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Diversity of Entomopathogenic Fungi (EPF) in Soil Across Adjacent Forest and Pasture Habitats and their Interactions with Arthropod Hosts

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Michelle Guerrero

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Abstract

Entomopathogenic fungi (EPF) are natural pathogens of insects that play a critical role in regulating arthropod populations and maintaining ecological balance. These fungi are commonly found in the soil, where they interact with invertebrates and with other microorganisms, making soil an essential reservoir for collecting EPF isolates.

Understanding the distribution and prevalence of EPF across different habitats is crucial for their effective utilization in the biological control of agricultural pests. In this thesis, the diversity of entomopathogenic fungi (EPF) in adjacent forest and pasture habitats of the North Island, New Zealand, was comprehensively investigated. This study utilized an integrative approach, combining traditional techniques such as insect baiting and semi-selective plating with advanced molecular methods, including metagenomics and metabarcoding, to explore EPF communities across these contrasting habitats.

In Chapter 1, I introduced the current understanding of EPF and identified key research gaps, setting the foundation for the study. Chapter 2 focused on the incidence of EPF across forest, forest-pasture boundary, and pasture habitats and the association with soil invertebrates. The results showed the highest EPF prevalence in forest soils, followed by boundary zones and finally pastures, demonstrating the influence of habitat conditions on EPF diversity. In Chapter 3, I characterized EPF isolates, primarily *Beauveria* spp. and *Metarhizium* spp., and found that forest and boundary soils caused up to 80% mortality in *Tenebrio molitor* larvae within seven days, indicating their insecticidal potential. Chapter 4 expanded the analysis by applying metagenomic and metabarcoding approaches, revealing additional fungal taxa, such as *Cordyceps*, *Lecanicillium* and *Ophiocordyceps*, which traditional methods could not detect. Chapter 5 assessed the efficacy of EPF isolates against agricultural pests, with pasture-derived isolates achieving higher pathogenicity and potential as cost-effective biocontrol agents. Finally, in Chapter 6, I synthesized these findings, emphasizing the ecological importance of soil habitats as reservoirs for EPF diversity and the adaptability of local isolates to specific environments. I recommended future research to expand studies into transitional zones, conduct field trials, and investigate the evolutionary mechanisms underpinning EPF adaptation and pathogenicity.

This thesis provides a comprehensive understanding of EPF diversity, habitat-specific dynamics, and their potential for sustainable pest management, contributing valuable insights to microbial ecology and integrated pest management strategies.

Thesis structure

This thesis is focused on the diversity of entomopathogenic fungi (EPF) in forests and pasture transition in the North Island of New Zealand using cultural and molecular approaches. The thesis has been written in a manuscript form with stand-alone chapters. As a consequence, there has been repetition in some sections between chapters, mainly on the materials and methods. The thesis comprises six chapters, four of which are experimental.

Chapter 1 – Introduction

This chapter presents the current body of knowledge about EPF and the research gap that needs to be addressed. In addition, the research aims of the thesis are presented in this chapter.

Chapter 2 – Incidence of entomopathogenic fungi in adjacent forest and pasture habitats and at varying elevations, and their association with soil invertebrates.

In New Zealand, the natural diversity of EPF in forest and agricultural soils is understudied. There is a need to explore diverse habitats as potential sources of local strains of EPF, which could potentially lead to promising and virulent fungal strains. Soil invertebrates and soil fungi play an essential role in the dynamics of the soil. This chapter focused on the incidence of EPF in adjacent habitats (forest interior, forest edge and pasture) using insect bait and dilution plating methods. Determining the soil microarthropods in soils with identified entomopathogens adds to current knowledge on the ecology of EPF.

Chapter 3 – Entomopathogenic fungi (*Beauveria* spp. and *Metarhizium* spp.) in adjacent pasture and forest soil: their occurrence, identification, and speed of kill assessed using *Tenebrio molitor* larvae.

This chapter deals with the molecular identification and microscopic characterization of EPF obtained in various habitats. In addition, this chapter presents the speed of kill at which the

soils kill insects reflecting the suppressive potential of the soil in a particular habitat through bait insect mortality.

Chapter 4 – Comparative microbiomics of the soil in adjacent native forest and exotic pasture habitats of NZ North Island

Metagenomics provides an unbiased way to discover biodiversity within the environment and soil samples. Soil DNA was extracted in forest and pasture habitats, and whole genome shotgun and amplicon sequencing was conducted. Bioinformatic analysis of amplicon sequencing resulted in the identification of EPF genera present in soil samples, while shotgun data provided information on the soil microorganisms present in concert with EPF.

Chapter 5 – Efficacy of *Beauveria* isolates collected in adjacent forest and pasture habitats in the North Island, NZ against selected agricultural pests

This chapter focuses on assessing the efficacy of the local isolates of EPF collected from adjacent forest and pasture habitats in North Island, New Zealand. These isolates were obtained as described in the Chapter 2 of the thesis. The laboratory efficacy assays were conducted to evaluate the potential of EPF isolates as biological control agents against below-ground pest (larvae of grass grub beetle *Costelytra giveni*) and above-ground insects (giant willow aphid, locust, and mealworm).

Chapter 6 – General discussion and recommendations for future work.

This chapter presents the overall findings of the thesis. Building on these findings, future research should include a more comprehensive investigation of the boundary as a source of native EPF isolates and incorporate metagenomic analysis of boundary soils. Conducting greenhouse and field trials of collected isolates is also recommended.

List of presentations where the PhD work or a portion of it was presented

- Guerrero, MS. 2023. International guest lecturer for the topic "Important Pest and their Biocontrol Technology, " held at Trunojoyo University, Indonesia via Zoom. Organized by the Faculty of Agriculture, Trunojoyo University, Indonesia, 8 June 2023.
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- Guerrero, MS, S. Trewick, T. Glare and M. Minor. Diversity of entomopathogenic fungi in New Zealand and their potential for pest management. An oral presentation presented at the 69th New Zealand Entomological Society Conference held in Otago Museum, Dunedin, April 7-9, 2021.
- Guerrero, MS, S. Trewick, T. Glare and M. Minor. Molecular identification of Entomopathogenic Fungi (EPF) in cultivated and non-cultivated soil in New Zealand using isolation-based and metagenomic approach. An oral presentation presented at the 22nd Molecular Ecology conference held at Paekakariki, Kapiti Coast, New Zealand, Nov. 29- Dec. 1, 2019.
- Guerrero, MS, S. Trewick, T. Glare and M. Minor. Diversity of Entomopathogenic Fungi (EPF) in New Zealand and their utility for pest management. A poster paper presented during the "Pathways to the Future", a School of Agriculture and Environment's Symposium 2019. AgHort Bldg. B, SAE, Massey University, New Zealand, Nov. 14-15, 2019.

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To everyone who played a part in this story, thank you for helping me write the lyrics to this unforgettable journey. I had the time of my life with you all, and I will remember this journey “All Too Well.”

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Chapter One

General Introduction

Introduction

Entomopathogenic fungi (EPF) are naturally occurring microorganisms that infect and kill insects, making them valuable in pest management strategies (Vega et. al., 2009; Hussein et al., 2010; Mantzoukas et al., 2022). These fungi naturally occur in the soil, where they spend a significant portion of their life cycle (Jaronski, 2010; Kepler et al., 2015; Sharma et al., 2018). The soil serves as an excellent environmental shelter, protecting fungi from UV radiation and other adverse abiotic and biotic influences (Keller & Zimmermann, 1989). Studies have found that the genera *Beauveria*, *Metarhizium*, and *Isaria* are commonly found in the soil (Domsch et al., 1980; Sánchez-Peña et al., 2011). These fungi have been isolated from various agricultural and natural ecosystems, highlighting their wide distribution (Chandler et al., 1997; Quesada-Moraga et al. 2007; Meyling et al., 2009; Goble at al., 2010; Schneider et al., 2012; Sharma et al., 2018).

Understanding the diversity and communities of entomopathogenic fungi in different ecosystems is important for managing and conserving EPF (Meyling & Eilenberg, 2007; Sevim et al., 2009). Indigenous populations of EPF in soil can be managed to facilitate the control of pest insect populations within agroecosystems (Pérez-González et al., 2014; Kepler et al., 2015; Keyser et al., 2015). Isolating and characterizing indigenous EPF also provides insights into the naturally occurring fungal biodiversity and offers a pool of potential biological control agents for pest control purposes (Sánchez-Peña et al., 2011; Sharma et al., 2018).

Insect pests remain as one of the significant threats to food security due to the damages and losses they inflict on agriculture. These threats are exacerbated by climate change and the loss of natural enemies that control the pests. While chemical pesticides are the most popular management option to control insect pests, this approach is costly and poses high risks to farmers, non-target organisms, food consumers and the environment (Subramanyam, 1995; Sinha et al., 2016; Mantzoukas et al., 2020). In addition, developing resistance to pesticides makes insects even harder to control. Use of chemical pesticides is also difficult to reconcile with the New Zealand government's objectives to develop world-leading environmentally sustainable agriculture. With the recent global call to reduce carbon footprint, an alternative

and sustainable control measure to manage insect pests in agriculture is necessary (United Nations, n.d.).

Traditional isolation and identification methods for entomopathogenic fungi are laborious and time-consuming, which limits our understanding of their ecological role and diversity (Vega et al., 2009). The emergence of metabarcoding and metagenomics has revolutionized the field by enabling high-throughput and culture-independent approaches for studying microbial communities (Abdelfattah et al., 2018; Masoudi et al., 2020). For example, RNA shotgun metagenomic sequencing has been used to characterize the microbiota of mosquitoes, which includes entomopathogenic fungi (Chandler et al., 2015). This approach allows for the discovery of unknown members of the microbiota and provides insights into the composition and dynamics of entomopathogenic fungal communities.

Information regarding the local isolates of EPF using cultural and molecular approaches, their diversity and distribution, are paramount in conserving the native fungal species for natural control of insect pest populations within agroecosystems (Hussein et al., 2010). Despite the recognized importance of soil-dwelling entomopathogenic fungi in managing pests, their occurrence and natural diversity are not sufficiently studied in New Zealand. Collecting EPF species in the natural environments, including pasture and forest soils, will increase the discovery and selection of effective species and isolates against insect pests.

Soil as the reservoir of entomopathogenic fungi

The soil offers an ideal environment for microorganisms. The EPF genera, *Beauveria*, *Metarhizium* and *Isaria,* are considered weak saprophytes in the soil ecosystem. EPF spend a significant portion of their life cycle in the soil (Meyling et al., 2006; Jaronski, 2010; Fisher et al., 2011). The soil protects the EPF from UV radiation and acts as a buffer against temperature and moisture fluctuations. The optimum temperature requirement for *Beauveria*, *Metarhizium* and *Isaria* is typically within the range of 20-30°C. The soil also provides a habitat for many invertebrates that are potential hosts (Quesada-Moraga et al., 2007). Metabolites secreted by other soil microorganisms hinder the pathogenicity of EPF as these metabolites may impair fungal germination and growth, or be toxic (Sinha et al., 2016).

The type of soil habitat can significantly impact the occurrence of EPF. For example, *Metarhizium anisopliae* is associated with arable soils, whereas *Beauveria bassiana* is more common in natural soils. Abiotic factors such as soil moisture, pH, organic matter, and chemical properties also affect EPF occurrence (Rath et al., 1992; Padmavathi et al., 2003;

Issaly et al. 2005; Jabbour & Barbercheck, 2009). Herbicide use can also significantly impact EPF persistence (Yousef et al., 2015). Numerous biotic factors affect EPF dynamics in the soil; the principal biotic components that influence EPF persistence and efficacy the most are invertebrates, soil microorganisms, and plants (Sinha et al., 2016).

EPF diversity in natural habitats

Entomopathogenic fungi (EPF) play an essential role in the natural regulation of arthropod populations (Evans, 1982). Most of the EPF's life cycles are synchronized with insect host stages and environmental conditions (Shah & Pell, 2003). The life cycle of EPF starts when the spore of EPF contacts the arthropod cuticle, attaching initially due to nonspecific hydrophobic interaction between the spore wall and epicuticle, but then adheres more firmly and germinates within a few hours, forming a germ-tube (Vega et. al., 2012). The germ-tube comprises an appressorium, and the penetration peg penetrates the arthropod cuticle utilizing several enzymes and mechanical pressure (Sharma, 2019). Once in the hemocoel, yeast-like hyphal bodies called blastospores invade the haemolymph, and some toxins are produced during the infection process. As the host dies, the fungus rapidly transforms into mycelium and, under ideal conditions, emerges to form conidia on the insect's exterior.

EPF of the genera *Beauveria*, *Metarhizium*, and *Isaria* (=*Paecilomyces*) are commonly found in the soil (Domsch et al., 1980). The most common species of EPF infecting a wide range of insect hosts are *Beauveria bassiana* (Balsamo), Vuillemin (Ascomycota: Hypocreales) and *Metarhizium anisopliae* (Metchnikoff), Sorokin (Ascomycota: Hypocreales).

EPF can be found in various habitats, including agricultural, forest, pasture, and urban habitats (Sánchez-Peña, 1990; Lacey et al., 1996; Chandler et al., 1997). Wakil et al. (2013) explored the occurrence and diversity of EPF from soils collected in forest and agricultural habitats in Pakistan. They recovered *B. bassiana, M. anisopliae, Paecilomyces lilacinus* (Thom) Samson (Ascomycota: Hypocreales), *Beauveria bonginratii* (Sacc.) Petch (Ascomycota: Hypocreales), *Pochonia chlamydosporia* Zare and Gams (= *Verticillum chlamydosporium* Goddard) (Ascomycota: Hypocreales) and *Lecanicillium attenuatum* Zare and Gams (Ascomycota: Hypocreales) in their soil samples. The study reported that the diversity of entomopathogenic fungi was greater in soil samples from forests compared to vegetable and fruit fields (Wakil et al., 2013). They also reported that geographical attributes

(altitude, longitude, latitude) influenced the presence of entomopathogenic fungi (Wakil et al., 2013).

Bidochka et al. (1998) explored the presence of EPF in natural (deciduous–boreal forest) and cultivated agricultural habitats in Canada. The study reported that *B. bassiana* was predominantly isolated from soils of natural habitats, while *M. anisopliae* was often recovered in agricultural habitats. The study concluded that the occurrence of *M. anisopliae* and *B. bassiana* was not related to soil type or pH (Bidochka et al., 1998).

The natural diversity and distribution of species within the genus *Metarhizium* in forest ecosystems in China were investigated by Masoudi (2019). Soil sampling was conducted at 19 geographically separated locations in diverse forest regions. Molecular analysis was performed using the internal transcribed spacer (ITS rDNA including 5.8S), elongation factor 1-alpha and the β-tubulin region. Of the 173 *Metarhizium* isolates obtained using *T. molitor* larvae as baits, phylogenetic comparison identified and resolved that 75 belonged to *M. pingshaense*, 44 to *M. robertsii*, 10 to *M. brunneum,* 4 to *M. guizhouense*, 3 to the *M. flavoviride* species complex (identified as *M. bibionidarum*) and 37 to a segregated, unknown *Metarhizium* cluster.

In New Zealand, the natural diversity of EPF in forest and agricultural soils have been understudied. The diversity of the genera *Beauveria* and *Isaria* in the native forest of New Zealand was studied by Cummings (2009) but using targeted visual sampling of diseased insects. Cummings reported for the first time the presence of *Beauveria caledonica* and *Beauveria malawiensis* in the New Zealand native forest. Blond et al. (2018) investigated the aspects of the ecology and population dynamics of the *Beauveria bassiana* strain F418 in the soil and reported that the presence of non-host larvae negatively impacts the persistence of *B. bassiana* in the soil. Even though EPF is known to be globally distributed, it is necessary to identify the natural distribution and local patterns of EPF abundance as it could be potentially different across habitats. To date, no studies have been conducted to determine the biological diversity of soil EPF in adjacent forest and pasture habitats in New Zealand. Moreover, there is a need to explore diverse habitats as potential sources of local strains of entomopathogenic fungi that could detect promising and virulent fungal strains.

EPF isolation: Insect bait method

Several methods are being extensively used to isolate EPF. Undoubtedly, the insect bait method is the most commonly used for EPF isolation (Zimmermann, 1986; Vega et al., 2012; Lacey et al., 2015; Sharma et al., 2018). The larvae of *Galleria mellonella* Linnaeus

(Lepidoptera: Pyralidae), first described for isolating EPF by Zimmermann (1986), have been used for EPF isolations in numerous studies (Bidochka et al., 1998; Meyling & Eilenberg, 2006; Quesada-Moraga et al., 2007; Jabbour & Barbercheck, 2009; Gan & Wickings, 2017). Wakil et al. (2013) studied the occurrence and diversity of EPF in cultivated and uncultivated soils using the *Galleria* bait method and successfully obtained 168 fungal isolates. In the study of Tuininga et al. (2009), it was concluded that the *Galleria* bait method efficiently isolates EPF from soil samples.

Tenebrio molitor Linnaeus (*Coleoptera: Tenebrionidae*) larvae are also widely-used insect bait for EPF isolation (Sánchez-Peña et al., 2011; Steinwender et al., 2014). Some studies have used both *G. mellonella* and *T. molitor* to isolate EPF, as particular baits can be sensitive to infection by one specific EPF genus (Hughes et al., 2004; Meyling et al., 2012). Sharma et al. (2018) recommended using the *Galleria*-*Tenebrio*-bait method to prevent underestimation of abundance and diversity as *G. mellonella* is more sensitive towards the infections by *B. bassiana* s.l. and *T. molitor* for *M. robertsii.*

EPF isolation: Plating method

Plating soil suspension on agar-based selective medium is another method of obtaining EPF species. Tkaczuk et al. (2019) determined the density of the infectious colony-forming unit (CFU) of EPF in the soil using dilution plating in semi selective media. Clifton et al. (2015) quantified the abundance of EPF in organic and conventional fields using the serial soil dilution plating method. Posadas et al. (2012) recommended a selective medium to isolate EPF. However, insect baiting is a more targeted and sensitive method for isolating EPF compared to using selective media to culture soil suspensions (Keller et al., 2003; Imoulan et al., 2011; Keyser et al., 2015).

EPF and soil invertebrates

Soil invertebrates play a role in the EPF dynamics in the soil. Most of the EPF's life cycles are synchronized with insect host stages and environmental conditions (Shah & Pell, 2003). The interactions between soil fungi and soil invertebrates occur in every ecosystem where they coexist (Gange & Brown, 2002). Relationships and associations between fungi and invertebrates in the soil, including predatory, mutualistic, parasitic and pathogenic interactions, have been widely studied (Maraun et al., 2003; Lilleskov & Bruns, 2005; Renker et al., 2005).

Soil-dwelling invertebrates, such as collembolans, mites, and nematodes, play an essential role as decomposers by consuming and transforming organic matter in the soil (Pflug & Wolters, 2002). Non-entomopathogenic fungi, on the other hand, represent a high-quality food source consumed by a wide variety of insects and nematodes as they contain proteins, vitamins and lipids. Acari, collembolans and nematodes rely on fungal hyphae, fruiting bodies and spores as food sources (Schigel, 2012). The lack of motility and high nutritional value attract fungivorous animals to use fungi as prey. Ingestion of EPF spores by collembolan *Proisotoma minute* Tullberg (Collembola: Isotomidae) leads to the loss of conidial viability of *M. anisopliae* after digestion (Dromph, 2001). Naturally, fungi defend themselves with mechanical defences (Böllmann et al., 2010) or chemical defences, including producing toxic secondary metabolites, peptides and proteins (Rohlfs & Churchill, 2011), making them less palatable and less attractive as prey. Production of metabolites for defence costs energy to fungi, such that they will produce these metabolites only when attacked by fungivores or in stressed conditions (Rohlfs, 2015).

The presence of host arthropods in the soil ecosystem is significant for EPF's lifecycle to continue. Kessler et al. (2004) reported that persistence of *B. brongniartii* decreased by 88% in the absence of the host insect *Melolontha melolontha* Linnaeus (Coleoptera: Scarabaeinae) compared to a decline of 27 % on average for sites with the host. Walstad et al. (1970) observed that *B. bassiana* was restricted to the host arthropods for their development as *B. bassiana* did not grow saprophytically in non-sterile soil. In the absence of hosts, EPF viability could be affected by nonspecific attachment and germination on the cuticle of the non-host arthropods, defined as arthropods not susceptible at a specific inoculum level to a particular EPF (Blond et al., 2018). Boucias et al. (1988) reported that *B. bassiana* and other EPF could attach to non-host cuticles due to binding forces. The non-host cuticle was infected, but the internal infection was not observed (Smith et al., 1981), suggesting that EPF attachment to non-host arthropods was in the absence of the arthropod immune system. *Lecanicillium lecanii* (Zimm.) Zare & W. Gams (Ascomycota: Ascomycetes) could attach and germinate onto the cuticle of non-host coleopteran *Bembidion obtusum* Audinet-Serville (Coleoptera: Carabidae) (Sitch & Jackson, 1997). Nonspecific attachment and germination of EPF in non-host arthropods cuticle are disadvantageous to the persistence and virulence of EPF due to a greater chance of conidial germination loss and prevention of the opportunity to find a suitable host (Blond et al., 2018). In contrast, Dromph (2003) reported that ingestion of EPF by non-host arthropods could lead to the dispersion of EPF to soil zones where the fungus was not formerly present and could increase the chance of contact with host arthropods.

The diversity of soil invertebrates in New Zealand is not well studied. Similarly, the presence of the soil invertebrates and EPF in the same local soil samples and their possible associations and dynamics are understudied. Therefore, there is a need to investigate the local co-existence of soil invertebrates and EPF, and possibly identify the association of these invertebrates with the identified EPF.

Entomopathogenic fungi interactions with plant roots

Entomopathogenic fungi (EPF) are traditionally known for their capacity to infect and kill insects; however, recent research highlights their broader ecological roles, including their interactions with plant roots and the rhizosphere(Zitlalpopoca-Hernandez et al., 2017; Gange et al., 2019; Ravnskov et al., 2020). Several EPF genera, notably *Beauveria* and *Metarhizium,* can establish close associations with plants as rhizosphere colonizers or endophytes (Jaber and Ownley, 2018; Gange et al., 2019). These associations may provide the plant with multiple benefits such as protection against insect herbivores and pathogens, growth promotion, and enhanced tolerance to abiotic stress. The protective effects are often mediated through the activation of plant defense mechanisms and the induction of systemic resistance (Rivas-Franco et al., 2020; Jensen et al., 2020)

Recent studies have demonstrated the potential for functional complementarity when EPF co-occur with other beneficial root-associated fungi such as arbuscular mycorrhizal fungi (AMF). For example, *Metarhizium brunneum*, *M. robertsii*, and *Beauveria bassiana* were shown to interact with the AMF *Funneliformis mosseae* in tomato (*Solanum lycopersicum*), leading to improved plant growth and suppression of the foliar pathogen *Botrytis cinerea* (Zitlalpopoca-Hernandez et al., 2022). EPF contributed to pathogen suppression, while AMF enhanced plant biomass production. Such findings illustrate the complexity of below-ground fungal interactions and their potential in integrated plant health management (Rivas-Franco et al., 2020; Zitlalpopoca-Hernandez et al., 2022).

Moreover, EPF can function as endophytes within plant tissues, where they influence plant physiology and immunity. Endophytic colonization facilitates both direct and indirect benefits, including nutrient acquisition, phytohormone production, synthesis of secondary metabolites, and induction of resistance against pathogens and pests (Raad et al., 2019; Ahsan et al., 2024). EPF are increasingly recognized not solely as insect pathogens but as multifunctional symbionts that promote plant health, influence soil processes, and contribute to the stability and resilience of terrestrial ecosystems (Zitlalpopoca-Hernandez et al., 2017; Gange et al., 2019; Ravnskov et al., 2020).

Assessment of entomopathogenic fungi (EPF) diversity in soil using metagenomics

The diversity of EPF in substrates such as soil and leaf litter is often assessed using a culturing method such as insect bait and direct plating. Before the advent of molecular methods, fungal classification depended on morphological, taxonomic and phenotypic characteristics. Still, these methods alone do not allow reliable identification of fungi at lower taxonomic levels. Thus, a molecular-based approach is necessary to identify microbial diversity without needing culturing strategies.

The nuclear ribosomal DNA (rDNA) has been the commonly used region for fungal identification and systematics (Bruns & Shefferson, 2004). The internal transcribed spacer (ITS) regions within the 45S rDNA cassette have been adopted as fungi's universal fungal barcode sequence (Schoch et al., 2012). These non-coding, non-conserved rDNA segments are transcribed but eventually excised and are multicopy, making them relatively easy to amplify using standard PCR techniques. The different rates at which ribosomal genes and ITS evolves can be informative at different taxonomic levels. The relatively low evolutionary pressure on non-coding and non-functional sequences accounts for the high degree of variation even in closely related species (Seifert et al., 1995). In some genera, closely related species show little sequence difference (Seifert et al., 1995; Bruns & Shefferson, 2004). In contrast, the highly conserved l8S and 28S genes are used to examine broad phylogenetic relationships at or above the generic level (Bruns et al., 1991). In New Zealand, DNA metabarcoding was recently used to describe the diversity and composition of plant pathogens in five different land uses (natural forest, planted forest, low-producing grassland, high-producing grassland, and perennial cropland) using soil, roots and leaves as substrates (Makiola et al., 2019). The authors reported that most plant pathogen communities (fungi, oomycetes, and bacteria) responded strongly to substrate type and land use.

Next-Generation sequencing (NGS) technologies have led to numerous studies on microbial diversity and their ubiquitous role in host ecology, physiology, and evolution (Foster et al., 2012; McFall-Ngai et al., 2013). The use of whole-metagenome (WMG) shotgun sequencing to investigate microbial communities opens new possibilities to identify a microbiome's taxonomic composition and metabolic potentials (Quince et al., 2017). Microbiome refers to the genetic material of all the microorganisms present in a particular environment or sample (Lear et al., 2018). Metagenomics involves direct sequencing of genome-wide DNA from mixed communities, allowing biological diversity studies without

cultivating the individual organisms. In whole genome shotgun sequencing, the total DNA of a sample is sheared into smaller fragments before being sequenced at random (Lear et al., 2018). One of the advantages of metagenomics over targeted gene methods is that it can capture all taxa, concurrently permitting the study of both prokaryotic and eukaryotic diversity (Lear et al., 2018).

The study of fungal diversity using whole genome shotgun metagenomic data has been applied rarely to fungi (Donovan et al., 2018). The fungal component of microbial communities is called mycobiota, while their genomes are called mycobiome (Orellana, 2013). Some studies in fungal diversity using the metagenomic approach in host-associated environments revealed the influence of soil stratification and vegetation coverage on fungal diversity (Buée et al., 2009; Baldrian et al., 2012). Samples from soil litter and organic horizons from forests planted with spruce (*Picea abies*) in Central Europe and plantations from the Morvan Mountains in France revealed Basidiomycota and Ascomycota as the prevalent fungal sequences (Orellana, 2013). In a mycobiome study in Italy and France, Ascomycota was reported to be the most abundant fungal kingdom, accounting for 36.7 to 93% of all OTUs, for most samples from different ecosystems. In the evaluation of the microbial communities in soil by Uroz et al. (2013) using a combined 454 and Illumina sequencing approach, it was revealed that only 0.2% of the annotated reads have a significant match to fungi which are found more abundant in the soil's organic horizon rather than mineral horizon.

Accurate identification of EPF from soil samples is crucial for studying these organisms and their potential applications in pest control. However, the efficient extraction of high-quality DNA from soil can be challenging due to the complex nature of soil matrices and the presence of various inhibitors. Several DNA extraction methods have been developed and used for this purpose, including the CTAB-based method, bead-beating, and commercial kits (Feinstein et al., 2009). Each DNA extraction method has its advantages and limitations. The choice of method depends on various factors, including the type of soil, the desired DNA yield and purity, and the downstream applications. It is important to consider these factors when selecting a DNA extraction method for studying entomopathogenic fungi in soil samples.

To date, studies on the diversity of EPF in soil samples using metagenomic approaches are minimal. Therefore, there is a need to investigate the natural distribution of EPF in forest and pasture habitats, including those that cannot be identified through direct culture, insect bait method and target visualized sampling of diseased insects.

Utilization of EPF against arthropod pests

Primary requirements in our understanding of EPF for their utilization as biocontrol agents are the susceptibility of relevant insect species and the virulence of the fungi. The entomopathogenic fungi in the genera *Beauveria* and *Metarhizium*, commonly found in soil, have been used and evaluated against insect pests of pasture such as grass grub, *Costelytra gave* (White) (Coleoptera: Scarabaeidae) and Corina, *Wiseana* spp. (Lepidoptera: Hepialidae). In the study of Nelson et al. (2004), a strain of *B. bassiana* F418 showed high virulence against a soil-dwelling pest, clover root weevil, *Sitona lepidus* Gyll (Coleoptera: Curcolionidae). However, field trials showed that post-application, *B. bassiana* F418 populations in soil had high variability in the performance between different sites, time of application, and treatments (Brownbridge et al., 2006).

Development of the fungi as a control agent depends on selecting a stable strain with specific efficacy for the target host, as the virulence of fungal strains differs within the same species. Sánchez-Peña (2011) tested six *B. bassiana* isolates against Cuban laurel thrips, *Gynaikothrips uzeli* Zimmerman (Thysanoptera: Phlaeothripidae) and greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae). Among the six isolates, only one showed significant virulence against *G. uzeli* and *T. vaporariorum* causing 81.3% and 76% mortality after six days. Since the virulence of EPF strains differs within species, and local strains are often more effective than introduced ones, collecting native species and testing the native strains against target pests is essential to discovering effective isolates for biological control.

Study Objectives

This thesis research aims to characterize the diversity of entomopathogenic fungi (EPF) naturally occurring in the North Island of New Zealand by analyzing soil samples collected from pastures, forests, and pasture-forest transition zones.

Specifically, the objectives of this study were:

1. To determine the incidence and diversity of soil entomopathogenic fungi (EPF) across adjacent forest, boundary, and pasture habitats and at varying elevations, and to examine their association with soil invertebrates.
2. To isolate and identify EPF species occurring in forest–pasture transition soils using microscopic and molecular techniques.
3. To assess the diversity of EPF and other microbial communities in forest and pasture habitats using metagenomic and metabarcoding analysis.
4. To evaluate the pathogenicity of soil EPF isolates against selected agricultural pests.

Chapter Two

Incidence of entomopathogenic fungi in adjacent forest and pasture habitats and at varying elevations, and their association with soil invertebrates

Introduction

Entomopathogenic fungi (EPF) are naturally occurring fungi that can infect and kill insects, making them potential biocontrol agents for insect pests (Vega et. al., 2009; Mantzoukas et. al., 2022). With increased environmental awareness, biological pest control methods such as biopesticides based on EPF have gained attention as alternatives to chemical pesticides (Jaronski, 2010). These fungi are distributed in various habitats, including forests, agricultural lands, pastures, deserts, and urban areas (Sánchez-Peña et al., 2011). Some EPFs have shown habitat-specific preferences (Bidochka et al., 1998; Quesada-Moraga et al., 2007). Understanding the differences in EPF abundances within different habitat types is critical for determining which fungal species is suitable for and would proliferate in a given habitat type (Quesada-Moraga et al., 2007).

Multiple factors affect the distribution of entomopathogenic fungi in different habitats. EPF survival in soils is shaped by both biotic and abiotic factors (Jaronski, 2007), soil properties, including pH and texture (Quesada-Moraga et al., 2007; Jabbour & Barbercheck, 2009; Clifton et al., 2015), temperature, and moisture (Garrido-Jurado et al., 2011a), have been key focuses of soil EPF research. Quesada-Moraga et al. (2007) investigated the effects of sand, silt, and clay content in Mediterranean soils on EPF, while Oddsdottir et al. (2010) explored correlations between total carbon, nitrogen, and the occurrence of *Metarhizium anisopliae* and *Beauveria bassiana*. Notably, Jabbour and Barbercheck (2009) and Jaronski (2010) found no significant impact of soil pH or CEC on *Metarhizium* presence.

Hypocreales (Ascomycota) comprise many EPF fungal species, which spend most of their life cycle in the soil, outside their host. The soil protects fungi from UV radiation and other abiotic and biotic factors (Keller et al., 1989), making soil a good EPF reservoir for studying EPF diversity (Meyling & Eilenberg, 2006, Quesada-Moraga et al., 2007).

Insect baiting and plating methods are commonly used for isolating EPF from soil samples. These methods allow for the selective isolation of entomopathogens among other soil microbes (Sharma et al., 2018; Bueno-Pallero et al., 2020). The insect baiting method uses a specific insect as bait to attract and isolate EPF. One of the most commonly used bait insects

is the larvae of *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidae), the greater wax moth (Zimmermann, 1986). Another insect used for baiting is larvae of *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae), commonly known as the mealworm beetle (Sharma et al., 2018). The main advantage of the insect baiting method is its selectivity in isolating entomopathogens. showed that insect baiting more effectively isolates soil EPF than culturing soil suspensions on selective media (Keller et al., 2003; Imoulan et al., 2011). The plating method is another commonly used technique for isolating EPF. This method involves diluting soil samples and plating them on a selective medium that promotes the growth of EPF (Bueno-Pallero et al., 2020). The plating process allows for the recovery of EPF from soil samples and provides a semi-quantitative assessment of fungal diversity and density (Jaronski, 2007). The plating method has been used together with insect baiting to detect different patterns of EPF recovery, occurrence, activity, and biodiversity (Bueno-Pallero et al., 2020).

Soil invertebrates play a role in the EPF dynamics in the soil. Some soil-dwelling invertebrates, such as collembolans, mites, and nematodes, play an essential role as decomposers by consuming and transforming organic matter in the soil, while others, such as many insects and their larvae, feed on plant roots (Pflug & Wolters, 2002). According to Shah and Pell (2003), EPF life cycles are synchronized with insect host stages and environmental conditions. The interactions between soil fungi and soil invertebrates occur in every ecosystem where they coexist (Gange & Brown, 2002), and the presence of host arthropods is a major factor for EPF dynamics in soil.

Despite the recognized importance of soil-dwelling entomopathogenic fungi in managing pests, their occurrence and natural diversity still need to be studied in New Zealand. Collecting EPF species in natural environments, including pasture and forest soils, will increase the discovery and selection of effective species and isolates against insect pests. The objective of this chapter was to investigate the incidence of entomopathogenic fungi in adjacent forest and pasture habitats, as well as at varying elevations, and to examine their association with soil invertebrates.

Materials and Methods

Study area

This study was conducted in 2019-2021 in three locations on New Zealand's North Island: Taranaki, Manawatu, and Whanganui regions (**Fig. 2.1).** Each of these selected sites

encompassed three adjacent habitats: pasture, boundary, and forest, with the fence line serving as the demarcation between the pasture and forest habitats.

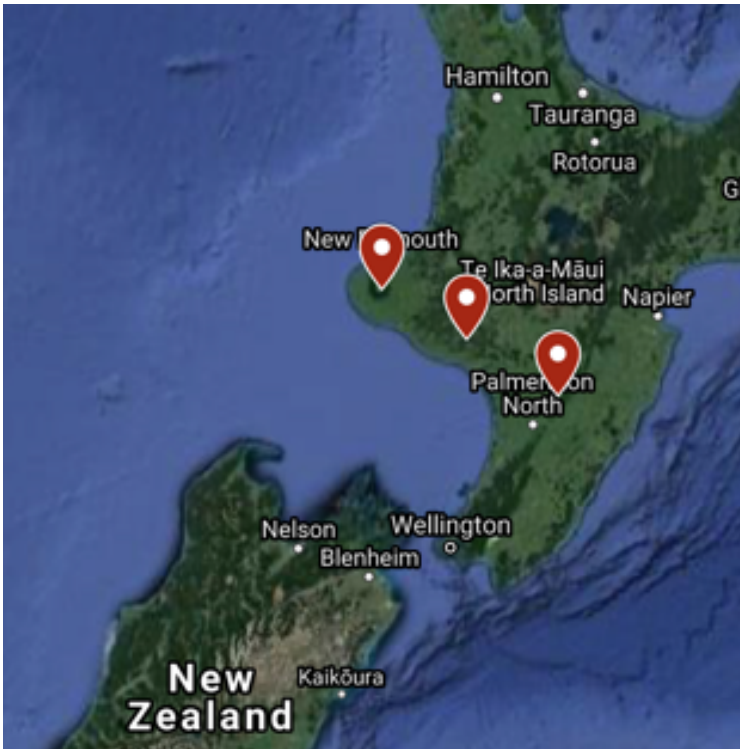


Figure 2.1. Soil collection sites in the North Island of New Zealand.

The Taranaki site (please see **Table 2.1** for coordinates) comprised a ryegrass pasture that undergoes annual fertilization and is grazed by young cows. The adjacent forest habitat is part of Mount Egmont National Park in Taranaki, New Zealand. The forest is characterized by native podocarp and broadleaf trees, including pukatea (*Laurelia novae-zelandiae*), kahikatea (*Dacrycarpus dacrydioides),* and kamahi (*Pterophylla racemose),* within a climate zone that ranges from warm to cold (Singers & Rogers, 2014).

The Whanganui site (**Table 2.1**) was in Bushy Park, Tarapuhi, Kai Iwi, Whanganui. Cows actively graze the pasture in this area, but it is not fertilized annually. The forest habitat primarily consists of a broadleaf podocarp complex, dominated by tawa (*Beilschmiedia tawa*) and pukatea (*L. novae-zelandiae*), along with rimu (*Dacrydium cupressinum*) and northern rata (*Metrosideros robusta*). Since 2005, the area has been designated a forest sanctuary, protected from mammalian predators except mice. The sanctuary has approximately 160 plant species, exhibiting significant structural diversity and complexity in the ground layer, sub-canopy, and emergent canopy (https://bushypark.nz/forest/). This diverse forest with a moderate climate,

small streams, and protection from most mammalian predators and competitors provides exceptional habitat for numerous indigenous and endemic species (https://bushypark.nz/forest/).

The sampling site in Manawatu was situated in the Totara Reserve, Pohangina Valley (**Table 2.1**). The forest is characterized by the presence of massive totara (*Podocarpus totara*) trees scattered throughout the area, indicating its historical significance as a timber source (Knight, 2008). Other tree species can also be observed, along with abundant seedling pukatea (*L. novae-zelandiae)* and juvenile nikau (*Rhopalostylis sapida*) as well as groupings of mamaku (*Cyathea medullaris*). Pockets of bracken and light-demanding shrubs such as koromiko (*Hebe salicifolia*) and karamu (*Coprosma robusta*) persist, although the surrounding vegetation gradually overshadows them.

Table 2.1. Details of the collection sites in Taranaki, Manawatu and Whanganui, North Island of New Zealand (2019-2021).

Study Site	Habitat	Collection Date	GPS	Elevation (m a.s.l.)
Taranaki	Forest (Native)	June 2019; August 2021	39°19' 12"S; 174°10' 16"E	563
	Boundary	June 2019; August 2021	39°19' 12"S; 174°10' 17"E	563
	Pasture	June 2019; August 2021	39°19' 12"S; 174°10' 18"E	562
Whanganui	Forest (Native)	June 2020; August 2021	39°47' 54"S; 174°55' 48"E	253
	Boundary	June 2020; August 2021	39°47' 54"S; 174°55' 49"E	255
	Pasture	June 2020; August 2021	39°47' 53"S; 174°55' 49"E	257
Manawatu	Forest (Native)	Oct 2020; August 2021	40°09' 06"S; 175°50' 37"E	141
	Boundary	Oct 2020; August 2021	40°09' 06"S; 175°50' 36"E	141
	Pasture	Oct 2020; August 2021	40°09' 07"S; 175°50' 35"E	141

Soil samples were also collected at various elevations within Egmont National Park to investigate the incidence of EPF, as detailed in **Table 2.2**. Four sampling sites were selected, located at elevations of 512, 680, 880, and 1024 meters above sea level (a.s.l.). These sites correspond to mean annual temperatures of 10.5°C, 9.1°C, 8.2°C, and 7.3°C, respectively. Appendix I (**Figs. A.1** to **A.4**) shows the sampling sites of the study.

Table 2.2. Collection sites in the Mt. Egmont Forest, Taranaki, New Zealand for assessing effect of elevation on diversity and abundance of enthomopathogenic fungi, 2019.

Collection Site	GPS Coordinates	Elevation (meters a.s.l.)
Site 1	39°19'15.82"S; 174°11'18.05"E	512
Site 3	39°18'46.43"S; 174° 8'47.20"E	680
Site 5	39°18'20.46"S; 174° 7'8.83"E	880
Site 7	39°18'21.00"S; 174° 6'27.12"E	1024

Field sampling and soil collection

Soil samples were collected from three sites in the North Island, New Zealand—Taranaki, Whanganui, and Manawatu. At each site, three parallel 40-m transects were established perpendicular to the forest–pasture edge, covering three habitat zones: forest interior, boundary (edge), and pasture. Each transect had three sampling points, one per habitat, spaced 20 m apart. The distance between adjacent transects was also 20 m to ensure spatial independence (**Fig 2.2**). At each sampling point, five soil cores (5 × 5 cm, 10 cm deep) (**Fig. 2.3**) were taken and combined to form a composite sample, giving a total of 45 soil cores per site (3 transects × 3 habitats × 5 cores) per sample collection.

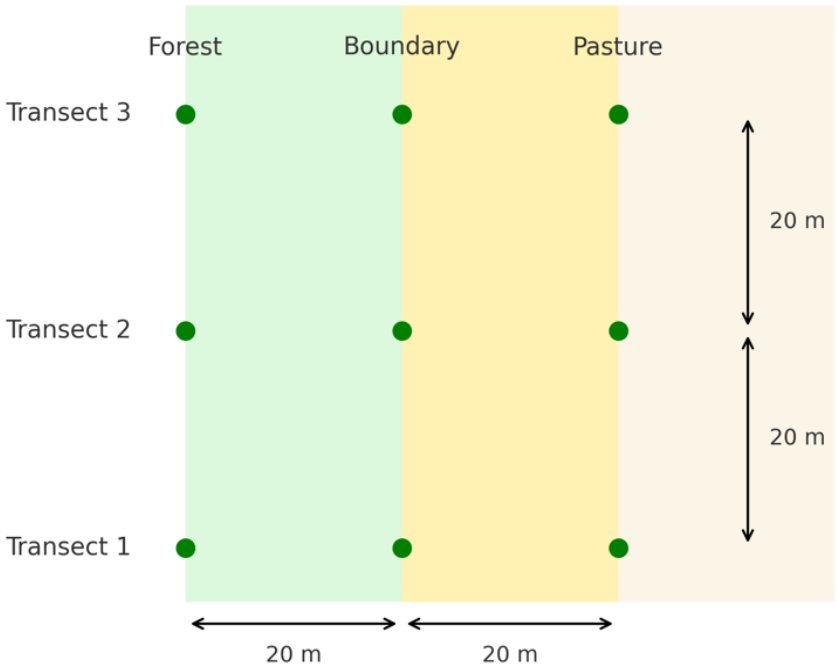


Figure 2.2. Layout of the field sampling design showing three parallel 40-m transects across adjacent habitats: forest, boundary, and pasture. Each transect had three sampling points spaced 20 m apart. Distance between transects was 20 m. Five soil cores were collected in each sampling point for subsequent analyses (insect baiting, plating, invertebrate extraction, and soil DNA extraction).

For elevation sampling, one sampling point per elevation was used, and five soil cores per elevation were collected.

A rectangular soil corer 5x5 cm with 10 cm depth was used to obtain soil samples in all soil collection sites. All soil samples were labelled and stored in clean plastic bags at 5°C until used. Two cores out of the five were used for insect baiting (this Chapter), the third core for the plating method (this Chapter), and the fourth core for extraction of soil invertebrates (this Chapter). The fifth core was used for soil DNA extraction (Chapter 4).

At the time of sample collection, soil temperature and moisture were recorded using a QM7216 Digital Stem Thermometer and TDR 300 Soil Moisture Probe (Spectrum Technologies Inc., USA), respectively. Elevation and GPS coordinates were recorded using a handheld GPS device. The plants surrounding each habitat were recorded. Soil collection in the Egmont National Park, Taranaki, New Zealand was done under the permit issued by the NZ Department of Conservation (authorization number 69401-RES).

Figure 2.3. Soil sample collection in adjacent habitats in Taranaki, NZ, June 2019.

Insect bait method

To assess the presence of entomopathogenic fungi (EPF) in the soil samples, I employed the insect bait method using wax moth *Galleria mellonella,* and mealworm *Tenebrio molitor* larvae as susceptible baits. One soil sample from each sampling point (whole core) was gently broken apart. Any stones, twigs, and roots were discarded, leaving only the soil, which was then transferred to a plastic container (8.5 cm diameter, 10 cm height). Five mealworm larvae were introduced into the soil at the middle depth (~ 5cm). Similarly, the second soil sample from each sampling point was placed in a separate container and five wax moth larvae were placed in the soil at middle depth. The containers were then incubated in the dark at a constant temperature of 25°C ± 1 for 21 days. I examined the samples on days 7, 14, and 21 to monitor infected larvae.

While published literature (Zimmermann, 1986; Barker & Barker 1998; Bidochka et al. 1998; Quesada-Moraga et al., 2007) suggested that *G. mellonella* is a highly susceptible host for this method, my initial experiments revealed that only five larvae (1.9%) out of 270 exposed to the soil died from EPF infection. I consistently observed a lack of infection by EPF using the *Galleria* bait method across all sampling sites and baiting times. Therefore, due to the low success rate, I decided not to employ *G. mellonella* larvae for further experiments. Instead, the subsequent setups only used mealworm larvae (*T. molitor*) as the susceptible bait.

The dead larvae showing visible external fungal growth were collected. Using a sterile wire loop, I transferred the fungal spores from the bait insects to Petri dishes containing sterilized potato dextrose agar (PDA) medium supplemented with antibiotics to prevent

bacterial contamination. The isolates growing on these Petri dishes were observed and documented for subsequent analysis.

Plating in semi-selective media

Another method employed to identify and quantify the presence of EPF in the soil samples was serial dilution and plating. For one soil core in each sampling point, two replicates of 20g soil were suspended in 180 ml of 0.01% Triton X-100 and shaken in an orbital shaker at 250 rpm for 45 minutes. Serial dilutions of the suspension were prepared using 0.01% Triton X-100, ranging from 10^{-2}, 10^{-3}, and 10^{-4}. These dilutions were then cultured on Petri dishes containing a semi-selective medium. To prepare the semi-selective medium, one litre of potato dextrose agar (PDA) was supplemented with antibiotics: 3.33 ml of tetracycline chloride (15 mg/ml solution in methanol, stored in the freezer) and 3.5 ml of streptomycin sulfate (100 mg/ml stock solution in distilled water). A 100 µl of each dilution was poured into the Petri dish, and this process was replicated twice. A total of 12 Petri plates per soil core was used, for a total of 36 Petri plates per transect and 108 Petri plates per site in forest-pasture boundary study, 12 Petri plates per elevation in elevation study. After ten days of incubation, the colonies were examined under a light microscope, and the EPF colonies were counted. The EPF colonies were then transferred to fresh Petri dishes with PDA for subculturing and obtaining pure cultures. The workflow is shown in **Fig. 2.4.**

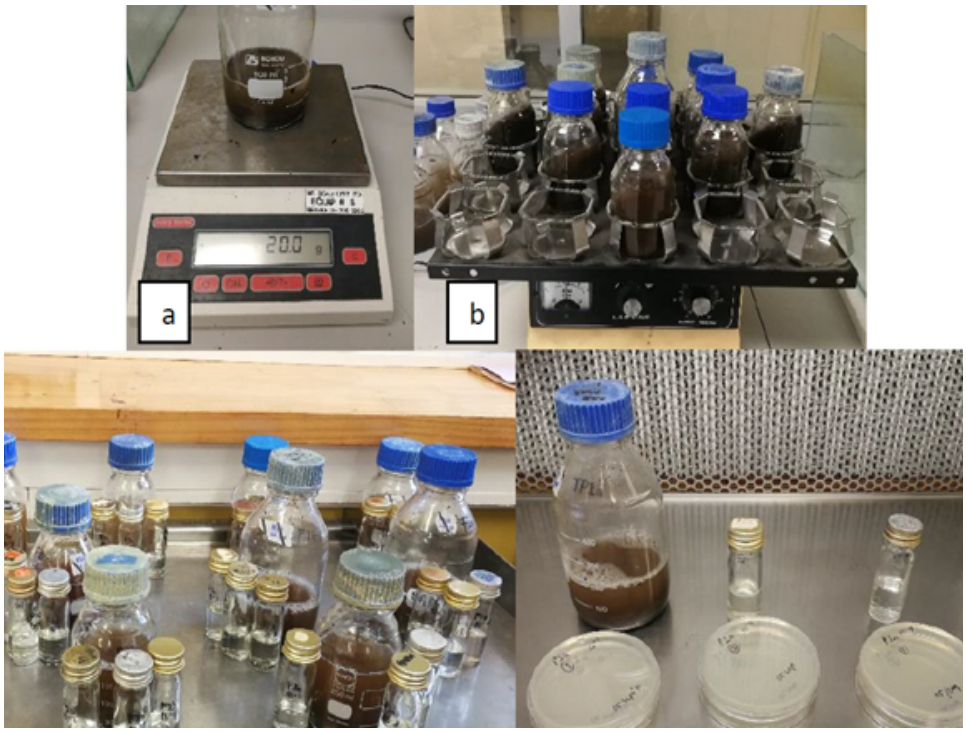


Figure 2.4. Workflow for determining EPF density per gram of soil leading to EPF isolation. a) Weighing 20 g of soil in 180 ml 0.001 TritonX. b) Shaking samples in an orbital shaker. c) Serial dilution of samples. d) Plating in Petri dishes with the semi-selective medium.

Soil invertebrates

One soil core per sampling point was allocated to explore the diversity of soil microarthropods in adjacent habitats. Soil microarthropods were extracted from the cores using a modified Berlese-Tullgren apparatus described by Oliver and Beattie (1996). The extraction was conducted for seven days. Soil microarthropods were collected into glass vials with 70% ethanol for counting and identification. Mites, Collembola, spiders and insects were counted and identified at the group level without further taxonomic specification. A category labelled 'Others' encompasses additional taxa, such as symphylans, among others, which were counted but not classified under the four main groups. The total invertebrate counts reflect the sum of the four groups plus the 'Others' category.

Statistical analysis

Baiting and plating

Statistical analysis was performed using R version 4.1.3 (R Development Core Team, 2022). Generalized Linear Mixed Models (GLMM) were used to analyse the relationship between habitat and the number of infected insects from baiting. The effect of habitat on the number of colony-forming units (CFU) from the plating method was also analysed. A nested GLMM with a Poisson distribution was fitted to the data using the glmer() function from the lme4 package. The response variables – "Infected Bait" and "CFU" per soil sample – were modelled as a function of habitat. The random intercept for "Site/Habitat" was included in the model to account for any non-independence in the data due to the nested structure. An example of the model used in this analysis is below:

```
glmm_Bait <- glmer(InfectedBait ~ Habitat + (1 | Site/Habitat),
                   data = Baits, family = poisson())
```

The Akaike Information Criterion (AIC) was assessed to determine whether including the "Habitat" variable improved the model's predictive ability. This approach allowed a rigorous exploration of the influence of habitat types on the count of EPF infections and the number of CFUs. When a significant difference was found in the model, post-hoc test was performed using the *multcomp* package. To assess whether there was a statistical difference in the efficacy of these two methods for detecting EPF, a Wilcoxon signed-rank test was conducted.

Elevation

A generalized linear model (GLM) with a Poisson distribution was employed to investigate the relationship between EPF infection using insect baiting and elevation. The GLM was fitted using the glm() function from the lme4 package. To perform multiple comparisons for infected bait at different elevations, the *lsmeans* and *multcomp* packages were utilized. The lsmeans() function was applied to the fitted GLM to obtain the least-squares means for each elevation level. The resulting means were subjected to the cld() function, which performed pairwise comparisons using the Sidak adjustment method. The significance levels, denoted by letters, were determined using a significance level of 0.05. Similar analyses were conducted to

investigate the relationship between CFU counts and elevation. The model comparisons and multiple comparisons were performed as described above.

Soil invertebrates

To assess the relationship between the total count of invertebrates and habitat, a Generalized Linear Model (GLMM) with Poisson distribution was generated with site/habitat as random effects. The relationship between the number of infected baits and the total number of invertebrates per sampling point was examined using Pearson correlation. The cor() function was utilized to calculate the correlation coefficients. Similarly, the relationship between the density of EPF (CFU 10^3 per gram of soil) and the total invertebrate count per sampling point was assessed using Pearson correlation. The relationship between invertebrate taxa counts and habitat was visualized using PCA plot. To determine if the invertebrate community composition significantly differed among habitats, PERMANOVA was conducted using the adonis function in *vegan* package in R.

Results

Detecting EPF presence in adjacent habitats (pasture, boundary, and forests)

In this study, 108 soil samples were collected from the forest-pasture transition in three sites to assess the presence of entomopathogenic fungi (EPF). Among these samples, 79 (73.15%) tested positive for EPF. I employed two different isolation methods, the insect bait method and plating in semi-selective media, to investigate the occurrence of EPF in the soil samples. From the insect bait method, 42 soil samples (38.89%) resulted in successful infections, indicating the presence of EPF. Similarly, 37 soil samples (34.26%) were positive for EPF through plating.

Both insect baiting and plating methods effectively isolated entomopathogenic fungi (EPF) across all examined sites and habitats. The Wilcoxon signed-rank test suggested that there is no significant difference between insect baiting and soil plating ($W = 3.0$, $P = 0.09$) for detecting EPF in soil.

When examining the individual habitats with positive presence of EPF, 30 soil samples (27.8%) from forests were positive for EPF, and 31 samples (28.7%) from the forest-pasture boundary, while only 18 pasture samples were positive for EPF (16.7%). The distribution of positive samples per habitat using both isolation methods is summarized in Appendix I (**Fig. A.5**).

Assessing EPF distribution in adjacent habitats through insect baiting

Insect baiting was used to assess the incidence of EPF in adjacent habitats. Of the 270 larvae exposed to the soil from the three sites, 120 (44.4%) were successfully infected (**Fig. 2.5**). Some *Tenebrio molitor* larvae also died due to other biotic factors such as entomopathogenic nematodes and bacterial infection.

The site as an independent predictor did not yield any significant effect; hence, it was excluded as an independent factor in the subsequent analysis but included as a random effect in the nested structure of the model. The findings revealed a significant association between habitat type and the count of infected bait insects ($F_{2,51} = 5.519$, $P < 0.05$). Post-hoc comparisons revealed that while the difference between forest and boundary habitats was not statistically significant ($Z = 0.892$, $P = 0.644$), the difference between pasture and boundary habitats was significant ($Z = -2.497$, $P = 0.033$), and so was the difference between pasture and forest ($Z = -3.308$, $P = 0.003$) (**Fig. 2.6**).

Figure 2.5. Example of EPF infection of *Tenebrio molitor* larvae in soil samples from the insect baiting set-up.

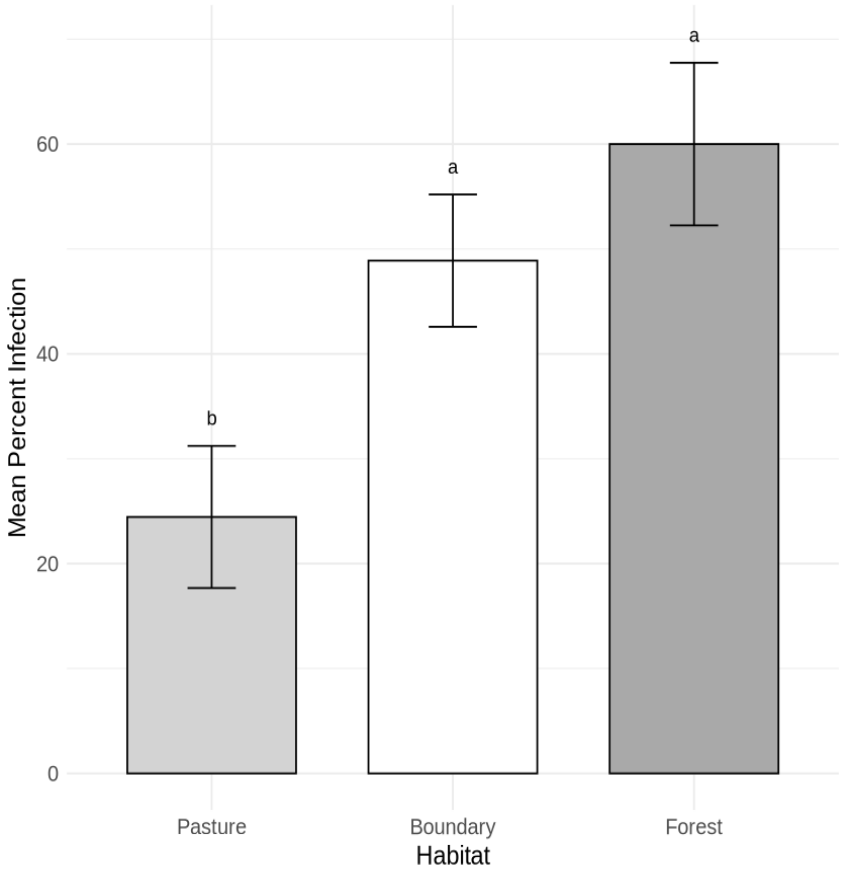


Figure. 2.6. Proportion of *Tenebrio molitor* larvae (mean ±se) infected with EPF in habitats across three forest-pasture transitions, NZ North Island. A total of 90 *T. molitor* larvae were baited per habitat. The values are mean ± se. Different letters indicate statistically significant differences at $\alpha = 0.05$, Tukey's HSD test.

Density of EPF across adjacent habitats assessed through soil plating

To determine the density of EPF in the collected soil samples, the number of colony-forming units (CFU) per gram of soil was quantified using a PDA medium supplemented with antibiotics. The results revealed a significant influence of habitat type on CFU counts (Likelihood ratio test: $\chi^2(2) = 36.01$, $P < 0.001$). The site was not significant as an independent predictor of CFU counts, as shown in Appendix I (Fig. A.6).

The findings emphasize the substantial impact of habitat type on CFU counts, with forest habitat exhibiting a marginal effect and pasture habitat showing significantly lower CFU counts than boundary and forest habitats. The results indicated no significant difference in CFU counts between boundary and forest habitats ($P = 0.644$). However, pasture habitats displayed significantly lower CFU counts compared to both boundary ($P = 0.033$) and forest ($P = 0.003$) (**Fig. 2.7**).

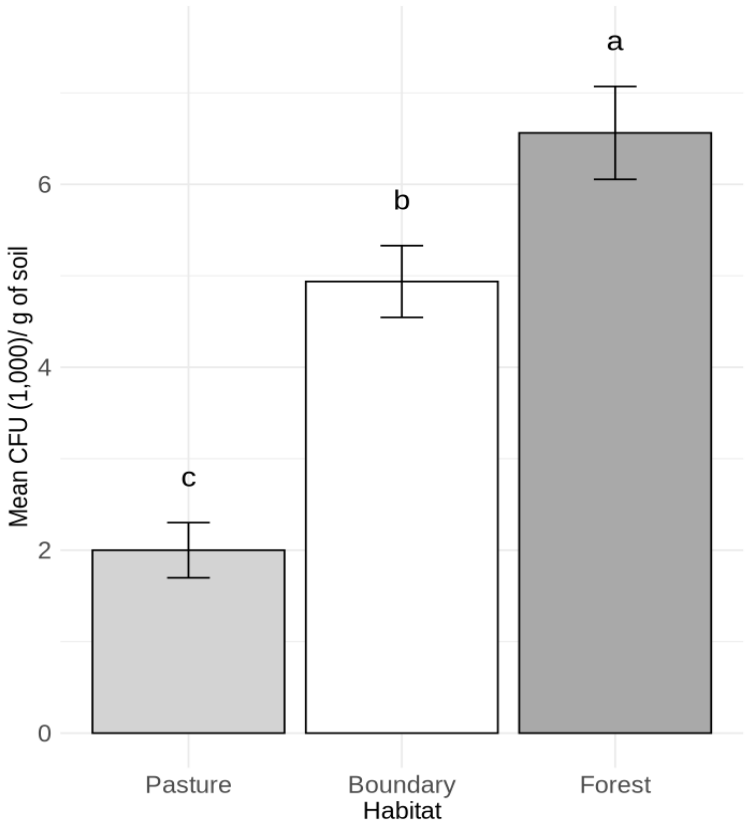


Figure 2.7. Mean density of EPF (1000 CFU per g soil) quantified using soil dilutions and direct plating in soil samples across three forest-pasture transitions habitats, NZ North Island. The values are mean ±se. Different letters indicate statistically significant differences at $\alpha = 0.05$, Tukey's HSD test.

Soil EPF and invertebrates in adjacent habitats

Of the 2,811 collected soil invertebrates from a total of 54 soil samples, 1,421 (50.55%) were collected from the forests, 896 (31.87%) from the boundary, and only 494 (17.57%) were collected from pastures. **Table 2.3** provides the invertebrate counts per group. Generally, results indicate that the composition of the soil arthropods was dominated by Collembola (49.6%), followed by mites (26.7%). Appendix I (Fig. A.7) illustrates the difference in invertebrate group count per habitat type per square meter.

There was a significant influence of habitat on the total invertebrate count ($\chi^2(2) = 49.44$, $P < 0.001$) with forest habitat significantly higher in abundance of soil invertebrates than pasture or boundary (**Fig. 2.8**).

Table 2.3. Counts of soil invertebrate groups (Collembola, mites, spiders, insects, and others) across pasture, boundary, and forest habitats at different sites (Manawatu, Taranaki, and Whanganui), 2019- 2021.

Habitat	Site	Collembola	Mites	Spiders	Insects	Others
Pasture	Manawatu	89	44	8	24	20
Boundary	Manawatu	141	59	16	24	33
Forest	Manawatu	289	168	22	43	31
Pasture	Taranaki	80	46	8	7	12
Boundary	Taranaki	167	71	12	24	31
Forest	Taranaki	186	157	17	45	46
Pasture	Whanganui	65	34	9	24	24
Boundary	Whanganui	166	70	14	32	36
Forest	Whanganui	211	101	15	49	41
	Total	1394	750	121	272	274

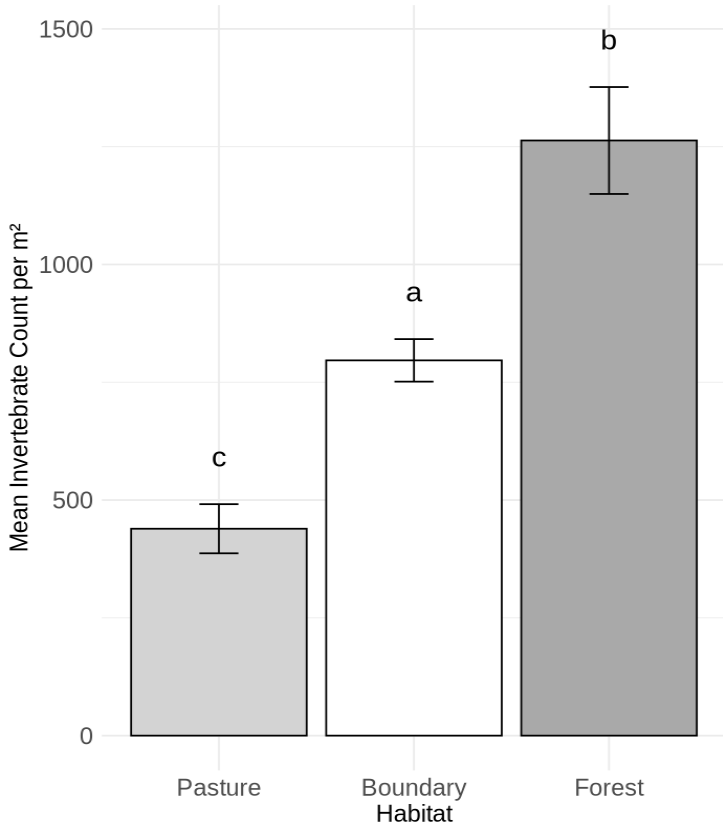


Figure 2.8. Soil invertebrate abundance (ind. per m^2) in adjacent forests, pastures, and boundary habitats. The values are mean ±se. Different letters indicate statistically significant differences across habitat type (Tukey's HSD test, $\alpha = 0.05$).

The PCA biplot **(Fig. 2.9)** illustrates the distribution of invertebrate group counts across habitats. Principal Component 1 (PC1) accounts for 53.26% of the total variance, while Principal Component 2 (PC2) accounts for 18.61%. Together, the PC1 and PC2 explain 71.87% of the variance in the dataset. PC1 primarily differentiates between forest habitats and other habitats based on invertebrate counts. The strong negative loading of Collembola and Mites along PC1 highlights their prevalence in forest habitats. PC2 captures additional variance, distinguishing subtle differences within boundary and pasture habitats. Other invertebrate groups (Spiders, Insects, and Others) were present across multiple habitats but with varying densities. The separation of forest habitats along PC1 and the partial separation of boundary and pasture habitats suggest habitat-specific invertebrate assemblages.

The PERMANOVA results indicated a significant effect of habitat type on the community structure of soil invertebrates ($F_{2,51} = 3.96$, $P = 0.001$). The analysis showed that habitat type explained 33.16% of the total variation in community structure ($R^2 = 0.3316$).

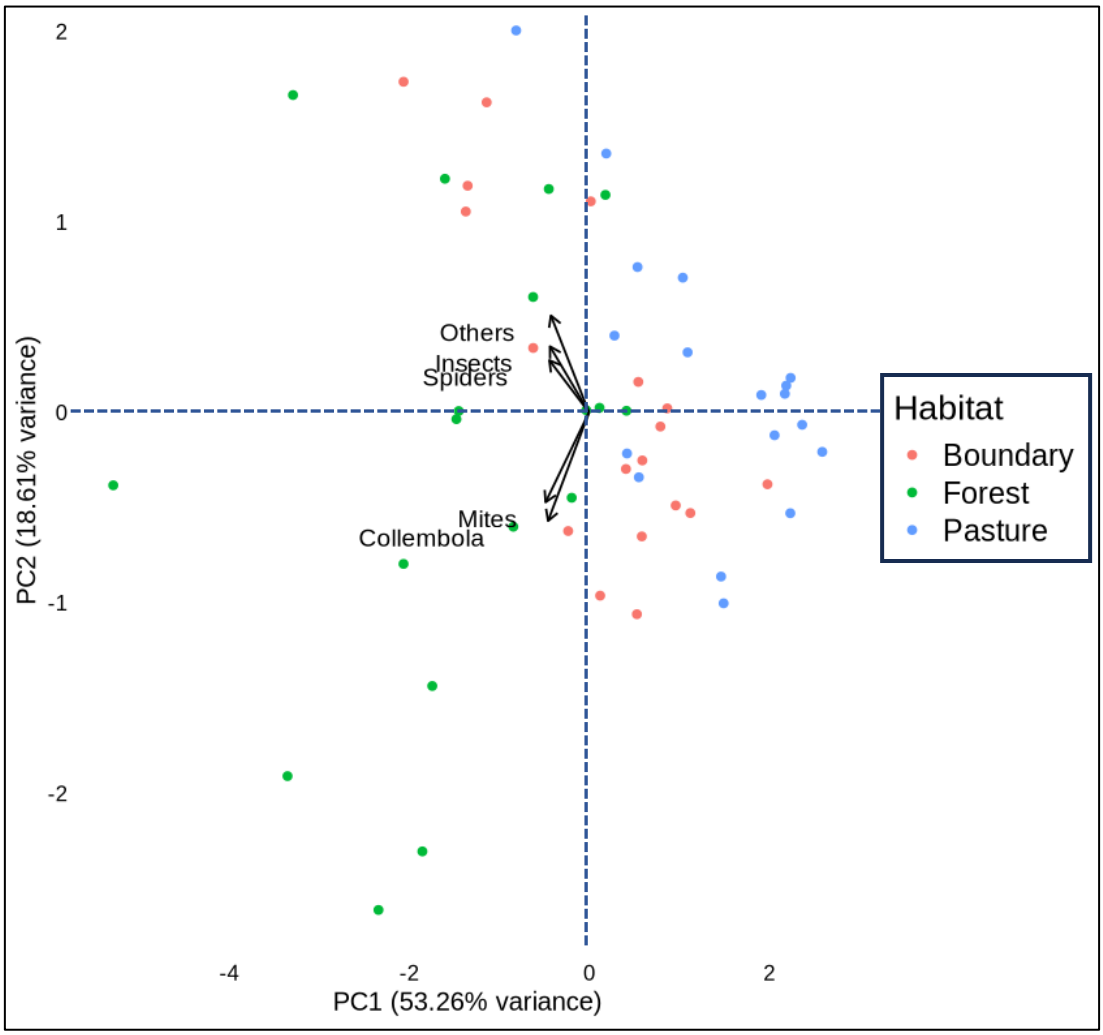


Figure 2.9. PCA plot of soil invertebrate community compositions among the sampled sites within the forest-pasture transition, NZ North Island, 2019, 2020, and 2021. Points represent individual observations coloured by habitat: Boundary (red), Forest (green), and Pasture (blue). Vectors indicate the weighting of different invertebrate groups on the principal components.

Correlation between invertebrate counts, infected baits and EPF density in the soil

The correlation analysis between the total invertebrate count, the counts of Infected Bait and CFU revealed several important associations. Notably, there was a moderate positive correlation between the total invertebrate count and the number of infected baits (r = 0.341). This suggests that as the count of total invertebrates increases, the occurrence of infected EPF also tends to increase. This positive association implies that invertebrate presence may promote conditions favourable for EPF infections or that EPF infections might proliferate in environments with higher invertebrate activity. Conversely, the correlation between total

invertebrate counts and CFU was very weak (r = -0.034), indicating that there was almost no relationship between the number of total invertebrates and the CFU levels in the soil.

Evaluation of EPF presence and density across differing elevations

Insect baiting and plating were used to study the presence and density of EPF in soil samples from native forests at various altitudes within Mt. Egmont National Park (512, 680, 880, and 1024 meters above sea level). A Generalized Linear Model (GLM), employing a Poisson distribution with a log link function, was formulated to explore the correlation between the presence of infected and various elevations. An examination of the deviance table revealed that including 'Elevation' as a variable significantly enhanced the model's fit compared to the null model ($\chi^2(3) = 39.954$, $P < 0.001$). The analysis of infected baits at different elevations (512, 680, 880, and 1024 meters above sea level) revealed significant differences, with lower elevations (512 and 680 m a.s.l.) having higher infection rates compared to higher elevations (880 and 1024 m a.s.l.). As shown in **Fig. 2.10**, the mean percent infection was highest at 512 m a.s.l. and decreased with increasing elevation. Infection rates at 512 and 680 m a.s.l. were not significantly different from each other, nor were infection rates at 880 m and 1024 m a.s.l. However, infection rates at lower elevations (512 m and 680 m a.s.l.) were significantly higher compared to higher elevations (880 m and 1024 m a.s.l.).

Elevation also significantly influenced CFU counts ($\chi^2(3) = 77.18$, $P < 0.001$). Similar to what was seen for insect baits, the fungal CFU count was notably higher at lower elevation levels (512 m and 680 m a.s.l.) compared to 880 m and 1024 m a.s.l. (**Fig. 2.11**)

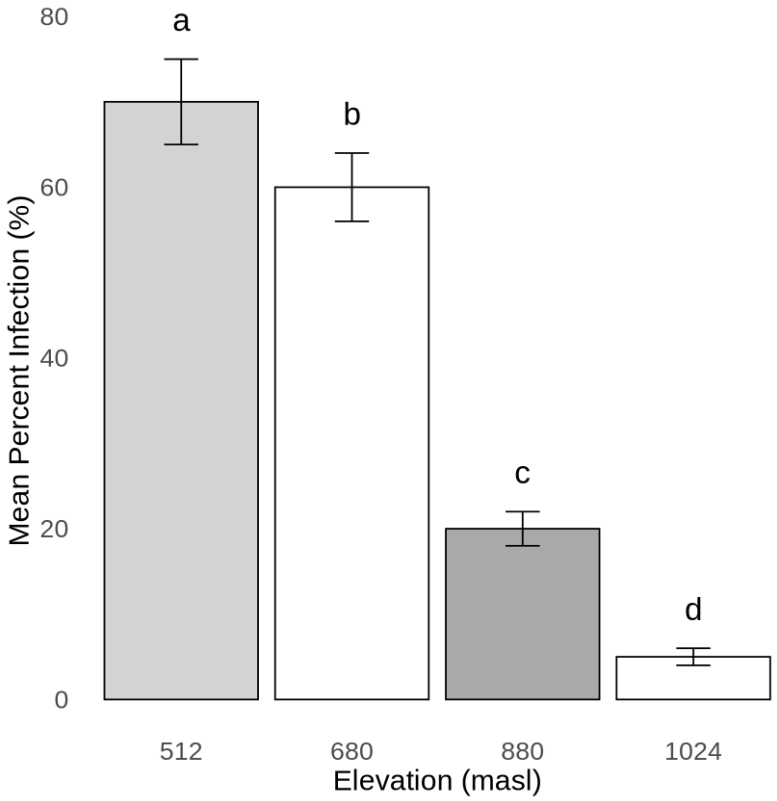


Figure 2.10. Proportion *Tenebrio molitor* larvae infected with EPF using soil from native forests at different elevations (meters a.s.l.) in Tongariro National Park, NZ, 2019. A total of 40 *T. molitor* larvae were baited per elevation. The values are mean percent infection ±se. Different letters indicate statistically significant differences at $\alpha = 0.05$, Tukey's HSD test.

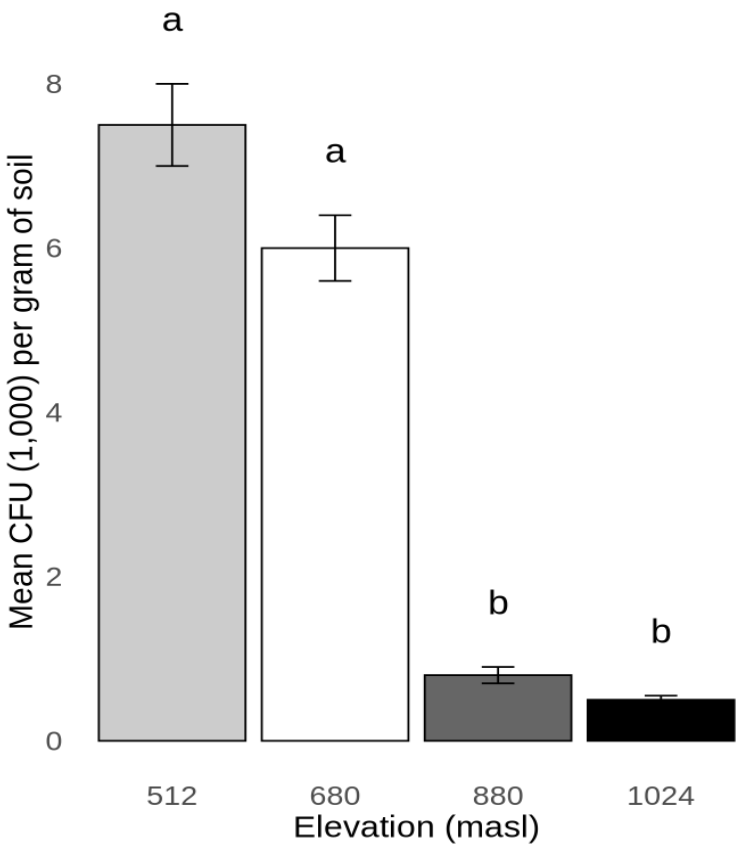


Figure 2.11. The density of EPF (1000 CFU per gram of soil) observed using soil dilutions and direct plating in soil samples from native forests at different elevations (meters a.s.l.) in Tongariro Nat. Park, NZ, 2019. The values are mean ±se. Different letters indicate statistically significant difference at $\alpha = 0.05$, Tukey's HSD test.

Discussion

Abundance of Entomopathogenic Fungi (EPF) in soil samples across adjacent habitats

The presence and abundance of entomopathogenic fungi (EPF) in soil samples from adjacent pasture and forest habitats were assessed, revealing that habitat type plays a crucial role in determining EPF distribution. There was a considerable presence of EPF in the adjacent habitats in decreasing order: Forests > Boundary >Pastures. To my knowledge, this is the first report of EPF abundance in adjacent habitats in New Zealand.

Entomopathogenic fungi (EPF) in different habitats have been the subject of intense research, given the significant role that these fungi play in controlling pest populations (Shah and Pell, 2003). The present study, along with recent studies from Sharma et al. (2018) in

Portugal and from Meyling and Eilenberg (2006) in Denmark, confirms that the presence and abundance of entomopathogenic fungi can vary between different habitats within proximity.

Our results align with the findings of Sánchez-Peña et al. (2011), who reported that the number of insects infected by EPF was significantly higher in forest soil compared to agricultural soil. Specifically, Sánchez-Peña et al. (2011) observed 51.4% infected insects in forest soil, whereas only 16.2% were found in agricultural soil.

Considering the substantial number of soil samples collected in this study (108) and the observed occurrence of EPF in different habitats, our findings (73.2% EPF-positive soil samples) are comparable to previous studies conducted by Klingen et al. (2002), Quesada-Moraga et al. (2007), Sun et al. (2008), Imoulan et al. (2011) and Schneider et al. (2012). For instance, Quesada-Moraga et al. (2007) reported a 71.7% occurrence of EPF-positive soil samples in both natural and cultivated habitats, which aligns with the findings of this study. The high percentage of EPF presence in soil samples underscores the ubiquity of these fungi in various environments.

In my study, only 28.7% of the total soil samples with positive EPF infection were from boundary habitats, which was comparable with 20.5% and 15.8% of positive soil samples from hedgerows habitats examined by Meyling & Eilenberg (2006) and Sharma et al. (2018), respectively. Semi-natural habitats, such as mid-field woodlots, have been found to play an essential role as a source of biodiversity of entomopathogenic fungi in agricultural landscapes (Tkaczuk et al., 2013).

The density of entomopathogenic fungi (EPF) is significantly higher in forest ecosystems than in agricultural pastures. Forest habitats, including agroforestry systems (Lin, 2007), contribute to shaded soils, regulate microclimatic conditions, conserve moisture, and enhance ecosystem stability by reducing disturbances (Jose, 2009). Moreover, the microclimate in forests, characterized by higher humidity and stable temperatures, further supports the survival and reproduction of EPF. These fungi thrive in forest soils, where they effectively infect their insect hosts and serve as crucial biotic factors in regulating arthropod populations within forest ecosystems (Gouli et al., 2013).

In contrast, agricultural pastures experience frequent soil disturbances due to tillage, crop rotation, and the application of chemical fertilizers and pesticides, which can disrupt the natural soil structure and microbial communities (Tkaczuk et al., 2014; Clifton et al., 2015). This disruption can lead to a decline in the abundance and diversity of EPF. Birkhofer et al. (2008) observed that fertilizers impact both aboveground and belowground systems, ultimately weakening internal biological control mechanisms. This suggests that the low abundance of

EPF in pasture habitats may be attributed to the effects of fertilization, as the pasture habitats collected in this study is fertilized. Additionally, in agricultural settings, the application of herbicides and fungicides can adversely affect EPF populations. These chemicals, while intended to manage pests, often have non-specific effects that harm beneficial organisms (Yousef et al., 2015; Bamisile et al., 2021).

The significance of higher EPF densities in forests compared to agricultural pastures extends beyond mere ecological observations, as it has significant implications for pest management. EPF act as a natural biocontrol agents, effectively regulating insect populations. These fungi are commonly found in forest areas, national parks, and nature reserves, where they have demonstrated successful and acceptable levels of control under laboratory and simulated field conditions (Barta et al., 2019). Their presence can help mitigate pest outbreaks, thereby reducing the need for chemical pesticides in adjacent agricultural lands. This natural pest control mechanism is especially vital in sustainable agriculture, where the focus is on reducing chemical inputs (Bamisile et al., 2021) and promoting biodiversity. Additionally, EPF contribute to soil health through their role in nutrient cycling (Sharma, 2019) and in regulating processes such as pest and disease control (Klingen and Haukeland, 2006). Their functions further support the sustainable management of agroecosystems (Barrios, 2007).

Ecotones in soil are dynamic boundaries where physical and chemical properties, such as pH, salinity, and air capacity, gradually shift with changes in habitat and biodiversity (Marfo et al., 2019). These zones, highly sensitive to environmental changes, are crucial in ecological research as they connect biomes and exhibit significant biological diversity, offering high conservation value (Korpela et al., 2015). The boundary habitat, considered as the transition zone, or ecotone, between forest and pasture in this study presents a unique ecological niche that supports a higher abundance of entomopathogenic fungi (EPF) compared to the pasture alone. This can be attributed to several interrelated factors, including habitat heterogeneity, the edge effect, and reduced disturbance.

Firstly, the ecotone is characterized by greater habitat heterogeneity and diversity, which not only supports fungal growth but also enhances the overall biodiversity of the area. The blend of environmental conditions from both the forest and pasture—such as increased organic matter from the forest and moderate sunlight exposure from pasture—creates a favorable microenvironment for fungal growth. Research indicates that soil properties, especially organic matter content, play a crucial role in shaping the diversity and abundance of EPF (Rath et al., 1992; Jabbour and Barbercheck, 2009; Sharma, 2019). For example, soils with higher organic matter content have been associated with greater EPF diversity and better

conidia absorption (Quesada-Moraga et al., 2007; Majchrowska-Safaryan et al., 2017), as these fungi benefit from the abundant organic inputs which are higher in boundary than pasture.

Secondly, the edge effect plays a significant role in promoting biodiversity within the ecotone. The interface between two distinct habitats often supports a greater variety of species, including a wider range of insect hosts (Korpela et al., 2015). The presence of host arthropods is a critical factor influencing the dynamics of entomopathogenic fungi (EPF) in soil (Walstad et al., 1970). Increased host availability is essential, as EPF depend on invertebrates to complete their life cycles (Islam et al., 2021). The forest-pasture boundary acts as a convergence zone for species from both ecosystems, thereby increasing the likelihood of EPF encountering suitable hosts

Moreover, the boundary area may experience less agricultural disturbance compared to the interior of the pasture (Meyling et al., 2009). Meyling et al. (2009) reported that the diversity of EPF was higher in seminatural hedgerow habitats. This was attributed to the greater abundance and diversity of insect hosts, higher humidity levels, reduced ultraviolet exposure, and enhanced long-term environmental stability compared to the structurally simpler, more exposed, and disturbed conditions found in agricultural fields.

Our results demonstrate the critical role of habitat in shaping the EPF and invertebrate dynamics in the soil, reinforcing the significance of habitat characteristics in structuring ecological communities. These results imply that the pasture habitat may be subject to environmental or ecological constraints that impede the occurrence and growth of EPF. Such constraints could be intrinsic to the pasture ecosystem or arise from interactions unique to this environment. Conversely, the forest habitat did not significantly differ from the boundary, suggesting similar conditions for EPF prevalence between these two habitats. Understanding the interplay between adjacent habitats and entomopathogenic fungi can provide valuable insights for managing and conserving these beneficial fungi in agricultural and forest environments.

Effect of elevation on the abundance of entomopathogenic fungi

Elevation can influence the distribution and abundance of entomopathogenic fungi (EPF) due to its impact on environmental factors such as temperature, humidity, and vegetation. The distribution of EPF can vary with elevation, as shown in studies conducted in Mexico. For example, in Mexico, Dela Rosa et al. (2000) observed that the average percentage of mycosis varied with altitude. At 450 m above sea level (a.s.l.), *B. bassiana* exhibited 14.3%

mycosis and *M. anisopliae* 6.3%. At 880 m a.s.l., these values increased to 40.6% and 12.6%, respectively, while at 1,100 m a.s.l., *B. bassiana* showed 33.9% and *M. anisopliae* 22.1%. Similarly, studies in Pakistan have shown that the highest number of EPF isolates were found at altitudes greater than 600 meters (Wakil et al., 2013).

Conversely, in our study, a considerable presence of EPF was observed at lower elevations of 512 m and 680 m a.s.l., while at deeper forest with elevations of 880 m and 1024 m a.s.l., EPF were scarce or not detected. The abundance of EPF at lower elevations can be attributed to several interrelated ecological factors that enhance their growth and efficacy as biological control agents. Increased activity and diversity of insect populations at lower elevations provide a more abundant host base for EPF (Islam et al., 2021). The combination of favorable temperatures and abundant insect hosts creates a synergistic environment that enhances the prevalence of EPF. The ecological advantages of EPF at lower elevations have significant implications for agricultural systems. The higher abundance of EPF can lead to enhanced biological control of pest populations, reducing the reliance on chemical pesticides (Mantzoukas et al., 2022). With effective pest suppression facilitated by EPF, agricultural productivity can increase, contributing to food security (Imoulan et al., 2011).

Changes in environmental conditions and vegetation can have profound effects on the distribution and abundance of insects in the soil, consequently impacting the distribution and abundance of entomopathogenic fungi. Previous research highlights progress in understanding elevation-based distribution patterns of soil microbes, facilitated by advancements in sequencing platforms (Zheng et al., 2024). While various global distribution patterns have been observed, definitive conclusions remain challenging due to the locality-specific factors influencing these patterns (Li & Ma, 2018).

Invertebrates and entomopathogenic fungi in the same soil system

In the present study, forest soils hosted a large abundance and diversity of soil fauna and also exhibited higher infection and density of soil EPF, suggesting a positive correlation between the two. The rich diversity of potential hosts in forest soils likely contributes to the elevated densities of EPF observed in these habitats, as supported by previous research emphasizing the importance of biodiversity in maintaining ecosystem functions (Duffy, 2008). The low abundance and diversity of invertebrates in pastures corresponded to the lower presence of EPF in this habitat. The interaction between EPF and invertebrates, particularly certain insect species, can significantly influence the occurrence and diversity of these fungi

within the ecosystem. Walstad et al. (1970) observed that *B. bassiana* relies on host arthropods for its development and does not grow saprophytically in non-sterile soil. In the absence of hosts, the viability of EPF can be affected by non-specific attachment and germination on the cuticle of non-host arthropods, which refers to arthropods that are not susceptible to a specific EPF at a particular level of inoculum (Blond et al., 2018). Some insect species act as preferred hosts for EPF, providing a constant source of potential hosts for the fungi. Kessler et al. (2004) found that the absence of the host insect significantly reduced the persistence of EPF compared to sites with a host.

Cows regularly grazed the pasture sites in Taranaki and Manawatu in this study, and Liu et al. (2019) reported that animal trampling and grazing had a detrimental effect on soil microarthropod abundance. The decline in soil invertebrates in pasture soils could have led to a lower presence of entomopathogenic fungi, making pasture soils more vulnerable to insect pest infestations. This finding highlights the crucial role of habitat in shaping the association between the EPF and invertebrates' presence, underscoring the significance of habitat characteristics in structuring ecological communities.

Conclusions

The presence and abundance of entomopathogenic fungi (EPF) in soil samples varied across habitats, with forest soils exhibiting higher EPF abundance than boundary and pasture soils. This supports previous studies indicating the influence of habitat on EPF occurrence. The unique conditions present at the forest-pasture boundary create an ecotone that is more favorable for the abundance of entomopathogenic fungi than pasture. The presence and abundance of EPF can also vary with elevation, with lower elevations showing higher EPF occurrence than higher elevations. This study observed that the forest habitats with higher abundance and diversity of soil invertebrates also have higher soil EPF abundance, indicating a positive association.

APPENDIX I

Fig. A.1. Collection site in Taranaki showing adjacent habitats.

Fig. A.2. Soil sample collection in adjacent native mixed forest and pasture habitats in Bushy Park conducted in June 2020 and August 2021.

Fig. A.3. Soil collection in adjacent habitats Totara Reserve (Manawatu, NZ) encompassing pasture-forest transition was conducted in October 2020 and August 2021.

Fig. A.4. Collection site in Egmont National Park across elevation gradient conducted in October, 2019

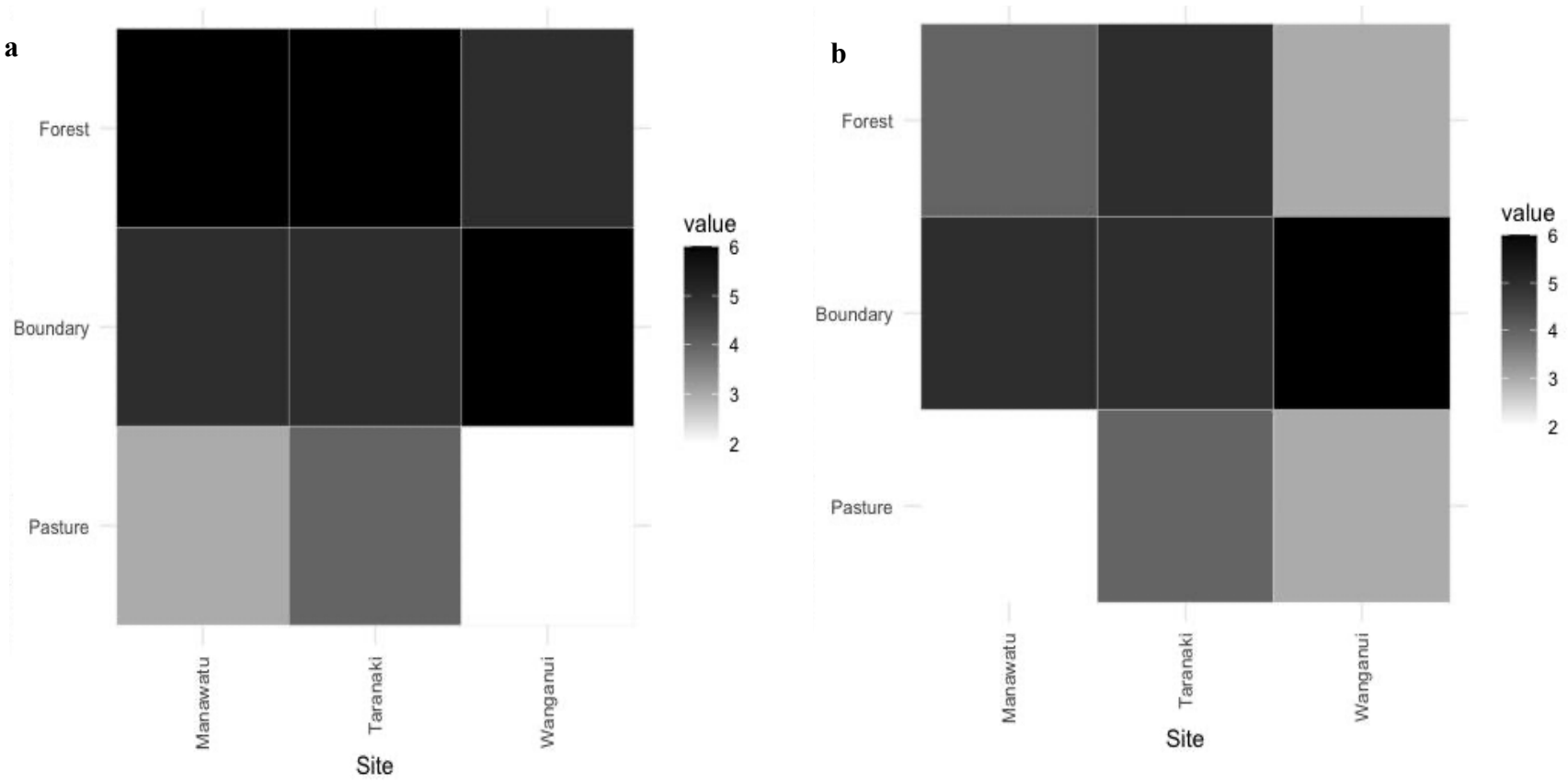


Fig. A.5. Heatmap of positive soil samples per site and habitat using a) insect baiting and b) plating methods.

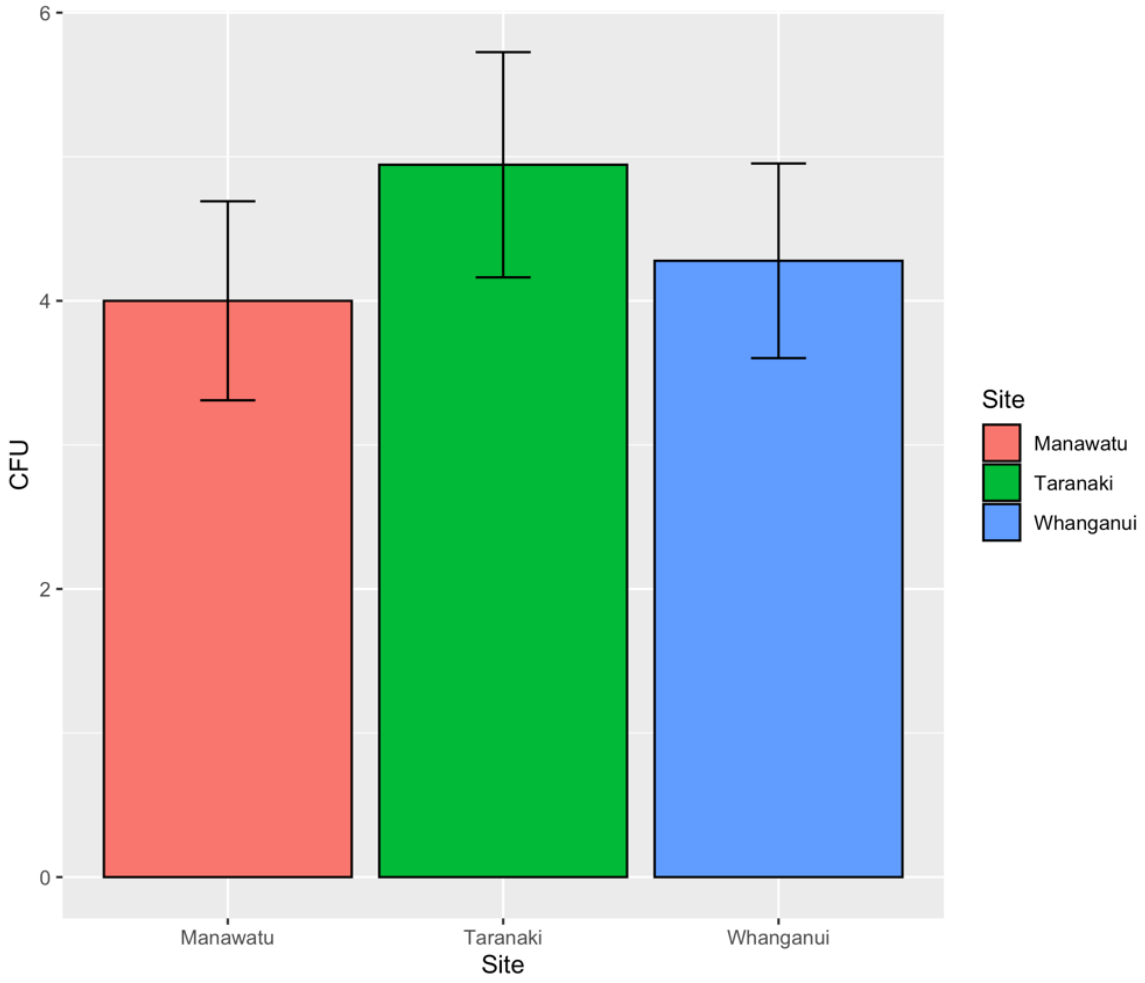


Fig. A.6. The density of EPF was observed using soil dilutions and direct Plating in soil samples from forests, pastures, and pasture-forest boundaries in different collection sites. The values are mean ±se. Different letters indicate statistically significant difference at 0.05, Tukey's HSD test.

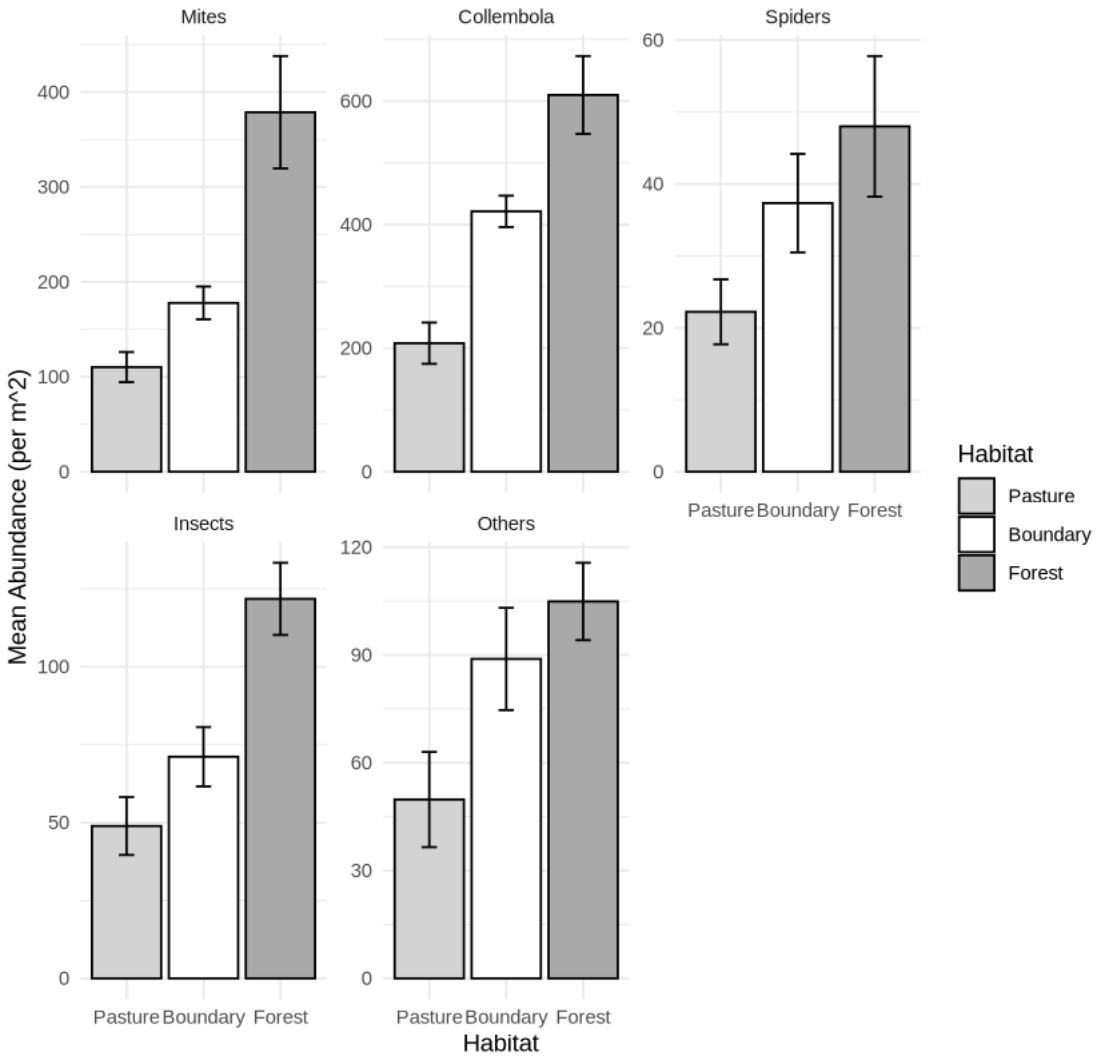


Fig. A.7. Mean abundance of invertebrate groups in different habitats (per m^2).

Chapter Three

Entomopathogenic fungi (*Beauveria* spp. and *Metarhizium* spp.) in adjacent pasture and forest soil: their occurrence, identification and speed of kill assessed using *Tenebrio molitor* larvae

Introduction

Entomopathogenic fungi (EPF) represent a diverse group of naturally occurring microorganisms that primarily target a wide range of insect species (Lovett & St. Leger, 2017; Masoudi et al., 2020). Among these fungi, those belonging to the order Ascomycetes and order Hypocreales, notably, the genera *Beauveria* (family Cordycipitaceae) and *Metarhizium* (family Clavicipitaceae) are extensively studied for their roles in biological pest control, showcasing their potential as effective agents against insect pests (Zimmermann, 2007; Lacey & Shapiro-Ilan, 2008; Vega et al., 2009; Lacey et al., 2015). The ecological importance and ubiquitous distribution of *Beauveria* and *Metarhizium* have made them integral to soil microbial diversity and functionality studies them as central subjects in research focused on soil microbial biodiversity and functional dynamics, extending beyond their well-known role in pest management. Their presence across various habitats, including agricultural and natural ecosystems, highlights their significance in integrated pest management (IPM) strategies (Vega et al., 2009; Skinner et al., 2014). Investigating the distribution, diversity and ecological interactions of these fungal genera in different soil environments is crucial for optimizing their use in sustainable pest control and advancing our understanding of soil ecosystem functionality.

Detailed knowledge of the morphology and microscopic features of entomopathogenic fungi is vital for their accurate identification, classification, and understanding of their ecological roles (Hibbet et al., 2011; Bich et al., 2021). However, *Beauveria* spp. and *Metarhizium* spp. are widely recognized as complexes of cryptic species, making it difficult to distinguish between individual species within these genera based on morphology alone (Bischoff et al., 2009; Fisher et al., 2011; Rehner et al., 2005), necessitating the use of molecular and genetic techniques for proper differentiation.

Molecular identification techniques are essential for the accurate identification of entomopathogenic fungi. These methods rely on analyzing specific DNA regions to determine the genetic identity of fungal isolates (Gebremariam et al., 2021). For instance, various studies have employed molecular approaches to identify species within the genus *Beauveria*. Rehner

et al. (2011) utilized a multi-gene sequencing approach, targeting traditional nuclear loci used in fungal phylogenetics, including genes encoding the largest (rpb1) and second largest (rpb2) subunits of RNA polymerase II, along with ITS (Internal Transcribed Spacer) and tef1-α (translation elongation factor 1-alpha). Additionally, a nuclear intergenic spacer region known as BLOC was sequenced (Rehner et al., 2006b). Genetic studies on *Beauveria* population across various regions including Brazil (Fernandes et al., 2009), China (Imoulan et al., 2016), Cuba (Ramos et al., 2017), Denmark (Meyling et al., 2009) and Greenland (Meyling et al., 2012) have employed combinations of genetic loci including nrLSU introns, rpb1, rpb2, tef1-α, β-tubulin, ITS, and BLOC, to identify and classify fungal species accurately.

Similarly, molecular techniques have been extensively used to study species within the genus *Metarhizium*. Various methods, such as RAPD (Random Amplified Polymorphic DNA) markers, AFLP (Amplified Fragment Length Polymorphism) (Inglis et al., 2008) and multiplexed microsatellite markers (Mayerhofer et al., 2015) have been employed to distinguish between *Metarhizium* species. Liang et al. (1991) found that based on ITS phylogeny, *Metarhizium* and *Cordyceps* are closely related. Before the reclassification by Sung et al. (2007a), where the teleomorphs of *Metarhizium* were transferred from *Cordyceps* to *Metacordyceps*, *Metarhizium* species were believed to be the anamorphs (asexual reproductive forms) of *Cordyceps*. These molecular insights have greatly advanced the understanding of fungal phylogeny and taxonomy, highlighting the complex evolutionary relationships within these genera.

Entomopathogenic fungi in different soil habitats exhibit varying "speed of kill", referring to the rate at which they infect and kill insect baits. Moreira et al. (2019) utilized the insect bait method combined with survival analysis to evaluate the speed of kill of soil EPF in agroforestry and full-sun coffee plantations. Their findings revealed that agroforestry coffee soils enhanced the insect-suppressive potential of entomopathogenic fungi compared to full-sun soils. Factors such as a diversified landscape, stable microclimatic conditions, and reduced soil disturbance in agroforestry systems were suggested to contribute to the increased suppressive capacity of these fungi. Moreira et al. (2019) also introduced the "bait survival technique" which uses the mortality rate of bait insects as a measure of EPF pest-suppressive potential.

This Chapter presents the identification of fungal isolates initially described in Chapter 2, alongside the insect bait "speed of kill" data. These data link the speed of kill to the prevalence of different EPF genera across various habitats. The study aims to explore the

occurrence and distribution of *Beauveria* and *Metarhizium* in distinct environments, offering valuable insights into their ecological roles and potential applications in biocontrol strategies.

Materials and Methods

Soil collection

As detailed in Chapter 2, soil samples were collected from 2019 to 2021 in three regions of New Zealand North Island: Taranaki, Manawatu, and Whanganui. Each site included three adjacent habitats (pasture, boundary, and forest). Three parallel 40 m transects spaced 20 m apart were established per site, each transect consisted of one sampling point for forest, boundary and pasture, also 20 m apart. Soil cores (5x5 cm, 10 cm depth, five at each sampling point) were taken using a soil corer and stored at 5°C. At each collection, soil temperature, moisture, GPS coordinates, and surrounding vegetation were recorded.

Isolation and genus assignment of entomopathogenic fungi from soil samples

To assess the presence of entomopathogenic fungi (EPF) in soil samples, I used two methods: the insect bait method and serial dilution plating (see details in Chapter 2). For the insect bait method, soil samples were cleaned of debris, placed in containers and baited with mealworm *Tenebrio molitor* larvae. The containers were incubated in the dark at 25°C ± 1 for 21 days, and infected larvae were monitored on days 7, 14, and 21. Larvae showing visible fungal growth were collected, and fungal spores were transferred to Petri dishes containing potato dextrose agar (PDA) with antibiotics to prevent bacterial contamination. For serial dilution plating, 20g soil samples were shaken in 0.01% Triton X-100 solution, serially diluted (10^{-2} to 10^{-4}), and cultured on semi-selective PDA media with antibiotics. The resulting infections and plated colonies were preliminarily identified at the genus level based on key observable characteristics, such as colony colour and typical morphological features associated with each genus.

Genomic DNA extraction and molecular identification of EPF isolates

Representative samples of purified entomopathogenic fungi (EPF) isolates collected from three regions, Taranaki (coded as TP, TB and TF samples), Whanganui (coded ad BP, BB and BF samples), and Manawatu (coded as ToP, ToB and ToF samples) were used for

genomic DNA extraction, employing two different methods based on the availability of supplies. Genomic DNA isolation was primarily performed using the Animal and Fungi DNA Preparation Kit (Jena Bioscience) following the manufacturer's protocol. Fungal isolates were first cultured in potato dextrose broth (PDB) to obtain sufficient mycelial biomass. The key steps in DNA extraction included cell lysis, protein precipitation, DNA precipitation, and DNA hydration. In addition, the Chelex 100 rapid DNA extraction method was also utilized when necessary. For this procedure, genomic DNA was extracted from pure fungal cultures grown on potato dextrose agar (PDA). The Chelex 100 method (Hennequin et al., 1999; Walsh et al., 2013; Alizadeh et al., 2017) involved dissolving 5% (2 g) of Chelex-100 resin in 40 mL of sterile water. Fungal colonies were scraped into centrifuge tubes, and 500 µL of the Chelex-100 solution was added. The samples were incubated on ice for 30 minutes, then the mycelium was ground with a sterile pestle. The mixture was vortexed, heated in a dry block incubator, and cooled to room temperature. After centrifugation at 13,000 rpm for 20 minutes, 200 µL of the supernatant was transferred to a new tube and stored at -20°C for future use.

For the amplification of DNA, the internal transcribed spacer (ITS) region of *Beauveria* and *Metarhizium* was targeted using primer pairs ITS5 and ITS4. PCR amplification was performed in a total reaction volume of 10 µL, consisting of 10 pmol of each primer, 0.1 µL Taq polymerase, 1 µL buffer, 1 µL dNTPs, 2 to 3 µL of genomic DNA, and nuclease-free water. Amplification was carried out using a Bio-Rad T100 PCR thermal cycler following the protocol described by White et al. (1990). Additional target genes included the translation elongation factor 1-alpha (tef1-α) for *Metarhizium* and the BLOC nuclear intergenic region for Beauveria. For tef1-α, primers EF349 (5'-TGGCCACCAGCACTCACTAC-3') and EF1685R (5'-ATGTCACGGACGGCGAAA-3') were used, amplifying an internal fragment of approximately 1350 bp (Reay et al., 2010). PCR conditions included 40 cycles of 45 seconds at 95°C, 45 seconds at 55°C, and 2 minutes at 72°C. The amplification of translation elongation factor 1-alpha (tef1-α) has been challenging even when using protocols and primers from published references The gel electrophoresis resulted in multiple bands repeatedly, even after troubleshooting PCR thermal profiles. Hence, this region was removed from further exploration.

The BLOC gene was amplified using primers B22U (5'-GTCGCAGCCAGAGCAACT-3') and B822L (5'-AGATTCGCAACGTCAACTT-3') to produce an approximately 400 bp fragment. PCR conditions included an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of 45 seconds at 95°C, 45 seconds at 57°C for annealing, and 2 minutes at 72°C, with a final extension at 72°C for 7 minutes (Glare et al.,

2008; Rehner & Buckley, 2005; Rojas et al., 2010). Positive controls (previously amplified samples) and negative controls (dH2O) were included in each PCR run. The amplified DNA fragments were visualized using a Bio-Rad GelDoc system. Electrophoresis was performed with molecular weight markers and negative controls to confirm the presence and size of PCR products. Samples with the expected molecular size were sent to the Massey Genome Service (MGS, Massey University) for Sanger sequencing. Proper labelling and parafilm sealing were ensured for secure transport.

Sequence data were analyzed using Geneious Prime® 2023.1.2 (Biomatters Ltd., Auckland, New Zealand) (Kearse et al., 2012). Forward and reverse reads were assembled to create consensus sequences, trimming low-quality regions. The identity of each isolate was confirmed using BLASTN (Altschul et al., 1990). The ITS and BLOC consensus sequences were aligned, and a phylogenetic tree was constructed using the Neighbor-Joining method with the Jukes-Cantor distance model. A bootstrap analysis with 1000 replications and a 50% support threshold was performed in the Geneious tree builder to ensure robust phylogenetic inference.

Speed of kill

To evaluate the speed of kill of soil-derived EPF from different habitats, the data collected during insect insect baiting (*T. molitor* larvae mortality in soil on day 7, day 14, and day 21, please see Chapter 2 for the details of baiting method) were analyzed. Statistical analysis to compare observation days and habitats was performed using R version 4.1.3 (R Development Core Team, 2022), with the packages *tidyverse*, *caret*, *multcomp* and *lsmeans*. A generalized linear model (GLM) with a binomial distribution was fitted to the data using the `glm` function. The model results were summarized using the `summary` function, which provided coefficients, standard errors, z-values, and p-values for the factors analyzed. Post-hoc comparisons among the habitats were performed using the `lsmeans` and `cld` functions to determine significant differences in the speed of kill between habitats. Additionally, the speed of kill for each fungal genus was plotted to highlight comparative differences between *Beauveria* and *Metarhizium*.

Scanning electron microscopy of Beauveria bassiana *and* Metarhizium novozealandicum

Molecular identification confirmed that all isolates collected from both plating and baiting belonged to two species: *Beauveria bassiana* and *Metarhizium novozealandicum.* Due to resource and time constraints, scanning electron microscopy (SEM) to obtain detailed morphological images was performed only on isolates of these species collected from the forest habitat in Taranaki. For SEM preparation, spores were harvested from culture plates using a sterile wire loop and placed into 5 mL glass vials containing a fixative solution of 3% glutaraldehyde and 2% formaldehyde in 0.1M sodium phosphate buffer. The spores were then sandwiched between circular filter papers and dehydrated through a graded series of ethanol concentrations (50%, 70%, 80%, 90%, and 95%). After dehydration, critical point drying was performed, followed by gold coating of the spores using sputter coating. High-resolution images of the fungal morphology were captured using SEM at an accelerating voltage of 20 kV, allowing for detailed visualization of the spore structures.

Results

Incidence of Beauveria *and* Metarhizium *in adjacent forest-pasture soils*

Out of 108 total soil samples, 79 showed the presence of entomopathogenic fungi (EPF). Specifically, *Beauveria* was detected in 34 samples (31.48%), with 16 samples identified through baiting and 18 through plating. *Metarhizium* was identified in six samples (5.56%), with four detected through baiting and two through plating. Both *Beauveria* and *Metarhizium* were found together in 39 soil samples (36.11%), with 23 detected through baiting and 16 through plating (**Table 3.1**).

Table 3.1. Count of soil samples with *Beauveria* and *Metarhizium* genera across three forest-pasture transitions, North Island of New Zealand (2019-2021). EPF detected by insect baiting and semi-selective PDA plating methods.

Habitat	Method	*Beauveria* sp. only	*Metarhizium* sp. only	*Beauveria* and *Metarhizium*
Pasture	Baiting	7	0	2
	Plating	9	0	0
Boundary	Baiting	2	2	12
	Plating	5	0	9
Forest	Baiting	7	2	9
	Plating	4	2	7
Total		34	6	39

Figure 3.1 shows the relative abundance of *Beauveri*a and *Metarhizium* across three habitats: pasture, boundary, and forest. The data combines results from both baiting and plating methods from a total of 108 soil samples (54 from baiting and 54 from plating). In the pasture habitat, predominantly detected EPF were *Beauveria* spp., with minimal presence of *Metarhizium* spp. and no co-occurrence of both genera. In the boundary habitat, *Beauveria* spp. were still dominant, but there was a noticeable presence of *Metarhizium* spp. and some co-occurrence of both genera. In the forest habitat, the relative abundance of both *Beauveria* and *Metarhizium* spp. increased, with a significant proportion of samples showing co-occurrence of both genera. The forest habitat displayed the highest diversity of EPF genera among the three habitats. *Beauveria* was present in all sites and all habitats. *Metarhizium* was present in all areas except for the pasture habitat in Whanganui.

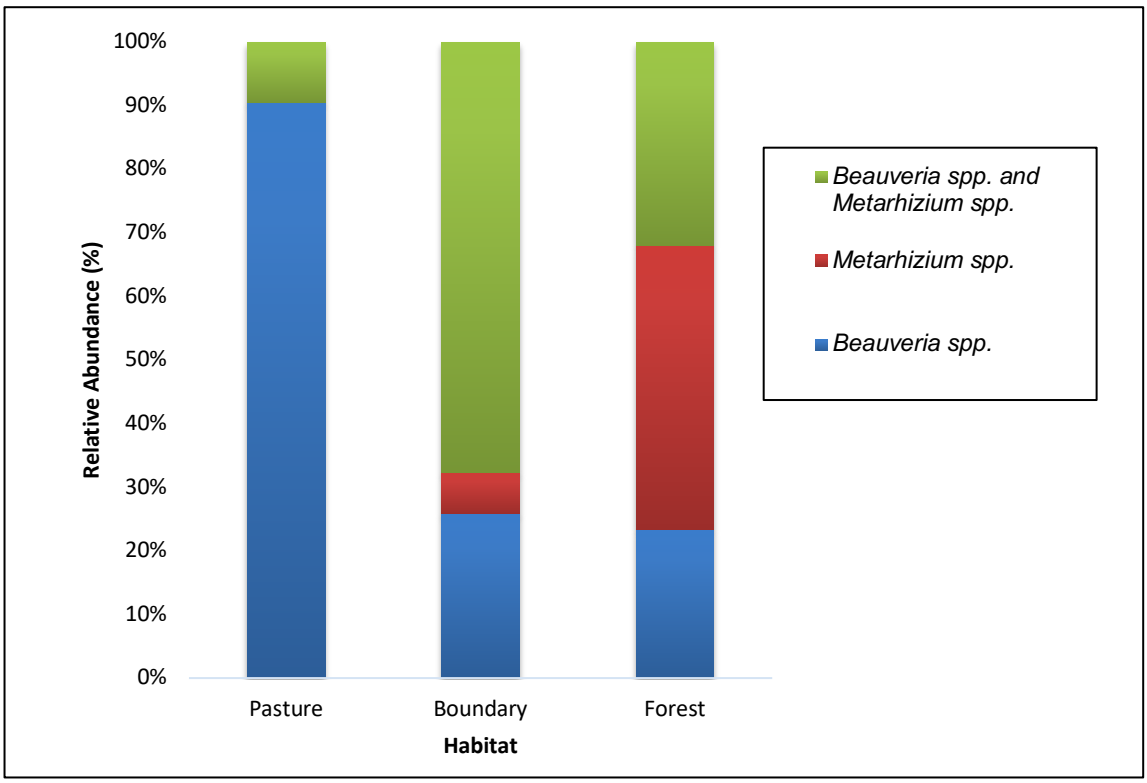


Figure 3.1. Occurrence of *Beauveria* and *Metarhizium* in soil samples collected across forest-pasture transitions, North Island of New Zealand, 2019-2021. Data from insect baiting (N=54) and semi-selective PDA plating (N=54).

In insect baiting, among the 120 *T. molitor* larval deaths, 73 (60.8%) were attributed to *Beauveria* infection, while 47 (39.2%) were associated with *Metarhizium*. *Beauveria* infections were characterized by a white coloration on the insects, while *Metarhizium* infections were distinguished by a vivid green coloration of conidia on the external cuticle (**Figure 3.2**). Notably, each insect bait exhibits infection by a single fungus type, with no cases of mixed infections per mycosed cadaver.

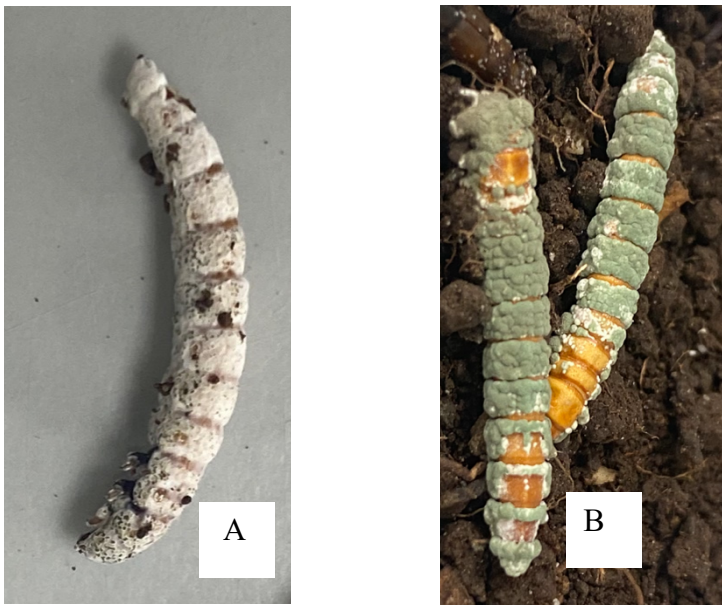


Figure 3.2. Insect cadavers (*Tenebrio molitor* larvae) infected by the entomopathogenic fungi from genera *Beauveria* (A) and *Metarhizium* (B).

Speed of kill

The soils from various habitats demonstrated differing abilities to infect mealworm larvae with naturally occurring EPF spores. Significant differences in the proportions of dead larvae were observed across the different observation days ($Z = 2.982$, $P = 0.003$), indicating significant variation in the "Speed of Kill" (infection speed) across the soil samples. Additionally, deviance analysis confirmed that both habitat and day significantly contributed to the model's goodness of fit ($P < 0.001$ for both Habitat and Day), highlighting characteristic infection speeds of soils from different habitats.

Forest soils reached a peak *T. molitor* larvae infection (40%) by day 7, whereas boundary soils peaked on day 14 and then plateaued. In contrast, pasture soils exhibited slower infection rates, with mealworm larvae infection peaking only on day 21. Day 21 had a significantly higher cumulative mean mortality of *T. molitor* larvae (0.45) than the other two days when the data were analysed using cumulative deaths. The analysis showed no significant difference in infection rates between the forest and boundary habitats ($P > 0.05$); however, both these habitats exhibited significantly faster infection rates compared to the pasture habitat ($P < 0.05$), as illustrated in **Figure 3.3**.

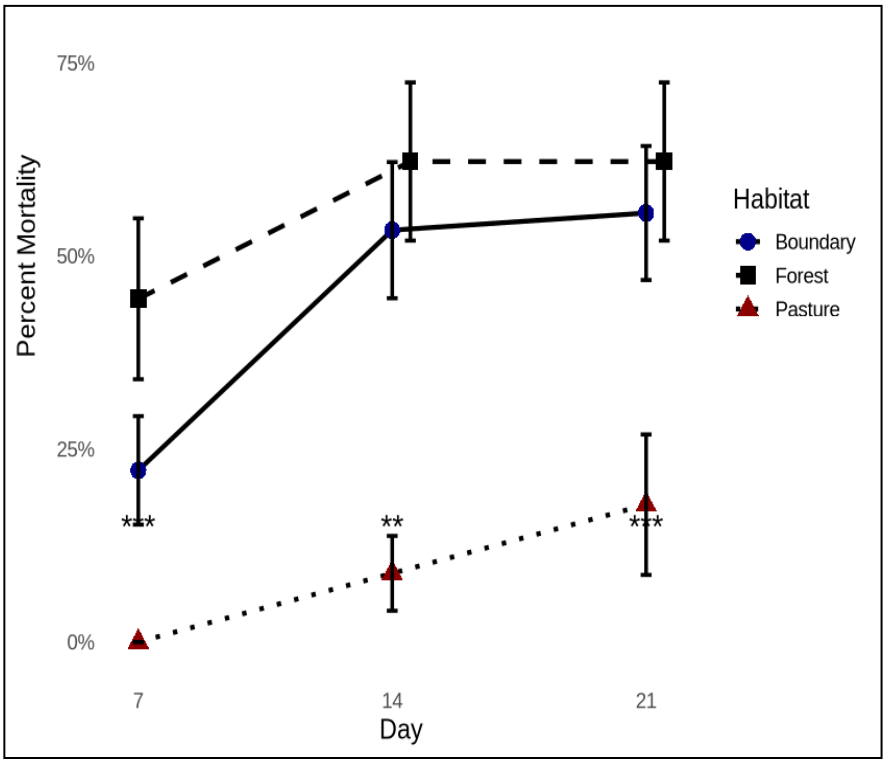


Figure 3.3. "Speed of Kill" analysis: cumulative mortality (%) of *Tenebrio molitor* larvae in soils collected across forest-pasture transitions, North Island of New Zealand, 2019-2021. Asterisks (*) indicate statistically significant differences ($p < 0.05$) between habitats on each respective day.

The percent mortality of insect baits (*T. molitor* larvae) was recorded over time. From the deceased larvae, the EPF genera *Beauveria* and *Metarhizium* were isolated, and their recovery was expressed as a percentage, calculated as the percent recovery of each EPF genus. The recovery rates of *Beauveria* and *Metarhizium* from insect baits varied significantly across pasture, boundary, and forest habitats (**Fig. 3.4**). In pasture habitats, *Beauveria* exhibited a steady increase in recovery, peaking on day 21, albeit at lower rates compared to other habitats. *Metarhizium* showed a sharper increase by day 14, then plateaued, suggesting rapid adaptation but limited growth potential. The boundary habitat supported a consistent upward trend for both fungi, with *Beauveria* and *Metarhizium* showing marked recovery by day 21, indicative of favorable transitional environmental conditions. In contrast, the Forest habitat, with soil rich in organic matter and with more stable microclimate, was most conducive to *Beauveria*, which displayed robust growth in infection throughout the period. *Metarhizium* also increased gradually in the forest, although the infection rates did not reach the levels of *Beauveria,* highlighting different habitat preferences between the fungi. These observations underscore the

influence of specific habitat characteristics on EPF success, which is crucial for optimizing their use in biological control strategies.

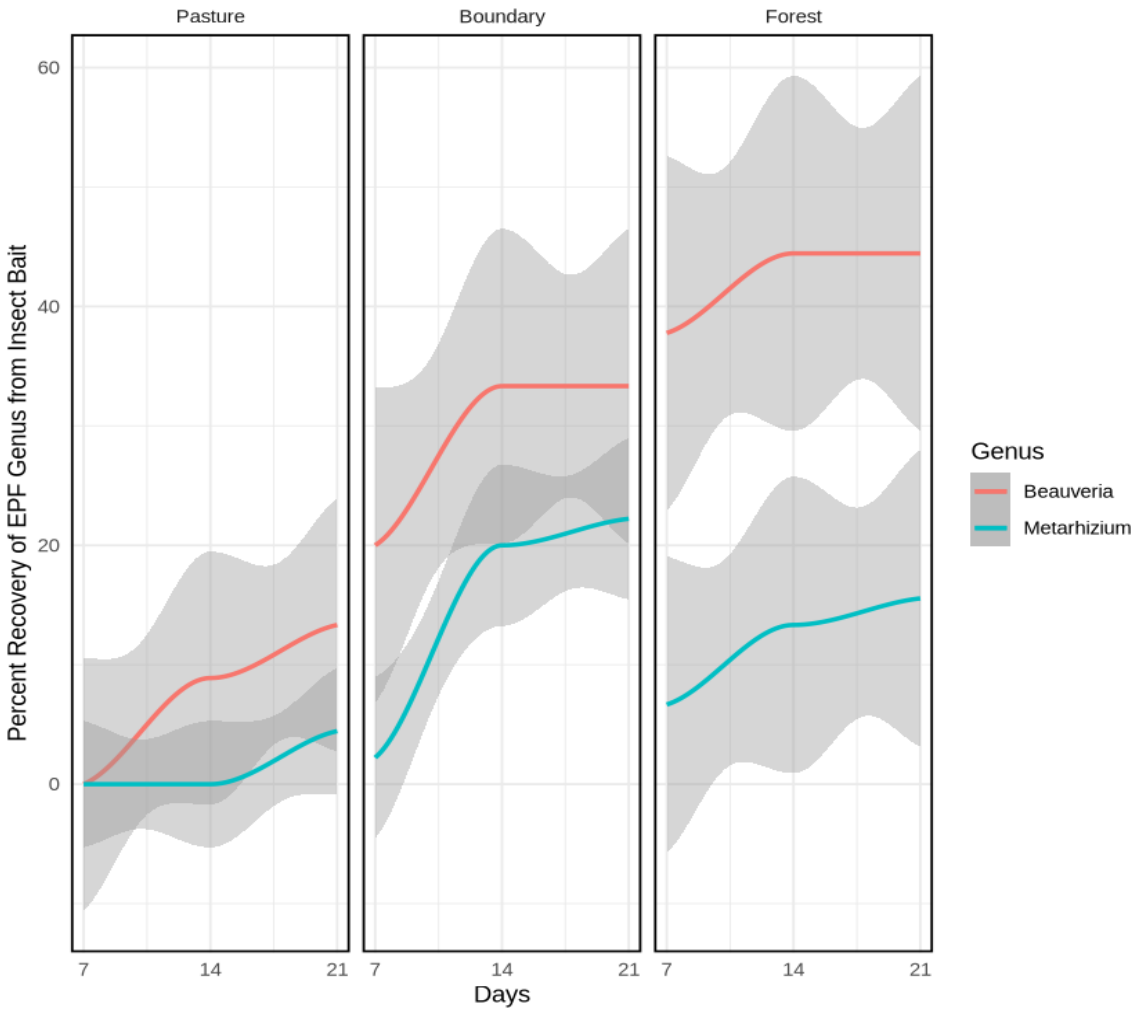


Figure 3.4 Temporal dynamics of entomopathogenic fungi *Beauveria* and *Metarhizium* recovery from infected insect baits (*T. molitor* larvae) in soils collected across pasture, boundary and forest habitats, North Island of New Zealand, 2019-2021.The smoothed lines, accompanied by confidence intervals (shaded region), trace the variability and trends in fungal recovery over three observation periods—days 7, 14, and 21.

Molecular identification of species present in isolates

The gene region encompassing ITS1-5.8S-ITS2 was amplified to identify the species of *Beauveria* and *Metarhizium* present in the samples from Taranaki, Whanganui and Manawatu sites (**Fig. 3.5**). BLASTn of the samples resulted to 100 percent identity of the

Metarhizium isolate to *Metarhizium flavoviride* var *novozelandicum* isolate F530 (GenBank Acc. No. DQ385622.1) while *Beauveria* isolates resulted in 99.6-100% identity with *Beauveria bassiana* (MH165266.1).

Although *Metarhizium* was identified as *Metarhizium flavoviride* var *novozelandicum*, the problematic taxonomy of this group suggests using a coding gene, such as translation elongation factor 1-alpha (tef1-α) as a molecular marker to obtain more confidence and consensus on the identity of obtained isolates. Unfortunately, the amplification of tef1-α has proven to be challenging, hence the identification of *Metarhizium* isolates in this study was based only on ITS. On the other hand, the use of ITS region and BLOC gene provided a reliable identification for the *Beauveria* species obtained in this study. Using BLOC locus for B*eauveria* isolates resulted in 99% to 100% identity with *Beauveria bassiana* isolates J2, Mo1 and J18 from New Zealand (GenBank Acc. Nos. MZ703285, MZ703284 and MZ703283).

A 560 bp alignment of the ITS region using ten *Beauveria* (TP3, TF3, BP3, BB2, TB2, TB1, ToP1, TB3, BF1 and TP1) and six *Metarhizium* (BB2-G, TF3-G, ToB2-G, BB1-G, TF1-G and ToF1-G) sequences obtained in this study with eight reference sequence from GenBank were used to construct a phylogenetic tree using the Neighbor-Joining method and Jukes-Cantor as a phylogenetically mathematical distance model, with 1000 Bootstrap was created **(Fig. 3.6).** Similarly, the phylogenetic tree using BLOC locus with the representative isolates from sites and habitats is presented in **Figure 3.7**.

Figure 3.5. Amplified fragments of the ITS1-5.8S-ITS2 region, approximately 600 bp, from EPF isolates collected in Taranaki. (lanes 2- 6, 8-9, and 11-15) A 1kb+ ladder (M) was used as a molecular size standard.

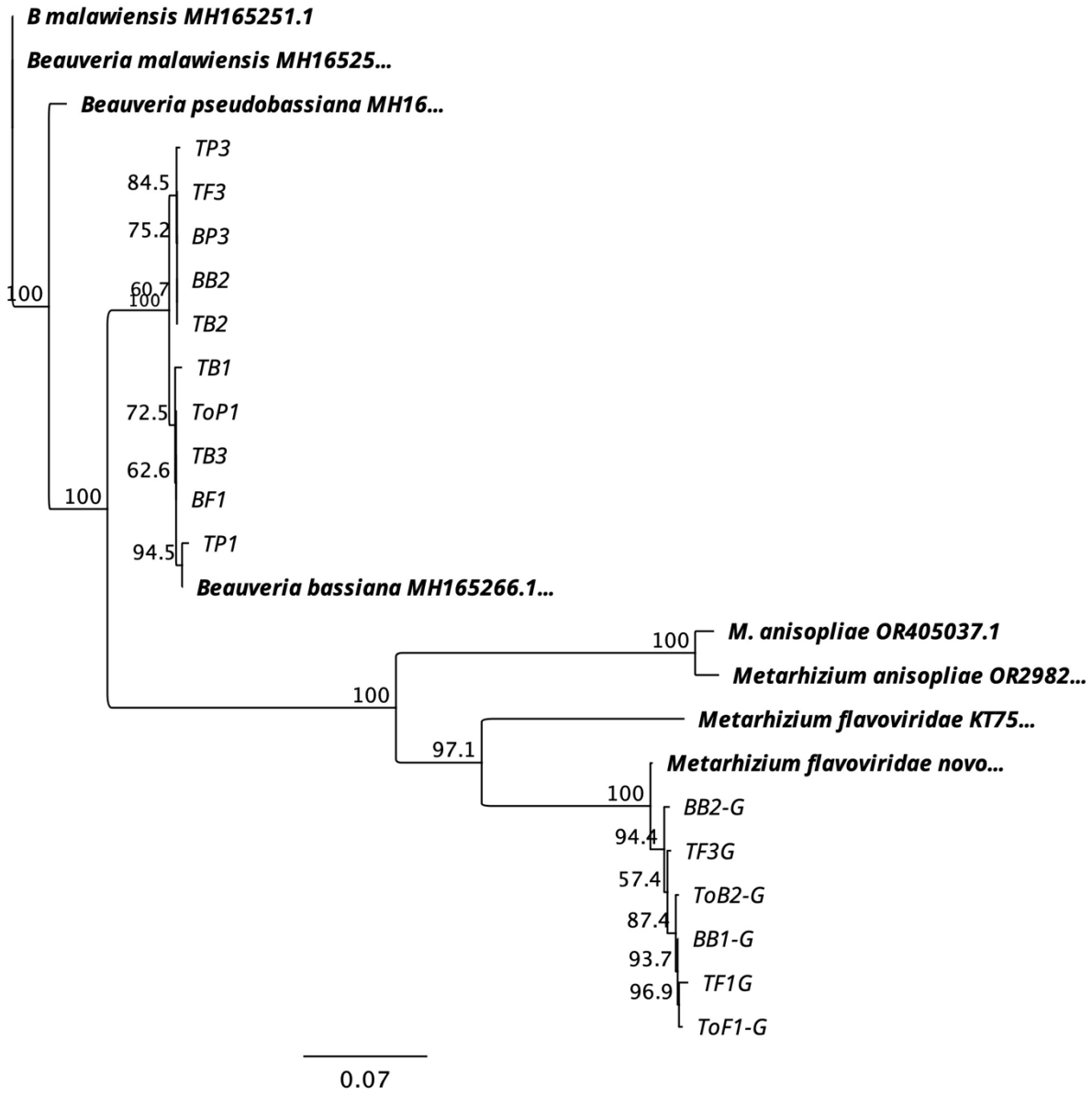


Figure. 3.6. The phylogenetic tree derived from the partial ITS1-5.8S-ITS2 region from EPF isolates collected in Taranaki, Whanganui and Manawatu, New Zealand was inferred based on the Neighbor-Joining method using Juke-Cantor as model. A bootstrap of 1000 replicates was implemented in Geneious tree builder. The isolates in bold are reference isolates from GenBank. *Beauveria bassiana* sample codes: TP1, TP3 – Taranaki Pasture; TB1, TB2 – Taranaki Boundary; TF3 – Taranaki Forest; BP3 – Whanganui Pasture; BB2 – Whanganui Boundary; BF1 – Whanganui Forest; ToP1 – Manawatu Pasture. *Metarhizium flavoviridae* var *novozealandicum* sample codes: TF1G, TF3G – Taranaki Forest; BB1-G, BB2-G – Whanganui Boundary; ToF1-G – Manawatu Forest; ToB2-G – Manawatu Boundary.

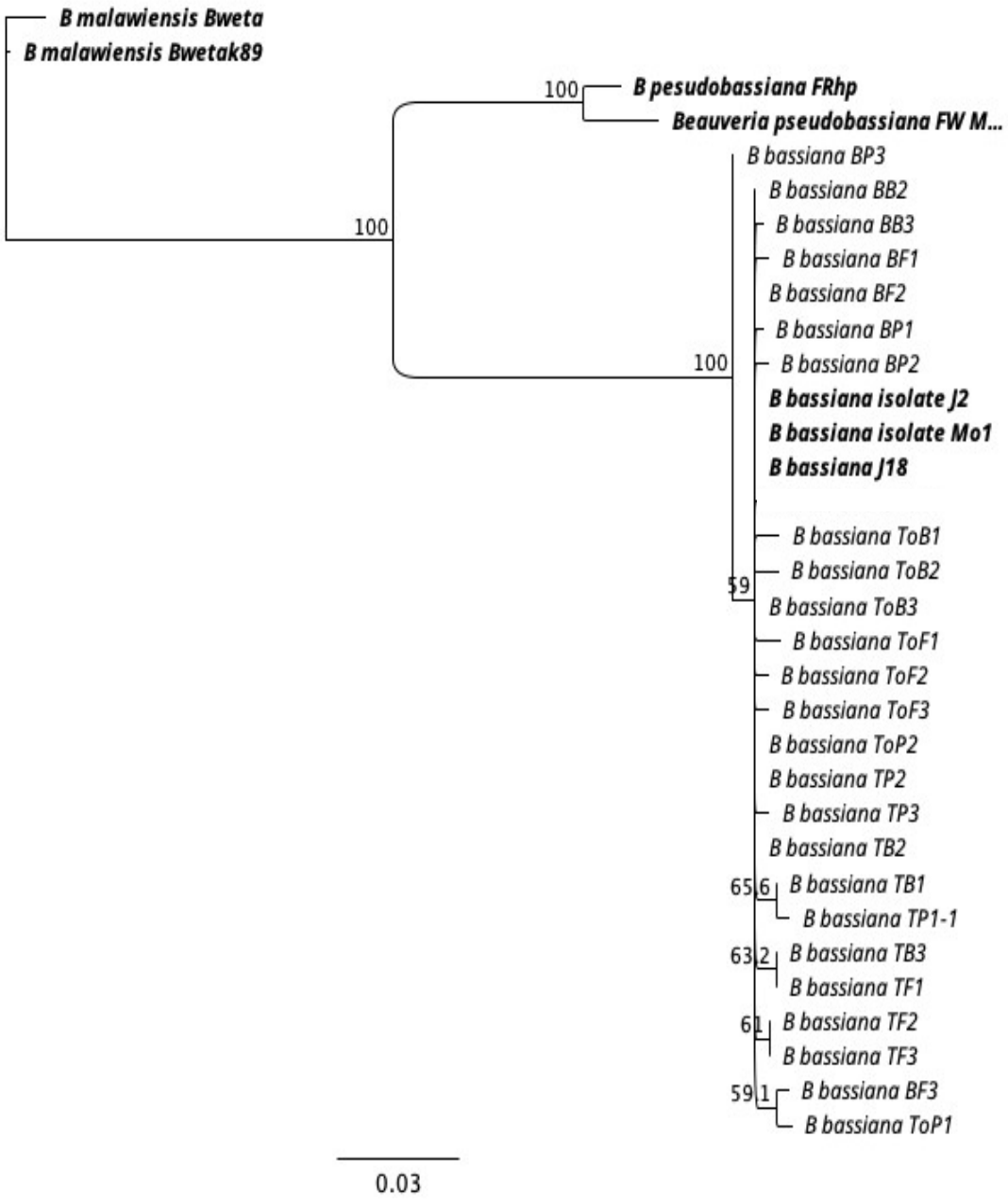


Figure 3.7. The phylogenetic tree derived from the partial BLOC sequences from purified *Beauveria* isolates collected in Taranaki, Whanganui and Manawatu, New Zealand. Tree was inferred based on the Neighbor-Joining method using Juke-Cantor as a phylogenetically mathematical distance model, no outgroup, with 1000 replicates bootstrap and the support threshold of 50% in Geneious tree builder. The isolates in bold are reference isolates from GenBank. Sample codes: TP1-1, TP2, TP3 are from Taranaki Pasture; TB1 and TB2 – Taranaki Boundary; TF1, TF2 and TF3 – Taranaki Forest; BP1, BP2 and BPR – Whanganui Pasture; BB2 and BB3 – Whanganui Boundary; BF1 and BF2 – Whanganui Forest; ToP1 and ToP2 – Manawatu Pasture; ToB1, ToB2 – Manawatu Boundary; ToF1, ToF2 and ToF3 – Manawatu Forest.

***Ultrastructure observations of* Beauveria bassiana *and* Metarhizium flavoviride *var* novozelandicum**

Following molecular identification that verified that only two EPF species were present, Scanning Electron Microscopy (SEM) was conducted on representative *Beauveria bassiana* and *Metarhizium flavoviride* var *novozelandicum* isolates from Taranaki Forest samples to obtain detailed images. *Beauveria bassiana* conidia have a distinctive, uniform, round to oval shapes and smooth texture **(Fig 3.8)**.

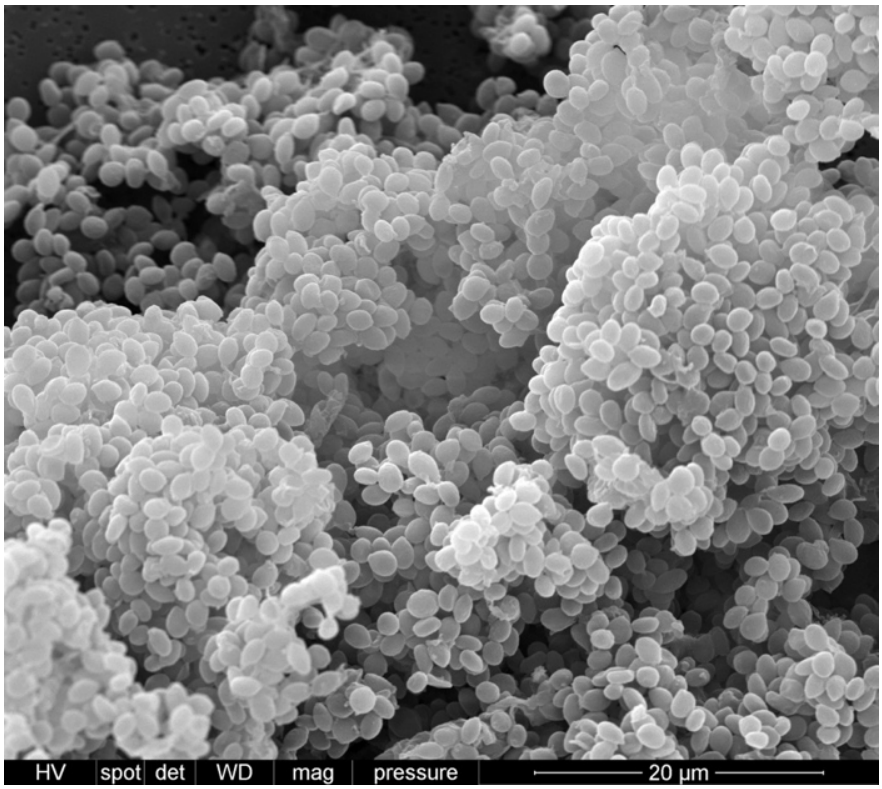


Figure 3.8. *Beauveria bassiana* conidia obtained through insect baiting from soil in a forest habitat, Taranaki, NZ. Magnification: 2,500x. SEM was conducted in 2022.

Figure 3.9 shows germinating *B. bassiana* conidia showing the initial stage where the conidium transitions into active growth by developing a germ tube. *Beauveria bassiana* with a prominent hyphal filament extending from a conidium is shown in **Figure 3.10** indicating the progression of fungal growth and the beginning of colonization, as the fungus establishes its filamentous network.

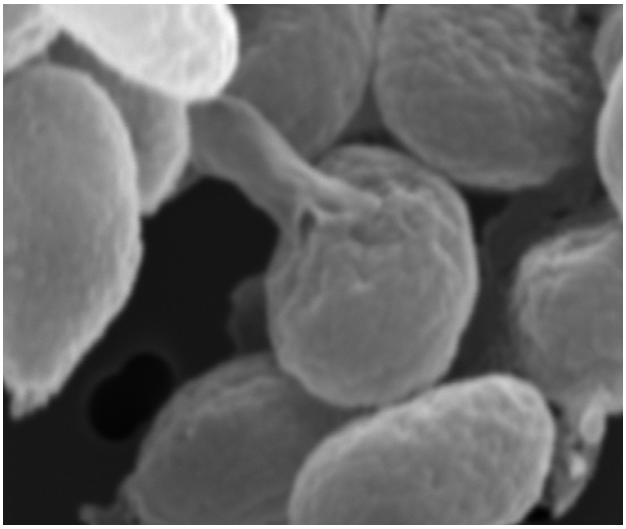


Figure 3.9. Germinating *Beauveria bassiana* conidia from the forest soil in Taranaki, NZ at 33,000X magnification. SEM was conducted in 2022.

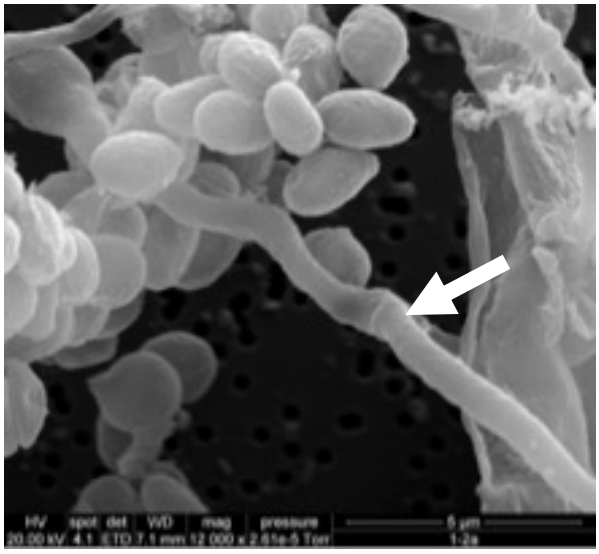
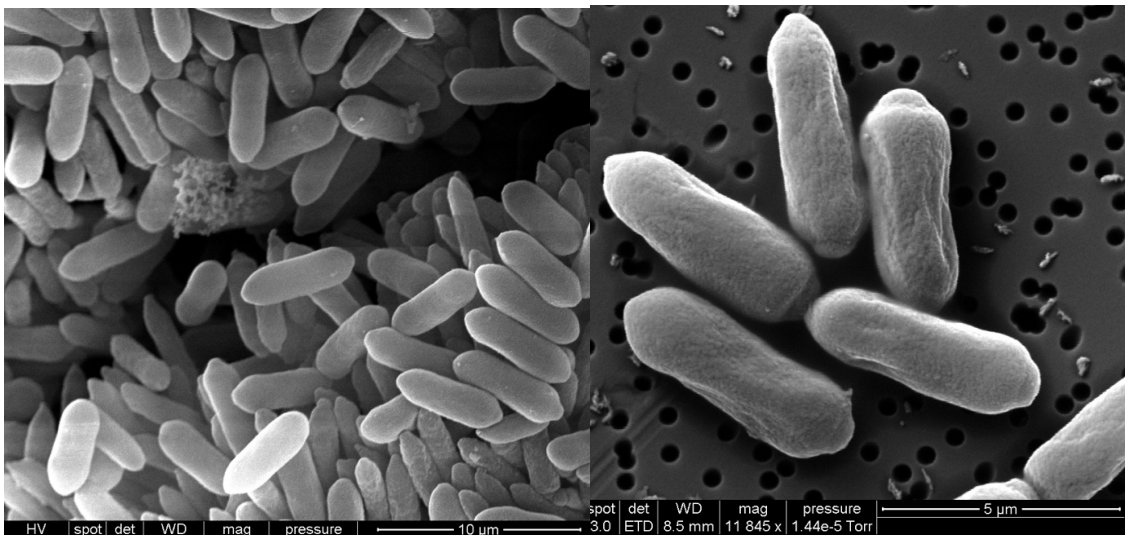


Figure 3.10. *Beauveria* bassiana conidia from forest soil in Taranaki, showing a prominent hyphal filament (indicated by an arrow) viewed at 12000X. SEM was conducted in 2022.

The conidia of *Metarhizium flavoviride* var. *novozelandicum* isolated from forest soil in Taranaki exhibit an elongated, cylindrical morphology, which is characteristic of the genus *Metarhizium* **(Fig. 3.11)**.



A B

Figure 3.11. *Metarhizium novozealandicum* conidia obtained by insect baiting from soil collected in forest habitat, Taranaki, NZ A) 10,000X magnification. B) Closer look at 11,000X magnification.

Discussion

Occurrence of Beauveria *and* Metarhizium *in adjacent habitats*

The study of entomopathogenic fungi (EPF), particularly *Beauveria* and *Metarhizium*, in adjacent forest-pasture soils of New Zealand's North Island reveals significant insights into their ecological distribution and potential applications in biological control. The observed prevalence of *Beauveria* (31.5%) compared to *Metarhizium* (5.6%) in positive soil samples aligns indicates greater ecological adaptability of *Beauveria* across diverse environments (Sánchez-Peña et al., 2011; Sharma et. al., 2018). The prevalence of *Beauveria* aligns with the findings of Majchrowska-Safaryan and Tkaczuk (2021) as well as those of Medo and Ludovít (2011).

The habitat-specific distribution of EPF genera observed in this study underscores the importance of environmental factors in shaping fungal communities. The higher prevalence of both genera in forest and boundary soils (vs. pasture) is consistent with previous research that

highlights the role of soil organic matter and microclimate in supporting fungal diversity (Quesada-Moraga et al., 2007). Studies have shown that soils with high organic matter tend to support greater fungal diversity (Hallouti et al., 2020). Forest soils, characterized by higher organic matter content and favorable microclimatic conditions, exhibit the highest diversity and abundance of both *Beauveria* and *Metarhizium* (Safitri et al., 2018). The co-occurrence of both fungal species (in 36.1% of samples) , especially in forest and boundary habitats, suggests that these fungi may engage in complementary ecological interactions that enhance their survival and infectivity. Fungal species occurrence across different geographical locations varies, emphasizing the need for localized studies to understand the ecological dynamics of EPF and optimize their use in pest management strategies (Vega et al. 2009; Parker et al., 2015; Vaicekonyte & Keesing, 2012).

The methodology employed in this study, utilizing both insect baiting and semi-selective medium plating techniques, proved essential for a comprehensive survey of fungal presence and diversity. Baiting, in particular, was found to be slightly more effective. Vega et al. (2012) stated that the primary benefit of the insect baiting method is its ability to selectively isolate entomopathogens from other soil microorganisms. Insect baiting is a more effective technique for isolating entomopathogenic fungi (EPF) compared to culturing soil suspensions on selective media (Imoulan et al. 2011; Keyser et al. 2015). This dual-method approach is supported by findings from other studies which indicate that different sampling methods can yield distinct species from the same environmental samples, highlighting the complexity of fungal ecology (Bidochka et al., 1998; Sharma et al., 2018). The implications of these findings are significant for biological control programs, as targeting the application of *Beauveria* and *Metarhizium* in environments where they are naturally abundant could enhance the sustainability and effectiveness of pest management strategies (Keyser et al., 2015).

Investigating speed of kill and temporal recovery patterns of **Beauveria** *and* **Metarhizium** *in soil samples from adjacent habitats*

The analysis of the speed of kill in soil samples from different habitats aimed to investigate the speed at which the soils kill insects using the "bait survival technique" introduced by Moriera et al. (2019). This technique indicates that differential insect survival reflects the suppressive potential of the soil in a particular habitat, providing a practical approach to assess pest-suppressive capabilities through bait insect mortality, although the correlation between the speed of kill and natural biological control of soil-dwelling insect pests

in the field was not directly tested by Moriera et al. (2019). Previous studies have predominantly focused on EPF abundance or species compositions rather than the activity of the soil (Klingen et al., 2002; Meyling & Eilenberg, 2006; Garrido-Jurado et al., 2015).

In this study, the observation day factor (i.e., length of incubation) has been a significant factor in the mortality of *Tenebrio* larvae exposed to the soil from different habitats. Forest soils showed the fastest speed of kill, with significant infection (0.40) on day 7, when boundary soils had 0.2 infection rate and pasture soils had the minimal infection. By day 14, boundary soils started contributing to peak mortality, and pasture soils began to catch up. On day 21, the mortality rate in forest soils gradually decreased but an infection could still be recovered from pasture soils, indicating a slow speed of kill. The variation in the speed of kill across habitats is consistent with Moriera et al. (2019) highlighting the importance of habitat characteristics in determining the success of EPF colonization and effectiveness, which is critical for their application in biological control programs. These findings can help inform strategic placement of EPF treatments in landscape management for pest control. In Chapter 4, we further tested these isolates from different habitats against various insects to explore this concept.

The trends observed in this study, suggest that both fungal genera have adapted to specific habitat conditions, with *Beauveria* generally performing better in more stable, organic-rich environments such as forests, while *Metarhizium* showing considerable adaptability but excelling in transitional zones, in our case boundaries. The recovery of *Beauveria* in pasture habitats was characterized by a slow increase, peaking at day 21, and overall recovery rates remained lower compared to other habitats. This suggests that pastures may not provide optimal conditions for *Beauveria* growth (Hallouti et al., 2020). Previous studies have noted that entomopathogenic fungi thrive better in soils with higher organic matter (Hallouti et al., 2020). Forest habitat, characterized by stable microclimates, proves to be the most conducive environment for *Beauveria*, where it exhibited robust and sustained recovery throughout the study period, highlighting the resource-rich environment that promotes its establishment. Conversely, *Metarhizium* in pastures showed a sharper increase in recovery by day 14 before plateauing, indicating a rapid adaptation to the pasture environment, although its growth potential appears to be limited by the habitat's characteristics (Bidochka et al., 1998). Although *Metarhizium* also showed gradual increases in recovery within the forests, it did not reach the levels observed for *Beauveria*, indicating differing habitat preferences and ecological niches between these two fungi (Bidochka et al., 1998).

The boundary habitat, acting as a transitional zone between forest and pasture, demonstrated a consistent upward trend in recovery for both fungi. This habitat's moderate

organic content and stable microclimate create favourable conditions for the proliferation of both *Beauveria* and *Metarhizium,* as evidenced by their markedly higher recovery by day 21. The boundary habitat serves as a critical zone for diverse EPF, facilitating their adaptability and sustained growth, which is essential for effective pest management strategies (Lee et al., 2018a).

Species identification of entomopathogenic fungi Beauveria *and* Metarhizium

The identification of entomopathogenic fungi (EPF) isolates through molecular characterization is essential for advancing our understanding of their diversity, classification, and evolutionary relationships. Recent advancements in molecular identification techniques have significantly expanded the recognized diversity within the genera *Beauveria* and *Metarhizium*, enabling more precise species identification and a better understanding of their ecological roles (Sánchez-Peña et al., 2011; Litwin et al., 2020).

In this study, *Beauveria bassiana* and *Metarhizium flavoviride var. novozelandicum* showed habitat preferences, which is consistent with findings that *B. bassiana* strains are more closely adapted to specific habitat types than to particular hosts (Bidochka et al., 2002; Hallouti et al., 2020). Conversely, Sharma et al. (2018) found that the type of bait insect used plays a more significant role than habitat type in isolating *B. bassiana* from soils (Fisher et al., 2011). This discrepancy highlights the complex ecology of EPF and the need for further research to clarify the factors influencing their distribution and abundance.

The sequencing of genomes for *Beauveria* and *Metarhizium* has opened new avenues for molecular research, providing valuable resources for exploring genetic diversity and pathogenicity (Lee et al., 2018b; Rizal et al., 2024). These genomic insights are crucial for optimizing the use of these fungi as biopesticides, as they can inform breeding programs aimed at enhancing virulence and environmental resilience (Safitri et al., 2018). The presence of EPF in diverse habitats, as observed in this study, suggests that microclimatic variations and habitat characteristics may significantly influence their infection rates and overall effectiveness as biological control agents (Rusch et al., 2010; Ennis et al., 2019) Factors such as soil pH, temperature, and vegetation density have been documented to affect the distribution of EPF in soil (Vaicekonyte & Keesing, 2012). While these factors were considered in this study, they were excluded from the generalized linear mixed model (GLM) due to concerns about model fitness and reliability. Future studies should aim to incorporate these environmental variables into more robust models to better understand their influence on EPF dynamics.

Reliable species identification of *Beauveria* and *Metarhizium* requires amplifying both coding and non-coding genes. In this study, *Metarhizium* was identified using ITS, while *Beauveria* was identified with ITS and the BLOC gene. Although *Metarhizium* was resolved as *Metarhizium flavoviride var. novozelandicum*, the complex taxonomy of this group underscores the need to include coding genes, such as elongation factor 1-alpha (ef1α), to enhance accuracy and establish consensus in species identification.

While elongation factor 1-alpha was utilized in this study, the PCR yielded multiple bands, complicating interpretation. This challenge has been documented in a similar work by Reason et al. (2020), where multiple bands were also observed. To resolve this, Reason et al. excised and purified the desired amplicon from the gel. However, this additional step was not conducted in the present study. Incorporating such refinement techniques in future work could improve reliability and consistency in molecular identification.

Conclusions

The detection of *Beauveria bassiana* and *Metarhizium flavoviride var. novozelandicum* across diverse habitats underscores their broad ecological distribution and adaptability. This suggests that these fungi are versatile and capable of thriving in various environmental conditions, enhancing their potential utility in biological control applications. Additionally, the investigation into the "speed of kill" of soil samples provides valuable insights into the interactions between habitats and the pest-suppressive capabilities of EPF. Notably, forest soil demonstrated significantly higher infectivity towards insect baits, indicating that forest environments, with their rich organic matter and stable microclimates, may enhance the efficacy of EPF. These findings emphasize the need to consider habitat-specific factors when deploying EPF as biocontrol agents, to maximize their effectiveness in pest management strategies.

Chapter Four

Comparative microbiomics of the soil in adjacent native forest and exotic pasture habitats of NZ North Island

Introduction

Soil is one of the most diverse biomes that host large reservoir of microbial diversity (Dimitrov et al., 2017). Soil serves as an excellent environmental shelter, protecting microbiota from UV radiation and other adverse abiotic and biotic influences (Sánchez-Peña et al., 2011). Recently, soil microbial communities have been characterized by molecular approaches since most soil bacteria and fungi cannot be cultured using traditional laboratory techniques (Feinstein et al., 2009).

The diversity of bacteria in the soil is influenced by various factors, including biotic factors and the microbiome's functional profile (Xu et. al. 2023). The fungal component of microbial communities is referred to as mycobiota while their genomes are called mycobiome (Orellana, 2013). Fungi heavily influence soil dynamics by the decomposition of organic substrates by soil saprophytic fungi (Setälä & McLean, 2004), symbiotic mycorrhizal interactions (Sun et al., 2016; Wen et al., 2023) and invasion of the rhizosphere by fungal plant pathogens. The entomopathogenic fungi (EPF) is one component in soil microbial communities. An improving understanding and documentation of genomic diversity includes EPF, which indicates comparative data are becoming available. Among insect-pathogenic fungi, the Hypocreales (Ascomycota) is by far the best-studied group with genomic data allowing insight into the molecular details underlying hypocrealean infection processes and interactions with the host immune system (Kepler et al., 2017). Studies have found entomopathogenic fungi in the genera *Beauveria*, *Metarhizium*, and *Isaria* commonly found in soil (Peña et al., 2011; Sharma et al., 2018). These fungi have been isolated from various agricultural and natural ecosystems, highlighting their wide distribution (Safitri et al., 2018; Budiarti & Nuryanti, 2022). Genome of several EPF such as *Beauveria bassiana*, *Cordyceps militaris*, *Ophiocordyceps sinensis* and several species of *Metarhizium* have been sequenced and currently available in the GenBank (Devi et al., 2006; Gao et al., 2011; Zheng et al., 2012; Wichadakul et al., 2015; Xia et al., 2017).

Understanding the diversity of soil microbial communities and soil fungi in different ecosystems are essential for their management and conservation (Sevim et al., 2009; Tedersoo

et al., 2022). Native populations of entomopathogenic fungi in soil can be managed to facilitate the control of pest insect populations within agroecosystems. Isolating and characterizing indigenous entomopathogenic fungi also provides insights into the naturally occurring fungal biodiversity and offers a pool of potential biological control agents for pest control (Sevim et al., 2009).

Efficient extraction of high-quality DNA from soil is imperative due to the complex nature of soil matrices and various inhibitors. Several DNA extraction methods have been developed and used for this purpose, including the CTAB-based method, bead-beating, and commercial kits (Feinstein et al., 2009). Two types of approaches are commonly used to probe the genomic diversity of environmental samples: metagenomics uses non-targeted sequencing of fragmented genomic DNA in the sample, while metabarcoding (amplicon sequencing) uses Polymerase Chain Reaction (PCR) to target a particular locus (Lear et al., 2018). These methods provide the means to discover otherwise invisible biological diversity within environmental samples such as fresh water (Bradford et al. 2013; Mao et al., 2014), animal guts (Leray et al., 2013; Hibert et al. 2013) and soils (Makiola et al., 2019; Lear et al., 2018).

Metabarcoding from amplicon sequencing and metagenomics from shotgun sequencing have revolutionized soil microbiome research by enabling high-throughput and culture-independent approaches for studying microbial communities (Lear et al., 2018). Metabarcoding utilizes short DNA markers, is a useful technique for identifying and quantifying multiple taxa simultaneously (Escobar-Zepeda et al. 2015). In amplicon sequencing, the environmental DNA is extracted, and the gene of interest is PCR-amplified using taxonomically informative primers such as 16S rRNA gene for bacteria and intergenic transcribed spacers (ITS)/18S for fungi (Lear et al., 2018; Tedersoo et al., 2022). The ITS region is highly variable and widely used for fungal identification (Tedersoo et al., 2022). Amplicon sequencing captures the diversity of a single gene of interest, but limitation includes the universality of the PCR primers chosen for the analysis (Lear et al., 2018; Tedersoo et al., 2022). These limitations can lead to PCR biases. One potential solution to address these biases is using PCR-free methods (Miller et al., 2011).

Metagenomics (shotgun sequencing) can provide a more comprehensive and taxonomically more complete view of the microbial community by sequencing a wide range of genomic elements and potentially all the DNA present in a sample (Handelsman et al., 1998; Miller et al., 2011). It is a powerful approach that provides a comprehensive view of the genetic diversity within a microbial community, including entomopathogenic fungi, but is strongly influenced by the scale of data generated per sample (read depth). Metagenomics opens new

possibilities to identify taxonomic composition and metabolic potentials of a microbiome allowing biological diversity studies without cultivating the individual organisms (Quince et al., 2017).

In shotgun sequencing, total DNA of a sample is sheared into smaller fragments before being sequenced at random. One of the advantages of shotgun sequencing is that it can capture all taxa, permitting the study of both prokaryotic and eukaryotic diversity concurrently (Lear et al., 2018). In addition, shotgun metagenomics allow species level classification since wide range of phylogenetic markers can be used to perform taxonomic annotation unlike the ribosomal markers used in the amplicon sequencing approach (Tedersoo et al., 2022). The workflow of shotgun metagenomics includes several steps, such as sample collection, library preparation, sequencing platforms, and bioinformatics analysis (Lear et al., 2018; Tedersoo et al., 2022).

The diversity of entomopathogenic fungi (EPF) was initially explored in this study using insect baiting and plating techniques, as detailed in Chapter 2. The species identity of these EPFs were further established in Chapter 3, providing a foundational understanding of their presence in adjacent habitats. However, these traditional approaches are insufficient and limited in their ability to capture the full spectrum of EPF in the soil. To overcome this limitation, this chapter employs metagenomics and metabarcoding to reveal the hidden diversity of EPF and other microorganisms in the soils of forest and pasture habitats. Building on earlier findings, this chapter expands detection and analysis through advanced molecular methods, offering a more comprehensive understanding of the diversity and composition of soil microbiome in concert with EPF.

Materials and Methods

Site description

Samples were collected from three regions in the central North Island of New Zealand: Taranaki, Whanganui, and Manawatu (**Fig. 4.1**). The sampling locations were in Taranaki (Mount Egmont National Park in Taranaki), Whanganui (Bushy Park Tarapūhi near Kai Iwi) and Manawatu (Totara Reserve in Pohangina Valley, Manawatu) (**Table 4.1**).

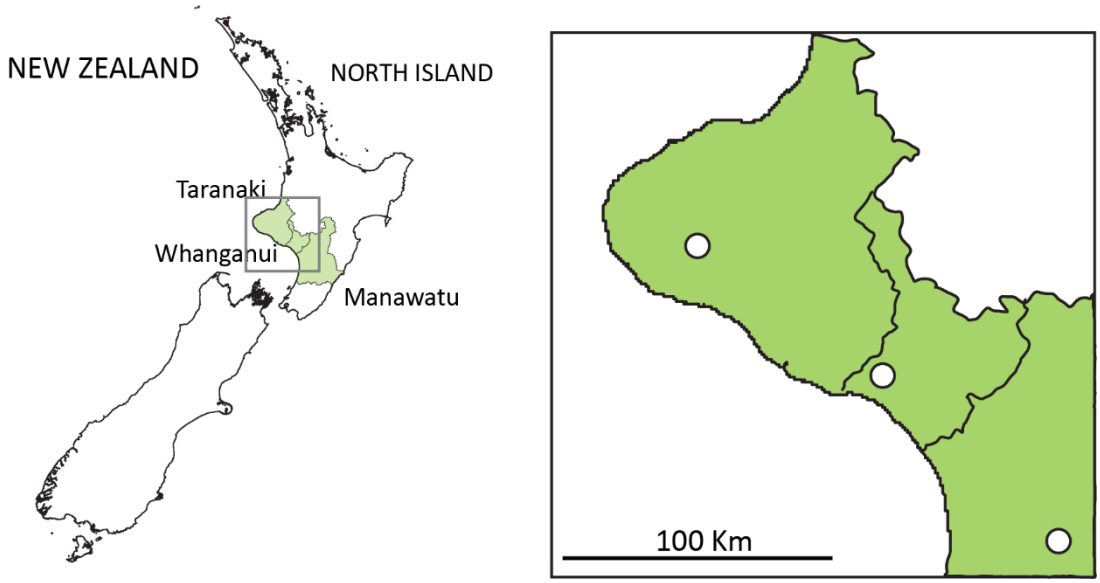


Figure 4.1. Soil collection locations in central North Island, New Zealand, showing Taranaki (Mt. Egmont National Park), Whanganui (Bushy Park Tarapuhini) and Manawatu (Totara Reserve, Pohangina) locations.

The Taranaki forest site situated in Mount Egmont National Park, Taranaki, New Zealand, features a podocarp-broadleaf ecosystem with species such as pukatea (*Laurelia novae-zelandiae*), kahikatea (*Dacrycarpus dacrydioides*), and kamahi (*Weinmannia racemosa*), within a climate ranging from warm to cold (Singers & Rogers, 2014). Adjacent to the forest site is a pasture that is fertilized annually and grazed by young cows. The site in Whanganui, Bushy Park Tarapuhini, has been a protected area since 2005 and comprises a diverse podocarp-broadleaf forest dominated by tawa (*Beilschmiedia tawa*), pukatea (*Laurelia novae-celandine*), rimu (*Dacrydium cupressinum*), and northern rata (*Metrosideros robusta*) (https://bushypark.nz/forest/). The site in Manawatu, Totara Reserve in Pohangina Valley, consists of mature totara (*Podocarpus totara*), juvenile nikau, and clusters of mamaku (*Cyathea medullaris*), along with bracken and shrubs such as koromiko (*Hebe salicifolia*) and karamu (*Coprosma robusta*) (Knight, 2008)

Soil collection in Mount Egmont National Park, Taranaki, was conducted under a permit issued by the New Zealand Department of Conservation (authorization number 69401-RES). Environmental data, including soil temperature, moisture, and elevation, were recorded at each collection site (**Table 4.1**). Soil temperature and moisture were measured using a QM7216 Digital Stem Thermometer and a TDR 300 Soil Moisture Probe (Spectrum Technologies Inc., USA), respectively. Elevation was recorded using a handheld GPS device. The DNA sequencing approach used for each sample is also detailed in Table 4.1.

Table 4.1. Environmental and sequencing details of ten soil samples from exotic pasture and native forest sites across North Island, New Zealand, collected for metagenomic analysis. Shotgun (Shot.) and ITS2 amplicon (Amp.) sequencing methods were used to generate data.

Code	Location	Habitat	Latitude S	Longitude E	Soil temp. (°C)	Soil moisture (%)	Year	Elevation (masl)	Method
TP1	Taranaki	Pasture	39°19'12"	174°10'18"	5	24.8	2019	562	Shot., Amp.
TP3	Taranaki	Pasture	39°19'12"	174°10'18"	5.7	23.4	2019	562	Shot., Amp.
TF1	Taranaki	Forest	39°19'12"	174°10'16"	7.7	30.4	2019	563	Shot., Amp.
TF3	Taranaki	Forest	39°19'12"	174°10'16"	7.1	29.8	2019	563	Shot., Amp.
TFS5	Taranaki	Forest	39°18'20.46"	174°7'8.83"	5	33.2	2019	880	Shot., Amp.
TFS7	Taranaki	Forest	39°18'21.00"	174°6'27.12"	3	32.5	2019	1024	Shot., Amp.
BF1	Whanganui	Forest	39°47'54"S	174°55'48"	9.1	26.5	2020	259	Amp.
BF3	Whanganui	Forest	39°47'54"S	174°55'48"	9.5	28.8	2020	259	Amp.
ToF1	Manawatu	Forest	40°09'06"S	175°50'37"	11.1	19.5	2020	138	Amp.
ToF3	Manawatu	Forest	40°09'06"S	175°50'37"	11.1	19.5	2020	138	Amp.

At Taranaki, four samples were taken 40 meters apart, two samples under native forest (TF1, TF3) and two in nearby pasture (TP1, TP3). These forest (39°19'12"S; 174°10'16"E) and pasture (39°19'12"S; 174°10'18"E) samples were collected approximately 20 m from the boundary fence near Pembroke Road access to the Mount Egmont National Park. Two further forest soil samples were collected approximately 5 km further into the Taranaki Forest and at slightly higher elevation (TFS5, TFS7). At Whanganui (BF1, BF3) and Manawatu (ToF1, ToF2) paired samples were collected approximately 20 m apart. This design allowed for comparison of the soil microbiome under native and exotic vegetation within the same soil horizon, and between locations with similar native vegetation. A rectangular soil corer (5 × 5 cm) with a depth of 10 cm was used to collect soil samples at all sites. Each soil sample was labelled and placed in clean plastic bags and stored at 5°C until use.

Soil DNA extraction and quantification

Genomic DNA was extracted from ten individual soil samples (six from Taranaki, two from Manawatu, and two from Whanganui) using the DNeasy PowerMax Soil Kit (Qiagen, Germany). Each extraction utilized soil from a single core, with no pooling of samples, ensuring that the DNA represented specific, localized soil conditions. Prior to extraction, soil samples were carefully cleaned by removing any debris, such as stones, twigs or other organic material, to enhance DNA purity. For each sample, 10 grams of soil were processed according

to the manufacturer's protocol, following established methods (Frey et al., 2016; Resch et al., 2021) to optimize DNA yield and quality. The extracted genomic DNA was then assessed for quality by agarose gel electrophoresis using a Bio-Rad electrophoresis system (Bio-Rad, USA) and visualized on a Bio-Rad molecular imager, verifying DNA integrity. DNA concentration and purity were quantified using a Qubit Fluorometer (Thermo Fisher Scientific, USA) to confirm suitability for downstream sequencing. The quantified DNA samples were then prepared and sent to third-party sequencing service provider for shotgun and/or amplicon sequencing.

Taranaki samples: shotgun sequencing and data analysis

Due to financial limitations, the shotgun sequencing approach was applied only to six soil DNA samples collected from Mount Egmont, Taranaki (**Table 4.1**). The DNA from pasture samples TP3 and TP1 were sent to Macrogen (Macrogen Inc, South Korea) while samples TF3, TF1, TES7 and TES5 were sent to BGI-Hong Kong (BGI Hong Kong Co., Ltd.) for 150 PE shotgun NGS using HiSeq 2500 system of Illumina (Illumina, Inc., USA). The soil DNA were processed through massive parallel, high-throughput sequencing using Illumina® TruSeq Nano DNA Kit. Post-sequencing, reads were de-multiplexed using standard indexes. The resulting sequences was trimmed of adapters and passed through standard quality filters using the software Fastp (Chen et al., 2018). Taxonomic profiling of the sequences was used the Metagenome Analyzer, MEGAN (Community-edition) (Huson et al., 2007). MEGAN implements sequence-based search (using BLAST) against the reference database and the lowest common ancestor (LCA) that best matches against each database is assigned to the sequence. For these raw data, sequences were aligned using DIAMOND, resulting in a .daa (DIAMOND alignment archive) file. This .daa file was directly transformed into an RMA6 format, which houses both reads and matches associated from query sequence and reference database. The RMA6 files were then imported into MEGAN for a refined visualization and interpretation based on BLASTX-derived alignment. The relative abundances were visualized in MEGAN.

To obtain resolution to EPF genera and species, KAIJU (http://kaiju.binf.ku.dk/), a program for sensitive taxonomic classification of high throughput sequencing reads from metagenomic shotgun sequencing was used with default parameters. Taxonomic assignment to species level followed the classification of entomopathogenic fungal genera of Sharma (2018).

Species level was identified as present or absent in the habitat and relative abundance was not taken into consideration due to low read numbers compared to the whole dataset.

Analysis of alpha and beta diversity was conducted using the Shannon index and Bray-Curtis dissimilarity, respectively. The non-parametric Wilcoxon rank sum exact test was applied to assess the significance of individual Shannon index results. For beta diversity at the phylum level, Bray-Curtis dissimilarity was computed using the vegdist() function in the *vegan* package (Oksanen et al., 2020) in R (R Development Core Team, 2023). Following the dissimilarity computation, a hierarchical clustering approach was employed to create a dendrogram, visually representing the similarities and disparities among samples. The Analysis of Similarities (ANOSIM) was then applied to determine the statistical significance of the observed groupings.

ITS2 metabarcoding and data analysis

DNA from all ten soil samples from native forests and from pastures (**Table 4.1**) were subject to amplicon sequencing. The polymerase chain reaction primers ITS3 (GCATCGATGAAGAACGCAGC; White et al., 1990) and ITS4 (TCCTCCGCTTATTGATATGC; White et al., 1990) were used to amplify the ITS2 region of the 45S rRNA cassette and the products sequenced on the Illumina Hiseq2500 platform. Raw data consisting of 300bp paired-end reads were filtered to remove adapters and generate high quality clean reads using in-house scripts (BGI-Hongkong). Consensus sequences were generated for paired end reads that overlap with each other using FLASH (Fast Length Adjustment of Short reads, v1.2.11). Filtered and demultiplexed sequences were quality checked using FastQC (Andrews, 2010).

Amplicon sequence analysis was conducted using the New Zealand eScience Intrastructure (NeSI- https://www.nesi.org.nz/) using Jupyter and terminal-based processing. Fasta files from each sample were combined into a single file, with sequences renamed according to sample ID to maintain unique identifiers across samples. The dataset was dereplicated to group identical sequences and unique sequences was identified based on abundance using VSEARCH. Subsequently, sequences were denoised to refine the core dataset, followed by the creation of a frequency table that recorded the occurrence of each sequence across samples. This table provided essential data for analyzing operational taxonomic units (OTUs) across different sites.

For taxonomic assignment, sequences were imported into QIIME2 (Bolyen et al, 2019) and a consensus BLAST approach was employed to match OTUs to known fungal taxa. The UNITE database (UNITE version 8.3, Abarenkov et al., 2023; https://doi.org/10.15156/BIO/1264708) a curated fungal ITS region database, was used for reference to enhance fungal specificity and reliability of taxonomy assignments. Output data were further processed and visualized in R using the *phyloseq* (McMurdie et al., 2013) and supplementary packages for data manipulation and graphical representation.

The alpha diversity (Shannon and Simpson index) of the fungal communities was calculated using the vegan package (Oksanen et al., 2020) in the R environment (R Core Team, 2023). To assess the significance, the non-parametric Wilcoxon rank sum exact test was used. For the beta-diversity analysis, the fungal composition was analyzed using the UniFrac distance in *phyloseq*. A Principal Coordinates Analysis (PCoA) ordination based on the Bray-Curtis dissimilarity matrix was subsequently performed to visualize the compositional differences in the microbial communities. To understand the influence of habitat, permutation multivariate analysis of variance (PERMANOVA) using adonis function in *vegan* was employed with the number of permutations fixed at 999. The significance level of 0.05 was used for all tests.

Results

Taranaki metagenome: Taxonomic domain composition in forest and pasture soils

The taxonomic domain composition of six metagenomes derived from forest and pasture soils in Taranaki, New Zealand, revealed a predominance of bacterial sequences across all samples. Out of the 90,914,628 (~91 million) paired-end reads obtained from the six metagenomes, 45.4% (41,308,825 paired-end reads) were classified into taxonomic domains. The vast majority of classified sequences were identified as bacteria, accounting for 98–99% of the total reads across all samples (**Table 4.2**).

Archaeal sequences comprised a minor proportion (<1%), with the highest abundance observed in TF3 (1%) from forest soil. Similarly, fungal and other eukaryotic sequences were rare, contributing <1% to the total reads for each sample. Notably, TF1 and TES5, both from forest habitats, exhibited slightly higher fungal representation at 1%, potentially indicating localized fungal activity or distinct microbial dynamics in these sites. The remaining reads (unassigned) constituted a negligible portion of the data, ranging from 0.09% to 0.2% across samples.

Table 4.2. Relative abundance (%) of taxonomic domains in soil samples from exotic pasture and native forest habitats in Taranaki, New Zealand, based on shotgun sequencing data.

Sample (Habitat)	Bacteria	Archaea	Fungi and other eukaryotes	Unassigned
TP1 (Pasture)	98	0.7	0.6	0.2
TP3 (Pasture)	98	0.6	0.5	0.19
TF1 (Forest)	98	0.7	1	0.10
TF3 (Forest)	98	1	0.8	0.10
TES5 (Forest)	98	0.1	1	0.10
TES7 (Forest)	98	0.1	0.9	0.10

Phyla such as Acidobacteria, Proteobacteria, Verrucomicrobia, Chloroflexi, Bacteroidetes, Planctomycetes, Actinobacteria and Gemmatimonadetes were consistently found across all six samples. Their ubiquity underscores their adaptability and potentially essential functions in the soil microbial community. The Acidobacteria were the most abundant group, contributing a substantial 54.1% of the core microbiome. Proteobacteria represented 27.6% of the core microbiome, while Verrucomicrobia and Chloroflexi accounted for 8.9% and 5.1%, respectively. Other phyla, such as Bacteroidetes, Planctomycetes, Actinobacteria, and Gemmatimonadetes comprosed less than 1%.

The phylum Actinobacteria and Protecobacteria exhibited a higher relative abundance in both pasture and forest metagenome of Taranaki samples. Actinobacteria were noticeably higher in pasture soils compared to forest soils (**Fig. 4.2**). Chloroflexi were notably more abundant in pasture samples and lower elevation forests compared to high elevation forest soils, where they decreased dramatically to 1.7% in TES7 and 2.2% in TF1 (**Fig. 4.2**). Planctomycetes showed consistent relative abundance across both habitats. Certain phyla, such as Deinococcus-Thermus, were exclusively observed in pasture samples (1.1% in TP1 and 1.0% in TP3). In contrast, Mucoromycota (fungi) were restricted to lower-elevation forest samples (TF1 and TF3), while Basidiomycota were detected only in high-elevation forest soils (TES5 and TES7). Unique microbial groups, such as Candidatus Melainabacteria, were identified exclusively in TES5 and TES7. Similarly, Candidatus Saccharibacteria was detected only in TES7, the sample collected at the highest elevation (1,081 m asl). DNA sequences attributed to Arthropoda were limited to forest soils at lower elevations.

At the phylum level there was no statistically significant difference in the alpha diversity (Shannon index) between the forest and pasture metagenomes (Wilcoxon rank sum test, $P = 0.1333$). For beta diversity at the phylum level, a dendrogram (**Fig. 4.3**) visually represents the (dis)similarities among the samples, reflecting their spatial location. For example, pasture samples TP1 and TP3 clustered together with the adjacent sample, TF1. The samples from forests at higher elevations, TES5 and TES7, are also clustered. The Analysis of Similarities (ANOSIM) suggesting a moderate degree of separation between the groups ($R = 0.6429$), however, observed differences were not statistically significant ($P = 0.1333$).

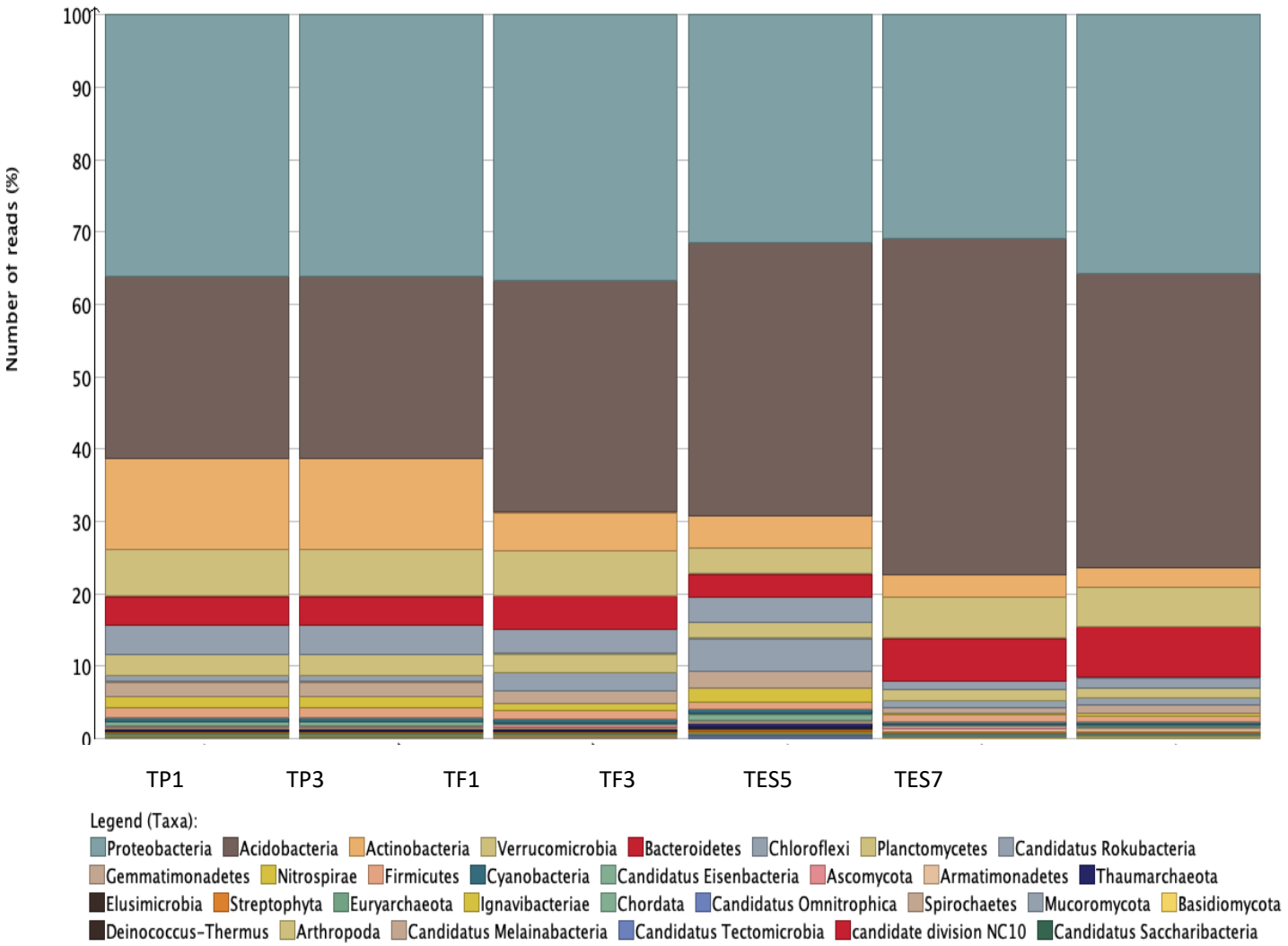


Figure 4.3 Relative abundance (%) of sequences classified by Phylum in soil samples from native forest (TF) and exotic pasture (TP) at the Taranaki site.

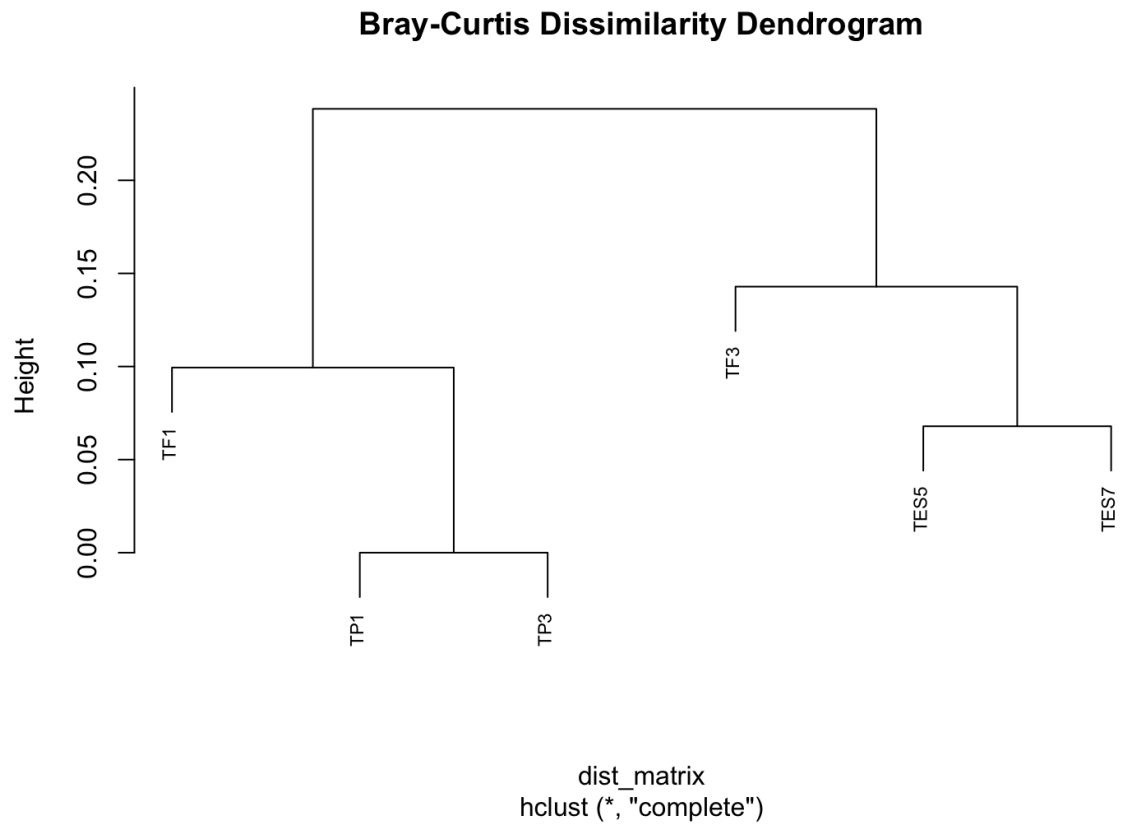


Figure 4.3 Hierarchical clustering of Bray-Curtis Dissimilarities among exotic pasture (TP3 and TP1) and native forest (TF1, TF3, TES5 and TES7) soil metagenomes from Taranaki soil samples.

Taranaki metagenome: Detection of entomopathogenic fungi using KAIJU

Table 4.3 summarizes the entomopathogenic fungi (EPF) identified through KAIJU analysis of metagenome sequences from six soil samples collected in Taranaki, New Zealand. EPF were successfully detected in both pasture and forest soils in Taranaki, despite fungi constituting only a minute proportion of the soil microbiome. The metagenomic analysis revealed that most EPF genera belonged to the order Hypocreales, which displayed a broader distribution compared to the more specialized order Entomophthorales. Among the Hypocreales, several species from the family Clavicipitaceae, particularly the genus *Metarhizium*, were consistently identified in both habitat types. In contrast, other genera exhibited habitat-specific patterns. For example, *Beauveria caledonica* was exclusively found in pasture soils, whereas *Hypocrella schizostachyi* was specific to forest soils. Although the representation of the order Entomophthorales was limited, several species from this group were detected in both forest and pasture.

Table 4.3. Entomopathogenic fungi (EPF) identified through KAIJU analysis of metagenome sequences from six soil samples collected in Taranaki, New Zealand. Species were detected in native forest and exotic pasture samples, forest only or pasture only.

Order	Family	Genus	Species
Hypocreales	Clavicipitaceae	*Metarhizium*	*M. anisopliae*
			M. robertsii
			M. brunneum
			M. acridum
			M. rileyi
			M. guizhouense
			M. flavoviride
			M. majus
Hypocreales	Clavicipitaceae	*Hypocrella*	*H. siamensis*
			H. schizostachyi
Hypocreales	Clavicipitaceae	*Claviceps*	*C. purpurea*
			C. paspali
			C. gigantea
Hypocreales	Clavicipitaceae	*Epichloe*	*E. festucae*
Hypocreales	Cordycipitaceae	*Cordyceps*	*C. fumosorosea*
			C. militaris
			C. javanica
Hypocreales	Cordycipitaceae	*Beauveria*	*B. bassiana*
			B. brongniartii
			B. malawiensis
			B. caledonica
Hypocreales	Cordycipitaceae	*Lecanicillium*	*L. psalliotae*
			L. longisporum
Hypocreales	Ophiocordycipitaceae	*Paecilomyces*	*P. divaricatus*
Hypocreales	Ophiocordycipitaceae	*Purpureocillium*	*P. lilacinum*
Hypocreales	Ophiocordycipitaceae	*Ophiocordyceps*	*O. sinensis*
			O. unilateralis s.l.,
			O. australis
Hypocreales	Ophiocordycipitaceae	*Tolypocladium*	*T. ophioglossoides*
			T. capitatum
			T. paradoxum
Entomophthorales	Ancylistaceae	*Conidiobolus*	*C. coronatus*
			C. heterosporus
Entomophthorales	Entomophthoraceae	*Entomophthora*	*E. muscae*

ITS2 metabarcoding: Entomopathogenic fungi

The metabarcoding approach, employing amplicon sequencing of the ITS2 region from ten soil DNA samples collected across Taranaki, Manawatu and Whanganui, yielded a total of 671,632 reads, with an average frequency of 67,132 reads per sample (maximum: 67,926; minimum: 67,132). This dataset generated 2,750 operational taxonomic units (OTUs). Rare sequences, likely resulting from sequencing errors, were excluded from the analysis, removing sequences with fewer than 600 reads. This filtering reduced the number of OTUs to 109. Rarefaction curve analysis was performed to evaluate whether Illumina HiSeq sequencing sufficiently recovered OTUs (**Figure 4.4**). The resulting rarefaction curves showed that most samples reached a saturation plateau, indicating that the sequencing depth of the ITS2 region was adequate for capturing the diversity of fungal communities.

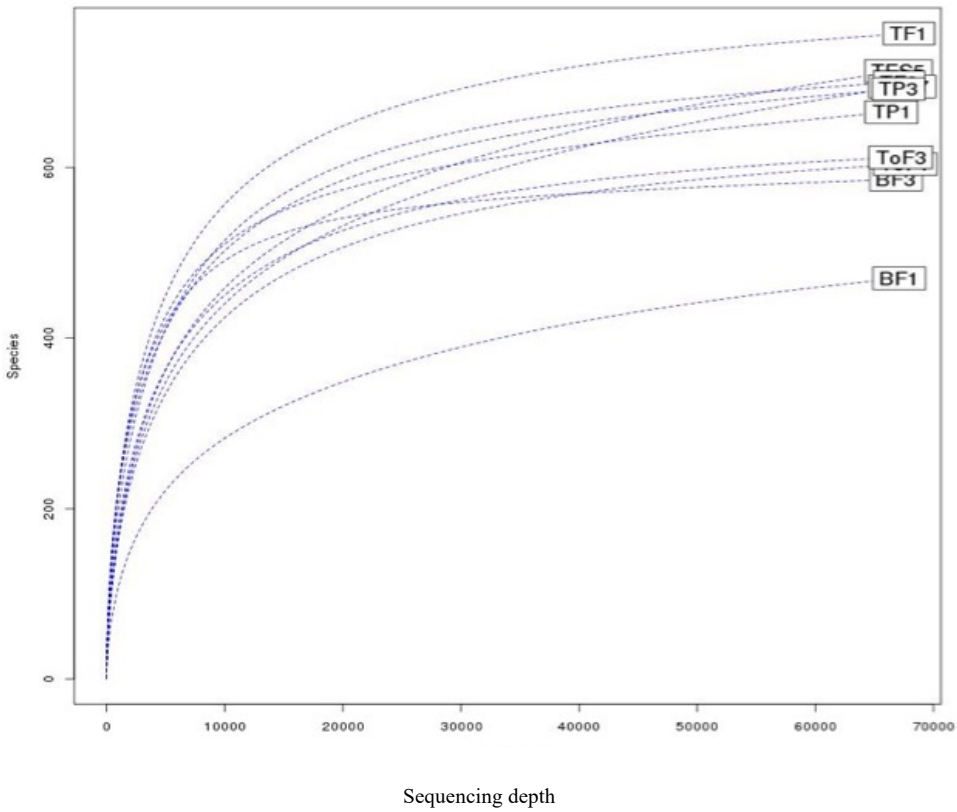


Figure 4.4 Rarefaction curve analysis of OTU recovery using Illumina HiSeq amplicon sequencing from exotic pasture and native forest soil samples. Abbreviations: TP1, TP3 - Taranaki pasture; TF1, TF3, TES5, TES7 – Taranaki forest; BF1, BF3 – Whanganui forest; ToF1, ToF3 – Manawatu forest.

At the operational taxonomic unit (OTU) level, alpha diversity was higher in pasture soils (Shannon index 4.72, Simpson index 0.98) compared to forest soils (Shannon: 3.94, Simpson: 0.92). However, the differences were not statistically significant ($P = 0.089$ and $P = 0.178$, respectively). For the beta-diversity, PCoA revealed that Axis 1 accounted for 19.5% of the total variation in the dataset, while Axis 2 explained an additional 16.6% (**Fig. 4.5**). Together, these axes cumulatively captured 36.1% of the variation in the data, providing significant insight into the microbial community structure across different habitats. Further, a permutation-based analysis was conducted to examine the significance of habitat on the sample data. Habitat was found to significantly influence the sample data ($F_{1,8} = 1.986$, $P = 0.044$) after 999 permutations. The habitat explained 19.9% ($R^2 = 0.1989$) of the variance in the sample data, while the residuals accounted for the remaining 80.1% ($R^2 = 0.8011$).

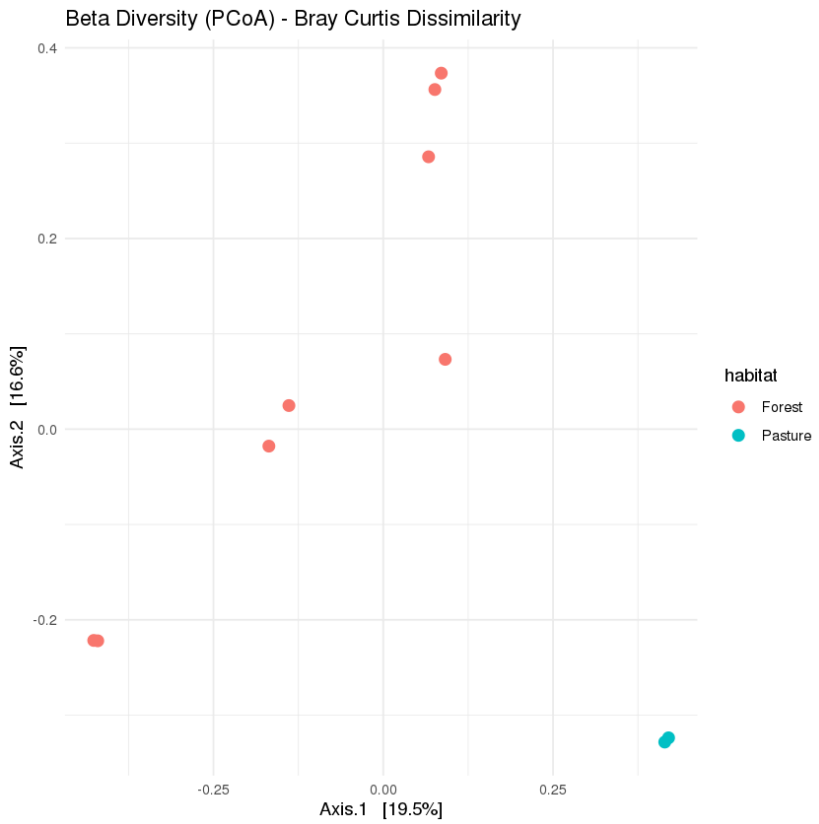


Figure 4.5. Principal Coordinate Analysis (PCoA) of fungal OTU composition in forest soils (Taranaki, Whanganui, Manawatu) and pasture soils (Taranaki). Bray-Curtis dissimilarity measure on metabarcoding data.

At the order level, Hypocreales, which includes entomopathogenic fungal genera, were present across all samples (**Fig. 4.6**). Their highest abundances were observed in Manawatu forest samples ToF1 (41.3%) and ToF3 (31.1%). In pasture soils, Hypocreales had a mean abundance of 14%, whereas their presence was minimal in high-elevation forest soils TES5 (1.1%) and TES7 (1.6%). Agaricales, another order within Agaricomycetes, were highly abundant in forest samples from Taranaki (TES5) and Whanganui (BF1). Interestingly, Auriculariales were uniquely present in lower-elevation Taranaki forest samples TF1 and TF3, emphasizing the impact of elevation on fungal community composition.

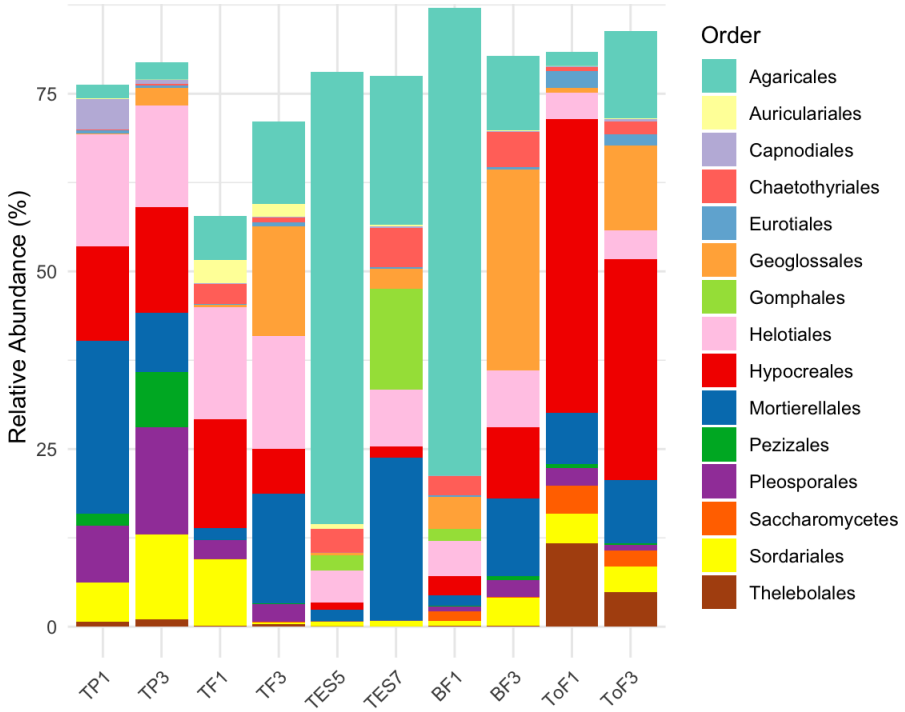


Figure 4.6. Relative abundance (%) of ITS2 sequences at the order level in pasture and forest soil samples based on metabarcoding analysis. Samples: TP1, TP3 – Taranaki pasture; TF1, TF3, TES5, TES7 – Taranaki forest; BF1, BF3 – Whanganui forest; ToF1, ToF3 – Manawatu forest.

Amplicon sequences analyzed through QIIME2 with the UNITE database, successfully detected EPF genera, albeit in low abundance. The three most frequently EPF genera detected as a proportion of total OTUs were *Lecanicillium*, *Metarhizium* and *Beauveria,* but there was also signal for *Ophiocordyceps*, *Tolypocladium*, *Hirsutella*, and *Paecilomyces* (**Figure 4.7**). Notably, the genus *Lecanicillium* exhibited the highest number of reads among the detected genera, despite not being detected in baiting method and rarely observed in cultures (Chapter 2). *Lecanicillium* was most abundant in exotic pasture soils (4.4%) while native forest soils in

Taranaki and Whanganui showed lower abundances (<0.9%). For the genus *Metarhizium*, reads were detected in seven out of ten samples, with Taranaki forest soils exhibiting the highest abundance (1.2%). Interestingly, only *Metarhizium marquandii* was identified from amplicon sequence, a species not detected through shotgun sequencing analyzed with KAIJU. The genus *Beauveria* exhibited the lowest abundance among the three most frequently detected EPF genera, which contrasts with the finding of higher prevalence of this genus where culturing methods were used (Chapter 3). *Beauveria* was detected in only three samples (0.6% TP1, 0.1% TF1, 0.1%TF3).

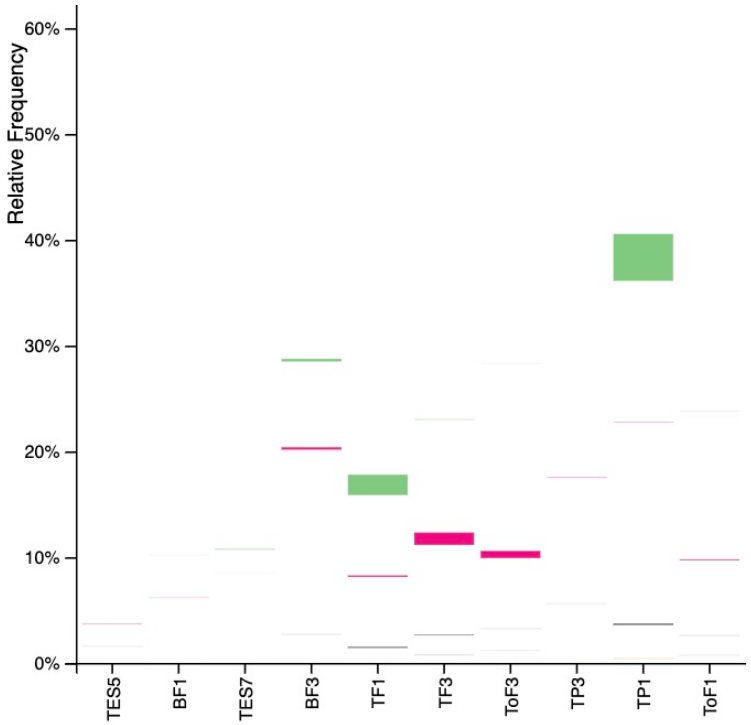


Figure 4.7. Relative frequency (%) of the most abundant EPF genera, *Lecanicillum* (green) *Metarhizium* (pink) and *Beauveri*a (gray) across different soil samples based on Metabarcoding Analysis. Samples: TP1, TP3 – Taranaki pasture; TF1, TF3, TES5, TES7 – Taranaki forest; BF1, BF3 – Whanganui forest; ToF1, ToF3 – Manawatu forest.

ITS2 metabarcoding: Taxonomic composition of soil fungi - higher taxa

Phylum Ascomycota dominated most samples, except for the high-elevation Taranaki deep native forest samples TES5 and TES7, and BF1 from Bushy Park. It was particularly abundant in BF3 (64.6%), TF1 (66.7%), TP1 (60.1%), TP3 (75.6%), ToF1 (76.4%), and ToF3 (70.2%) (**Figure 4.8**). Basidiomycota comprised a higher proportion at 68.1% and 67.0% for forest samples BF1 and TES5, respectively. The Basidiomycota phylum was notably scarce in samples TP1 and TP3 as also reflected in the metagenomics results where Basidiomycota is only detected in deep native forest samples.

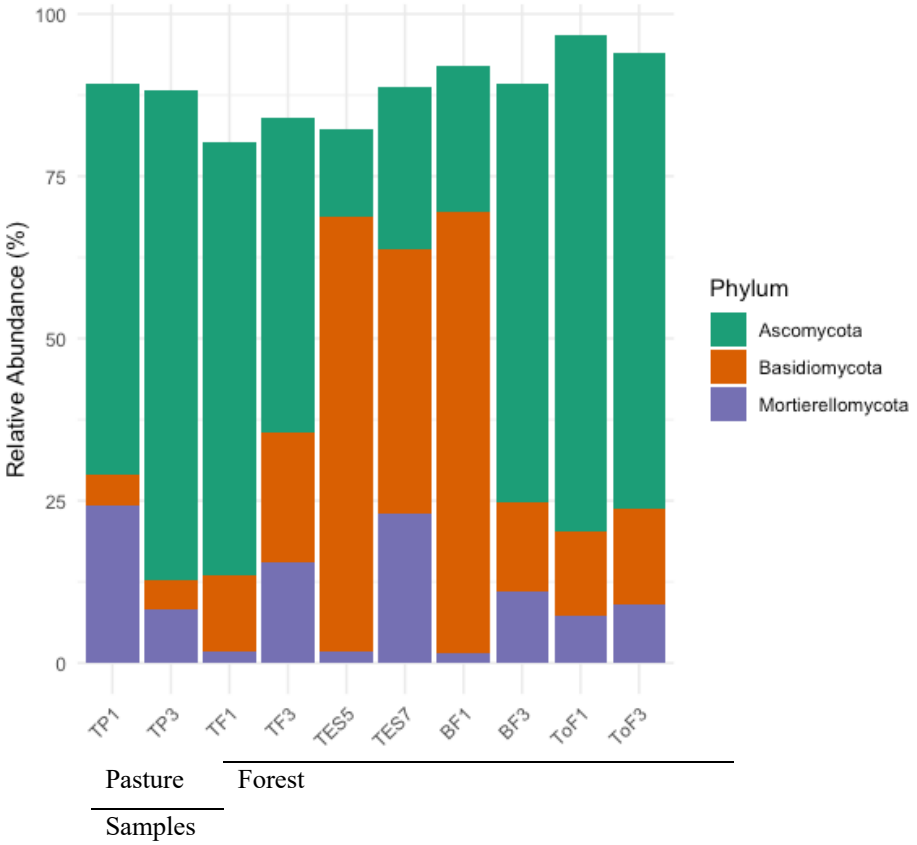


Figure 4.8. Relative abundance (%) of ITS2 sequences assigned to fungal phyla in exotic pasture and native forest soil samples based on metabarcoding analysis. Samples: TP1, TP3 – Taranaki pasture; TF1, TF3, TES5, TES7 – Taranaki forest; BF1, BF3 – Whanganui forest; ToF1, ToF3 – Manawatu forest.

At the class level, fungal classes such as Sordariomycetes, which includes entomopathogenic fungi, and Agaricomycetes exhibited the most variability among samples, with some showing exceptionally high values (**Fig. 4.8**). Sordariomycetes are highly abundant in exotic pasture samples TP1 (25.0%) and TP3 (30.6%), as well as in Manawatu forest samples ToF1 (49.2%) and ToF3 (37.0%). Their abundance was moderate in native forest samples TF1 (25.4%) and BF3 (18.1%) but low in TF3, TES5, TES7, and BF1. Agaricomycetes, on the other hand, were in high abundance in forest samples TES5 (66.9%) and BF1 (68.0%), with moderate levels in TF3 (17.3%) and ToF3 (13.8%), while being consistently low in exotic pasture soils. Tremellomycetes was notably high only in ToF1 (14.0%) and low in all other samples. Other classes, such as Leotiomycetes and Mortierellomycetes, displayed moderate and consistent levels in specific samples, while Dothideomycetes, Archaeosporomycetes, and Pezizomycetes were recorded in pasture soils but were minimal to absent in forest soils.

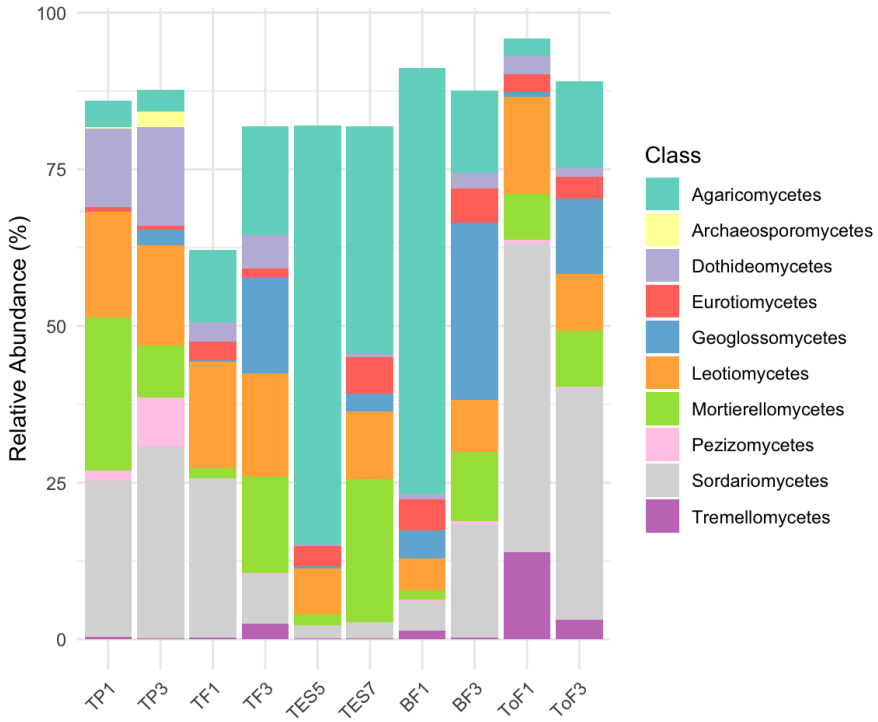


Figure 4.9. Relative abundance (%) of ITS2 sequences assigned to fungal classes in exotic pasture and native forest soil samples based on metabarcoding Analysis. Samples: TP1, TP3 – Taranaki pasture; TF1, TF3, TES5, TES7 – Taranaki forest; BF1, BF3 – Whanganui forest; ToF1, ToF3 – Manawatu forest.

Discussion

Entomopathogenic fungal diversity in forest and pasture: Insights from metagenomics and metabarcoding

Recent advancements in molecular methodologies have significantly enhanced our understanding of entomopathogenic fungal (EPF) diversity in soil ecosystems (Masoudi et al., 2020). Microorganisms in the soil, such as bacteria, fungi, archaea, viruses, are essential for preserving soil health and fertility (Islam et al., 2020). These microorganisms are fundamental to soil ecosystem processes, including the decomposition of organic matter, nutrient recycling, and the prevention of soil-borne diseases (Khaziev, 2011). Soil ecosystems are complex microbial hubs, with EPF playing a critical role in regulating arthropod populations.

This study used metagenomics and metabarcoding approaches to identify EPF genera not detected through traditional baiting and plating methods. Shotgun sequencing revealed the dominance of EPF within the order Hypocreales, particularly in both pasture and forest soils, while the order Entomophthorales was represented in lower abundance. This aligns with findings from previous studies highlighting Hypocreales as the dominant entomopathogenic group in terrestrial ecosystems, particularly in subtropical and temperate regions, where they regulate insect populations and contribute to ecosystem balance (Masoudi et al., 2020). Habitat-specific distributions of certain EPF species were found in this chapter. For example, *Beauveria caledonica* was exclusively detected in pasture soils, while *Hypocrella schizostachyi* was found solely in forest soils. These patterns suggest that specific ecological conditions, such as soil physicochemical properties, organic matter content, and microclimatic variables, may influence the habitat preferences of EPF (Kepler et al., 2015; Sharma et al., 2019). Such findings underscore the role of habitat heterogeneity in shaping microbial community structure and emphasize the potential ecological drivers determining fungal distribution.

The genus *Metarhizium* (family Clavicipitaceae) was consistently present across both habitats. *Metarhizium* species are widely documented for their ability to persist in diverse soil environments, exhibiting resilience to fluctuating conditions such as temperature, pH, and organic matter availability (Bidochka et al., 1998; Quesada-Moraga et al. 2007; Sharma et al., 2019; Fernández-Bravo et al., 2021). The relatively higher abundance of *Metarhizium* in Taranaki Forest soils suggests it may play a key role in regulating arthropod populations in these ecosystems. Interestingly, *Beauveria,* despite being prevalent EPF genus detected in previous chapters of this thesis (which used culturing methods), exhibited lower relative

abundances in the soil when assessed via metabarcoding. This discrepancy reflects methodological differences, as culturing may favour fast-growing and culturable EPF strains, whereas metabarcoding provides a broader, culture-independent snapshot of fungal diversity (Masoudi et al., 2020).

For metabarcoding, the dominance of Ascomycota in exotic pasture and native forest margin highlights their crucial role in terrestrial ecosystems, where they contribute to nutrient cycling and organic matter decomposition through their diverse lifestyles, including saprotrophic, pathogenic, and mutualistic functions (Li et al., 2022). In high-elevation deep native forest soils, the notable presence of Basidiomycota can be attributed to the availability of woody debris, which serves as a substrate for their enzymatic degradation of complex organic materials (Liu et al., 2022; Zheng, 2024). The heterogeneity in fungal community composition is further evidenced by the prevalence of Hypocreales, which can indicate the presence of insect hosts or environmental conditions favourable for entomopathogenic fungi. The limited representation of Entomophthorales in both forest and pasture soils is also noteworthy. Entomophthorales have high host specificity, often limited to a single family or genus, and are challenging to mass-produce in artificial culture, but uniquely eject spores rapidly under favourable conditions, distinguishing them from Hypocrealeas (Steinkraus, 2007). While Entomophthorales are ecologically significant as insect pathogens, their often-obligate nature and narrow environmental requirements may explain their lower detectability compared to Hypocreales (Sharma et al., 2019; Fernández-Bravo et al., 2021).

Co-occurrence of bacterial communities and entomopathogenic fungi in soil microbiomes

The taxonomic composition of Taranaki pasture and forest soil metagenomes revealed that bacteria overwhelmingly dominate soil microbial communities, accounting for 98–99% of the relative abundance in all samples. This dominance highlights the pivotal role of bacteria in shaping soil ecosystems and maintaining functional stability. The phyla Proteobacteria and Acidobacteria were consistently dominant, aligning with their ubiquitous presence in soils (Vaicekonyte & Keesing, 2012). Proteobacteria exhibit remarkable metabolic versatility, encompassing species involved in nitrogen fixation, nutrient cycling, and pathogenic interactions (Tkaczuk et al., 2013). Acidobacteria, often underrepresented in culture-based studies, contribute significantly to nutrient cycling and organic matter decomposition (Khaziev, 2011; Islam et al., 2020). Bacterial diversity also serves as an indicator of soil ecosystem stability and resilience to environmental changes (Suman et al., 2022). Variations

in bacterial community composition across habitats may reflect differences in soil properties such as pH, carbon-to-nitrogen ratios, and phosphorus availability (Staves & Knell, 2010; Niu et al., 2019; Wilhelm et al., 2023). These microbial interactions underpin key ecosystem functions that enhances multifunctionality in terrestrial ecosystems (Suman et al., 2022).

EPF genera such as *Beauveria*, *Metarhizium* and *Lecanicillium* were detected alongside abundant bacterial taxa, suggesting potential relationships that may influence soil health and invertebrate population dynamics (Wakil et al., 2014; Dong et al., 2016). Co-occurrence of bacterial communities and entomopathogenic fungi within the same soil microbiome underscores microbial interactions influencing the persistence and activity of EPF (Studdert & Kaya, 1990; Brownbridge et al., 2006; Gouli et al., 2013; Cai et al., 2022). For instance, Huber (1958, cited in Clerk, 1969) observed that *Beauveria bassiana* conidia failed to germinate in fresh garden soil but were able to germinate once the soil was sterilized. Lockwood (1964) reviewed research on soil fungistasis, describing it as the inability of fungal spores to germinate in natural soil due to microbial competition for nutrients or the production of antibiotics (Blond, 2012). Clerk (1969) found similar fungistasis affecting *B. bassiana* and *Isaria farinose*, noting that this inhibition could be reduced by filtering soil extracts or adding extracts from *Bombyx mori* cocoon silk, a host of *B. bassiana* (Clerk, 1969) concluded that soil bacteria and other microorganisms were responsible for this fungistasis, but the presence of host arthropods could mitigate it. Similarly, Walstad et al. (1970) observed that *B. bassiana* and *Metarhizium anisopliae* conidia could not germinate in non-sterile soil or leaf litter unless the inhibition was counteracted. Soil microorganisms can also degrade EPF propagules (Brownbridge et al., 2006; Blond, 2012). These interactions highlight the complex role of soil microorganisms in modulating the survival, germination, and effectiveness of EPF (Blond, 2012; Cai et al., 2022).

While EPF directly regulate arthropod populations, bacteria contribute indirectly by modulating soil properties, such as pH and nutrient availability, which in turn affect fungal growth and activity (Safitri et al., 2018; Afandhi et al., 2020; Hallouti et al., 2020). Diversity analyses revealed comparable bacterial richness and evenness across forest and pasture soils, as indicated by alpha diversity indices. Beta diversity, assessed using Bray-Curtis dissimilarities, indicated subtle but non-significant differences between habitats (**Fig. 4.3**) This observation supports previous findings that microbial diversity reflects habitat-specific environmental conditions (Silva et al., 2015). Forest habitats uniquely supported wood-decomposing fungi such as Basidiomycota, particularly at higher elevations, likely due to the presence of lignocellulosic substrates (Vaicekonyte & Keesing, 2012). This suggests that soil

bacteria and EPF may exhibit complementary ecological functions, with bacteria driving nutrient cycling and EPF targeting invertebrate hosts (Petzold-Maxwell et al., 2011; Mantzoukas et al., 2019; Mehrmoradi et al., 2022).

Conclusions

Insect baiting and plating techniques, commonly used to isolate EPF from the soil, may not fully represent the EPF diversity, especially of temporarily non-infective species. Metagenomics and metabarcoding, however, provide a more holistic view, allowing for a broader assessment of the EPF composition in soils. In this Chapter, DNA extracted from forest and pasture soils underwent shotgun and amplicon sequencing targeting the ITS2 region. Notable differences in microbial community structure, abundance, and functionality were detected between forest and pasture habitats. Bacteria overwhelmingly dominated the soil samples, constituting 98–99% of the microbial content, with Acidobacteria, Proteobacteria, and Actinobacteria being the most prevalent phyla. Shotgun sequencing highlighted the presence of various EPF species, especially from the Hypocreales order. Although the MEGAN-based metagenomic analysis did not identify EPF genera, analysis using KAIJU revealed species exclusive to either pasture or forest habitats, as well as species that were widespread. Amplicon sequencing of soil DNA, focusing on the ITS2 region, predominantly revealed Ascomycota among fungal phyla. EPF genera, undetected through baiting and plating, were also confirmed through amplicon sequencing. The findings illuminate the diversity of soil microbial community in different habitats and the importance of using a range of sampling methods to detect full diversity of EPF.

Entomopathogenic fungi (EPF) diversity further highlighted the utility of molecular techniques in uncovering previously undetected species and habitat-specific distributions. These findings emphasize the significance of both culture-based and DNA-based methods to gain a better understanding of the diversity of soil fungi.

Chapter Five

Efficacy of *Beauveria* isolates collected in adjacent forest and pasture habitats in the North Island, NZ against selected agricultural pests

Introduction

The utilization of entomopathogenic fungi (EPF), such as *Metarhizium* and *Beauveria*, has gained prominence as an eco-friendly alternative for pest control in agriculture. Entomopathogenic fungi play an important role in the natural regulation of arthropod populations (Evans, 1982). EPF are naturally found in the soil which serves as an excellent environmental shelter, protecting fungi from UV radiation and other adverse abiotic and biotic influences (Keller et al., 1989). Most insect orders are susceptible to fungal diseases, making EPF an important resource in microbial control of insect pests.

The interactions between soil fungi and arthropods occur in every ecosystem where they coexist in nature (Gange & Brown, 2002). The virulence of EPF is always strain-specific; it can differ within the same fungal species and is influenced by the ecological context of the site, impacting the pathogenicity and host range. Development of the EPF as biological control agents depends on the selection of a virulent strain. Therefore, collection of native species and testing the native strains against a target pest is essential for the discovery of effective isolates to be used in biological control.

The interaction between EPF and their hosts is a complex process that begins with spore attachment to the insect cuticle, followed by germination, penetration, colonization, and ultimately the death of the host. The success of these steps depends not only on the virulence of the fungus but also on the environmental conditions that can either enhance or inhibit fungal development and virulence (Vega et al., 2009). Meyling and Eilenberg (2007) emphasized the significance of environmental factors and host specificity in shaping the virulence traits of EPF populations, suggesting that isolates from habitats with high pest densities may evolve greater virulence towards those pests. Similarly, Quesada-Moraga et al. (2014) demonstrated that EPF isolates collected from agricultural soils exhibited specific adaptations that enhanced their pathogenicity against prevalent pests in those environments, highlighting the potential of selecting habitat-specific isolates for targeted pest control.

Understanding the interactions between fungus, target insect pest, and the environment is imperative in the control of insect pests using EPF (Vega et al., 2009; Islam et al., 2021). Isolation of the fungus from the soil or from the infected insects and subsequently employing biological assays on the target pest are the first step in the selection of EPF strains as microbial biological control agents. Characterization of the ecological constraints of the candidate isolates relative to the environment in which insect pests are targeted is an additional parameter in selecting successful EPF isolates.

This thesis chapter aims to elucidate the efficacy of local *Beauveria* isolates against a range of economically significant below-ground and above-ground insect pests, including the grass grub *Costelytra giveni* White (Coleoptera: Scarabaeidae), giant willow aphid *Tuberolachnus salignus* (Gmelin, 1790) (Hemiptera: Aphididae), locust *Locusta migratoria* (Linnaeus, 1758) (Orthoptera: Acrididae), and mealworm *Tenebrio molitor* Linnaeus 1758 (Coleoptera: Tenebrionidae). By comparing the efficacy of these isolates against both these below-ground and above-ground pests, this research seeks to identify potential habitat-specific adaptations that will contribute to valuable insights into the potential application of these fungi in integrated pest management strategies.

Materials and Methods

Fungal isolates

Cultures of EPF were obtained from the soil samples collected from pasture, boundary, and forest areas within Mt. Egmont National Park, Taranaki, Bushy Park Sanctuary, Whanganui, and Totara Reserve, Manawatu (Chapter 2), and were utilized in the bioassays. Specifically, isolates retrieved from insect baiting (Chapter 2) were chosen for these experiments. Additionally, commercially available EPF products – Com-A and Com-B were included for comparison. One representative isolate from each site/habitat combination were used for *Beauveria* bioassays. *Metarhizium* isolates were not utilized for this chapter as infection by insect baiting was not achieved across all sites.

Conidia were harvested from 15 days old fungal colonies (isolates) grown in PDA plates at 20 ± 2 °C, with eight to ten plates for each isolate. A 5 ml of 0.01% Triton X-100 solution was added onto each plate and conidia suspended in liquid were collected, yielding approximately 5 ml per plate. Conidial suspensions were quantified using a Neubauer chamber and adjusted to desired concentration of conidia/ml.

Insects used in bioassays

Insects utilized for bioassays included below-ground pest – grass grub *Costelytra giveni*, and above-ground pests – giant willow aphid *Tuberolachnus salignus*, locust *Locusta migratoria*, and mealworm *Tenebrio molitor*.

Grass grub. *Costelytra giveni*, formerly misrepresented as *C. zealandica* White, are soil dwelling beetle larvae which cause substantial damage in pastures. The grass grub adults are only present for a few weeks in spring/early summer each year, when they feed on the young foliage of a wide range of plants (Specialty Seed Brochure, 2020). Grass grub larvae were collected from the turf at the Research Dairy Farm at Lincoln University and at Manawatu Golf Course in Palmerston North in April 2021 and July 2021, respectively (**Fig. 5.1**). Second and third instar larvae collected from the field underwent preliminary testing for feeding activity, ensure free of bacterial disease. Larvae were placed individually in wells in 6-well or 12-well plates, with each well containing ~7.5 mm carrot cubes as feed (**Fig. 5.1**) and left overnight at room temperature (20° C). Larvae were checked for feeding and mortality after three days. Only larvae displaying active feeding behaviour were used for bioassays.

Figure 5.1. A and B: Field collection of grass grub in Manawatu Golf Course, Palmerston North, July 2021. C: Grass grub larvae dug up from Manawatu Golf Course, PN. D: Cell culture plates used for maintaining individual larvae in the laboratory with carrot cubes as food.

Giant Willow Aphid. The giant willow aphid (GWA) *Tuberolachnus salignus* is a large stem-feeding aphid and is one of the most problematic insects in willow production in New Zealand. Apart from hindering physiological processes of willow plants from direct feeding, GWA produces black sooty mould on the leaves due to honeydew deposition which further aggravates optimum willow production. For mass rearing of giant willow aphid, stem cuttings (1 cm diameter, 25 cm height) of the willow clone NZ 04-116-168 were collected from the National Willow Collection of the New Zealand Poplar & Willow Research Trust, at the Plant Growth Unit of Massey University (Palmerston North, New Zealand) in summer 2020 and 2021. The willows were grown in screw cap plastic containers (8.5 cm diameter, 10 cm height), with 1.5 cm hole in the centre to accommodate the willow cuttings. Approximately three-fourths of the plastic container was filled with 25% strength Hoagland's hydroponic solution that was

replenished as required. Two-month-old stem cuttings were infested with 20 adult GWA as a starting colony. A total of 40 cuttings were maintained for the experiment.

Mealworm. The mealworm *Tenebrio molitor* is a damaging pest in stored grain products, but also serves as a valuable model organism for scientific research and has potential as a sustainable protein source in animal feed and human nutrition (Moruzzo et al., 2021) Mealworms used in the experiment were purchased from Biosuppliers (Auckland, NZ) and were maintained in containers (15 x 10 cm) with wheat bran in a temperature-controlled room (20°C) until use for assays.

Locust. Locusts are primarily outdoor pests characterized by their swarming behavior and significant potential to cause agricultural devastation (Le Gall et al., 2019). Locusts can transition from solitary to gregarious forms, leading to large swarms capable of consuming vast amounts of crops and pasture. This behaviour makes them one of the most destructive migratory pests in the world, with the capacity to affect food security and livelihoods on a large scale. Effective control measures are critical to prevent and mitigate the extensive damage caused by locust outbreaks. Locusts were also purchased from Biosuppliers (Auckland, NZ) and were fed with fresh grass every other day and were maintained in small cages in a temperature-controlled room (20°C) until use for assays (**Fig. 5.2**).

Figure 5.2. Locusts maintained in a cage in a temperature-controlled lab (20^{o} C) for use in bioassay.

Bioassays

Bioassay for grass grub larvae were conducted using the method of Glare (1994) with some modifications. Soil was semi-sterilized at 90°C for 48 hrs and then 10 grams of soil was added to 50 ml screw cap glass vials. One ml of conidial suspension was added to the soil. After soil and conidial suspension were mixed, one grass grub larva was added and the cap was loosely closed to allow gas exchange. Larvae were kept in the dark for at least 35 days at 20 ± 2 °C and were checked at day 7, 14, 21 and 35 for mortality and fungal infection (mycosis). Dead larvae were kept in the glass vials with the lid closed tight to keep the humidity in the system and were checked for mortality. At the time of assays for grass grubs, commercially available EPF products were not yet available, hence, only isolates from soil collection were used. Conidial concentrations of 10^6, 10^7 and 10^9 conidia/ml was used. This experiment had ten treatments (nine *Beauveria* isolates and positive control), with four replicates per treatment. This experiment was repeated three times with new sets of larvae (so overall 12 replicates per treatment, per concentration). A total of 360 field-collected grass grubs were utilized for the experiment.

For giant willow aphid (GWA), the bioassay protocol for green peach aphid *Myzus persicae*, developed by the Bio-Protection Research Centre at Lincoln University, was adapted (Nguyen, 2021). Modifications were made, including the substitution of stem cuttings instead of leaves, which proved to be more suitable for GWA application. A 100 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. Conidial concentrations of 10^6, 10^7 and 10^8 conidia/ml were used for this assay. Five third-instar aphid nymphs were placed in Petri dishes with a cut out piece of willow stem. A total of ten treatments (nine *Beauveria* strains plus positive control) were used for this assay with four replicates, a total of 20 GWA per treatment. For inoculation, the conidial suspension was sprayed onto the stem cuttings using an airbrush sprayer (Paasche). The numbers of live and dead nymphs were recorded at day 3, 7 and 10. A pilot study showed that aphids are able to survive in this set-up for 12+ days. The experiment was conducted in a temperature-controlled room at 20^o C with photoperiod of 16L: 8D.

For locust bioassays, a conidial concentration of 10^9 conidia/ml was used. For the bioassay setup, containers suitable for housing locusts were prepared. A small plant (*Phormium* sp.) was housed inside the container as feed for the locusts for the duration of assay. Subsequently, 1 ml of 10^9 conidial suspension was applied to the locust body using a micropipette. Locusts were monitored over 3, 7 and 14 days to assess mortality rates and fungal

infection. This experiment had 12 treatments: nine field-collected *Beauveria* strains, two commercially available products (Com-A and Com-B) and one control (0.01% TritonX-100), with three replicates per treatment and with five locusts in each replicate (cage) with a total of 15 locusts per treatment. Experiment was conducted in a temperature-controlled room at 20^{o} C with photoperiod of 16L: 8D.

For mealworm bioassay, *Beauveria* isolates were used at 10^9 conidia/ml concentration. For the bioassay, 100 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. Five mealworm larvae were placed in each dish. For inoculation, 300 µl of the conidial suspension was sprayed directly onto the larvae using an airbrush sprayer. For a negative control, 300 µl of sterile 0.01% TX-100 solution was used. Plates were incubated at 25°C with a 16:8 h light: dark cycle for the duration of the experiment. There were 12 treatments: nine field collected *Beauveria* strains, two commercially available products, Com-A and Com-B and one control, with eight replicates per treatment and with five mealworms in each replicate (total 40 mealworm larvae per treatment). Data were recorded at day 3,7 and 10. Experiment was conducted in a temperature-controlled room at 20^{o}C.

Statistical analysis

The LC50 values, indicating the concentration required to achieve 50% mortality was collected for giant willow aphids (GWA) using probit regression analysis. Although three concentrations were tested on grass grubs, LC50 values could not be determined because mortality did not reach 50% at most concentrations. As a result, calculating the LC50 was deemed unnecessary. Abbott's formula, [(%Treatment mortality - %Control mortality) / (100 - %Control mortality)] was applied to correct for natural mortality before determining LC50 values (Abbott, 1925).

Prior to further statistical analysis, the distribution of the dataset was assessed to check for normality, which is a common assumption for many parametric statistical tests. This was done using the Shapiro-Wilk test in R. To analyze the effects of habitat, isolate, and dose concentration on mortality, a Generalized Linear Model (GLM) with Gaussian family and identity link function was employed to assess the effect of isolate and concentration on mortality rates. The dependent variable (mortality) was modeled as a function of independent variables: isolate, habitat (where EPF was collected) and dose concentration. Post-hoc

comparisons were conducted using Tukey's HSD test to identify significant differences at $p < 0.05$ between isolates at each concentration level.

Ultrastructural observations of EPF in target insects

Scanning electron microscopy (SEM) was used to obtain details of EPF infection in tested insects. SEM imaging was conducted for GWA, locusts and mealworms; grass grubs were not imaged with SEM as the assay was concluded before SEM services were accessible. Small segments of EPF infected insects were fixed in 0.1M sodium phosphate buffer with 3% glutaraldehyde and 2% formaldehyde. These samples were then dehydrated through a graded ethanol series (50, 70, 80, 90, and 95%). Following dehydration, critical point drying was performed and the samples were sputter-coated with gold. In some instances, if the samples seemed adequately dry, they were directly gold-coated without undergoing critical point drying. The samples were imaged using FEI Quanta 200 SEM operated at a 20 kV accelerating voltage.

Results

Grass grub bioassay using field-collected Beauveria *isolates*

Cumulative mortality. The earliest larval death was observed at 10^6 conidia/ml conidial concentrations on day 21. The maximum mortality achieved at day 35 at this concentration was only 12.5% using pasture isolate TP and forest isolate ToF (**Fig. 5.3**). Mortality at higher EPF concentrations of 10^7 and 10^9 conidia/ml started on day 7. Increasing the inoculum to 10^7 conidia/ml increased grass grub mortality to a maximum of 50% using TP isolate. At the highest concentration of 10^9 conidia/ml, 100% mortality of grass grub larvae using pasture isolate TP was observed at 35 days after treatment.

Mean mortality by isolate. The mortality rates varied significantly among *Beauveria* isolates, with the effect being concentration-dependent. At the lowest concentration (10^6 conidia/ml), a significant effect of isolate was observed (χ^2 (8) = 27.0, $P < 0.05$) with pasture isolates TP and ToP causing significantly higher mortality compared to other isolates (**Fig. 5.4**). Mortality rates at 10^6 conidia/ml concentration remained relatively low across all isolates, suggesting this concentration may be below the effective threshold for consistent control. At the medium concentration (10^7 conidia/ml), significant differences among isolates remained (χ^2 (8) = 38.4, $P < 0.05$), with some isolates causing notably higher mortality rates. The highest concentration (10^9 conidia/ml) showed the most pronounced differences in virulence among isolates (χ^2 (8) = 22.9, $P < 0.05$). At this concentration, grass grub larval mortality rates were generally higher across all isolates, with pasture isolate ToP achieving up to 100% mortality. The pasture isolates TP and ToP consistently performed well across all concentrations and particularly at higher concentrations.

Mean mortality by habitat. Pasture isolates caused the highest mortality rate (55%) in grass grub larvae across all conidial concentrations, compared to isolates from boundary (30%) and forest habitats (23%) (χ^2 (2)= 9.87, $P < 0.005$) (**Fig. 5.5**). Boundary isolates showed intermediate mortality rates, which were not significantly different from either pasture or forest.

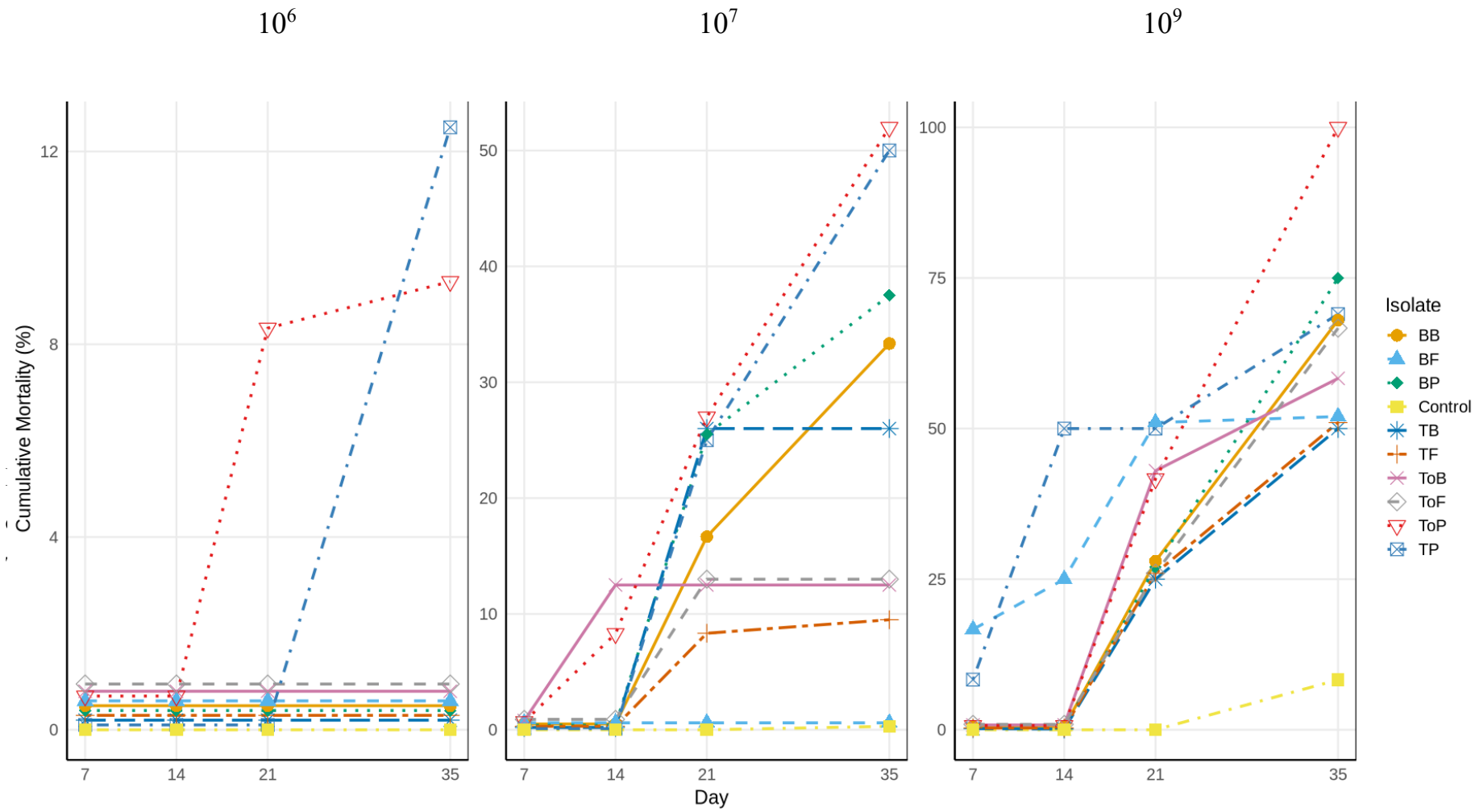


Figure 5.3. Cumulative mortality of grass grub, *Costelytra giveni* larvae using field collected *Beauveria* isolates at three levels of conidia concentrations, 10^6, 10^7, and $10^{9.}$ Isolate codes: BB – Whanganui boundary, BF – Whanganui forest, BP – Whanganui pasture, TB – Taranaki boundary, TF –Taranaki forest, TP– Taranaki pasture, ToB – Manawatu boundary, ToF – Manawatu forest, ToP – Manawatu pasture.

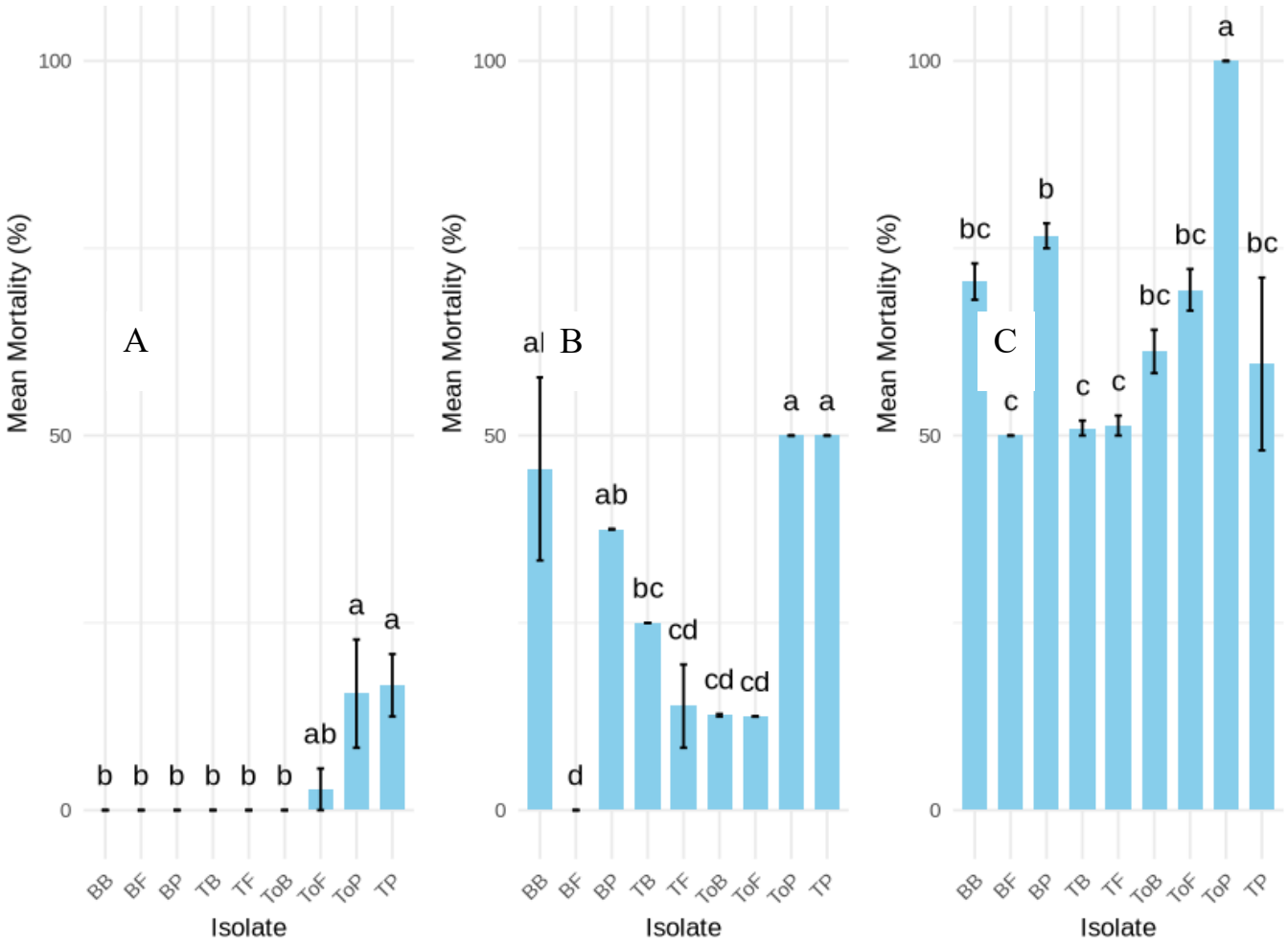


Figure 5.4. Mean mortality (% ± SE) of grass grub *Costelytra giveni* larvae after 35 days exposure to different *Beauveria* isolates at three concentrations: (A) 10^6 conidia/ml, (B) 10^7 conidia/ml, (C) 10^9 conidia/ml. Different letters above bars within each concentration indicate significant differences between isolates according to Tukey's HSD test ($P < 0.05$). Isolate codes: BB – Whanganui boundary, BF – Whanganui forest, BP – Whanganui pasture, TB – Taranaki boundary, TF – Taranaki forest, TP – Taranaki pasture, ToB – Manawatu boundary, ToF – Manawatu forest, ToP – Manawatu pasture.

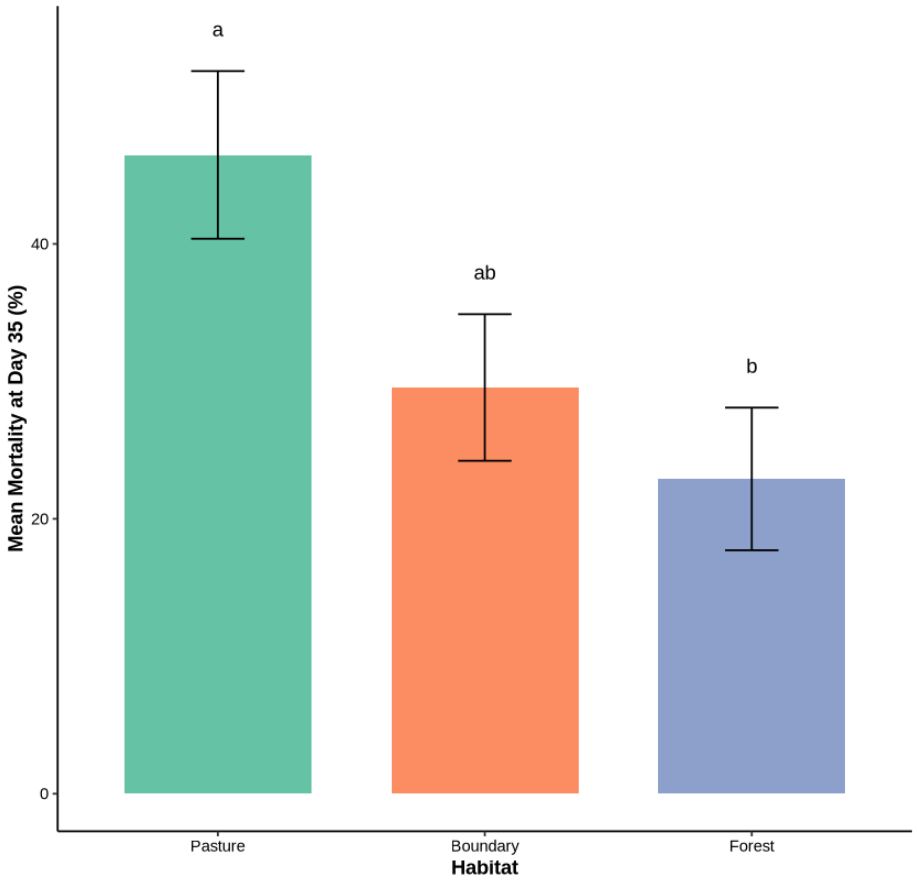


Figure 5.5. Mean mortality of grass grub *Costelytra giveni* larvae at day 35, after exposure to *Beauveria* isolates obtained in different habitats through insect baiting. Different letters indicate significant differences according to Tukey's HSD test ($P < 0.05$).

Giant Willow Aphid (GWA) bioassay using field-collected Beauveria *isolates*

Cumulative mortality. At the lowest conidial concentration of 10^6 conidia/ml**,** mortality of GWA began on day 7 with a maximum mortality of 15% using a pasture isolate from Whanganui (isolate BP) (**Fig. 5.6**). On day 10, interestingly, boundary isolates BB and TB from Whanganui and Taranaki exhibited the highest mortality in GWA (20%). Increasing the conidial concentration to 10^7 conidia/ml markedly enhanced mortality rates, with pasture isolates BP and ToP achieving a mortality rate of 50% by day 10. At the highest tested concentration of 10^8 conidia/ml, the pasture isolates TP and ToP demonstrated significant lethality to GWA, causing 80% and 85% mortality, respectively.

Mean mortality by isolate. At the lowest conidial concentration (10^6 conidia/ml), there were no significant differences in the mean mortality rates of GWA induced by the isolates. At 10^7

conidia/ml concentration, pasture isolates, particularly TP and ToP, consistently caused higher mean mortality rates in GWA, although the differences among isolates at this concentration were not statistically significant (**Fig. 5.7**). At the highest concentration (10^8 conidia/ml), the pasture isolates TP and ToP achieved mortality rates exceeding 80%, while isolates from the boundary and forest areas showed lower efficacy, with GWA mortality rates generally remaining below 50%. High variability was observed across the data, as indicated by the error bars in **Fig. 5.7**. The differences in GWA mortality across isolates were not statistically significant (χ^2 (8) = 3.44, P = 0.90).

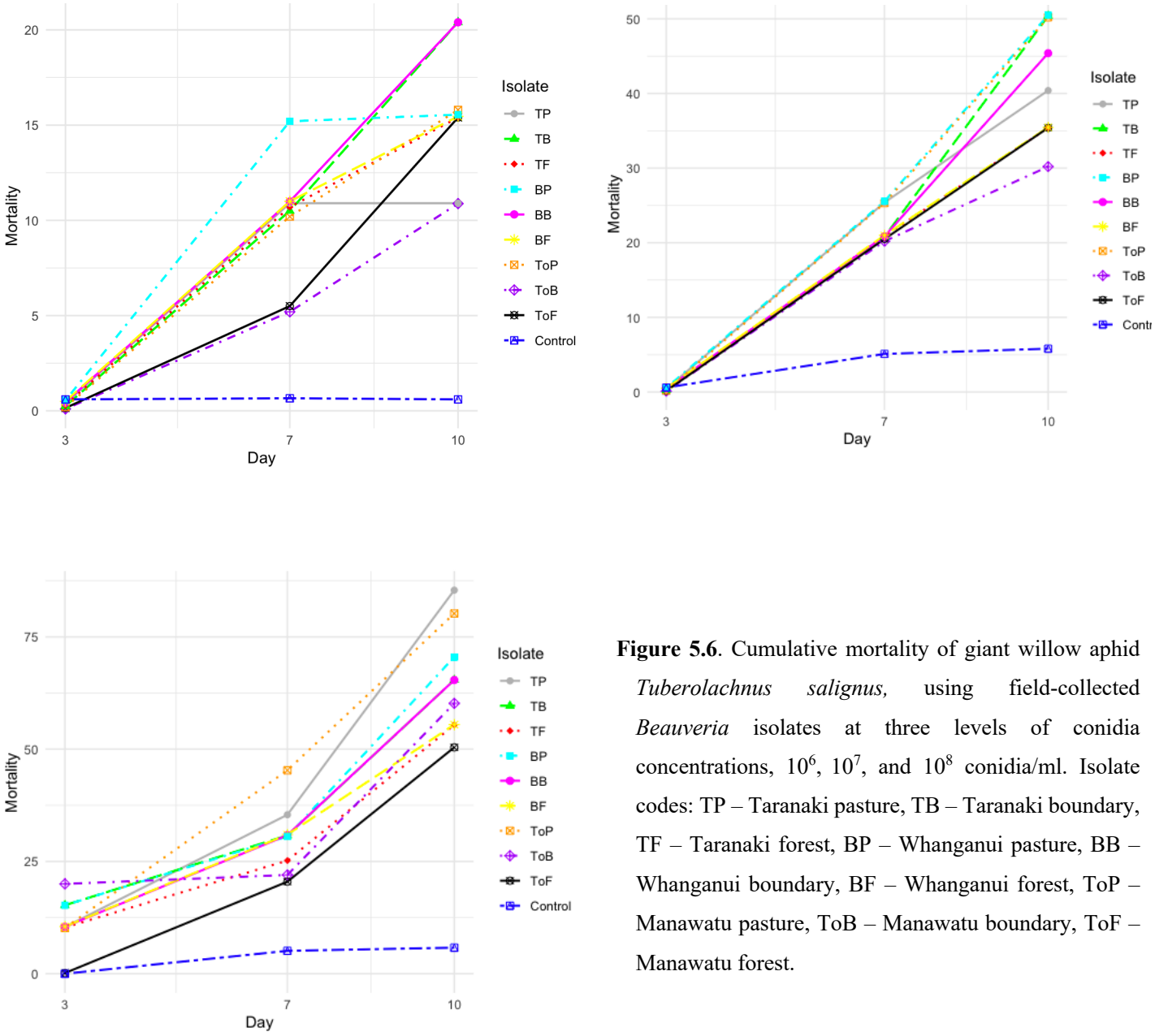


Figure 5.6. Cumulative mortality of giant willow aphid *Tuberolachnus salignus,* using field-collected *Beauveria* isolates at three levels of conidia concentrations, 10^6, 10^7, and 10^8 conidia/ml. Isolate codes: TP – Taranaki pasture, TB – Taranaki boundary, TF – Taranaki forest, BP – Whanganui pasture, BB – Whanganui boundary, BF – Whanganui forest, ToP – Manawatu pasture, ToB – Manawatu boundary, ToF – Manawatu forest.

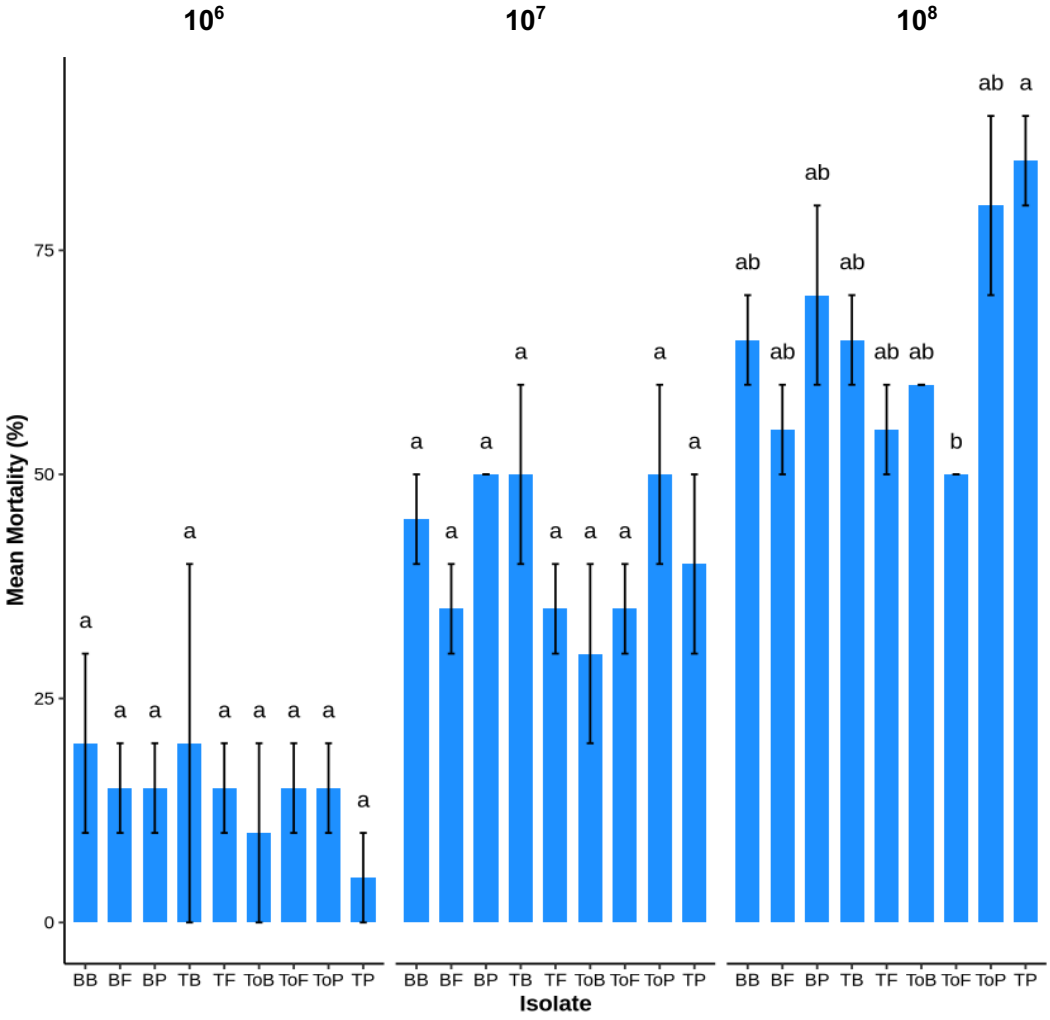


Figure 5.7. Mean mortality (% ± SE) of giant willow aphid *Tuberolachnus salignus* larvae after 14 days exposure to different *Beauveria* isolates at three concentrations: (A) 10^6 conidia/ml, (B) 10^7 conidia/ml, (C) 10^9 conidia/ml. Different letters above bars within each concentration indicate significant differences between isolates according to Tukey's HSD test ($P < 0.05$). Isolate codes: TP – Taranaki pasture, TB – Taranaki boundary, TF – Taranaki forest, BP – Whanganui pasture, BB – Whanganui boundary, BF – Whanganui forest, ToP – Manawatu pasture, ToB – Manawatu boundary, ToF – Manawatu forest.

Mean mortality by habitat. In all concentrations, the *Beauveria* isolates from pasture habitat tended to cause higher mortality of GWA. However, the mean mortality of GWA from *Beauveria* isolates sourced from different habitats was not significantly different across all concentrations ($\chi^2(2) = 0.22$, $P = 0.125$) (**Fig. 5.8**).

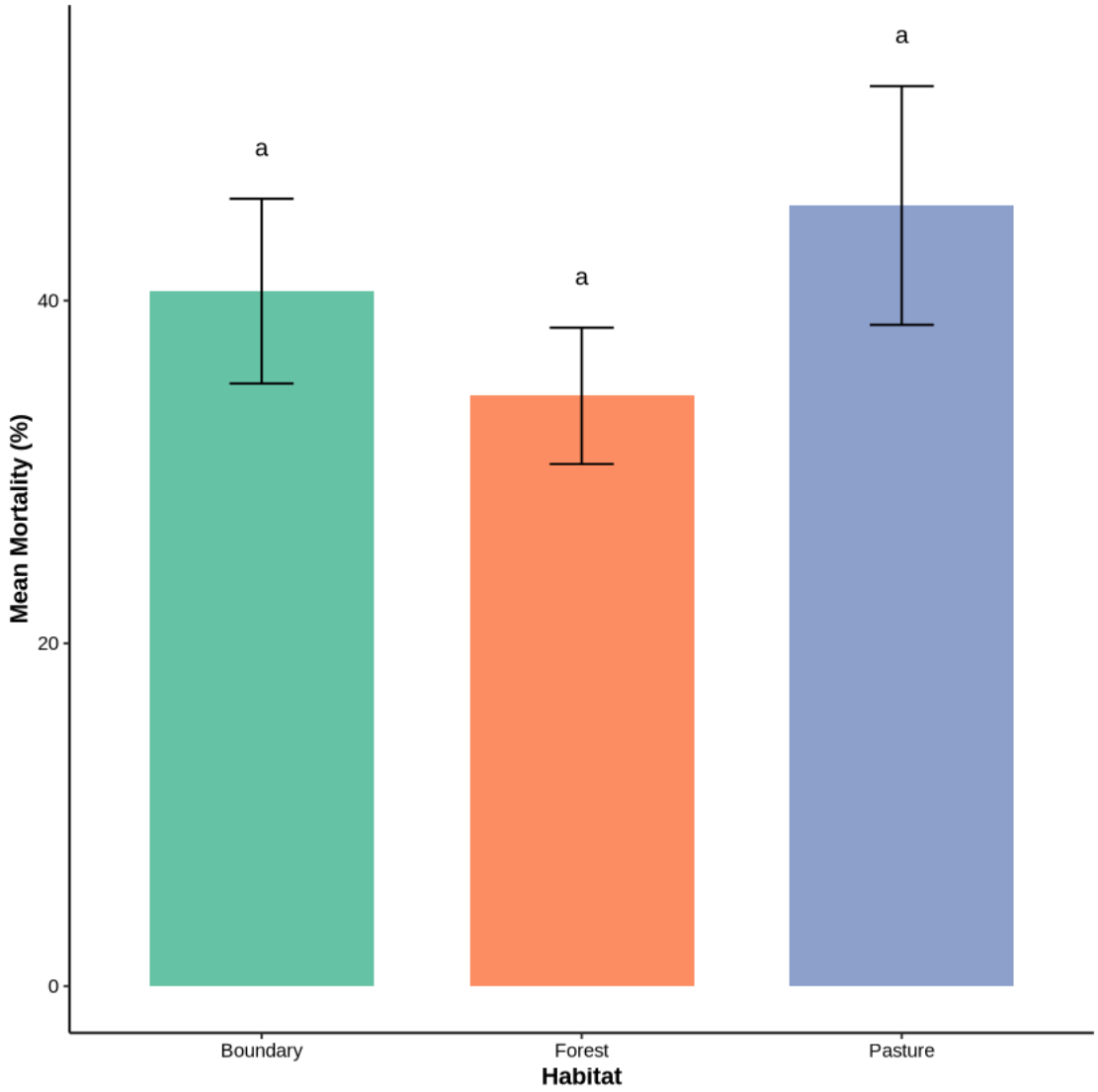


Figure 5.8. Mean mortality of giant willow aphid *Tuberolachnus salignus* at day 10, using *Beauveria* isolates obtained in different habitats through insect baiting. Different letters above bars within each concentration indicate significant differences between isolates according to Tukey's HSD test ($P < 0.05$).

Median lethal concentration (LC50) of *Beauveria* isolates against GWA. The comparative virulence of *Beauveria* isolates against GWA was assessed by determining the median lethal concentration (LC50) necessary to achieve 50% mortality. The results showed that pasture isolates, namely TP, ToP and BP, exhibited the highest virulence. These isolates required a concentration of 10^7 conidia/ml to reach the 50% mortality threshold (**Fig. 5.9**). Boundary isolates TB and BB had a moderate level of virulence, although the LC50 for both was achieved at a concentration of 10^7 conidia/ml as well, they were not as virulent as the pasture isolates. Forest isolates displayed the lowest virulence among the tested groups – to induce a 50% mortality rate within the GWA population, a concentration of more than 10^8 conidia/ml was required (**Fig. 5.9**).

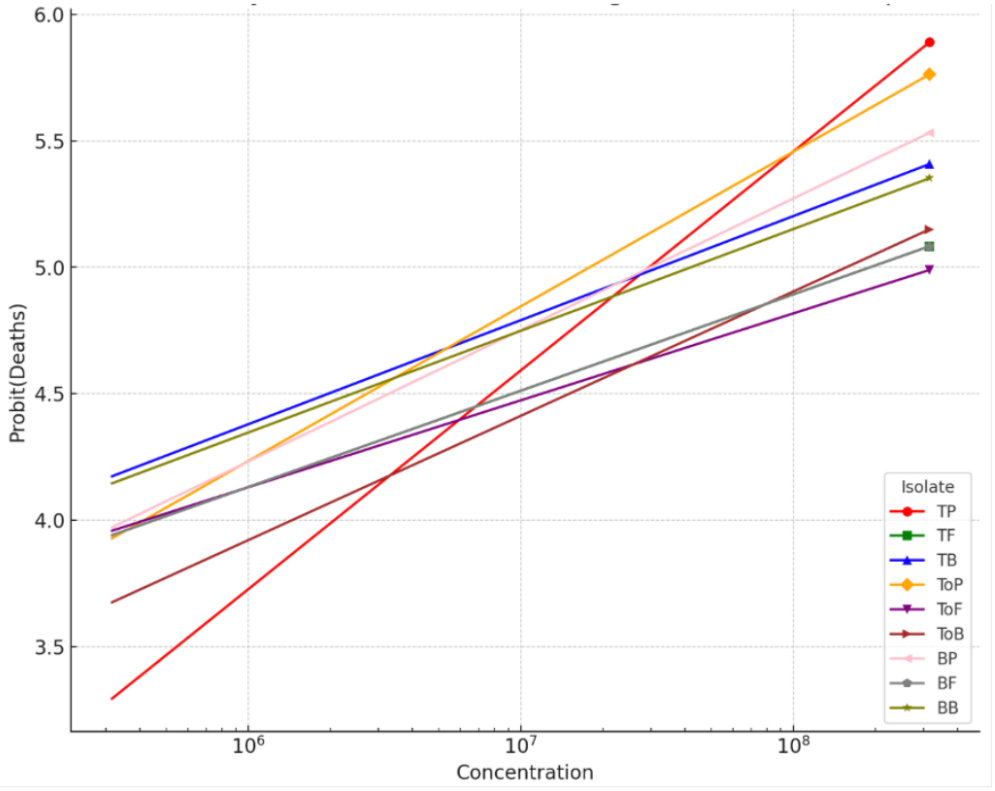


Figure 5.9. Dose-response relationships for the determination of LC50 values against giant willow aphid *Tuberolachnus salignus* using field-collected *Beauveria* isolates. Isolate codes: TP – Taranaki pasture, TB – Taranaki boundary, TF – Taranaki forest, BP – Whanganui pasture, BB – Whanganui boundary, BF – Whanganui forest, ToP – Manawatu pasture, ToB – Manawatu boundary, ToF – Manawatu forest.

Locust bioassay using field-collected* Beauveria *isolates and two commercially available EPF products.

Cumulative mortality. The mortality of locusts started at day 7 with most isolates causing lethal effects (**Fig. 5.10**). Mortality increased at day 10 with all isolates showing marked increase. On the last day of observation (day 14), isolates TP, ToP, and BP caused more than 50% mortality.

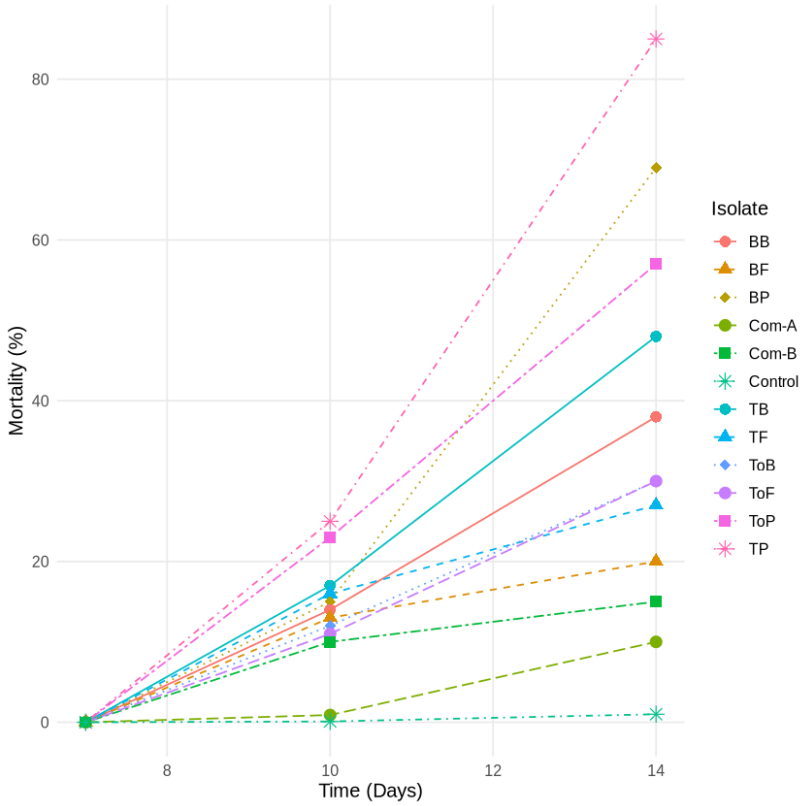


Figure 5.10. Cumulative mortality of locust *Locusta migratoria* exposed to field-collected *Beauveria* isolates at 10^9 conidia/ml concentration and to two commercially available EPF products, Com-A and Com-B. Isolate codes: BB – Whanganui boundary, BF – Whanganui forest, BP – Whanganui pasture, Com-A – Commercial Product A, Com-B – Commercial Product B, TP – Taranaki pasture, TB – Taranaki boundary, TF – Taranaki forest, ToP – Manawatu pasture, ToB – Manawatu boundary, ToF – Manawatu forest.

Mean mortality by isolate and comparison with commercial products. The isolates exhibited a wide range of virulence levels, with locust mortality rates ranging from approximately 17% to 75% (**Fig. 5.11**). The highest locust mortality rate was observed in pasture BP isolate (75% ± 3.2%), which was significantly higher than most other isolates (χ^2 (10)= 41.6, $P < 0.05$). This was followed by a pasture TP isolate, which caused 63% ± 2.8% mortality, demonstrating the second-highest virulence level among all tested isolates. Notably, the commercial isolates (Com-A and Com-B) demonstrated the lowest virulence levels among all tested isolates. Com-A and Com-B induced mortality rates of 17.3% ± 1.9% and 30% ± 2.1%, respectively, significantly lower than the BP and TP isolates (**Fig. 5.11**).

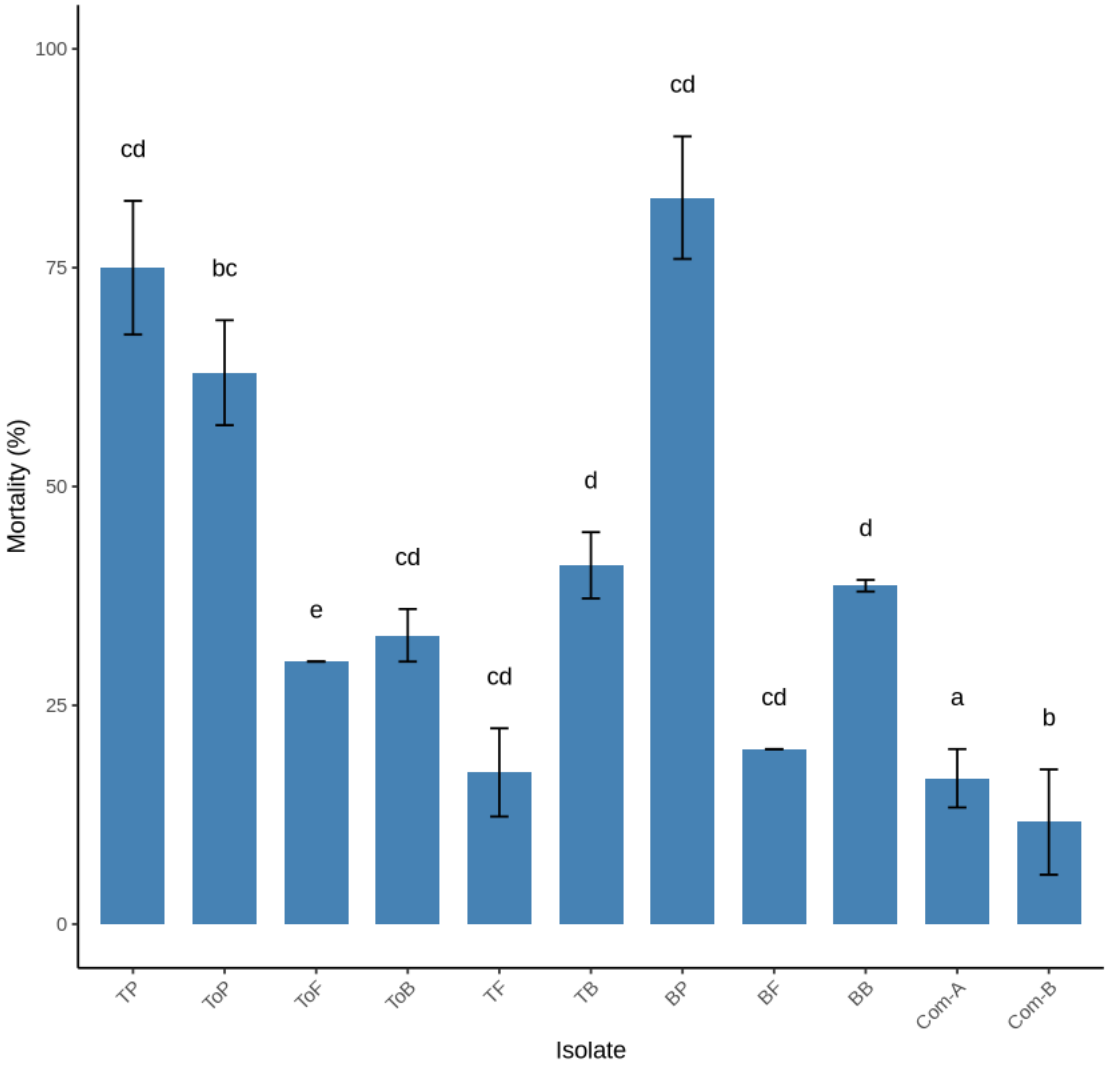


Figure 5.11 Mean mortality (% ± SE) of locust, *Locusta migratoria* at day 14 using field-collected *Beauveria* isolates at 10^9 conidia/ml concentration and to two commercially available EPF products, Com-A and Com-B. Isolate codes: BB – Whanganui boundary, BF – Whanganui forest, BP – Whanganui pasture, Com-A – Commercial Product A, Com-B – Commercial Product B, TP – Taranaki pasture, TB – Taranaki boundary, TF – Taranaki forest, ToP – Manawatu pasture, ToB – Manawatu boundary, ToF – Manawatu forest. Different letters above bars indicate significant differences between treatments (Tukey's HSD test, $P < 0.05$).

Mean mortality by habitat and comparison with commercial products. The generalized linear model (GLM) with a binomial distribution and logit link function revealed significant differences in locust mortality rates exposed to *Beauveria* isolates from different habitat types ($\chi^2(2) = 18.46$, $P < 0.001$; **Fig. 5.12**). Mortality rates in locusts exposed to pasture isolates were significantly higher than from other field-collected isolates and from commercial EPF products ($P < 0.001$). Mortality from forest isolates and commercial products was not significantly different ($P = 0.338$). The boundary isolates showed intermediate virulence between forest and pasture isolates.

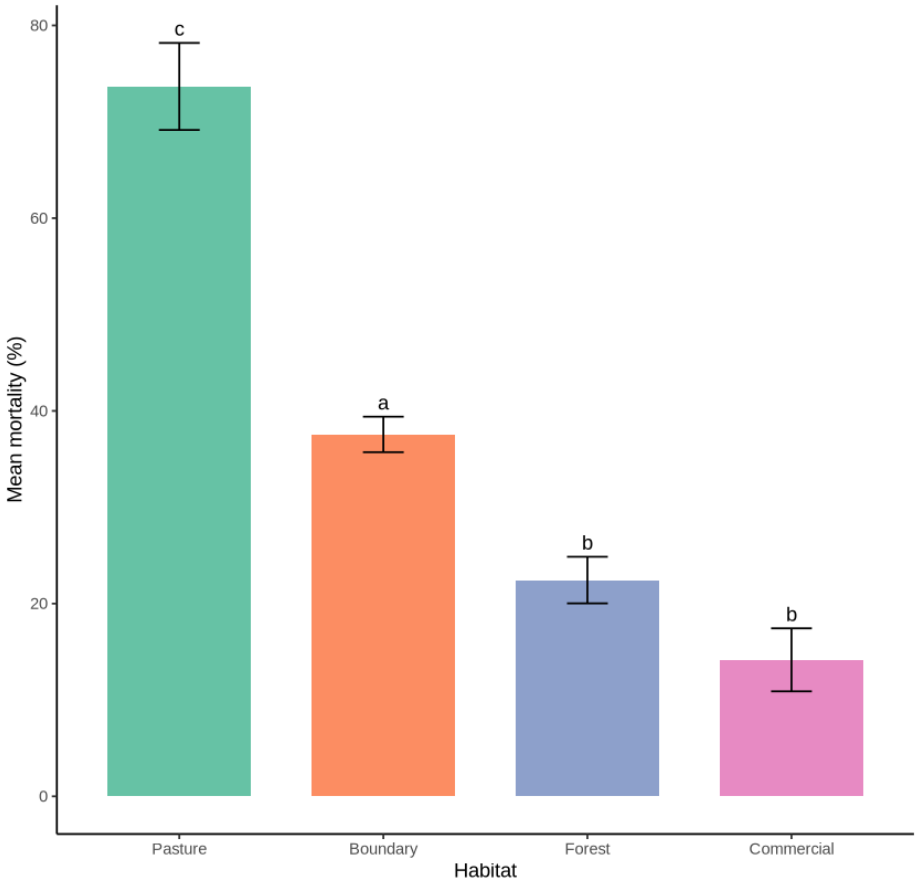


Figure 5.12 Mean mortality of locust *Locusta migratoria* at day 14 using *Beauveria* isolates obtained in different habitats through insect baiting. Different letters above bars within each concentration indicate significant differences between isolates according to Tukey's HSD test ($P < 0.05$).

Mealworm bioassay using field-collected* Beauveria *isolates and two commercially available EPF products.

Cumulative mortality. The mortality of mealworm began at 7 days after inoculation with the *Beauveria* isolates at 10^8 concentration (**Fig. 5.13**). There was a steady and progressive increase in mortality as the days progressed, with increases in mortality consistent across the various isolates. Isolates TP, TF, and TB yielded the highest mortality rates among the mealworms.

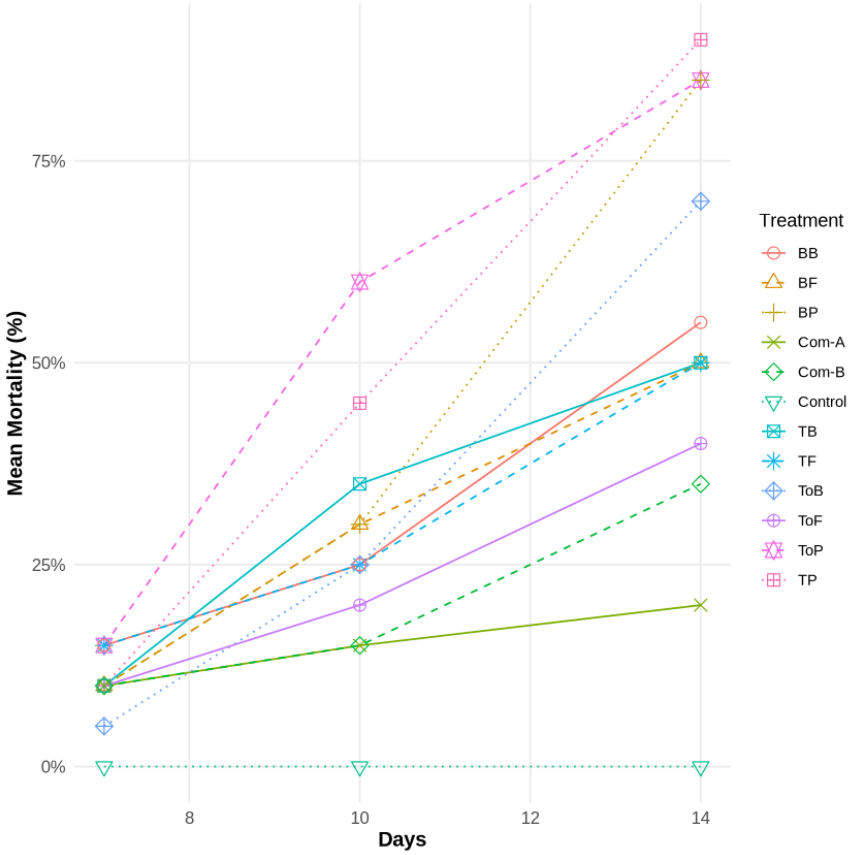


Figure 5.13. Cumulative mortality of *Tenebrio molitor*, when exposed to field-collected *Beauveria* isolates at 10^8 conidia/ml concentration and to two commercially available EPF products, Com-A and Com-B. Isolate codes: BB – Whanganui boundary, BF – Whanganui forest, BP – Whanganui pasture, Com-A – Commercial Product A, Com-B – Commercial Product B, TP – Taranaki pasture, TB – Taranaki boundary, TF – Taranaki forest, ToP – Manawatu pasture, ToB – Manawatu boundary, ToF – Manawatu forest.

Mean mortality by isolate and comparison with commercial products. The Generalized Linear Model revealed a highly significant effect of isolate on mealworm mortality (χ^2 (10) = 33.7, $P <$ 0.001), indicating that mortality rates varied substantially among isolates (Fig. 5.14). Analysis of mealworm mortality rates following exposure to different *Beauveria* isolates revealed significant variation (**Fig. 5.14**). Pasture isolate TP showed the highest mortality rates demonstrating significantly greater effectiveness ($P < 0.05$), compared to other treatments. Conversely, commercially available product, Com-A exhibited the lowest mortality rate, significantly differing from most other isolates ($P < 0.05$), indicating its comparatively limited efficacy. The pasture isolates BP and ToP showed marginally significant increase in mealworm mortality rates (+ 30%). Other isolates showed varying levels of virulence but were not significantly different ($P > 0.05$) (**Fig. 5.15**).

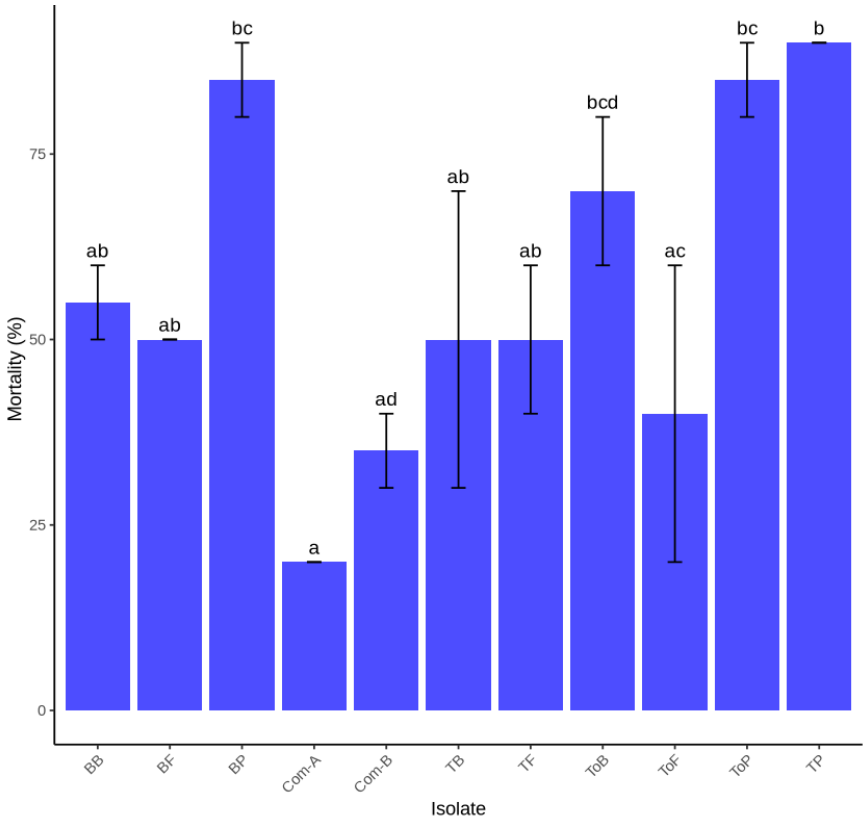


Figure 5.14. Mean mortality of mealworm *Tenebrio molitor,* after 14 days exposure to field-collected *Beauveria* isolates at 10^8 conidia/ml. Isolate codes: BB – Whanganui boundary, BF – Whanganui forest, BP – Whanganui pasture, Com-A – Commercial Product A, Com-B – Commercial Product B, TP – Taranaki pasture, TB – Taranaki boundary, TF – Taranaki forest, ToP – Manawatu pasture, ToB –

Manawatu boundary, ToF – Manawatu forest. Different letters indicate significant differences between means (Tukey's HSD test, $P < 0.05$).

Mean mortality by habitat and comparison with commercial products. The Generalized Linear Model analysis of mortality rates of mealworms exposed to field-collected *Beauveria* isolates from different habitats and commercial EPF products revealed significant differences among treatments ($\chi^2(3) = 32.5$, $P < 0.001$) (**Fig. 5.15**). With the baseline mortality rate of 58.33%, pasture isolates caused significantly higher (+28.33%) mortality ($P < 0.01$), followed by forest and boundary isolates with intermediate levels, and commercial products producing the lowest (30.83%) mortality rates ($P < 0.01$)**.**

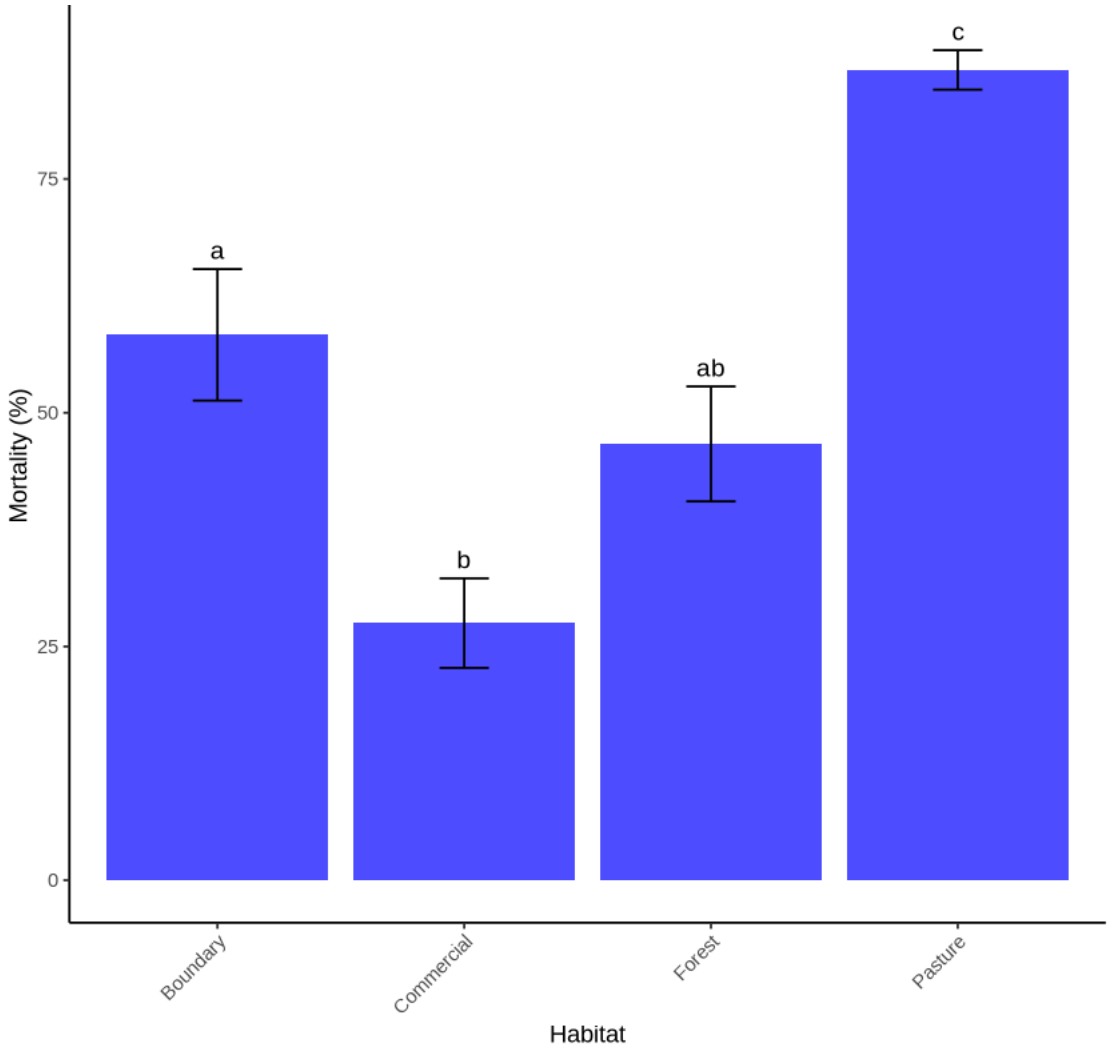


Figure 5.15. Mean mortality of mealworm *Tenebrio molitor* at day 10 using *Beauveria* isolates obtained in different habitats through insect baiting. Different letters above bars within each concentration indicate significant differences between isolates according to Tukey's HSD test ($P < 0.05$).

Ultrastructural insights of Beauveria *pathogenicity in locust and mealworm hosts*

The locust treated with the local *Beauveria* isolate TP exhibited abnormal moulting, characterized by incomplete shedding of the exoskeleton and disfigured wings (**Fig. 5.16A**). While this locust had died, no visible spore growth was observed on its cadaver. Although the death cannot be directly attributed to the EPF, it may be correlated with previous findings suggesting that EPF can disrupt the moulting cycle. A mycosed locust caused by the *Beauveria* soil isolate TP is shown in **Figure 5.16B**, displaying fungal colonization and sporulation on the cadaver.

Figure 5.16. Morphological effects of *Beauveria bassiana* pasture isolate TP from Taranaki on *Locusta migratoria*. **A.** Irregularly moulted locust with visible malformation. **B.** Mycosed cadaver exhibiting fungal colonization and sporulation, covering most of the body surface.

Scanning electron microscopy of a mycosed locust leg (**Fig. 5.17**) reveals conidia attached to rachi, indicating ongoing fungal growth and the progression toward spore dispersal. The conidia are yet to be released, suggesting that dispersal has not occurred at this stage. A dense network of mycelia on the leg demonstrates extensive colonization of the host by the fungus. The locust abdomen was also visualized in SEM from infection at day 14 (**Fig. 5.18**) to see what it looks like internally. The cross-sectional image of EPF infection revealed the extensive infiltration of fungal

hyphae within the locust's abdominal cavity. The filamentous structures of the hyphae are clearly visible, intertwined with the host's tissues, indicating a high level of colonization.

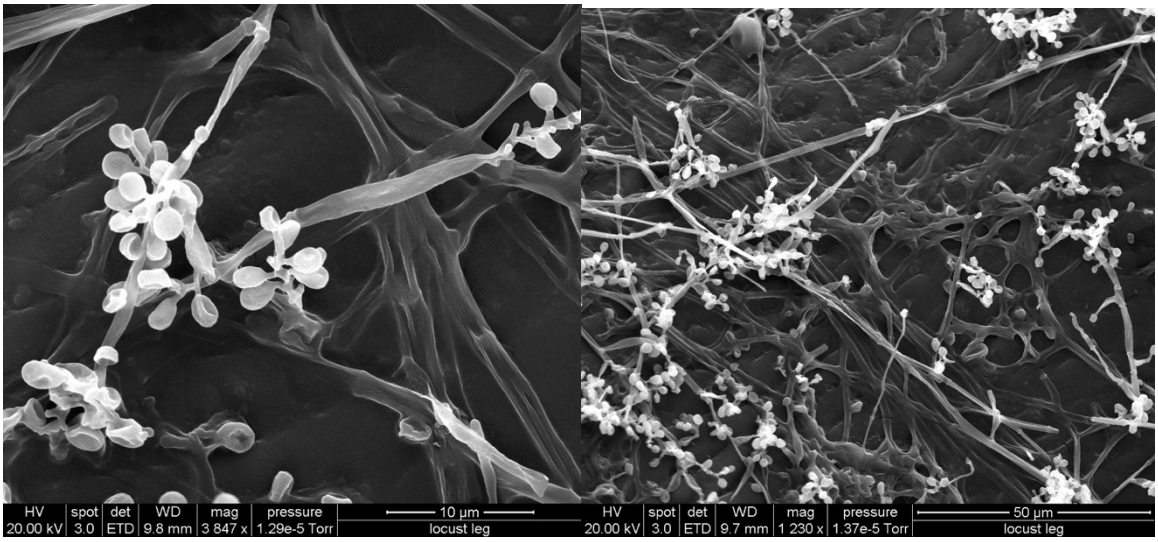


Figure 5.17. Locust leg post- *Beauveria bassiana* infection, visualized using SEM. **A**. Conidia attached to rachi. **B**. Hyphal network on the leg surface.

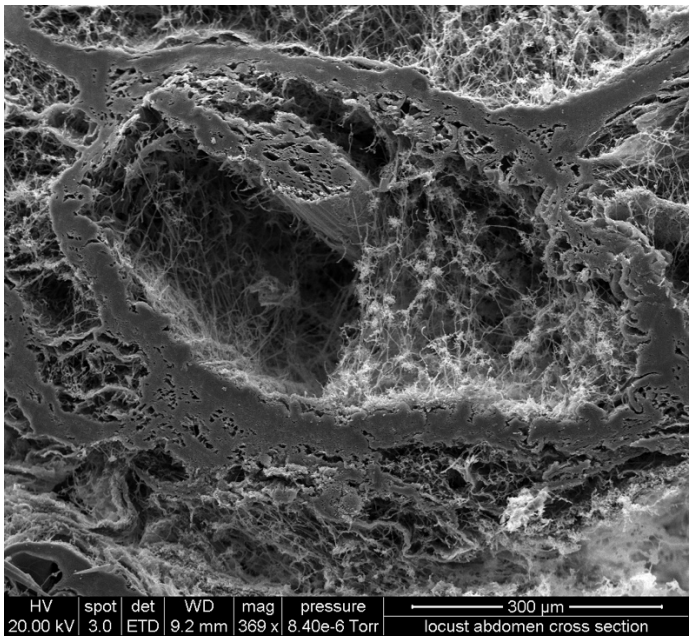


Figure 5.18. Locust (*L. migratoria*) infected with *Beauveria bassiana* pasture isolate TP from Taranaki, NZ. The cross-section of locust abdomen, showing extensive infiltration of fungal hyphae within the locust's abdominal cavity.

The SEM also provides visualization of the pathogenic interaction between *Beauveria bassiana* and the mealworm host. The SEM image reveals conidia lodged within the spiracles (**Fig. 5.19**), which are the respiratory openings of the mealworm.

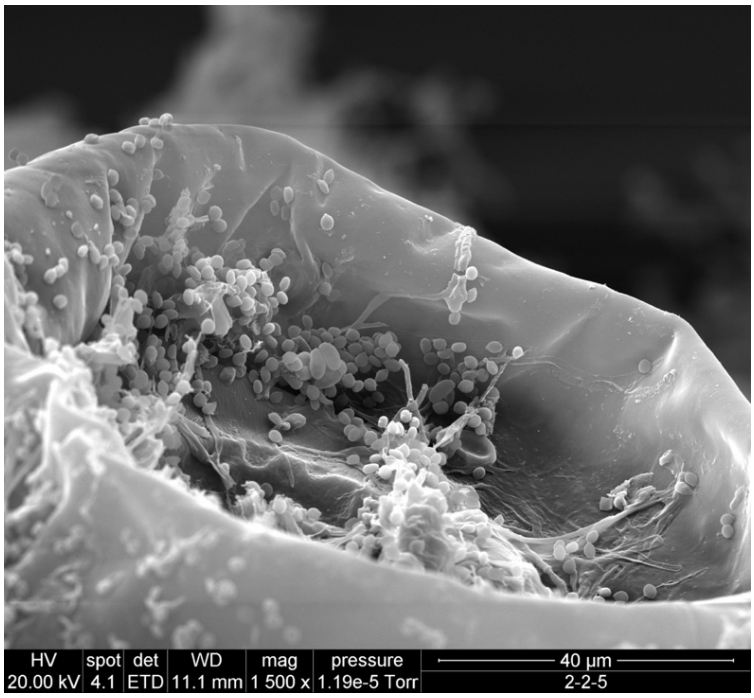


Figure 5.19. *Beauveria bassiana* hyphal growth and sporulation on mealworm (*Tenebrio molitor*) spiracle.

Discussion

Differences between isolates and sources of variation in pathogenecity

The pathogenicity of *Beauveria* isolates demonstrated significant variability, highlighting isolate-specific and host-specific nature of pathogenicity. For instance, pasture isolates such as TP and ToP consistently exhibited higher efficacy across multiple hosts, including grass grubs, locusts, and mealworms, while isolates BF and TF showed markedly lower virulence, even at elevated conidial concentrations. These findings align with those of Zemek et al. (2021) who highlighted the strain-specific characteristics of *Beauveria* virulence. The differences observed are

likely driven by genetic variability, environmental adaptation, and specific interactions with local host species, as suggested by Bidochka et al. (1998).

The observed dose-dependent mortality patterns further elucidate the relationship between conidial concentration and pathogenicity. For example, isolate TP achieved 100% mortality at 10^9 conidia/ml against grass grub larvae, but significantly lower mortality was observed at 10^6 conidia/ml, suggesting a critical threshold below which effective pest control is unattainable. Ullah et al. (2023) similarly reported that sub-lethal concentrations may not adequately overcome host immune defenses, limiting the efficacy of entomopathogenic fungi (EPF) in biocontrol applications. These findings reinforce the notion that both isolate selection and conidial concentration are critical factors in optimizing *Beauveria*-based treatments for pest control. Higher concentrations of conidia generally yield better control across most isolates, as evidenced by the significant mortality rates achieved with elevated doses. This observation is consistent with previous studies that have demonstrated a direct correlation between spore concentration and pathogenicity in various entomopathogenic fungi, including *Beauveria bassiana* (Hassan et al., 2019; Mantzoukas et al., 2019). The efficacy of higher concentrations can be attributed to the increased likelihood of successful infection and subsequent fungal proliferation within the host, which is essential for effective biocontrol.

The origin of the isolates, particularly those recovered from mealworm infections in insect bait setups, is also a significant factor influencing their virulence. These isolates demonstrated high efficacy across multiple host species, suggesting that they may possess adaptations that confer broad-spectrum virulence. This finding aligns with the work of Quesada-Moraga et al. (2024) who noted that the selection of isolates based on their environmental origins can enhance their effectiveness against a wider range of pests. The use of insect baiting as a strategy for sourcing robust biocontrol agents is particularly promising, as it allows for the identification of isolates that have evolved in the presence of diverse pest challenges, thereby enhancing their potential for effective pest management (Sharma et al., 2019).

Moreover, the adaptability of these isolates to various environmental conditions further underscores their potential utility in integrated pest management strategies. Research has shown that isolates derived from environments with high pest pressure tend to exhibit greater virulence, likely due to natural selection favouring those strains that can effectively exploit available hosts (Ullah et al., 2022). This adaptability not only enhances their efficacy but also supports the

sustainability of pest control measures by reducing reliance on chemical pesticides (Bamisile et al., 2021).

Differences in pathogenicity across habitats

A comparative analysis of *Beauveria* isolates derived from different habitats reveals significant variations in pathogenicity, with pasture-derived isolates generally demonstrating superior efficacy. For instance, pasture isolates TP and ToP consistently achieved high mortality rates across multiple hosts, such as grass grubs, locusts, and mealworms. In grass grubs, pasture isolates achieved the highest mean mortality (55%), although this was not statistically different from boundary isolates (30%), suggesting shared virulence factors between these habitats potentially due to ecological overlap or genetic similarities (Bueno-Pallero et al., 2020). Conversely, forest isolates demonstrated the lowest efficacy, likely reflecting reduced exposure to target pests in these environments.

For giant willow aphids, mortality rates were not significantly different across isolates from various habitats, indicating that habitat-specific effects were less pronounced for this host. However, in locusts and mealworms, pasture isolates significantly outperformed both forest and boundary isolates ($p < 0.05$), reflecting the influence of habitat-driven selective pressures in shaping virulence. Pasture ecosystems, characterized by high densities of soil-dwelling pests, appear to drive the evolution of more virulent fungal strains, as noted by Xiao et al. (2012). Bidochka et al. (1998) further emphasized that the distribution of EPF species could be strongly influenced by habitat-specific ecological pressures.

Boundary isolates exhibited intermediate pathogenicity, suggesting that they possess traits derived from both adjacent habitats. The transitional nature of boundary habitats may expose isolates to a mix of environmental conditions and host species, resulting in a hybrid pathogenicity profile. These findings show the importance of understanding the ecological factors that influence EPF diversity and pathogenicity, particularly in transitional zones (Bueno-Pallero et al., 2020).

The superior performance of pasture isolates could also be attributed to their adaptation to agricultural ecosystems, where soil-dwelling pests like *C. giveni* exert strong selective pressures. This evolutionary dynamic would favour fungal strains capable of overcoming host defenses, enabling them to achieve higher efficacy across a range of pests (Bava et al., 2022). Furthermore, the efficacy of pasture isolates in pest suppression supports the potential for using

these isolates as targeted biocontrol agents in agricultural systems, particularly in pastures and golf courses where soil pest damage is a significant challenge (Bueno-Pallero et al., 2020).

Interestingly, despite the observed trends, habitat-specific effects were not universal across hosts. For example, giant willow aphid mortality rates were comparable across habitats, suggesting that some pests are less influenced by habitat-derived differences in EPF pathogenicity. This variability highlights the complex interplay between host susceptibility and fungal pathogenicity and underscores the need for host-specific studies when developing biocontrol strategies (Vega et al., 2009).

The critical role of habitat-specific factors in shaping EPF virulence and their implications for biological pest control is evident in the results of this chapter. The superior efficacy of pasture-derived isolates highlights their potential as robust biocontrol agents, while the intermediate performance of boundary isolates suggests a promising role in transitional zones. These results underscore the necessity of integrating ecological insights into the development of targeted pest management strategies (Clifton et al., 2015).

Ultrastructural insights

Scanning Electron Microscopy (SEM) analysis has provided critical insights into the infection mechanisms employed by *Beauveria* isolates. In locusts, SEM revealed extensive hyphal colonization within the abdominal cavity, indicative of systemic fungal invasion leading to host mortality. These findings align with the work of Camara et al. (2022), who studied the effectiveness of *Beauveria* strains against locusts, highlighting the role of fungal invasion in host mortality.

In mealworms, conidia were found lodged within spiracles, disrupting respiratory functions and providing an alternative route for infection. This mechanism highlights the adaptive strategies of *Beauveria* in circumventing host defenses, showcasing its ability to exploit respiratory entry points. The ability to utilize multiple infection routes emphasizes the potential of pasture-derived isolates for broad-spectrum pest control, as noted by Bustamante et al. (2019). This adaptability not only enhances the virulence of these isolates but also suggests a robust evolutionary response to the selective pressures imposed by their environments.

Moreover, the interaction between *Beauveria* isolates and their insect hosts is not merely a one-way process; the host's immune response also plays a significant role in shaping the infection dynamics. Research has shown that insects possess a range of immune mechanisms to combat fungal infections, including the production of antimicrobial peptides and the activation of phagocytic cells (Wang et al., 2021). The ability of *Beauveria* to evade or suppress these immune responses is crucial for its success as a biocontrol agent. For example, the production of specific enzymes that degrade chitin (Raya-Díaz et al., 2017) This enzymatic activity not only aids in overcoming physical barriers but also contributes to the overall virulence of the fungus (Wang et al., 2021).

Superior performance of field-collected isolates and implications for pest management

The superior performance of pasture-derived *Beauveria* isolates, such as TP and ToP, compared to commercial EPF products underscores their significant potential for targeted pest management strategies. Commercial products like Com-A and Com-B exhibited limited efficacy against tested hosts, including locusts and mealworms, with mortality rates ranging from 0% to 30%. These highlights the ineffectiveness of the NZ commercially available EPF products compared to the field-collected isolates against locusts. These findings raise concerns about the efficacy of current commercial products in biological control applications (Mark et al., 2006).

Field-collected isolates, particularly TP and ToP, consistently demonstrated superior efficacy, achieving mortality rates as high as 100% at optimal concentrations. Qayyum et al. (2021) emphasized that native isolates often demonstrate higher virulence due to their adaptation to local pests and environmental conditions, further supporting the use of field-collected strains in pest management strategies. The potential of pasture-derived isolates to serve as cost-effective alternatives to current products is particularly relevant for managing high-value crops or pest-prone ecosystems. Soth et al. (2022) further demonstrated that mixtures of *Beauveria* isolates could enhance pest control efficacy, suggesting that combining field-collected isolates with complementary biocontrol agents may provide synergistic benefits. Moreover, indigenous *Beauveria* strains, such as those studied by Bueno-Pallero et al. (2020) and Soth et al. (2022), have shown significant virulence against a range of pests. Their adaptability to local environmental conditions makes them particularly effective for biological control applications.

Conclusions

Soil habitat plays a crucial role in the efficacy and virulence of EPF against insect pests. Variability in EPF virulence and efficacy is influenced by the fungi's origin and the target pest, highlighting the need for selecting habitat-specific strains. Pasture-derived EPFs, with their heightened virulence, are particularly effective against soil grubs, suggesting an evolutionary adaptation for pest control. The superior efficacy of pasture-derived isolates as biological control agents can be attributed to the evolutionary pressures within pasture ecosystems that select for aggressive strains capable of overcoming host defenses. Utlizing habitat-collected isolates presents a valuable opportunity for targeted pest management, especially in agricultural settings where rapid and high-impact solutions are crucial.

Chapter Six

General discussion and recommendations for future work

Comprehensive assessment of entomopathogenic fungi diversity through integrated approaches

Assessing the diversity of entomopathogenic fungi (EPF) through an integrative approach that combines traditional insect baiting, semi-selective medium plating, and advanced molecular techniques such as metagenomics and metabarcoding offers a robust and comprehensive understanding of EPF diversity within soil ecosystems. This comprehensive approach, as emphasized by Masoudi et al. (2020), not only facilitates the identification of dominant culturable EPF isolates but also uncovers a significantly broader spectrum of fungal taxa, including rare and fastidious species that traditional methods alone fail to detect, offering a more complete and nuanced understanding of EPF diversity and population dynamics. Such an integrative strategy highlights the importance of employing complementary methods to capture the complexity of EPF biodiversity within soil ecosystems (Campos-Herrera & Lacey, 2018; Bueno-Pallero et al., 2020).

This thesis provided a more holistic understanding of EPF diversity and their ecological roles, affirming the necessity of these integrative methodologies in microbial ecology research. Insect baiting and plating techniques were used to establish a foundational understanding of EPF presence in the adjacent habitats (Chapter 2). Chapter 3 resolved the species, which were limited to primarily *Beauveria bassiana* and *Metarhizium novozealandicum*.

Molecular advancements, such as metabarcoding enhances our understanding of EPF diversity in soil ecosystems (Masoudi et al., 2020). In Chapter 4, advanced molecular tools revealed the further hidden diversity of EPF in forest and pasture soils. Shotgun sequencing revealed that EPF within the order Hypocreales were predominant in both forest and pasture soils, while the order Entomophthorales appeared in lower abundance. Metagenomics also identified additional *Beauveria* species, including *B. brongniartii*, *B. malawiensis,* and *B. caledonica*, as well as a wide range of *Metarhizium* species, such as *M. anisopliae*, *M. robertsii*, *M. brunneum*, *M. acridum*, *M. rileyi*, *M. guizhouense*, *M. flavoviride*, and *M. majus*. Other EPF genera, such as *Cordyceps*, *Lecanicillium*, and *Ophiocordyceps*, were also detected. Complementary metabarcoding analyses using ITS2 amplified DNA revealed that *Lecanicillium* species were more

abundant than *Metarhizium* and *Beauveria*, offering further insights into community composition. The high abundance of *Lecanicillium* only in the Taranaki region underscores the site-specific nature of EPF distribution, which may be attributed to unique ecological or environmental conditions. Masoudi (2020) demonstrated that *Metarhizium anisopliae* (sensu lato), *Isaria farinosa*, and *Beauveria bassiana* were consistently abundant as core operational taxonomic units in their soil sampling sites.

High-throughput sequencing (HTS) has become a useful tool for exploring microbial diversity in both agricultural and natural environments (Abdelfattah et al., 2017). For instance, metagenomics has been successfully employed by Friedl & Druzhinina (2012) to detect *Trichoderma*, a fungal genus widely recognized for its role in biological control (Howell, 2003), providing valuable data for the development of products targeting soil-borne pathogens. Similarly, profiling EPF in soils using both metagenomics and metabarcoding, as demonstrated in this thesis, not only identifies potential reservoirs for biocontrol agents but also provides valuable insights for developing strategies to integrate these fungi into effective pest management systems.

Comprehensive methods are essential for detecting and understanding the distribution of EPF, as this knowledge is valuable for developing effective biological control strategies. The integration of traditional and molecular methodologies provides a robust framework for assessing EPF diversity. This comprehensive approach not only addresses the limitations of conventional techniques but also advances our understanding of the ecological roles and habitat-specific dynamics of EPF, contributing valuable knowledge to microbial ecology and sustainable pest management.

Habitat influence on EPF abundance

This thesis highlights the critical role of habitat type in shaping the abundance and distribution of entomopathogenic fungi (EPF) across adjacent forest, boundary, and pasture soils, with the highest EPF prevalence observed in forests, followed by forest-pasture boundary zones, and the lowest in pastures. Forest habitats provide nutrient-rich organic matter and stable microclimatic conditions, such as high humidity and consistent temperatures, which support fungal growth and activity (Li, 2022). In contrast, agricultural practices in pastures, including soil disturbance, chemical inputs, and reduced organic content, disrupt EPF populations and limit their diversity (Tkaczuk et al., 2013; Clifton et al., 2015). The boundary habitat, or ecotone, benefits

from habitat heterogeneity and the edge effect, which enhance biodiversity and create favourable conditions for EPF, supported by the convergence of species from forest and pasture ecosystems These findings are consistent with previous studies showing higher EPF prevalence in undisturbed or semi-natural habitats compared to agricultural lands (Qeusada-Moraga et al., 2007; Sharma et al., 2018). The ecological implications of these results underscore the importance of forested and transitional zones as reservoirs for EPF, which serve as natural pest regulators and contribute to soil health through nutrient cycling and organic matter decomposition (Li, 2022). Understanding these dynamics is crucial for integrating EPF into sustainable pest management strategies and enhancing biodiversity in both agricultural and natural systems.

Importance of local EPF isolates for biological control

This thesis highlights the enhanced pathogenicity of local entomopathogenic fungi (EPF) isolates. For instance, pasture-derived isolates consistently achieved high mortality rates across multiple pest species, showcasing their potential as robust, cost-effective alternatives to commercial biocontrol agents (Imoulan et al., 2011; Mehrmoradi et al., 2022). These findings align with the growing evidence that local strains, shaped by habitat-specific ecological pressures, offer distinct advantages in biological pest management (Rosa et al., 2020; Gul et al., 2022). A deeper understanding of entomopathogen ecology is essential for crafting well-informed strategies to enhance the field effectiveness of these fungi. The implications of utilizing local isolates for pest control are profound, as they not only enhance pest management efficacy but also promote ecological sustainability by reducing reliance on synthetic pesticides (Wakil et al., 2013). Interestingly, boundary isolates exhibited intermediate pathogenicity, reflecting their transitional nature and exposure to mixed environmental conditions and host species from adjacent habitats However, habitat-specific effects were not universal across all hosts, emphasizing the importance of understanding habitat-driven ecological dynamics and tailoring biocontrol strategies to target specific pests and environments (Bueno-Pallero et al., 2020).

Future work

The study of EPF in their natural habitats has yielded considerable insights into their biodiversity and potential as biological control agents. However, there is much more to add to the

current understanding of EPF distribution, interaction with hosts, and effectiveness in pest control. Future research should focus on several key areas to enhance our knowledge and application of EPF in various ecological and agricultural settings:

1. **Expanded metagenomics and metabarcoding analyses and utilizing metatransciptomics.** Future studies should incorporate comprehensive metagenomics, metabarcoding, and metatranscriptomics analyses across a broader sample set to capture a more detailed and dynamic understanding of EPF community structure, diversity, and functionality. Samples for High Throughput Sequencing (HTS) should include not only adjacent habitats such forests, and pastures but also transitional zones, which could reveal critical ecological interactions and gradients. Expanding the metabarcoding approach with additional molecular markers could enhance taxonomic resolution and better characterize fungal communities. Furthermore, integrating metatranscriptomics would provide valuable insights into gene expression patterns and functional activity of EPF under different environmental and land-use conditions.

2. **Ecotone and edge effect dynamics.** The boundary habitat, or ecotone, presents a unique ecological niche that warrants further investigation. Future work should focus on the edge effect, habitat heterogeneity and reduced disturbance in these zones to understand their role in supporting EPF diversity and intermediate pathogenicity. Examining changes in EPF populations in boundary habitats over time could provide insights into how these transitional zones act as reservoirs for both biodiversity and pest management.

3. **Field-Level efficacy studies.** Expanding on the laboratory-based findings, field trials are essential to evaluate the efficacy of local EPF isolates in the field against key agricultural pests. These studies should consider diverse agricultural systems and environmental conditions to assess the isolates' performance, environmental persistence, and compatibility with existing integrated pest management strategies. Collaborating with farmers and agricultural practitioners could enhance the relevance and scalability of the findings.

4. **Formulation development for pasture-derived isolates.** Given the demonstrated high pathogenicity of pasture-derived isolates, future research should focus on developing and

testing formulations optimized for field application. This includes evaluating shelf life, ease of application, and environmental stability under various conditions. Formulations should also be tested for compatibility with other biocontrol agents and for their potential to reduce reliance on chemical pesticides.

5. **Bioefficacy trials and evolutionary dynamics.** Further bioefficacy trials are needed to validate the observed pathogenicity trends and confirm whether pasture habitats exert selective pressures favouring more virulent EPF strains. Integrating evolutionary and genetic analyses could reveal adaptations specific to pasture-derived isolates, shedding light on the ecological and evolutionary dynamics driving these patterns. Comparative studies between isolates from different habitats and pest species would further refine our understanding of EPF-host interactions and inform the development of targeted pest management strategies.

References

Abarenkov, K., Nilsson, R.H., Larsson, K.-H., Taylor, A.F.S., May, T.W., Frøslev, T.G., Pawlowska, J., Lindahl, B., Põldmaa, K, Truong, C., Vu D., Hosoya, T., Niskanen, T., Piirmann, T., Ivanov, F., Zirk, A., Peterson, M., Cheeke, T.E., Ishigami, Y., Jansson, A.T., Jeppesen, T.S., Kristiansson E., Mikryukov V., Miller J.T., Oono, R., Ossandon, F.J., Paupério, J., Saar, I., Schigel, D., Suija, A., Tedersoo, L., & Kõljalg, U. (2023). The UNITE database for molecular identification and taxonomic communication of fungi and other eukaryotes: sequences, taxa and classifications reconsidered. *Nucleic Acids Research*, https://doi.org/10.1093/nar/gkad1039

Abbott, W. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*, 18(2), 265-267.

Abdelfattah, A., Malacrino A., Wisniewski M., & Cacciola S. (2018). Metabarcoding: A powerful tool to investigate microbial communities and shape future plant protection strategies. *Biological Control*, 120, 1-10.

Afandhi, A., Pertiwi, E., Purba, D., Widjayanti, T., & Leksono, A. (2020). The diversity of entomopathogenic fungi collected from leaves and rhizospheres of rice implementing integrated pest management. *Biodiversitas Journal of Biological Diversity*, 21(6).

Ahsan, S. M., Injamum-Ul-Hoque, M., Das, A. K., Rahman, M. M., Mollah, M. M. I., Paul, N. C., & Choi, H. W. (2024). Plant–entomopathogenic fungi interaction: recent progress and future prospects on endophytism-mediated growth promotion and biocontrol. *Plants, 13*(10), 1420.

Alizadeh, H., Kandula, D. R., Hampton, J. G., Stewart, A., Leung, D. W., Edwards, Y., & Smith, C. (2017). Urease producing microorganisms under dairy pasture management in soils across New Zealand. *Geoderma Regional, 11*, 78-85.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., & Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, 215(3), 403-410.

Andrews S. (2010). FastQC: a quality control tool for high throughput sequence data. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Attua, E. M., & Pabi, O. (2013). Tree species composition, richness and diversity in the northern forest-savanna ecotone of Ghana. *Journal of Applied Biosciences*, 69, 5437-5448.

Bava, R., Castagna, F., Piras, C., Musolino, V., Lupia, C., Palma, E., Britti, D., & Musella, V. (2022). Entomopathogenic fungi for pests and predators control in beekeeping. *Veterinary Sciences*, 9(2), 95. https://doi.org/10.3390/vetsci9020095

Baldrian, P., Kolařík, M., Štursová, M., Kopecký, J., Valášková, V., Větrovský, T., . . . Vlček, Č. (2012). Active and total microbial communities in forest soil are largely different and highly stratified during decomposition. *The ISME journal,* 6(2), 248-258.

Bamisile, B.S., Akutse, K.S., Siddiqui, J.A., & Xu, Y. (2021). Model application of entomopathogenic fungi as alternatives to chemical pesticides: Prospects, challenges, and insights for next-generation sustainable agriculture. *Frontiers in Plant Science*, *12*, 741804. https://doi.org/10.3389/fpls.2021.741804

Barker, C.W., & Barker, G.M. (1998). Generalist entomopathogens as biological indicators of deforestation and agricultural land use impacts on Waikato soils. *New Zealand Journal of Ecology, 22*, 189-196.

Barrios, E. (2007). Soil biota, ecosystem services and land productivity. *Ecological Economics*, *64*, 269-285.

Barta, M., Lalík, M., Rell, S., Kunca, A., Horáková, M.K., Mudrončeková, S., & Galko, J. (2019). Hypocrealean Fungi Associated with *Hylobius abietis* in Slovakia, Their Virulence against Weevil Adults and Effect on Feeding Damage in Laboratory. *Forests*, *10*(8), 634.

Bich, G.A., Castrillo, M.L., Kramer, F.L., Villalba, L., & Zapata, P.D. (2021). Morphological and molecular identification of entomopathogenic fungi from agricultural and forestry crops. *Floresta e Ambiente, 28*(2). https://doi.org/10.1590/2179-8087-floram-2018-0086

Bidochka, M.J., Kasperski, J.E., & Wild, G.A. (1998). Occurrence of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* in soils from temperate and near-northern habitats. *Canadian Journal of Botany, 76*(7), 1198-1204.

Birkhofer, K., Bezemer, T.M., Bloem, J., Bonkowski, M., Christensen, S., Dubois, D., Ekelund, F., Fließbach, A., Gunst, L., Hedlund, K., Mäder, P., Mikola, J., Robin, C., Setälä, H., Tatin-Froux, F., Van der Putten, W.H., & Scheu, S. (2008). Long-term organic farming fosters below and aboveground biota: Implications for soil quality, biological control and productivity. *Soil Biology and Biochemistry,* 40, 2297-2308.

Bischoff, J.F., Rehner, S.A., & Humber, R.A. (2009). A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia*, 101(4), 512–530.

Blond, C.L., Ridgway, H.J., Brownbridge, M., Chapman, R.B., Condron, L.M., Saville, D.J., & Glare, T.R. (2018). Non-host larvae negatively impact persistence of the entomopathogen *Beauveria bassiana* in soil. *Journal of Invertebrate Pathology, 156*, 19-28.

Blond, C. (2012). *Aspects of the ecology and population dynamics of the fungus Beauveria bassiana strain F418 in soil*(PhD thesis). Lincoln University, New Zealand.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., …

Caporaso, J.G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology, 37*(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Böllmann, J., Elmer, M., Wöllecke, J., Raidl, S., & Hüttl, R.F. (2010). Defensive strategies of soil fungi to prevent grazing by *Folsomia candida* (Collembola). *Pedobiologia, 53*(2), 107-114.

Boucias, D.G., Pendland, J.C. & Latge, J.P. (1988). Nonspecific factors involved in attachment of entomopathogenic deuteromycetes to host insect cuticle. *Applied and Environmental Microbiology*, 54(7), 1795-1805.

Bradford, T.M., Morgan, M.J., Lorenz, Z., Hartley, D.M., Hardy, C.M., & Oliver, R.L. (2013). Microeukaryote community composition assessed by pyrosequencing is associated with light availability and phytoplankton primary production along a lowland river. *Freshwater Biology,* 58, 2401–2413.

Brownbridge, M., Nelson, T., Hackell, D., Eden, T., Wilson, D., Willoughby, B., & Glare, T. (2006). Field application of biopolymer-coated *Beauveria bassiana* F418 for clover root weevil (*Sitona lepidus*) control in Waikato and Manawatu. *New Zealand Plant Protection, 59*, 304–311.

Bruns, T.D., White, T.J., & Taylor, J.W. (1991). Fungal molecular systematics. *Annual Review of Ecology and Systematics, 22*(1), 525-564.

Bruns, T.D., & Shefferson, R.P. (2004). Evolutionary studies of ectomycorrhizal fungi: recent advances and future directions. *Canadian Journal of Botany, 82*(8), 1122-1132.

Budiarti, L. & Nuryanti, N. (2022). Entomopathogenic fungus from rice rhizosphere soil in Politeknik Negeri Lampung Land. *Iop Conference Series Earth and Environmental Science*, 1012(1), 012059.

Buée, M., Reich, M., Murat, C., Morin, E., Nilsson, R., Uroz, S., & Martin, F. (2009). 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. *New Phytologist,* 184(2), 449-456.

Bueno-Pallero, F.Á., Blanco-Pérez, R., Vicente-Díez, I., Rodríguez Martín, J.A., Dionísio, L., & Campos-Herrera, R. (2020). Patterns of occurrence and activity of entomopathogenic fungi in the Algarve (Portugal) using different isolation methods. *Insects*, 11*(6),* 352. https://doi.org/10.3390/insects11060352

Bustamante, D.E., Oliva, M., Leiva S., Mendoza, J.E., Bobadilla, L., Angulo, G., & Calderon, M.S. (2019). Phylogeny and species delimitations in the entomopathogenic genus *Beauveria* (Hypocreales, Ascomycota), including the description of *B. peruviensis* sp. nov. *MycoKeys*. 9, 58, 47-68. doi: 10.3897/mycokeys.58.35764. PMID: 31565026; PMCID: PMC6746742.

Cai, Q., Tian, L., Xiè, J., & Keyhani, N. (2022). Contributions of a histone deacetylase (sirt2/hst2) to *Beauveria bassiana* growth, development, and virulence. *Journal of Fungi*, 8(3), 236. https://doi.org/10.3390/jof8030236

Camara, I., Cao, K., Sangbaramou, R. *et al.* (2022). Virulence analysis of novel *Beauveria bassiana* strains isolated from three different climatic zones against *Locusta migratoria*. *International Journal of Tropical Insect Science, 42*, 2877–2886.

Campos-Herrera, R., & Lacey, L. A. (2018). Methods for studying the ecology of invertebrate diseases and pathogens. In A. E. Hajek & D. I. Shapiro-Ilan (Eds.), *Ecology of invertebrate diseases* (pp. 19–48). Cornell University, Ithaca, NY, & USDA-ARS, Byron, GA.

Chen, Q., Yang, F., & Cheng, X. (2022). Effects of land use change type on soil microbial attributes and their controls: Data synthesis. *Ecological Indicators*, *138*, 108852. https://doi.org/10.1016/j.ecolind.2022.108852

Clerk, G.C. (1969). Influence of soil extracts on the germination of conidia of the fungi *Beauveria bassiana* and *Paecilomyces farinosus*. *Journal of Invertebrate Pathology*, 13, 120-124.

Clifton, E.H., Jaronski, S.T., Hodgson, E.W., & Gassmann, A.J. (2015). Abundance of soil-borne entomopathogenic fungi in organic and conventional fields in the Midwestern USA with an emphasis on the effect of herbicides and fungicides on fungal persistence. *PLoS ONE, 10*(7)

Chandler, D., Hay, D., & Reid, A. (1997). Sampling and occurrence of entomopathogenic fungi and nematodes in UK soils. *Applied Soil Ecology, 5*(2), 133-141.

Chandler, J.A., Liu, R.M., & Bennett, S.N. (2015). RNA shotgun metagenomic sequencing of northern California (USA) mosquitoes uncovers viruses, bacteria, and fungi. *Frontiers in Microbiology*, *6*, 129043. https://doi.org/10.3389/fmicb.2015.00185

Cummings, N.J. (2009). *Entomopathogenic fungi in New Zealand native forests: The genera Beauveria and Isaria* (PhD thesis). University of Canterbury, New Zealand.

Dela Rosa, W., Alatorre, R., Barrera, J. F., & Toriello, C. (2000). Effect of *Beauveria bassiana* and *Metarhizium anisopliae* (Deuteromycetes) upon the coffee berry borer (Coleoptera: Scolytidae) under field conditions. *Journal of Economic Entomology, 93*(5), 1409–1414.

Deka, B., Baruah, C., & Babu, A. (2021). Entomopathogenic microorganisms: their role in insect pest management. *Egyptian Journal of Biological Pest Control*, 31(1). https://doi.org/10.1186/s41938-021-00466-7

Devi, K. U., Reineke, A., Reddy, N. N. R., Rao, C. U. M., & Padmavathi, J. (2006). Genetic diversity, reproductive biology, and speciation in the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin. *Genome, 49*, 495-504.

Dimitrov, M. R., Veraart, A. J., Smidt, H., & Kuramae, E. E. (2017). Successive DNA extractions improve characterization of soil microbial communities. *PeerJ, 5*, e2915. https://doi.org/10.7717/peerj.2915

Domsch, K.H., Gams, W., & Anderson, T.H. (1980). *Compendium of soil fungi*. Academic Press.

Donovan, P.D., Gonzalez, G., Higgins, D.G., Butler, G., & Ito, K. (2018). Identification of fungi in shotgun metagenomics datasets. *PLoS ONE, 13*(2), e0192898. https://doi.org/10.1371/journal.pone.0192898

Dong, T., Zhang, B., Jiang, Y., & Hu, Q. (2016). Isolation and classification of fungal whitefly entomopathogens from soils of Qinghai-Tibet plateau and Gansu corridor in China. PLoS ONE, 11(5), e0156087. https://doi.org/10.1371/journal.pone.0156087

Dromph, K.M. (2001). Dispersal of entomopathogenic fungi by collembolans. *Soil Biology and Biochemistry*, 33(15), 2047-2051.

Dromph, K. M. (2003). Collembolans as vectors of entomopathogenic fungi. *Pedobiologia*, 47(3), 245-256.

Duffy, J.E. (2009). Why biodiversity is important to the functioning of real-world ecosystems. *Frontiers in Ecology and the Environment, 7*(8), 437–444

Ennis, K. K., & Philpott, S. M. (2019). Seasonal and microhabitat differences alter ant predation of a globally disruptive coffee pest. *Agriculture, Ecosystems & Environment*, *284*, 106597.

Escobar-Zepeda, A., Vera-Ponce de León, A., & Sanchez-Flores, A. (2015). The road to metagenomics: from microbiology to DNA sequencing technologies and bioinformatics. *Frontiers in Genetics,* 6, 348.

Evans, H.C. (1974). Natural control of arthropods, with special reference to ants (Formicidae), by fungi in the tropical high forest of Ghana. *Journal of Applied Ecology*, *11*(1), 37–49. https://doi.org/10.2307/2402003

Evans, H. (1982). Entomogenous fungi in tropical forest ecosystems: an appraisal. *Ecological Entomology, 7*(1), 47-60.

Feinstein, L.M., Sul, W.J., & Blackwood, C.B. (2009). Assessment of bias associated with incomplete extraction of microbial DNA from soil. *Applied and Environmental Microbiology, 75*(16), 5428-5433. https://doi.org/10.1128/AEM.00120-09

Fernandes, É.K.K., Moraes, Á.M.L., Pacheco, R.S., Rangel, D.E.N., Miller, M.P., Bittencourt, V. R.E.P., & Roberts, D.W. (2009). Genetic diversity among Brazilian isolates of *Beauveria bassiana*: Comparisons with non-Brazilian isolates and other *Beauveria* species. *Journal of Applied Microbiology, 107*, 760-774.

Fernández-Bravo, M., Gschwend, F., Mayerhofer, J., Hug, A., Widmer, F., & Enkerli, J. (2021). Land-use type drives soil population structures of the entomopathogenic fungal genus *Metarhizium*. *Microorganisms*, *9*, 1380. https://doi.org/10.3390/microorganisms9071380

Fisher, J.J., Rehner, S.A., & Bruck, D.J. (2011). Diversity of rhizosphere associated entomopathogenic fungi of perennial herbs, shrubs and coniferous trees. *Journal of Invertebrate Pathology, 106*, 289-295.

Foster, J.A., Bunge, J., Gilbert, J.A., & Moore, J.H. (2012). Measuring the microbiome: perspectives on advances in DNA-based techniques for exploring microbial life. *Briefings in bioinformatics, 13*(4), 420-429.

Frey, B., Rime, T., Phillips, M., Stierli, B., Hajdas, I., Widmer, F., & Hartmann, M. (2016). Microbial diversity in European alpine permafrost and active layers. *FEMS Microbiology Ecology*, 92(3), fiw018. https://doi.org/10.1093/femsec/fiw018

Friedl, M.A., & Druzhinina, I.S. (2012). Taxon-specific metagenomics of *Trichoderma* reveals a narrow community of opportunistic species that regulate each other's development. *Microbiology*, 158, 69-83.

Galus-Barchan, A., & Paśmionka, I. (2014). The occurrence of selected microorganisms in the soil under forest with particular reference to mould fungi. *Polish Journal of Agronomy*, *17*, 11-17.

Gan, H., & Wickings, K. (2017). Soil ecological responses to pest management in golf turf vary with management intensity, pesticide identity, and application program. *Agriculture, Ecosystems & Environment*, 246, 66-77.

Gange, A.C., & Brown, V.K. (2002). Actions and Interactions of Soil Invertebrates and Arbuscular Mycorrhizal Fungi in Affecting the Structure of Plant Communities. In: van der Heijden, M.G.A., Sanders, I.R. (eds) Mycorrhizal Ecology. Ecological Studies, vol 157. Springer, Berlin, Heidelberg

Gange, A. C., Koricheva, J., Currie, A. F., Jaber, L. R., & Vidal, S. (2019). Meta-analysis of the role of entomopathogenic and unspecialized fungal endophytes as plant bodyguards. *New Phytologist, 223*(4), 2002–2010.

Gao, Q., Jin, K., Ying, S.-H., Zhang, Y., Xiao, G., Shang, Y., Duan, Z., Hu, X., Xie, X.-Q., Zhou, G., Peng, G., Luo, Z., Huang, W., Wang, B., Fang, W., Wang, S., Zhong, Y., Ma, L.-J., St. Leger, R.J., Zhao, G.-P., Pei, Y., Feng, M.-G., Xia, Y., & Wang, C. (2011). Genome

sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. *PLOS Genetics, 7*, e1001264.

Garrido-Jurado, I., Valverde-García, P., & Quesada-Moraga, E. (2011). Use of a multiple logistic regression model to determine the effects of soil moisture and temperature on the virulence of entomopathogenic fungi against pre-imaginal Mediterranean fruit fly *Ceratitis capitata*. *Biological Control,* 59, 366-372.

Gebremariam, A., Chekol, Y., & Assefa, F. (2021). Phenotypic, molecular, and virulence characterization of entomopathogenic fungi, *Beauveria bassiana* (Balsam) Vuillemin, and *Metarhizium anisopliae* (*Metschn*.) Sorokin from soil samples of Ethiopia for the development of mycoinsecticide. *Heliyon, 7*(2021)

Glare, T.R. (1994). Stage-dependent synergism using *Metarhizium anisopliae* and *Serratia entomophila* against *Costelytra zealandica*. *Biocontrol Science and Technology, 4*(3), 321-329.

Glare, T.R., Reay, S.D., Nelson, T.L., & Moore, R. (2008). *Beauveria caledonica* is a naturally occurring pathogen of forest beetles. *Mycological Research, 112*(3), 352-360.

Goble, T.A., Dames, J.F., Hill, M., & Moore, S.D. (2010). The effects of farming system, habitat type and bait type on the isolation of entomopathogenic fungi from citrus soils in the eastern Cape province, South Africa. *BioControl, 55*, 399-412.

Gouli, V., Gouli, S., Marcelino, J.A., Skinner, M., & Parker, B.L. (2013). Entomopathogenic fungi associated with exotic invasive insect pests in northeastern forests of the USA. *Insects*, *4*(4), 631-645.

Gul, E., Demirci, F., & Babaroğlu, N. (2022). Characterization of *Lecanicillium psalliotae* and *Akanthomyces muscarium* from sunn pests (*Eurygaster* spp.). *Journal of Central European Agriculture*, 23(3), 526-532. https://doi.org/10.5513/jcea01/23.3.3556

Handelsman, J., Rondon, M. R., Brady, S. F., Clardy, J., & Goodman, R. M. (1998). Molecular biological access to the chemistry of unknown soil microbes: A new frontier for natural products. *Chemistry & Biology*, 5(10), R245–R249. https://doi.org/10.1016/s1074-5521(98)90108-9

Hassan, F.R., Abdullah, S.K., & Assaf, L.H. (2019). Pathogenicity of the entomopathogenic fungus, *Beauveria bassiana* (Bals.) Vuill. Endophytic and a soil isolate against the squash beetle, *Epilachna chrysomelina* (F.) (Coleoptera: Coccinellidae). *Egyptian Journal of Biological Pest Control*, *29*(1), 1-7. https://doi.org/10.1186/s41938-019-0169-x

Hansen, A. J., & DiCastri, F. (Eds.). (2012). *Landscape boundaries: consequences for biotic diversity and ecological flows* (Vol. 92). Springer Science & Business Media.

Hallouti, A., Hamza, M., Zahidi, A., Hammou, R., Bouharroud, R., Aoumar, A & Boubaker, H. (2020). Diversity of entomopathogenic fungi associated with mediterranean fruit fly (*Ceratitis capitata* (Diptera: Tephritidae)) in Moroccan Argan Forests and nearby area: impact of soil factors on their distribution. *BMC Ecology*, 20(1). https://doi.org/10.1186/s12898-020-00334-2

Hennequin, C., Abachin, E., Symoens, F., Lavarde, V., Reboux, G., Nolard, N., & Berche, P. (1999). Identification of *Fusarium* species involved in human infections by 28S rRNA gene sequencing. *Journal of Clinical Microbiology,* 37(11), 3586-3589.

Hibbett, D.S., Ohman, A., Glotzer D., Nuhn, M., Kirk, P., Nilsson, R.H. (2011). Progress in molecular and morphological taxon discovery in Fungi and options for formal classification of environmental sequences. *Fungal Biology Reviews*, 25(1), 38-47.

Hibert, F., Taberlet, P., Chave, J., Scotti-Saintagne, C., Sabatier, D., & Richard-Hansen, C. (2013). Unveiling the diet of elusive rainforest herbivores in next generation sequencing era? The tapir as a case study. *PLoS ONE* 8: e60799

Howell, C. (2003). Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Disease, 87*, 4-10.

Huber, J. (1958). Untersuchungen zur Physiologie insektentötender Pilze. *Arch. Mikrobiol*., 29, 257-276.

Huson, D.H., Auch, A.F., Qi, J., & Schuster, S.C. (2007). MEGAN analysis of metagenomic data. *Genome Res*.,17(3). http://dx.doi.org/10.1101/gr.5969107.

Hussein, K.A., Abdel-Rahman, M.A., Abdel-Mallek, A.Y., El-Maraghy, S.S., & Joo, J.H. (2010). Climatic factors interference with the occurrence of *Beauveria bassiana* and *Metarhizium anisopliae* in cultivated soil. *African Journal of Biotechnology, 9*(45), 7674-7682.

Hughes, W.O.H., Thomsen, L., Eilenberg, J., & Boomsma, J.J. (2004). Diversity of entomopathogenic fungi near leaf-cutting ant nests in a neotropical forest, with particular reference to *Metarhizium anisopliae var. anisopliae*. *Journal of Invertebrate Pathology, 85*, 46-53.

Imoulan, A., Alaoui, A., & El Meziane, A. (2011). Natural occurrence of soil-borne entomopathogenic fungi in the Moroccan endemic forest of *Argania spinosa* and their pathogenicity to *Ceratitis capitata*. *World Journal of Microbiology and Biotechnology, 27*, 2619-2628.

Inglis, G.D., Duke, G.M., Goettel, M.S., & Kabaluk, J.T. (2008). Genetic diversity of *Metarhizium anisopliae var. anisopliae* in southwestern British Columbia. *Journal of Invertebrate Pathology, 98*, 101-113.

Islam, W., Noman, A., Naveed, H., Huang, Z., & Chen, H.Y. (2020). Role of environmental factors in shaping the soil microbiome. *Environmental Science and Pollution Research*, *27*, 41225-41247.

Islam, W., Adnan, M., Shabbir, A., Naveed, H., Abubakar, Y. S., Qasim, M., Tayyab, M., Noman, A., Nisar, M. S., Khan, K. A., & Ali, H. (2021). Insect-fungal-interactions: A detailed review on entomopathogenic fungi pathogenicity to combat insect pests. *Microbial Pathogenesis*, *159*, 105122. https://doi.org/10.1016/j.micpath.2021.105122

Issaly, N., Chauveau, H., Aglevor, F., (2005). Influence of nutrient, pH and dissolved oxygen on the production of *Metarhizium flavoviride* Mf189 blastospores in submerged batch culture. *Process Biochemistry*, 40, 1425–1431.

Jabbour, R. & Barbercheck, M.E. (2009). Soil management effects on entomopathogenic fungi during the transition to organic agriculture in a feed grain rotation. *Biological Control, 51*, 435-443. https://doi.org/10.1016/j.biocontrol.2009.08.004

Jaber, L. R., & Ownley, B. H. (2018). Can we use entomopathogenic fungi as endophytes for dual biological control of insect pests and plant pathogens? *Biological Control, 116*, 36–45.

Jaronski, S.T. (2007). Soil Ecology of the Entomopathogenic Ascomycetes: A Critical Examination of What We (Think) We Know. In: Maniana K, Ekesi S (Eds) Use of Entomopathogenic Fungi in Biological Pest Management. Research SignPosts, Trivandrum, India, 91-144. https://www.ars.usda.gov/research/publications/publication/?seqNo115=209733

Jaronski, S.T. (2010). Ecological factors in the inundative use of fungal entomopathogens. *BioControl, 55*, 159-185.

Jose, S. (2009). Agroforestry for ecosystem services and environmental benefits: An overview. *Agroforestry Systems*, 76, 1–10.

Khaliq, A., Perveen, S., Alamer, K. H., Rafique, Z., Alsudays, I. M., Althobaiti, A. T., Saleh, M. A., Hussain, S., & Attia, H. (2021). Arbuscular mycorrhizal fungi symbiosis to enhance plant–soil interaction. *Sustainability*, *14*(13), 7840. https://doi.org/10.3390/su14137840

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., . . . Duran, C. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics, 28(12), 1647-1649.

Keller S,. & Zimmermann G. (1989) Mycopathogens of soil insects. In: Wilding N, Collins NM, Hammond PM, Webber JF (Eds) Insect-Fungus Interactions. Academic Press, London, United Kingdom, 239–270.

Keller, S., Kessler, P., & Schweizer, C. (2003). Distribution of insect pathogenic soil fungi in Switzerland with special reference to *Beauveria brongniartii* and *Metharhizium anisopliae*. *BioContro*l, 48, 307-319.

Kepler, R.M., Ugine, T.A., Maul, J.E., Cavigelli, M.A., & Rehner, S.A. (2015). Community composition and population genetics of insect pathogenic fungi in the genus *Metarhizium* from soils of a long-term agricultural research system. *Environmental Microbiology, 17*, 2791-2804.

Kepler, R.M., Maul, J.E., & Rehner, S.A. (2017). Managing the plant microbiome for biocontrol fungi: Examples from Hypocreales. *Current Opinion in Microbiology, 37*, 48–53

Kessler, P., Enkerl, J., Schweize, C., & Keller, S. (2004). Survival of *Beauveria brongniartii* in the soil after application as a biocontrol agent against the European cockchafer *Melolontha melolontha*. *BioControl, 49*, 563-581.

Keyser, C. A., De Fine Licht, H. H., Steinwender, B. M., & Meyling, N. V. (2015). Diversity within the entomopathogenic fungal species *Metarhizium flavoviride* associated with agricultural crops in Denmark. *BMC Microbiology, 15*, Article 249.

Khaziev, F.K. (2011). Soil and biodiversity. *Russian Journal of Ecology*, *42*, 199-204.

Kim, S. (2023). Correlation of fruit tree rhizosphere soils with entomopathogenic fungi. *Entomological Research*, 53(9), 333-342. https://doi.org/10.1111/1748-5967.12666

Klingen, I., Eilenberg, J., & Meadow, R. (2002). Effects of farming system, field margins and bait insect on the occurrence of insect pathogenic fungi in soils. *Agriculture, Ecosystems and Environment,* 91, 191-198.

Klingen I. & Haukeland S. (2006) The soil as a reservoir for natural enemies of pest insects and mites with emphasis on fungi and nematodes. In: Eilenberg J, Hokkanen HMT (Eds) An Ecological and Societal Approach to Biological Control. Springer Netherlands, Dordrecht, 145-211.

Knight, C. (2008). Totara Reserve: a window into the Manawatu's environmental history. *The Manawatu Journal of History* (4), 50-58.

Korpela, E. L., Hyvönen, T., & Kuussaari, M. (2015). Logging in boreal field-forest ecotones promotes flower-visiting insect diversity and modifies insect community composition. *Insect Conservation and Diversity*, *8*(2), 152-162.

Kovač, M., Tkaczuk, C., & Pernek, M. (2021). First report of entomopathogenic fungi occurrence in forest soils in Croatia. *Forests*, 12(12), 1690. https://doi.org/10.3390/f12121690

Lacey, L., Frutos, R., Kaya, H., & Vail, P. (2001). Insect pathogens as biological control agents: do they have a future? *Biological Control, 21*(3), 230-248.

Lacey, L.A., & Shapiro-Ilan, D.I. (2008). Microbial control of insect pests in temperate orchard systems: potential for incorporation into IPM. *Annual Review of Entomology, 53*, 121–144. https://doi.org/10.1146/annurev.ento.53.103106.093419

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M., and Goettel, M. S. (2015). Insect pathogens as biological control agents: back to the future. *Journal of Invertebrate Pathology*, 132, 1–41. https://doi.org/10.1016/j.jip.2015.07.009

Le Gall, M., Overson, R., & Cease, A. (2019). A global review on locusts (Orthoptera: Acrididae) and their interactions with livestock grazing practices. *Frontiers in Ecology and Evolution*, *7*, 457930. https://doi.org/10.3389/fevo.2019.00263

Lear, G., Dickie, I., Banks, J., Boyer, S., Buckley, H., Buckley, T., . . . Holdaway, R. (2018). Methods for the extraction, storage, amplification and sequencing of DNA from environmental samples. *New Zealand Journal of Ecology,* 10.20417/nzjecol.42.9.

Lee, S., Yang, Y., Kim, S., Lee, M., Kim, J., Park, S., & Nai, Y. (2018a). Transcriptional response of bean bug (*Riptortus pedestris*) upon infection with entomopathogenic fungus, *Beauveria bassiana* jef-007. *Pest Management Science*, 75(2), 333-345. https://doi.org/10.1002/ps.5117

Lee, S.J., Lee, M.R., Kim, S. *et al.* (2018b). Genomic analysis of the insect-killing fungus *Beauveria bassiana* JEF-007 as a biopesticide. *Scientific Reports, 8*, 12388. https://doi.org/10.1038/s41598-018-30856-1

Leray, M., Yang, J. Y., Meyer, C. P., Mills, S. C., Agudelo, N., Ranwez, V., Boehm, J. T., & Machida, R. J. (2013). A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: Application for characterizing coral reef fish gut contents. *Frontiers in Zoology, 10*(1), Article 34.

Li, X., Qu, Z., Zhang, Y., Gao, Y., & Sun, H. (2022). Soil fungal community and potential function in different forest ecosystems. *Diversity*, 14(7), 520. https://doi.org/10.3390/d14070520

Li, G.X. & Ma, K.M. (2018). Progress in the study of elevational patterns of soil microbial diversity. *Acta Ecologica Sinica*, 38, 1521–1529.

Liang, Z.-Q., Liu, A.-Y., & Liu, J.-L. (1991). A new species of the genus *Cordyceps* and its *Metarhizium* anamorph. *Acta Sinica, 10*, 257-262.

Lilleskov, E. A., & Bruns, T. D. (2005). Spore dispersal of a resupinate ectomycorrhizal fungus, *Tomentella sublilacina*, via soil food webs. *Mycologia, 97*(4), 762-769.

Lin, B. B. (2007). Agroforestry management as an adaptive strategy against potential microclimate extremes in coffee agriculture. *Agricultural and Forest Meteorology*, 144, 85–94.

Litwin, A., Nowak, M., & Różalska, S. (2020). Entomopathogenic fungi: unconventional applications. *Reviews in Environmental Science and Bio/Technology*, *19*(1), 23-42.

Liu, R., Meller, R., & Steinberger, Y. (2019). Changes in a soil microarthropod community in the vicinity of dominant tree species under trampling management at the Safari Zoological Center, Israel. *Applied Soil Ecology, 59*(1), 33–45.

Liu, H., Cheng, J., Jin, H., Xu, Z., Yang, X., Deng, M.,& Qin, B. (2022). Characterization of rhizosphere and endophytic microbial communities associated with *Stipa purpurea* and their correlation with soil environmental factors. *Plants*, 11(3), 363. https://doi.org/10.3390/plants11030363

Lockwood, J.L. (1964). Soil fungistasis. *Annual Review of Phytopathology, 2*, 341-362.

Lovett, B., & St. Leger, R. J. (2017). The insect pathogens. *Microbiol. Spectr.* 5, 923–943. https://doi.org/10.1128/microbiolspec.FUNK-0001-2016

Majchrowska-Safaryan, A., Tkaczuk, C., & Symanowicz, B. (2017). Effects of the application of a mineral-and-organicfertilizer produced from brown coal on the occurrence and infectious

potential of entomopathogenic fungi in soil. *Journal of Ecological Enginering, 18*, 140-148.

Majchrowska-Safaryan, A., & Tkaczuk, C. (2021). Abundance of entomopathogenic fungi in leaf litter and soil layers in forested habitats in Poland. *Insects*, *12*(2), 134. https://doi.org/10.3390/insects12020134

Makiola, A., Dickie, I., Holdaway, R., Wood, J., Orwin, K., & Glare, T. (2019). Land use is a determinant of plant pathogen alpha- but not beta-diversity. *Molecular Ecology, 28*(16), 3786-3798. 10.1111/mec.15177

Mantzoukas, S., Zikou, A., Triantafillou, V., Lagogiannis, I., & Eliopoulos, P. (2019). Interactions between *Beauveria bassiana* and *Isaria fumosorosea* and their hosts *Sitophilus granarius* (l.) and *Sitophilus oryzae* (l.) (Coleoptera: Curculionidae). *Insects, 10*(10), 362. https://doi.org/10.3390/insects10100362

Mantzoukas, S., Kitsiou, F., Natsiopoulos, D., & Eliopoulos, P. A. (2022). Entomopathogenic Fungi: Interactions and Applications. *Encyclopedia*, *2*(2), 646-656. https://doi.org/10.3390/encyclopedia2020044

Mantzoukas, S., Lagogiannis, I., Ntoukas, A., Eliopoulos, P.A., Kouretas, D., Karpouzas, D.G., & Poulas, K. (2020). Trapping entomopathogenic fungi from vine terroir soil samples with insect baits for controlling serious pests. *Applied Sciences*, *10*(10), 3539.

Mao, D. Q., Luo, Y., Mathieu, J., Wang, Q., Feng, L., Mu, Q. H., Feng, C. Y., & Alvarez, P. J. J. (2014). Persistence of extracellular DNA in river sediment facilitates antibiotic resistance gene propagation. *Environmental Science & Technology, 48*(1), 71–78. https://doi.org/10.1021/es404280v

Maraun, M., Martens, H., Migge, S., Theenhaus, A., & Scheu, S. (2003). Adding to 'the enigma of soil animal diversity': fungal feeders and saprophagous soil invertebrates prefer similar food substrates. *European Journal of Soil Biology, 39*(2), 85-95.

Marfo, T., Datta, R., Vranová, V., & Ekielski, A. (2019). Ecotone dynamics and stability from soil perspective: forest-agriculture land transition. *Agriculture, 9*(10), 228.

Mark, G. L., Morrissey, J. P., Higgins, P., & O'gara, F. (2006). Molecular-based strategies to exploit *Pseudomonas* biocontrol strains for environmental biotechnology applications. *FEMS Microbiol. Ecol., 56*, 167–177. https://doi.org/10.1111/j.1574-6941.2006.00056.x

Masoudi, A., Wang, M., Zhang, X., Wang, C., Qiu, Z., Wang, W., & Liu, J. (2020). Meta-analysis and evaluation by insect-mediated baiting reveal different patterns of Hypocrealean entomopathogenic fungi in the soils from two regions of China. *Frontiers in Microbiology*, 11. https://doi.org/10.3389/fmicb.2020.01133

McMurdie, P.J., Holmes, S. (2013). phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* 8(4): e61217. https://doi.org/10.1371/journal.pone.0061217.

Medo, J. & Ludovít, C. (2011). Factors affecting the occurrence of entomopathogenic fungi in soils of Slovakia as revealed using two methods. *Biological Control*, 59(2), 200–208

Mayerhofer, J., Lutz, A., Widmer, F., Rehner, S.A., Leuchtmann, A., & Enkerli, J. (2015). Multiplexed microsatellite markers for seven *Metarhizium* species. *Journal of Invertebrate Pathology*, 132, 132-134.

McFall-Ngai, M., Hadfield, M.G., Bosch, T.C., Carey, H.V., Domazet-Lošo, T., Douglas, A.E., . . . Gilbert, S. F. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences, 110*(9), 3229-3236.

McGuire, K., Allison, S., Fierer, N., & Treseder, K. (2013). Ectomycorrhizal-dominated boreal and tropical forests have distinct fungal communities, but analogous spatial patterns across soil horizons. *PLoS ONE*, 8(7), e68278. https://doi.org/10.1371/journal.pone.0068278

Mendes, L.W., Braga, L.P.P., Navarrete, A.A., Souza, D.G. d., Silva, G.G., & Tsai, S.M. (2017). Using metagenomics to connect microbial community biodiversity and functions. *Current Issues in Molecular Biology, 24*, 103-118.

Mehrmoradi, H., Jamali, S., & Pourian, H. (2022). Isolation of entomopathogenic fungi from cultivated and uncultivated soils and evaluation of virulence against *Callosobruchus maculatus* (f.) (Coleoptera: Chrysomelidae). *Journal of Entomological Society of Iran*, 42(1), 29-53. https://doi.org/10.52547/jesi.42.1.3

Meng, Y., Don, P., & Wang, D. (2022). A new strain of *Lecanicillium uredinophilum* isolated from Tibetan plateau and its insecticidal activity. *Microorganisms*, 10(9), 1832. https://doi.org/10.3390/microorganisms10091832

Meyling, N.V., & Eilenberg, J. (2006). Occurrence and distribution of soil borne entomopathogenic fungi within a single organic agroecosystem. *Agriculture, Ecosystems & Environment, 113*, 336-341.

Meyling, N.V., Eilenberg, J. (2007). Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: potential for conservation biological control. *Biol. Cont.,* 43,145–155.

Meyling, N.V., Lubeck, M., Buckley, E.P., Eilenberg, J., & Rehner, S.A. (2009). Community composition, host range and genetic structure of the fungal entomopathogen *Beauveria* in adjoining agricultural and seminatural habitats. *Molecular Ecology, 18*, 1282- 1293.

Miller, C.S., Baker, B.J., Thomas, B.C., Singer, S.W., Banfield, J.F. (2011). EMIRGE: reconstruction of full-length ribosomal genes from microbial community short read sequencing data. *Genome Biology* 12: R44.

Moreira, C.C., Celestino, D., Guerra Sobrinho, T., Cardoso, I.M., & Elliot, S.L. (2019). Agroforestry coffee soils increase the insect-suppressive potential offered by entomopathogenic fungi over full-sun soils: A case proposing a "bait survival technique." *Ecology and Evolution, 9*(19), 10777–10787.

Moruzzo, R., Riccioli, F., Diaz, S.E., Secci, C., Poli, G., & Mancini, S. (2021). Mealworm (*Tenebrio molitor*): Potential and challenges to promote circular economy. *Animals : An Open Access Journal From MDPI*, *11*(9), 2568. https://doi.org/10.3390/ani11092568

Nelson, T.L., Willoughby, B.E., Wilson, D.J., Eden, T. & Glare, T.R. (2004). Establishing the fungus *Beauveria bassiana* in pasture for clover root weevil (*Sitona lepidus*) control. *New Zealand Plant Protection*, 57, 314-318.

Nguyen, N. T. (2021). *Metarhizium strains associated with grass grub (Costelytra giveni) in New Zealand, and their potential as biocontrol agents* (PhD thesis). Lincoln University.

Niu, X., Xie, W., Zhang, J., & Hu, Q. (2019). Biodiversity of entomopathogenic fungi in the soils of South China. *Microorganisms*, 7(9), 311. https://doi.org/10.3390/microorganisms7090311

Norjmaa, U., Nasamdulam, D., Enkhjargal, B., & Dalai, B. (2019). Morphological and molecular identification of *Beauveria bassiana* from agricultural soils. *Mongolian Journal of Agricultural Sciences*, 27(02), 20-24. https://doi.org/10.5564/mjas.v27i02.1280

Oddsdottir, E. S., Nielsen, C., Sen, R., Harding, S., Eilenberg, J., & Halldorsson, G. (2010). Distribution patterns of soil entomopathogenic and birch symbiotic ectomycorrhizal fungi

across native woodland and degraded habitats in Iceland. *Icelandic Agricultural Sciences, 23*, 37–49.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E., & Wagner, H. (2020). vegan: Community Ecology Package. R package version 2.4-4. URL https://CRAN.R-project.org/package=vegan

Oliver, I. & Beattie, A. J. (1996). Designing a cost-effective invertebrate survey: a test of methods for rapid assessment of biodiversity. *Ecological Applications*, 6(2), 594- 607.

Orellana, S. C. (2013). Assessment of fungal diversity in the environment using metagenomics: a decade in review. *Fungal Genomics & Biology, 03*(02) 10.4172/2165-8056.1000110

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., Edwards, R. A., Gerdes, S., Parrello, B., Shukla, M., Vonstein, V., Wattam, A. R., Xia, F., & Stevens, R. (2013). The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Research*, *42*(D1), D206-D214. https://doi.org/10.1093/nar/gkt1226

Padmavathi, J., Devi, K.U., & Rao, C.U.M. (2003). The optimum and tolerance pH range is correlated to colonial morphology in isolates of the entomopathogenic fungus *Beauveria bassiana*, a potential biopesticide. *World Journal of Microbiology & Biotechnology,* 19, 469–477.

Parker, B. L., Skinner, M., Gouli, S. Y., Gouli, V. V., Tobi, D., & Kim, J. S. (2015). Persistence of *Beauveria bassiana* sensu lato and *Metarhizium anisopliae* sensu lato in Vermont (USA) forest soil. *Biocontrol Science and Technology*, *25*(7), 768–788.

Pérez-González, V. H., Guzmán-Franco, A. W., Alatorre-Rosas, R., Hernández- López, J., Hernández-López, A., Carrillo-Benítez, M. G., & Baverstock, J. (2014). Specific diversity

of the entomopathogenic fungi *Beauveria* and *Metarhizium* in Mexican agricultural soils. *Journal of Invertebrate Pathology, 119*, 54-61.

Petzold-Maxwell, J., Jaronski, S., & Gassmann, A. (2011). Tritrophic interactions amongbtmaize, an insect pest and entomopathogens: effects on development and survival of western corn rootworm. *Annals of Applied Biology*, 160(1), 43-55. https://doi.org/10.1111/j.1744-7348.2011.00515.x

Pflug, A., & Wolters, V. (2002). Collembola communities along a European transect. *European Journal of Soil Biology, 38*(3-4), 301-304.

Posadas, J.B., Comerio R.M., Mini, J.I., Nussenbaum, A.L., & Lecuona, R.E. (2012). A novel dodine-free selective medium based on the use of cetyl trimethyl ammonium bromide (CTAB) to isolate *Beauveria bassiana*, *Metarhizium anisopliae* sensu lato and *Paecilomyces lilacinus* from soil. *Mycologia*, 104, 974-980. https://doi.org/10.3852/11-234

Qayyum, M. A., Saeed, S., Wakil, W., Nawaz, A., Iqbal, N., Yasin, M., Chaurdhry, M. A., Bashir, M. A., Ahmed, N., Riaz, H., Bilal, H., Hashem, M., & Alamri, S. (2021). Diversity and correlation of entomopathogenic and associated fungi with soil factors. *Journal of King Saud University - Science, 33*(6), 101520.

Quesada-Moraga, E., Navas-Cortés, J.A., Maranhao, E.A.A., Ortiz-Urquiza, A., & Santiago-Álvarez, C. (2007). Factors affecting the occurrence and distribution of entomopathogenic fungi in natural and cultivated soils. *Mycological Research, 111*, 947-966.

Quesada-Moraga, E., González-Mas, N., Yousef-Yousef, M. *et al.* (2024). Key role of environmental competence in successful use of entomopathogenic fungi in microbial pest control. *J. Pest Sci., 97*, 1–15 https://doi.org/10.1007/s10340-023-01622-8

Quince, C., Walker, A.W., Simpson, J.T., Loman, N.J., & Segata, N. (2017). Shotgun metagenomics, from sampling to analysis. *Nature Biotechnology, 35*(9), 833.

Raad, M., Glare, T. R., Brochero, H. L., Müller, C., & Rostás, M. (2019). Transcriptional reprogramming of *Arabidopsis thaliana* defence pathways by the entomopathogen *Beauveria bassiana* correlates with resistance against a fungal pathogen but not against insects. *Frontiers in Microbiology, 10*, 615.

Ramos, Y., Portal, O., Lysøe, E., Meyling, N. V., & Klingen, I. (2017). Diversity and abundance of *Beauveria bassiana* in soils, stink bugs and plant tissues of common bean from organic and conventional fields. *Journal of Invertebrate Pathology, 150*, 114-120.

Rath, A.C., Koen, T.B., Yip, H.Y. (1992). The influence of abiotic factors on the distribution and abundance of *Metarhizium anisopliae* in Tasmanian pasture soils. *Mycological Research,* 96, 378-384.

Ravnskov, S., Cabral, C., & Larsen, J. (2020). Mycorrhiza-induced tolerance in *Cucumis sativus* against root rot caused by *Pythium ultimum* depends on fungal species in the arbuscular mycorrhizal symbiosis. *Biological Control, 141*, 104133.

Reason, A., Bulgarella, M., & Lester, P. (2022). Identity, prevalence, and pathogenicity of entomopathogenic fungi infecting invasive *Polistes* (Vespidae: Polistinae) paper wasps in New Zealand. *Insects*, 13(10), 922. https://doi.org/10.3390/insects13100922

Reay, S.D., Brownbridge, M., Gicquel, B., Cummings, N.J., & Nelson, T.L. (2010). Isolation and characterization of endophytic *Beauveria* spp. (Ascomycota: Hypocreales) from *Pinus radiata* in New Zealand forests. *Biological Control*, *54*, 52-60

Raya-Díaz, S., Sánchez-Rodríguez, A.R., Segura-Fernández, J.M., Del Campillo, M.D.C., & Quesada-Moraga, E. (2017). Entomopathogenic fungi-based mechanisms for improved Fe nutrition in sorghum plants grown on calcareous substrates. *PLoS One, 12*, e0185903.

Rehner, S.A., & Buckley, E. (2005). A *Beauveria* phylogeny inferred from nuclear ITS and EF1-α sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. *Mycologia*, *97*(1), 84-98.

Rehner, S.A., Posada, F., Buckley, E.P., Infante, F., Castillo, A., & Vega, F.E. (2006b). Phylogenetic origins of African and neotropical *Beauveria bassiana* s.l. pathogens of the coffee berry borer, *Hypothenemus hampei*. *Journal of Invertebrate Pathology, 93*, 11-21.

Rehner, S.A., Minnis, A.M., Sung, G.H., Luangsa-Ard, J.J., Devotto, L., & Humber, R.A. (2011). Phylogeny and systematics of the anamorphic, entomopathogenic genus *Beauveria*. *Mycologia, 103*, 1055-1073.

Renker, C., Otto, P., Schneider, K., Zimdars, B., Maraun, M., & Buscot, F. (2005). Oribatid mites as potential vectors for soil microfungi: study of mite-associated fungal species. *Microbial Ecology, 50*(4), 518.

Resch, M. C., Schütz, M., Graf, U., Wagenaar, R., van der Putten, W. H., & Risch, A. C. (2019). Does topsoil removal in grassland restoration benefit both soil nematode and plant communities? *Journal of Applied Ecology, 56*(7), 1782–1793

Rivas-Franco, F., Hampton, J. G., Narciso, J., Rostás, M., Wessman, P., Saville, D. J., Jackson, T. A., & Glare, T. R. (2020). Effects of a maize root pest and fungal pathogen on entomopathogenic fungal rhizosphere colonization, endophytism and induction of plant hormones. *Biological Control, 150*, 104347.

Rizal, L. M., Hereward, J. P., Brookes, D. R., Furlong, M. J., & Walter, G. H. (2024). Hidden diversity within *Beauveria* and *Metarhizium* – Comparing morphology, barcoding, multilocus phylogenies, and whole-genome sequences. *Fungal Ecology, 67,* 101304.

R Development Core Team. (2023). R: a language and environment for statistical computing. R foundation for statistical computing. Vienna, Austria. Retrieved from http://www.R-project.org/.

Rohlfs, M., & Churchill, A. C. (2011). Fungal secondary metabolites as modulators of interactions with insects and other arthropods. *Fungal Genetics and Biology, 48*(1), 23-34.

Romdhane, S., Spor, A., Banerjee, S. *et al.* Land-use intensification differentially affects bacterial, fungal and protist communities and decreases microbiome network complexity. *Environmental Microbiome, 17*, https://doi.org/10.1186/s40793-021-00396-9

Rojas, E. I., Rehner, S. A., Samuels, G. J., Van Bael, S. A., Herre, E. A., Cannon, P., . . . Zhang, Y. (2010). *Colletotrichum gloeosporioides* sl associated with *Theobroma cacao* and other plants in Panama: multilocus phylogenies distinguish host-associated pathogens from asymptomatic endophytes. *Mycologia*, 102(6), 1318-1338.

Rosa, E., Ekowati, C., Handayani, T., Ikhsanudin, A., & Apriliani, F. (2020). Characterization of entomopathogenic fungi as a natural biological control of American cockroaches (*Periplaneta americana*). *Biodiversitas Journal of Biological Diversity*, 21(11). https://doi.org/10.13057/biodiv/d211131

Rusch, A., Valantin-Morison, M., Sarthou, J. P., & Roger-Estrade, J. (2010). Biological control of insect pests in agroecosystems: Effects of crop management, farming systems, and seminatural habitats at the landscape scale: A review. *Advances in Agronomy, 109*, 219–259. https://doi.org/10.1016/S0065-2113(10)09006-1

Safitri, A., Herlinda, S., & Setiawan, A. (2018). Entomopathogenic fungi of soils of freshwater swamps, tidal lowlands, peatlands, and highlands of South Sumatra, Indonesia. *Biodiversitas Journal of Biological Diversity*, 19(6), 2365-2373. https://doi.org/10.13057/biodiv/d190647

Saminathan, N., & Subramanian, J. (2024). Entomopathogenic fungi: translating research into field applications for crop protection. *Arthropod-Plant Interactions* 19(8). https://doi.org/10.1007/s11829-024-10110-4

Sánchez-Peña, S.R., Lara, J.S.-J., Medina, R.F. (2011). Occurrence of entomopathogenic fungi from agricultural and natural ecosystems in Saltillo, México, and their virulence towards thrips and whiteflies. *Journal of Insect Science*, 11, https://doi.org/10.1673/031.011.0101

Schneider, S., Widmer, F., Jacot, K., Kölliker, R., & Enkerli, J. (2012). Spatial distribution of *Metarhizium* clade 1 in agricultural landscapes with arable land and different semi-natural habitats. *Applied Soil Ecology, 52*, 20-28.

Schigel, D. S. (2012). Fungivory and host associations of Coleoptera: a bibliography and review of research approaches. *Mycology, 3*(4), 258-272.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., . . . Consortium, F. B. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences, 109*(16), 6241-6246.

Seifert, K. A., Wingfield, B. D., & Wingfield, M. J. (1995). A critique of DNA sequence analysis in the taxonomy of filamentous Ascomycetes and ascomycetous anamorphs. *Canadian Journal of Botany, 73*(S1), 760-767.

Setälä, H., McLean, M.A. (2004). Decomposition rate of organic substrates in relation to the species diversity of soil saprophytic fungi. *Oecologia,* 139, 98–107.

Sevim, A., Demir, I., Höfte, M., Humber, R.A., Demirbag, Z. (2009). Isolation and characterization of entomopathogenic fungi from hazelnut-growing region of Turkey. *BioControl,* 55, 279-297. https://doi.org/10.1007/s10526-009-9235-8

Shah, P., & Pell, J. (2003). Entomopathogenic fungi as biological control agents. *Applied Microbiology and Biotechnology, 61*(5-6), 413-423.

Sharma, L. (2019). *Diversity and ecology of entomopathogenic fungi in the Douro wine region of Portugal* (PhD dissertation). University of Trás-os-Montes and Alto Douro, Vila Real, Portugal.

Sharma, L., Oliveira, I., Torres, L., & Marques, G. (2018). Entomopathogenic fungi in Portuguese vineyards soils: suggesting a '*Galleria*-*Tenebrio*-bait method' as bait-insects *Galleria* and *Tenebrio* significantly underestimate the respective recoveries of *Metarhizium (robertsii)* and *Beauveria (bassiana)*. *Mycokeys, 38*, 1-23.

Silva, A., Alves, R., Lima, E., & Lima, V. (2015). Bioformulations in pest control – a review. *Annual Research & Review in Biology*, 5(6), 535-543. https://doi.org/10.9734/arrb/2015/12395

Singers, N. J. D., & Rogers, G. M. (2014). *A classification of New Zealand's terrestrial ecosystems. Science for Conservation, 325.* Department of Conservation. ISSN 1177-9241 (web PDF), ISBN 978-0-478-15013-1 (web PDF).

Sinha, K. K., Choudhary, A. K., & Kumari, P. (2016). Entomopathogenic fungi. In Omkar (Ed.), *Ecofriendly pest management for food security* (pp. 475-505). San Diego: Elsevier Academic Press Inc.

Skinner, M., Gouli, S. Y., Gouli, V. V., Tobi, D., & Kim, J. S. (2015). Persistence of *Beauveria bassiana* sensu lato and *Metarhizium anisopliae* sensu lato in Vermont (USA) forest soil. *Biocontrol Science and Technology*, *25*(7), 768–788.

Skinner, M., Parker, B. L., & Kim, J. S. (2013). Role of entomopathogenic fungi in integrated pest management. *Integrated Pest Management*, 169-191. https://doi.org/10.1016/B978-0-12-398529-3.00011-7.

Soth, S., Glare, T. R., Hampton, J. G., Card, S. D., & Brookes, J. J. (2022). Biological control of diamondback moth—increased efficacy with mixtures of *Beauveria* fungi. *Microorganisms*, *10*(3), 646. https://doi.org/10.3390/microorganisms10030646

Staves, P. and Knell, R. (2010). Virulence and competitiveness: testing the relationship during inter- and intraspecific mixed infections. *Evolution*, 64(9), 2643-2652. https://doi.org/10.1111/j.1558-5646.2010.00999.x

Steinkraus, D. C. (2007). Documentation of naturally occurring pathogens and their impact in agroecosystems. In L. A. Lacey, & H. K. Kaya (Eds.), *Field manual of techniques in invertebrate pathology: application and evaluation of pathogens for control of insects and other invertebrate pests* (pp. 267-281). Dordrecht: Springer Netherlands.

Steinwender, B. M., Enkerli, J., Widmer, F., Eilenberg, J., Thorup-Kristensen, K., & Meyling, N. V. (2014). Molecular diversity of the entomopathogenic fungal *Metarhizium* community within an agroecosystem. *Journal of Invertebrate Pathology, 123*, 6-12.

Studdert, J. P., & Kaya, H. K. (1990). Water potential, temperature, and clay-coating of *Beauveria bassiana* conidia - effect on *Spodoptera exigua* pupal mortality in 2 soil types. *Journal of Invertebrate Pathology*, 56(3), 327-336.

Subramanyam, B. (Ed.). (1995). *Integrated management of insects in stored products*. CRC Press.

Suman, J., Rakshit, A., Ogireddy, S. D., Singh, S., Gupta, C., & Chandrakala, J. (2022). Microbiome as a key player in sustainable agriculture and human health. *Frontiers in Soil Science*, 2, 821589. https://doi.org/10.3389/fsoil.2022.821589

Sun, B.-D., & Liu, X.-Z. (2008). Occurrence and diversity of insect-associated fungi in natural soils in China. *Applied Soil Ecology, 39*, 100-108.m

Sun, B.-D., Yu, H.-Y., Chen, A.J., Liu, X.-Z. (2008). Insect-associated fungi in soils of field crops and orchards. *Crop Protection,* 27, 1421-1426.

Sung, G.-H., Hywel-Jones, N. L., Sung, J.-M., Luangsa-Ard, J. J., Shrestha, B., & Spatafora, J. W. (2007a). Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. *Studies in Mycology, 57*, 5-59.

Tardy, V., Spor, A., Mathieu, O., Lévèque, J., Terrat, S., Plassart, P., Regnier, T., Bardgett, R. D., van der Putten, W. H., Roggero, P. P., Seddaiu, G., Bagella, S., Lemanceau, P., Ranjard, L., & Maron, P.-A. (2015). Shifts in microbial diversity through land use intensity as drivers of carbon mineralization in soil. *Soil Biology and Biochemistry, 90*, 204–213.

Tedersoo, L., Bahram, M., Toots, M., Diédhiou, A., Henkel, T., Kjøller, R., & Kõljalg, U. (2012). Towards global patterns in the diversity and community structure of ectomycorrhizal fungi. *Molecular Ecology*, 21(17), 4160-4170. https://doi.org/10.1111/j.1365-294x.2012.05602.x

Tedersoo, L., Bahram, M., Zinger, L., Nilsson, R. H., Kennedy, P. G., Yang, T., Anslan, S., & Mikryukov, V. (2022). Best practices in metabarcoding of fungi: From experimental design to results. *Molecular Ecology.* https://doi.org/10.1111/mec.16450

Tkaczuk, C. (2008). Occurrence and infective potential of entomopathogenic fungi in soils of agrocenoses and seminatural habitats in the agricultural landscape. *Scientific Dissertation*, (94).

Tkaczuk, C., Krzyczkowski, T., & Wegensteiner, R. (2013). The occurrence of entomopathogenic fungi in soils from mid-field woodlots and adjacent small-scale arable fields. *Acta Mycologica*, 47(2), 191-202. https://doi.org/10.5586/am.2012.024

Tkaczuk, C., Król, A., Majchrowska-Safaryan, A., & Nicewicz, Ł. (2014). The occurrence of entomopathogenic fungi in soils from fields cultivated in a conventional and organic system. *Journal of Ecological Engineering, 15*, 137-144.

Tkaczuk, C., Tipping, C., Majchrowska-Safaryan, A., Krol, A., Wyrzykowska, M., & Wegensteiner, R. (2019). The occurrence of entomopathogenic fungi in soils from apple orchards protected in different farming systems. *Fresenius Environmental Bulletin, 28*(11), 7906-7914.

Tuininga, A. R., Miller, J. L., Morath, S. U., Daniels, T. J., Falco, R. C., Marchese, M., . . . Stafford, K. C., 3rd. (2009). Isolation of entomopathogenic fungi from soils and *Ixodes scapularis* (Acari: Ixodidae) ticks: prevalence and methods. *Journal Of Medical Entomology, 46*(3), 557-565.

Ullah, M. I., Qadeer, M., Arshad, M., Khalid, S., Saleem, U., Riaz, M. A., Mumtaz, A., Rizwan, M., Sayed, S. M., Alhelaify, S. S., Alharthy, O. M., & Mushtaq, M. (2023). Insights into sublethal effects of *Metarhizium anisopliae* on the biotic potentials of *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) on maize. *Egyptian Journal of Biological Pest Control*, *33*(1), 1-9. https://doi.org/10.1186/s41938-023-00741-9

United Nations. (n.d.). *Net-Zero Coalition*. Retrieved August 27, 2024, from https://www.un.org/en/climatechange/net-zero-coalition

Uroz, S., Ioannidis, P., Lengelle, J., Cébron, A., Morin, E., Buée, M., & Martin, F. (2013). Functional assays and metagenomic analyses reveals differences between the microbial communities inhabiting the soil horizons of a Norway spruce plantation. *PLoS ONE, 8*(2)

Vaicekonyte, R. and Keesing, F. (2012). Effects of garlic mustard (*Alliaria petiolata*) removal on the abundance of entomopathogenic fungi. *Invasive Plant Science and Management*, 5(3), 323-329. https://doi.org/10.1614/ipsm-d-12-00006.1

Vega, F. E., Goettel, M. S., Blackwell, M., Chandler, D., Jackson, M. A., Keller, S., Koike, M., Maniania, N.K., Monzón, A., Ownley, B.H., Pell, J.K., Rangel, D.E.N., & Roy, H.E. (2009). Fungal entomopathogens: new insights on their ecology. *Fungal Ecology, 2*, 149-159.

Villamizar, L., Barrera, G., Hurst, M., & Glare, T. (2021). Characterization of a new strain of *Metarhizium novozealandicum* with potential to be developed as a biopesticide. *Mycology: An International Journal on Fungal Biology*, 12(4), 261-278. https://doi.org/10.1080/21501203.2021.1935359

Vega, F. E., Meyling, N. V., Luangsa-Ard, J. J., & Blackwell, M. (2012). Fungal entomopathogens. In F. E. Vega, & H. K. Kaya (Eds.), *Insect pathology* (pp. 171-220). San Diego: Elsevier Academic Press Inc.

Wakil, W., Ghazanfar, M. U., Riasat, T., Kwon, Y. J., Qayyum, M. A., & Yasin, M. (2013). Occurrence and diversity of entomopathogenic fungi in cultivated and uncultivated soils in Pakistan. *Entomological Research, 43*(1), 70-78. 10.1111/1748-5967.12003

Walsh, P. S., Metzger, D. A., & Higuchi, R. (2013). Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques*, 54(3), 134-139.

Walstad, J. D., Anderson, R. F. & Stambaugh, W. J. (1970). Effects of environmental conditions on two species of muscardine fungi (*Beauveria bassiana* and *Metarrhizium anisopliae*). *Journal of Invertebrate Pathology*, 16(2), 221-226.

Wang, H., Peng, H., Li, W., Cheng, P., & Gong, M. (2021). The toxins of *Beauveria bassiana* and the strategies to improve their virulence to insects. *Frontiers in Microbiology*, *12*, 705343. https://doi.org/10.3389/fmicb.2021.705343

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications, 18*(1), 315-322.

Wilhelm, R. C., Amsili, J. P., Kurtz, K. S., Van Es, H. M., & Buckley, D. H. (2023). Ecological insights into soil health according to the genomic traits and environment-wide associations of bacteria in agricultural soils. *ISME Communications*, *3*(1), 1-11. https://doi.org/10.1038/s43705-022-00209-1

Wichadakul, D., Kobmoo, N., Ingsriswang, S., Tangphatsornruang, S., Chantasingh, D., Luangsa-Ard, J. J., & Eurwilaichitr, L. (2015). Insights from the genome of *Ophiocordyceps polyrhachis-furcata* to pathogenicity and host specificity in insect fungi. *BMC Genomics, 16*, 881.

Xia, E., Yang, D., Jiang, J., Zhang, Q., Liu, Y., Liu, Y., Zhang, Y., Zhang, H., Shi, C., Tong, Y., Kim, C., Chen, H., Peng, Y., Yu, Y., Zhang, W., Eichler, E. E., & Gao, L. (2017). The caterpillar fungus, *Ophiocordyceps sinensis*, genome provides insights into highland adaptation of fungal pathogenicity. *Scientific Reports*, *7*(1), 1-11. https://doi.org/10.1038/s41598-017-01869-z

Xiao, G., Ying, S., Zheng, P., Wang, Z., Zhang, S., Xie, X., Shang, Y., J., R., Zhao, G., Wang, C., & Feng, M. (2012). Genomic perspectives on the evolution of fungal entomopathogenicity in *Beauveria bassiana*. *Scientific Reports*, 2(1). https://doi.org/10.1038/srep00483

Xu, M., Liao, H., Qin, F., Gao, S., Ma, S., Chen, W., Huang, Q., & Hao, X. (2023). Deciphering the role of biotic factors in community assembly and diversity patterns of soil bacterial communities under different land use. *Ecological Indicators, 157,* 111273.

Yousef, M., Quesada-Moraga, E., & Garrido-Jurado, I. (2015). Compatibility of herbicides used in olive orchards with a *Metarhizium brunneum* strain used for the control of preimaginal stages of tephritids in the soil. *Journal of Pest Science, 88*, 605-612.

Zemek, R., Konopická, J., Jozová, E., & Skoková Habuštová, O. (2021). Virulence of *Beauveria bassiana* strains isolated from cadavers of Colorado Potato Beetle, *Leptinotarsa decemlineata*. *Insects*, *12*(12), 1077. https://doi.org/10.3390/insects12121077

Zheng, P., Xia, Y., Xiao, G., & et al. (2012). Genome sequence of the insect pathogenic fungus *Cordyceps militaris*, a valued traditional Chinese medicine. *Genome Biology, 12*(11), R116. https://doi.org/10.1186/gb-2011-12-11-r116

Zheng, J., Shi, J., & Wang, D. (2024). Diversity of soil fungi and entomopathogenic fungi in subtropical mountain forest in southwest China. *Environmental Microbiology reports*, *16*(4), e13267. https://doi.org/10.1111/1758-2229.13267.

Zimmermann, G. (1986). The *Galleria* bait method for detection of entomopathogenic fungi in soil. *Journal of Applied Entomology, 102*, 213-215.

Zimmermann, G. (2007). Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol Sci. Technol.* 17, 553–596. doi: 10.1080/09583150701309006

Zitlalpopoca-Hernandez, G., Najera-Rincon, M. B., del-Val, E., Alarcon, A., Jackson, T., & Larsen, J. (2017). Multitrophic interactions between maize mycorrhizas, the root-feeding insect *Phyllophaga vetula*, and the entomopathogenic fungus *Beauveria bassiana*. *Applied Soil Ecology, 115*, 38–43.

Zitlalpopoca-Hernandez, G., Pozo, M. J., Hauser, T. P., & Meyling, N. V. (2022). Combined effects of root-associated entomopathogenic and mycorrhizal fungi on the foliar pathogen *Botrytis cinerea* in tomato. *Biological Control*, *175*, 105034. https://doi.org/10.1016/j.biocontrol.2022.105034

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