgResearch (Lincoln) were used in bioassays and the the results are presented in appendix 5.4. For this experiment, all bacteria were grown in 7 ml of LB media overnight. Cell counts were done using 10^2 dilutions. Twenty µl of the diluted culture was pipetted into a hemocytometer and viewed under 40x using a Leica Microscope. Cell count per ml was computed as: Cell per ml: [(Average x 16)/0.00002] x 100 (dilution). The cell concentrations of bacteria isolates are presented in Table 5.2. Fungal spores were collected directly from PDA plates into sterile 0.01% TX-100 solution. The spore solution were quantified with an improved Neubauer chamber, adjusted to 10^4, 10^6 and 10^8 spores/10 g dry soil, and used immediately.

Field collected second and third instar larvae were pretested for feeding activity by placing them individually in compartments of a 12-well plate with carrot squares for food and overnight at room temperature (20°C). Only actively feeding larvae were selected for use in bioassays. Wakanui soil semi-sterilised at 90°C for three days. Ten g of soil was placed in a universal vial with a screw lid. Each single pathogen treatment received 1 ml of a pathogen solution added directly to the soil and 1 ml of 0.01% TX-100. Treatments with both pathogens received 1 ml of each pathogen suspension. Untreated controls received 2 ml of 0.01% TX-100. All vials were shaken to mix soil and solutions thoroughly before the larvae were added. Larvae were placed individually in the soil, with a carrot cube for food, then incubated at 22°C in environmental cabinets using a 16-h photoperiod.

The vials were arranged in a randomized complete block design (RCBD). There were 16 treatments including seven treatments of C14 and bacteria isolates, seven treatments of bacteria isolates, one

treatment of C14 and one control. Each treatment had five replicates, with 4 larvae in each replicate (total =20 larvae per treatment). Data on larval mortality were taken 1, 3, 5, 7, 10, 14, 21, 28 and 35 days after the inoculation. Mortality of larvae was compared as a binary state variable: each larva was either dead (0) or alive (1) at the time of data recording. The data were corrected for control mortality by using Abbott's formula (Abbott, 1925). The results were analysed by a general ANOVA in GenStat®, 18th edition by comparison of mean values of the treatments using Fisher's unprotected least significance difference.

Table 5.2 Bacterial strain cell concentrations used in bioassays

Bacterial strains	Concentration (cell/vial)
2-2	1.7×10^{9} - 1.4×10^{10}
3-1	5×10^{7} – 2.58×10^{8}
3-2	4.8×10^{7} - 1.09×10^{8}
3-3	3.37×10^{7} - 2.6×10^{8}
5-1	1.25×10^{7} – 5×10^{7}
6-1	5.8×10^{9} - 1.13×10^{11}
S3	6.55×10^{7}–8.7×10^{8}

Isolate 6-1 and 3-2 were selected based on the results from the first experiment and then applied at different rates (10^{4}, 10^{6}, 10^{8} cells/ml) to determine concentrarion had the potential to affect the virulence of *M. novozealandicum* (C14) against grass grub these results are presented in Appendix 5.4.

5.2.8 Assay of chitinase production by the bacterial strains

A colloidal chitin suspension was prepared from Sigma practical grade crab shells (Sigma-Aldrich) for use in the preparation of chitin detection assay media, according to the methods described by Rodriguez *et al*. (1983). Ten g of crustacean chitin flakes were ground using a mill for 30 seconds and added to 150 ml of 36% HCl. The solution was agitated with a magnetic stirrer and to improve dissolving, the mixture was left overnight at 20°C. One thousand four hundred ml milli Q water was poured into a 2 L glass container, the solution was added and then more milli Q water was added to achieve a volume of 1.7 L. The suspension was left standing overnight to allow the chitin suspension to settle and the supernatant was discarded. One L of tap water was then added, and the suspension was left overnight before the supernatant was discarded. This was repeated 4 times with tap water and was repeated 4 more times with ddH2O. The acidity of the solutions was checked at the end to determine whether the acid had been sufficiently washed off. A range of pH between 5.5-6.0 was considered acceptable. The final suspension was filtered through a Whatmann filter paper (No. 31). The white gel of chitin from the Whatmann filter paper was collected and stored in the dark at 4°C.

Chitin agar was prepared using a modification of the method of Hsu & Lockwood (1975). Ten g bacto agar was mixed with 1 L water containing minimal salts including: Na_2HPO_4 6g, KH_2PO_4 3g, NH_2Cl 1g, NaCl 0.5 g, Yeast 0.5 g and 2% chitin. This was then autoclaved and stirred vigorously before dispensing in plates. Plates were left to dry for a minimum of 2 hours before a plug (3 mm) of a growing fungus cut from a three day old colony was placed at the centre of each plate. The inoculated plates were then incubated at 30°C for 2-5 days and appearance of a clear zone around the colonies was considered as a positive reaction. The clear zone diamters were measured using a ruler and recorded.

The experiment included ten treatments: Nine bacterial isolates/strains, 2-2, 3-1, 3-2, 3-3, 5-1, 6-1, s3, A1MO2 and 145WT (Two additional disease causing bacteria strains identified as A1MO2 (*S. entomophila*) and 145WT (*S. proteamaculans*) were obtained from AgResearch) and one control (no bacteria). The plates were arranged in a randomized complete block design (RCBD) with five replicates per treatment.

5.2.9 Assay measuring protease production of bacterial strains

Protease assay plates containing 1% casein or 1% gelatin, or 1% casein and 1% gelatin in a 1.5% agar medium were prepared following the method of Montville (1983). The casein solution was created by dissolving casein in 0.02 M NaOH in water and stirring until it became translucent. Once all compounds were added, the media were adjusted to a pH of 7.0 with 1 N HCl. Media were autoclaved at 121°C for 15 min and stirred vigorously before plates were poured separately.

The experiment included ten treatments: nine bacteria isolates, 2-2, 3-1, 3-2, 3-3, 5-1, 6-1, s3, A1MO2 and 145WT, and a positive control (proteinase K (Sigma-Aldrich)). All strains were grown overnight on LB agar plates in an incubator at 30°C in a 16-h photoperiod and used immediately. For each replicate, a 1 cm diameter agar plug containing a growing bacteria colony was taken from one of these plates and placed in the centre of a casein or gelatin or casein and gelatin agar plate. Plugs of agar containing proteinase K (20 mg/ml) were used as a positive control. The plates were arranged in a randomized complete block design (RCBD) with five replicates per treatment. All plates were incubated at 37°C in a 16-h photoperiod and observed after 24 h.

5.2.10 Assays of bacterial volatile compounds

5.2.10.1 Comparing effect of volatiles of nine bacterial strains on radial growth of C14 in culture

This experiment was designed to test the effect of nine bacterial isolates, 2-2, 3-1, 3-2, 3-3, 5-1, 6-1, S3, A1MO2 and 145WT, on radial growth of *M. novozealandicum* (C14) in culture and was performed according to a method described by Ajith & Lakshmidevi (2010). These assays were conducted using

inverted Petri dishes with bacteria and C14 in different plates (above and below), not in contact with each other. Nine bacterial strains were grown overnight on PDA plates in an incubator at 30°C in a 16-h photoperiod and used immediately. These bacteria could grow on both media (LB agar and PDA) so PDA medium was chosen for the growth of bacterial isolates and C14. *M. novozealandicum* (C14) was grown on PDA in an incubator at 25°C with a 16-h photoperiod and used after four days. This experiment had 10 treatments with four replicates per treatment; nine bacteria strains and fungus, and a no bacteria control. A one cm diameter PDA plug was excised at the margin of the growing *M. novozealandicum* (C14) and each bacterial strain placed at the centre of separate petri plates containing PDA. Both plates were then placed facing each other with the lids removed so there was no barrier between the two agar surfaces (and no contact), and the plates were sealed together. This was repeated for each replicate for all bacterial strains. For the control, a PDA plate without bacteria was used. The plates were arranged in a randomized complete block design (RCBD) and incubated at 25°C for 22 days after which the fungal colony diameter was measured. Radial growth data were analysed using a general ANOVA in GenStat®, 19th edition by comparison of mean values of the treatments using Fisher's unprotected least significance difference.

5.2.10.2 Assay of the antagonistic effect of the bacterial metabolites on *Metarhizium novozealandicum* (C14)

This experiment was designed to test the direct effect of nine bacterial isolates: 2-2, 3-1, 3-2, 3-3, 5-1, 6-1, s3, A1MO2 and 145WT on the growth of *M. novozealandicum* (C14). Production cultures were grown for the bacteria strains and *M. novozealandicum* to provide inocula for the experimental plates. Bacteria strains were grown overnight on LB agar plates in an incubator at 30°C with a 16-h photoperiod and used immediately. *Metarhizium novozealandicum* (C14) was grown on PDA agar in an incubator at 25°C with a 16-h photoperiod and used after 4 days.

With nine bacteria strains and a no-bacteria control, this experiment had 10 treatments and there were four replicates per treatment. From the production cultures, a 1 cm diameter plug was excised from the margin of *M. novozealandicum* (C14) and placed 1 cm from the side of a PDA plate, and one of nine bacterial strains was streaked with a swab directly from the production culture on the opposite side. For the controls, bacteria alone were streaked onto one side of a plate plus a *M. novozealandicum* (C14) control was created by placing a fungal plug alone one cm from the edge of a plate. The plates were incubated for 17 days at 25°C and then checked for any growth inhibition.

5.3 Results

5.3.1 Isolation of bacteria from infected *Costelytra giveni* larvae and soil

Fifteen bacterial isolates were isolated from *C. giveni* larvae and three from soil. The isolation source and putative identity of each is presented in Table 5.3. Results of the sequencing comparisons

showed that among the 18 isolates, four were identified as *Serratia* species, four as *Yersinia* species, three as *Carnobacterium maltaromaticum*, two as *Bacillus megaterium*, two as *Pseudomonas lundensis*, and one each of *Oerskovia emterophila, Chryseobacterium* sp. and *Stenotrophomonas* sp.

Table 5.3 The source and putative identity of the bacteria strains isolated.

Insect	Bacteria	Origin/Source	Species
1	1	*Costelytra giveni* larvae	*Serratia proteamaculans*
1	2		*Serratia proteamaculans*
2	1		*Serratia* sp.
2	2		*Yersinia frederiksenii*
2	3		*Serratia proteamaculans*
2	4		*Yersinia frederiksenii*
3	1		*Carnobacterium maltaromaticum*
3	2		*Bacillus megaterium*
3	3		*Oerskovia emterophila*
4	1		*Carnobacterium maltaromaticum*
4	2		*Carnobacterium maltaromaticum*
5	1		*Pseudomonas lundensis*
5	2		*Pseudomonas lundensis*
6	1		*Yersinia enterocolitica*
6	2		*Yersinia frederiksenii*
S1		Soil	*Bacillus megaterium*
S2			*Chryseobacterium* sp.
S3			*Stenotrophomonas* sp.

5.3.2 Visualisation of the megaplasmid pADAP in *Serratia* spp. and *Yersinia* spp.

The results of the tests for the presence of the megaplasmid pADAP are presented in Table 5.4. Gel visualizations (Appendix D.5.2) revealed the presence of large plasmid bands just below the agar well which are bigger than the chromosomal DNA. Chromosomal DNA generally migrates in a gel at around 23 Kb in size. Isolates 1-1, 1-2 and 6-1 did not show the plasmid bands.

5.3.3 Detection of *sep* and *afp* virulence encoding regions in the bacteria isolates

Primers designed to a portion of each region were used to determine if the gene regions were present in the bacteria, as these regions correlate with grass grub disease causing ability. The expected size of the *S. entomophila* pADAP derived SepC TC-A component target gene region was approximately 550 bp. Only *Y. frederiksenii* isolates 2-2, 2-4 and 6-2 had a 550 bp PCR product (Table 5.4). *Yersinia* isolate 6-1 had two bands with a different size of 200 bp and 1325 bp. *Serratia* isolates 1-1, 2-1 and 2-3 had two bands estimated as 300 bp and 780 bp; isolate 1-2 also had two bands but at 200 bp and 800 bp. Other than the three *Carnobacterium maltaromaticum* isolates (3-1, 4-1 and 4-2) and one of the two *Bacillus megaterium* isolates (3-2), all the remaining strains had at least one band. Isolates identified as the same species often, but not always, had identical band patterns.

Isolates 5-1, 5-2 and s1 had 500 bp and 1200 bp bands. There were 200 bp bands found in isolates 3-3, S2 and S3. Isolate 3-3 also had a 500 bp band and S2 a 750 bp band. The gel resulting from the above amplification is shown Appendix D 5.2. Strong bands of approximately 550 bp in length indicate the toxin encoding genes being present (see the indicated band in isolates 2-2, 2-4 and 6-2 in (Appendix D 5.2).

Table 5.4 Size of bands obtained from PCR products amplified from 18 bacteria isolates using primers targeting the *sep* and *afp* regions of the virulence associated plasmid pADAP. Strong bands are shown in bold font.

Insect	Isolate	Species	Megaplasmid	Estimated band size (in bp) for Sep primer	Estimated band size (in bp) for Afp primer
1	1	*Serratia proteamaculans*	Absent	300, 780	**800**
1	2	*Serratia proteamaculans*	Absent	200, 800	1325, **800**, 400
2	1	*Serratia* sp.	Not tested	300, 780	**800**
2	2	*Yersinia frederiksenii*	Present	**550**	1825
2	3	*Serratia proteamaculans*	Present	300, 780	**800**, 200
2	4	*Yersinia frederiksenii*	Present	**550**	1825, **800**
3	1	*Carnobacterium maltaromaticum*		No band	No band
3	2	*Bacillus megaterium*		No band	No band
3	3	*Oerskovia emterophila*		200, 500	No band
4	1	*Carnobacterium maltaromaticum*		No band	No band
4	2	*Carnobacterium maltaromaticum*		No band	No band
5	1	*Pseudomonas lundensis*		500, 1200	No band
5	2	*Pseudomonas lundensis*		500, 1200	No band
6	1	*Yersinia enterocolitica*	Absent	200, 1325	400, 200
6	2	*Yersinia frederiksenii*	Present	**550**	1850
s1		*Bacillus megaterium*		500, 1200	No band
s2		*Chryseobacterium* sp.		200, 750	No band
s3		*Stenotrophomonas* sp.		200	No band

The expected band size of the PCR product targeting the Afp18 toxin encoding gene was approximately 800 bp. Bands of this length were found in isolates 1-1, 1-2, 2-1, 2-3 and 2-4 (Table 5.4). Additionally, *Serratia* isolate 1-2 had 1325 bp and 400 bp length bands, while isolates 2-3 and 2-4 had additional bands 200 bp and 1825 bp in length respectively. Some of the isolates in which 800 bp bands did not occur had bands of other sizes. Isolates 2-2 and 6-2 also had a band corresponding

to 1825 bp, similar to that of isolate 2-3, but an 800 bp band was absent in these isolates. There were 400 bp and 200 bp bands in isolate 6-1. The remaining isolates produced no bands. Appendix D 5.2 shows the gels resulting from amplification using the primer pair Afp18F and Afp18R. The strong bands seen in isolates 1-1, 1-2 and 2-3 mostly indicate the presence of the toxin encoding genes. Bands of any length only appeared in isolates of species from the genera *Serratia* and *Yersinia*. Target bands were only strong in the *Serratia* species.

5.3.4 Bioassays of *Costelytra giveni* larvae using bacteria and *Metarhizium novozealandicum* (C14) alone and in combination

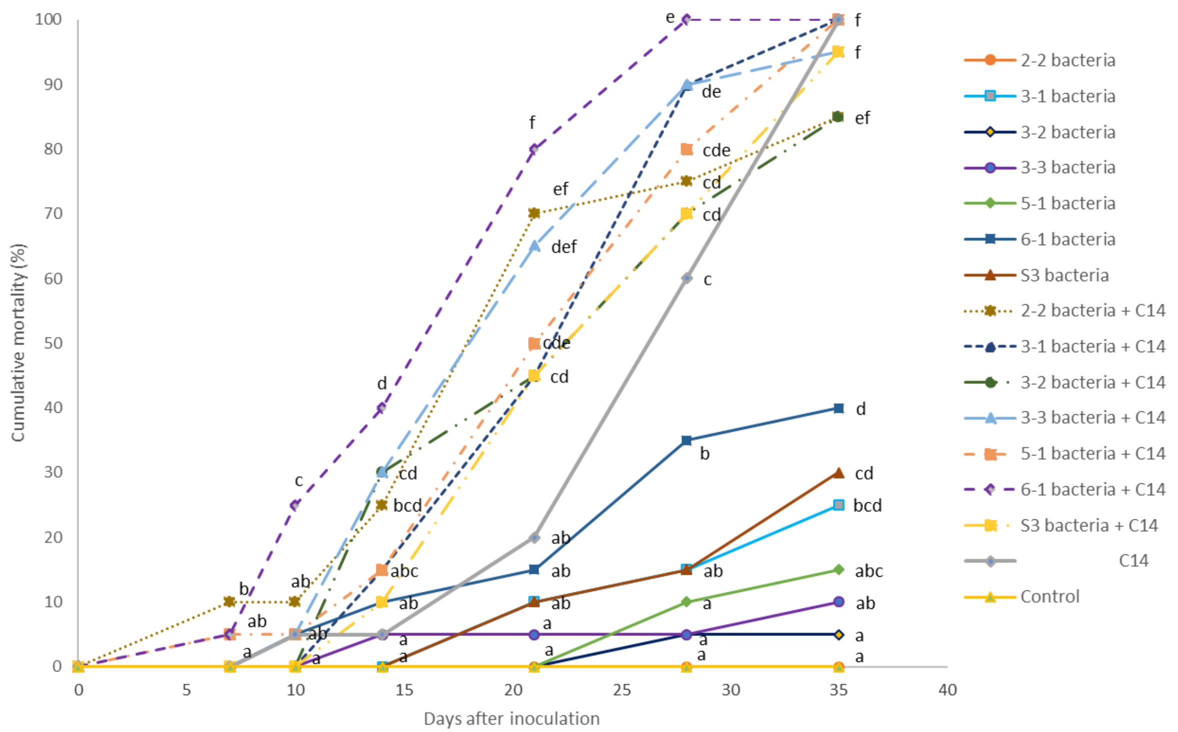
The cumulative mortalities of second and third instar *C. giveni* larvae over 35 days when treated with *M. novozealandicum* (C14) and bacteria are shown in Figure 5.1a & b. No second or third instar larvae in the control died during the bioassay. C14 alone began to kill larvae between day 10 and day 14. These deaths were significantly ($P<0.01$) greater than the control. By day 35 C14 had killed 100% of second instar and 80% of third instar larvae (Figure 5.1a, b). C14 had a significantly faster rate of mortality for third instar larvae compared to that of the second instar larvae (Figure 5.1, Table 5.5).

Against second instar larvae, the bacterial strains alone achieved mortalities ranging from 5% to 35% (Figure 5.1a). However, when combined with C14, these mortalities increased to between 80 and 100% (Figure 5.1a). The rate of larval mortality was greater for all the combined treatments than for C14 alone (Figure 5.1a).

Against third instar larvae, there was only a small, non-significant difference in mortality rates between the *M. novozealandicum* (C14) only treatment and each of the combined treatments, although this was mainly due to the increased virulence of the fungus against 3rd instar larvae (Figure 5.1b).

The time taken for 50% of larvae to die (LT_{50}) was calculated for each of the treatments in each experiment (Table 5.5). For both second and third instar larvae, the LT_{50} was lower (most virulent) in the 6-1 (*Yersinia* sp.) + *M. novozealandicum* (C14) treatment compared with the other combination treatments. For second instar larvae, all combination treatments produced significantly lower LT_{50} values than the *M. novozealandicum* only treatment except for the 3-1 + C14 treatment. Treatment 6-1 + C14 also produced a significantly lower LT_{50} than that of treatment 3-1 + C14. The LT_{50} was not significantly different among the other combination treatments, but there was a significant difference between combined treatment 6-1 + C14 and the *M. novozealandicum* (C14) only treatment. For third instar larvae, only 6-1 + C14 had a lower LT_{50} than C14 alone, and LT_{50} did not differ among the combinations (Table 5.5).

a) second instar



b) third instar

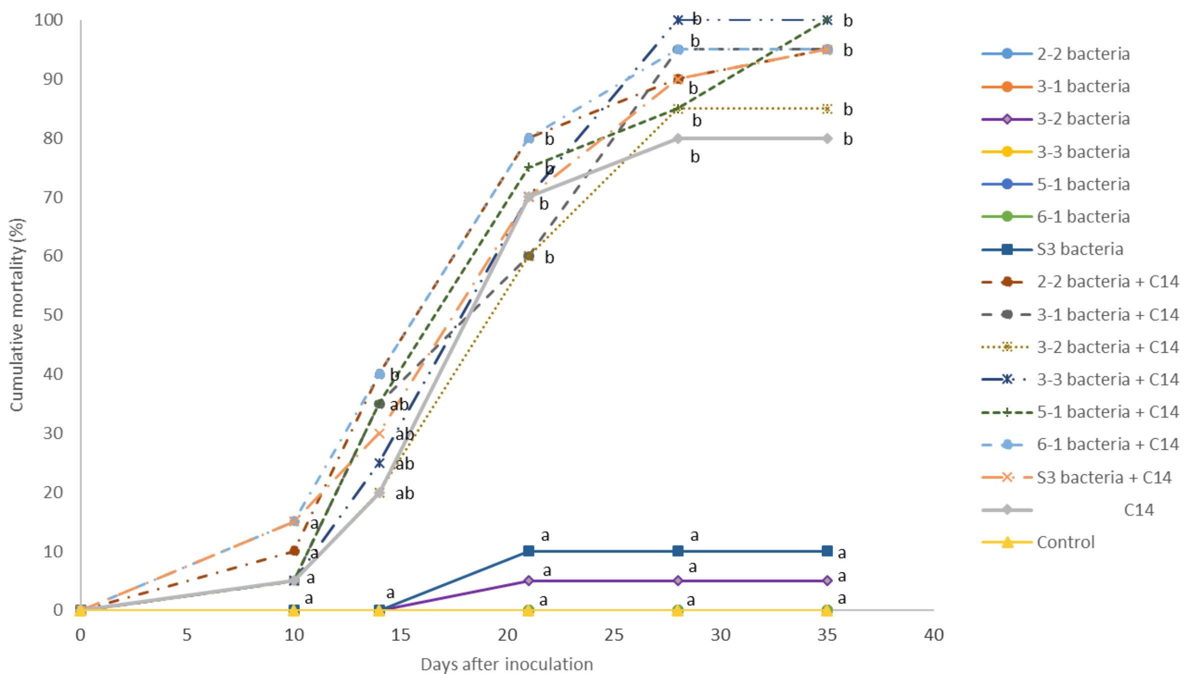


Figure 5.1 The cumulative mortality of (a) second instar and (b) third instar *Costelytra giveni* larvae resulting from the single and combined inocula of *Metarhizium novozealandicum* (C14) (10^8 spores/ml) and seven bacterial isolates (2-2, 3-1, 3-2, 3-3, 5-1, 6-1 and S3; 10^8 cells/ml) at 22°C. Note that data points for the third instar (b) for baterial isolates 2-2, 3-1, 3-3, 5-1, 6-1, S3) and the control overlap. Letters indicate significance groupings based on Unprotected LSD test statistic: at each assessment data means with no letters in common are significantly different (P<0.05).

Table 5.5 The time taken for 50% of *Costelytra giveni* larvae to die (LT_{50}) for all combined treatments and the *Metarhizium novozealandicum* (C14) only treatment for second and third instar larvae. Letters following the mean values indicate the results of pairwise Fisher's unprotected LSD tests conducted within each instar factor: means not marked with the same letter are significantly different.

Treatment	LT_{50} (days)-second instar	LT_{50} (days)-third instar
6-1 bacterium + C14	13.2a	14.83a
3-3 bacterium + C14	17.5ab	17.03ab
S3 bacterium + C14	17.5ab	16.8ab
2-2 bacterium + C14	17.73ab	15.7ab
3-2 bacterium + C14	18.9ab	19.13ab
5-1 bacterium + C14	19.13ab	16.57ab
3-1 bacterium + C14	21bc	16.07ab
C14	26.6c	21.53b
LSD (5%)	7.3	6.7

5.3.5 Chitinase production by bacterial strains

All bacterial isolates had significant clearing zones around the colonies on chitin-containing medium at day ten after inoculation, except for isolates 2-2, 5-1 and 3-1, which exhibited no obvious halos (Figure 5.3). After 3 days, these clearing zones were significantly larger compared with isolate 2-2, 5-1, 3-1 and control treatments ($P<0.05$). Isolate A1MO2 produced a larger chitinase clearing zone than isolates 6-1 and 145WT, which were the same as each other, but both were significantly greater than 3-3, 3-2 and S3 ($P<0.05$) which were not significantly different from each other. After 6 and 10 days, the clearing zone around colonies of all bacteria isolates had grown significantly larger compared with zones at 3 days (Figure 5.2). Isolate A1MO2 still produced a larger clear zone than the remaining strains at this time point. After day 10, isolates A1MO2 and 6-1 had similar chitinase activity which was significantly greater than the rest of the isolates ($P<0.05$). There was a significant difference between all other isolates in chitinase production ($P<0.05$). A1MO2 and 145WT isolates, which cause amber disease of grass grub (Hurst *et al.*, 2007) are known chitinase producers, and were used in this assay to compare with other bacterial isolates. Isolate 6-1 produced more chitinase compared to the other tested bacterial isolates (Figure 5.2 & 5.3).

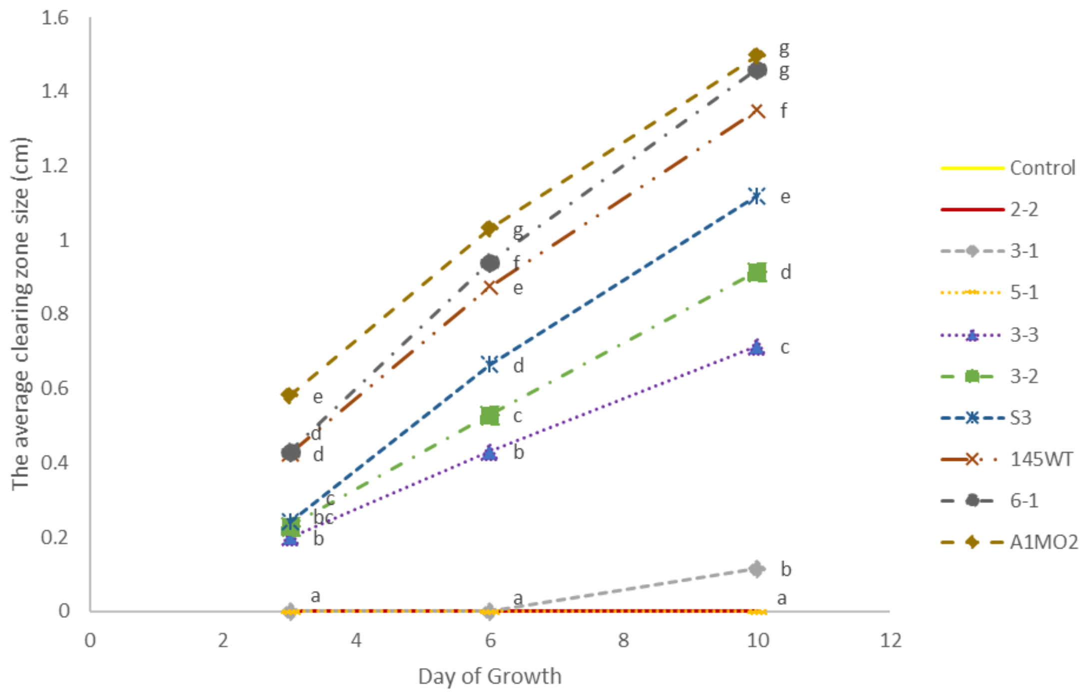


Figure 5.2 Clearing zone of nine bacteria isolates grown on chitin agar plates 3, 6 and 10 days after inoculation. Isolates 3-1, 5-1, 2-2 and control exhibited zero or close to zero clearing. Letters indicate significance groupings based on Unprotected LSD test statistic: at each assessment data means with no letter in common are significantly different (P<0.05).

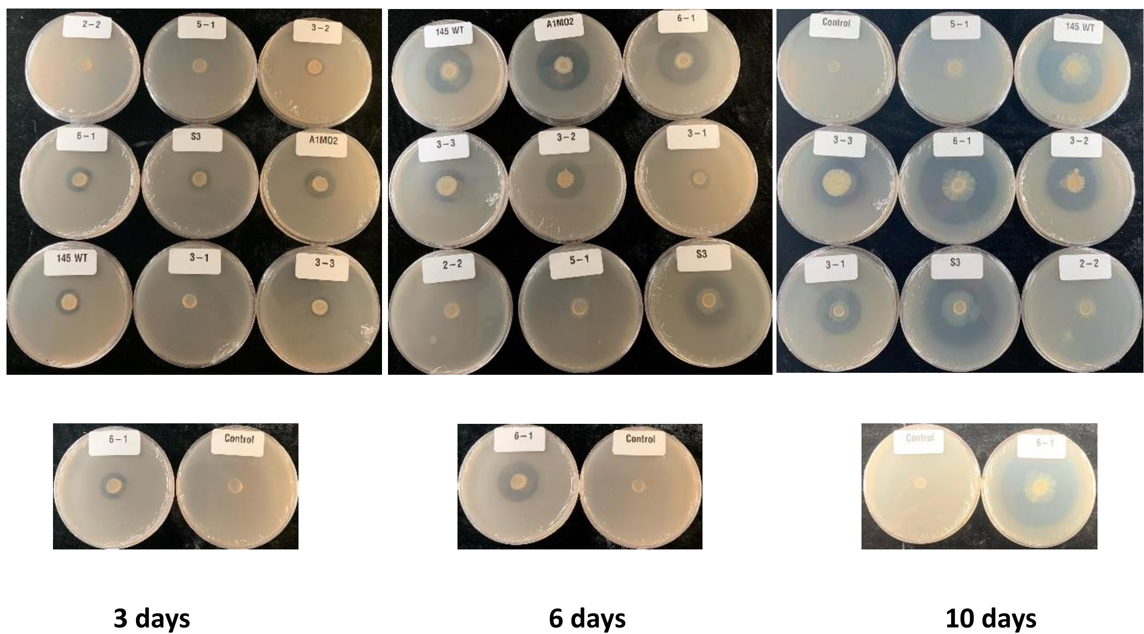


Figure 5.3 Zones cleared of chitin surrounding the colonies of nine bacterial isolates on chitin agar plates 3, 6 and 10 days after transfer.

5.3.6 Protease production by bacterial isolates

Some of the bacterial isolates (6-1, A1MO2) which increased C14 virulence in the interaction between the fungus and bacteria did not produce protease, while other bacterial strains (S3, 5-1) which had less virulence to grass grub produced protease on plates containing casein (Figure 5.4).

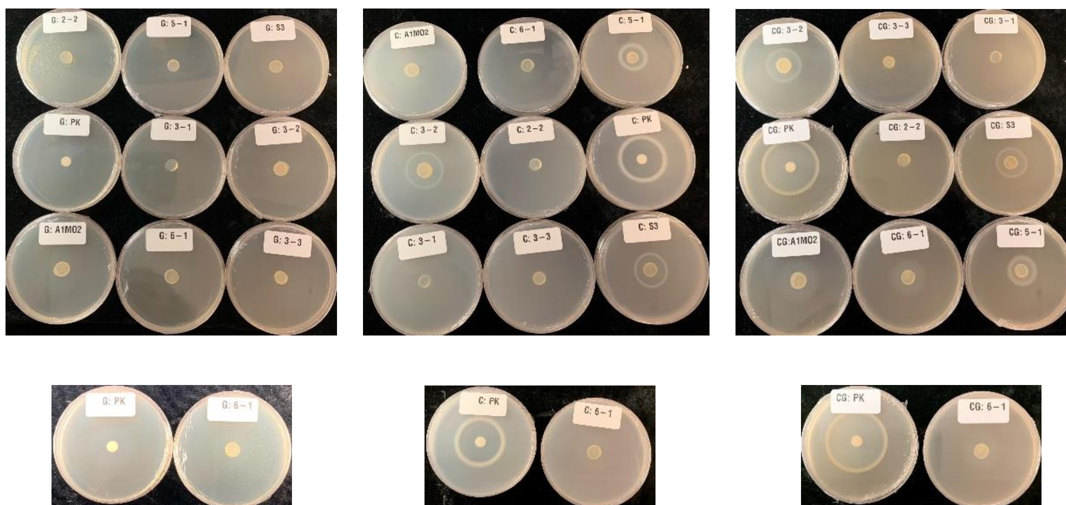


Figure 5.4 Twenty-four hour growth of nine bacterial isolates on plates containing gelatin (G), casein (C) or casein plus gelatin (CG). Proteinase K (PK) was used as the positive control.

5.3.7 Effect of bacterial volatile compounds on C14

5.3.7.1 Effect of volatile compounds released from nine bacterial strains on the radial growth of *Metarhizium novozealandicum* (C14)

The aim of this experiment was to determine if some bacterial isolates produce volatile compounds able to inhibit the growth of *M. novozealandicum* (C14) on plates *in vitro*.

Isolates A1MO2 and 145WT significantly reduced C14 radial growth but the other isolates did not (Figure 5.5). Radial growth reduction did not differ between A1MO2 and 145WT, but that reduced by A1MO2 was significantly greater than the other seven bacterial isolates (Figure 5.5 & 5.6).

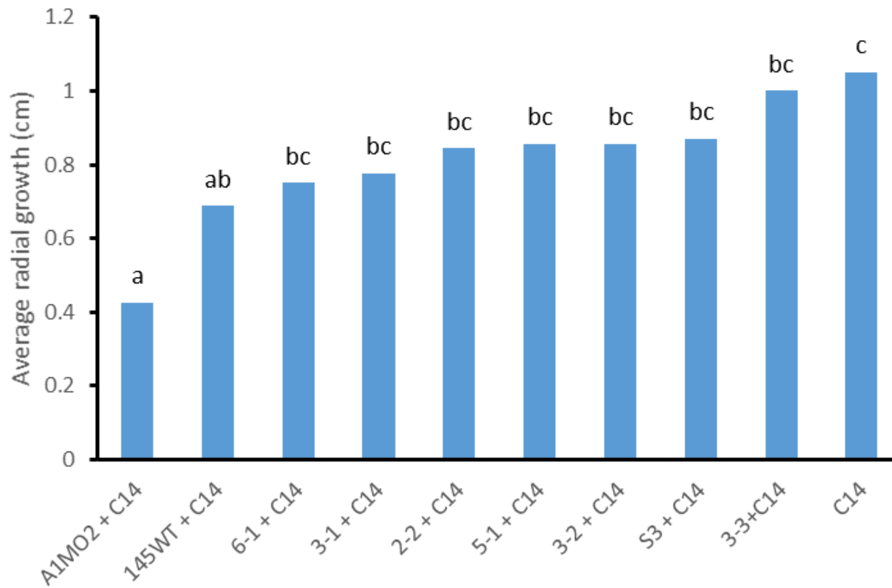


Figure 5.5 Effect of volatile compounds released by nine bacteria isolates on radial growth of *Metarhizium novozealandicum* (C14) after 22 days. Letters indicate significance groupings based on Unprotected LSD test statistic: at each assessment data means with no letters in common are significantly different (P<0.05).

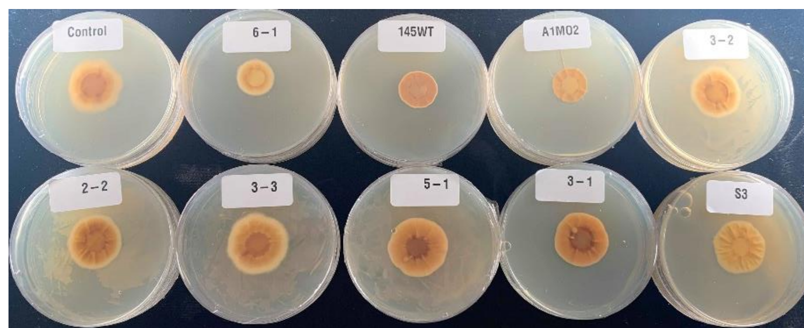


Figure 5.6 Effect of the volatile compounds produced by bacteria on the growth of *Metarhizium novozealandicum* (C14) after 22 days. Control = no bacteria.

5.3.7.2 Antagonistic effect of the bacterial metabolites on *Metarhizium novozealandicum* (C14)

The aim of this experiment was to see if some bacterial isolates could produce metabolites which could inhibit the growth of *M. novozelandicum* (C14). No growth inhibition of the fungus was observed in the medium, indicating that no metabolite released by any of the bacteria was able to reduce the growth of *Metarhizium.*

5.4 Discussion

This chapter reported investigations of bacteria isolated from infected grass grub larvae and from soil to determine if the selected bacteria could affect the pathogenicity of *M. novozealandicum* (C14).

The combination of fungus and bacteria in some cases increased grass grub mortality over that of C14 alone. The aforementioned bacteria were not necessarily themselves pathogenic to the larvae, but they produced a number of enzymes such as chitinase that have the potential to degrade the cuticle of grass grub. There was a synergistic interaction between C14 and all bacterial isolates against the second instar larvae, but it was not observed on the third instar larvae in all experiments. The lack of difference with third instars was likely due to the increased virulence of the fungus alone against the older larvae.

The combination of 6-1 (*Y. enterocolitica*) or 3-2 (*B. megaterium*) with C14 caused significantly faster second larvae instar mortality than the remaining combined isolates. Isolates 6-1 and 3-2 did not have the Afp and Sep toxin genes which cause amber disease in grass grub. However, these isolates produced more chitinase, but not protease, compared with the remained strains. The mechanism of this synergism is unknown, but it could be attributed to the production of chitinase enzyme involved in affecting or inhibiting cuticle formation in grass grub. This suggests that these isolates may be synergistic with the fungus through enzyme activity, although more research, such as potentially knocking out the chitinase genes from the bacteria, would be needed to confirm this hypothesis.

Bacterial isolates can infect larvae through ingestion as reported by Jackson *et al.* (1993), who showed that amber disease (caused by *S. entomophila*) resulted from ingestion of pathogenic bacterial cells while *M. anisopliae* attacked directly through the host's cuticle (Glare & Milner, 1991). It is possible that those bacterial isolates modify larval behaviour and influence their contact with the fungal inoculum, or possibly weaken the larvae and allow fungal infection to proceed more rapidly. Another possible cause of the synergism between bacteria and fungus could be the weakening of the pest cuticle to allow a greater fungal germ tube penetration. There is no obvious theory about how the combination of bacteria and fungus could affect larvae via the alimentary canal or through the cuticle and haemocoel. Possibly it is an indirect mechanism. Bacteria entering the haemocoel may trigger immune responses and weaken the individual by using up resources at the expense of defence against *Metarhizium*.

One non-pathogenic 6-1 bacterial isolate combined with C14 produced a synergistic effect against second instar larvae. No significant difference between pathogenic and non-pathogenic bacterial isolates when applied with C14 was observed. Most bacterial isolates produced volatiles that could inhibit the growth of C14 on plates *in vitro*. Volatiles released from A1MO2, 145WT and 6-1 isolates showed a greater fungal inhibition than the remaining isolates and the control. Interestingly, these isolates were also the more effective in increasing C14 virulence. Bacteria are known to produce volatiles which inhibit the growth of fungi (Ebadzadsahrai et al., 2020; Mannaa & Kim, 2018; Xie *et al.,* 2020; Zhou *et al.,* 2019). *Bacillus subtilis* produces volatiles which have the potential to inhibit the

mycelial growth of the fungus *Alternaria solani* in potato (Zhang *et al.,* 2020). Similarly, Rajaofera *et al.* (2019) reported that *Bacillus atrophaeus* HAB-5 produced volatiles that inhibited the growth of *Colletotrichum gloeosporioides.* In the current study, volatiles released from bacteria limited the growth of *Metarhizium* in plates, but when incorporated into media, did not affect fungal growth. Kriukov *et al*. (2009) also showed that there was no antagonism on artificial nutrient media between the bacterium *Bacillus thuringiensis* and the fungus *Metarhizium anisopliae*. The effect of volatiles on the fungal growth in the soil environment needs more investigation. *Metarhizium* generally exists as conidia in the soil if not infecting insects, so it is unclear if this antagonism would impact the ecology of *Metarhizium.*

At 22ºC, treatment of the second instar larvae simultaneously with both C14 and bacteria isolates increased mortality of larvae over the C14 treatment alone. This synergistic effect was larval stage-dependent, as it was not found with the third instar larvae. The third instar larvae were more susceptible to C14 than the second instars. The mortality in the fungus alone treatment at high concentration against the third instar had the same mortality as with the combination treatments. There are some studies which have demonstrated synergism between microbial pathogens. Glare (1994) showed a synergism between *M. anisopliae* and *S. entomophila* against the second instar larvae of grass grub. Moreover, some studies showed interactions between nematodes and fungi affecting their virulence towards insect hosts (Ansari *et al*., 2004; Davies, 2005). Similarly, a pathogen combination between *Metarhizium brunneum* and the microsporida *Paranosema locustae* to control the migratory grasshopper was shown to be synergistic under laboratory and greenhouse conditions (Dakhel *et al*., 2019).

Third instar grass grub larvae were more susceptible to C14 than the second instar. It is a well-established phenomenon that insects often differ in susceptibility to pathogens at different stages of their development, and that was the reason why C14 treatments had a fluctuating rate of mortality between the experiments. Older instars were reported to be more susceptible to *M. anisopliae* compared with younger instars (Glare & Milner, 1991; Glare, 1994). No synergistic effect from simultaneous inoculation with both C14 and those bacterial strains was found against the third instar, even at lower inoculum levels of the fungus.

Chitinase is an enzyme which can degrade the grass grub cuticle. Chitinase can be produced by bacteria and could potentially have synergistic impacts on grass grubs when using with *M. novozealandicum.* The bacterial strains were grown on media containing colloidal chitin to induce chitinase production. Chitinase activity was assayed qualitatively by observing the formation of a clear zone around colonies and most bacteria isolates produced clear zones after three days incubation. A clear zone was the result of chitin hydrolysis in the specific period. Chitinase produced

by chitinolytic bacteria could degrade chitin into monomers, and an extracellular enzyme that might play an important role in the hydrolysis of chitin on the cuticle of grass grub. Isolates 6-1, 145WT, A1MO2 produced more chitinase than the remaining strains while 145WT and A1MO2 isolates are able to cause amber disease on grass grub.

There was no presence of the protein clearing zones by most of the bacteria in this study that had high synergism with fungus against grass grub, while other bacterial isolates with less combined virulence to grass grub produced abundant protease. I am not able to comment on this with our current data, but any interaction could be because of the specific effect of the protease on *Metarhizium* proteins rather than those of grass grub.

Overall, the combination of fungus and bacteria produced a significant synergistic response to mortality of second larval instar of grass grub, possibly because of chitinase production by the bacteria. *Yersinia enterocolitica* Isolate 6-1 could be a promising addition to C14 for biological control in the future. *Yersinia enterocolitica* caused about 117,000 illnesses, 640 hospitalizations, and 35 deaths in the United States every year (CDC, https://www.cdc.gov/yersinia/), so the strain would need to be checked for any potential negative impacts on human or animal health, but the potential for bacterial enhancement of *Metarhizium* has been demonstrated.

Chapter 6

Exploring the host range of a grass grub-active *Metarhizium novozealandicum* (C14)

6.1 Introduction

Entomopathogenic fungi can have a wide host range and can infect insect species belonging to orders Coleoptera, Lepidoptera, Diptera, Homoptera, and Hymenoptera in agricultural environments (Sinha *et al.*, 2016). There are between 750-1000 insect species known to be attacked by *Metarhizium* (Lacey *et al.*, 2001; Meyling & Eilenberg, 2007; Schneider *et al.*, 2011; Vega *et al.*, 2012; Keyser, 2015). Some strains are reported to infect important agricultural pests such as locusts, grasshoppers, termites, noctuids, scarab beetle larvae, and spittlebugs and other hemipterans (St. Leger, 1993; Zimmermann, 2007). Host range may vary between different *Metarhizium* species and strains. For example, *M. album* and *M. acridum* have narrow reported host ranges, while *M. robertsii, M. flavoviride* and *M. anisopliae* generally have wide host ranges (Wang *et al.*, 2016).

In one of the few summaries of the host range of a *Metarhizium* species, Veen (1968) listed 204 species infected in nature by *M. anisopliae sensu lato,* mostly belonging to order Coleoptera. In 2009, *M. anisopliae* was recognized as a species complex, and was divided into several species (Bischoff *et al.*, 2009). Research has since expanded the known host range. Sinha *et al.* (2016) reported that *M. anisopliae sensu stricto* had a broad host range including insect pests belong to orders Coleoptera, Lepidoptera, Diptera, Hemiptera, and Hymenoptera. Strains from the *M. anisopliae* species complex have been used to control species of Acari (Ixodidae, Tetranychidae), Blattodea (Blattidae, Blattellidae), Coleoptera (Curculionidae, Nitidulidae, Scarabaeidae), Diptera (Ephydridae, Mycetophilidae, Sciaridae, Tipulidae), Hemiptera (Aphididae, Cercopidae, Cicadellidae, Delphacidae, Miridae, Pentatomidae), Isoptera (Kalotermitidae, Rhinotermitidae, Termopsidae), Hymenoptera (Formicidae), Lepidoptera (Crambidae, Noctuidae), Orthoptera, Siphonaptera (Pulicidae), and Thysanoptera (Thripidae) in different parts of the world (St Leger, 1993; Huang *et al.,* 2005; Faria & Wraight, 2007; Zimmermann, 2007; Brunner-Mendoza *et al*., 2018; Gutierrez *et al*., 2019). *Metarhizium* spp. (*M. album, M. acridum, M. anisopliae, M. flavoviride, M. novozealandicum, M. pemphigi, M. brasiliense, M. lepidiotae, M. majus, M. minus, M. pingshaense* and *M. robertsii)* have been successfully isolated from insects of the orders Hemiptera, Orthoptera, Coleoptera, Lepidoptera, and Isoptera (Barranco *et al*., 2019).

In a summary of entomopathogenic fungi and their recorded hosts in New Zealand, *M. anisopliae sensu lato* was listed as associating with insect pests belonging to orders Coleoptera and Lepidoptera

while another species, *M. flavoviride sensu lato,* has been isolated from order Coleoptera (Glare *et al*., 1993). In addition, recently *M. brunneum* (a species from the *M. anisopliae* complex) has been isolated from *Heteronychus arator* (Coleoptera) and *Stethaspis longicornis* (Coleoptera), and *M. novozealandicum* (a species originally split from the *M. flavoviride* complex [Tóthné Bogdányi *et al.*, 2019]) was isolated from *Costelytra zealandica* (= *C. giveni*, Coleoptera) and the porina moth caterpillars *Wiseana* spp. (Lepidoptera) (Liu *et al.,* 2020). However, this is likely to be only a small fraction of their true range in New Zealand, as there has been no comprehensive assessment of the host range of any *Metarhizium* species in New Zealand.

6.1.1 Aim

The aim of this chapter was to explore the host range of isolate C14 of *M. novozealandicum*, a strain isolated from kiwifruit soil in the Nelson region, New Zealand, with the intent to investigate its use in the control of insect pests. This chapter describes bioassays against five insect species (*Plutella xylostella, Helicoverpa armigera, Wiseana* sp*., Myzus persicae, Tenebrio molitor*) from three orders that represent common New Zealand agricultural insect pests with a range of differing life history and ecological traits. This knowledge will provide useful information for assessing isolate C14's use as an insect biocontrol agent, and its ability to remain in the insect populations longer if alternate hosts are present. In addition, commercial development of biopesticides can be more successful if the active agent is able to infect multiple pest species.

6.1.2 Fungal pathogens of other insect pests

6.1.2.1 Mealworm

The larvae of mealworms, *Tenebrio molitor* (Coleoptera: Tenebrionidae) are susceptible to *Beauveria bassiana* (Chun-Sheng *et al*., 2006; Rodriguez-Gomez *et al*., 2008; Batta *et al*., 2010; Lee *et al*., 2014; Lee *et al*., 2015; Lestari & Rao, 2016; Kilic, 2019) and *Metarhizium* spp. (Chun-Sheng *et al*., 2006; Bharadwaj & Stafford, 2011; Steinwender *et al*., 2015; Lestari & Rao, 2016; Kilic, 2019; Moonjely & Bidochka, 2019; Adatia *et al*., 2020). The larvae of *T. molitor* have been successfully used as insect baits for entomopathogenic fungi in nature (Meyling & Eilenberg, 2007). These species are model test insects due to their susceptibility to entomopathogenic fungi. The larvae of both insect pests are commercially available in New Zealand.

6.1.2.2 Diamondback Moth (DBM)

Diamondback moth (DBM), *Plutella xylostella* (Lepidoptera: Plutellidae) is an important pest of Brassica crops in the world causing serious damage (Herrick *et al*., 2008; Saenz-Aponte *et al*., 2020). The life cycle of DBM has four stages; egg-larvae-pupae-adult (Butts & McEwen, 1981; Rosario & Cruz, 1986; Kim & Lee, 1991; Oouchi, 2005; Faithpraise *et al.*, 2014; Huaripata & Sanchez, 2019). The

larva is the main damaging stage of the insect because it feeds on the leaves of plants (Dosdall, 1994). The larvae of DBM are readily infected by *M. anisopliae* (Silva *et al.*, 2003; Embaby & Lotfy, 2015). At 10^8 conidia/ml spore concentration, *M. anisopliae* and *Beauveria bassiana* have high virulence against larvae of *P. xylostella* (Godonou *et al.,* 2009).

6.1.2.3 Aphids

Green peach aphid, *Myzus persicae* (Hemiptera: Aphididae) is a sap sucking insects which can be found in different parts of the world (Dixon, 1977; Capinera, 2001), and are an important pest of most cruciferous vegetables including cabbage, beet, and cauliflower (Capinera, 2001; Duarte *et al*., 2011). The life cycle has three stages, namely egg-nymph-adult (Dixon, 1977; Capinera, 2001). A high density of aphids on young plant tissue causes water stress, wilting and reduced plant growth (Capinera, 2001). Asi *et al*. (2009) reported that *M. anisopliae* L6 with LC_{50} values at 2.2 x 10^6 and concentration of 5.3 x 10^6 conidia/ml was effective against the aphid, *Brevicoryne brassicae* after 7 days. Moreover, *M. anisopliae* CPD5 achieved 100% mortality of the cowpea aphid, *Aphis craccivora* after 7 days at a concentration of 10^8 conidia/ml (Ekesi *et al*, 2000).

6.1.2.4 Bollworms

Cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae), also known as the cotton bollworm, pod borer, gram podborer and fruit borer is a major and cosmopolitan pest of many crops (Suganthy, 2000; Gopali *et al*., 2009; Sarkar *et al*., 2015; Haddad *et al*., 2017). The life cycle of *H. armigera* has four stages: eggs, larvae, pupae and adults (Haddad *et al*., 2017). The larval stage is the important stage of the insect and cause damage to different parts of plant (Haddad *et al*., 2017; Guazina *et al*., 2019). *Metarhizium anisopliae* at a concentration of 10^8 conidia/ml caused 76.6% mortality in *H. armigera in vitro* (Khare & Gupta, 2019) while it reached 90.5% mortality in gram pod borer at concentration of a 10^{10} conidia/ml under field conditions (Adsure & Mohite, 2015).

6.1.2.5 Porina

Porina*, Wiseana* spp. (Lepidoptera: Hepialidae) is an important pasture pest in New Zealand (Bourner *et al*., 1996; Atijegbe *et al*., 2016, 2017). The life cycle of porina has four stages: eggs, larvae, pupae and adults (Atijegbe, 2019). The larva is the main damaging stage of the insect because it tunnels in the soil and feeds on foliage near the soil surface (Atijegbe, 2019). Latch (1983) showed a 90% mortality of porina larvae after 30 days by baiting with *M. anisopliae* under field conditions.

6.2 Materials and methods

6.2.1 Inoculum

M. novozealandicum (C14) was grown on PDA plates at room temperature (20°C). After 15 days, fungal conidia were harvested directly from the plates. The conidial density was adjusted to 10^8 conidia /ml as described in Chapter 2.

6.2.2 Bioassays

For each experiment at least five replicates were used. For some experiments more replicates, depending on times, condition and resource were used.

6.2.2.1 *Plutella xylostella* (Lepidoptera)

A laboratory colony of Diamondback Moth (DBM), originally collected from a cabbage field in Lincoln, New Zealand, was used as a source of second and third instar larvae, and cabbage (green cabbage, *Brassica oleracea* var. *capitata*) was used as a food source and to deliver the inocula during the experiment. A 30 mm diameter cabbage leaf disc was added to a plastic container (Huhtamaki, 35 ml) containing filter paper wetted with 100 µl sterile water (Autoclaved Milli-Q water) to provide moisture. Five caterpillars were added to each container. For inoculation, the C14 solution (300 µl) was sprayed directly onto the caterpillars and on one side of the leaf in each container using a handheld airbrush sprayer (Paasche). For a negative control, 300 µl of sterile 0.01% TX-100 was used. The containers were then left for 2 min to air dry in a laminar flow cabinet, and were covered with lids. Subsequently, the containers were placed in a Contherm incubator at 25°C with a 16:8 h light: dark cycle. The number of live and dead larvae per container was recorded every 24 hours until 6 days. This experiment had two treatments and five replicates with 5 larvae in each replicate (total =25 larvae per treatment).

6.2.2.2 *Helicoverpa armigera* (Lepidoptera)

Caterpillars were purchased from Plant and Food Research Ltd (Auckland). The second larval instar was used for the bioassay which was conducted using the method described above for *P. xylostella*. Five larvae were placed on one side of the leaf in each container. The containers were placed in a Contherm incubator incubated at 25°C with a 16:8 h light dark cycle. The number of live larvae was recorded every 24 hours until ten days. This experiment had two treatments and seven replicates with 5 larvae in each replicate (total = 35 larvae per treatment).

6.2.2.3 *Wiseana* sp. (Lepidoptera)

Adult female porina moths were hand collected between 21:00 h and 1:30 h from a light trap located at the AgResearch farm on Springs Road, Lincoln. Eggs were hatched and reared using the method

developed by Atijegbe *et al*. (2017). The larvae were fed weekly with carrot pieces for 12 weeks prior to the bioassay. Because porina moth larvae are soil insects, this bioassay was conducted using soil as the medium. Ten g of semi-sterilized Wakanui silt loam soil (see chapter 3 for soil details) was placed in a universal vial which had a screw lid. For sterilization, soil was heated to 90°C for 24 hours in a soil oven. Each treatment vial then received 1 ml of isolate C14 inoculation solution and 1 ml of 0.01% TX-100, applied with a pipettor. Untreated controls received 2 ml of 0.01% TX-100. This amount served to deliver inoculum and to rewet the soil. All vials were shaken to mix soil and solutions thoroughly before the larvae were added. Six larvae were placed individually in the soil with a carrot cube for food. A screw lid was placed on each vial before they were placed in a Contherm incubator and incubated at 25°C with a 16:8 light: dark cycle. Larval mortality was recorded 1, 5, 10, 14, 21, 28 and 35 days. This experiment had two treatments and six replicates with 6 larvae in each replicate (total =36 larvae per treatment).

6.2.2.4 *Myzus persicae* (Hemiptera)

Mixed age *M. persicae* were used from a colony maintained in the Bio-Protection Research Centre, originally collected from a cabbage field in Lincoln. The aphids were placed on sections of cabbage leaves in empty standard 100 mm diameter Petri dishes and left to settle at room temperature (20°C) overnight. These leaves were not cut to specific dimensions but were roughly the same size. The number of remaining healthy aphids was counted and reduced to 20 on one side of the leaf, and the other side was cleaned with sterile tissue paper soaked in 70% ethanol. The leaf and aphids were then placed on water agar (2%) in a separate 100 mm Petri dish. For inoculation, 300 µl of C14 containing spore solution was sprayed onto the surface of the cabbage leaf and aphids using an airbrush sprayer. Three hundred µl of sterile 0.01% TX-100 was applied to cabbage sections to create a negative control. The leaves were left to air-dry in a laminar flow cabinet and checked to make sure no aphids were lost in this process. Plates were covered with a vented lid and placed in a Contherm incubator at 25°C with a 16:8 h light: dark cycle. The number of live and dead aphids was recorded every 24 hours until six days. This experiment had two treatments and ten replicates with 20 aphids in each replicate (total = 200 aphids per treatment). Complicating the design, aphids reproduced through parthenogenesis after the experiment commenced. Reproduction slowed after the first day and only one plate showed any reproduction after day four. Since this is part of the natural lifecycle of aphids, and since the mean total number of aphids per plate did not differ significantly by the end of the experiment, the percent mortality data were analysed without trying to take the reproduction into account (*i.e.*, as a straightforward percent between 1 and 100).

6.2.2.5 *Tenebrio molitor* (Coleoptera)

Mealworm larvae were purchased from Biosuppliers (Auckland, NZ). They were maintained in containers with cornmeal at room temperature (20°C) and allowed to grow to the fourth instar

before use in the bioassay. One hundred µl of sterile water was spread onto the surface of a 90 mm diameter filter paper which was then placed in a standard 100 mm diameter Petri dish. Four larvae were placed in each dish. For inoculation, 300 µl of C14 inoculation solution was sprayed onto the paper and directly onto the larva using an airbrush sprayer. For a negative control, 300 µl of sterile 0.01% TX-100 solution was used. On the second day of the experiment a spoonful of cornmeal (approximately 10 g) was added to each plate to provide food and 100 µl of sterile water was again added to the paper to prevent drying. Plates were placed in a Contherm incubator incubated at 25°C with a 16:8 h light: dark cycle for the duration of the experiment. The numbers of live and dead larvae were recorded every 24 hours until seven days. This experiment had two treatments and ten replicates with 4 larvae in each replicate (total = 40 larvae per treatment).

6.2.3 Statistical analysis

All five experiments were analysed as randomized complete block designs with each iteration of the experiment treated as a block. Mortality of larvae was compared was a binary state variable: each larva was either dead (0) or alive (1) at the time of data recording. The data were corrected for control mortality by using Abbott's formula (Abbott, 1925). Percent mortality was analysed using an ANOVA in GenStat®, 19th edition, and each recorded day after inoculation was analysed separately, starting with the first day mortality was recorded.

6.3 Results

6.3.1 *Plutella xylostella*

Mean percent mortality of DBM larvae treated with C14 showed an increasing trend between 2 and 6 days after inoculation (Figure 6.1). Diamondback moth mortality reached 80% with C14 while the control was 40% six days after inoculation. LT_{50} (time of 50% mortality) was reached after 2.7 days. Although mortality of the treated larvae was greater than that of the control for the duration of the experiment, differences were only statistically significant at $P<0.05$ on day 5 and 6. *Plutella xylostella* cadavers were infected by *M. novozealandicum* (C14) which had produced conidia.

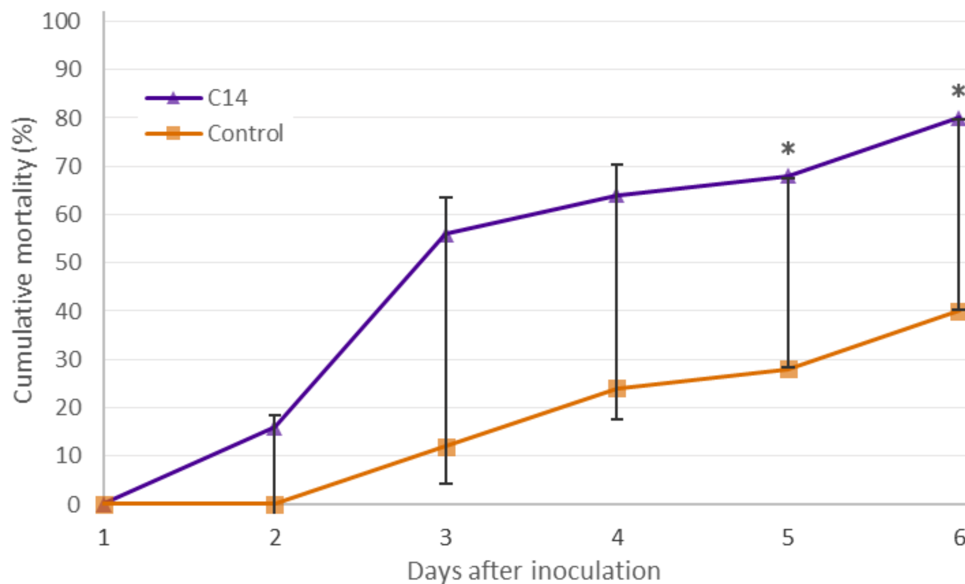


Figure 6.1 Mean percent mortality of second instar *Plutella xylostella* larvae treated with 10^8 conidia/ml of *Metarhizium novozealandicum* (C14) compared with mortality for a non-inoculated negative control. Means were statistically compared within each day post inoculation, but not between different days. Bars represent Fisher's Unprotected Least Significant Difference (LSD) test statistic based on five replicates per treatment and an acceptable error rate of α=0.05. Asterisks (*) denote recording days at which the treatment mean was significantly greater than that of the control.

6.3.2 *Helicoverpa armigera*

Mean cumulative percent mortality of larvae increased from day 3 to 10 in the C14 treatment, reaching 100% at 10 days while there was no mortality in the 0.01% TX-100 control (Figure 6.2). LT_{50} was reached at about 4.5 days. There was a significant difference in the mortality rate between C14 treated larvae and control larvae across the same period. Isolate C14 infected and produced conidia on the caterpillars (Figure 6.3).

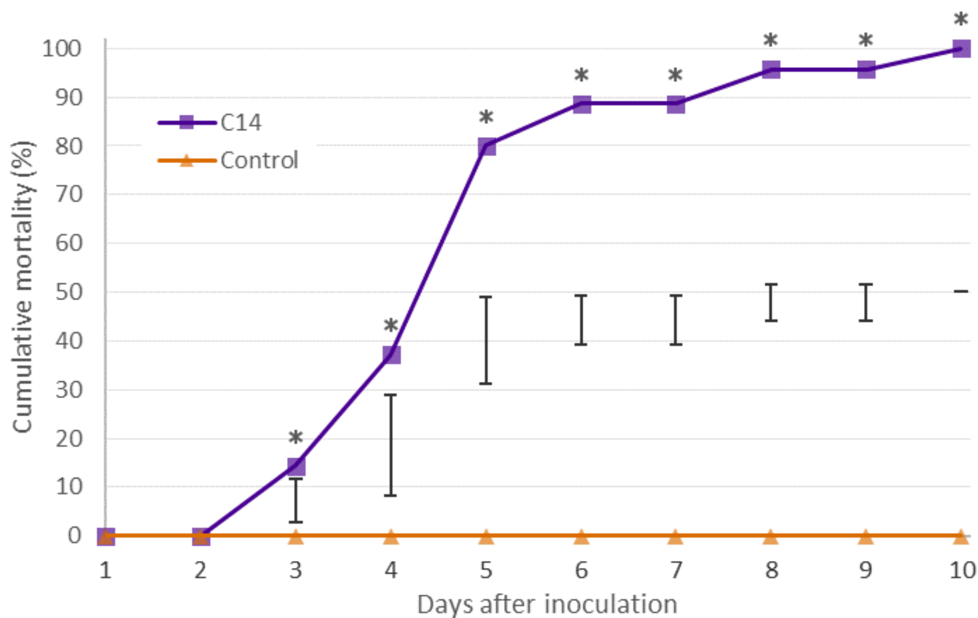


Figure 6.2 Mean percent mortality of *Helicoverpa armigera* larvae treated with 10^8 conidia/ml of *Metarhizium novozealandicum* (C14) compared with mortality for a non-inoculated negative control. Means were statistically compared within each day post inoculation but not between different days. Bars represent Fisher's Unprotected Least Significant Difference (LSD) test statistic based on seven replicates per treatment and an acceptable error rate of α=0.05. Asterisks (*) denote recording days at which the treatment mean was significantly greater than that of the control.

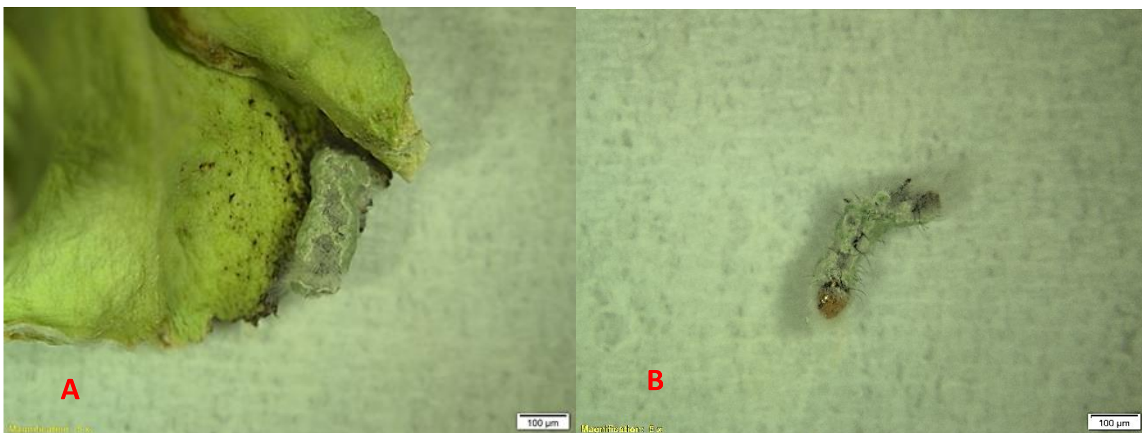


Figure 6.3 *Helicoverpa armigera* infected by *Metarhizium novozealandicum* (C14) on cabbage leaf disc (A) and *Helicoverpa* larva showing mycosis and conidiation (B). Bar = 100 µm

6.3.3 *Wiseana* sp.

Wiseana sp. mortality was not observed until 14 days after inoculation, after which mean cumulative percent mortality of C14 inoculated larvae increased for the duration of the experiment (Figure 6.4). Mortality was significantly different between the inoculated treatments and control ($P<0.05$) at 14, 21 and 28 days. Mortality in the C14 treatment reached 75% at 28 days, with the LT_{50} occurring at around 18 days. Some mortality had occurred in the triton treated control on the 28th and last day of

the experiment, with 25% of larvae dead. Mycosis caused by isolate C14 and conidiation were observed on cadavers.

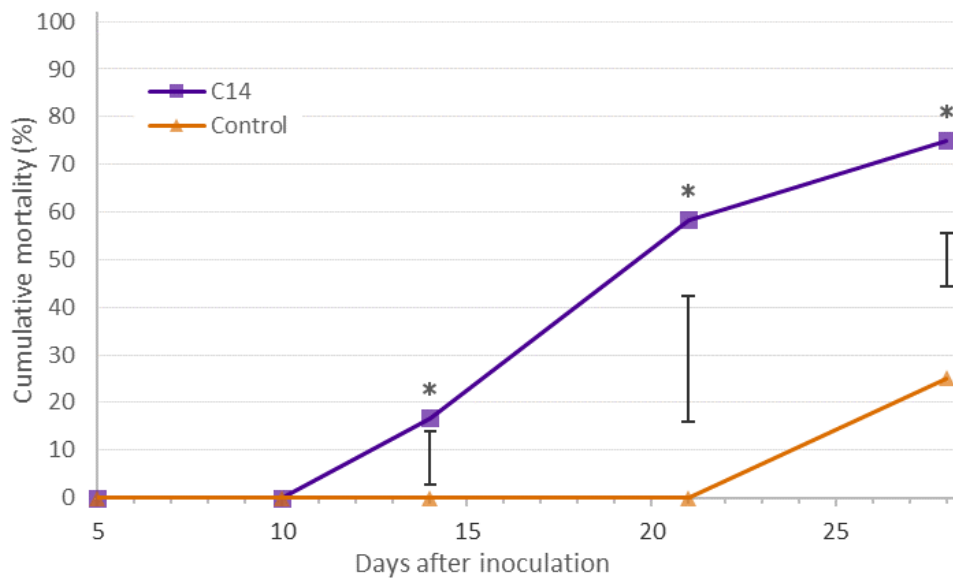


Figure 6.4 Mean percent mortality of porina larvae (*Wiseana* sp.) treated with 10^8 conidia/ml *Metarhizium novozealandicum* (C14) compared with mortality for a non-inoculated negative control. Means were statistically compared within each day post inoculation but not between different days. Bars represent Fisher's Unprotected Least Significant Difference (LSD) test statistic based on 5 replicates per treatment and an acceptable error rate of α=0.05. Asterisks (*) denote recording days at which the treatment mean was significantly greater than that of the control.

6.3.4 *Myzus persicae*

Mean cumulative percent mortality of the aphids increased from the second day after inoculation and continued to increase for the duration of the experiment for both the fungus- and no-fungus treatments (Figure 6.5). However, C14 treated plates showed significantly greater mortality than the controls from day three on ($P<0.05$). The C14 treatment reached 95% mean mortality six days after inoculation, with an LT_{50} of about 2.7 days. Infected aphids from the isolate C14 treated plates displayed mycosis and conidiation (Figure 6.6).

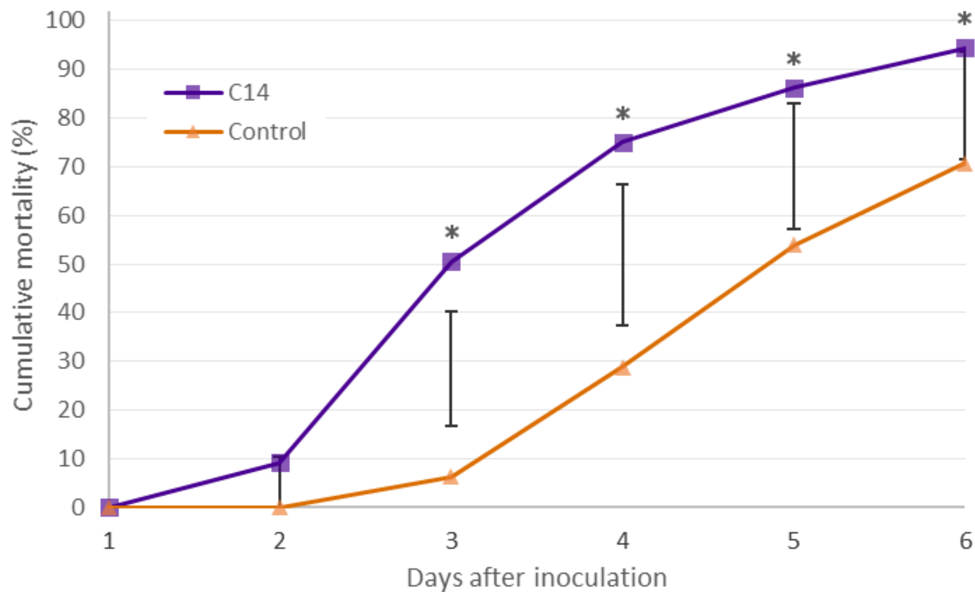


Figure 6.5 Mean percent mortality of mixed age *Myzus persicae* treated with 10^8 conidia/ml of *Metarhizium novozealandicum* (C14) compared with mortality for a non-inoculated negative control. Means were statistically compared within each day post inoculation but not between different days. Bars represent Fisher's Unprotected Least Significant Difference (LSD) test statistic based on 5 replicates per treatment and an acceptable error rate of α=0.05. Asterisks (*) denote recording days at which the treatment mean was significantly greater than that of the control.

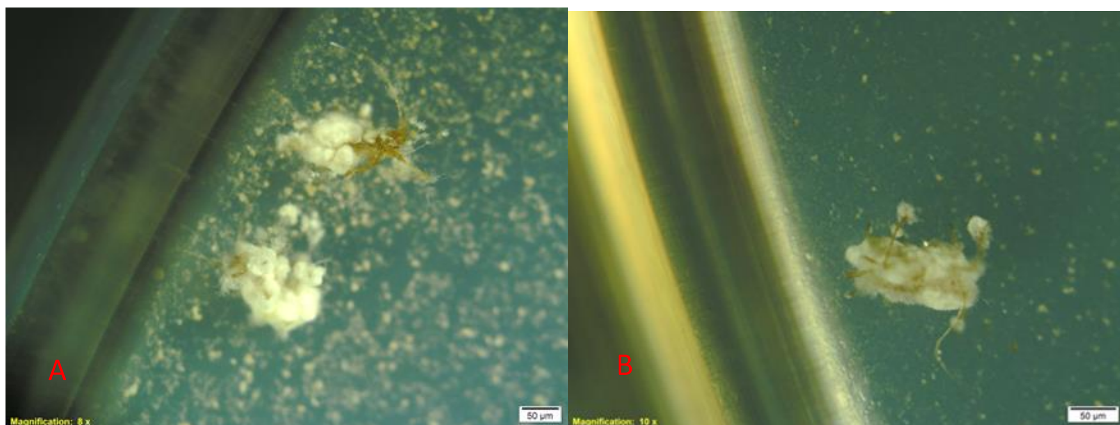


Figure 6.6 *Myzus persicae* infected by *Metarhizium novozealandicum* (C14), displaying mycosis and conidiation (A and B). Bar = 50 µm

6.3.5 *Tenebrio molitor*

Mean cumulative percent mortality of mealworm larvae increased from the third day, reaching 92.5% on the seventh and final day of observation in the C14 treatment (Figure 6.7). The LT_{50} was reached after about 3.5 days. There was no mortality in the triton-treated control. Mortality was statistically significantly higher than that of the control treatment from day three onward. The mealworm larvae were infected by the fungus and supported conidiation (Figure 6.8).

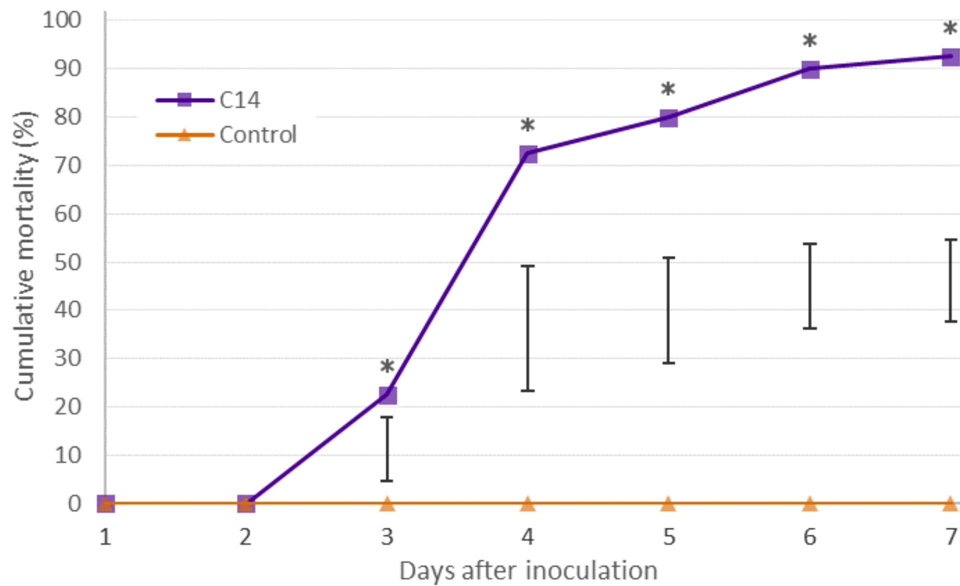


Figure 6.7 Mean percent mortality of mealworm (*Tenebrio molitor*) larvae treated with 10^8 conidia/ml of *Metarhizium novozealandicum* (C14) compared with mortality for a non-inoculated negative control. Means were statistically compared within each day post inoculation but not between different days. Bars represent Fisher's Unprotected Least Significant Difference (LSD) test statistic based on 5 replicates per treatment and an acceptable error rate of α=0.05. Asterisks (*) denote recording days at which the treatment mean was significantly greater than that of the control.

Figure 6.8 Mealworm (*Tenebrio molitor*) infected with *Metarhizium novozealandicum* (C14), showing mycosis (A) and conidiation (B). Bar = 100 µm

6.4 Discussion

The aim of this study was to explore the virulence and entomopathogenicity of *M. novozealandicum* (C14) against a range of insect larvae to provide some indication of a basic host range among pest species. This was not an extensive host range test, as that was beyond what was possible with the available insects and time, but all insects tested were susceptible. The bioassays, using a relatively

high dose of 10^8 conidia/ml, showed isolate C14 had activity against representative species of Lepidoptera, Hemiptera and Coleoptera. Moreover, infected larvae of all the species were observed supporting conidiation after death, showing that infection following exposure to 10^8 conidia/ml can result in spread of new conidia under the right conditions.

Overall, *M. novozealandicum* (C14) infected three different lepidopteran species. Both *P. xylostella* and *H. armigera* are common, widespread and economically important pests, and there have been a number of studies assessing the virulence of *Metarhizium* isolates against them. Isolate C14 was effective against DBM (*P. xylostella*) larvae, with a LT_{50} of 2.7 days and 80% mortality at day 6, while control had 40% mortality. This result could be explained, based on the life cycle of *P. xylostella,* that the larval stage for this experiment finished after five days before they pupate or die (Huaripata and Sanchez, 2019). Other reports have found similar results using *M. anisopliae sensu lato*, a species complex with a wide host range, with 84% mortality using a 10^8 conidia/ml concentration (Correa-Cuadros *et al.*, 2014) and mortality rates between 46 and 82% and LT_{50} values between 3.86 and 2.26 days using a 10^6 conidia/ml concentration (Nunilahwati *et al*., 2012). Silva *et al*. (2003) tested five *M. anisopliae sensu lato* strains and found four of the strains produced mortality ranging from 70 to 96% after eight days with the last strain inducing only 31% mortality using a 10^8 conidia/ml.

In the current study, isolate C14 was highly virulent against *H. armigera* with a LT_{50} of 4.5 days, an 80% mortality by day eight and a 100% mortality at day ten. These results suggest a higher virulence than most previously tested *M. anisopliae sensu lato* strains under the same inoculation concentrations (10^8 conidia/ml) and incubation periods. Tahir *et al*. (2019) tested ten *Metarhizium* strains on second instar larvae and got results ranging from 48 to 84% mean mortality using a 10^8 conidia/ml concentration. Fite *et al.* (2020) tested three *Metarhizium* strains on third instar larvae and measured mean mortality of 20, 56 and 73% using a 10^8 conidia/ml concentration for one larvae. Vijayavani *et al.* (2010), however, tested two highly virulent *Metarhizium* strains achieving 100 and 92% mortality by day eight using the same concentration. In another study that tested 62 *Metarhizium* strains, mostly from unidentified species, using a 10^7 conidia/ml inoculation solution for one larvae and 14 day incubation period, a wide mortality range from 36 to 92% was found against *H. armigera*, but the three strains producing the highest mortality were identified as *M. anisopliae sensu stricto* (Kulkarni *et al.*, 2008).

Metarhizium rileyi (until recently known as *Nomuraea rileyi* (Kepler *et al.*, 2014)) a *Metarhizium* species that has a relatively narrow host range primarily limited to the lepidopteran families Noctuidae, Erebidae, and Nymphalidae is considered a prospective biocontrol agent (Binneck *et al.*, 2019). Although it may be expected that host specialization would affect virulence, *M. rileyi* has not consistently shown greater or lesser virulence than related host generalists when tested against

either *P. xylostella* or *H. armigera*. Although some studies have shown very high virulence (*P. xylostella*: Duarte *et al.*, 2016; *H. armigera*: Iqtiat *et al*., 2009) others have produced results on the mid to lower ends of the mortality ranges achieved using *M. anisopliae* (*H. armigera*: Iqtiat *et al*., 2009; Hatting, 2012). In a study including both strains of *M. anisopliae* and a strain of *M. rileyi*, the *M. rileyi* strain did not stand out as especially virulent (Jun, 2000). The mortality caused by isolate C14 in this experiment was comparable to the mortality caused by *M. rileyi* in both insects in these previous studies. Field tests have been performed using *M. anisopliae sensu lato* against *P. xylostella* (Nguyen & Vo, 2007) and *M. rileyi* against *H. armigera* (Iqtiat *et al*., 2009), and both authors found significant reductions in both larvae/m^2 and damage in cruciferous and tomato crops, respectively. Given the comparable laboratory results of isolate C14 to both of these fungi, this suggests that C14, and other isolates of *M. novozealandicum*, also have potential for use in the field.

The bioassay on porina moth (*Wiseana* sp.) larvae showed that isolate C14 was also infective and at least moderately virulent against porina. Although the different inoculum application method makes comparisons to the other insects tested difficult; this application method was used because porina larvae spend much of their time underground, coming out on the grass surface at night to feed, and this bioassay method better approximated field application. Mortality of porina was slightly lower but still close to the mortality isolate C14 produced in *C. giveni* (see Chapter 2), tested under similar inoculation conditions. Porina are a different case than the other lepidopteran insects in this study. As a pest localized to New Zealand, it has had relatively little study regarding biocontrol. The study of Latch & Kain (1983) is the only one that tested *Metarhizium*, a strain from *M. anisopliae sensu lato*, against porina, and the methods used therein render it incomparable to the current study because it was affected by soil temperature. However, when testing this *Metarhizium* strain in a field trial using an inoculum bait method, they found significant increases in infected larvae compared with the non-inoculated control.

The bioassay on the green peach aphid, *M. persicae*, produced high levels of both parthenogenesis (clonal reproduction) in both treatments and mortality in the control treatment. When performing bioassays with *M. persicae*, reproduction is not uncommon, and previous studies have left the nymphs in the plates and calculations (Vu *et al*., 2007, for example), or taken them out as they appear (Shan & Feng, 2010, for example). Some mortality in control treatments is not unusual, but the level recorded in this study was high; 70% mortality in the control at six days after inoculation because some aphids gave birth after one day and died to finish the life cycle. Shan & Feng (2010) tested 23 *Metarhizium* isolates and recorded levels of mortality from 5 – 20% under similar conditions. Regardless of these issues, there was a significant increase in mortality over most of the incubation period. This shows that isolate C14 was at least moderately virulent towards *M. persicae*. Abbott’s formula (Abbott, 1925) for corrected mortality is useful when comparing studies with

mortality in the control treatment, although generally not for mortality levels in the control of over 20%. In this study, mortality after six days was 95% corresponding to an 83% corrected mortality. This is comparable to that of a number of other tested strains of *M. anisopliae sensu lato* (Shan & Feng, 2006; Vu *et al.*, 2007; Shan & Feng, 2010; Lefort *et al.*, 2015; Mohammed *et al.*, 2018) and *Metarhizium acridum* (Shan & Feng, 2010). Other studies have found very high or total mortality using other strains of *M. anisopliae sensu lato* (Butt *et al.*, 1994; Loureiro & Moino Jr., 2006; Shan & Feng, 2006; Lee *et al.*, 2015; Yun *et al.*, 2017) and *M. flavoviridae sensu lato* (Lee *et al.*, 2015). Vu *et al.* (2007) tested a strain of *M. rileyi*, a species generally considered to be host specific to the lepidopteran families Noctuidae, Erebidae, and Nymphalidae, against *Myzus persicae* using a 10^7 conidia/ml concentration and reported 87% corrected mortality.

Mealworms (*T. molitor*) have been of interest to researchers for many years for a variety of reasons. A major reason they are used is that they are very easy to rear in large numbers. They are a pest of stored food products and also are produced as food for both humans and livestock (Grau *et al.*, 2017; Vigneron *et al.*, 2019). They are also used as trap insects for isolating entomopathogenic fungi (Jia *et al.*, 2006; Sharma *et al.* 2018), proxies to test the virulence of entomopathogenic fungi for use on other insects (*e.g.*, Bharadwaj & Stafford, 2011) and even as model organisms for the study of human fungal diseases (Canteri de Souza *et al*., 2018). As such, a number of studies have tested the virulence of *Metarhizium* species against mealworms. In the current study, *M. novozealandicum* C14 was highly virulent against the mealworm larvae with an LT_{50} of 3.5 days and 92.5% mortality after seven days incubation. *Metarhizium anisopliae sensu lato* can be very virulent towards *T. molitor*, reaching 95 to 100% mortality or higher after 7 days, even at lower inoculum concentrations than used in this study (Skrobek *et al.*, 2008; Skalický *et al.*, 2014; Lestari & Rao, 2016). *Metarhizium brunneum*, a species from the *M. anisopliae* complex, also has produced 100% mortality in other studies (Bharadwaj & Stafford, 2011; Lestari & Rao, 2016). Other studies have found lower mortality or slower rates of mortality (Oreste *et al.*, 2012; Simamora *et al.*, 2013; Skalický *et al.*, 2014), and in one study, neither of two tested strains achieved on Abbott-corrected mortality of over 11% (Adatia *et al.*, 2010). Skalický *et al.* (2014) tested eight *M. anisopliae sensu lato* strains and found that five were highly virulent (97.3 to 100% mortality after 7 days) while the other three achieved only low mortality. Kos *et al.* (2020) found that *M. guizhouense*, another species from the *M. anisopliae* complex, failed to infect *T molitor* at all, although it successfully infected the waxworm, *Galleria mellonella* in the same study. These studies highlight the importance of strain and species host-sensitivity in *Metarhizium*. Chapter 2 of this thesis reported differences between strains and species: isolate C14 and another isolate of *M. novozealandicum* were also found to be highly virulent against another coleopteran species, *C. giveni*, while a third *M. novozealandicum* strain in the same test produced much lower mortality.

These laboratory bioassays to characterise its host range demonstrated that isolate C14 can infect insects belonging to three different orders and produce conidia on the cadavers. In all tests, strain C14 was comparable to virulence reported in the literature of both broad host-range and more host specific *Metarhizium* strains from other species. This suggests that isolate C14, and *M. novozealandicum* as a species, has a broad host range and likely has the potential to infect insects outside of the orders Lepidoptera, Hemiptera and Coleoptera as well, although this was not tested. This also shows the potential of isolate C14 to be useful as a biocontrol agent. This broad host range means that it can be used for a wide range of pests and may be able to remain in the environment longer due to the availability of alternate hosts, but this also requires a need for caution. A broad host range creates concerns about impacts on non-target species and a greater need to investigate impacts on beneficial invertebrates before testing in the field.

Chapter 7

Determining the in-field distribution of *Metarhizium*

7.1 Introduction

Beneficial soil-borne organisms are an important component of healthy agricultural soils (Magdoff, 2001). Soil borne entomopathogenic fungi can contribute to the control of insect pests (Keller & Zimmerman, 1989; Fuxa, 1998; Lacey *et al.*, 2001; Shah & Pell, 2003). *Beauveria bassiana* and *Metarhizium anisopliae*, for example, are the natural enemies of many insect pests in agriculture (Meyling & Eilenberg, 2007, Vega *et al.,* 2009, McGuire & Northfield, 2020).

Metarhizium spp. are usually one of the most common entomopathogenic fungi isolated from infected insects and agricultural soils (Vanninen, 1995; Barker & Barker, 1998; Bidochka *et al.*, 1998; Sosa-Gomez *et al.*, 2001; Asensio *et al.*, 2003; Keller *et al.*, 2003; Bruck, 2004; Sapieha-Waszkiewicz *et al.*, 2005; Meyling and Eilenberg, 2006; Quesada-Moraga *et al.*, 2007; Derakhshan, 2009; Goble *et al.*, 2010; Hussein *et al.*, 2010; Meyling *et al.*, 2011; Shin *et al.*, 2013; Clifton *et al.*, 2015; Tkaczuk, 2019) and forest soils (Imoulan *et al.*, 2019) across the world, from the subarctic to the subantarctic (Jaronski, 2007). When comparing agricultural fields to adjacent natural areas such as hedgerows, *M. anisopliae* *sensu lato* abundance was often not affected by cultivation while species from other genera usually were negatively affected (Bidochka *et al.*, 1998; Klingen *et al.*, 2002; Meyling & Eilenberg, 2006; Jaronski, 2007; Goble *et al.*, 2010; Meyling *et al.*, 2011; Ingilis *et al.*, 2019). *M. anisopliae* has often been positively associated with agriculture in large-scale surveys (Vanninen, 1995; Barker & Barker, 1998; Rodrigues *et al.*, 2005; Quesada-Moraga *et al.*, 2007; Shin *et al.*, 2013).

There is some understanding about the importance of interactions between abundance of *Metarhizium* spp. and habitat type. Although clear, predictable patterns have not yet been elucidated due at least in part to the ubiquity of the genus, the sensitivity of detection methods (Klingen *et al.*, 2002; Schneider *et al.*, 2011) and unclear species boundaries, cultivation methods and environmental factors have had demonstrable effects. Therefore, studying the distribution of *Metarhizium* spp. across a wide geographic scale and variety of habitats is necessary to capture the true diversity and distribution.

Some agricultural practices have been shown to affect *Metarhizium*, although results are sometimes conflicting. There was not a difference in the abundance of *M. anisopliae* between highly organic and less organic soil in agroecosystems in Denmark (Meyling *et al.*, 2011), but soils harboured a greater abundance of the same fungus in the U.S. Midwest (Clifton *et al.*, 2015) and in northern Norway

(Klingen *et al.,* 2002). Results from studies looking at tillage practices have also shown a significant but inconsistent pattern with Sosa-Gomez *et al.* (2001) and Clifton *et al.* (2015) finding that no-till practices increased populations of *M. anisopliae* and Kepler *et al.* (2015) finding the opposite. Field applications of herbicides and fungicides have been reported to have little effect on *M. anisopliae* (Jaronski, 2007; Clifton *et al.*, 2015), but Tkaczuk and Majchrowska-Safaryan (2019) found that herbicide had a negative effect. Although *Metarhizium* is often less or equally abundant in hedgerows and field borders when compared to neighbouring pasture, Meyling and Eilenberg (2007) suggest that these landscape features may serve as refugia for insect hosts, thus affecting populations of *Metarhizium*, a genus known for its wide host range (Roberts & St Leger, 2004). Supporting this idea, studies have found that permanent grasslands (Keller *et al.*, 2003, Rodrigues *et al*., 2005; Schneider *et al.*, 2012) and improved field margins (Schneider *et al.*, 2012) harbour an abundance of *Metarhizium* species, and these researchers suggest that having these habitat types in the agricultural landscape benefits insect control. *Metarhizium anisopliae* has shown enhanced persistence in the rhizosphere of plants (Hu & St Leger, 2002), and several *Metarhizium* species are known to be endophytes as well as entomopathogens (Hu & Bidochka, 2019).

Habitat factors have direct effects on *Metarhizium*. Combining *in vitro* characterization of fungi with environmental data, Bidochka *et al*. (2001) found that two separate clades in the *M. anisopliae* species complex could be significantly associated with different traits. One clade with higher UV and heat tolerance was associated with cultivated field sites while the other clade showed higher cold tolerance and was associated with forests. In large scale surveys, *Metarhizium* species have been less frequently encountered as latitude (Vanninen, 1995; Ingilis *et al.,* 2019) and altitude (Ingilis *et al.*, 2019) increased. In both studies, entomopathogenic fungi of other genera were either not affected or even showed positive correlations with these variables.

Regarding specific biotic and abiotic factors, several studies have reported multivariate analyses on *M. anisopliae* populations surveyed in the field. Rath *et al*. (1992), Bidochka *et al.* (1998) and Meyling *et al.* (2011) found no significant effect of any variables tested, but Rath *et al*. (1992) noted that fungi were isolated from loam and clay loam soils more frequently than from sandy loam and clay in Australian pasture soils. Several other studies found effects of soil conditions on *M. anisopliae* abundance. For example, a positive correlation with organic matter content, but no correlation with pH or soil conductivity was found by Quesada-Moraga *et al.* (2007). Clifton *et al*. (2015) found a positive correlation with organic fertilizers and higher silt composition, but negative correlations with nitrogen content and Jaronski (2007) showed that both high clay and high sand content in soils adversely affected insect mortality due to *M. anisopliae*. In another study, soil type had no effect on occurrence of any entomopathogenic fungi (Vanninen, 1995). The weather has been found to affect the occurrence of *Metarhizium*. In one study, *M. anisopliae* was found in higher numbers during

spring and autumn seasons. Minimum relative humidity and both minimum and maximum air temperature correlated with *M. anisopliae* abundance while soil temperatures had no significant effect (Hussein *et al.*, 2010). This suggests that these weather effects were not directly acting upon the fungi but affecting their insect hosts.

The grass grub *C. giveni* is an important pasture pest in New Zealand, and there is interest in *Metarhizium* as a biological control agent for use in its control. In addition to *M. anisopliae*, *M. novozealandicum* has been isolated from *C. giveni* larvae (Chapter 2). Generally, 5-10% of collected grass grubs are infected by *Metarhizium* (Glare pers. comm.), which suggests that the spatial distribution is patchy.

In this chapter, the soil distribution of *Metarhizium*, a genus of fungi best known for their role as entomopathogens and biocontrol agents (Aw & Hue, 2017; Brunner-Mendoza *et al.*, 2019) was examined, with the goal of better understanding their ecological niche as control agents of insects in agroecosystems. There was little information about the distribution of entomopathogens in pasture soils, so the aim of this chapter was to study the spatial and temporal distribution of *Metarhizium* in three different regions of New Zealand, and to explore the relationship of the distribution to grass grub populations.

7.2 Material and methods

7.2.1 Methodology for soil sampling

For this study, soil samples were collected from the South Island of New Zealand within three different locations: Lincoln (at the Lincoln University Dairy Research Farm on the edge of the town of Lincoln), Oxford (near Eyrewell Forest) and West Coast (in the Atarau area of the West Coast region) during three seasons (summer, autumn and winter) in 2019. The geographical details of the sites were recorded using global positioning system (GPS) equipment, and their positions are given in Figure 7.1.

Figure 7.2 shows the sampling times and how they relate to the annual lifecycle of *C. giveni*. During the summer season, samples were collected from 18th to 25th February 2019. During the autumn season, samples were collected from 5th to 25th May 2019. During the winter season, samples were collected from 10th to 28th August 2019. At each site, different farming regimes were practiced. Ryegrass pasture had been grown for about six years at Lincoln and West Coast (Atarau) and for four years at Oxford, prior to sampling. Lincoln and Oxford were regularly irrigated whilst the West Coast site was not irrigated. These sites were chosen to investigate the number of grass grub larvae in different environments.

Figure 7.1 Map of New Zealand showing the location of the three sampling sites. The base map was modified from NZ 10 m Satellite Imagery (2018-2019) created by Land Information New Zealand (LINZ), inset modified from NZ Digital Elevation Map created by Manaaki Whenua Landcare Research NZ Ltd. Used under the Creative Commons Attribution 4.0 International License and the Landcare Data Use License, respectively.

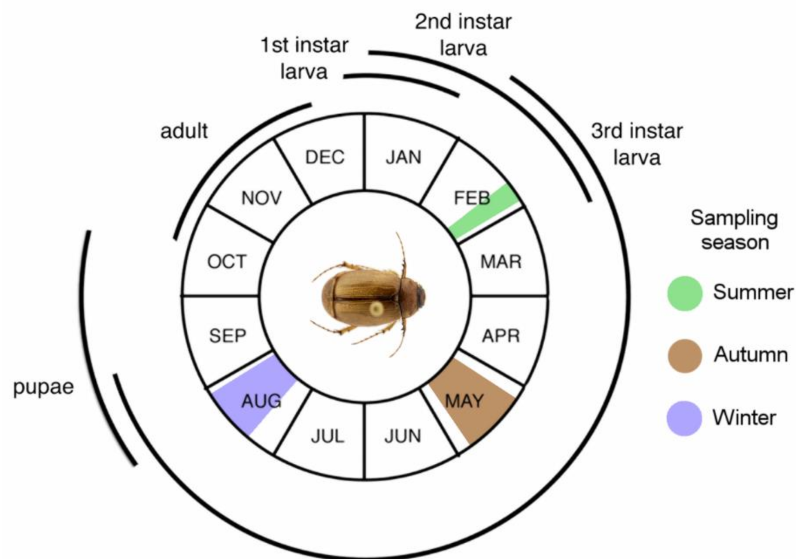


Figure 7.2 The sampling periods used in this study overlaid on the annual, univoltine life cycle of *Costelytra giveni.* Modified with permission from Lefort (2013).

At each site, soil samples were taken from several locations in the field in a zig-zag pattern. A 15 cm by 15 cm square area of soil was excavated to count grass grub larvae. Two soil samples were collected from two different depths, 0-5 cm and 5 - 10 cm, from each of these larger soil samples, with a further 200 grams of soil collected using a spoon and following the guidelines of Imoulan *et al.* (2019). The soil (200 grams of soil from each depth) was stored at 4°C for later use in quantifying and identifying *Metarhizium*. Between each use, the spoon was dipped in 70% ethanol to avoid cross contamination. Following this, *C. giveni* larvae in the larger samples were counted to test whether there was a spatial relationship between *Metarhizium* presence and larval counts in the field. This was repeated in each of the three seasons to account for and characterize seasonal changes in the abundance of *Metarhizium* strains. The smaller 200 g samples were placed in polyethylene bags and then immediately transferred to Lincoln University and stored at 4°C for no longer than one week before further processing. There were 450 soil samples, 25 each at three field sites, across three seasons and two depths. Samples of each soil type (500 g) were collected from 5 random positions and mixed, and sent to Hill Laboratories (Hamilton, New Zealand) for determination of general chemical characteristics, including pH, contents of phosphorus (P), potassium (K), calcium (Ca),

magnesium (Mg) and sodium (Na); cation exchange capacity (CEC), total base saturation, and organic matter and total carbon contents (Table 7.1).

Table 7.1 Characteristics of three soil types within three different locations in the South Island of New Zealand (Hill Laboratories)

Soil parameters		Location (Soil type)		
		Lincoln (Templeton silt loam)	West Coast (Stillwater loam)	Oxford (Lismore silt loam)
pH		5.5	6	6
Olsen Phosphorus (mg/L)		22	43	35
Potassium (me/100g)		0.43	0.55	0.81
Calcium (me/100g)		7.3	15.4	14.2
Magnesium (me/100g)		0.98	0.81	2.47
Sodium (me/100g)		0.18	0.25	0.12
CEC (me/100g)		15	24	25
Total Base Saturation (%)		61	71	70
Volume Weight (g/mL)		0.88	0.72	0.73
Sulphate Sulphur (mg/kg)		9	17	24
Potentially Available Nitrogen (15cm depth) (kg/ha)		103	341	106
Anaerobically Mineralisable N (µg/g)		79	316	96
Organic Matter (%)		5.7	13.1	11.4
Total Carbon (%)		3.3	7.6	6.6
Total Nitrogen (%)		0.26	0.61	0.47
C/N Ratio		12.7	12.6	14.3
Anaerobically Mineralisable N/Total N ratio (%)		3	5.2	2.1
Soil Sample Depth (mm)		100	100	100
Base Saturation %	K	2.9	2.3	3.2
	Ca	50	65	56
	Mg	6.7	3.4	9.9
	Na	1.2	1.1	0.5
MAF Units	K	8	8	12
	Ca	8	14	13
	Mg	19	13	41
	Na	7	8	4

7.2.2 Isolation of *Metarhizium* species

Each soil sample was plated on selective medium to determine quantity of *Metarhizium*. Ten grams of soils was removed from each soil sample collected from two different depths (0-5 cm and 5 - 10

cm) and mixed into 90 ml of sterile 0.01% TX-100. The soil + Triton (TX) suspensions were homogenised using a Stuart flask shaker (Staffordshire, UK) at 300-400 rpm for 30 min at room temperature. The initial soil suspension was considered the 10^0 dilution from which further dilutions were prepared. Serial dilutions from 10^{-1} to 10^{-4} were made by taking 1 ml of each dilution and adding it to 9 ml of sterile 0.01% TX-100. Each dilution was plated on MSM (see Chapter 2) with two plates per dilution. One hundred µl of each dilution was spread over the surface of a MSM plate and incubated at 25°C with a 16h light/8h dark photoperiod. The inoculated plates were observed daily for 6-7 days after which *Metarhizium* colonies were visually identified, counted to calculate colony forming units (CFU) per g of soil and individual colonies transferred to fresh PDA medium for further identification. To confirm their identification as *Metarhizium,* conidia were observed using a Leica DM2500 microscope with an Olympus SC100 camera. For CFU/g soil counts, either 10^{-3} or 10^{-4} dilutions were used due to excessive non-target microbial growth on the less diluted plates; only plates representing one dilution were counted for each sample. The raw counts were then multiplied by 1000 (to scale the 100 µl aliquot to the 100 ml initial soil suspension), divided by the dilution and divided by ten (grams of soil) to arrive at the final value.

7.2.3 Molecular identification of recovered *Metarhizium*

Metarhizium isolates were selected based on morphology and identified using polymerase chain reaction (PCR) followed by Sanger sequencing. Elongation factor 1-alpha (EF1α) (Bischoff *et al*., 2009; Kepler *et al*., 2014; Stielow *et al*., 2015; Meyer *et al*., 2019) was used as barcodes for fungal identification (Chapter 2). There were 39 samples in total, including 15 samples from Lincoln, 15 samples from West Coast and nine samples from Oxford. DNA was extracted using the method of Alizadeh *et al*. (2017).

PCR was run as described in Chapter 2 with the primer pair used to target the EF1α gene region were 983F (GCY CCY GGH CAY CGT GAY TTY AT and 2218R (ATG ACA CCR ACR GCR ACR GTY TG (Rehner & Buckley 2005).

All amplified products were sequenced at the Lincoln University Sequencing Unit (Lincoln, NZ) and the sequences generated were edited and assembled using ChromasPro software (http://www.technelysium.com.au/ChromasPro.html) before being compared to the nucleotide GenBank database (Alizadeh *et al*., 2017).

7.2.4 Data analyses

The data were expressed as mean grass grub larvae per square metre for larval abundance and colony-forming units (CFU) per gram of soil and percentage of positive soil samples for *Metarhizium* abundance and incidence. A standard error of the mean (SE) was calculated to show the variability

within each field on each sampling date. However, with just one field per area, these SEs cannot be used to draw general inferences about how one area compares to another (as an average over all possible fields and years). For each season, the relationship between (*y*) grass grub larval density and (*x*) *Metarhizium* colony-forming units (CFU) per gram of soil was examined by fitting a regression to a plot of *y* versus *x*, using the three site means as points.

7.3 Results

7.3.1 *Metarhizium* abundance

Overall, the trends varied across seasons (Figure 7.3), but Lincoln soil consistently had the highest CFU counts at both depths in all seasons. While Oxford generally had few *Metarhizium*, this changed in the winter season. Colony forming unit counts increased in winter at Lincoln and Oxford but there was little change over the seasons in the West Coast soil and in summer and autumn at Lincoln. In the summer collection, Lincoln and West Coast produced roughly equal CFU counts when comparing soil depths within sites while the Oxford site produced very few and none at the 0-5 cm and 5-10 cm depths respectively. In autumn, Lincoln produced the most *M. novozealandicum* colonies at both soil depths. At the 0-5 cm depth, West Coast and Oxford produced similar numbers of CFU, and at the 5-10 cm depth, West Coast produced fewer, but still appreciable, CFU counts while the Oxford soil produced none. Contrary to the pattern found in the other seasons, winter CFU counts were greater at Oxford than at West Coast at both soil depths and in total.

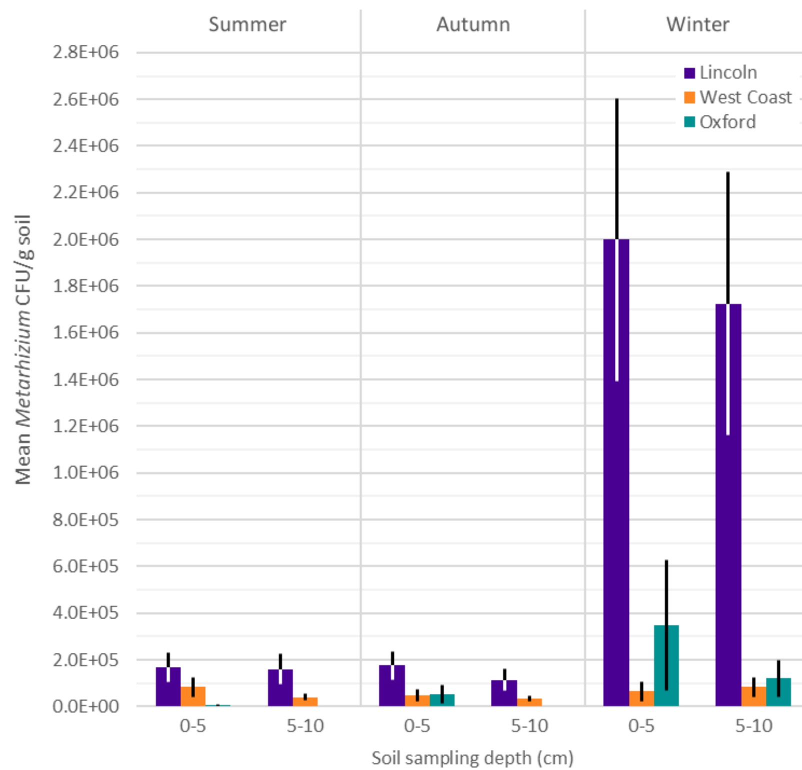


Figure 7.3 ***Metarhizium*** **CFU over 25 soil samples collected across three seasons on the three different field sites at two depths. Each SE measures the variability within one field on one sampling data in just one year, so cannot be used for extrapolating to other fields within the same area.**

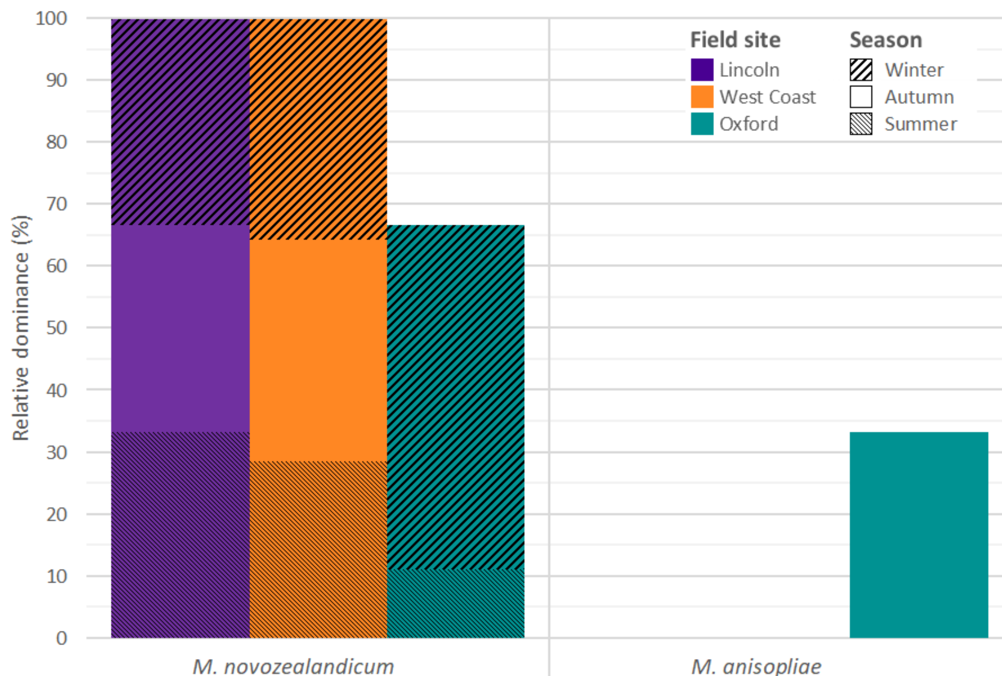


Figure 7.4 Recovery of *M. novozealandicum* and *M. anisopliae* at three field sites across three sampling seasons.

Only two *Metarhizium* species were found across all soil samples: *M. novozealandicum* and *M. anisopliae* (Figure 7.4). The most common species found was *M. novozealandicum*, which was the

only species detected at all sites except for one site in one season; *M. anisopliae* colonies were found in the autumn season at Oxford but were not found in any other season nor in any other site.

7.3.2 Grass grub abundance

In general, the mean abundances of grass grub larvae were higher at a depth of 0-5 cm while there were usually small numbers or none at 5-10 cm (Figure 7.5). Only during the summer season were grass grubs found at both depths, but abundance was nevertheless higher at 0-5 cm than at 5-10 cm. In the autumn and winter season samples, grass grubs were only found in the 0-5 cm samples. In all seasons and depths, when larvae were present at all, larval abundance was highest at Oxford. At 0-5 cm, Lincoln harboured the second highest abundance while West Coast had the lowest except for in the winter season where Lincoln's larva count dropped to equal that of West Coast's. Overall, the Oxford soil harboured by far the highest grass grub abundance and West Coast the lowest with very few across all seasons.

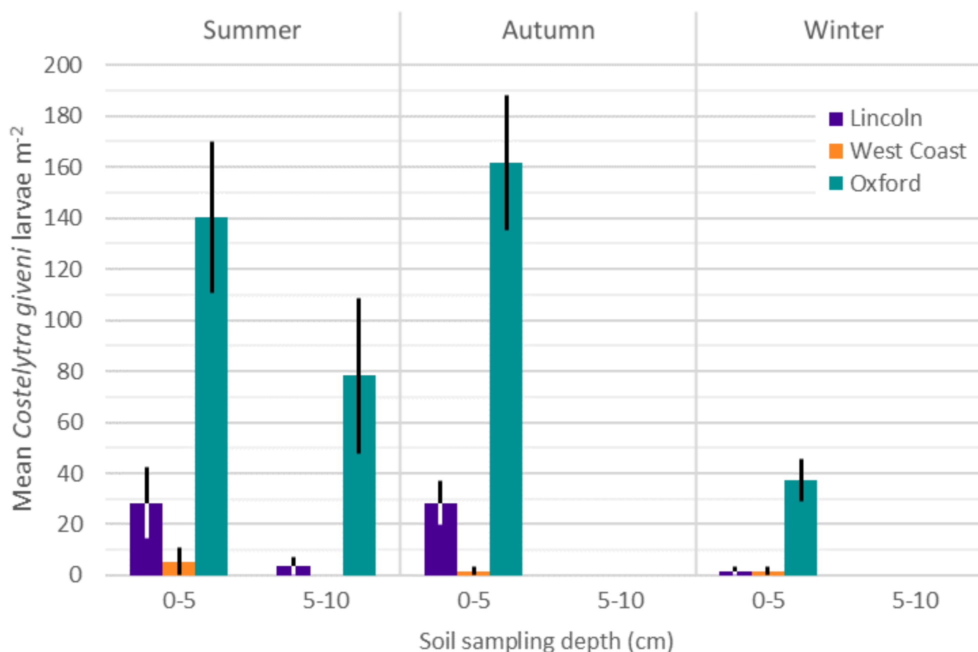


Figure 7.5 Mean grass grub abundance over 25 soil samples collected across three seasons on the three different field sites. Each SE measures the variability within one field on one sampling date in just one year, so cannot be used for extrapolating to other fields within the same area.

7.3.3 Comparing grass grub and *Metarhizium* presence and abundance across the three seasons

Grass grubs were found in a much higher proportion of soil samples at 0-5 cm than at a depth of 5-10 cm across all seasons (Figure 7.6). At the lower soil depth, they were only found, albeit in a much smaller proportion, in the summer season. The grass grub presence data followed almost identical trends to those of the larval abundance data.

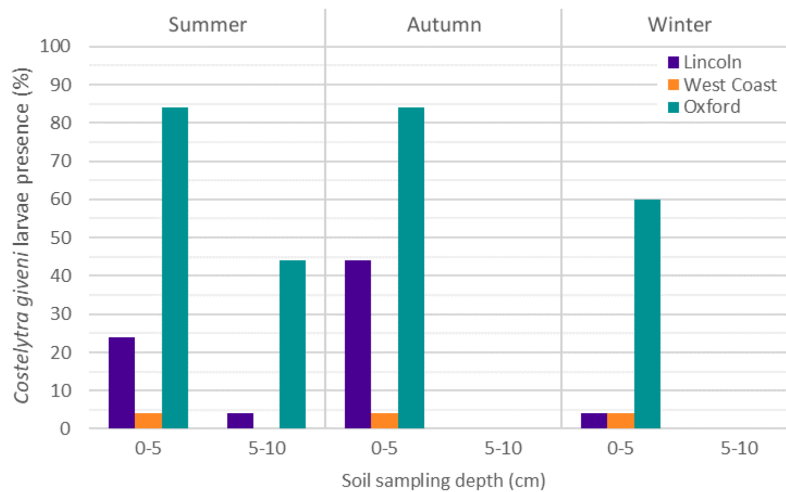


Figure 7.6 Grass grub presence presented as the percentage of total soil samples containing at least one grass grub from 25 samples collected across three seasons at two depths.

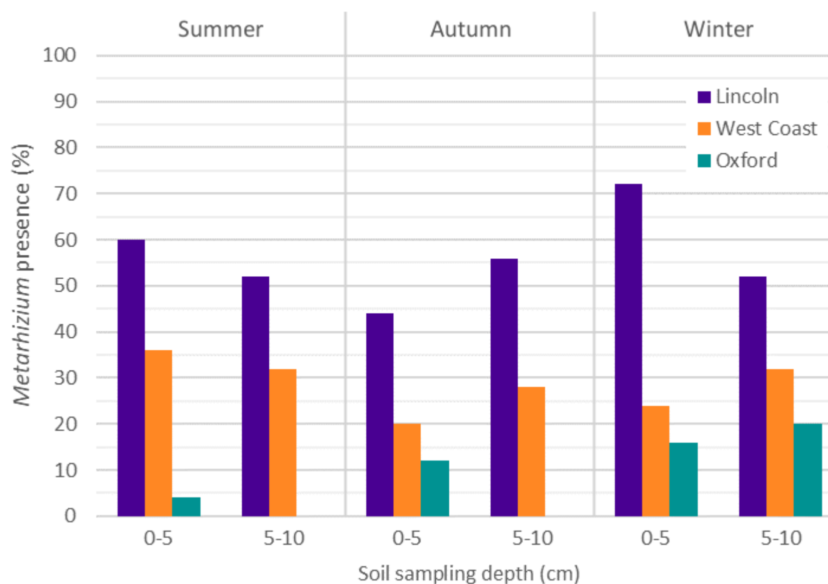


Figure 7.7 *Metarhizium* presence presented as the percentage of total soil samples producing at least one colony in inoculated agar plates. Twenty-five soil samples were collected from three sites across three seasons at two depths

The *Metarhizium* presence data followed similar trends to the abundance data but the relative differences between sites was somewhat attenuated (Figure 7.7). The high increases seen in the winter sampling in the abundance data were not seen in the presence data, and in collections where Oxford produced more CFU/g of soil than West Coast. However, *Metarhizium* at Oxford was found in fewer soil samples than West Coast. Overall, this suggests that the large disparities between sites seen in the abundance data was the result of greater concentrations within soil samples rather than *Metarhizium* being found in more soil samples, *i.e.*, greater patchiness rather than less patchiness.

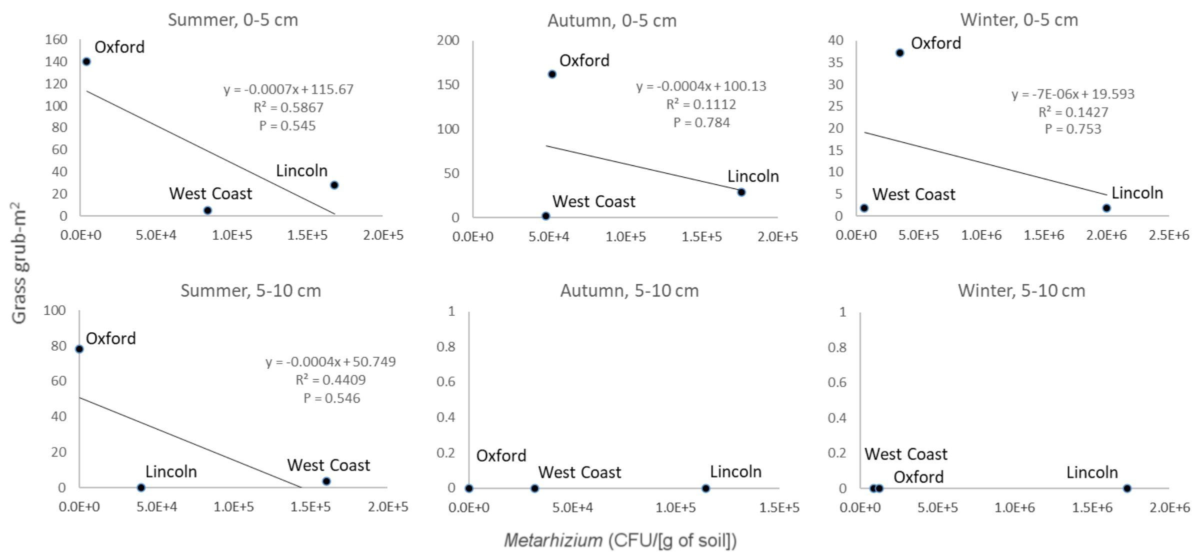


Figure 7.8 Correlation between grass grub and *Metarhizium* colony abundance at both depths and in three different seasons

Correlations between grass grub abundance and *Metarhizium* colony abundance were analysed separately at each of the three sites in each of the three different seasons (Figure 7.8). No significant correlation between grass grub density and *Metarhizium* colonies density was found in any analysis.

7.3.4 Environmental conditions at three field sites in three seasons

Temperature maximum and minimum (^{o}C), relative humidity (%) and soil moisture (%) were recorded at the three field sites during the three sampling seasons (Figure 7.9). However, there was no rainfall during the sample collection. The mean temperature was higher at the Lincoln and West Coast sites than the Oxford site in each season. In addition, relative humidity at the Lincoln site was lower than the West Coast and Oxford sites in the summer and autumn. Soil moisture at West Coast was highest in all three seasons.

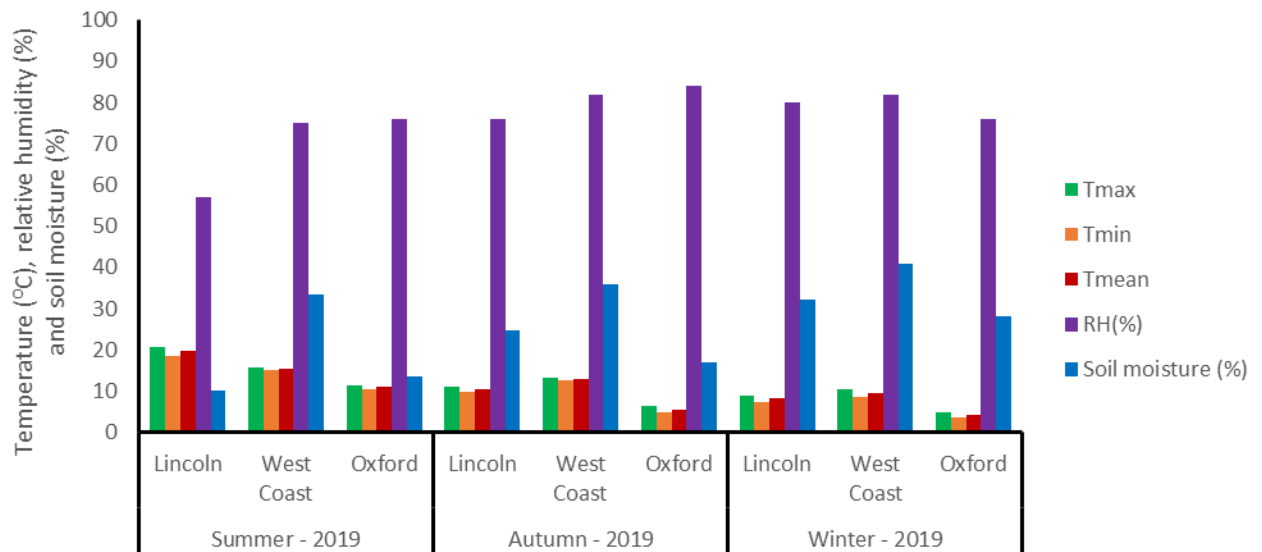


Figure 7.9 Environmental conditions at three field sites across three sampling seasons in 2019 (Cliflo-niwa, 2021)

7.4 Discussion

In this study, the extent of the relative prevalence of *Metarhizium* species at two soil depths, across three different seasons and at three geographically separate pasture field sites was determined to provide some indication of distribution. *M. novozealandicum* and *M. anisopliae* were the only *Metarhizium* species identified, with *M. novozealandicum* found at all soil depths at all three sites, and *M. anisopliae* found in the 0-5 cm deep soil layer in the autumn season only at Oxford. However, the sample size was small in this study, sampling from more sites is required to test whether *M. novozealandicum* is dominant in soils of South Island New Zealand pastures. Both species have been recorded as pathogens of grass grubs in New Zealand (Glare *et al.,* 1993a; Liu *et al.*, 2020). Moreover, *M. novozealandicum* has also been isolated from larvae of *Wiseana* spp. in New Zealand (Liu *et al.,* 2020), and *M. anisopliae* has been recorded from *Wiseana* sp., *Crambus* sp. (Lepidoptera*), Mythimna separata, Persectania aversa, Scellodes cordalis, Heteronychus arator, Pericoptus truncatus, Listronotus bonariensis* (Coleoptera) and unidentified wireworm larvae (Coleoptera) (Glare *et al.*, 1993a). *Wiseana* and *Listronotus* also occur in South Island pastures (Morris *et al*., 2016).

The soils tested in this study yielded high and variable CFU/g estimates whenever *Metarhizium* were present. Although high variability may have in part been due to the need to count CFUs from the more dilute plates (a single CFU on a 10^3 dilution plate, the lowest dilution used, represents 10000 CFU/g of soil), this does not explain the high values found in this study, particularly in the Lincoln site, which could reflect high levels of infection and sporulation on a host in these soils, such as grass grub. Additionally, *Metarhizium* may attack other hosts in the soil (see Chapter 6). Barranco *et al*. (2019) reported that *Metarhizium* spp. (*M. album, M. acridum, M. anisopliae, M. flavoviride, M.*

novozealandicum, M. pemphigi, M. brasiliense, M. lepidiotae, M. majus, M. minus, M. pingshaense and *M. robertsii)* have been isolated from orders Hemiptera, Orthoptera, Coleoptera, Lepidoptera and Isoptera, and in New Zealand, *Metarhizium* has been isolated from natural diseased insect pests belonging to orders Coleoptera and Lepidoptera (Glare *et al*., 1993a; Liu *et al.*, 2020). The estimates of soil concentrations reported herein are generally greater than CFU counts found in agricultural soils in other studies that used similar estimation methods, which were generally of magnitudes of 10^2 to 10^4 (Yip, 1990; Rath *et al.*, 1992; Sosa-Gomez *et al.*, 2001; Clifton *et al.*, 2015, Kepler *et al.*, 2015; Nishi *et al.*, 2018; Tkaczuk, 2019). The two highest estimates, those found at Lincoln between 0 and 5 cm in the autumn and winter, were magnitudes greater, while the soil information in Table 7.1 showed that the Lincoln soil had the lowest pH, organic matter and nitrogen of all three sites. It also had lower Ca, K, Mg, P, CEC, S and total carbon. *Metarhizium* growth increased with decreasing pH and the optimum was reported to be pH = 4.5 (Rousk *et al*., 2009). The lower pH at the Lincoln site may have contributed to the greater *Metarhizium* abundance.

As expected of a root feeder, grass grub larvae were more abundant in the surface soil layer than at the 5-10 cm depth at all sites and in every season. Generally, abundance decreased in the winter which is when the larval stage finishes before they pupate or die (Cottier, 1962; Kain, 1975). Overall, grass grub density and presence, across both depths, was highest at Oxford in every season. Based on the soil information in Table 7.1, the Oxford soil had the highest potassium, magnesium, sulphate sulphur and C/N ratio which would help the growth of grass roots. In addition, the mean temperature at the Oxford site was lowest in each season (Figure 7.9). Some studies also showed that temperature and farming practice affect grass grub density. Barlow *et al*. (1996) reported that temperature had an important effect on grass grub density in outbreaks in Canterbury, New Zealand, where the Lincoln and Oxford sites were in this study, and Brock (1986) reported that grass grub density increased rapidly with changing management practises (such as the removal of organochlorine insecticides) on a pasture ecosystem near Palmerston North on the North Island of New Zealand.

Radcliffe (1970) reported that in a pot trial there was significantly reduced plant growth at a grass grub density of 108 larvae/m^2, and greater reductions at 323 larvae/m^2 on all pasture species tested: perennial ryegrass (*Lolium perenne* L.), browntop (*Agrostis tenuis* Sibth.), Yorkshire fog (*Holcus lanatus* L.), and white clover (*Trifolium repens* L.). Grass grubs were present in all seasons at two sites (Lincoln and Oxford), but at the West Coast site were low. Based on the soil moisture information in Figure 7.9, the West Coast soil moisture was the highest (> 33%) in each season which may have affected the grass grub larvae. Joan (1971) showed that soil moisture affected the survival of grass grub larvae; in dry soil the larvae were more prevalent than in wet soil. In addition, Prestidge and East (2012) reported that using nitrogen had little or no influence on abundance of grass grub.

Moreover, Townsend *et al.* (2013) found that most damage to pastures on the West Coast region of New Zealand was caused by manuka beetle larvae (*Pyronta* spp.*)*, and only infrequently by grass grub. The farmer at the West Coast site used the pesticide Lorsban for porina control, and this might also have affected grass grub populations there.

The Lincoln site had high *Metarhizium* CFU counts and relatively low larval counts while the Oxford site had low CFU counts and high larval counts, but it would take much greater replication at the site level to show a statistically significant trend. Additionally, the West Coast site produced low counts of both. Nevertheless, this may be an indication of the potential of *M. novozealandicum* to infect and kill grass grubs and further sampling in the future may confirm the trend observed here.

This study has provided initial information suggesting several areas that deserve further study greater numbers of New Zealand pasture sites. A trend of increasing natural density of *Metarhizium* with decreasing grass grub density, and the opposite trend of increasing grass grub density coupled with decreasing *M. novozealandicum* density, if shown to be significant with further studies, would be a strong indication that this *Metarhizium* species would be an effective biocontrol tool for controlling grass grubs in pasture soils.

Chapter 8

General discussion

8.1 Overview of the study

Grass grub (*C. giveni*) is a major pest of improved pasture and causes an estimated economic damage of between NZ$215-$585M/year in New Zealand (Ferguson *et al*., 2019). Several management strategies to reduce the damage caused by grass grub on pasture have previously been reported (East & Pottinger, 1975; Lauren, 1979; Dearing *et al*., 1980; Stewart & Toor, 1983; Zydenbos *et al*., 2016). However, the inappropriate use of chemical insecticides to control grass grub can affect human health and damage the environment over time (Jackson, 1990). The use of biological control agents such as parasitic nematodes, bacteria, viruses and fungi (Villalobos-Hernandez, 1994; Lacey *et al*., 2001; Hajek, 2004) can be safe to the environment and have less impact on beneficial natural pest enemies (Roberts & St Leger, 2004). However, challenges are associated with their use, such as the high costs involved in production and formulation, product shelf life, and the sometimes negative impacts of environmental conditions (Glare *et al.,* 1994; Georgis *et al*., 2006; Abbaszadeh *et al.,* 2011; Lacey *et al*., 2015; Lengai & Muthomi, 2018).

Metarhizium species are considered to be promising entomopathogens for use against grass grub because the larvae have shown susceptibility to some *Metarhizium* strains in nature (Glare *et al*., 1993a; Jackson, 1990). However, no commercial product based on *Metarhizium* has been developed in New Zealand for grass grub control, and few studies have examined the endemic strains for their potential against grass grub, outside of simple pathogenicity studies. Therefore, the objectives of this thesis were to:

I) Isolate and identify *Metarhizium* spp. that are pathogenic to *C. giveni* (Chapter 2)

II) Assess the pathogenicity of *Metarhizium* against grass grub in different soil types (Chapter 3)

III) Assess the effect of the presence of the meadow fescue (*Festuca pratensis*) endophyte (*Epichloë uncinatum*) on the pathogenicity of *M. novozealandicum* (C14) to grass grub (Chapter 4)

IV) Determine the effect of selected soil bacteria on the virulence of *M. novozealandicum* (C14) to grass grub (Chapter 5)

V) Explore the host range of a grass grub-active *M. novozealandicum* (C14) (Chapter 6)

VI) Determine the in-field distribution of *Metarhizium* (Chapter 7)

8.2 Major findings

8.2.1 Isolate and identify *Metarhizium* spp. that are pathogenic to *Costelytra giveni*

The hypothesis in Chapter 2 that some *Metarhizium* strains have the potential to control *C. giveni* grass grub was accepted. *Metarhizium* isolates from field-infected larvae, soils and from existing collections were cultured and identified. These isolates from existing culture collections belonged to the species, *M. anisopliae, M. novozealandicum, M. robertsii, M. guizhouense, M. pemphigi, M. brunneum, M. frigidum* and *M. pinghaense*. The species isolated from field-collected grass grub were isolates of *M. anisopliae* and *M. novozealandicum* which caused up to 5% mortality in the field collected larvae. Glare *et al*. (1993a) reported that these species were present in New Zealand. Other studies (Lacey *et al*., 2001; Meyling & Eilenberg, 2007; Schneider *et al.*, 2011; Vega *et al*., 2012; Keyser, 2015) have reported *Metarhizium* spp. attacking between 750-1000 insect species. Many of these records were for *M. anisopliae sensu lato* which has since been recognized as a species complex with nine different species including: *M*. *anisopliae*, *M*. *acridum*, *M*. *brunneum*, *M*. *globosum*, *M*. *guizhouense*, *M*. *lepidiotae*, *M*. *majus*, *M*. *pingshaense* and *M*. *robertsii* (Bischoff *et al.*, 2009).

Pathogenicity to grass grub

M. novozealandicum (C14) and *M. pinghaense* (JK) originally isolated from a kiwifruit orchard in Nelson, New Zealand, *M. novozealandicum* (F99) and *M. anisopliae* (M2) originally isolated from *Costelytra giveni* larvae from Canterbury, *M. anisopliae* (F672) originally isolated from endophyte of *Pinus radiata* roots, New Zealand all had higher pathogenicity than other *Metarhizium* isolates against second and third instar grass grub larvae at high spore concentration (10^7 conidia/g dry soil) under laboratory conditions. These five *Metarhizium* isolates were used to investigate the effect of different soil types on pathogenicity of *Metarhizium* against grass grub (Chapter 3).

M. novozealandicum (C14) had higher pathogenicity compared with the other *Metarhizium* isolates in both semi-sterilised and non-sterilised soils using different spore concentrations. A concentration of 10^8 conidia per 10 g dry soil caused almost 100% mortality. In addition, the LT_{50} and LC_{50} values of *M. novozealandicum* (C14) were lower than for other *Metarhizium* isolates (Chapter 3), indicating strongly that C14 has the potential to control grass grub as it kills more quickly.

8.2.2 The effect of soil type on the pathogenicity of *Metarhizium* against grass grub

The hypothesis in Chapter 3 that soil type influences the pathogenicity of *Metarhizium* to grass grub was refuted. In addition, the hypothesis that soil microbial community alters the infectivity and pathogenicity of *Metarhizium* spp. against soil dwelling insects was also refuted. The interaction

between soil type and the pathogenicity of five *Metarhizium* isolates against grass grub larvae was investigated. The experiments were carried out under controlled conditions (soil moisture and temperature) to understand any influence on larval infection in both sterilised and non-sterilised soils.

Soil type did not affect the pathogenicity of *Metarhizium* isolates against grass grub, even though two were a silt loam and one a clay loam. There was a slight decrease of some components such as pH, potassium, magnesium and sodium while there was an increase in olsen phosphorus, calcium, CEC and sulphate sulphur in semi-sterilised Wakanui soil compared to non-sterilised Wakanui soil, but the larval mortality caused by *Metarhizium* isolates did not differ between sterilised and non-sterilised soils, indicating that physical and biological properties of soil did not greatly affect the pathogenicity of *Metarhizium*. The microbial community (*Bacillus megaterium, Chryseobacterium* sp. and *Stenotrophomonas* sp. were collected from the Wakanui, Templeton and Temuka soils) may have been low, as the soil samples were collected after a drought and this could have affected microbial communities. Also, based on the results from the direct effect of some soil microbes on *Metarhizium* pathogenicity in bioassays in chapter 5, the pathogenicity of *Metarhizium* was not affected by soil physical properties or soil microbial communities of the three soil types against second and third instar larvae. Garrido-Jurado *et al.* (2011) and Peciulyte & Dirginciute-Volodkiene (2012) have also shown that the activity of *M. anisopliae* in soils was not affected by soil type, $CaCl_2$ concentration or trace elements (copper and zinc).

Wakanui soil was used for assessing the interaction between endophyte of meadow fescue and C14 against grass grub (Chapter 4) and also the experiments of grass grub and soil microbial community. As the strongest performing candidate, *M. novozealandicum* (C14) was selected to investigate its biocontrol potential against grass grub in the next chapters (Chapters 4, 5, and 6).

8.2.3 The effect of the presence of the meadow fescue (*Festuca pratensis*) endophyte (*Epichloë uncinatum*) on the pathogenicity of *Metarhizium novozealandicum* (C14) to grass grub

The relationship between meadow fescue endophyte (E^+) and *M. novozealandicum* (C14) against grass grub was unknown. In Chapter 4, no interaction was found between meadow fescue endophyte (E^+) and the pathogenicity of *M. novozealandicum* (C14) at a low spore concentration (10^5 conidia/ml). Also, it was shown that plant dry weight could not explain clearly the potential of meadow fescue endophyte to control grass grub, as grass grub damaged roots of the plant, and there was little or no effect on the feeding activity of grass grub on roots of meadow fescue grasses when *M. novozealandicum* (C14) was applied at the low spore concentration. Patchett *et al*. (2008) showed that grass grub attack of roots led to the production by E^+ of a high loline concentration inside the

root, decreasing the feeding activity of larvae. In addition, Popay *et al.* (2003) recorded that root consumption of grass grub was significantly lower in meadow fescue with endophyte (E+) than meadow fescue without endophyte (E-). Further study is required to determine effects on grass grub larval mortality and meadow fescue root dry weight when there was a combination between C14 at a high dose and endophyte.

C14 was confirmed to be present as an endophyte of meadow fescue grass at a high spore concentration. The presence of other fast-growing fungi within the plants was observed in uninoculated controls with and without endophyte in both experiments, but not in C14-inoculated treatments, suggesting that the presence of *Metarhizium* could inhibit the growth of other fungi. The occurrence of other endophytes is likely as plants were grown in non-sterilised soil. There is a need to isolate fungi from non-sterilised soil and check for any antagonism between these fungi and *Metarhizium*. Previous studies found *M. robertsii* and *M. brunneum* inhibited the growth of *Fusarium solani in vitro* (Sasan & Bidochka, 2013; Jaber & Alananbeh, 2018). It is possible that C14 has the potential to inhibit some pathogenic fungi of fescue, which would be an added benefit of the use of a *Metarhizium*-based biopesticide. Previous studies have shown potential of *Metarhizium* to reduce plant disease (Keyser *et al.,* 2015; Lozano-Tovar *et al*., 2017; Canassa *et al*., 2020).

Fluorescent microscopy was also used to determine the presence of C14, but as the technique was not specific to the *M. novozealandicum* strain, it was not possible to confirm that the endophyte was C14. The soil used in this experiment was non-sterilised and could have contained other fungi which entered the plant tissues. Further experiments are required to determine the endophytic ability of *M. novozealandicum* C14 in meadow fescue by using green fluorescent protein tags (Stretton *et al*., 1998) to tag *Metarhizium* to confirm it as an endophyte in grass. Rivas-Franco (2018) used this method to detect *M. anisopliae* hyphae within the roots of maize.

8.2.4 The effect of selected soil bacteria on the virulence of *Metarhizium novozealandicum* (C14) to grass grub

The hypothesis in Chapter 5 that interactions between some soil common bacteria and *Metarhizium* influence the biological control of *C. giveni* was accepted. Some bacterial isolates of *Serratia proteamaculans*, *Carnobacterium maltaromaticum, Bacillus megaterium, Oerskovia emterophila, Pseudomonas lundensis, Yersinia enterocolitica* and *Stenotrophomonas* sp*.* were isolated from dead larvae and soil, and two amber disease-causing bacterial strains (*S. entomophila* 145WT and *S. proteamaculans* A1MO2 isolates, originally from infected insects) were used to determine if they could affect the pathogenicity of C14 in second and third instar grass grubs. Most bacterial isolates in combination with C14 had a positive effect on second instar larvae mortality, particularly at 10^8 conidia/ml, but no synergism was observed against third instar larvae. Glare (1994) had previously

reported synergism between *M. guizhouense* (F16) and *S. entomophila,* a cause of amber disease, against the second instar larvae of grass grub, but not with the third instar larvae. One reason could be an increased virulence of the fungus alone against the third instar larvae, compared to its virulence against second instar, obscuring any additional effect. Two bacterial isolates had a greater effect on C14 pathogenicity than the others, 6-1 (*Yersinia enterocolitica*) and 3-2 (*Bacillus megaterium*). These two bacterial isolates were non-pathogenic to grass grub when used alone. In addition, the bacteria 6-1 and 3-2 when combined with C14 caused faster larval mortality than other combinations of C14 and bacterial isolates, and there was no significant difference between non-pathogenic and pathogenic bacterial isolates combined with C14 against grass grub.

The reason why the bacteria increased C14 related mortality could have several causes. Bacterial isolates through the production of enzymes such as chitinase could modify larval behaviour to produce more convenient conditions for fungal infection or make the cuticle of grass grub weaker to increase fungal germ tube penetration. Juliya (2020) showed that there was a correlation of the pathogenicity of *M. pingshaense* and *M. guizhouense* with chitinase activity when these fungi grew on the cowpea aphid cuticle. Also, some previous studies have shown that chitinase activity of *M. anisopliae* contributed to its bioactivity against diamondback moth, *Plutella xylostella* (Wu *et al*., 2010), whitefly, *Bemisia tabaci* (Anwar *et al*., 2019), and cotton bollworm, *Helicoverpa armigera* (Nahar *et al*.,2004) through softening the larval cuticle to help mycelial penetration.

Isolates 6-1 and 3-2 produced more chitinase in culture (but not protease) than the other isolates, potentially contributing to C14 virulence. Jackson *et al.* (1993) reported that amber disease acted through ingestion of pathogen bacterial cells, while Glare & Milner (1991) have shown the fungus attacking through the host's cuticle. There have been no previous reports of synergism for fungal and bacterial interactions when the entry is through either the cuticle or the alimentary canal. Ansari *et al*. (2004) and Davies (2005) found synergism between nematodes and fungi against *Hoplia philanthus* larvae.

In this study, most bacterial isolates produced volatiles that inhibited the growth of C14 when they were cultured together in a Petri plate. The unknown volatiles still need to be identified. Isolate 6-1 and other isolates did not differ in inhibition of C14 except 145WT and A1MO2, but isolate 6-1 was more effective in increasing C14 virulence, indicating that volatiles produced by the bacterium did not affect the combined impact of bacteria and fungus against grass grub. Nieto-Jacobo *et al*. (2017) reported that *Trichoderma* strains (*T. virens* Gv29.8, *T. atroviride* IMI206040, *T*. sp. "*atroviride* B" LU132, and *T. asperellum* LU1370) produced volatile organic compounds such as 1-Octen-3-ol and 3-Octanone, β-Elemene, ε-Amorphene, 1,3-Octadiene, Limonene and β-Eudesmol + Valerianol. Also, Li *et al*. (2018) showed that *T. virens* and *T. harzianum* produced volatile compounds such as 1-octen-3-

ol, 3-octanone, and acetic acid to inhibit *Fusarium oxysporum* growth. However, isolate 6-1 did not affect C14 when grown together directly on PDA medium, suggesting it did not produce metabolites that could directly affect the fungus. This is consistent with results of Kriukov *et al*. (2009) who reported that *Bacillus thuringiensis* did not produce metabolites antagonistic to *M. anisopliae* on artificial nutrient media.

Isolate 6-1, when combined with C14, has the potential for an improved biological control approach. However given that *Y. enterocolitica* has caused about 117,000 illnesses, 640 hospitalizations, and 35 deaths in the United States every year (CDC, https://www.cdc.gov/yersinia/), it will be necessary to evaluate if it is safe to use, and have few deleterious impacts on either the soil beneficial microbial community or beneficial natural insect enemies in the field.

8.2.5 The host range of a grass grub-active *Metarhizium novozealandicum* (C14)

The virulence of *M. novozealandicum* (C14) at 10^8 conidia/ml was assessed on five insect pests belonging to orders Lepidoptera, Hemiptera and Coleoptera under laboratory conditions. *Plutella xylostella* larvae had high mortality for both inoculated (80% mortality after 6 days) and control (40% mortality after 6 days) treatments, indicating that C14 only had a limited measurable effect on *P. xylostella.* This result could be explained based on the life cycle of *P. xylostella*, the larval stage for the experiment finished after five days, after which it begins to enter the prepupal stage or die (Huaripata & Sanchez, 2019). A previous study by Correa-Cuadros *et al.* (2014) showed using *M. anisopliae sensu lato* against *P. xylostella* produced 84% mortality at 10^8 conidia/ml. However, Silva *et al*. (2003) reported that one strain of *M. anisopliae sensu lato* caused only 31% mortality against *P. xylostella* at a 10^8 conidia/ml concentration. This could be attributed to differences in strain ability to control the pest assuming other things are equal such as dose received.

The endemic porina moth, (*Wiseana* sp.) larvae had a mortality of 75% after 28 days when *M. novozealandicum* (C14) was applied at 10^8 conidia/ml. The study of Latch & Kain (1983) showed that a strain from *M. anisopliae sensu lato* was effective against porina when testing this *Metarhizium* strain in a field trial using an inoculum bait method. However, this study is difficult to compare with the current study because of the inability to control soil temperatures in the field.

Helicoverpa armigera (Lepidoptera) was one of these three pests which had the highest larval mortality (100% at day ten) when *M. novozealandicum* (C14) was applied. Previous studies of *M. anisopliae sensu lato* strains (Fite *et al.*, 2020; Tahir *et al*., 2019) reported lower ranges of mortality, from 20%-84%, under the same inoculation concentrations (10^8 conidia/ml). However, the reports of Vijayavani *et al*. (2010) and Kulkarni *et al.* (2008) showed some *M. anisopliae* strains achieved 92%-100% mortality using a 10^7-10^8 conidia/ml concentration.

The aphid *Myzus persicae* belongs to the order Hemiptera and had high mortality in both the inoculated and control treatment. However because some aphids gave birth after one day and died to finish the life cycle, C14 was less effective against *M. persicae* than the percentage mortality suggests, and the strain may not provide effective control in the field. *M. persicae* has previously been shown to be susceptible to some strains of *M. anisopliae sensu lato* (Butt *et al.*, 1994; Loureiro & Moino Jr., 2006; Shan & Feng, 2006; Lee *et al.,* 2015; Yun *et al.,* 2017) and *M. flavoviridae sensu lato* (Lee *et al.*, 2015). Moreover, Rasool *et al*. (2020) showed that *M. robertsii* reduced aphid population when wheat and bean seeds were inoculated while *M. brunneum* increased aphid densities under *in vitro* conditions.

Similar to *H. armigera* larvae, *Tenebrio molitor* larvae (Coleoptera) were susceptible to *M. novozealandicum* (C14) and 92.5% died after seven days, which was similar to previous studies (Skrobek *et al.*, 2008; Skalický *et al.*, 2014; Lestari & Rao, 2016) which reported that there was high larval mortality (95-100%) after seven days using *M. anisopliae sensu lato* against *T. molitor* larvae. Skalický *et al.* (2014) reported some strains of *M. anisopliae sensu lato* caused low mortality (5.3-46.7%) against *T. molitor* larvae. This indicates different strains could have different ability to control the pest.

Isolate C14 can infect insect pests belong to the orders Lepidoptera, Hemiptera and Coleoptera, indicating that isolate C14, and *M. novozealandicum* as a species, has a broad host range, and may possibly infect insect pests outside of these three orders (such as Diptera, Orthoptera, Hymenoptera, Thysanoptera). Therefore, C14 may have potential as a multi-pest control option in some situations. However most of these pests other than grass grub are pests above ground, so application method would need to be considered so that pests both above and below ground could be targeted. A pathogen with a wide range of pest hosts would be able to remain in the environment for a longer time due to the availability of alternate hosts, but it would be necessary to check their effect on beneficial insects as well. In other words, a broad host range creates concerns about impacts on non-target species and a greater need to investigate impacts on beneficial invertebrates before testing in the field.

8.2.6 In-field distribution of *Metarhizium*

The distribution and abundance of *Metarhizium* in pasture soils is unknown in most cases, so a single season comparison was made to examine any indicative correlation between *Metarhizium* and grass grub. This was done by isolating *Metarhizium* from soil, counting grass grub, and comparing with farming practice and soil type. The distribution of *Metarhizium* species was determined at two soil depths, across three different seasons on three pasture sites in the South Island of New Zealand. Both *M. novozealandicum* and *M. anisopliae* were isolated from these soils but *M. novozealandicum*

was dominant, which is similar to the findings of Glare *et al.* (1993a) and Liu *et al.* (2020). This suggests that these *Metarhizium* species are naturally occurring pathogens of grass grub in New Zealand, as grass grub is generally one of the most common insects in these pastures. Also, a higher population of grass grub was found in the soil surface layer (0-5 cm) compared with the 5-10 cm depth at all sites and seasons. Higher larvae distribution in the soil surface layer can be because of the presence of more of the root system of perennial ryegrass at the surface layer compared with the deeper layers. Therefore, larvae tend to move to the surface layer to feed on the root of plants.

The Oxford site had the highest abundance of the grass grub. Lincoln had high CFUs in all seasons. One reason for having a higher population of grass grub at the Oxford site could be the higher potassium, magnesium, sulphate sulphur contents and also C/N ratio (Table 6.1 of Chapter 6) which would help the growth of grass roots. Also, based on the information of farming practice in three sites, farmers used the pesticide Lorsban for porina control at the West Coast site while the Lincoln and Oxford sites did not.

Barlow *et al*. (1996) reported that grass grub density was affected by temperature in outbreaks in Canterbury, New Zealand, where the Lincoln and Oxford sites were in this study, and Brock (1986) also reported that changing management practises (such as the removal of organochlorine insecticides) led to increase grass grub density on a pasture ecosystem near Palmerston North on the North Island of New Zealand. The grass grub presence at the Lincoln and Oxford sites in all seasons would cause damage to pasture, while grass grub levels at the West Coast site were very low or nil. The current study found results similar to the study of Townsend *et al.* (2013) who indicated that the damage to pasture on the West Coast was caused mainly by manuka beetle larvae (*Pyronta* spp.*)* and damage due to grass grub was very low. Additionally, CFU counts of *Metarhizium* was higher at the Lincoln site than the two remaining sites. This does not explain the high values found in this study but could reflect high levels of infection and sporulation on a host in these soils, such as grass grub. The soil type did not affect the presence of *Metarhizium* (based on the soil information in Table 7.1), a result consistent with the reports of Pecuilyte & Dirginciute-Volodkiene (2012) and Rajapaksha *et al*. (2004). Hussein *et al.* (2010) also has found that weather affected the occurrence of *Metarhizium.* A correlation between high *Metarhizium* abundance and low grass grub abundance at Lincoln and Oxford sites could not be confirmed because of the low number of samples from each site. More replicate from large areas would be needed to confirm this correlation. It could be attributed to death of the third instar and multiplication of fungi on their body.

8.2.7 The potential of C14 and 6-1 combinations for grass grub control

This study confirmed that C14 had potential as a biocontrol agent against grass grub and that its virulence was improved when it was combined with bacteria, such as *Y. enterocolitica* isolate 6-1.

C14 seems to be appropriate for use as biocontrol agent in agricultural systems because this *Metarhizium* species was dominant in pasture soil in three different regions of Canterbury and was highly virulent in bioassays. Further investigation of other sites is required to determine the abundance of this fungus in pasture soils across New Zealand as there might be some differences between sites because of temperature, soil moisture or types. Moreover, C14 could also be endophytic in the roots of grass when applied at high doses, suggesting it has the potential to control other insect pests attacking the plant roots. It may also elicit an induced defence response in the plant or even have direct action, which could help protect the plant against soil pathogenic fungi, but this requires investigation. Ahmad *et al*. (2020) showed that *M. robertsii* was endophytic in maize and contributed to the promotion of maize growth, prevention of insect pests, and altering gene expression in plant defence. Kern *et al*. (2010) also showed that the chitinase gene from *M. anisopliae* in tobacco plant was active against the soil-borne pathogen *Rhizoctonia solani*. Moreover, Rivas-Franco *et al*. (2019) reported that coating maize seed with *M. anisopliae* improved plant growth and reduced infection by *Fusarium graminearum* and root feeding by *C. giveni* larvae.

This study showed that application of *Y. enterocolitica*, isolate 6-1 and C14 improved biological control of grass grub. However, Nesbakken *et al*. (2006) and Fredriksson-Ahomaa *et al*. (2007) have recorded that the pathogenic *Y. enterocolitica* was found on pigs. Also, *Y. enterocolitica* caused about 117,000 illnesses, 640 hospitalizations, and 35 deaths in the United States every year (CDC, https://www.cdc.gov/yersinia/). Therefore, the effect of the isolate on human and animal health needs to be investigated before developing a biocontrol agent. If both microorganisms are safe to the environment and humans, and also have few deleterious impacts on both soil beneficial microbial communities or beneficial natural insect enemies (this particularly applies to the fungus which has got a wide host range) in the field, they could be developed as a product to be used for a sustainable ecosystem in pasture soils. A number of experiments need to be conducted to find the most effective concentrations of *Y. enterocolitica* 6-1 and C14 against grass grub in field trials, to determine the extent of control achievable.

8.3 Recommendations for future research

- ✓ Determine the effect of other soil types on the pathogenicity of *Metarhizium* spp. against grass grub. Although the three soil types did not affect the pathogenicity of *Metarhizium* in this study, other soils should also be tested to get an indication to determine the ability of *Metarhizium* to persist in New Zealand soils.
- ✓ Confirm whether *Metarhizium* can become endophytic in grasses and therefore become an effective biocontrol agent.

- ✓ Determine whether *M. novozealandicum* (C14) inhibits other grass endophytic fungi (including *Epichloë* spp. and plant pathogens). Some endophytes are beneficial, and this study will help to see if C14 will remove beneficial endophytes or conversely reduce plant disease causing fungi.
- ✓ Determine if the application of two microbes (fungus and bacteria) together to pasture soil at the start of the year when grass grub is present is beneficial for control. The feasibility of using the two microbes at relatively high doses will also need to be determined. Both microbes could be formulated in one product and soil applied. However, their impacts on other beneficial soil microorganisms and the safety of the bacteria needs to be determined.
- ✓ Determine the mode of action for the synergism between *Metarhizium* and and *Y. enterocolitica* 6-1, for example, measuring cuticle thickness of grass grub or using mutated strains of the bacterium.
- ✓ Determine the pathogenicity of *M. novozealandicum* (C14) to other insects to determine how broad is its host range. This would need to include beneficial soil residing invertebrates to determine to environment and ecosystem safety.
- ✓ Determine if coating ryegrass seed with *M. novozealandicum* (C14) before sowing can provide control of the pest.
- ✓ Further study to determine the abundance of *Metarhizium* in New Zealand pasture soils from all pests of New Zealand. The current study showed that *Metarhizium* has potential to be used as a biocontrol agent against grass grub. Further study could help to get an indication regarding natural population of the fungus in New Zealand pasture soils.

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Appendix A for Chapter 2

2.1 Material for experiments

2.1.1 *Metarhizium* selective medium (MSM)

Dissolved in 1 L of distilled water:

Potato dextrose agar (Merck) 39 g/L

Then autoclave at 121 ^{0}C at 15 SI for 15 min

Before pouring into Petri plates, add to each L of PDA

Tetracycline chloride 1.5 % 3.33 ml

Streptomycin sulphate 10 % 3.5 ml

Cycloheximide 1.56 % (filter sterilise) 8 ml

PDA + A therefore contains 50 mg/l of tetracycline chloride, 250 mg/l of streptomycin sulphate and 125 mg/l of cycloheximide.

2.1.2 Haemocytometer counts and spore concentration

Original spore concentration/ml = mean x 25 x 10^4 x DF

Total the spore number for the grids and take the average (mean). Then multiply the average by 25 (as there are 25 squares in haemocytometer), multiply this by 1x10^4 (spores per ml, volume of slide), multiply by dilution factor (DF) (x10 or 100).

2.1.3 Formula for calculating volume about to be diluted

$$C1 \times V1 = C_2 \times V_2.$$

C_1 = original spore concentration of the solution, before it gets watered down or diluted.
C_2 = final spore concentration of the solution, after dilution.
V_1 = volume about to be diluted
V_2 = final volume after dilution

2.2 Statistical analyses

2.2.1 Analysis of variance for the pathogenicity of nine *Metarhizium* strains against second grass grub larvae after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	450.0	112.5	1.00	
Treatment	9	950.0	105.6	0.94	0.505
Residual	36	4050.0	112.5		
Total	49	5450.0			

2.2.2 Analysis of variance for the pathogenicity of nine *Metarhizium* strains against second grass grub larvae after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	325.0	81.2	0.23	
Treatment	9	17012.5	1890.3	5.37	<.001
Residual	36	12675.0	352.1		
Total	49	30012.5			

2.2.3 Analysis of variance for the pathogenicity of nine *Metarhizium* strains against second grass grub larvae after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate stratum	4	1825.0	456.2	0.78	
Treatment	9	34762.5	3862.5	6.57	<.001
Residual	36	21175.0	588.2		
Total	49	57762.5			

2.2.4 Analysis of variance for the pathogenicity of nine *Metarhizium* strains agianst second grass grub larvae after 35 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate stratum	4	3375.0	843.8	1.46	
Treatment	9	54500.0	6055.6	10.44	<.001
Residual	36	20875.0	579.9		
Total	49	78750.0			

2.2.5 Analysis of variance for the pathogenicity of nine *Metarhizium* strains against third grass grub larvae after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	2	6.67	3.33	0.06	
Treatment	9	2263.33	251.48	4.20	0.005
Residual	18	1076.67	59.81		
Total	29	3346.67			

2.2.6 Analysis of variance for the pathogenicity of nine *Metarhizium* strains against third grass grub larvae after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	2	61.67	30.83	0.31	
Treatment	9	15936.67	1770.74	17.82	<.001
Residual	18	1788.33	99.35		
Total	29	17786.67			

2.2.7 Analysis of variance for the pathogenicity of nine *Metarhizium* strains against third grass grub larvae after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	2	86.67	43.33	1.34	
Treatment	9	35320.00	3924.44	121.79	<.001
Residual	18	580.00	32.22		
Total	29	35986.67			

2.2.8 Analysis of variance for the pathogenicity of nine *Metarhizium* strains against third grass grub larvae after 35 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	2	46.67	23.33	0.93	
Treatment	9	41896.67	4655.19	184.84	<.001
Residual	18	453.33	25.19		
Total	29	42396.67			

Appendix B for Chapter 3

3.1 Materia and method

Table 3.1: The soil moisture content (%) of three air-dried soil types after placed in an oven at 90°C for three days.

Sample	Soil type	Air-dried soil (g)	Oven-dried soil (g)	Soil moisture content (%)
1	Wakanui	50	48.84	2.32
2	Wakanui	50	48.91	2.18
3	Wakanui	50	48.70	2.6
4	Temuka	50	48.61	2.78
5	Temuka	50	48.75	2.5
6	Temuka	50	48.55	2.9
7	Templeton	50	49.06	1.88
8	Templeton	50	49.15	1.70
9	Templeton	50	49.09	1.82

3. 2 Statistical analyses

3.2.1 C14 strain for the second instar larvae

3.2.1.1 Analysis of variance of three sterilised soil types with four spore concentrations of C14 strain against grass grub after 7 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	116.67	58.33	1.08	0.348
Spore_concentration	4	383.33	95.83	1.77	0.148
Soil_type.Spore_concentration					
	8	216.67	27.08	0.50	0.851
Rep	4	216.67	54.17	1.00	0.415
Residual	56	3033.33	54.17		
Total	74	3966.67			

3.2.1.2 Analysis of variance of three sterilised soil types with four spore concentrations of C14 strain against grass grub after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	116.67	58.33	0.63	0.535
Spore_concentration	4	4250.00	1062.50	11.52	<.001
Soil_type.Spore_concentration					
	8	300.00	37.50	0.41	0.912
Rep	4	583.33	145.83	1.58	0.192
Residual	56	5166.67	92.26		
Total	74	10416.67			

3.2.1.3 Analysis of variance of three sterilised soil types with four spore concentrations of C14 strain against grass grub after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	266.7	133.3	0.65	0.525
Spore_concentration	4	13616.7	3404.2	16.62	<.001
Soil_type.Spore_concentration					
	8	2733.3	341.7	1.67	0.127
Rep	4	2783.3	695.8	3.40	0.015
Residual	56	11466.7	204.8		
Total	74	30866.7			

3.2.1.4 Analysis of variance of three sterilised soil types with four spore concentrations of C14 strain against grass grub after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	716.7	358.3	1.83	0.170
Spore_concentration	4	65950.0	16487.5	84.19	<.001
Soil_type.Spore_concentration					
	8	1950.0	243.7	1.24	0.291
Rep	4	1783.3	445.8	2.28	0.072
Residual	56	10966.7	195.8		
Total	74	81366.7			

3.2.1.5 Analysis of variance of three sterilised soil types with four spore concentrations of C14 strain against grass grub after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	150.0	75.0	0.36	0.698
Spore_concentration	4	95716.7	23929.2	115.35	<.001
Soil_type.Spore_concentration					
	8	2183.3	272.9	1.32	0.255
Rep	4	383.3	95.8	0.46	0.763
Residual	56	11616.7	207.4		
Total	74	110050.0			

3.2.1.6 Analysis of variance of three sterilised soil types with four spore concentrations of C14 strain against grass grub after 35 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	816.7	408.3	1.53	0.226
Spore_concentration	4	84300.0	21075.0	78.94	<.001
Soil_type.Spore_concentration					
	8	1100.0	137.5	0.52	0.840
Replicate	4	550.0	137.5	0.52	0.725
Residual	56	14950.0	267.0		
Total	74	101716.7			

3.2.1.7 Analysis of variance of LT_{50} value of three sterilised soil types with four spore concentrations of C14 strain against grass grub

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	101.88	50.94	1.84	0.172
Spore_concentration	3	3636.24	1212.08	43.67	<.001
Soil_type.Spore_concentration					
	6	203.83	33.97	1.22	0.312
Rep	4	12.68	3.17	0.11	0.977
Residual	44	1221.28	27.76		
Total	59	5175.91			

3.2.1.8 Analysis of variance of LC_{50} value of three sterilised soil types with four spore concentrations of C14 strains against grass grub at 28 days

Source	d.f.	deviance	deviance	ratio
Regression	6	84.79	14.1316	24.16
Residual	68	39.77	0.5849	
Total	74	124.56	1.6833	

3.2.2 F99 strain for the second instar larvae

3.2.2.1 Analysis of variance of three sterilised soil types with four spore concentrations of F99 strain against grass grub after 7 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	66.67	33.33	2.67	0.078
Spore_concentration	4	133.33	33.33	2.67	0.042
Soil_type.Spore_concentration					
	8	266.67	33.33	2.67	0.015
Rep	4	50.00	12.50	1.00	0.415
Residual	56	700.00	12.50		
Total	74	1216.67			

3.2.2.2 Analysis of variance of three sterilised soil types with four spore concentrations of F99 strain against grass grub after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	50.00	25.00	0.44	0.648
Spore_concentration	4	2550.00	637.50	11.16	<.001
Soil_type.Spore_concentration					
	8	450.00	56.25	0.98	0.458
Rep	4	50.00	12.50	0.22	0.927
Residual	56	3200.00	57.14		
Total	74	6300.00			

3.2.2.3 Analysis of variance of three sterilised soil types with four spore concentrations of F99 strain against grass grub after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.

Soil_type	2	116.7	58.3	0.36	0.698
Spore_concentration	4	7050.0	1762.5	10.93	<.001
Soil_type.Spore_concentration					
	8	2050.0	256.2	1.59	0.149
Rep	4	966.7	241.7	1.50	0.215
Residual	56	9033.3	161.3		
Total	74	19216.7			

3.2.2.4 Analysis of variance of three sterilised soil types with four spore concentrations of F99 strain against grass grub after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	650.0	325.0	1.47	0.239
Spore_concentration	4	37116.7	9279.2	41.96	<.001
Soil_type.Spore_concentration					
	8	1683.3	210.4	0.95	0.483
Rep	4	866.7	216.7	0.98	0.426
Residual	56	12383.3	221.1		
Total	74	52700.0			

3.2.2.5 Analysis of variance of three sterilised soil types with four spore concentrations of F99 strain against grass grub after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	1316.7	658.3	4.08	0.022
Spore_concentration	4	72300.0	18075.0	112.05	<.001
Soil_type.Spore_concentration					
	8	2350.0	293.7	1.82	0.092
Rep	4	716.7	179.2	1.11	0.361
Residual	56	9033.3	161.3		
Total	74	85716.7			

3.2.2.6 Analysis of variance of three sterilised soil types with four spore concentrations of F99 strain against grass grub after 35 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	516.7	258.3	1.06	0.352
Spore_concentration	4	88133.3	22033.3	90.61	<.001
Soil_type.Spore_concentration					
	8	816.7	102.1	0.42	0.904
Rep	4	383.3	95.8	0.39	0.812
Residual	56	13616.7	243.2		
Total	74	103466.7			

3.2.2.7 Analysis of variance of LT_{50} value of three sterilised soil types with four spore concentrations of F99 strain against grass grub

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	134.48	67.24	5.33	0.010
Spore_concentration	2	1601.94	800.97	63.44	<.001
Soil_type.Spore_concentration					
	4	73.32	18.33	1.45	0.240
Rep	4	36.42	9.10	0.72	0.584
Residual	32	404.04	12.63		
Total	44	2250.19			

3.2.2.8 Analysis of variance of LC_{50} value of three sterilised soil types with four spore concentrations of F99 strain against grass grub at 28 days

Source	d.f.	deviance	deviance	ratio
Regression	4	76.45	19.1133	27.01
Residual	70	49.53	0.7076	
Total	74	125.99	1.7025	

3.2.3 M2 strain for the second instar larvae

3.2.3.1 Analysis of variance of three sterilised soil types with four spore concentrations of M2 strain against grass grub after 7 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	50.0	25.0	0.23	0.792
Spore_concentration	4	3033.3	758.3	7.12	<.001
Soil_type.Spore_concentration					
	8	616.7	77.1	0.72	0.670
Rep	4	533.3	133.3	1.25	0.300
Residual	56	5966.7	106.5		
Total	74	10200.0			

3.2.3.2 Analysis of variance of three sterilised soil types with four spore concentrations of M2 strain against grass grub after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	16.7	8.3	0.08	0.928
Spore_concentration	4	3283.3	820.8	7.39	<.001
Soil_type.Spore_concentration					
	8	816.7	102.1	0.92	0.507
Rep	4	783.3	195.8	1.76	0.149
Residual	56	6216.7	111.0		
Total	74	11116.7			

3.2.3.3 Analysis of variance of three sterilised soil types with four spore concentrations of M2 strain against grass grub after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	816.7	408.3	2.12	0.129
Spore_concentration	4	10216.7	2554.2	13.26	<.001
Soil_type.Spore_concentration					
	8	433.3	54.2	0.28	0.969
Rep	4	1466.7	366.7	1.90	0.122
Residual	56	10783.3	192.6		
Total	74	23716.7			

3.2.3.4 Analysis of variance of three sterilised soil types with four spore concentrations of M2 strain against grass grub after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	866.7	433.3	1.95	0.152
Spore_concentration	4	37716.7	9429.2	42.41	<.001
Soil_type.Spore_concentration					
	8	2633.3	329.2	1.48	0.185
Rep	4	1550.0	387.5	1.74	0.153
Residual	56	12450.0	222.3		
Total	74	55216.7			

3.2.3.5 Analysis of variance of three sterilised soil types with four spore concentrations of M2 strain against grass grub after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	316.7	158.3	0.75	0.478
Spore_concentration	4	64966.7	16241.7	76.65	<.001
Soil_type.Spore_concentration					
	8	2683.3	335.4	1.58	0.151
Rep	4	383.3	95.8	0.45	0.770
Residual	56	11866.7	211.9		
Total	74	80216.7			

3.2.3.6 Analysis of variance of three sterilised soil types with four spore concentrations of M2 strain against grass grub after 35 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	116.7	58.3	0.20	0.818
Spore_concentration	4	65283.3	16320.8	56.36	<.001
Soil_type.Spore_concentration					
	8	1216.7	152.1	0.53	0.833
Rep	4	783.3	195.8	0.68	0.611
Residual	56	16216.7	289.6		
Total	74	83616.7			

3.2.3.7 Analysis of variance of LT_{50} value of three sterilised soil types with four spore concentrations of M2 strain against grass grub

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	1.88	0.94	0.03	0.970
Spore_concentration	2	1707.05	853.53	27.49	<.001
Soil_type.Spore_concentration					
	4	117.42	29.35	0.95	0.451
Rep	4	108.64	27.16	0.87	0.490
Residual	32	993.48	31.05		
Total	44	2928.46			

3.2.3.8 Analysis of variance of LC_{50} value of three sterilised soil types with four spore concentrations of M2 strain against grass grub at 28 days

Source	d.f.	deviance	deviance	ratio
Regression	6	75.35	12.5578	17.23
Residual	68	49.55	0.7286	
Total	74	124.89	1.6878	

3.2.4 JK strain for the second instar larvae

3.2.4.1 Analysis of variance of three sterilised soil types with four spore concentrations of JK strain against grass grub after 7 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	66.67	33.33	2.67	0.078
Spore_concentration	4	133.33	33.33	2.67	0.042
Soil_type.Spore_concentration					
	8	266.67	33.33	2.67	0.015
Rep	4	50.00	12.50	1.00	0.415
Residual	56	700.00	12.50		
Total	74	1216.67			

3.2.4.2 Analysis of variance of three sterilised soil types with four spore concentrations of JK strain against grass grub after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	316.67	158.33	1.71	0.191
Spore_concentration	4	3716.67	929.17	10.01	<.001
Soil_type.Spore_concentration					
	8	433.33	54.17	0.58	0.787
Rep	4	50.00	12.50	0.13	0.969
Residual	56	5200.00	92.86		
Total	74	9716.67			

3.2.4.3 Analysis of variance of three sterilised soil types with four spore concentrations of JK strain against grass grub after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	216.67	108.33	1.16	0.320
Spore_concentration	4	6450.00	1612.50	17.31	<.001
Soil_type.Spore_concentration					
	8	450.00	56.25	0.60	0.771
Rep	4	533.33	133.33	1.43	0.236
Residual	56	5216.67	93.15		
Total	74	12866.67			

3.2.4.4 Analysis of variance of three sterilised soil types with four spore concentrations of JK strain against grass grub after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	600.0	300.0	1.35	0.268
Spore_concentration	4	25366.7	6341.7	28.49	<.001
Soil_type.Spore_concentration					
	8	2733.3	341.7	1.53	0.166
Rep	4	2283.3	570.8	2.56	0.048
Residual	56	12466.7	222.6		
Total	74	43450.0			

3.2.4.5 Analysis of variance of three sterilised soil types with four spore concentrations of JK strain against grass grub after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	716.7	358.3	1.67	0.198
Spore_concentration	4	60033.3	15008.3	69.75	<.001
Soil_type.Spore_concentration					
	8	866.7	108.3	0.50	0.849
Rep	4	1450.0	362.5	1.68	0.166
Residual	56	12050.0	215.2		
Total	74	75116.7			

3.2.4.6 Analysis of variance of three sterilised soil types with four spore concentrations of JK strain against grass grub after 35 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	1050.0	525.0	1.45	0.242
Spore_concentration	4	66783.3	16695.8	46.25	<.001
Soil_type.Spore_concentration					
	8	866.7	108.3	0.30	0.963
Rep	4	533.3	133.3	0.37	0.829
Residual	56	20216.7	361.0		
Total	74	89450.0			

3.2.4.7 Analysis of variance of LT_{50} value of three sterilised soil types with four spore concentrations of JK strain against grass grub

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	36.54	18.27	1.07	0.355
Spore_concentration	2	1678.04	839.02	49.08	<.001
Soil_type.Spore_concentration					
	4	50.21	12.55	0.73	0.575
Rep	4	19.78	4.95	0.29	0.883
Residual	32	546.99	17.09		
Total	44	2331.55			

3.2.4.8 Analysis of variance of LC_{50} value of three sterilised soil types with four spore concentrations of JK strain against grass grub at 28 days

Source	d.f.	deviance	deviance	ratio
Regression	6	70.42	11.7366	16.38
Residual	68	48.72	0.7164	
Total	74	119.14	1.6100	

3.2.5 F672 strain for the second instar larvae

3.2.5.1 Analysis of variance of three sterilised soil types with four spore concentrations of F672 strain against grass grub after 7 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	50.00	25.00	0.47	0.626
Spore_concentration	4	616.67	154.17	2.91	0.029
Soil_type.Spore_concentration					
	8	1033.33	129.17	2.44	0.024
Rep	4	283.33	70.83	1.34	0.268
Residual	56	2966.67	52.98		
Total	74	4950.00			

3.2.5.2 Analysis of variance of three sterilised soil types with four spore concentrations of F672 strain against grass grub after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	316.67	158.33	2.51	0.090
Spore_concentration	4	1216.67	304.17	4.82	0.002
Soil_type.Spore_concentration					
	8	1433.33	179.17	2.84	0.010
Rep	4	216.67	54.17	0.86	0.495
Residual	56	3533.33	63.10		
Total	74	6716.67			

3.2.5.3 Analysis of variance of three sterilised soil types with four spore concentrations of F672 strain against grass grub after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	0.0	0.0	0.00	1.000
Spore_concentration	4	7550.0	1887.5	12.51	<.001
Soil_type.Spore_concentration					
	8	250.0	31.3	0.21	0.988
Rep	4	300.0	75.0	0.50	0.738
Residual	56	8450.0	150.9		
Total	74	16550.0			

3.2.5.4 Analysis of variance of three sterilised soil types with four spore concentrations of F672 strain against grass grub after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	0.0	0.0	0.00	1.000
Spore_concentration	4	23583.3	5895.8	22.90	<.001
Soil_type.Spore_concentration					
	8	1166.7	145.8	0.57	0.801
Rep	4	833.3	208.3	0.81	0.525
Residual	56	14416.7	257.4		
Total	74	40000.0			

3.2.5.5 Analysis of variance of three sterilised soil types with four spore concentrations of F672 strain against grass grub after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	50.0	25.0	0.07	0.930
Spore_concentration	4	37383.3	9345.8	27.14	<.001
Soil_type.Spore_concentration					
	8	2366.7	295.8	0.86	0.556
Rep	4	1216.7	304.2	0.88	0.480
Residual	56	19283.3	344.3		
Total	74	60300.0			

3.2.5.6 Analysis of variance of three sterilised soil types with four spore concentrations of F672 strain against grass grub after 35 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	716.7	358.3	1.01	0.370
Spore_concentration	4	57033.3	14258.3	40.33	<.001
Soil_type.Spore_concentration					
	8	1116.7	139.6	0.39	0.919
Rep	4	950.0	237.5	0.67	0.614
Residual	56	19800.0	353.6		
Total	74	79616.7			

3.2.5.7 Analysis of variance of LT_{50} value of three sterilised soil types with four spore concentrations of F672 strain against grass grub

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_type	2	53.59	26.80	0.64	0.534
Spore_concentration	2	2125.67	1062.83	25.42	<.001
Soil_type.Spore_concentration					
	4	219.65	54.91	1.31	0.286
Rep	4	73.24	18.31	0.44	0.780
Residual	32	1338.21	41.82		
Total	44	3810.36			

3.2.5.8 Analysis of variance of LC_{50} value of three sterilised soil types with four spore concentrations of F672 strain against grass grub at 28 days

Source	d.f.	deviance	deviance	ratio
Regression	6	42.96	7.1602	7.91
Residual	68	61.57	0.9055	
Total	74	104.53	1.4126	

3.2.6 C14 strain for the third instar larvae

3.2.6.1 Analysis of variance of three non-sterilised soil types with four spore concentrations of C14 strain against grass grub after 7 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_Type	2	50.00	25.00	0.97	0.387
Spore_Concentration	4	133.33	33.33	1.29	0.286
Soil_Type.Spore_Concentration					
	8	116.67	14.58	0.56	0.803
Rep	4	50.00	12.50	0.48	0.748
Residual	56	1450.00	25.89		
Total	74	1800.00			

3.2.6.2 Analysis of variance of three non-sterilised soil types with four spore concentrations of C14 strain against grass grub after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_Type	2	116.67	58.33	1.02	0.367
Spore_Concentration	4	216.67	54.17	0.95	0.443
Soil_Type.Spore_Concentration					
	8	133.33	16.67	0.29	0.966
Rep	4	300.00	75.00	1.31	0.277
Residual	56	3200.00	57.14		
Total	74	3966.67			

3.2.6.3 Analysis of variance of three non-sterilised soil types with four spore concentrations of C14 strain against grass grub after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_Type	2	1816.7	908.3	4.28	0.019
Spore_Concentration	4	1366.7	341.7	1.61	0.184
Soil_Type.Spore_Concentration					
	8	1683.3	210.4	0.99	0.453
Rep	4	616.7	154.2	0.73	0.578
Residual	56	11883.3	212.2		
Total	74	17366.7			

3.2.6.4 Analysis of variance of three non-sterilised soil types with four spore concentrations of C14 strain against grass grub after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_Type	2	316.7	158.3	0.38	0.687
Spore_Concentration	4	36250.0	9062.5	21.67	<.001
Soil_Type.Spore_Concentration					
	8	2100.0	262.5	0.63	0.751
Rep	4	833.3	208.3	0.50	0.737
Residual	56	23416.7	418.2		
Total	74	62916.7			

3.2.6.5 Analysis of variance of three non-sterilised soil types with four spore concentrations of C14 strain against grass grub after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_Type	2	116.7	58.3	0.15	0.860
Spore_Concentration	4	51416.7	12854.2	33.35	<.001
Soil_Type.Spore_Concentration					
	8	1633.3	204.2	0.53	0.829
Rep	4	1916.7	479.2	1.24	0.303
Residual	56	21583.3	385.4		
Total	74	76666.7			

3.2.6.6 Analysis of variance of three non-sterilised soil types with four spore concentrations of C14 strain against grass grub after 35 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_Type	2	16.7	8.3	0.03	0.969
Spore_Concentration	4	63550.0	15887.5	60.52	<.001
Soil_Type.Spore_Concentration					
	8	650.0	81.3	0.31	0.959
Rep	4	1300.0	325.0	1.24	0.305
Residual	56	14700.0	262.5		
Total	74	80216.7			

3.2.6.7 Analysis of variance of LT_{50} value of three non-sterilised soil types with four spore concentrations of C14 strain against grass grub

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_Type	2	31.03	15.52	0.44	0.650
Spore_Concentration	3	1097.06	365.69	10.27	<.001
Soil_Type.Spore_Concentration					
	6	75.68	12.61	0.35	0.904
Rep	4	68.60	17.15	0.48	0.749
Residual	44	1567.46	35.62		
Total	59	2839.82			

3.2.6.8 Analysis of variance of LC_{50} value of three non-sterilised soil types with four spore concentrations of C14 strain against grass grub at 28 days

Source	d.f.	deviance	deviance	ratio
Regression	6	35.76	5.9604	6.18
Residual	68	65.57	0.9643	
Total	74	101.33	1.3693	

3.2.7 F99 strain for the third instar larvae

3.2.7.1 Analysis of variance of three non-sterilised soil types with four spore concentrations of F99 strain against grass grub after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_Type	2	50.00	25.00	0.61	0.548
Spore_Concentration	4	116.67	29.17	0.71	0.588
Soil_Type.Spore_Concentration					
	8	783.33	97.92	2.38	0.028
Rep	4	200.00	50.00	1.22	0.314
Residual	56	2300.00	41.07		
Total	74	3450.00			

3.2.7.2 Analysis of variance of three non-sterilised soil types with four spore concentrations of F99 strain against grass grub after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_Type	2	216.67	108.33	1.60	0.212
Spore_Concentration	4	1783.33	445.83	6.57	<.001
Soil_Type.Spore_Concentration					
	8	2116.67	264.58	3.90	0.001
Rep	4	450.00	112.50	1.66	0.173
Residual	56	3800.00	67.86		
Total	74	8366.67			

3.2.7.3 Analysis of variance of three non-sterilised soil types with four spore concentrations of F99 strain against grass grub after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_Type	2	266.7	133.3	0.30	0.743
Spore_Concentration	4	46450.0	11612.5	26.05	<.001
Soil_Type.Spore_Concentration					
	8	2650.0	331.2	0.74	0.653
Rep	4	1283.3	320.8	0.72	0.582
Residual	56	24966.7	445.8		
Total	74	75616.7			

3.2.7.4 Analysis of variance of three non-sterilised soil types with four spore concentrations of F99 strain against grass grub after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_Type	2	116.7	58.3	0.17	0.845
Spore_Concentration	4	57133.3	14283.3	41.48	<.001
Soil_Type.Spore_Concentration					
	8	1966.7	245.8	0.71	0.678
Rep	4	1466.7	366.7	1.06	0.383
Residual	56	19283.3	344.3		
Total	74	79966.7			

3.2.7.5 Analysis of variance of three non-sterilised soil types with four spore concentrations of F99 strain against grass grub after 35 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_Type	2	116.7	58.3	0.14	0.869
Spore_Concentration	4	56366.7	14091.7	33.87	<.001
Soil_Type.Spore_Concentration					
	8	1133.3	141.7	0.34	0.946
Rep	4	1950.0	487.5	1.17	0.333
Residual	56	23300.0	416.1		
Total	74	82866.7			

3.2.7.6 Analysis of variance of LT_{50} value of three non-sterilised soil types with four spore concentrations of F99 strain against grass grub

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_Type	2	5.19	2.59	0.08	0.924
Spore_Concentration	2	1696.54	848.27	25.84	<.001
Soil_Type.Spore_Concentration					
	4	42.96	10.74	0.33	0.858
Rep	4	112.64	28.16	0.86	0.500
Residual	32	1050.62	32.83		
Total	44	2907.96			

3.2.7.7 Analysis of variance of LC50 of three non-sterilised soil types with four spore concentrations of F99 strain against grass grub at 28 days

Source	d.f.	deviance	deviance	ratio
Regression	6	69.87	11.645	10.91
Residual	68	72.61	1.068	
Total	74	142.48	1.925	

3.2.8 M2 strain for the third instar larvae

3.2.8.1 Analysis of variance of three non-sterilised soil types with four spore concentrations of M2 strain against grass grub after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_Type	2	316.67	158.33	2.92	0.062
Spore_Concentration	4	216.67	54.17	1.00	0.415
Soil_Type.Spore_Concentration					
	8	183.33	22.92	0.42	0.902
Rep	4	216.67	54.17	1.00	0.415
Residual	56	3033.33	54.17		
Total	74	3966.67			

3.2.8.2 Analysis of variance of three non-sterilised soil types with four spore concentrations of M2 strain against grass grub after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_Type	2	1050.0	525.0	2.93	0.062
Spore_Concentration	4	10616.7	2654.2	14.79	<.001
Soil_Type.Spore_Concentration					
	8	3533.3	441.7	2.46	0.023
Rep	4	200.0	50.0	0.28	0.891
Residual	56	10050.0	179.5		
Total	74	25450.0			

3.2.8.3 Analysis of variance of three non-sterilised soil types with four spore concentrations of M2 strain against grass grub after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_Type	2	1116.7	558.3	1.79	0.177
Spore_Concentration	4	49000.0	12250.0	39.20	<.001
Soil_Type.Spore_Concentration					
	8	1300.0	162.5	0.52	0.836
Rep	4	1500.0	375.0	1.20	0.321
Residual	56	17500.0	312.5		
Total	74	70416.7			

3.2.8.4 Analysis of variance of three non-sterilised soil types with four spore concentrations of M2 strain against grass grub after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_Type	2	716.7	358.3	0.94	0.396
Spore_Concentration	4	70216.7	17554.2	46.19	<.001
Soil_Type.Spore_Concentration					
	8	2533.3	316.7	0.83	0.577
Rep	4	716.7	179.2	0.47	0.756
Residual	56	21283.3	380.1		
Total	74	95466.7			

3.2.8.5 Analysis of variance of three non-sterilised soil types with four spore concentrations of M2 strain against grass grub after 35 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_Type	2	516.7	258.3	0.75	0.477
Spore_Concentration	4	82283.3	20570.8	59.69	<.001
Soil_Type.Spore_Concentration					
	8	566.7	70.8	0.21	0.989
Rep	4	700.0	175.0	0.51	0.730
Residual	56	19300.0	344.6		
Total	74	103366.7			

3.2.8.6 Analysis of variance of LT_{50} value of three non-sterilised soil types with four spore concentrations of M2 strain against grass grub

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soil_Type	2	188.42	94.21	1.31	0.285
Spore_Concentration	2	2010.88	1005.44	13.93	<.001
Soil_Type.Spore_Concentration					
	4	139.30	34.82	0.48	0.748
Rep	4	93.34	23.34	0.32	0.860
Residual	32	2309.05	72.16		
Total	44	4740.98			

3.2.8.7 Analysis of variance of LC_{50} of three non-sterilised soil types with four spore concentrations of M2 strain against grass grub at 28 days

Source	d.f.	deviance	deviance	ratio
Regression	6	93.21	15.535	12.70
Residual	68	83.16	1.223	
Total	74	176.37	2.383	

3.3 Analyses of three field soil type used for the experiment 1 and 2 by Hill Laboratories

Hill Laboratories
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ANALYSIS REPORT

Client:	Lincoln University	**Lab No:**	1925637 shpv1
Address:	Finance Department	**Date Received:**	15-Feb-2018
	PO Box 85094	**Date Reported:**	19-Feb-2018
	Lincoln University	**Quote No:**	
	Lincoln 7647	**Order No:**	LU456288
		Client Reference:	
Phone:	03 325 2811	**Submitted By:**	Nghia Thi Nguyen

Temuka

Analysis	Units	Level Found	Medium Range
pH	pH Units	6.0	5.7 - 6.2
Olsen Phosphorus	mg/L	5	20 - 30
Potassium	me/100g	0.43	0.30 - 0.60
Calcium	me/100g	7.8	5.0 - 12.0
Magnesium	me/100g	1.91	0.60 - 1.20
Sodium	me/100g	0.16	0.00 - 0.30
CEC	me/100g	15	12 - 25
Total Base Saturation	%	70	50 - 85
Volume Weight	g/mL	0.95	0.60 - 1.00
Sulphate Sulphur	mg/kg	2	10 - 20
Potentially Available Nitrogen (15cm Depth)*	kg/ha	73	100 - 150
Anaerobically Mineralisable N*	µg/g	51	
Organic Matter*	%	5.4	7.0 - 17.0
Total Carbon*	%	3.1	
Total Nitrogen*	%	0.30	0.30 - 0.60
C/N Ratio*		10.4	
Anaerobically Mineralisable N/Total N Ratio*	%	1.7	3.0 - 5.0
Soil Sample Depth*	mm	0-150	
Base Saturation %		K 2.9 Ca 53	Mg 13.0 Na 1.1
MAF Units		K 8 Ca 9	Mg 41 Na 7

Templeton

Analysis	Units	Level Found	Medium Range
pH	pH Units	5.7	5.7 - 6.2
Olsen Phosphorus	mg/L	23	20 - 30
Potassium	me/100g	0.56	0.30 - 0.60
Calcium	me/100g	5.6	5.0 - 12.0
Magnesium	me/100g	0.87	0.60 - 1.20
Sodium	me/100g	0.15	0.00 - 0.30
CEC	me/100g	11	12 - 25
Total Base Saturation	%	63	50 - 85
Volume Weight	g/mL	1.03	0.60 - 1.00
Sulphate Sulphur	mg/kg	7	10 - 20
Potentially Available Nitrogen (15cm Depth)*	kg/ha	43	100 - 150
Anaerobically Mineralisable N*	µg/g	28	
Organic Matter*	%	3.7	7.0 - 17.0
Total Carbon*	%	2.2	
Total Nitrogen*	%	0.17	0.30 - 0.60
C/N Ratio*		12.5	
Anaerobically Mineralisable N/Total N Ratio*	%	1.6	3.0 - 5.0
Soil Sample Depth*	mm	0-150	
Base Saturation %		K 4.9 Ca 49	Mg 7.7 Na 1.3
MAF Units		K 12 Ca 7	Mg 20 Na 7

Wakanui

Analysis	Units	Level Found	Medium Range
pH	pH Units	6.3	5.7 - 6.2
Olsen Phosphorus	mg/L	14	20 - 30
Potassium	me/100g	1.36	0.30 - 0.60
Calcium	me/100g	6.5	5.0 - 12.0
Magnesium	me/100g	1.51	0.60 - 1.20
Sodium	me/100g	0.14	0.00 - 0.30
CEC	me/100g	13	12 - 25
Total Base Saturation	%	72	50 - 85
Volume Weight	g/mL	0.93	0.60 - 1.00
Sulphate Sulphur	mg/kg	1	10 - 20
Potentially Available Nitrogen (15cm Depth)*	kg/ha	82	100 - 150
Anaerobically Mineralisable N*	µg/g	58	
Organic Matter*	%	5.5	7.0 - 17.0
Total Carbon*	%	3.2	
Total Nitrogen*	%	0.31	0.30 - 0.60
C/N Ratio*		10.1	
Anaerobically Mineralisable N/Total N Ratio*	%	1.9	3.0 - 5.0
Soil Sample Depth*	mm	0-150	
Base Saturation %		K 10.3 Ca 50	Mg 11.5 Na 1.0
MAF Units		K 26 Ca 8	Mg 32 Na 6

Sterilized Wakanui

Analysis	Units	Level Found	Medium Range
pH	pH Units	5.9	5.7 - 6.2
Olsen Phosphorus	mg/L	31	20 - 30
Potassium	me/100g	0.96	0.30 - 0.60
Calcium	me/100g	7.2	5.0 - 12.0
Magnesium	me/100g	1.33	0.60 - 1.20
Sodium	me/100g	0.13	0.00 - 0.30
CEC	me/100g	14	12 - 25
Total Base Saturation	%	67	50 - 85
Volume Weight	g/mL	1.04	0.60 - 1.00
Sulphate Sulphur	mg/kg	6	10 - 20
Potentially Available Nitrogen (15cm Depth)*	kg/ha	131	100 - 150
Anaerobically Mineralisable N*	µg/g	84	
Organic Matter*	%	5.5	7.0 - 17.0
Total Carbon*	%	3.2	
Total Nitrogen*	%	0.27	0.30 - 0.60
C/N Ratio*		11.6	
Anaerobically Mineralisable N/Total N Ratio*	%	3.1	3.0 - 5.0
Soil Sample Depth*	mm	0-150	
Base Saturation %		K 6.6 Ca 50	Mg 9.2 Na 0.9
MAF Units		K 20 Ca 9	Mg 31 Na 6

Appendix C for chapter 4

4.1 Analysis of variance of plant dry weight after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Endophyte	1	0.012960	0.012960	4.36	0.044
Metarhizium	1	0.005760	0.005760	1.94	0.172
Endophyte.Metarhizium	1	0.002890	0.002890	0.97	0.331
Residual	36	0.106980	0.002972		
Total	39	0.128590			

Appendix D for chapter 5

5.1 Material for experiments

5.1.1 Bacteria general culture medium

A bacterial general culture medium, essentially Luria-Bertani Miller (LB) with 1.5% agar with antibiotics (recipe below), was used for culturing of bacteria isolated from soil and larvae.

- ❖ Bacteria General Culture Medium

Dissolved in 1 L of distilled water:

Luria-Bertani, Miller (Difco)	25 g
Bacteriological agar (1.5%)	15g

Then autoclaved at 121 ^{0}C at 15 SI for 15 min

Bacteria were plated as described in sections 5.3.1 and 5.3.2 using the following extraction solutions:

5.1.2 Bacterial extraction solution used for larval extractions

For isolating bacteria from dead grass grub larvae a phosphate buffered solution with the following ingredients was used:

- ❖ Phosphate buffered solution

Dissolved in 1 L of distilled water:

NaCl	8 g
KCl	0.2g
Na_2HPO_4	1.44g
KH_2PO_4	0.24g

Adjust pH to 7.4 with HCl

5.1.3 Bacterial extraction solution used for soil extractions

For the extraction of bacteria from soil through the flask method the following solutions were used:

- ❖ Soil extraction solutions

Peptone water (0.1%): 1g bacteriological peptone in 1L dH_2O.

Phosphate buffer (0.1M):

Working stock:	Solution A (K_2HPO_4)	65 ml
	Solution B (KH_2PO_4)	35 ml
	dH_2O	900 ml

Dispensed into 500ml Schott bottles.

Then autoclaved at 121 ^{0}C at 15 SI for 15 min

5.2 Gel photos

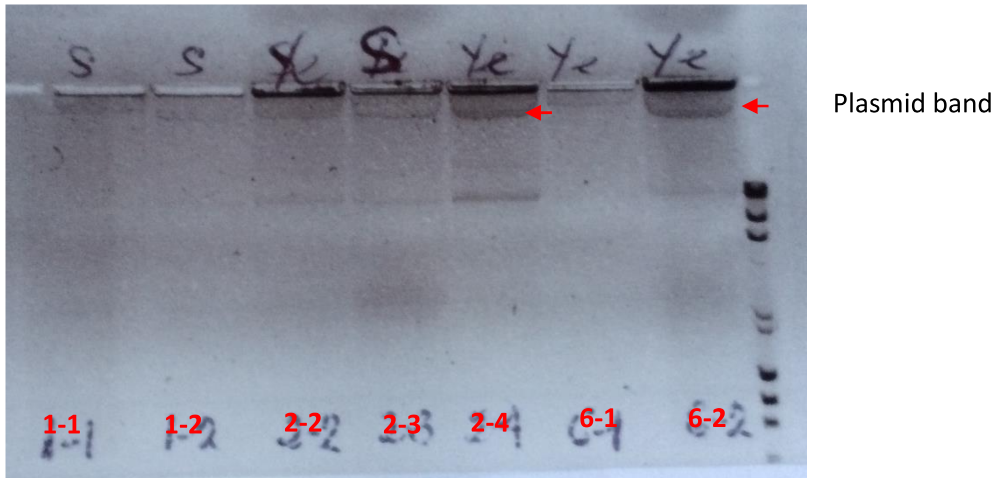


Figure 1: Visualization of plasmids (as indicated by the arrow) in *Serratia* spp. isolates 1-1, 1-2 and 2-3 and *Yersinia* spp. isolates 2-2, 2-4 and 6-2.

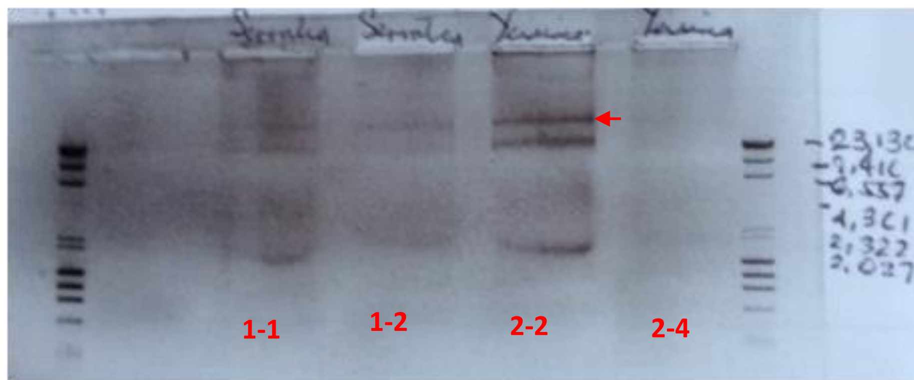


Figure 2: Visualization of plasmids (as indicated by the arrow) in *Serratia* spp. isolates 1-1 and 1-2 and *Yersinia* spp. isolates 2-2 and 2-4.

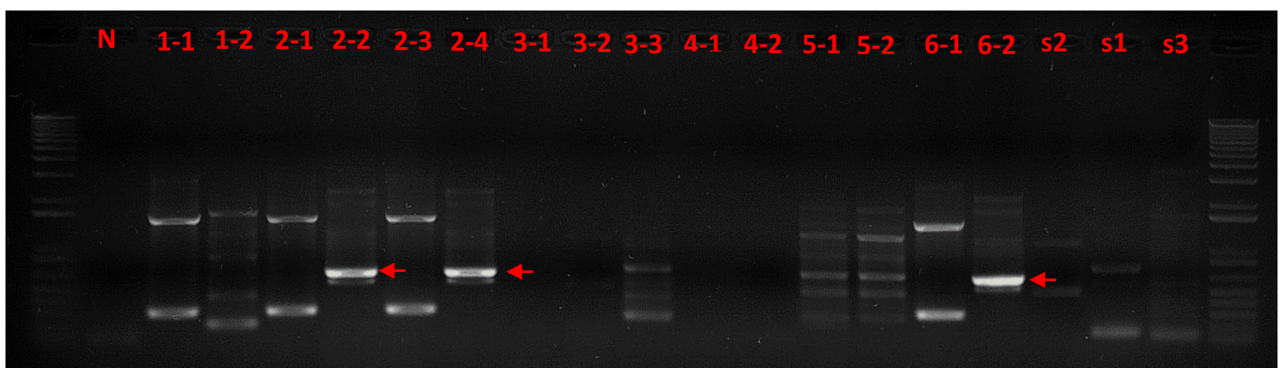


Figure 3: Gel photo of the PCR products of the 18 bacteria isolates following amplification with primers SepCF and SepCR. Strong bands of approximately 550bp in length, as pointed by the arrow which indicates the presence of *S. entomophila* pADAP derived SepC TC-A toxin encoding genes.

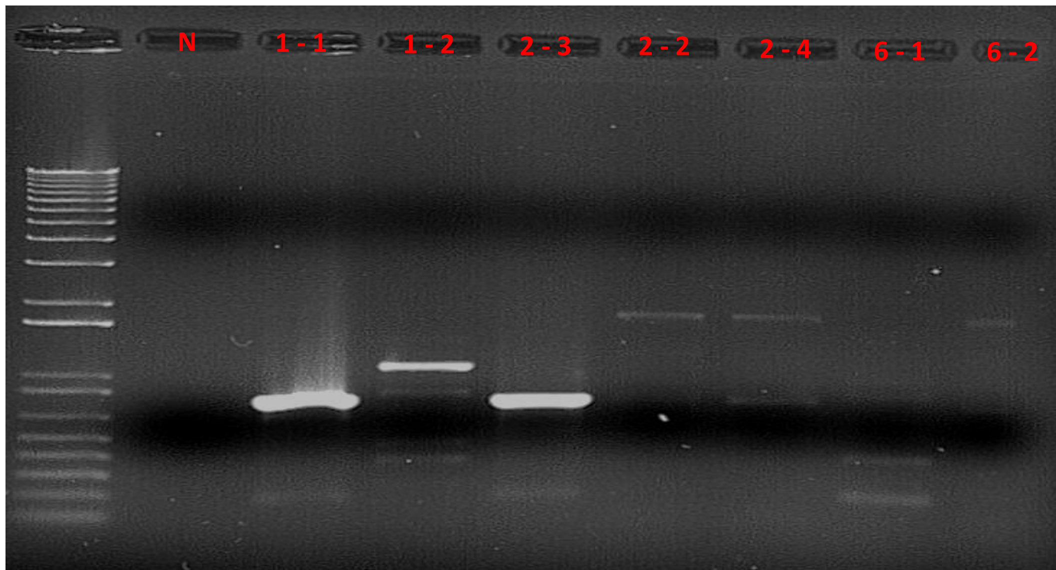


Figure 4: Gel photo of PCR products of seven *Serratia* spp. and *Yersinia* spp. isolates amplified with primers Afp18F and Afp18R. Strong bands of approximately 800bp in length, as pointed by the arrows which indicates the presence of the putative Afp18 toxin encoding genes.

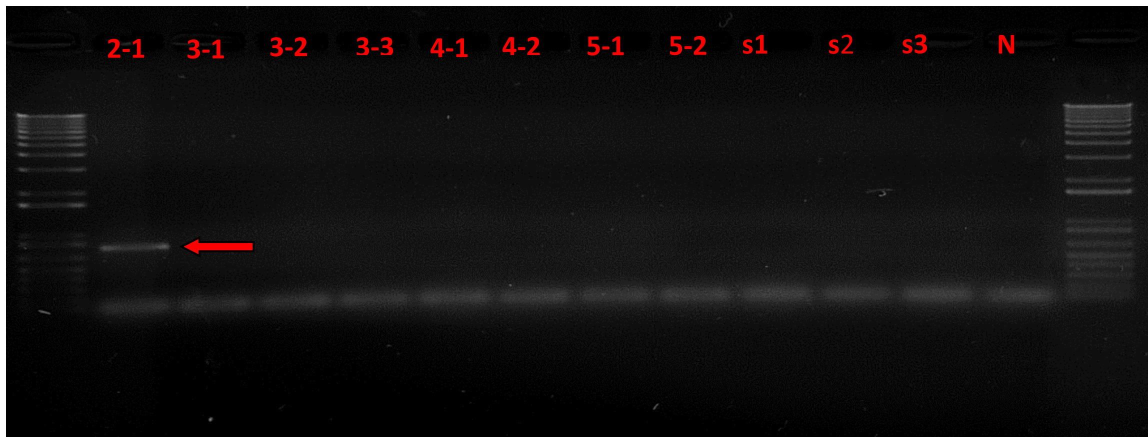


Figure 5: Gel photo of PCR products of bacterial isolates with primers: Afp18F and Afp18R. Isolate 2-1 was identified as a *Serratia spp* and showed the band size of approximately 800bp pointed by the arrow indicating the presence of the putative Afp18 toxin encoding genes.

5.3 Statistical analyses

5.3.1 The combination of C14 and eight bacterial strains against second instar larvae

5.3.1.1 Analysis of variance of the combination of C14 and eight bacterial strains against second larvae after 7 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	236.11	59.03	1.60	
Treatment	17	750.00	44.12	1.19	0.294
Residual	68	2513.89	36.97		
Total	89	3500.00			

5.3.1.2 Analysis of variance of the combination of C14 and eight bacterial strains against second larvae after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	694.44	173.61	2.03	
Treatment	17	3812.50	224.26	2.63	0.003
Residual	68	5805.56	85.38		
Total	89	10312.50			

5.3.1.3 Analysis of variance of the combination of C14 and eight bacterial strains against second larvae after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1013.9	253.5	1.15	
Treatment	17	13000.0	764.7	3.47	<.001
Residual	68	14986.1	220.4		
Total	89	29000.0			

5.3.1.4 Analysis of variance of the combination of C14 and eight bacterial strains against second larvae after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1180.6	295.1	0.99	
Treatment	17	60305.6	3547.4	11.87	<.001
Residual	68	20319.4	298.8		
Total	89	81805.6			

5.3.1.5 Analysis of variance of the combination of C14 and eight bacterial strains against second larvae after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	791.7	197.9	0.61	
Treatment	17	113312.5	6665.4	20.41	<.001
Residual	68	22208.3	326.6		
Total	89	136312.5			

5.3.1.6 Analysis of variance of the combination of C14 and eight bacterial strains against second larvae after 35 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1069.4	267.4	1.24	
Treatment	17	144138.9	8478.8	39.27	<.001
Residual	68	14680.6	215.9		
Total	89	159888.9			

5.3.1.6 Analysis of variance of LT_{50} value of the combination of C14 and eight bacterial strains against second larvae

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	163.19	40.80	1.27	
Treatment	8	538.85	67.36	2.10	0.066
Residual	32	1027.21	32.10		
Total	44	1729.25			

5.3.2 The combination of C14 and eight bacterial strains against third instar larvae

5.3.2.1 Analysis of variance of the combination of C14 and eight bacterial strains against third larvae after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	281.2	70.3	0.61	
Treatment	15	2054.7	137.0	1.18	0.312
Residual	60	6968.8	116.1		
Total	79	9304.7			

5.3.2.2 Analysis of variance of the combination of C14 and eight bacterial strains against third larvae after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	578.1	144.5	0.49	
Treatment	15	21117.2	1407.8	4.78	<.001
Residual	60	17671.9	294.5		
Total	79	39367.2			

5.3.2.3 Analysis of variance of the combination of C14 and eight bacterial strains against third larvae after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	890.6	222.7	0.69	
Treatment	15	97125.0	6475.0	20.07	<.001
Residual	60	19359.4	322.7		
Total	79	117375.0			

5.3.2.4 Analysis of variance of the combination of C14 and eight bacterial strains against third larvae after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1140.6	285.2	1.58	
Treatment	15	157304.7	10487.0	57.94	<.001
Residual	60	10859.4	181.0		
Total	79	169304.7			

5.3.2.5 Analysis of variance of the combination of C14 and eight bacterial strains against third larvae after 35 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	828.1	207.0	1.25	
Treatment	15	168750.0	11250.0	68.03	<.001
Residual	60	9921.9	165.4		
Total	79	179500.0			

5.3.2.5 Analysis of variance of LT_{50} value of the combination of C14 and eight bacterial strains against third larvae

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	102.44	25.61	0.97	
Treatment	7	161.20	23.03	0.87	0.542
Residual	28	741.07	26.47		
Total	39	1004.71			

5.3.3 The combination of C14 and isolate 6-1 against second instar larvae

5.3.3.1 Analysis of variance of the combination of C14 and isolate 6-1 against second instar larvae after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	93.8	23.4	0.12	
Treatment	7	1000.0	142.9	0.74	0.640
Residual	28	5406.2	193.1		
Total	39	6500.0			

5.3.3.2 Analysis of variance of the combination of C14 and isolate 6-1 against second instar larvae after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	250.0	62.5	0.16	
Treatment	7	2609.4	372.8	0.97	0.471
Residual	28	10750.0	383.9		
Total	39	13609.4			

5.3.3.3 Analysis of variance of the combination of C14 and isolate 6-1 against second instar larvae after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1031.2	257.8	0.60	
Treatment	7	7484.4	1069.2	2.50	0.040
Residual	28	11968.8	427.5		
Total	39	20484.4			

5.3.3.4 Analysis of variance of the combination of C14 and isolate 6-1 against second instar larvae after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	3531.2	882.8	2.20	
Treatment	7	21750.0	3107.1	7.75	<.001
Residual	28	11218.8	400.7		
Total	39	36500.0			

5.3.3.5 Analysis of variance of the combination of C14 and isolate 6-1 against second instar larvae after 35 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	7343.8	1835.9	7.18	
Treatment	7	36984.4	5283.5	20.67	<.001
Residual	28	7156.2	255.6		
Total	39	51484.4			

5.3.3.6 Analysis of variance of LT_{50} value of the combination of C14 and isolate 6-1 against second instar larvae

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	31.58	7.89	0.13	
Treatment	3	136.55	45.52	0.73	0.554
Residual	12	749.32	62.44		
Total	19	917.45			

5.3.4 The combination of C14 and isolate 6-1 against third instar larvae

5.3.4.1 Analysis of variance of the combination of C14 and isolate 6-1 against third instar larvae after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	250.00	62.50	1.40	
Treatment	7	234.38	33.48	0.75	0.633
Residual	28	1250.00	44.64		
Total	39	1734.38			

5.3.4.2 Analysis of variance of the combination of C14 and isolate 6-1 against third instar larvae after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1156.2	289.1	1.14	
Treatment	7	8687.5	1241.1	4.90	0.001
Residual	28	7093.8	253.3		
Total	39	16937.5			

5.3.4.3 Analysis of variance of the combination of C14 and isolate 6-1 against third instar larvae after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1000.0	250.0	0.90	
Treatment	7	54750.0	7821.4	28.26	<.001
Residual	28	7750.0	276.8		
Total	39	63500.0			

5.3.4.4 Analysis of variance of the combination of C14 and isolate 6-1 against third instar larvae after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	218.8	54.7	0.28	
Treatment	7	68687.5	9812.5	49.67	<.001
Residual	28	5531.2	197.5		
Total	39	74437.5			

5.3.4.5 Analysis of variance of LT_{50} value of C14 and isolate 6-1 against third instar larvae

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	7.131	1.783	0.22	
Treatment	3	66.806	22.269	2.81	0.085
Residual	12	95.125	7.927		
Total	19	169.061			

5.3.5 The combination of C14 and isolate 6-1 against second instar larvae

5.3.5.1 Analysis of variance of the combination of C14 and isolate 3-2 against second instar larvae after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	250.00	62.50	2.33	
Treatment	7	187.50	26.79	1.00	0.452
Residual	28	750.00	26.79		
Total	39	1187.50			

5.3.5.2 Analysis of variance of the combination of C14 and isolate 3-2 against second instar larvae after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	843.8	210.9	0.75	
Treatment	7	18187.5	2598.2	9.20	<.001
Residual	28	7906.2	282.4		
Total	39	26937.5			

5.3.5.3 Analysis of variance of the combination of C14 and isolate 3-2 against second instar larvae after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1093.8	273.4	0.63	
Treatment	7	41359.4	5908.5	13.61	<.001
Residual	28	12156.2	434.2		
Total	39	54609.4			

5.3.5.4 Analysis of variance of the combination of C14 and isolate 3-2 against second instar larvae after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1500.0	375.0	0.75	
Treatment	7	39437.5	5633.9	11.27	<.001
Residual	28	14000.0	500.0		
Total	39	54937.5			

5.3.5.5 Analysis of variance of LT_{50} value of C14 and isolate 3-2 against second instar larvae

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	108.09	27.02	1.15	
Treatment	3	28.11	9.37	0.40	0.756
Residual	12	281.32	23.44		
Total	19	417.52			

5.3.6 The combination of C14 and isolate 3-2 against third instar larvae after 28 days

5.3.6.1 Analysis of variance of the combination of C14 and isolate 3-2 against third instar larvae after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	218.75	54.69	1.00	
Treatment	7	500.00	71.43	1.31	0.284
Residual	28	1531.25	54.69		
Total	39	2250.00			

5.3.6.2 Analysis of variance of the combination of C14 and isolate 3-2 against third instar larvae after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	406.2	101.6	0.45	
Treatment	7	14750.0	2107.1	9.30	<.001
Residual	28	6343.8	226.6		
Total	39	21500.0			

5.3.6.3 Analysis of variance of the combination of C14 and isolate 3-2 against third instar larvae after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	562.5	140.6	0.35	
Treatment	7	33109.4	4729.9	11.84	<.001
Residual	28	11187.5	399.6		
Total	39	44859.4			

5.3.6.4 Analysis of variance of the combination of C14 and isolate 3-2 against third instar larvae after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate stratum	4	625.0	156.2	0.30	
Treatment	7	32484.4	4640.6	8.88	<.001
Residual	28	14625.0	522.3		
Total	39	47734.4			

5.3.6.5 Analysis of variance of of LT_{50} value of the combination of C14 and isolate 3-2 against third instar larvae

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	91.64	22.91	2.06	
Treatment	3	63.25	21.08	1.89	0.184
Residual	12	133.58	11.13		
Total	19	288.47			

5.3.7 The combination of C14 and isolate A1MO2 against second instar larvae

5.3.7.1 Analysis of variance of the combination of C14 and isolate A1MO2 against second instar larvae after 3 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	218.75	54.69	1.00	
Treatment	7	500.00	71.43	1.31	0.284
Residual	28	1531.25	54.69		
Total	39	2250.00			

5.3.7.2 Analysis of variance of the combination of C14 and isolate A1MO2 against second instar larvae after 5 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	312.50	78.12	1.30	
Treatment	7	734.38	104.91	1.74	0.140
Residual	28	1687.50	60.27		
Total	39	2734.38			

5.3.7.3 Analysis of variance of the combination of C14 and isolate A1MO2 against second instar larvae after 7 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	312.50	78.12	0.81	
Treatment	7	2937.50	419.64	4.37	0.002
Residual	28	2687.50	95.98		
Total	39	5937.50			

5.3.7.4 Analysis of variance of the combination of C14 and isolate A1MO2 against second instar larvae after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1625.0	406.2	1.05	
Treatment	7	9984.4	1426.3	3.67	0.006
Residual	28	10875.0	388.4		
Total	39	22484.4			

5.3.7.5 Analysis of variance of the combination of C14 and isolate A1MO2 against second instar larvae after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1156.2	289.1	1.86	
Treatment	7	20109.4	2872.8	18.52	<.001
Residual	28	4343.8	155.1		
Total	39	25609.4			

5.3.7.6 Analysis of variance of the combination of C14 and isolate A1MO2 against second instar larvae after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	4468.8	1117.2	3.28	
Treatment	7	33359.4	4765.6	14.00	<.001
Residual	28	9531.2	340.4		
Total	39	47359.4			

5.3.7.7 Analysis of variance of the combination of C14 and isolate A1MO2 against second instar larvae after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	875.0	218.8	0.60	
Treatment	7	41359.4	5908.5	16.34	<.001
Residual	28	10125.0	361.6		
Total	39	52359.4			

5.3.7.8 Analysis of variance of LT_{50} value of the combination of C14 and isolate A1MO2 against second instar larvae

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	64.16	16.04	0.90	
Treatment	5	819.49	163.90	9.25	<.001
Residual	20	354.54	17.73		
Total	29	1238.19			

5.3.8 The combination of C14 and isolate A1MO2 against third instar larvae

5.3.8.1 Analysis of variance of the combination of C14 and isolate A1MO2 against third instar larvae after 7 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	93.75	23.44	0.72	
Treatment	7	187.50	26.79	0.83	0.573
Residual	28	906.25	32.37		
Total	39	1187.50			

5.3.8.2 Analysis of variance of the combination of C14 and isolate A1MO2 against third instar larvae after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	468.75	117.19	1.30	
Treatment	7	984.38	140.62	1.56	0.190
Residual	28	2531.25	90.40		
Total	39	3984.38			

5.3.8.3 Analysis of variance of the combination of C14 and isolate A1MO2 against third instar larvae after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	406.2	101.6	0.69	
Treatment	7	7000.0	1000.0	6.84	<.001
Residual	28	4093.8	146.2		
Total	39	11500.0			

5.3.8.4 Analysis of variance of the combination of C14 and isolate A1MO2 against third instar larvae after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1343.8	335.9	1.31	
Treatment	7	15500.0	2214.3	8.66	<.001
Residual	28	7156.2	255.6		
Total	39	24000.0			

5.3.8.5 Analysis of variance of the combination of C14 and isolate A1MO2 against third instar larvae after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	406.2	101.6	0.30	
Treatment	7	23609.4	3372.8	9.84	<.001
Residual	28	9593.8	342.6		
Total	39	33609.4			

5.3.8.6 Analysis of variance of the combination of C14 and isolate A1MO2 against third instar larvae after 35 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	156.2	39.1	0.16	
Treatment	7	30187.5	4312.5	17.64	<.001
Residual	28	6843.8	244.4		
Total	39	37187.5			

5.3.8.7 Analysis of variance of LT_{50} value of the combination of C14 and isolate A1MO2 against third instar larvae

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	55.51	13.88	0.83	
Treatment	5	1323.82	264.76	15.91	<.001
Residual	20	332.80	16.64		
Total	29	1712.13			

5.3.9 The combination of C14 and isolate 145WT against third instar larvae

5.3.9.1 Analysis of variance of the combination of C14 and isolate 145WT against second instar larvae after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	218.75	54.69	1.00	
Treatment	7	2687.50	383.93	7.02	<.001
Residual	28	1531.25	54.69		
Total	39	4437.50			

5.3.9.2 Analysis of variance of the combination of C14 and isolate 145WT against second instar larvae after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	406.2	101.6	0.30	
Treatment	7	11109.4	1587.1	4.63	0.002
Residual	28	9593.8	342.6		
Total	39	21109.4			

5.3.9.3 Analysis of variance of the combination of C14 and isolate 145WT against second instar larvae after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	2593.8	648.4	1.26	
Treatment	7	29109.4	4158.5	8.08	<.001
Residual	28	14406.2	514.5		
Total	39	46109.4			

5.3.9.4 Analysis of variance of the combination of C14 and isolate 145WT against second instar larvae after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	2406.2	601.6	1.67	
Treatment	7	27484.4	3926.3	10.89	<.001
Residual	28	10093.8	360.5		
Total	39	39984.4			

5.3.9.5 Analysis of variance of LT_{50} value of the combination of C14 and isolate 145WT against second instar larvae

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	66.56	16.64	0.72	
Treatment	5	1024.18	204.84	8.89	<.001
Residual	20	460.90	23.04		
Total	29	1551.64			

5.3.10 The combination of C14 and isolate 145WT against third instar larvae

5.3.10.1 Analysis of variance of the combination of C14 and isolate 145WT against third instar larvae after 7 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	312.50	78.12	1.30	
Treatment	7	734.38	104.91	1.74	0.140
Residual	28	1687.50	60.27		
Total	39	2734.38			

5.3.10.2 Analysis of variance of the combination of C14 and isolate 145WT against third instar larvae after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	93.75	23.44	0.34	
Treatment	7	1609.38	229.91	3.38	0.010
Residual	28	1906.25	68.08		
Total	39	3609.38			

5.3.10.3 Analysis of variance of the combination of C14 and isolate 145WT against third instar larvae after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1156.2	289.1	1.67	
Treatment	7	7734.4	1104.9	6.39	<.001
Residual	28	4843.8	173.0		
Total	39	13734.4			

5.3.10.4 Analysis of variance of the combination of C14 and isolate 145WT against third instar larvae after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1781.2	445.3	1.39	
Treatment	7	7984.4	1140.6	3.56	0.007
Residual	28	8968.8	320.3		
Total	39	18734.4			

5.3.10.5 Analysis of variance of the combination of C14 and isolate 145WT against third instar larvae after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1656.2	414.1	1.02	
Treatment	7	17250.0	2464.3	6.08	<.001
Residual	28	11343.8	405.1		
Total	39	30250.0			

5.3.10.6 Analysis of variance of the combination of C14 and isolate 145WT against third instar larvae after 35 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
replicate stratum	4	406.2	101.6	0.20	
replicate.*Units* stratum					
Treatment	7	33250.0	4750.0	9.44	<.001
Residual	28	14093.8	503.3		
Total	39	47750.0			

5.3.10.7 Analysis of variance of LT_{50} value of the combination of C14 and isolate 145WT against third instar larvae

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	179.03	44.76	2.14	
Treatment	5	1125.05	225.01	10.76	<.001
Residual	20	418.22	20.91		
Total	29	1722.30			

5.3.11 The combination of C14 and isolate KIA against second instar larvae

5.3.11.1 Analysis of variance of the combination of C14 and isolate KIA against second instar larvae after 3 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1250.0	312.5	1.13	
Treatment	7	6000.0	857.1	3.10	0.015
Residual	28	7750.0	276.8		
Total	39	15000.0			

5.3.11.2 Analysis of variance of the combination of C14 and isolate KIA against second instar larvae after 5 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1031.2	257.8	1.12	
Treatment	7	45484.4	6497.8	28.13	<.001
Residual	28	6468.8	231.0		
Total	39	52984.4			

5.3.11.3 Analysis of variance of the combination of C14 and isolate KIA against second instar larvae after 7 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	62.5	15.6	0.09	
Treatment	7	61250.0	8750.0	52.27	<.001
Residual	28	4687.5	167.4		
Total	39	66000.0			

5.3.11.4 Analysis of variance of the combination of C14 and isolate KIA against second instar larvae after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	250.0	62.5	0.58	
Treatment	7	79734.4	11390.6	106.31	<.001
Residual	28	3000.0	107.1		
Total	39	82984.4			

5.3.11.5 Analysis of variance of the combination of C14 and isolate KIA against second instar larvae after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	531.2	132.8	1.16	
Treatment	7	69984.4	9997.8	86.97	<.001
Residual	28	3218.8	115.0		
Total	39	73734.4			

5.3.11.6 Analysis of variance of the combination of C14 and isolate KIA against second instar larvae after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1031.2	257.8	0.66	
Treatment	7	45984.4	6569.2	16.77	<.001
Residual	28	10968.8	391.7		
Total	39	57984.4			

5.3.11.7 Analysis of variance of the combination of C14 and isolate KIA against second instar larvae after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1031.2	257.8	0.83	
Treatment	7	36984.4	5283.5	16.97	<.001
Residual	28	8718.8	311.4		
Total	39	46734.4			

5.3.11.8 Analysis of variance of LT_{50} value of the combination of C14 and isolate KIA against second instar larvae

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	36.68	9.17	0.52	
Treatment	7	5800.98	828.71	47.43	<.001
Residual	28	489.27	17.47		
Total	39	6326.93			

5.3.12 The combination of C14 and isolate KIA against third instar larvae

5.3.12.1 Analysis of variance of the combination of C14 and isolate KIA against third instar larvae after 3 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	625.0	156.2	1.52	
Treatment	7	484.4	69.2	0.67	0.692
Residual	28	2875.0	102.7		
Total	39	3984.4			

5.3.12.2 Analysis of variance of the combination of C14 and isolate KIA against third instar larvae after 5 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1937.5	484.4	1.92	
Treatment	7	11687.5	1669.6	6.62	<.001
Residual	28	7062.5	252.2		
Total	39	20687.5			

5.3.12.3 Analysis of variance of the combination of C14 and isolate KIA against third instar larvae after 7 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	1468.8	367.2	1.24	
Treatment	7	37734.4	5390.6	18.23	<.001
Residual	28	8281.2	295.8		
Total	39	47484.4			

5.3.12.4 Analysis of variance of the combination of C14 and isolate KIA against third instar larvae after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	3343.8	835.9	1.25	
Treatment	7	43687.5	6241.1	9.37	<.001
Residual	28	18656.2	666.3		
Total	39	65687.5			

5.3.12.5 Analysis of variance of the combination of C14 and isolate KIA against third instar larvae after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	718.8	179.7	0.63	
Treatment	7	72750.0	10392.9	36.23	<.001
Residual	28	8031.2	286.8		
Total	39	81500.0			

5.3.12.6 Analysis of variance of the combination of C14 and isolate KIA against third instar larvae after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	156.2	39.1	0.25	
Treatment	7	73000.0	10428.6	67.22	<.001
Residual	28	4343.8	155.1		
Total	39	77500.0			

5.3.12.7 Analysis of variance of the combination of C14 and isolate KIA against third instar larvae after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	531.2	132.8	0.40	
Treatment	7	54687.5	7812.5	23.73	<.001
Residual	28	9218.8	329.2		
Total	39	64437.5			

5.3.12.8 Analysis of variance of the combination of C14 and isolate KIA against third instar larvae after 35 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	93.8	23.4	0.11	
Treatment	7	46750.0	6678.6	31.66	<.001
Residual	28	5906.2	210.9		
Total	39	52750.0			

5.3.12.9 Analysis of variance of LT_{50} value of the combination of C14 and isolate KIA against third instar larvae

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	45.91	11.48	0.95	
Treatment	6	5243.70	873.95	72.21	<.001
Residual	24	290.46	12.10		
Total	34	5580.07			

5.3.13 Analysis of variance of clearing zone of nine bacterial isolates

5.3.13.1 Analysis of variance of clearing zone of nine bacterial isolates grown on chitin medium after 3 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	0.0026750	0.0006687	0.89	
Treatment	9	2.0466125	0.2274014	302.36	<.001
Residual	36	0.0270750	0.0007521		
Total	49	2.0763625			

5.3.13.2 Analysis of variance of clearing zone of nine bacterial isolates grown on chitin medium after 6 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	0.009050	0.002262	1.91	
Treatment	9	8.100300	0.900033	758.81	<.001
Residual	36	0.042700	0.001186		
Total	49	8.152050			

5.3.13.3 Analysis of variance of clearing zone of nine bacterial isolates grown on chitin medium after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	4	0.010550	0.002637	1.14	
Treatment	9	18.321550	2.035728	878.20	<.001
Residual	36	0.083450	0.002318		
Total	49	18.415550			

5.3.14 Analysis of variance of volatile compounds released by nine bacteria isolates on radial growth of *M. novozealandicum* C14 after 22 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate	3	0.01756	0.00585	0.12	
Treatment	9	1.08244	0.12027	2.47	0.033
Residual	27	1.31369	0.04866		
Total	39	2.41369			

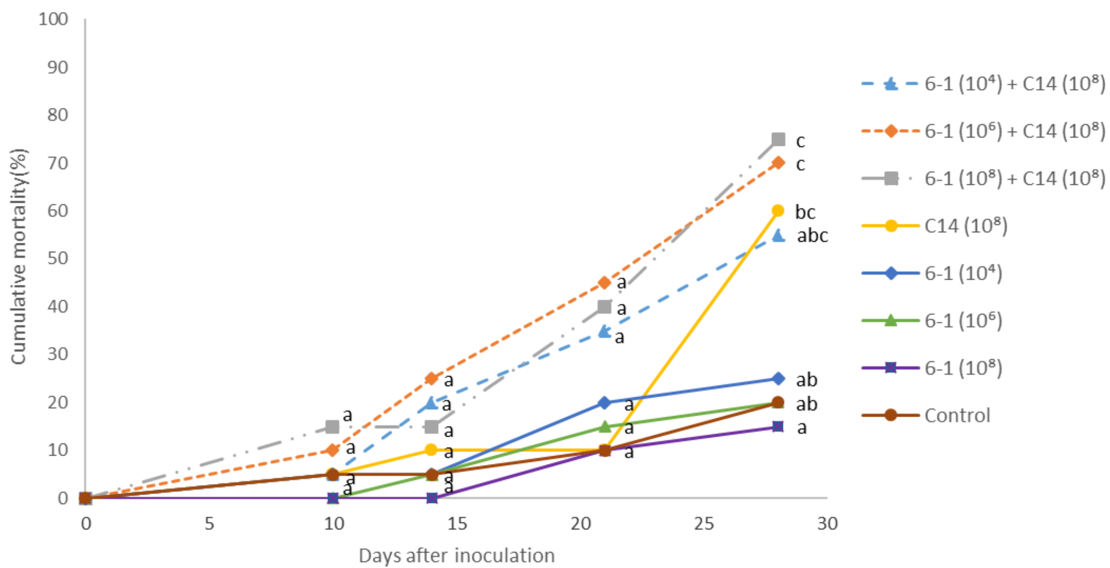
5.4 Additional results

5.4.1 The effect of different concentrations of *Yersinia enterocolitica* isolate 6-1 inoculum on *Metarhizium novozealandicum* (C14) pathogenicity to *Costelytra giveni*

Further bioassays were conducted using *Yersinia* isolate 6-1 which, in combination with C14, had the highest mortality for second instar larvae but not third instar larvae. *Yersinia* sp. isolate 6-1 alone had little effect on either second instar (Figure 5.1a) or third instar (Figure 5.1b) larvae at several bacterial inoculum concentrations. The *M. novozealandicum* treatment alone had a significant effect on cumulative mortality against both second instar larvae after day 21 ($P<0.05$) and third instar larvae after day 14 ($P<0.01$) compared to the untreated control, as did the combined treatments. There was

no difference in total mortality for either instar by the final day of observation (day 28) between the *M. novozealandicum* alone and the combined treatments.

a) second instar



b) third instar

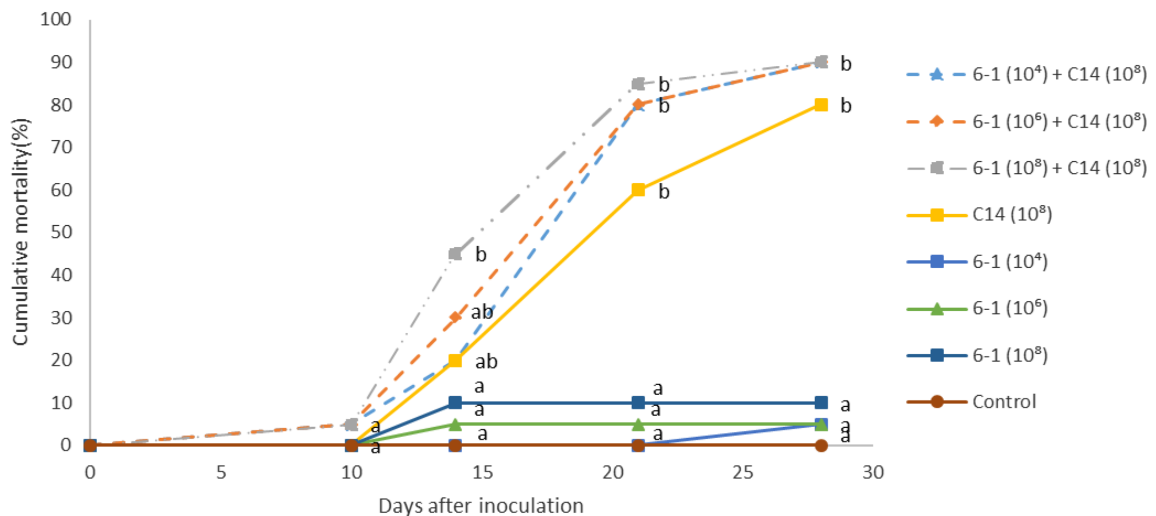


Figure 5.1 The cumulative mortality of (a) second instar and (b) third instar *Costelytra giveni* larvae resulting from single and combined inocula of *Metarhizium novozealandicum* (C14) (10^8 spores/ml) and *Yersinia* sp. isolate 6-1 at three concentration levels (10^4, 10^6 and 10^8 cells/ml). Letters indicate significance groupings based on Unprotected LSD test statistic: at each assessment data means with no letters in common are significantly different (P<0.05).

When *M. novozealandicum* was combined with the highest tested *Yersinia* isolate 6-1 concentration, the rate of larval mortality, measured as LT_{50}, was significantly greater than C14 alone for third instar larvae but not for second instar larvae (Table 5.1).

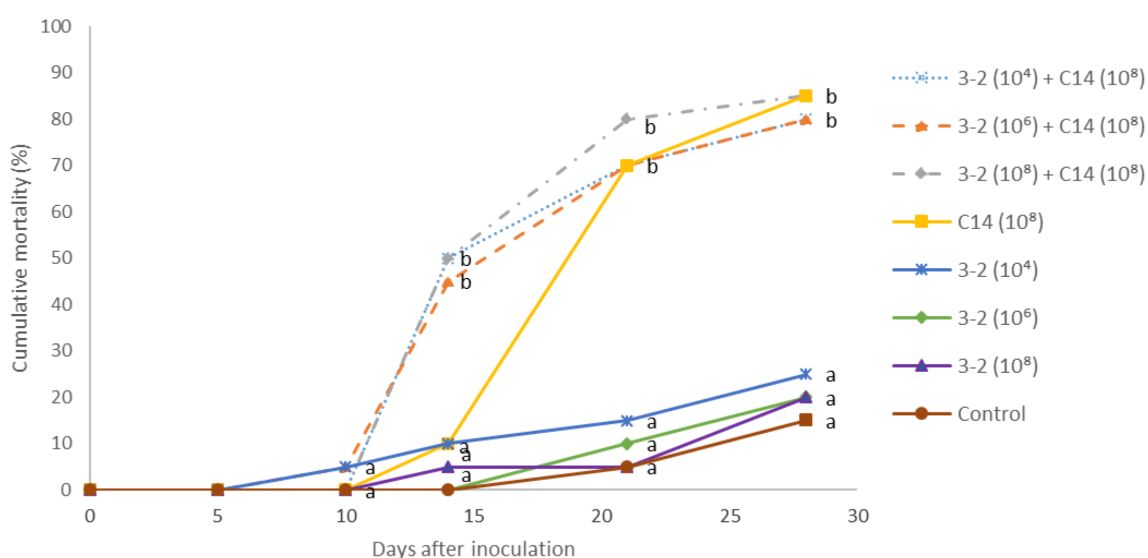
Table 5.1 The LT_{50} of second and third instar of *Costelytra giveni* larvae resulting from inoculation with *Metarhizium novozealandicum* alone and in combination with *Yersinia* isolate 6-1 at three different bacterial cell concentrations. Letters following the mean values indicate the results of pairwise Fisher's unprotected LSD tests conducted within each instar factor: means not marked with the same letter are significantly different.

Treatment	LT_{50} (days)-second instar	LT_{50} (days)-third instar
6-1 (10^4) + C14 (10^8)	23.1a	17.7ab
6-1 (10^6) + C14 (10^8)	20.9a	17.0ab
6-1 (10^8) + C14 (10^8)	20.9a	14.5a
C14 (10^8)	27.3a	19.6b
LSD (5%)	10.9	3.9

5.4.2 The effect of inoculum concentration of *Bacillus megaterium* isolate 3-2 with *M. novozealandicum* (C14) on pathogenicity to *Costelytra giveni*

Bacillus megaterium isolate 3-2 alone did not significantly increase cumulative mortality of either instar compared to the untreated control (Figure 5.2a, b). However, the *M. novozealandicum* C14 only treatment and all combination treatments between C14 and 3-2 isolate with different bacterial inocula levels produced significantly higher mortality than the control and all *B. megaterium* alone treatments with different inocula levels in both second and third instar larvae after day 14 ($P<0.01$). There was no difference between these treatments (C14 and C14 with 3-2 isolate). However, the C14 alone treatment caused higher mortality at day 21 than the combined treatments. There was no significant difference in mortality between C14 alone and the combined treatments after day 14 (Figure 5.2a, b).

a) second instar



b) third instar

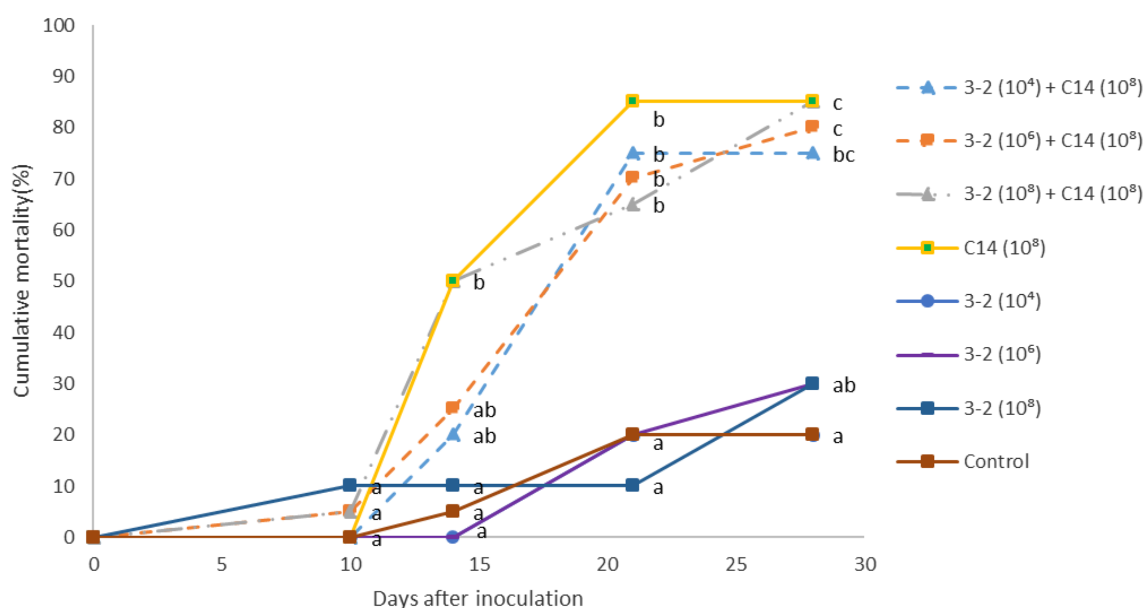


Figure 5.2 The cumulative mortality of (a) second instar and (b) third instar *Costelytra giveni* larvae resulting from single and combined inocula of *Metarhizium novozealandicum* (C14) (10^8 spores/ml) and *Bacillus megaterium* isolate 3-2 at three bacterial inoculum levels (10^4, 10^6 and 10^8 cells/ml). Letters indicate significance groupings based on Unprotected LSD test statistic: at each assessment data means with no letters in common are significantly different (P<0.05).

There were no significant differences in the LT_{50} values among any treatments in the experiment (Table 5.2). Interestingly, in this experiment two of the LT_{50} values were lower for the second instar than the third, unlike in the previous experiment (Table 5.1).

Table 5.2 The LT_{50} of second and third instar *Costelytra giveni* larvae resulting from inoculation with *Metarhizium novozealandicum* alone and in combination with *Bacillus megaterium* isolate 3-2 at three different bacterial cell concentrations. Letters following the mean values indicate the results of pairwise Fisher's LSD tests conducted within each instar factor: means not marked with the same letter are significantly different.

Treatment	LT_{50} (days)-second instar	LT_{50} (days)-third instar
3-2 (10^4) + C14 (10^8)	16.5a	18.7a
3-2 (10^6) + C14 (10^8)	15.4a	18.9a
3-2 (10^8) + C14 (10^8)	16.5a	15.1a
C14 (10^8)	18.7a	15.3a
LSD (5%)	6.7	4.6

5.4.3 Effect of inoculum concentration of *Metarhizium novozealandicum* (C14) and a single concentration of isolate A1MO2 alone and in combination for the control of *Costelytra giveni*

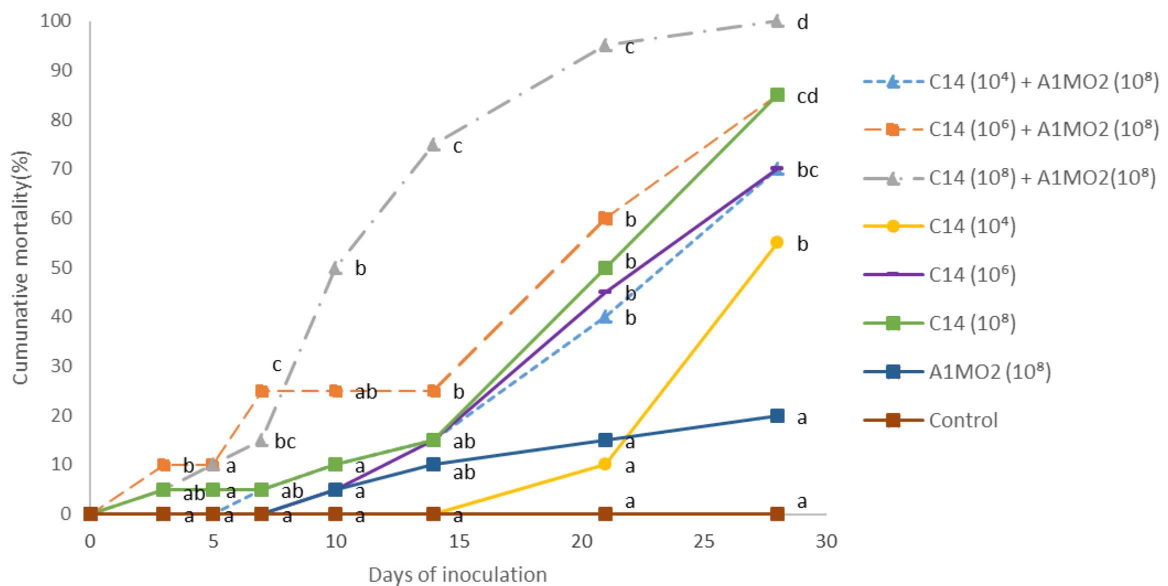
Serratia entomophila isolate A1MO2 alone had no significant effect on the mortality rates of either instar within the 28 days of the experiment, which was expected of this chronic disease-causing strain. Mortality due to amber disease can take over two months. All *M. novozealandicum*

inoculation concentration treatments significantly increased mortality of both second and third instar *C. giveni* larvae compared to the untreated control, and mortality significantly increased ($P<0.05$) with concentration (Figure 5.3a, b).

When applied to the second instar larvae, combined treatments with *M. novozealandicum* at inoculation concentrations of 10^6 and 10^8 produced higher mortality than the *M. novozealandicum* alone at the same concentrations after day 7 ($P<0.05$). Mortality reached over 50% at the highest combined treatment after day 10 and increased rapidly at 14 and 21 days. There was a combined interaction between C14 at the highest inoculation concentration and A1MO2 strain compared to each of three inoculation concentrations of C14 alone, separately, and the bacterium alone (Figure 5.3a).

Against third instar larvae, only when *M. novozealandicum* was inoculated at a concentration of 10^8 with bacteria did the combination treatment have a significantly higher mortality than the *M. novozealandicum* alone treatment, which occurred after day 14 ($P<0.05$). Mortality also rapidly increased at 21 and 28 days (Figure 5.3b).

a) second instar



b) third instar

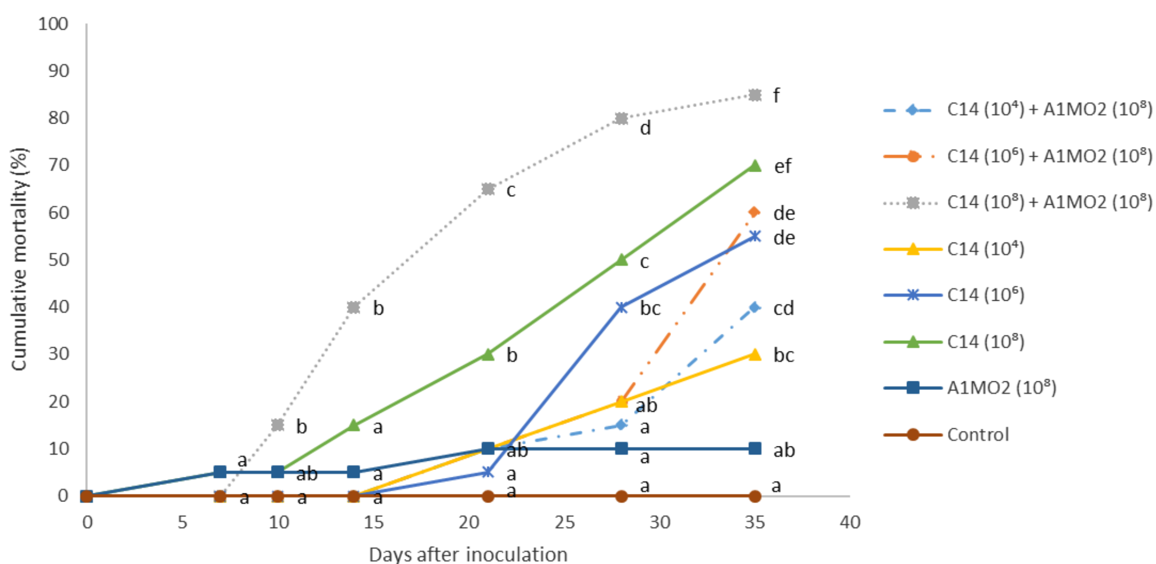


Figure 5.3 The cumulative mortality of (a) second instar and (b) third instar *Costelytra giveni* larvae resulting from inoculation with *Metarhizium novozealandicum* (C14) at three inoculum concentrations alone and in combination with *Serratia entomophila* A1MO2. Letters indicate significance groupings based on Unprotected LSD test statistic: at each assessment data means with no letters in common are significantly different (P<0.05).

For both instars, the highest inoculation concentration of *M. novozealandicum* produced significantly lower LT_{50} values compared with the lower concentration when applied alone or in combination with A1MO2 (Table 5.3). The highest concentration level was also the only level at which a significant synergist effect could be detected, as shown by the significantly lower LT_{50} in the combination treatment in both larval instars (P<0.01) (Table 5.3).

Table 5.3 The LT_{50} of second and third instar *Costelytra giveni* larvae resulting from the inoculation of *Metarhizium novozealandicum* at three conidial concentrations alone and in combination with bacteria isolate A1MO2. Letters following the mean values indicate the results of pairwise Fisher's unprotected LSD tests conducted within each instar factor: means not marked with the same letter are significantly different.

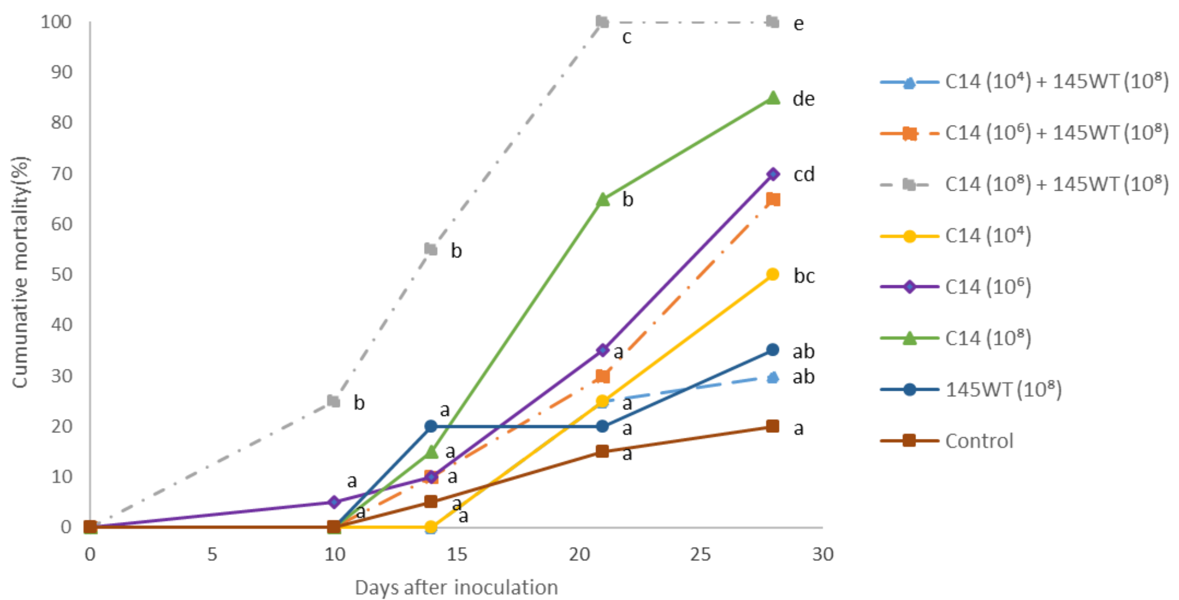
Treatment	LT_{50} (days)-second instar	LT_{50} (days)-third instar
C14 (10^6) + A1MO2 (10^8)	20.1b	32.2c
C14 (10^6)	21.7bc	30.3bc
C14 (10^8) + A1MO2 (10^8)	10.3a	15.8a
C14 (10^8)	21.0b	26.6b
LSD (5%)	5.6	5.3

5.4.4 Effect of inoculum concentration of *Metarhizium novozealandicum* (C14) and a single concentration of isolate 145WT alone and in combination for the control of *Costelytra giveni*

Serratia proteamaculans isolate 145WT, which causes chronic amber disease, alone had no significant effect on the mortality rates of either instar (Figure 5.4a, b). All *M. novozealandicum*

treatments with 10^8 spores/ml had a significantly higher mortality against both second and third instar larvae compared to the control, and each higher concentration produced significantly higher mortality than the lower concentration after day 10 ($P<0.05$). When applied to the second instar larvae, combined treatments with *M. novozealandicum* at the highest inoculation concentration, produced higher mortality than the *M. novozealandicum* alone at the same concentration after day 10 ($P<0.05$). Combination treatments had significantly higher larval mortality than the corresponding *M. novozealandica* alone treatment against both instars (Figure 5.4a, b). The highest inoculum concentration of *M. novozealandicum* produced lower LT_{50} values than the lower concentration treatments for third but not second larval instars (Table 5.4). The highest combination treatment resulted in lower LT_{50} values than the other treatments only when applied to second instar larva ($P<0.05$).

a) second instar



b) third instar

Fi

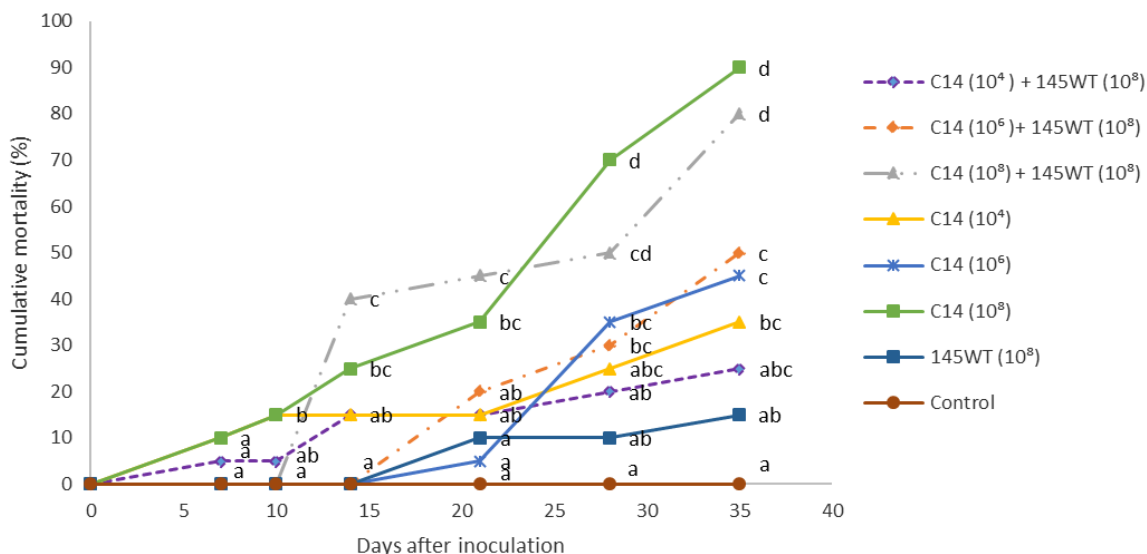


Figure 5.4 The cumulative mortality of (a) second instar and (b) third instar *Costelytra giveni* larvae resulting from inoculation with *Metarhizium novozealandicum* (C14) at three inoculum concentrations alone and in combination with bacteria isolate 145WT. Letters indicate significance groupings based on Unprotected LSD test statistic: at each assessment data means with no letters in common are significantly different (P<0.05).

Table 5.4 The LT_{50} of *Costelytra giveni* larvae resulting from the inoculation of *Metarhizium novozealandicum* at three conidia concentration levels alone and in combination with bacteria strain 145WT for the second and third instar larvae. Letters following the mean values indicate the results of pairwise Fisher's unprotected LSD tests conducted within each instar factor: means not marked with the same letter are significantly different.

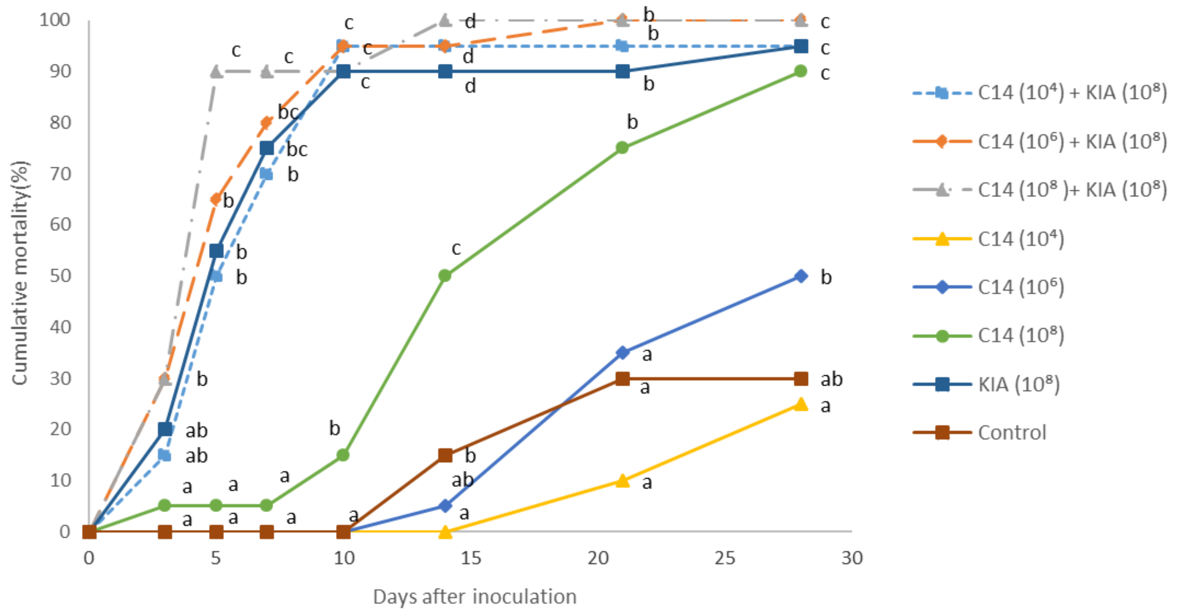
Treatment	LT_{50} (days)-second instar	LT_{50} (days)-third instar
C14 (10^6) + 145WT (10^8)	24.7bc	32.9b
C14 (10^6)	23.3bc	29.4b
C14 (10^8) + 145WT (10^8)	13.3a	17.7a
C14 (10^8)	19.8b	22.4a
LSD (5%)	6.3	6.0

5.4.5 Effect of inoculum concentration of *Metarhizium novozealandicum* (C14) and a single rate of isolate KIA alone and in combination for the control of *Costelytra giveni*

Yersinia entomophaga isolate KIA, a strain which causes rapid mortality of grass grub, dramatically and significantly increased both second and third instar *C. giveni* larval mortality whether alone (relative to the controls) or in combination with *M. novozealandicum* (after day 3 with the second instar larvae, P<0.05 and after day 5 with third instar larvae, P<0.01) (Figure 5.5a, b). When applied alone, the three higher concentrations of *M. novozealandicum* had a significantly higher cumulatvie mortality for both second and third instar larvae compared to the control (after day 7 with second instar larvae and after day 28 with third instar larvae, P<0.01), however there was no significant

difference with the lowest rate against the second or third instar larvae. For the *M. novozealanicum* treatments, each higher inoculation concentration produced significantly higher mortality than the lower concentration (after day 10 with second instar larvae and after day 21 with third instar larvae, P<0.01). However, only the highest rate of *M. novozealandicum* increased larval mortality to levels rivaling, and not significantly lower than that of the treatments containing bacterial isolate KIA (Figure 5.5a, b).

a) second instar



b) third instar

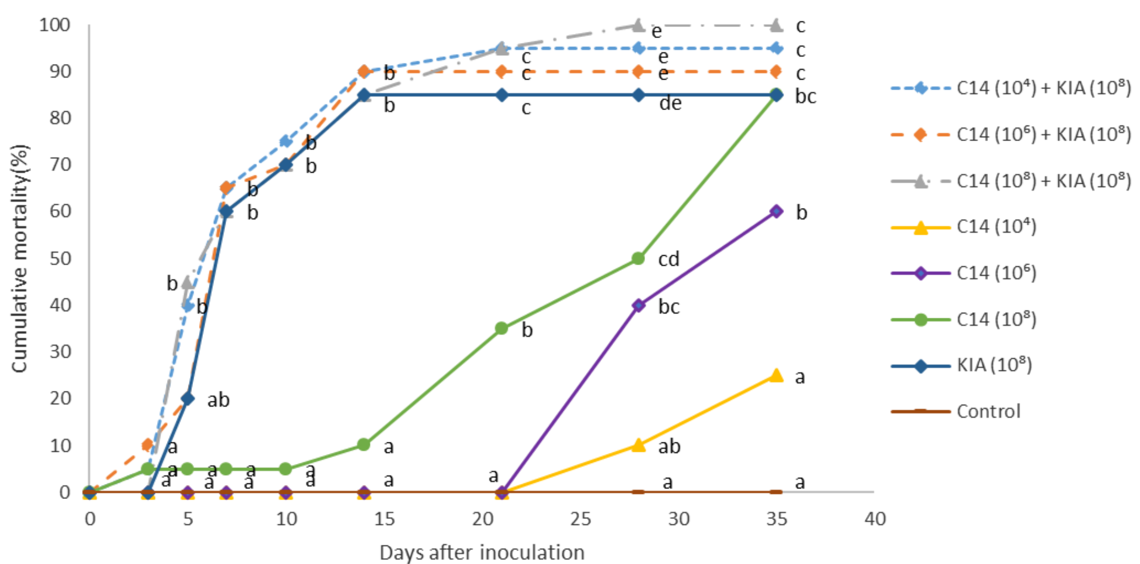


Figure 5.5 The cumulative mortality of (a) second instar and (b) third instar *Costelytra giveni* larvae resulting from inoculation with *Metarhizium novozealandicum* (C14) at three inoculum

concentrations alone and in combination with bacterium isolate KIA. Letters indicate significance groupings based on Unprotected LSD test statistic: at each assessment data means with no letters in common are significantly different (P<0.05).

Bacterial isolate KIA alone and combined with three different spore concentrations of C14 significantly decreased the LT_{50} values compared to all *M. novozealandicum* alone treatments (Table 5.5), but there was no significant difference among any of the bacteria alone and combined with fungus treatments. For the *M. novozealandicum*-only treatments applied to the second instar larva, increasing the inoculation concentration produced a significantly lower LT_{50} (P<0.01). There was no significant difference when these treatments were applied to the third instar larvae (Table 5.5).

Table 5.5 The LT_{50} of *Costelytra giveni* larvae resulting from the inoculation of *Metarhizium novozealandicum* at three spore concentration levels (10^4, 10^6 and 10^8 per ml) alone and in combination with bacteria isolate KIA for the second and third instar larvae. Letters following the mean values indicate the results of pairwise Fisher's unprotect LSD tests conducted within each instar factor: means not marked with the same letter are significantly different.

Treatment	LT_{50} (days)-second instar	LT_{50} (days)-third instar
C14 (10^4) + KIA (10^8)	4.8a	5.8a
C14 (10^6) + KIA (10^8)	4.1a	5.9a
C14 (10^8) + KIA (10^8)	3.5a	7.6a
C14 (10^6)	27.1c	30.3b
C14 (10^8)	15.0b	26.6b
KIA (10^8)	4.7a	6.1a
LSD (5%)	5.4	4.5

5.4.6 Comparing the C14 treatments over all the experiments

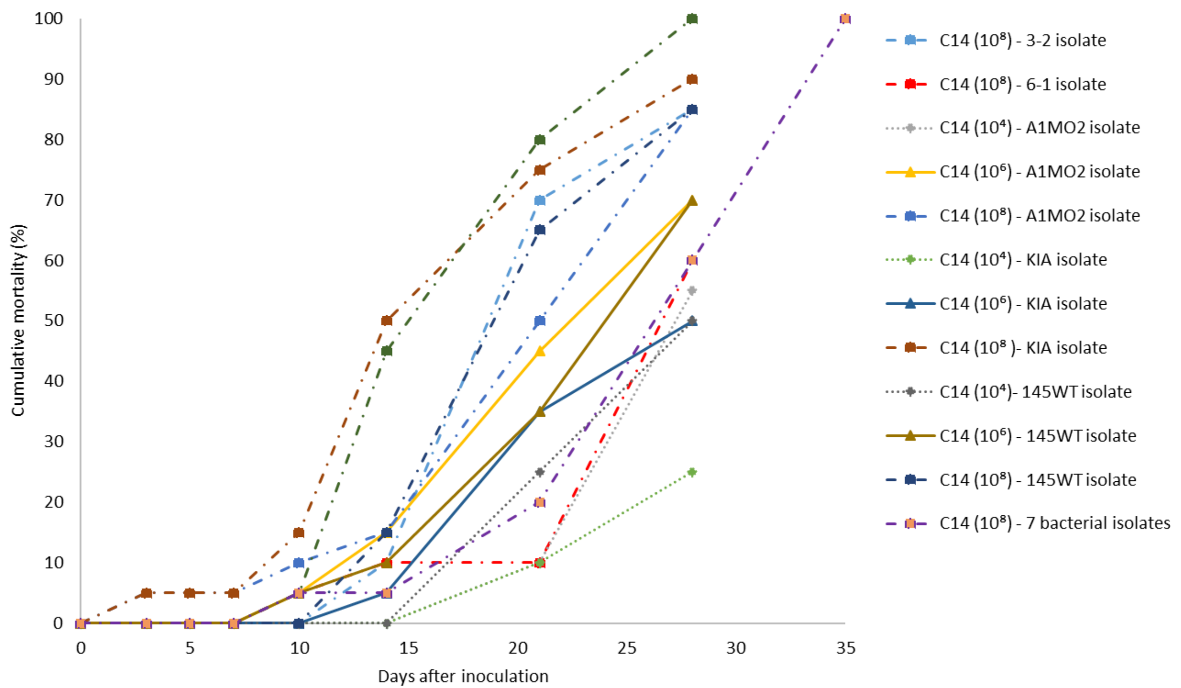
The experiments presented in this chapter were completed over the course of a season. Grass grub larvae go through three larval instars before pupation over January to September; the first instar from mid December to January, the second instar from January to mid March, and the third instar from February to mid September. All larvae were field collected. This appears to have led to some variation in response to the pathogens, as demonstrated by comparison between the C14 only treatments in each experiment.

As expected, the mean mortality of C14 treatments on the second instar larvae and third instar larvae in all experiments increased with higher spore concentration. For the second larval instar, most C14 treatments at the highest (10^8/ml) spore concentration showed the highest mortality in all experiments, except the experiment with seven bacterial isolates (Figure 5.1 in Chpater 5) or the experiment with isolate 6-1 (Figure 5.1) which had a slight increase in mortality before day 21. C14 treatments with a 10^6 spore concentration had the second highest mortality in three treatments with A1MO2 isolate (Figure 5.3) or K1A isolate (Figure 5.5) or 145WT isolate (Figure 5.4) and finally C14 treatments with 10^4 spore concentration in three experiments with A1MO2 isolate (Figure 5.3) or

K1A isolate (Figure 5.5) or 145WT isolate (Figure 5.4) (Figure 5.6a). The LT_{50} value is lower at high concentration than at low concentration of each C14 treatment over all experiments (Figure 5.7a, b).

For the third larval instar, the treatments with different spore concentrations were similar with second larval instar results above, but only in C14 treatment alone of the experiment with 3-2 isolate (Figure 5.2b) was the larval mortality higher than in the remaining experiments (Figure 5.1b in Chapter 5, 5.1b, 5.3b, 5.4b, 5.5b) at the highest spore concentration (Figure 5.7b)

a) second instar



b) third instar

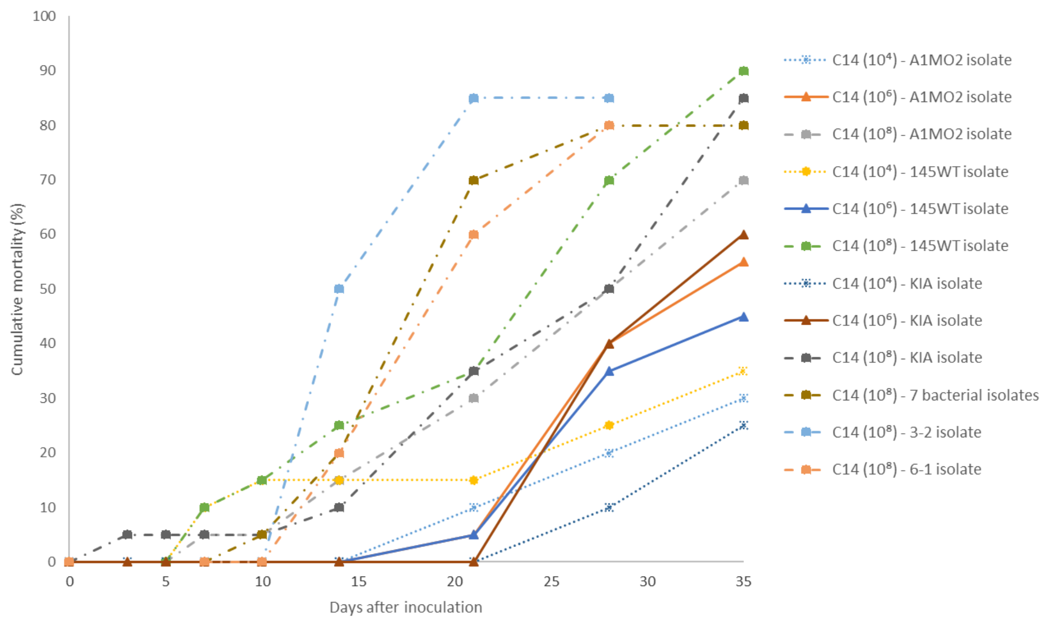
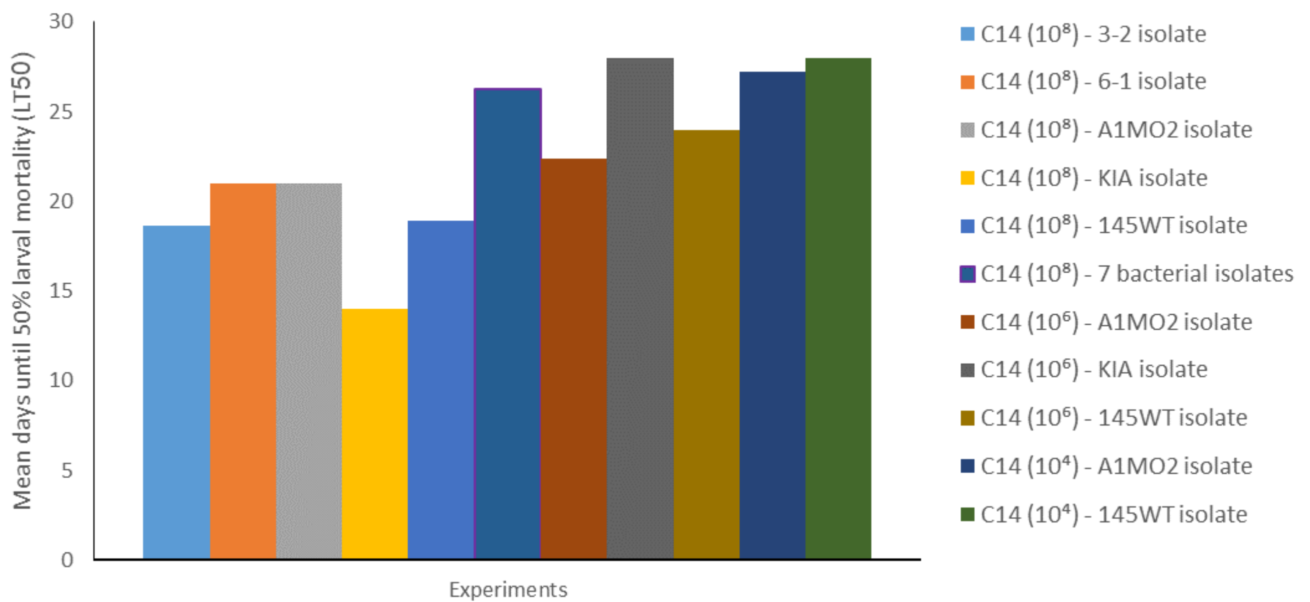


Figure 5.6 The bacterial strains label indicates in which experiment C14 was used alone in comparing all C14 only treatments in experiments using three different spore concentration on second instar (a) and third instar (b).

a) second instar



b) third instar

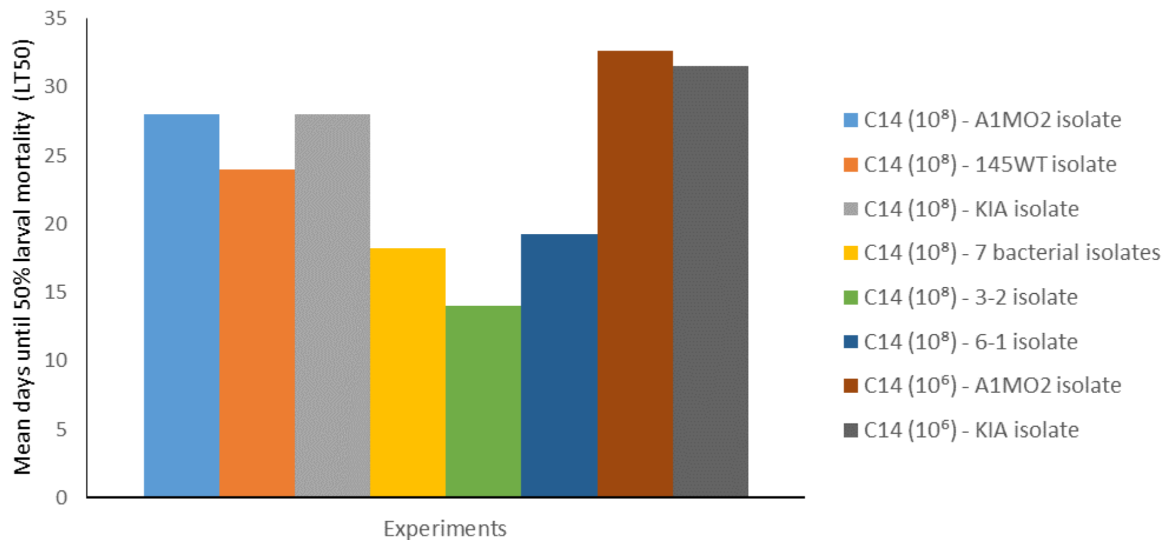


Figure 5.7 Comparing LT_{50} of all C14 only in experiments with different spore concentration levels on second instar larvae (a) and third instar larvae (b).

Fluctuation in the mortality of each C14 treatment with the same spore concentration in the different experiments did not affect the results of each experiment. The reasons for the fluctuation may be a biological factor of each grass grub, such as overall health of field collected larvae at the time of the experiment.

Appendix E for chapter 6

6.1 Statistical analyses

6.1.1 *Plutella xylostella*

6.1.1.1 Analysis of variance of the pathogenicity of C14 against *Plutella xylostella* after 2 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	4	560.0	140.0	1.00	
Treatment	1	640.0	640.0	4.57	0.099
Residual	4	560.0	140.0		
Total	9	1760.0			

6.1.1.2 Analysis of variance of the pathogenicity of C14 against *Plutella xylostella* after 3 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	4	2640.	660.	0.58	
Treatment	1	4840.	4840.	4.25	0.108
Residual	4	4560.	1140.		
Total	9	12040.			

6.1.1.3 Analysis of variance of the pathogenicity of C14 against *Plutella xylostella* after 4 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	4	5840.0	1460.0	1.62	
Treatment	1	4000.0	4000.0	4.44	0.103
Residual	4	3600.0	900.0		
Total	9	13440.0			

6.1.1.4 Analysis of variance of the pathogenicity of C14 against *Plutella xylostella* after 5 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	4	5360.0	1340.0	2.68	
Treatment	1	4000.0	4000.0	8.00	0.047
Residual	4	2000.0	500.0		
Total	9	11360.0			

6.1.1.5 Analysis of variance of the pathogenicity of C14 against *Plutella xylostella* after 6 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	4	2800.0	700.0	1.40	
Treatment	1	4000.0	4000.0	8.00	0.047
Residual	4	2000.0	500.0		
Total	9	8800.0			

6.1.2 *Helicoverpa armigera*

6.1.2.1 Analysis of variance of the pathogenicity of C14 against *Helicoverpa armigera* after 3 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	6	285.71	47.62	1.00	
Treatment	1	714.29	714.29	15.00	0.008
Residual	6	285.71	47.62		
Total	13	1285.71			

6.1.2.2 Analysis of variance of the pathogenicity of C14 against *Helicoverpa armigera* after 4 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	6	1471.4	245.2	1.00	
Treatment	1	4828.6	4828.6	19.69	0.004
Residual	6	1471.4	245.2		
Total	13	7771.4			

6.1.2.3 Analysis of variance of the pathogenicity of C14 against *Helicoverpa armigera* after 5 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	6	1100.0	183.3	1.00	
Treatment	1	22400.0	22400.0	122.18	<.001
Residual	6	1100.0	183.3		
Total	13	24600.0			

6.1.2.4 Analysis of variance of the pathogenicity of C14 against *Helicoverpa armigera* after 6 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	6	342.86	57.14	1.00	
Treatment	1	27457.14	27457.14	480.50	<.001
Residual	6	342.86	57.14		
Total	13	28142.86			

6.1.2.5 Analysis of variance of the pathogenicity of C14 against *Helicoverpa armigera* after 8 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	6	185.71	30.95	1.00	
Treatment	1	32064.29	32064.29	1035.92	<.001
Residual	6	185.71	30.95		
Total	13	32435.71			

6.1.2.6 Analysis of variance of the pathogenicity of C14 against *Helicoverpa armigera* after 10 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	6	0.0	0.0		
Treatment	1	35000.0	35000.0		
Residual	6	0.0	0.0		
Total	13	35000.0			

6.1.3 *Wiseana* sp

6.1.3.1 Analysis of variance of the pathogenicity of C14 against *Wiseana* sp after 14 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	5	277.78	55.56	1.00	
Treatment	1	833.33	833.33	15.00	0.012
Residual	5	277.78	55.56		
Total	11	1388.89			

6.1.3.2 Analysis of variance of the pathogenicity of C14 against *Wiseana* sp after 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	5	1597.2	319.4	1.00	
Treatment	1	10208.3	10208.3	31.96	0.002
Residual	5	1597.2	319.4		
Total	11	13402.8			

6.1.3.3 Analysis of variance of the pathogenicity of C14 against *Wiseana* sp after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	5	1111.11	222.22	4.00	
Treatment	1	7500.00	7500.00	135.00	<.001
Residual	5	277.78	55.56		
Total	11	8888.89			

6.1.4 *Myzus persicae*

6.1.4.1 Analysis of variance of the pathogenicity of C14 against *Myzus persicae* after 2 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	9	1139.8	126.6	1.00	
Treatment	1	423.6	423.6	3.34	0.101
Residual	9	1139.8	126.6		
Total	19	2703.3			

6.1.4.2 Analysis of variance of the pathogenicity of C14 against *Myzus persicae* after 3 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	9	5479.4	608.8	1.13	
Treatment	1	9785.5	9785.5	18.12	0.002
Residual	9	4860.8	540.1		
Total	19	20125.7			

6.1.4.3 Analysis of variance of the pathogenicity of C14 against *Myzus persicae* after 4 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	9	7544.9	838.3	1.02	
Treatment	1	10708.2	10708.2	13.03	0.006
Residual	9	7395.9	821.8		
Total	19	25648.9			

6.1.4.4 Analysis of variance of the pathogenicity of C14 against *Myzus persicae* after 5 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	9	6684.9	742.8	1.13	
Treatment	1	5176.7	5176.7	7.87	0.021
Residual	9	5922.5	658.1		
Total	19	17784.1			

6.1.4.5 Analysis of variance of the pathogenicity of C14 against *Myzus persicae* after 6 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	9	5609.8	623.3	1.30	
Treatment	1	2809.9	2809.9	5.87	0.038
Residual	9	4307.2	478.6		
Total	19	12726.9			

6.1.5 *Tenebrio molitor*

6.1.5.1 Analysis of variance of the pathogenicity of C14 against *Tenebrio molitor* after 3 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	9	1531.2	170.1	1.00	
Treatment	1	2531.2	2531.2	14.88	0.004
Residual	9	1531.2	170.1		
Total	19	5593.8			

6.1.5.2 Analysis of variance of the pathogenicity of C14 against *Tenebrio molitor* after 4 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	9	5906.2	656.2	1.00	
Treatment	1	26281.2	26281.2	40.05	<.001
Residual	9	5906.2	656.2		
Total	19	38093.8			

6.1.5.3 Analysis of variance of the pathogenicity of C14 against *Tenebrio molitor* after 5 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	9	4250.0	472.2	1.00	
Treatment	1	32000.0	32000.0	67.76	<.001
Residual	9	4250.0	472.2		
Total	19	40500.0			

6.1.5.4 Analysis of variance of the pathogenicity of C14 against *Tenebrio molitor* after 6 days

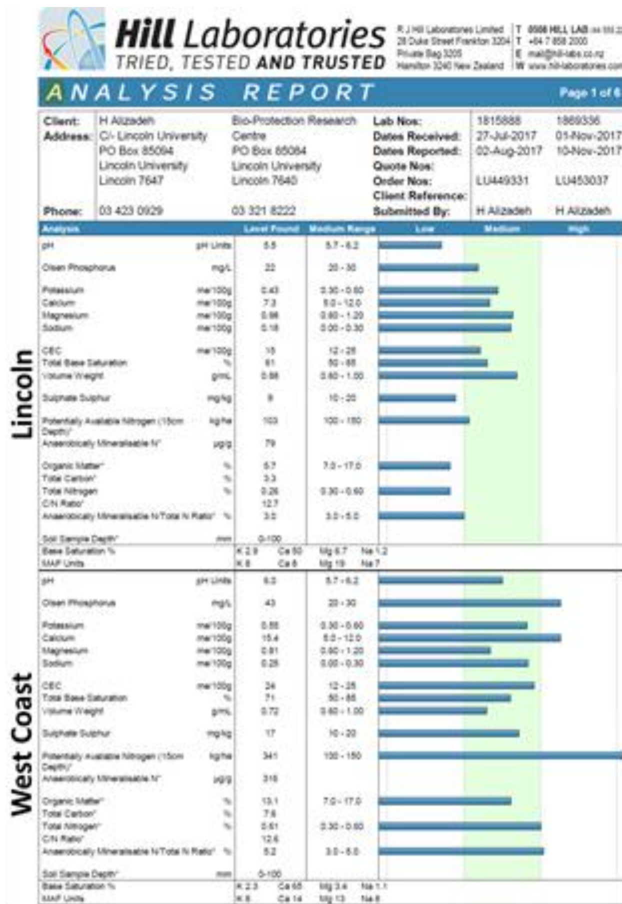
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	9	2625.0	291.7	1.00	
Treatment	1	40500.0	40500.0	138.86	<.001
Residual	9	2625.0	291.7		
Total	19	45750.0			

6.1.5.5 Analysis of variance of the pathogenicity of C14 against *Tenebrio molitor* after 7 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	9	2531.2	281.2	1.00	
Treatment	1	42781.2	42781.2	152.11	<.001
Residual	9	2531.2	281.2		
Total	19	47843.8			

Appendix F for Chapter 7

7.1 Analyses of three soil type at three sites (Lincoln, West Coast and Oxford) by Hill Laboratories

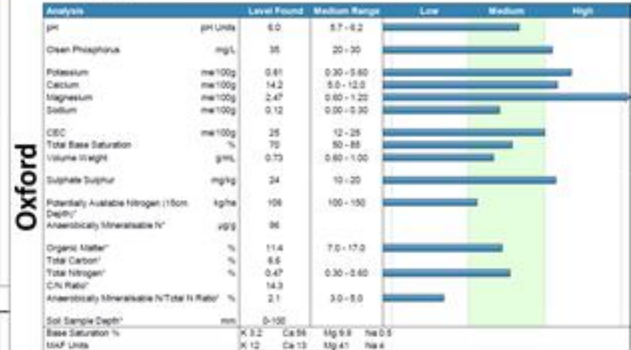


Hill Laboratories — TRIED, TESTED AND TRUSTED

ANALYSIS REPORT — Page 1 of 6

Client:	H Alizadeh	Bio-Protection Research	Lab Nos:	1815888 / 1869336
Address:	C/- Lincoln University, PO Box 85094, Lincoln University, Lincoln 7647	Centre, PO Box 85084, Lincoln University, Lincoln 7640	Dates Received:	27-Jul-2017 / 01-Nov-2017
			Dates Reported:	02-Aug-2017 / 10-Nov-2017
			Quote Nos:	
			Order Nos:	LU449331 / LU453037
			Client Reference:	
Phone:	03 423 0929	03 321 8222	Submitted By:	H Alizadeh / H Alizadeh

Analysis	Unit	Lincoln – Level Found	West Coast – Level Found	Medium Range
pH	pH Units	5.5	6.0	5.7 - 6.2
Olsen Phosphorus	mg/L	22	43	20 - 30
Potassium	me/100g	0.43	0.55	0.30 - 0.60
Calcium	me/100g	7.3	15.4	6.0 - 12.0
Magnesium	me/100g	0.98	0.81	0.80 - 1.20
Sodium	me/100g	0.18	0.25	0.00 - 0.30
CEC	me/100g	15	24	12 - 25
Total Base Saturation	%	61	71	50 - 85
Volume Weight	g/mL	0.88	0.72	0.60 - 1.00
Sulphate Sulphur	mg/kg	8	17	10 - 20
Potentially Available Nitrogen (15cm Depth)*	kg/ha	103	341	100 - 150
Anaerobically Mineralisable N*	µg/g	79	316	
Organic Matter*	%	5.7	13.1	7.0 - 17.0
Total Carbon*	%	3.3	7.6	
Total Nitrogen*	%	0.26	0.61	0.30 - 0.60
C/N Ratio*		12.7	12.6	
Anaerobically Mineralisable N/Total N Ratio*	%	3.0	5.2	3.0 - 5.0
Soil Sample Depth*	mm	0-100	0-100	
Base Saturation %		K 2.9 Ca 50 Mg 6.7 Na 1.2	K 2.3 Ca 65 Mg 3.4 Na 1.1	
MAF Units		K 8 Ca 6 Mg 19 Na 7	K 8 Ca 14 Mg 13 Na 8	



Oxford

Analysis	Unit	Level Found	Medium Range
pH	pH Units	6.0	5.7 - 6.2
Olsen Phosphorus	mg/L	35	20 - 30
Potassium	me/100g	0.61	0.30 - 0.60
Calcium	me/100g	14.2	6.0 - 12.0
Magnesium	me/100g	2.47	0.80 - 1.20
Sodium	me/100g	0.12	0.00 - 0.30
CEC	me/100g	25	12 - 25
Total Base Saturation	%	70	50 - 85
Volume Weight	g/mL	0.73	0.60 - 1.00
Sulphate Sulphur	mg/kg	24	10 - 20
Potentially Available Nitrogen (15cm Depth)*	kg/ha	106	100 - 150
Anaerobically Mineralisable N*	µg/g	86	
Organic Matter*	%	11.4	7.0 - 17.0
Total Carbon*	%	6.6	
Total Nitrogen*	%	0.47	0.30 - 0.60
C/N Ratio*		14.3	
Anaerobically Mineralisable N/Total N Ratio*	%	2.1	3.0 - 5.0
Soil Sample Depth*	mm	0-100	
Base Saturation %		K 3.2 Ca 56 Mg 9.8 Na 0.5	
MAF Units		K 12 Ca 13 Mg 41 Na 4	