Companion biota associated with *Leptospermum scoparium* (mānuka; Myrtaceae)

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Abstract

Leptospermum scoparium (mānuka; Myrtaceae) is involved in three crucial ecological interactions that might affect nectar production, and the New Zealand honey industry. First, these plants can be affected by scale insect infestation which have the potential to affect plant health, second, they provide nectar for honey bees (mānuka honey), and third, they are hosts for, and may receive benefits from, dual mycorrhizal fungal associations (both ecto- and endo-). The understanding of these interactions is very important for the honey industry as well as for New Zealand ecosystems. However, there is limited knowledge about the influence of scale insects and mycorrhizal fungi on plant growth and nectar production, and the influence of honey bee visitation on the honey making-process. To better understand the significance of these interactions, a variety of methods, including behavioural observations, histological, molecular, and taxonomic techniques, were used in this thesis.

Findings showed that the eriococcids *Acanthococcus campbelli* and *Acanthococcus leptospermi* are now the main species on *L. scoparium*, rather than *Acanthococcus orariensis*, which was the main causative agent of the mānuka blight in the 1940's and 1960's. Whereas the distribution of *A. leptospermi* was previously reported, the distribution of *A. campbelli* across New Zealand's islands was illustrated for the first time in this thesis. Other scale insect species classified within the families Coelostomidiidae, Diaspididae, and Pseudococcidae were also found, but their incidence and abundance was typically lower in comparison to the family Eriococcidae.

The number of eriococcids was reduced by the application of an Insect Growth Regulator (IGR) on six different cultivars in a split plot designed experiment, but cultivars differed in response to the insecticide treatment. Using the same common garden design, but just the unsprayed plants, honey bees showed a preference for the cultivar with the highest nectar sugar content and nectar DHA content. However, sugar, rather than DHA, was the best predictor of visitation pattern. The number of honey bee visits increased at midday as the day warmed up. The overall number of flowers estimated per plant was included in the model, but did not drive the visit number as, for example, it was found that the cultivar with the highest estimated number of flowers was less visited.

Bioinformatics analysis revealed the association of *L. scoparium* with at least 25 fungal classes, including 16 ectomycorrhizal (EcM) fungal lineages and eight arbuscular mycorrhizal (AM) families. The majority of mycorrhizal fungal lineages were shared among cultivated and wild plants at the three studied sites, which suggests that cultivated plants are naturally colonised by mycorrhizal fungi. The EcM fungal lineages /cortinarius, /laccaria, /tomentella-thelephora, and the AM families Glomeraceae and Claroideoglomeraceae were the most abundant. Among the EcM fungal species, *Laccaria glabripes* and the endemic EcM fungal species *Clavulina subrugosa*, *Cortinarius waiporianus* and *Dermocybe indotata* were revealed as the most abundant. The presence of the exotic EcM fungal species *Amanita muscaria* was limited and mainly found in cultivated plants, that had established on a site previously with *Pinus radiata*. The cosmopolitan AM fungal species *Rhizophagus irregularis* and *Claroideoglomus lamellosum* were the dominant species found in both cultivated and wild plants.

Among cultivated and wild plants, wild plants appeared to be colonised by a more diverse mycorrhizal fungal community. For instance, the lineage /russula-lactarius was more abundant in wild plants than in cultivated plants. The presence of /russula-lactarius and other lineages and species could be improving host performance (seed establishment, drought tolerance, pathogen resistance, and plant growth) on wild plants. However, the absence of some of the mycorrhizal fungal species from cultivated plants, which could be present on wild plants, could limit the potential yield of *L. scoparium* plantation. Finding suitable combinations of mycorrhizal fungal inoculum could help optimise the development of *L. scoparium*, nectar production, and subsequently the New Zealand mānuka honey industry.

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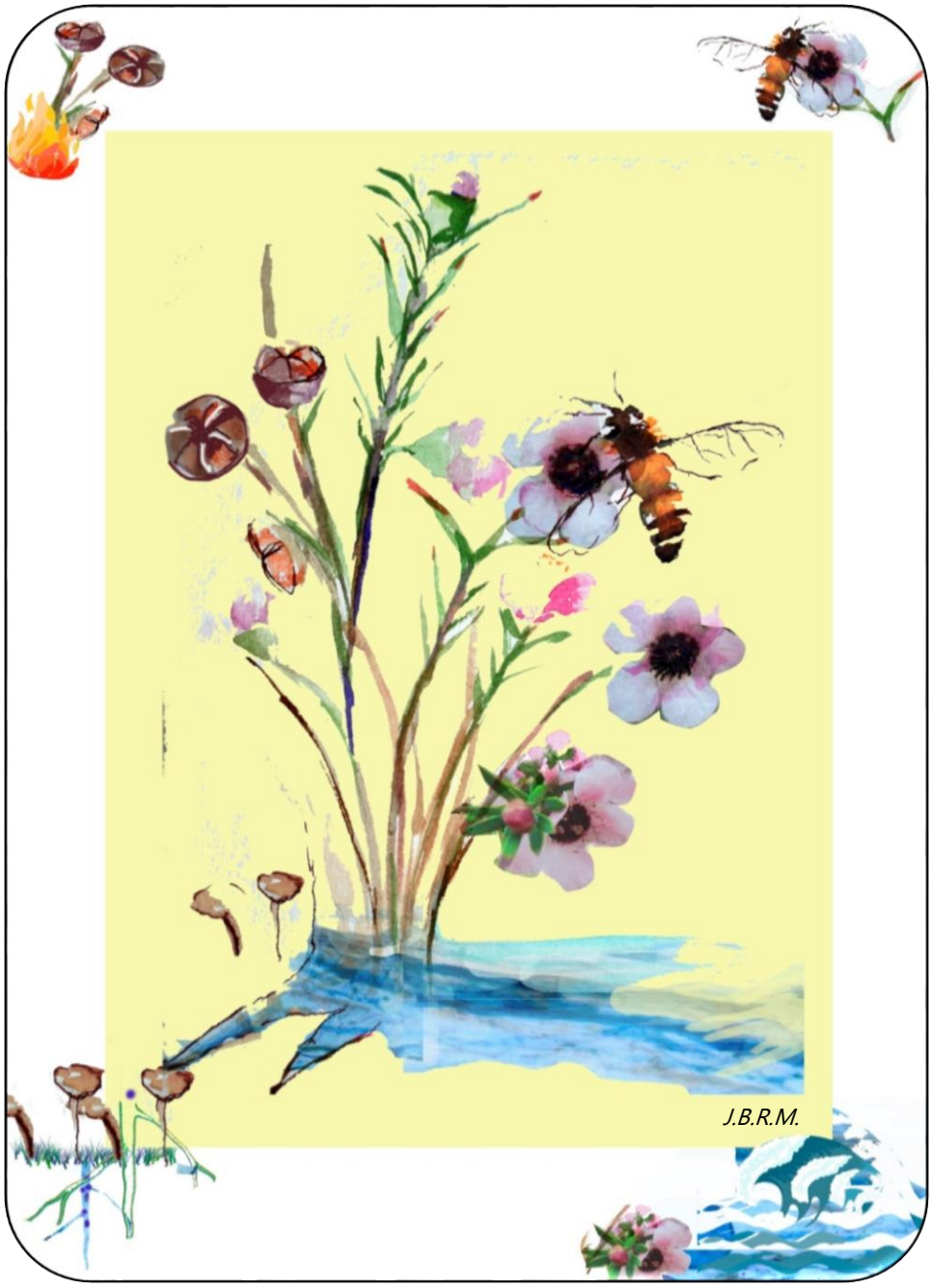
Kilometres do not make the distance, people make the distance.

To:

los pulgos Alessandro, Catalina, Cristina, and Eva



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Glossary of abbreviations

Abbreviation	Full name
AAs	Amino acids
acc. no.	Accession number
ACN	Acetonitrile
AGRF	Australian Genomic Research Facility
AK	Auckland
AM	Arbuscular mycorrhizal
am	Ante meridiem
ANOVA	Analysis of Variance
B	Blue (cultivar)
BA	Basal area
BIOM	Biological Observation Matrix
BLAST	Basic Local Alignment Search Tool
bp	Base Pairs
BP	Bay of Plenty
CL	Coromandel
Dev	Deviance
d.f.	Degrees of freedom
DHA	Dihydroxyacetone
DSE	Dark-septate endophyte
E	East
EcM	Ectomycorrhizal
GIS	Geographic Information System
GLM	Generalized Linear Model
GPS	Global Positioning System
H	Height
HA	Hydroxyacetone
HB	Hawkes Bay
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
IGR	Insect Growth Regulator
ITS	Internal Transcribed Spacer
KA	Kaikoura
KOH	Potassium hydroxide
LC	Lethal concentration
LG	Lime green (cultivar)
LOD	Limit of detection
LSU	Large Subunit
M	Molar
MG	Mint green (cultivar)
MGO	Methylglyoxal
N	North
NaOH	Sodium hydroxide
NC	North Canterbury
NCBI	National Center for Biotechnology Information
ND	Northland
NGS	Next Generation Sequencing

NIWA	National Institute of Water and Atmospheric
NMDS	Nonmetric Multidimensional Scaling
NN	Nelson
NPA	Nonperoxide activity
NZAC	New Zealand Arthropod Collection
NZGL	New Zealand Genomics Limited
O	Orange (cultivar)
OTU	Operational Taxonomic Unit
P	Pink (cultivar)
PCR	Polymerase Chain Reaction
PCRU	Pasture & Crop Research Unit
PermANOVA	Permutational multivariate analysis of variance
PFBHA	O-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine
PGP	Primary Growth Partnership
pH	Potential of hydrogen
Ph. As.	Phylum Ascomycota
Ph. Ba.	Phylum Basidiomycota
Ph. Ch.	Phylum Chytrydiomycota
Ph. Mu.	Phylum Mucoromycota
pm	Post meridiem
QIIME	Quantitative Insights into Microbial Ecology
Ra	Rangitatau
RI	Rangitikei
RI	Refractive Index
Ru	Ruatiti
rRNA	Ribosomal Nuclear A
S	South
SE	Standard error
sec	Seconds
SH	Species hypothesis
SI	Stewart Island
SSU	Small Subunit
Tut	Tutira
UMF	Unique Mānuka Factor
UV	Ultraviolet
VT	Virtual Taxa
W	West
WA	Wairarapa
WD	Westland
WI	Wanganui
Y	Yellow (cultivar)

Chapter 1.

General introduction

"What is seen above the root collar is a function of what happens below it, a fact that cannot be rationally ignored"

Trappe, 2004

1.1. Introduction

1.1.1. General background

In Spain, "The man of Bicorp", depicted in the Araña Cave, reveals the gathering of honey by humans at least 8000 years ago. From ancient times, humans used honey as traditional medicine (Zumla & Lulat, 1989), but it was in 1892 that its antibacterial properties were discovered by van Ketel (Dustmann, 1979). These antibacterial properties, mainly driven by hydrogen peroxide (White, Subers, & Schepartz, 1963; Allen, Molan, & Reid, 1991), encourage the use of honey for clinical treatment such as wound healing. However, hydrogen peroxide and the antibacterial properties associated with it dissipate in the presence of enzymes like catalase[1] (Allen et al., 1991; Cooper, Molan, & Harding, 1999). In Australasia, *Leptospermum scoparium* J.R.Forst. & G.Forst. (mānuka) is recognised for producing honey with additional antibacterial properties. The uniqueness of mānuka honey resides in its nonperoxide antibacterial activity (NPA), which is absent in the majority of the honeys (Molan & Russell, 1988; Allen, Molan, & Reid, 1991). Nonperoxide activity occurs mainly as a result of methylglyoxal (MGO), which has strong antibacterial activity (Adams et al., 2008). Consequently, mānuka honey is a natural antiseptic being used in clinical treatments such as wound dressings (Speer, Schreyack, & Bowlin, 2015).

1.1.2. From the natural source to the final product

New Zealand honeys are produced by honey bees that gather the nectar from different natural sources, including species such as *Trifolium repens* L. (clover), *Weinmannia racemosa* L. f. (kāmahi), *Kunzea ericoides* (A.Rich.) Joy Thomps. (kānuka), *L. scoparium*, *Metrosideros umbellata* Cav. (rātā), and *Knightia excelsa* R. Br. (rewarewa). From these final products, the New Zealand honey industry has raised its exports to $315 million in 2016, which triplicates the export benefits obtained in 2011 ($103 million) (Ministry for Primary Industries, 2016).

Among honeys, mānuka honey is regarded as a treasure due to the uniqueness of its antibacterial activity, which is originated from nectar dihydroxyacetone (DHA) that is

[1] Catalase: an enzyme present in all living organisms that decomposes hydrogen peroxide into oxygen and water

converted in the hive to MGO (Adams et al., 2008; Mavric et al., 2008; Adams et al., 2009), absent in the nectar of other plant species. Due to mānuka honey exclusivity, the price per kilogram of bulk mānuka honey fluctuated from $12 to $148 in 2016, while for light clover honey the price per kilogram ranged from $9.50 to $13 (Ministry for Primary Industries, 2016). Despite the importance of mānuka honey, there is limited understanding about the nectar produced by *L. scoparium* flowers (Smallfield, Joyce & Klink, 2018). Studies have shown that nectar DHA and nectar sugar are influenced by factors such as the genotype (Clearwater et al., 2018; Millner et al., 2016; Nickless et al., 2016) and environment (Clearwater et al., 2018; Williams et al., 2014). However, further study on a wide variety of scientific disciplines is needed to understand which potential factors influence nectar production, and subsequently, mānuka honey quantity and quality (Smallfield et al., 2018).

Besides the need of further research, there is a strong national and international demand of this honey and its derived products, while limited supplies exist to cover all demands (Ministry for Primary Industries, 2017). As result of the limited understanding of mānuka honey yield and its strong demand, a primary growth partnership[1] (PGP), led by Mānuka Research Partnership Limited and Comvita Limited, was established by the New Zealand government in 2011. This PGP programme has aimed to get a better understanding from the final product, honey, to its natural source, nectar, regarding environmental factors, companion biota associated with *L. scoparium*, and differences among *L. scoparium* provenances that can influence plant growth, flowering, nectar yield, and quality (Ministry for Primary Industries, 2017). Within the PGP programme, this PhD aims to get a better understanding of the companion biota associated with *L. scoparium*.

1.1.2.1. Mānuka honey characteristics

Honeys are characterised by low pH (White, 1975a), high osmolarity (high levels of sugars, particularly glucose and fructose) (White, 1975b), and hydrogen peroxide (promoter of antibacterial activity) (White et al., 1963; Bogdanov, 1984; Allen et al., 1991). Among these features, hydrogen peroxide is recognised as a driver of antibacterial activity (Molan, 1992). However, this peroxide activity is rapidly dissipated once it is in contact with enzymes such as catalase (Allen et al., 1991; Cooper et al., 1999), resulting

[1] http://www.mpi.govt.nz/funding-and-programmes/primary-growth-partnership/primary-growth-partnership-programmes/high-performance-manuka-plantations/

in reduced efficiency in wound-healing treatments. Along with these features, mānuka honey contains MGO, which is the main promoter of the NPA (Molan & Russell, 1988; Allen et al., 1991). Unlike hydrogen peroxide, MGO allows the continuation of the antibacterial activity once the honey is used for wound healing (Adams et al., 2008).

Although MGO plays a crucial role driving NPA (Adams et al., 2008), other honey compounds such phenolic compounds are being identified for their influence on antibacterial activity (Molan, 2015). This influence was validated by Molan (2015), who suggested a synergism exists between MGO and another compounds. To clarify the doubts and possible misunderstandings of antibacterial properties within mānuka honey, Molan demonstrated that MGO in the presence of water resulted in a low level of antibacterial activity in mānuka honey, which increased when MGO was combined with the phenolic compounds (Molan, 2015). These results demonstrated the importance of MGO and other compounds for evaluating the activity of mānuka honey.

1.1.2.2. Making and grading mānuka honey

Honey bees collect nectar and add the glucose-oxidase enzyme, which is responsible for generating hydrogen peroxide and gluconic acid (White et al., 1963). The peculiarity of mānuka honey is that during the honey making-process the additional compound DHA in the nectar is transformed to MGO (Adams et al., 2009). These two compounds have been widely used for grading mānuka honey (Atrott, Haberlau, & Henle, 2012), but currently additional chemical markers that can validate the authenticity of mānuka honey are being evaluated (Stephens et al., 2017). Recently, Stephens et al. (2017) identified two plant compounds, leptosperin[1] and lepteridine[2], that could be useful for defining mānuka honey. Leptosperin and lepteridine compounds are the precursors of the fluorescent emissions $_{ex}270–_{em}365$ nm (MM1) and $_{ex}330–_{em}470$ nm (MM2) respectively in mānuka honey (Stephens et al., 2017). Consequently, both fluorescence emissions are being suggested as potential markers for mānuka honey. However, MM1 and MM2 are not correlated with the nectar compound DHA and the honey compound MGO (Stephens et al., 2017), which complicates the evaluation of the antibacterial activity.

[1] Leptosperin: nectar derivative of methyl syringate 4-O-β-D-gentiobiose in mānuka honey.
[2] Lepteridine: nectar derivative of pteridine derivative 3, 6, 7-trimethyllumazine in mānuka honey.

1.1.2.3. Companion biota associated with Leptospermum scoparium

The root system is responsible for absorbing nutrients and water, which help determine the plant's above ground biomass (López-Bucio et al., 2003). Biomass is directly associated with photosynthetic capability and it can be indirectly correlated to plant features such as leaves, stems and flowers (Chaplin & Walker, 1982; Gange, Brown & Aplin, 2005). In addition, floral traits can be indirectly affected by this capability (Gange & Smith, 2005). For example, the root weight can be correlated with nectar production, suggesting an indirect response to the availability of plant biomass above ground (Pleasants, 1983; Gange & Smith, 2005). Root biomass is positively influenced by symbionts such as mycorrhizal fungi that uptake and transfer water and nutrients, important for stimulating plant growth (Hall, 1977a). However, other fungi associated with *L. scoparium* have shown a detrimental visible effect on the plant (Mulcock, 1954; Hoy, 1961). The fungus *Capnodium walteri* Sacc. is a causative agent of the sooty mould covering stems and leaves (Mulcock, 1954). This fungus appears following infestation by the scale insect *Eriococcus* (now *Acanthococcus*) *orariensis* Hoy, which sucks sap from the plant and excretes honeydew that allows the growth of sooty mould (Hoy, 1961). These two agents were responsible for the mānuka blight of the 1940s and 1060s. *Capnodium walteri* had a visual impact on plants due to the "black mantle"; subsequently, it was an indicator of honeydew and *A. orariensis* presence (Hoy, 1961). In contrast, *.A. orariensis* was suggested as the main agent that debilitated and caused the death of the plant (Hoy, 1961).

As previous studies have shown (Hoy, 1961; Hall, 1977a), scale insects and mycorrhizal fungi can damage or benefit *L. scoparium* development, affecting plant growth and may affect flowering, nectar yield, and quality. Furthermore, as nectar yield and quality vary among *L. scoparium* provenances (Stephens et al., 2010; Williams et al., 2014; Millner, Hamilton, Ritchie, & Stephens, 2016), honey bee visitation between these provenances could also differ, influencing honey yield and quality. However, even though there is a significant interest in increasing mānuka honey yield and quality (Ministry for Primary Industries, 2017), limited research has been carried out in these areas. Therefore, there is a need for further research determining the importance of these associated biotic elements for *L. scoparium* and the influence of different provenances on honey bee visitation. The results from these studies could lead to breeding programs where *L. scoparium*

provenances are selected according to their potential for improving mānuka honey yield and quality.

1.2. Literature review

This literature review summarises the biology of *L. scoparium* and comments on morphological features, which can play an important role in how this species fits into ecosystems. In addition, scale insects and mycorrhizal fungal species associated with *L. scoparium* are outlined and the main effects on the host plant are described. As honey bees are crucial during the mānuka honey-making process, the sensory processes that drive honey bees to a flower are described, and potential factors affecting their decision are outlined.

While this section is an overall review, a comprehensive review of each biotic element is presented in the introduction of each experimental chapter (Chapter 2, 3 and 4). Chapter 2 describes main causative agents of mānuka blight and their consequences. Chapter 3 introduces insecticide treatments applied to scale insects, the main factors affecting honey bee visitation, and the importance of *L. scoparium* nectar. Chapter 4 defines main mycorrhizal groups, their influence on hosts and approaches commonly used for determining mycorrhizal fungal communities.

1.2.1. Taxonomy and biology of Leptospermum scoparium

Leptospermum scoparium J.R. Forst et G. Forst (mānuka, tea tree or kahikatoa) is defined by the Greek terms "*leptos*" and "*sperma*", meaning, respectively, slender and thin, and the seed. The species term is derived from the Latin term "*scoparium*", which means broom. While *L. scoparium* was originally considered endemic to New Zealand (Allan, 1961), later studies demonstrated that it is also native to mainland Australia and Tasmania, as well as to New Zealand, where it is found from Cape Reinga to Stewart Island (Thompson, 1989). Initial suggestions were that this plant arrived in New Zealand during the Palaeocene period (56-66 Ma) (Fleming, 1975), but a recent study have shown that it probably arrived from Australia 2–3 Ma (Battersby et al., 2017).

Leptospermum scoparium belongs to the family Myrtaceae, which contains nearly 130 genera and 6000 species (Christenhusz & Byng, 2016). Of the 130 genera, the genus *Leptospermum* includes 87 species, but *L. scoparium* is recognised as the only native species within the genus *Leptospermum* recorded in New Zealand (Dawson, 1997). However, previously the species *Leptospermum ericoides* (kānuka) A. Rich was also included in this genus. This species was redefined as *Kunzea ericoides* (A. Rich) Joy after

the nomenclatural revision of the genus *Leptospermum* (Thompson, 1983). Then, it was subsequently segregated into ten species (de Lange, 2014).

Leptospermum scoparium is described as a shrub or small tree typically from 2 to 4 m in height (Allan, 1961). The bark is grey and peels off in long strips (Allan, 1961). It has leathery dark green leaves, lanceolate with a fine margin and measuring between 10 to 15 mm length and 1.2 mm of width (Allan, 1961; Thompson, 1989). The flowers are polymorphic, andromonoecious, symmetrical, solitary in leaf axils, and mostly white (Burrell, 1965; Primack & Lloyd, 1980). They measure up to 20 mm in diameter and have five white, pink or occasionally red petals (Burrell, 1965; Primack & Lloyd, 1980; Dawson, 1997). Flowers produce hexose-dominant nectar (Williams et al., 2014; Nickless et al., 2016), dominated by the monosaccharides fructose and glucose (Nicolson, Nepi, & Pacini, 2007).

Persistent woody fruit-valves (capsules) are a characteristic of *L. scoparium* (Thompson, 1989), which is a partially serotinous species. This ecological adaption is common in Australian and European species, but unusual in the New Zealand flora (Perry, Wilmshurst, & McGlone, 2014). After a fire event, woody fruit-valves in the serotinous forms of *L. scoparium* open and release their seeds, facilitating the natural regeneration and forest restoration (Thompson, 1989; Harris, 2002; Bond, Dickinson, & Mark, 2004; Bond, & Muethel, 2012; Perry et al., 2014). *Leptospermum scoparium* is also able to tolerate other stressful conditions such as waterlogging (Cook, Mark, & Shore, 1980). This resistance is conferred by the aerenchymatous layers present in the bark and in the root system (Cook et al., 1980). This root system is characterised by both adventitious roots and a large number of fine roots, which also increase its resistance to waterlogging conditions (Cook et al., 1980).

Leptospermum scoparium grows from sea level to approximately 1200 m (Burrows, 1973). It is a nurse species able to be established in extreme conditions (e.g. frost, wind, poor soils with low fertility) (Mulcock, 1954; Molloy, 1975; Primack & Lloyd, 1980; Ausseil & Dymond, 2010; Davis et al., 2013). Due to its capability for growing in diverse soil conditions, *L. scoparium* can be established from above the tree line to the sea level (Wardle, 1991). In New Zealand, it appears from gumlands in North Island (Burrows, Mcqueen, Esler, & Wardle, 1979) to wetlands in South Island (Wardle, Mark, & Baylis, 1973; Wardle, 1974). It is commonly found on New Zealand hill countries and specially

on land susceptible to erosion (Bergin, Kimberley, & Marden, 1995), and for growing at sites characterised by arsenic without absorbing this substrate (Craw et al.,2007).

The life span of *L. scoparium* ranges from 20 to 70 years (Rogers & Leathwick, 1994), being replaced by forest species such as *K. ericoides*, the life span of which ranges from 100 (Atkinson, 2004) to 250 years (Burrows, 1973). However, depending on altitude, the suppression of *L. scoparium* by the presence of *K. ericoides* can occur at different stages, being slower at higher altitudes (Rogers & Leathwick, 1994). For example, this suppression occurs at 20 years at Waitakere Ranges (Esler & Astridge, 1974), while both *L. scoparium* and *K. ericoides* start to coexist at 30 years in Otago (Allen et al., 1992). The suppression of *L. scoparium* occurs when the *K. ericoides* canopy starts covering *L. scoparium*, providing shading that limits *L. scoparium* growth. However, despite *K. ericoides* being regarded as successional to *L. scoparium*, studies have shown that during a perturbation event such as a fire, *L. scoparium* will be favoured compared with *K. ericoides* (Atkinson, 2004). Whereas *L. scoparium* seeds will be retained and open probably at high temperatures, *K. ericoides* seeds will probably not usually resist this perturbation (Atkinson, 2004). If both plant species are established in the same habitat, *L. scoparium* regeneration will be favoured, as it is a pioneer species present in the first stage of an ecological succession and is followed by forest (Mohan, Mitchell & Lovell, 1984).

Along with it ecological roles, *L. scoparium* is also known for different uses. These uses include boiling leaves for tea (Cooper & Cambie, 1991) and using mānuka oil as an antiseptic (Maddocks-Jennings et al., 2009; Chen et al., 2014). Tea from *L. scoparium* (tea-tree) was produced by Captain James Cook after his second voyage to New Zealand. Cook also used boiled leaves from *L. scoparium* and *Dacrydium cupressinum* Sol. ex Lamb. (rimu; Podocarpaceae) for brewing beer (Cooper & Cambie, 1991). *Leptospermum scoparium* wood was widely used for building tools and canoes by Māori (Cooper & Cambie, 1991). However, nowadays *L. scoparium* is mainly valued for its honey. Therefore, landowners are establishing plantations of *Leptospermum scoparium* on marginal hill country to help stabilise slopes (Primack & Lloyd, 1980; Ausseil & Dymond, 2010; Davis et al., 2013), and to produce mānuka honey. The mānuka honey industry earned $150 million from exports in 2016 (McPherson, 2016), and this export value is expected to rise to $1.2 billion by 2028 (Ministry for Primary Industries, 2017), indicating the importance of the mānuka honey industry. *Leptospermum scoparium* has

been important due to its different uses, and importantly due to its honey. However, ironically, this plant was considered a weed in the past, as it could reduce the value of the agricultural land (Hoy, 1961). Therefore, there was an interest in eradicating *L. scoparium* throughout New Zealand.

Mānuka honey yield depends directly on honey bee visitation, as they gather the nectar that is transformed afterward to honey (White et al., 1963). Besides the harvesting efficiency of the honey bee, which is the main biotic element influencing mānuka honey yield, two other biotic elements (scale insects, mycorrhizal fungi) associated directly with *L. scoparium* could influence plant growth, flowering, nectar yield, and quality, as it has been shown in other studies (Gange & Smith, 2005; Gehring & Bennett, 2009). Depending on their influence, mānuka honey yield and the subsequent benefits obtained by the mānuka honey industry could differ. Thus, these three elements are regarded as important potential drivers of mānuka honey yield, and, subsequently, as drivers of the profit obtained by the mānuka honey industry.

1.2.2. Companion biota associated with Leptospermum scoparium

1.2.2.1. The olfactory and visual stimuli of honey bees

The honey bee (*Apis mellifera* Linnaeus) is recognised as one of the 41 bees species present in New Zealand (Donovan, 2007). This species was first introduced into New Zealand from England in 1839 by Mary Anna Bumby in two straw hives (Barrett, 1996) and were established in Hokianga, Northland (Barrett, 1996).

The honey bee *Apis mellifera* belongs to the Order Hymenoptera, Family Apidae, and Subfamily Apinae. Its name is derived from the Latin term "*Apis*" meaning bee, and the Greek terms "*melli*" and "*ferre*", which means honey and bear respectively. Honey bees are long-tongue bees characterised for gathering pollen and nectar from the flowers as sources of proteins and carbohydrates respectively (Menzel, 1993; Goulson, 1999). They are eusocial, expressing their gathering experience through the "waggle dance" (von Frisch, 1967). The size of their brain is minimal, containing only approximately 960,000 neurons (Giurfa, 2007). However, they have the capability of learning a new colour in less than half an hour, a new odour in half a day, and a new route after 3 or 4 visits (Werner & Chalupa, 2004).

Two main organs, antennae and eyes, are responsible for the olfactory and visual stimuli that drive honey bee activity (Giurfa, 2007). Antennae are the main chemoreceptor organs, reacting to sucrose, which is probably the favoured nectar compound (Giurfa, 2007). Bees extend their proboscis to suck up the sucrose solution once their antennae are in contact with sucrose (Takeda, 1961). Eyes are the main photoreceptor organs involved in the flower selection process, which is influenced mainly by flower phenotype factors such as colour (Giurfa, 1991; Srinivasan, 2010), shape (Lehrer et al., 1995), and symmetry (Dafni & Kevan, 1996). Flower phenotype can directly influence honey bee visitation (Giurfa, 2007) and indirectly, nectar quality and quantity (Percival, 1961; Galetto & Bernardello, 2004; Witt, Jürgens, & Gottsberger, 2013). For example, flowers characterised by long-tube corollas can reduce evaporation and increase nectar volume (Witt et al., 2013). In addition, there are some plant families characterised by longer-tube corollas that have shown to produce sucrose-dominant nectar, rather than hexose-dominant nectar (Percival, 1961). Studies have found that sucrose-dominant nectar is usually more attractive to long-tongue bees such as honey bees (Wykes, 1952; Baker & Baker, 1983), which is a disadvantage for *L. scoparium* as it produces hexose-dominant nectar (Williams et al., 2014; Nickless et al., 2016).

Studies have evaluated nectar sugar and the carbohydrate dihydroxyacetone (DHA) in different *L. scoparium* cultivars, demonstrating that DHA differs among cultivars (Williams et al., 2014; Millner et al., 2016; Nickless et al., 2016). However, these studies did not examine the influence of *L. scoparium* nectar traits such as sugar and DHA content on honey bee visitation. Other studies have shown that sugars and other nectar compounds such as amino acids (Inouye & Waller, 1984; Hendriksma, Oxman, & Shafir, 2014), minerals (Waller, 1972), and phenolic compounds (Hagler & Buchmann, 1993) influence honey bee visitation. This suggests that DHA could affect the olfactory senses of honey bees. Along with nectar compounds, other factors (e.g. environmental conditions, habitat and other insect visits) can be determinant in the decision-making process of honey bees. For example, warmer temperatures can increase the number of visits (McCall & Primack, 1992; Totland, 1994) and native fly visits have shown to be negatively correlated with honey bee visits on *L. scoparium* flowers (Bennik, 2009).

Bees and flies are regarded as important insects responsible for plant pollination in New Zealand (Lloyd, 1985; Newstrom & Robertson, 2005). Specifically, native flies (Diptera)

and short-tongue bees (Hymenoptera) are regarded as major pollinator in the New Zealand native flora (Heine, 1937; Primack, 1978, 1983). More than 2000 species are recognised within Diptera, being the families Muscidae, Syrphidae, and Tachinidae the most representative (Macfarlane & Gurr, 1995; Crowe, 2002). Along with them, moths (Lepidoptera) and beetles (Coleoptera) are also well represented in New Zealand. However, the role of moths as pollinators is still uncertain, since the information available focuses on rather scant moth-plant observations, rather than pollen transfer by moths (Buxton et al, 2018). Other pollinators such as butterflies and hawkmoths, which are recognised worldwide, are limited in New Zealand habitats (Lloyd, 1985).

1.2.2.2. Scale insects

Scale insects belong to the Order Hemiptera and the superfamily Coccoidea. They were firstly named as coccids, derived from the Latin term "*Coccus*" meaning berries (Lambdin, 2004). During ancient times, scale insects were recognised as berries rather than insects. Therefore, Carl Linnaeus introduced this term, including the majority of scale insects into the genus "*Coccus*" (Kondo, Gullan, & Williams, 2008).

Globally there are approximately 8000 species classified into 1150 genera (García Morales et al., 2010). They are sap suckers that can be involved in a mutualistic relationship with their host and pollinators such as bees, as the honeydew excreted by them can be a source of food for these pollinators (Markwell, Kelly, & Duncan, 1993). Previous studies have shown that they can have an impact on the host both directly on host development (Dixon, 1971a, 1971b; Newbery, 1980; Vranjic & Gullan, 1990), and indirectly, by facilitating the development of a black sooty mould which grows on leaves and branches using the honeydew excreted from them as a food source, which smothers the leaves and stems. Scale insects can thus be important pests, causing ecological and economic impacts (Herren & Neuenschwander, 1991; Jordan et al., 1993; Hamon & Hodges, 2001; Cale et al., 2017). For example, *Cryptococcus fagisuga* Lindinger (Eriococcidae) have significantly affected the timber production of *Fagus grandifolia* Ehrh. (Fagaceae) in North America (Cale et al., 2017), and *Saissetia oleae* Olivier (Coccidae) have damaged olive trees in Tunisia (Mansour et al., 2017).

Of the nearly 8000 species, approximately 830 are found in Australia and 370 in New Zealand (García Morales et al., 2010). Among them, approximately 270 species are recognised as native to New Zealand (Henderson, 2009). They are classified into ten

families (Henderson, 2009), with Eriococcidae the largest family that includes most native species (ca 100 species) (Hoy, 1954, 1958, 1959, 1962; Hodgson, 1994; Hodgson & Henderson, 1996; Henderson, 2006, 2007a, 2007b; Henderson, Sultan, & Robertson, 2010). This family, commonly known as felt scale insects, is characterised by its diversity in the Southern Hemisphere, and is most often associated with host plants belonging to the Myrtaceae (Gullan, Miller, & Cook, 2004). For example, genera such as *Eucalyptus* L'Hér. and *Leptospermum* J.R.Forst. & G.Forst. are known for being associated with *Eriococcus* (now *Acanthococcus*) *coriaceus* Maskell (Morales & Bain, 1989) and *A. orariensis* (Hoy, 1954).

In New Zealand, three *Acanthococcus* species have been found associated with *L. scoparium*, *Acanthococcus orariensis* (Hoy, 1954), *A. campbelli* Hoy (Henderson, 2009) and *A. leptospermi* Maskell (Hoy, 1961), all of them reported as being dispersed from Australia (Hoy, 1959, 1961). However, whereas *A. orariensis* was well studied during the 1950s due to the mānuka blight, limited studies have reported the possible presence of *A. campbelli* and *A. leptospermi* (Gardner-Gee & Beggs, 2009; Henderson et al., 2010). *Acanthococcus leptospermi* seems to cause a minimal impact on *L. scoparium*, but *A. campbelli* is suggested as the potential successor of *A. orariensis* on *L. scoparium* in New Zealand (Hoy, 1961). This species could thought to have the potential to cause more losses in *L. scoparium* than could *A. orariensis* (Hoy, 1959).

Globally, a total of 23 species of scale insects are reported as being associated with *L. scoparium* (Table 1.1); 20 are present in New Zealand. Among these 20 species, *A. campbelli* is not included, as this species has been to date only definitively reported from Australia according to Scalenet (García Morales et al., 2010). The majority of species are reported only from New Zealand, and most are present on host plants classified as Myrtaceae, which suggest a high level of specificity between scale insects and this host family. Despite this high level of local specificity, there is a risk of more species dispersing from Australia. Therefore, there is a need for further research examining the species currently associated with *L. scoparium* and their distribution as this may also allow identification of novel species.

Table 1.1. Scale insect species associated with *L. scoparium* according to Scalenet (García Morales et al., 2010). Table includes author, year, distribution and host plant family associated to the respective scale insect species. *This generic placement requires further study; the species is usually placed in *Acanthococcus* (P.J. Gullan, pers. comm.).

Family	Genus	Species	Author; year	Distribution	Host family
Asterolecaniidae	*Asterolecanium*	*epacridis*	Maskell; 1882	Australia, New Zealand	Ericaceae, Myrtaceae
Coccidae	*Crystallotesta*	*leptospermi*	Maskell; 1882	New Zealand (NI, SI, TKI)	Myrtaceae
Coccidae	*Crystallotesta*	*ornata*	Maskell; 1885	New Zealand (NI)	Elaeocarpaceae, Lauraceae, Monimiaceae, Myrtaceae, Nothofagaceae, Podocarpaceae
Coccidae	*Crystallotesta*	*ornatella*	Henderson & Hodgson; 2000	New Zealand (NI, SI)	Ericaceae, Myrtaceae
Coccidae	*Plumichiton*	*pollicinus*	Henderson & Hodgson; 2000	New Zealand (NI, SI)	Myrtaceae
Coccidae	*Umbonichiton*	*bullatus*	Henderson & Hodgson; 2010	New Zealand (NI)	Cunoniaceae, Myrtaceae, Podocarpaceae
Coelostomidiidae	*Coelostomidia*	*jenniferae*	Morales; 1991	New Zealand (SI)	Araliaceae, Asteraceae, Cunoniaceae, Cupressaceae, Elaeocarpaceae, Myrtaceae, Nothofagaceae, Phyllocladaceae, Podocarpaceae, Winteraceae
Coelostomidiidae	*Coelostomidia*	*wairoensis*	Maskell; 1884	New Zealand	Myrtaceae, Xanthorrhoeaceae
Diaspididae	*Anzaspis*	*angusta*	Green; 1904	Australia, New Zealand	Myrtaceae, Primulaceae
Diaspididae	*Hemiberlesia*	*lataniae*	Signoret; 1869	109 countries (Australia and New Zealand included)	113 families (Myrtaceae included)
Diaspididae	*Hemiberlesia*	*rapax*	Comstock; 1881	71 countries (Australia and New Zealand included)	79 families (Myrtaceae included)

Diaspididae	*Poliaspis*	*media*	Maskell; 1880	6 countries (New Zealand included)	21 families (Myrtaceae included)
Diaspididae	*Symeria*	*intermedia*	Maskell; 1891	New Zealand (SI)	Myrtaceae
Diaspididae	*Symeria*	*leptospermi*	Maskell; 1882	New Zealand (NI, SI)	Myrtaceae
Eriococcidae	*Acanthococcus*	*campbelli*	Hoy; 1959	Australia	Myrtaceae
Eriococcidae	*Acanthococcus*	*gibbus*	Hoy; 1959	Australia	Myrtaceae
Eriococcidae	*Acanthococcus*	*leptospermi*	Maskell; 1891	Australia, New Zealand	Myrtaceae
Eriococcidae	*Acanthococcus*	*orariensis*	Hoy; 1954	Australia, New Zealand (NI, SI)	Myrtaceae
Eriococcidae	**Uhleria*	*mariannae*	Pellizzari & Germain; 2010	Corsica, Italy	Myrtaceae
Pseudococcidae	*Crisicoccus*	*tokaanuensis*	Cox; 1987	New Zealand	Myrtaceae
Pseudococcidae	*Paracoccus*	*leptospermi*	Cox; 1987	New Zealand	Myrtaceae
Pseudococcidae	*Paracoccus*	*miro*	de Boer; 1967	New Zealand	Elaeocarpaceae, Ericaceae, Myrtaceae, Podocarpaceae, Rousseaceae, Sapindaceae
Pseudococcidae	*Paracoccus*	*zealandicus*	Ezzat & McConnell; 1956	New Zealand	Achariaceae, Ericaceae, Myrtaceae, Nothofagaceae, Rutaceae

1.2.2.3. The fungi kingdom

The Fungi kingdom was recognised as an independent kingdom for the first time by Whittaker in 1969 (Whittaker, 1969). This kingdom is suggested to include at least 1.5 million species (Hawksworth, 1991), although further studies raise this number to 2.2 to 3.8 million (Hawksworth, 2001; Hawksworth & Lücking, 2017). Among them, approximately 40,000–50,000 are recognised as mycorrhizal fungi (Heijden, Martin, Selosse, & Sanders, 2015). Their classification as mycorrhizal is derived from the Greek terms "*mykós*" and "*rhiza*", meaning fungus and root respectively. This term was established by Professor Albert Bernhard Frank in 1885 (Trappe, 2004), when he described the symbiotic relationship between plants of the family Cupuliferae (Fagaceae) and mycorrhizal fungi (Frank & Trappe, 2005).

Mycorrhizal fungal species differ depending on their fungal structures and their role within the host. They are categorised into four main groups, arbuscular mycorrhiza (AM), ectomycorrhiza (EcM), orchid mycorrhiza and ericoid mycorrhiza (Smith & Read, 2008). Among these, orchid and ericoid mycorrhizal fungi are only found within two plant families, the family Orchidaceae and Ericaceae respectively (Heijden et al., 2015). Arbuscular mycorrhizal fungi are the most common, being present in more than 200,000 host plants. Host species numbers are about to 6,000, 20,000–35,000, and 3,900 for EcM, orchid mycorrhiza, and ericoid mycorrhiza, respectively (Heijden et al., 2015). In comparison with ericoid and orchid mycorrhiza, the groups AM and EcM, which were previously classified as endo and ecto-mycorrhizal respectively (Trappe, 2004), have been widely studied (Heijden et al., 2015). The initial distinction was based on the presence of a fungal mantle surrounding the host root, which was absent in endomycorrhizal fungi.

The mantle was the first mycorrhizal fungal structure recognised, along with the Hartig net, by Theodor Hartig in 1840 (Trappe, 2004). The Hartig net is an intercellular hyphal network, formed by finely branched hyphal strands that facilitates the contact between host and fungus (Trappe, 2004; Brundrett, 2009). These structures play a major role in the symbiotic relationships between host plants and mycorrhizal fungi (Harley & Smith, 1983). While the host plant receives mineral nutrients (e.g. nitrogen and phosphorus) from the fungi, the fungi receive resources such as sugars and water from their hosts (Harley & Smith, 1983). This nutrient exchange occurs through different specialised

mycorrhizal fungal structures depending on the mycorrhizal group (Smith & Read, 2008; Brundrett, 2009). For example, while the Hartig net is defined as the fundamental EcM fungal structure where this exchange occurs, arbuscules are the fundamental structure within AM fungi (Brundrett, 2009). As result of these associations, host plants may have increased drought tolerance (Michelsen & Rosendahl, 1990), pathogen resistance (Sikes, Cottenie, & Klironomos, 2009), growth (Tedersoo, May, & Smith, 2010; Suparno et al., 2015) and seedling survival (Heijden, 2004). These benefits depend both on the mycorrhizal group (Lodge, 2000), and also on the host plant identity (Nguyen et al., 2016; Kolaříková et al., 2017). Only a few plant genera are able to form a dual EcM and AM association (Harley & Harley, 1987; Brundrett & Bougher, 1996; Lodge, 2000). Among them, *Leptospermum* and *Kunzea* are recognised as the only two woody plant genera able to form this dual association in New Zealand (Hall, 1977b; Moyersoen & Fitter, 1999; Orlovich & Cairney, 2004; McKenzie, Johnston, & Buchanan, 2006).

1.2.2.3.1. Mycorrhizal fungi associated with Leptospermum scoparium in New Zealand

The ecological uniqueness of *L. scoparium* results from its dual association with EcM (Mcnabb, 1968; McKenzie et al., 2006) and AM fungi (Baylis, 1971; Hall, 1977b). Previous studies have shown that this association can improve the development and establishment of *L. scoparium* (Baylis, 1971; Hall, 1975) and the spread of other EcM hosts such as *Nothofagus* Blume (Wardle, 1980). The improvement of host dispersal and colonisation is suggested as a reciprocal benefit for both hosts, *L. scoparium* and *Nothofagus* (Cooper, 1976; Wardle, 1980), and also between *Kunzea ericoides* and *Nothofagus* (Dickie, Davis, & Carswell, 2012). However, despite its importance as a host for both EcM and AM mycorrhizal fungi, there are limited studies of the mycorrhizal fungal communities associated with *L. scoparium*.

Ectomycorrhizal fungal communities may be more abundant on older *L. scoparium* plants than in younger plants (Weijtmans et al., 2007). In their study, Weijtmans et al. highlighted the need of further research on the fungal communities associated with *L. scoparium* and *Nothofagus*, since these two hosts will have a reciprocal benefit when they share the same EcM fungal species. *Leptospermum scoparium* and *Nothofagus* share at least 30 EcM fungal genera (Orlovich & Cairney, 2004). Of the 30 genera, *Cortinarius* (Pers.) Gray species may be present on both hosts; which is not surprising, given that *Cortinarius* is globally the largest EcM fungal genus (Kirk et al., 2008) and one of the

most diverse and abundant in New Zealand (Taylor, 1970; Dickie, Bolstridge, Cooper, & Peltzer, 2010; Tedersoo et al., 2010). However, the abundance, identity, distribution, and influence of *Cortinarius* species and other EcM fungal species associated with *L. scoparium* are poorly understood (Tedersoo et al., 2010; Teasdale et al., 2013).

Similarly, limited research has been undertaken on the association between AM fungal communities and *L. scoparium*. It was in 1977 when Hall, using a morphological approach, revealed the association between *L. scoparium* and two AM species (*Acaulospora laevis* Gerd. & Trappe and *Glomus pallidum* I.R. Hall). These species were identified morphologically in the past by examination of the spores (Hall, 1977b), an approach also traditionally used for identifying EcM fungal species. However, there are limitations to classifying mycorrhizal fungal species using their morphological structures, as there are some fungal taxa that cannot be classified to species level without the use of molecular techniques (Schubler, 1999; Kim et al., 2003). The use of molecular techniques including next generation sequencing (NGS) has substantially increased the knowledge of mycorrhizal fungal diversity and distribution between hosts and geographical locations (Tedersoo et al., 2010; Krüger et al., 2012).

1.2.2.3.2. Next generation sequencing and bioinformatics analysis

Next generation sequencing, also known as high-throughput sequencing, has been widely used over the last decade, to help clarify novel mycorrhizal fungal lineages and their distribution worldwide (Öpik et al., 2013; Davison et al., 2015). Different NGS platforms, including 454-pyrosequencing (Öpik et al., 2009), Ion semiconductor (Taylor, Helgason, & Öpik, 2017), and Illumina (Smith & Peay, 2014), have been used for recovering DNA sequences. Prior to sequencing, DNA must be extracted and amplified(Lindahl et al., 2013; Hart et al., 2015) using specific markers from different nuclear ribosomal DNA (rDNA) regions (Ban et al., 2000; Schluenzen et al., 2000; Wimberly et al., 2000; Yusupov et al., 2001).

For eukaryotes, nuclear rDNA is composed of three conservative regions recognized as 18S (small subunit, SSU), 5.8S, and 28S (large subunit, LSU). It also includes two non-coding regions denominated as internal transcribed spacers (ITSs), ITS1 and ITS2. Both spacers are separated after the transcription phase occurs. Therefore, they are highly variable, so useful for phylogenetic studies, but often complex (Stockinger, Krüger, & Schüßler, 2010). These three regions (ITS, LSU and SSU) are widely used for researching

fungal communities, but the target region differs among studies, as different studies focus on different fungal clades and research objectives (Kohout et al., 2014). For example, the ITS region is the universal fungal barcode, since it usually performs better for amplifying DNA through PCR than the SSU or the LSU (Schoch et al., 2012). Therefore, the ITS region is commonly used for an overall perspective of fungal communities present in a source (e.g. roots and soil). However, the LSU (Krüger et al., 2009) and SSU (Öpik et al., 2013) are commonly used for targeting specific fungal clades (e.g. Glomeromycota) and for carrying out phylogenetic studies of AM fungi.

After DNA is amplified and amplicons are sequenced, bioinformatics analysis needs to be undertaken to identify fungal clades. To do so, different pipelines, such as QIIME (Caporaso et al., 2010) and USEARCH (Edgar, 2010), based on different algorithms, are generally used. Within this analysis, sequences with high similarity are clustered in a representative sequence (Edgar, 2010), commonly defined as the operational taxonomic unit (OTU), which is further assigned to a fungal taxa.. Fungal taxa classification is essential step for evaluating the presence, abundance and distribution of mycorrhizal fungal communities. In addition, phylogenetic studies are needed if the main aim lead to understanding the connection and evolution between fungal clades. To carried out phylogenetic studies on mycorrhizal fungal studies, sequence alignment is mainly performed when the target regions are LSU and SSU, as the ITS region is highly variable and phylogenetic studies are difficult to conduct (Stockinger, Walker, & Schüßler, 2009). Consequently, studies involving NGS and the combination of several rDNA regions often lead to a better understanding of different fungal clades, their distribution and phylogeny than single gene studies.

1.3. Research objectives and thesis outline

Previous studies have shown that the biotic elements scale insects and mycorrhizal fungi can influence the development of *Leptospermum scoparium*. In addition, the provenance of *L. scoparium* has been shown to influence nectar quality, leading to differences in honey bee visitation. Nectar contains the precursor of the antibacterial compound in mānuka honey and is a reward cue for honey bees (Fig.1.1). While mānuka honey and the potential parameters affecting its antibacterial properties are widely studied, there are limited studies on biotic elements associated with *L. scoparium*. Therefore, this thesis aims to:

- identify eriococcid species associated with *L. scoparium* and their effects on plant growth and nectar quality.
- determine the relative attractiveness of different *L. scoparium* cultivars to honeybees and factors influencing honey bee visitation.
- examine the diversity of mycorrhizal fungal communities on *L. scoparium* depending on plant provenance and geographical location.

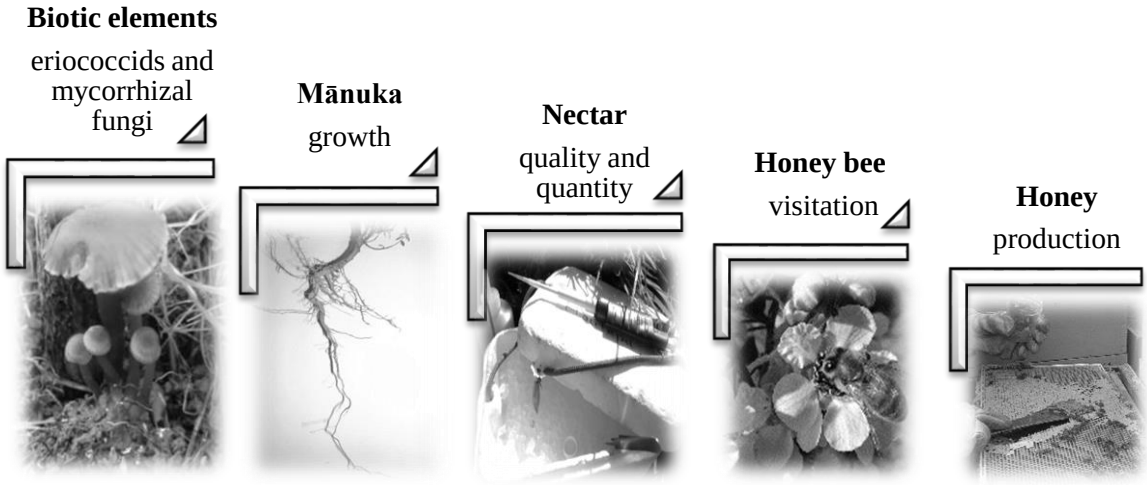

Fig. 1.1. Flow diagram indicating the relationship among the main research areas relevant to this study.

This thesis contains five chapters. An introduction and revision of the literature review is presented in Chapter 1. Chapters 2, 3, and 4 describe the experimental work and the final Chapter 5 presents a general discussion and conclusions.

Chapter 1 introduces this thesis and reviews the literature regarding *L. scoparium* and the association of scale insects and mycorrhizal fungi within this host. Furthermore, it

highlights the main components that influence the level of antibacterial properties in mānuka honey and reviews the main factors involved in the olfactory and visual stimuli of honey bees.

Chapter 2 is an evaluation of the level of eriococcid infestation and associated sooty mould on different *L. scoparium* cultivars and wild plants. The chapter also illustrates current eriococcid species associated with *L. scoparium* following a survey in a range of locations throughout New Zealand, using staining and microscope slide-mounting techniques for species identification.

Chapter 3 shows the efficiency of an insecticide treatment on eriococcid populations and eriococcid effects on plant growth and nectar quality for six *L. scoparium* cultivars. These six cultivars are also used to evaluate honey bee preference based on their flower density and nectar content. A sub-sampling approach using the plant basal area is validated to estimate flower density and HPLC technique is used to analyse nectar sugars and DHA in all six cultivars.

Chapter 4 explores the diversity of ectomycorrhizal and arbuscular mycorrhizal fungal communities associated with *L. scoparium*. The ITS and SSU rDNA regions are selected for amplifying DNA extracted from *L. scoparium* roots and amplicons are sequenced using Illumina MiSeq platform. The diversity of EcM fungal lineages and AM families are evaluated among *L. scoparium* cultivated and wild plants.

Chapter 5 is a pilot study that evaluates the effects of mycorrhizal fungal inoculation in *L. scoparium* seedlings from three different nurseries. Soil is collected from *L. scoparium* habitat colonised by mycorrhizal fungi, and added as soil inoculant to the seedlings.

Chapter 6 identifies and discusses the main findings obtained in the three experimental studies. It outlines limitations that have emerged during the studies and suggestions for further research.

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Chapter 2.

An update of eriococcids on *Leptospermum scoparium* in New Zealand

"*To be a useful natural agent of weed control, an insect must be capable of inflicting, directly or indirectly, decisive destruction of its plant host, thus determining the latter's abundance, and, reciprocally, its own abundance is then adjusted to the abundance of its host plant"*

Huffaker, 1957

2.1. Introduction

The genus *Leptospermum* J.R.Forst. & G.Forst. (Myrtaceae), well represented in the Southern Hemisphere, includes 87 species, mostly endemic to the Australasian region (Thompson, 1989; Bean, 1992; Lyne, 1993; Lyne & Crisp, 1996; Bean, 2004). *Leptospermum scoparium* J.R. Forst. et G. Forst. (mānuka), which is a common native species from Australia and New Zealand, is the only species in the genus *Leptospermum* present in New Zealand (Dawson, 1997). However, this morphologically very variable species has several named varieties and may include several cryptic species (Stephens et al., 2005).

Leptospermum scoparium is widespread in New Zealand, including Chatham, Three Kings and Stewart Island (Allan, 1961). As the species was considered a weed in the past, there was interest in reducing *L. scoparium* to increase the land area available for agricultural production (Gardiner, 1953). On the other hand, this species was important for its ecological value as a pioneer species (Campbell, 1953; Cook, Mark, & Shore, 1980; Primack & Lloyd, 1980). The discovery of a felt scale insect, described by Hoy (1954) as *Eriococcus* (now *Acanthococcus*) *orariensis* Hoy, which had become prominent as the primary cause of the disease mānuka blight, drew attention to this as a potential control agent for *L. scoparium* between the 1940s and the 1960s (Hoy, 1954; Mulcock, 1954; Hoy, 1961; Zondag, 1977). Mānuka blight started in the 1940s as a result of an association between *A. orariensis* and the fungus *Capnodium walteri* Sacc. (Fungi: Capnodiaceae), which was attracted to the honeydew excreted by this eriococcid (Mulcock, 1954). *Capnodium walteri* belongs to the sooty mould group, and its black mycelium grows on honeydew, coating leaves and stems and producing a black "mantle" on the plants. Affected plants of *L. scoparium* were thought to have their photosynthetic activity reduced (Hoy, 1961; Zondag, 1977); however, studies revealed the resistance of *L. scoparium* to the fungus in the absence of *A. orariensis* and suggested that the main factor causing plant death was nutrient removal from the host plant by *A. orariensis* (Hoy, 1961; Zondag, 1977). The recovery of *L. scoparium* began in 1954, perhaps resulting from the emergence of the entomogenous fungus *Myriangium thwaitesii* Petch (now *Angatia thwaitesii* (Petch) Arx) (Fungi: Myriangiaceae) (Crous et al., 2004) (Hoy, 1961). This fungus attacked *A. orariensis*, introducing its mycelium into the body of the eriococcids

and causing their death. Posteriorly, a mass of hyphal tissue grew in the insect's body, creating the visible fungal structure known as a stroma (Hoy, 1961).

Other felt insects have been worldwide protagonists of important diseases (Patel, 1971; Ülgentürk & Çanakçioğlu, 2004; Cale et al., 2017). Of the approximately 369 scale species (Hemiptera: Coccomorpha) recorded in New Zealand (García Morales et al., 2010), about 100 are felt scales (Eriococcidae) (Hoy, 1954, 1958, 1962; Hodgson, 1994; Hodgson & Henderson, 1996; Henderson, 2006, 2007a, 2007b; Henderson, Sultan, & Robertson, 2010). Only three of these felt insects are associated with *L. scoparium* according to Gardner-Gee & Beggs (2009), Henderson et al. (2010) and García Morales et al. (2010). However, there are other types of scale insects, such as armoured scales and mealybugs associated with *L. scoparium* in New Zealand. Specifically, there are 18 scales present on *L. scoparium* that belong to the families Diaspididae (6), Coccidae (5), Pseudococcidae (4), Coelostomidiidae (2) and Asterolecaniidae (1) (Appendix 2.1). Besides the scale insects, other insects such as mānuka beetle (*Pyronota festiva*; Scarabaeidae), a leaf-feeding mānuka moth (*Declana floccosa*; Geometridae), a webworm (*Heliostibes atychioides*; Oecophoridae), the wood-boring larvae of a longhorn beetle (*Ochrocudus huttoni*; Cerambycidae), a wood borer (*Amasa truncates*; Scolytidae), and a gall-inducing mite (*Aceria manukae*; Eriophyidae) have been reported as pest insect on *L. scoparium* in the past (Lamb, 1960; Molloy, 1975; Thomson, Miln, & Kain, 1979; Brockerhoff & Bain, 2000). Although these pests have been studied in the past, little research concerning their influence on *L. scoparium* has been carried out in recent decades.

Nowadays, *L. scoparium* is considered a valuable species due to its rising ecological and economic importance. Ecologically, this species plays an important role as a nurse plant in forest regeneration as it is able to control erosion and tolerate extreme conditions such as frost or soils with low fertility (Primack & Lloyd, 1980; Ausseil & Dymond, 2010). Most important, this plant is valued for the anti-bacterial and antioxidant properties of its honey and its significant contribution to the New Zealand honey industry (Stephens, Molan, & Clarkson, 2005; Ministry for Primary Industries, 2016). However, in comparison to the research that has been undertaken on mānuka honey, very little recent research has been carried out on the influence of biotic elements associated with this plant.

Biotic elements such as insects could influence the growth of the plant and consequently affect the nectar and thus the honey.

Most recent studies show that mānuka beetle and scale insects, which can damage the development of *L. scoparium* and associated vegetation, continue to have an important influence in New Zealand (Gardner-Gee & Beggs, 2009; Henderson et al., 2010; Willoughby, Beard, & Luketina, 2015). It has recently become apparent, however, that *A. orariensis* has been largely replaced by other felt scale species, such as *Acanthococcus campbelli* Hoy and *Acanthococcus leptospermi* Maskell (Epenhuijsen et al., 2000; Gardner-Gee & Beggs, 2009). In addition, one study showed that some *Leptospermum* cultivars are more resistant to eriococcids than other older cultivars. For instance, the hybrid *L. rotundifolium* (Maiden & Betche) F.A.Rodway 'Jervis Bay' was more resistant to *Acanthococcus* infestation than the hybrid *L. scoparium* 'Crimson Glory' (Bicknell, 1995).

In this study, the level of eriococcid infestation and associated sooty mould on different cultivars and wild plants of *L. scoparium* was evaluated to get a better understanding of the ecological and economic impacts of felt scales (Eriococcidae) on *L. scoparium*. The level of infestation on wild plants of *L. scoparium* was expected to be higher than on cultivars. Furthermore, a survey was undertaken to update the distribution of all scale insect species on *L. scoparium* in a range of locations in the North, South and Stewart Islands. Scale insect species were identified from their morphological features based on microscope slide preparations of the insects' cuticles. In doing so, the hypotheses that *A. orariensis* has been replaced by other *Acanthococcus* species was tested.

2.2. Material and methods

2.2.1. Survey of eriococcid infestation in Central North Island

Plants of *L. scoparium* were surveyed from three sites in the Wanganui and Hawke's Bay regions. Two sites were in the Wanganui region, where cultivars of *L. scoparium* from the same nursery were planted in 2011 (Rangitatau) and 2008 and 2012 (Ruatiti). Cultivars of *L. scoparium* from a different nursery were planted in 2013 in Hawke's Bay (Tutira). Sites and labelled plant material (CVT1, CVT2, CVT3, and CVT4) were provided by different stakeholders and Comvita Limited respectively. The three sites contained cultivated and wild plants of *L. scoparium*. However, not all cultivars were present at the three sites, and wild plants were scattered within each cultivar block, as they were established naturally in the field. Plantations were established with a plant spacing of 2.5 m × 2.5 m using similar plant techniques. Rangitatau was planted with three different cultivars (CVT1, CVT3, and CVT4) of *L. scoparium*. Ruatiti included the CVT1 and CVT4 cultivars, and Tutira included the cultivars CVT2, CVT3, and CVT4.

To determine the level of scale insect infestation stem samples were collected in January 2016 (Fig. 2.1). Ten plants infested with both sooty mould and scale insects were selected from each cultivar at each site. From each plant, a 20-cm length of stem was collected and placed in a ziplock plastic bag. Twenty wild plants were selected and sampled using the same procedure. In the lab, stems were observed under a dissecting microscope. The number of adult female eriococcids was counted for each 20-cm stem sample. In addition, the presence or absence of sooty mould, dead adult females enclosed in felted cotton sacs (killed, potentially, as a consequence of entomogenous fungi), stromata, and first-instar nymphs (called crawlers) were recorded for each sample.

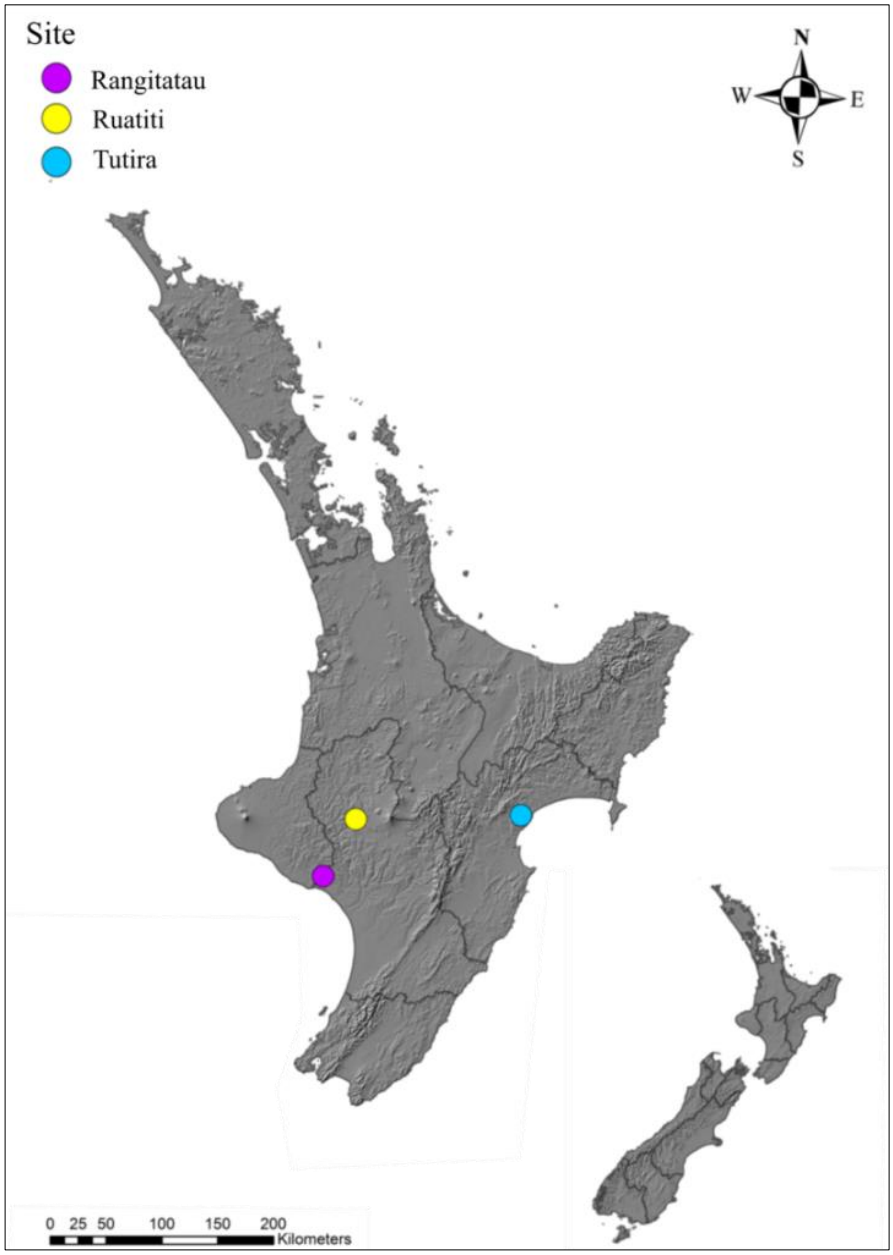


Fig. 2.1. Map showing the sites where *L. scoparium* stems were collected during the survey in the Wanganui (Rangitatau, Ruatiti) and Hawke's Bay (Tutira) region during 2016.

To proceed with the microscope slide-mounting protocol (Fig. 2.2), fine forceps were used to uncover the cotton sac of pupae, adult females, and males. Specimens at different life stages were preserved in 70% ethanol for identification. Adult females were mounted following the protocol of Gullan (1984). Specifically, adult females were opened in the marginal right side and were soaked over 24 hours in 10% KOH. Later, females were immersed in a solution of water and Decon detergent. Body contents were removed from adult females using a paintbrush under a light microscope. Females were immersed in a stained solution (3:1 acid alcohol: acid fuchsin), followed by three immersions in ethanol and three immersions of isopropanol. Females were soaked three times in xylene and

were further mounted under slides with Canada balsam. In addition, a few crawlers that belonged to the family Coelostomidiidae were mounted for identification. Slides were observed under ×40 objective on a compound microscope for species identification using taxonomic keys (Hoy, 1959, 1962; Morales, 1991).

For each mounted specimen, collector, date, location, Crosby area (Crosby, Dugdale, & Watt, 1998), and host plant were recorded. The software cellSens Dimension 1.6 was used to capture external and internal morphological features of the specimens (Fig. 2.3) (Appendix 2.2). Electron micrograph images of different life stages of representative scale insects were produced by the Manawatū Microscopy and Imaging Centre (Appendix 2.3).

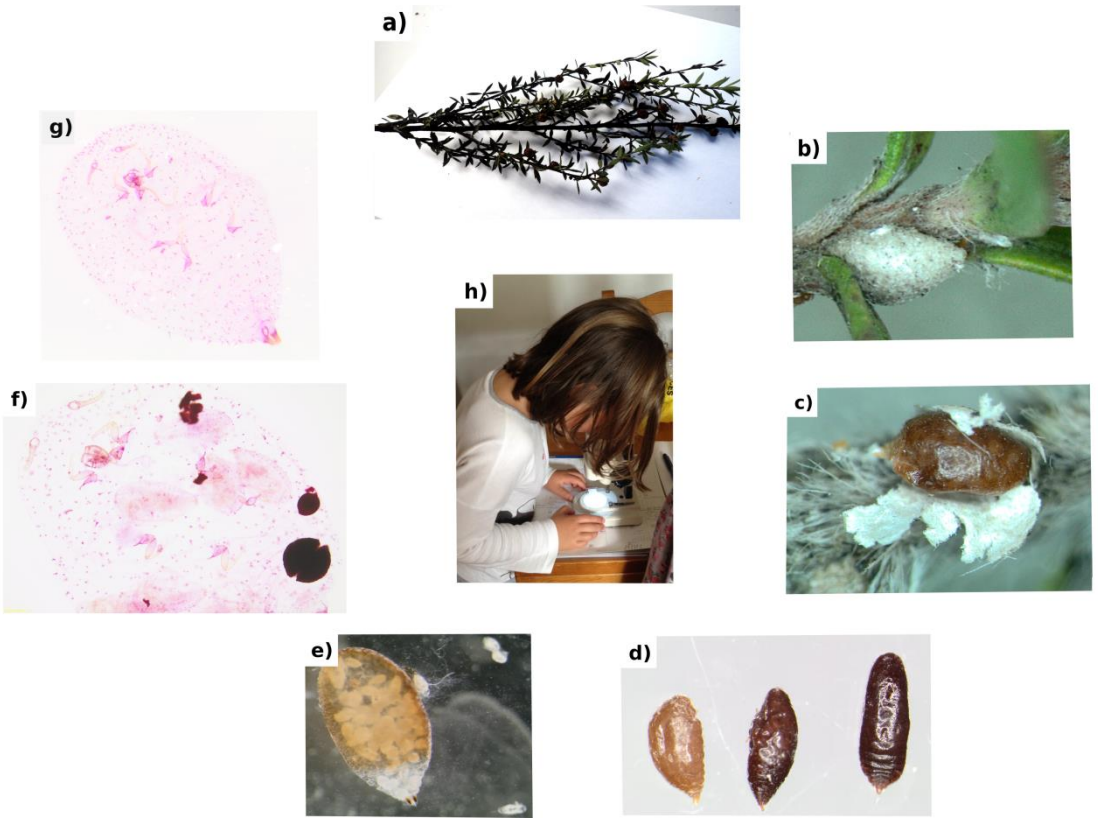


Fig. 2.2. Diagram flow showing several steps followed for microscope slide-mounting and taxonomic classification. a) stem sample; b) adult female covered with cotton sac; c) uncovered adult female; d) adult females ready for be cut on the right margin; e) crawlers removed from adult females; f) last body contents removed after stained; g) adult female stained and slide-mounted; h) species identification.

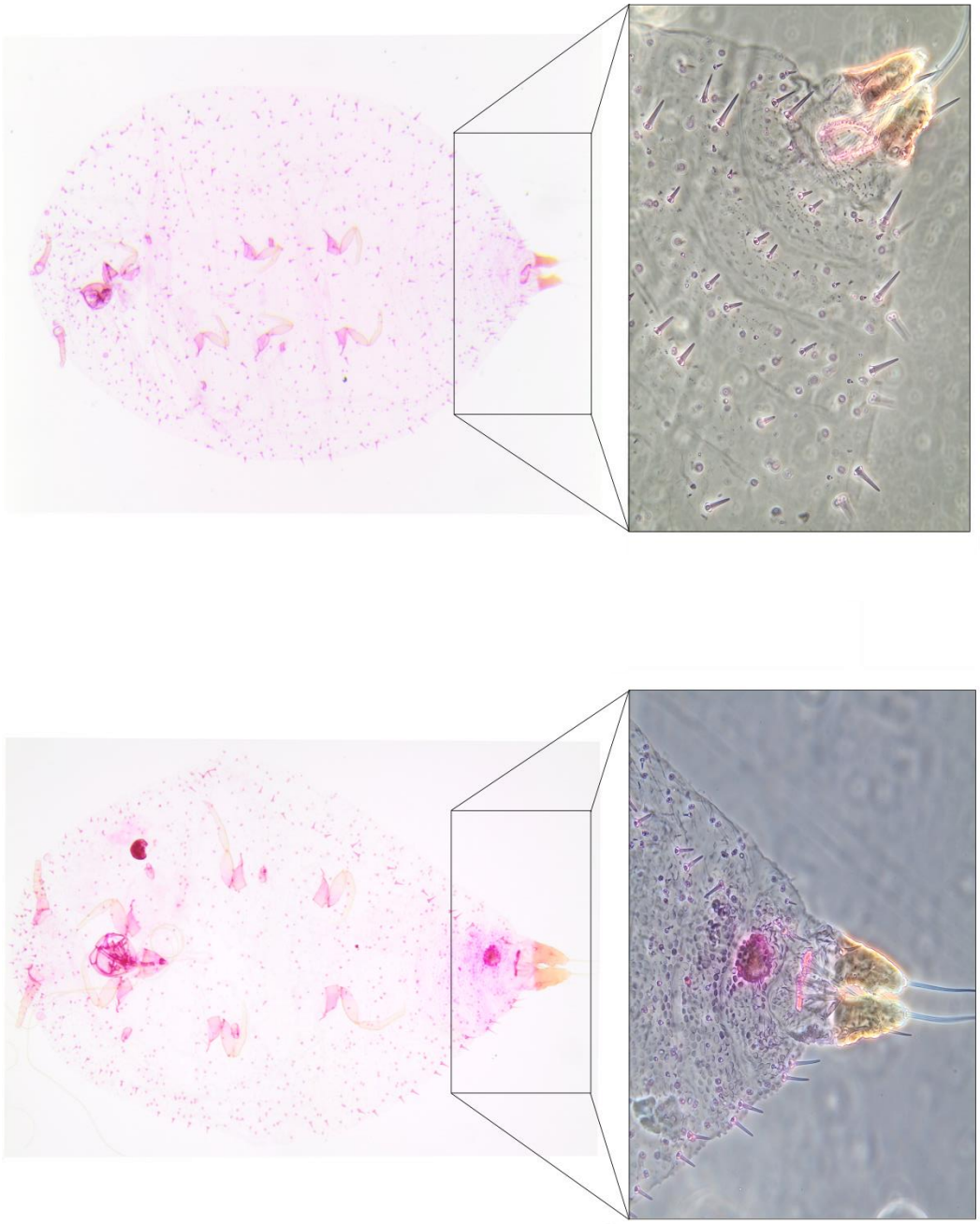


Fig. 2.3. Stained and slide-mounted adult females. left *Acanthococcus campbelli* (Eriococcidae); right: *Acanthococcus leptospermi* (Eriococcidae).

2.2.2. Statistical analysis

A Generalized Linear Model (GLM) model was used to evaluate the influence of site and provenance (CVT1, CVT2, CVT3, CVT4, wild) on adult scale insect female density. The model was fitted using a negative binomial distribution with logistic link function, as the data was over-dispersed. The interaction among the explanatory variables was included in the initial model, but it was dropped from the final model if was not significant ($P > 0.25$).

To evaluate the influence of the explanatory variables adult female density, site and plant variety on the presence of each of sooty mould, dead adult females enclosed in felted cotton sacs, and stromata, binomial models were fitted using a log link function. Dependent variables were considered as presence or absences. The interactions among the explanatory variables was included in the initial models, but was dropped form the final model if it was not significant ($P > 0.25$).

Data analysis was conducted using the software R 3.3.1 (R Core Team, 2016) within RStudio using the package "car", "ddply", "lrtest", "nlme" and "MASS". The binomial and Poisson models were fitted using the R function "glm", and the negative binomial was fitted using the function "glm.nb". The goodness of fit for selecting the most appropriate model was evaluated using Akaike criterion (AIC) values, likelihood ratio test and chi-square test based on the residual deviance. The R functions used were "AIC", lrtest", and "pchi" respectively. To obtain analysis of variance (ANOVA) tables and coefficients for the explanatory variables, the functions "Anova" and "summary" were used.

Distribution maps were generated using GIS software (ArcMap 10.4.1).

2.2.3. Survey of eriococcid species associated with Leptospermum scoparium

The diversity of scale insect species (Eriococcidae) feeding on *L. scoparium* were recorded from stem samples associated with sooty mould (Fig. 2.4) that were collected between March 2015 and January 2017 from 28 sites, which included sites in North, South and Stewart Islands (Appendix 2.4). Samples were stored in ziplock plastic bags until evaluation in the lab. For species identification, specimens were treated following

the protocol described above. For taxonomic identification, type specimen slides provided by New Zealand Arthropod Collection (NZAC) were examined to corroborate species identifications made from published keys.

Fig. 2.4. *Leptospermum scoparium* plants infested with sooty mould. Left: The Pinnacles (Martinborough, North Island); right: Lake Wilkie (Southland, South Island).

2.3. Results

2.3.1. Survey of eriococcid infestation in Central North Island

Wild plants of *L. scoparium* had higher numbers of adult females than did plantation plants. Wild plants presented a median of 1 adult females per 20 cm of stem, while CVT2 presented a median of 0.5 adult females, and the other cultivars were represented by 0 adult females (Fig. 2.5). Adult female density was significantly affected by provenance (χ^2 = 23.54, P < 0.0001), but not by site (χ^2 = 1.01, P < 0.603) (Appendix 2.5). The interaction between provenance and site was removed from the final model as it was not significant (P = 0.628).

Rangitatau had the lowest overall infestation rates and the lowest frequency of plants affected by the presence of crawlers, sooty mould, dead adult females enclosed in felted cotton sacs, and stromata (Table 2.1). Sooty mould was common, being recorded on 75% of all plants surveyed. Tutira showed the highest number of plants infested with sooty mould (94%). Ruatiti had the highest proportion (95%) of wild plants of *L. scoparium* affected by sooty mould. This site also had the highest number of plants that presented dead adult females enclosed in felted cotton sacs 57%, followed by Tutira and Rangitatau with 38% and 8%, respectively. Few samples (n = 20) were affected by stromata. Overall, Rangitatau was the least infected by scale insects and associated fungi.

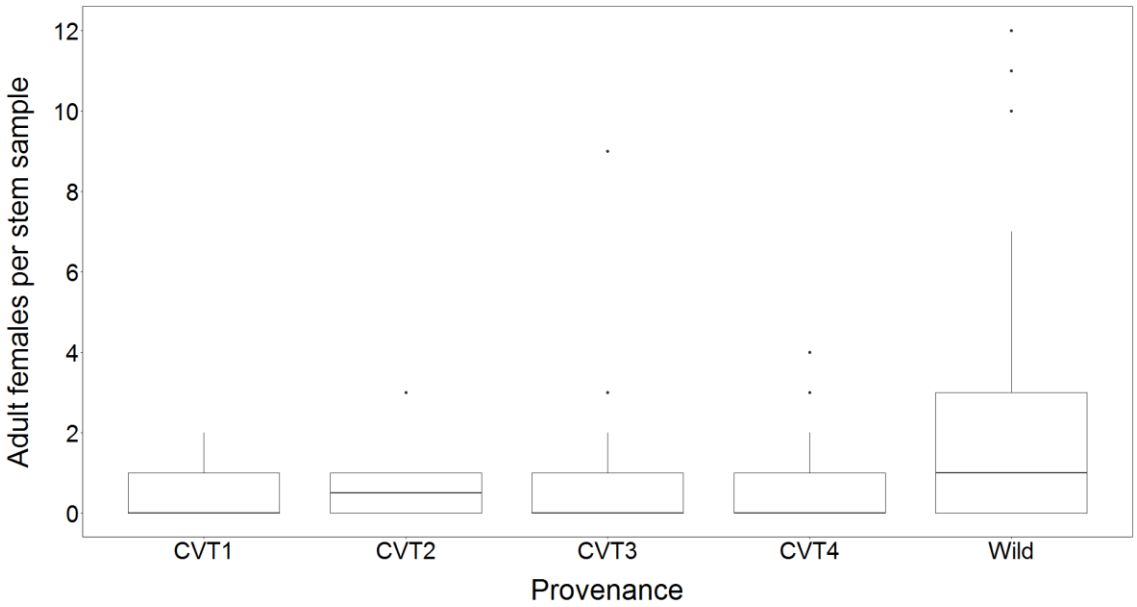
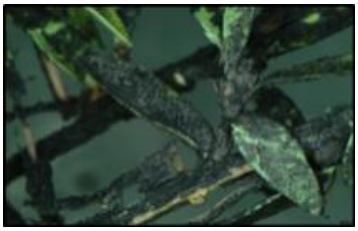


Fig. 2.5. Boxplots showing the median of adult females found per 20-cm of stem samples collected at Rangitatau (CVT1, CVT3, CVT4, and wild), Ruatiti (CVT1, CVT4, and wild), and Tutira (CVT2, CVT3, CVT4, and wild).

Table 2.1. Number of plants presenting crawlers, sooty mould, dead adult females enclosed in felted cotton sacs and stromata per 20-cm stem for planted cultivars and wild plants of *L. scoparium* at Rangitatau, Ruatiti, and Tutira. CVT1, CVT2, CVT3, CVT4 (*n* = 10 plants for each cultivar), wild plants of *L. scoparium* (*n* = 20 plants). (-: cultivar absent at that site). Ra: Rangitatau, Ru: Ruatiti, T: Tutira.

Presence		Site	CVT1	CVT2	CVT3	CVT4	Wild
Crawler		Ra	1	-	3	1	10
		Ru	3	-	-	6	11
		T	-	8	6	7	15
Sooty mould		Ra	6	-	4	1	11
		Ru	10	-	-	7	19
		T	-	10	10	10	17
Dead adult female enclosed in a felted cotton sac		Ra	0	-	2	0	2
		Ru	5	-	-	3	15
		T	-	2	5	2	10
Stromata		Ra	0	-	0	0	1
		Ru	7	-	-	0	10
		T	-	0	1	0	1

Results from the binomial model showed that site significantly affected the presence of sooty mould, stromata and dead adult females enclosed in felted cotton sacs ($P < 0.0001$) (Table 2.2) (Appendix 2.6). The presence of stromata ($P = 0.0019$) and dead adult female ($P = 0.0031$) on stem samples were also influenced by the provenance. The number of adult females did not influence the presence of associated fungi (Appendix 2.6) (Fig. 2.6)

Table 2.2. Significance effects of the explanatory variables adult female density, provenance and site on sooty mould, stromata and dead adult female enclosed in felted sac cotton. A GLM test with a binomial distribution was chosen to evaluate the influence of the explanatory variables.

Associated fungi	Explanatory variable	d.f.	χ^2	*P*
Sooty mould	Female adult density	1	2.02	0.1557
	Provenance	4	8.34	0.0798
	Site	2	38.74	**<0.0001**
Stromata	Female adult density	1	0.47	0.4913
	Provenance	4	17.00	**0.0019**
	Site	2	29.50	**<0.0001**
Dead adult female enclosed in a felted cotton sac	Female adult density	1	3.29	0.0698
	Provenance	4	15.95	**0.0031**
	Site	2	34.61	**<0.0001**

2.3.2. Survey of eriococcid species associated with Leptospermum scoparium

A total of 211 microscope slides were prepared from a subsample selection of the sites visited during the study. Although the focus of the study was to identify species from the family Eriococcidae associated with *L. scoparium*, species from the Coelostomidiidae, Pseudococcidae and Diaspididae were also found (Table 2.3). Of the 211 slides, 176 slides were mounted with specimens that belonged to the Eriococcidae, 24 to the Coelostomidiidae, nine to the Pseudococcidae, and two specimens to the Diaspididae.

Table 2.3. Specimens found during the survey that belong to the families Eriococcidae, Coelostomidiidae, Pseudococcidae and Diaspididae.

Family		
Eriococcidae		
	Acanthococcus campbelli	*Acanthococcus leptospermi*
Coelostomidiidae		
	Coelostomidia wairoensis	*Coelostomidia wairoensis*
Pseudococcidae		
Diaspididae		

A total of 185 felt scale insect specimens (genus *Acanthococcus*) were prepared during the survey. Three species were identified: *A. leptospermi*, *A. campbelli*, and *A. orariensis*. *Acanthococcus leptospermi* was the most widespread (absent from four locations only), followed by *A. campbelli*. *Acanthococcus orariensis* was the least common (Fig. 2.6). *Acanthococcus leptospermi* was found in 23 of 27 locations from the most northern part of North Island to Stewart Island. *Acanthococcus campbelli* was found in eight out of the 15 locations on North Island but only two of eight on South Island.

Acanthococcus campbelli was not recorded in the northern half of North Island. *Acanthococcus orariensis*, the main cause of the mānuka blight in the 1950s, was only found on the West Coast location and at Totaranui.

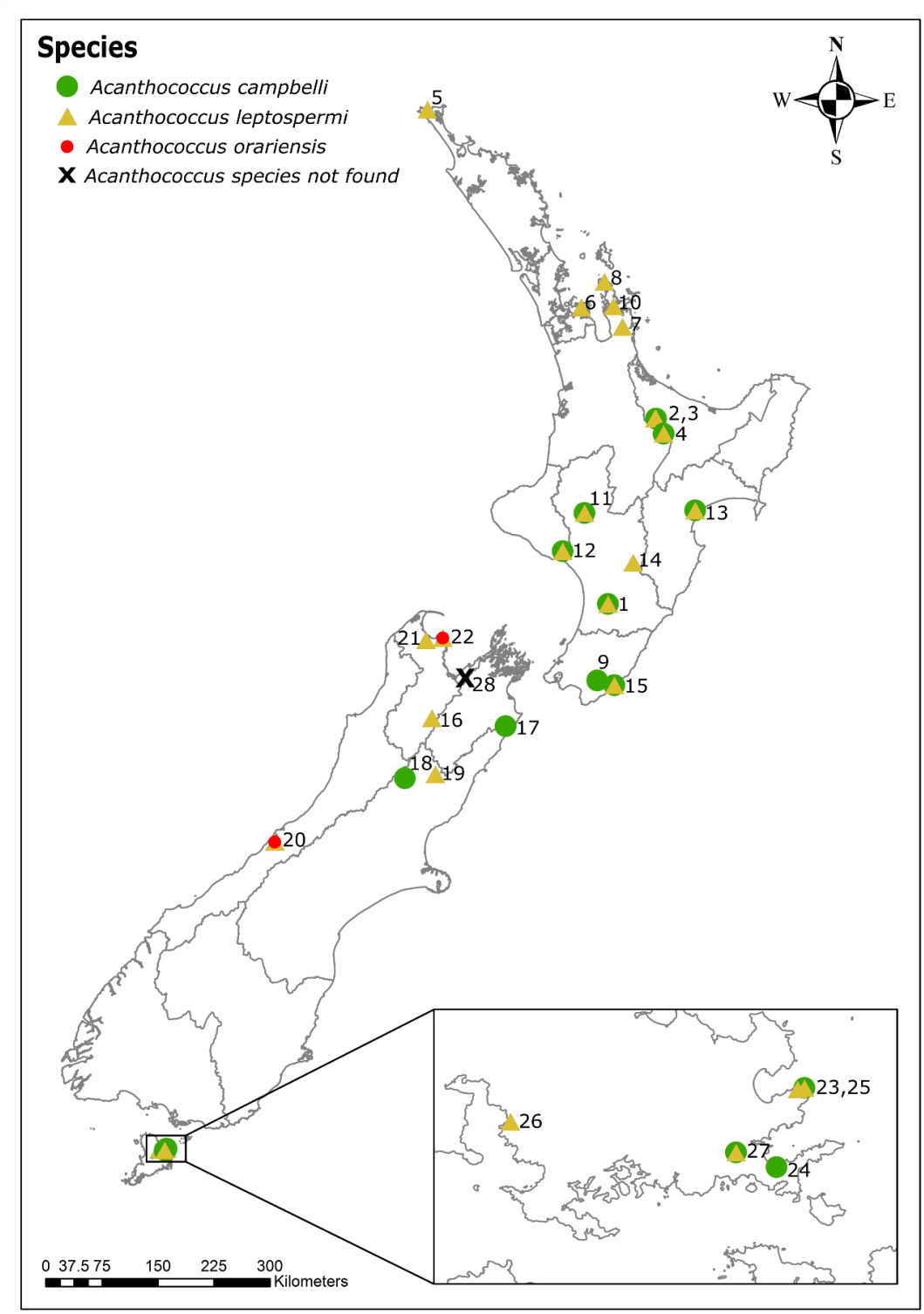


Fig. 2.6. Distribution of *Acanthococcus* species found in *Leptospermum scoparium* samples collected from 2015 to 2017 on North Island, South Island and Stewart Island. The distribution map also shows the one location where *Acanthococcus* species was searched for but not found. Numbers refer to locations listed in Appendix 2.4.

For the Coelostomidiidae, *Coelostomidia wairoensis* Maskell was found only in three locations: United Creek Nelson, Totaranui, and Pahoa River (Fig. 2.7). The samples collected in these three locations were highly infested by *C. wairoensis*. However, the number of locations where *C. wairoensis* was found was lower than locations where *Acanthococcus* was present. Thus, *Acanthococcus* is numerically much more important than *Coelostomidia*, at least in the 27 locations sampled during the survey. As was the case for the family Coelostomidiidae, a limited number of specimens within the family Diaspididae and Pseudococcidae were found. Two specimens within Diaspididae were found at Palmerston North and Ruatiti. At both sites, two specimens of the family Pseudococcidae were also found. Pseudococcidae was present in South Island at North Arm Hut (five specimens), and on Stewart Island at Oban (two specimens).

Fig. 2.7. Distribution of *Coelostomidia wairoensis* recorded in *L. scoparium* samples associated with sooty mould collected from 2015 to 2017 on North Island and South Island.

2.4. Discussion

2.4.1. Survey of eriococcid infestation in Central North Island

The results showed higher infestations of adult female eriococcids on wild plants of *L. scoparium* compared to the cultivated varieties. In addition, a high number of cultivars and wild plant samples were infected with sooty mould. This result was also observed by Gardner-Gee & Beggs (2009): 92% of the plants (*L. scoparium* and *Leptospermum* (now *Kunzea*) *ericoides* (A. Rich) J. Thompson (de Lange et al., 2010) in their survey were infested by sooty mould. This infestation, which started during the mānuka blight in the 1940s (Sewell, 1949; Zondag, 1977), appears to continue being a major element associated with *Leptospermum* species in New Zealand. However, the main species producing honeydew leading to the development of sooty mould was *A. orariensis* in the 1940s, which seems to be replaced by the species *A. campbelli* and *A. leptospermi* according to the findings from this study, and the most recent published studies (Epenhuijsen et al., 2000; Gardner-Gee & Beggs, 2009; Henderson et al., 2010).

Breeding programmes have improved the resistance of cultivated varieties of *Leptospermum* to the sooty mould and scale insect infestation (Epenhuijsen et al., 2000). This may explain the results of a higher density of adult females on wild plants of *L. scoparium* compared with the cultivated varieties. Another explanation is that host plant age could influence the establishment of adult females, as adult females were more abundant on older plants. Ruatiti was found to have a higher number of wild plant samples affected by sooty mould, dead adult females enclosed in felted cotton sacs, and stromata compared with cultivars. In contrast, cultivated varieties at Tutira had the highest incidence of sooty mould across all sites.

2.4.2. Survey of eriococcid species associated with Leptospermum scoparium

The main scale insect species found during the study belonged to the family Eriococcidae, which has a high proportion of endemic species in New Zealand (66%) (Henderson, 2009). Despite the number of endemic species, the species found during this survey (*A. leptospermi*, *A. campbelli* and *A. orariensis*) have been dispersed from Australia (Hoy, 1961, 1969). Among them, *Acanthococcus orariensis* is known as the most common species affecting *L. scoparium*; however, the most recent studies have shown it is being replaced by *A. leptospermi* and *A. campbelli* (Epenhuijsen et al., 2000; Gardner-Gee &

Beggs, 2009). Epenhuijsen et al. (2000) reported the presence of *A. leptospermi* in North and South Islands and Gardner's study (2009) showed the presence of both *A. campbelli* and *A. leptospermi* on *L. scoparium* in Auckland forests. However, until this study, there were limited recent widespread surveys that included actual eriococcid species present in both the North and South Islands.

During the present study, only two specimens identified as *A. orariensis* were found in the West Coast and Nelson region. This finding demonstrates the replacement by the species *A. leptospermi* and *A. campbelli*. Nelson and the West Coast region were shown by Hoy to be the last places to which *A. orariensis* was dispersed in 1958 (Hoy, 1961) (Appendix 2.7). Epenhuijsen et al. (2000) recorded the occurrence of *A. orariensis* in the South Island (Nelson, Mosgiel area and Monowai). Thus, according to Hoy (1961) and Epenhuijsen et al. (2000) findings, it might be that Nelson and the West Coast are the last two regions were *A. orariensis* is still present. Hoy indicated the coexistence of *A. leptospermi* and *A. orariensis* on *L. scoparium*. He suggested the potential future replacement of *A. orariensis* by *A. leptospermi*, given that *A. orariensis* is being attacked by the entomogenous fungus *A. thwaitesii* (Hoy, 1961; Ridley et al., 2000), while *A. leptospermi* was resistant to *A. thwaitesii*. In addition, Zondag (1977) confirmed the sporadic presence of the ladybird beetle (*Rhizobius ventralis*; Coccinellidae) on *A. orariensis*. However, predators or parasites were not indicated as possible associates of *A. leptospermi* in New Zealand, though Hoy (1961) suggested the possibility for *A. leptospermi* in Australia. Thus, it appears that these different pressures have been the main drivers of the decline of populations of *A. orariensis* and the increase in populations of *A. leptospermi*. As Hoy predicted, *A. leptospermi* has increased its populations, as shown by Epenhuijsen et al. (2000). Similar results were obtained in this study, with *A. leptospermi* being present in different locations in North, South and Stewart Islands. Hoy (1961) also highlighted the probability of a future association between *A. campbelli* and *L. scoparium*, suggesting this species as a possible means of control for the host plant. Hoy's study (1959) showed records of *A. campbelli* in Australia (New South Wales, Queensland, and Tasmania). The presence of *A. campbelli* on *L. scoparium* was confirmed in Auckland forest (New Zealand) by Gardner-Gee et al. (2009), corroborating Hoy's predictions. In the present study, *A. campbelli* was recorded at 13 locations that included from the Central North Island to Stewart Island, as well as few locations in the South Island.

Another important species associated with *L. scoparium* was *C. wairoensis*, which is endemic to New Zealand (Morales, 1991). *Coelostomidia wairoensis*, known as the mānuka giant scale, was recorded from only three sites. At these sites *C. wairoensis* was predominant compared with the *Acanthococcus* species. Gardner-Gee et al. (2009) indicated that the presence of this species was minor compared with eriococcids, though their study examined only Auckland forests (New Zealand). In their study, only 0.02% of the *L. scoparium* plants were associated with *C. wairoensis*. Hoy, however, indicated *C. wairoensis* as the most frequent scale insect species present on *Leptospermum* species. Hoy may have been referring to *K. ericoides*, since 21% of the plants identified as *K. ericoides* were infested with *C. wairoensis* in Auckland forests (Gardner-Gee & Beggs, 2009). According to previous studies, it appears that *C. wairoensis* has a minor effect on *L. scoparium* and has not been implicated in the death of plants (Sewell, 1949; Hoy, 1961). In contrast, *Acanthococcus* species have been shown previously to damage this host plant.

Previous studies have shown that the damage of *L. scoparium* was mainly caused by *A. orariensis*, which appeared to be controlled by *A. thwaitesii* by the end of 1954, when plants started to recover. In comparison to *A. orariensis*, *A. leptospermi* seems to be resistant to *A. thwaitesii* (Hoy, 1961), which could be the main explanation for its dispersion throughout New Zealand. Epenhuijsen et al. (2000) recorded infestations of another fungus, potentially species of *Myriangium* Mont. & Berk. on *A. leptospermi* on *L. scoparium* plants that belonged to a breeding program for *Leptospermum* species as well as on some plants in their field survey. However, the *Myriangium* species was not stated in their study. While the genus *Myriangium* is characterised as being saprobic, occupying dead leaves, stems, and the bark of the plants (Dissanayake et al., 2014), some species from this genus are said to be parasitic on scale insects (Fetch, 1924; Dissanayake et al., 2014). Although it is known that *A. thwaitesii* is only attracted to *A. orariensis*, and it has been implicated in controlling *A. orariensis*, no recent studies have identified the actual entomogenous fungi on *L. scoparium*. The present study regularly found dead adult female eriococcids, but it was not possible to identify the cause of death. It will be interesting to research possible entomogenous fungi associated with *A. leptospermi*, *A. campbelli* and their host plant in the future.

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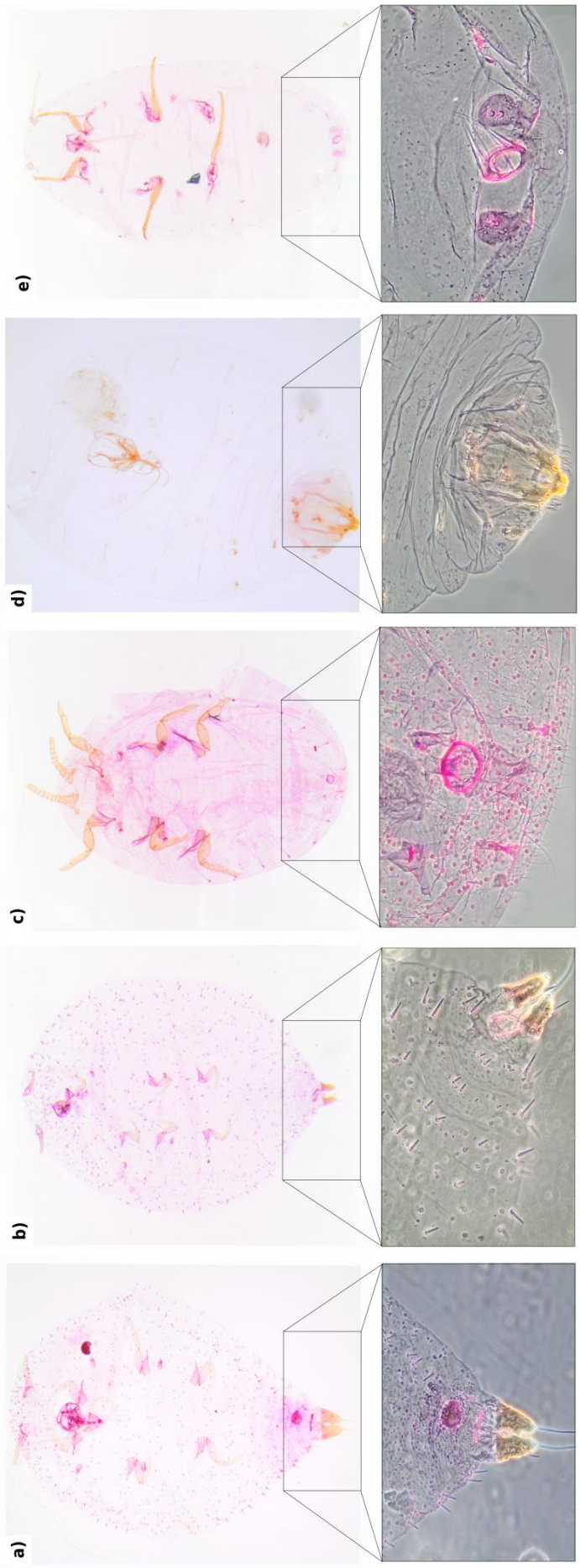
2.6. Appendices

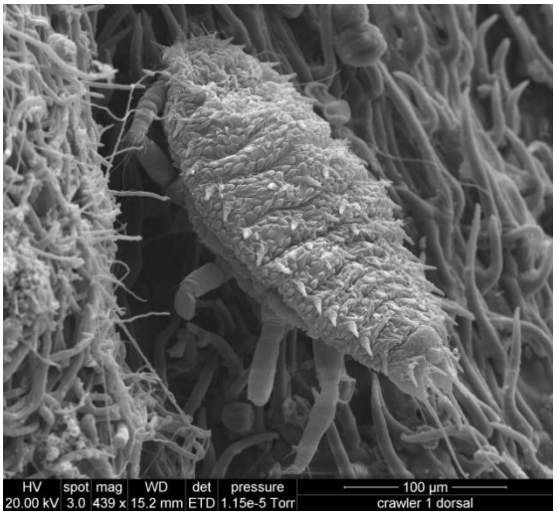
Appendix 2.1. Scale insect species associated with *Leptospermum scoparium* according to ScaleNet (García Morales et al., 2010). *This generic placement requires further study; the species is usually placed in *Acanthococcus* (P.J. Gullan, pers. comm.). **Scale species recorded in this study.

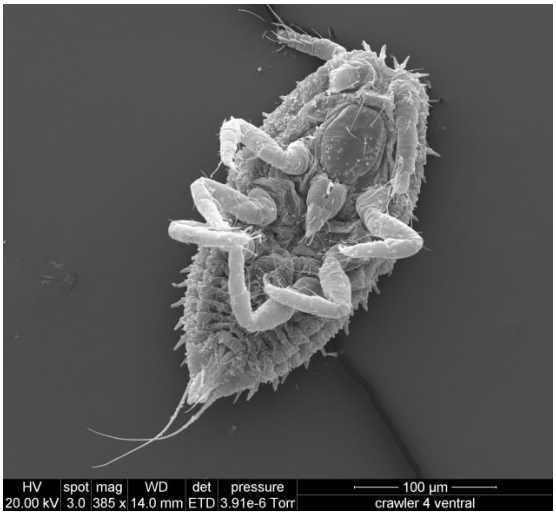
Family	Species	Geographic distribution
Asterolecaniidae	*Asterolecanium epacridis*	Australia, New Zealand
Coccidae	*Crystallotesta leptospermi*	New Zealand
Coccidae	*Crystallotesta ornate*	New Zealand
Coccidae	*Crystallotesta ornatella*	New Zealand
Coccidae	*Plumichiton pollicinus*	New Zealand
Coccidae	*Umbonichiton bullatus*	New Zealand
Coelostomidiidae	*Coelostomidia jenniferae*	New Zealand
Coelostomidiidae	*Coelostomidia wairoensis***	New Zealand
Diaspididae	*Anzaspis angusta*	Australia, New Zealand
Diaspididae	*Hemiberlesia lataniae*	109 countries (New Zealand included)
Diaspididae	***Hemiberlesia rapax*	71 countries (New Zealand included)
Diaspididae	*Poliaspis media*	6 countries (New Zealand included)
Diaspididae	*Symeria intermedia*	New Zealand
Diaspididae	*Symeria leptospermi*	New Zealand
Eriococcidae	*Acanthococcus campbelli***	Australia
Eriococcidae	*Acanthococcus gibbus*	Australia
Eriococcidae	*Acanthococcus leptospermi***	Australia, New Zealand
Eriococcidae	*Acanthococcus orariensis***	Australia, New Zealand
Eriococcidae	*Uhleria mariannae**	Italy and Corsica
Pseudococcidae	*Crisicoccus tokaanuensis*	New Zealand
Pseudococcidae	*Paracoccus leptospermi*	New Zealand
Pseudococcidae	*Paracoccus miro*	New Zealand
Pseudococcidae	*Paracoccus zealandicus*	New Zealand

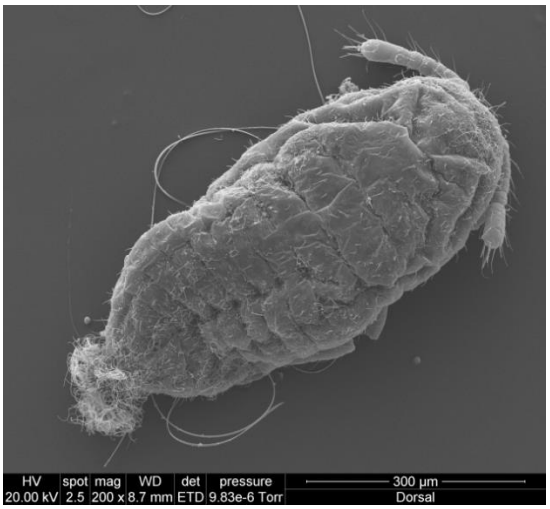


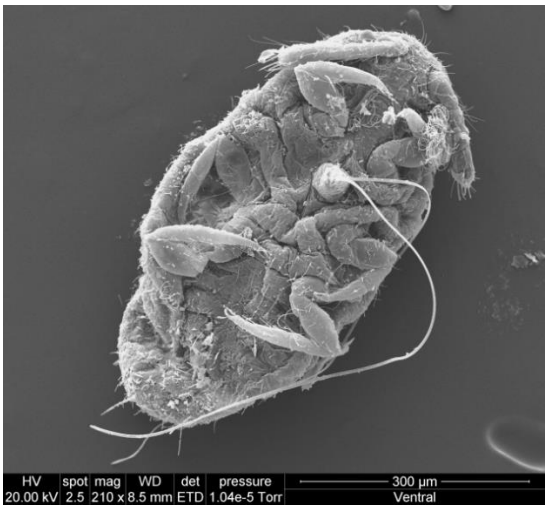
Appendix 2.2. Stained and slide-mounted adult females. a) *Acanthococcus campbelli* (Eriococcidae); b) *A. leptospermi* (Eriococcidae); c) *Coelostomidia wairoensis* (Coelostomidiidae); d) *Hemiberlesia rapax* (Diaspididae); e) species undetermined (Pseudococcidae).

Appendix 2.3. First-instar of nymphs belonging to the genus *Acanthococcus* (above) and *Coelostomidia* (below) found on *Leptospermum scoparium* during sample collection.









Appendix 2.4. Locations sampled between March 2015 and January 2017; includes map code assigned in figure 2, number of slide-mounted specimens per location, New Zealand Island and Crosby area code (AK: Auckland; BP: Bay of Plenty; BR: Buller; CL: Coromandel; KA: Kaikoura; NC: North Canterbury; ND: Northland; NN: Nelson; RI: Rangitikei; SI: Stewart Island; WA: Wairarapa; WD: Westland; WI: Wanganui).

Map code	Location	Number of specimens	Island	Crosby area code
1	Palmerston North	26	North	WI
2	Rotorua Museum	5	North	BP
3	Kuirau Park	4	North	BP
4	Rainbow mountain	3	North	BP
5	Pandora Bay	2	North	ND
6	Waiheke Island	3	North	AK
7	Pinnacles	4	North	CL
8	Stony Bay	4	North	CL
9	Ruakokoputuna r	7	North	WA
10	Castle Rock	7	North	CL
11	Ruatiti	12	North	RI
12	Rangitatau	14	North	WI
13	Tutira	14	North	HB
14	Renfrew Road	5	North	RI
15	Pahoa River	5	North	WA
16	Mount Roberts	7	South	BR
17	Waima Road	5	South	KA
18	Boyle R Outdoor	9	South	NC
19	Jollies Pass	8	South	NC
20	Wahapo Lake	6	South	WD
21	Pupu Hydro Walkway	3	South	NN
22	Totaranui	2	South	NN
23	Eastern Horseshoe	6	Stewart	SI
24	Peterson Hill	6	Stewart	SI
25	Western Horsehoe	9	Stewart	SI

26	North Arm Hut	4	Stewart	SI
27	Oban	5	Stewart	SI
28	United Creek	0	South	NN

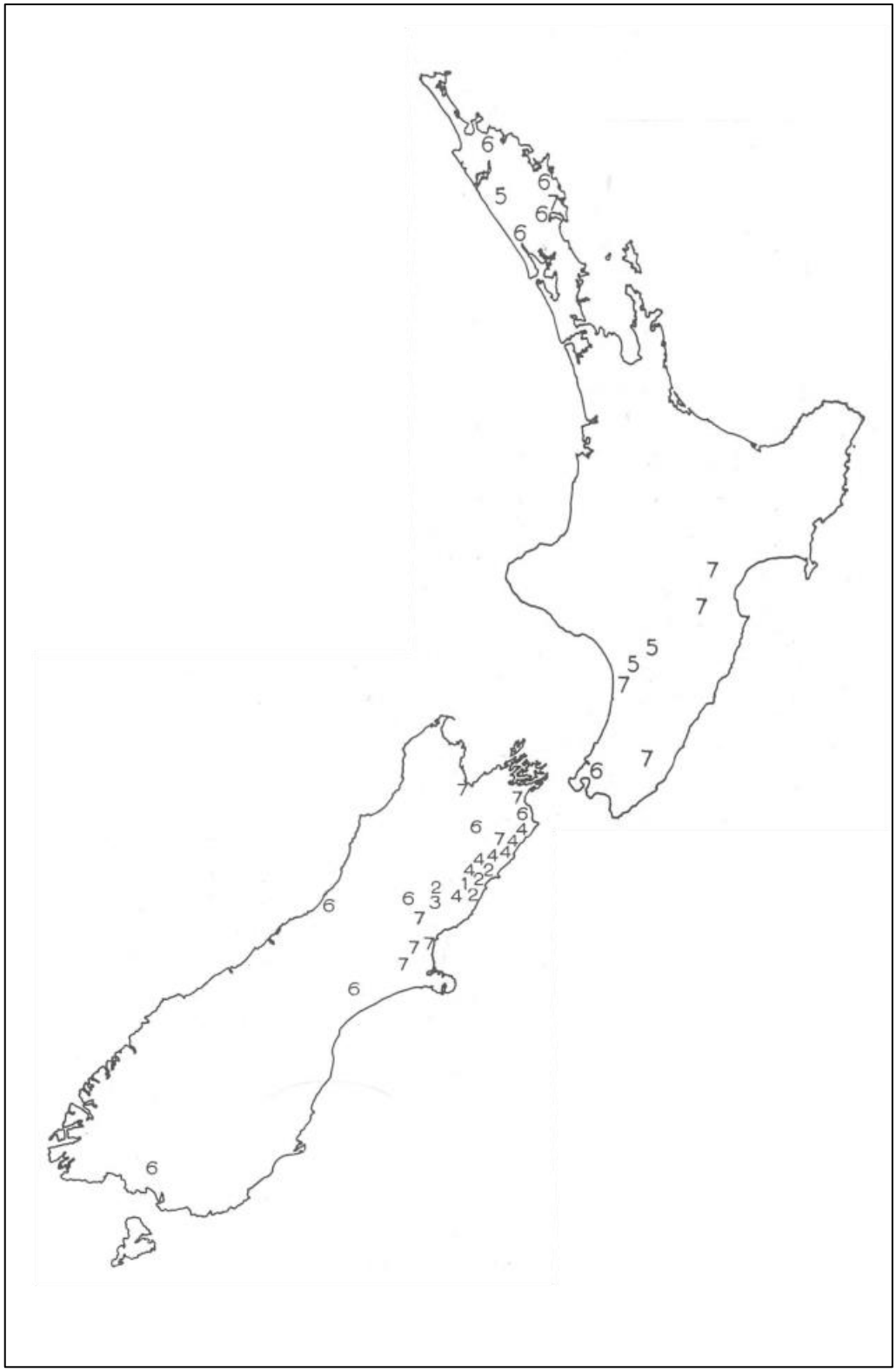
Appendix 2.5. Coefficients for the explanatory variables *L. scoparium* provenance and field location to the response variable adult female density.

Response, explanatory variables	Estimate ± SE	Z value	*P*
CVT2	0.54 ± 0.70	0.77	0.4399
CVT3	0.74 ± 0.57	1.30	0.1925
CVT4	0.41 ± 0.52	0.78	0.4350
Wild plants of *L. scoparium*	1.58 ± 0.46	3.45	**<0.0006**
Ruatiti	0.28 ± 0.32	0.87	0.3823
Tutira	0.28 ± 0.32	0.88	0.3794

Appendix 2.6. Coefficients for the explanatory variables adult female presence, *L. scoparium* provenance and field location to the response variables sooty mould, stromata and dead adult females enclosed in felted cotton sacs.

Response, explanatory variables	Estimate ± SE	Z value	*P*
Sooty mould			
Female presence	0.70 ± 0.50	1.41	0.1594
CVT2	13.81 ± 1235.62	0.01	0.9911
CVT3	–0.83 ± 0.86	–0.97	0.3311
CVT4	–2.00 ± 0.83	–2.41	**0.0158**
Wild plants of *L. scoparium*	–0.79 ± 0.73	–1.08	0.2812
Ruatiti	2.66 ± 0.67	3.95	**<0.0001**
Tutira	3.18 ± 0.72	4.40	**<0.0001**
Stromata			
Female presence	0.52 ± 0.771	0.68	0.4970
CVT2	–17.68 ± 3385.456	–0.01	0.9958
CVT3	–0.44 ± 1.417	–0.31	0.7568
CVT4	–19.08 ± 1667.352	–0.01	0.9909
Wild plants of *L. scoparium*	–0.91 ± 0.882	–1.03	0.3035
Ruatiti	4.04 ± 1.154	3.51	**<0.0004**
Tutira	1.31 ± 1.307	1.00	0.3176
Dead adult female enclosed in a felted cotton sac			
Female presence	–0.86 ± 0.49	–1.77	0.0775
CVT2	0.15 ± 1.16	0.13	0.8944
CVT3	1.63 ± 0.96	1.70	0.0897
CVT4	–0.32 ± 0.84	–0.38	0.7021
Wild plants of *L. scoparium*	1.71 ± 0.81	2.12	**0.0336**
Ruatiti	3.38 ± 0.71	4.78	**<0.0001**
Tutira	2.15 ± 0.64	3.35	**0.0008**

Appendix 2.7. Distribution of *Acanthococcus leptospermi* studied on *L. scoparium* and *Leptospermum* (now *Kunzea*) *ericoides* from 1949 to1959, as recorded by Hoy (1961). Numbers refer to years when samples were collected: 1 – 1949, 2 – 1953, 3 – 1954, 4 – 1955, 6 – 1958, 7 – 1959.



Chapter 3.

Scale insect presence, honey bee visitation and nectar yield of *Leptospermum scoparium* cultivars

"The brain of a bee is the size of a grass seed and is not made for thinking. The actions of bees are mainly governed by instinct"

von Frisch, 1962

3.1. Introduction

Scale insects (Hemiptera: Sternorrhyncha) are phytophagous insects characterised by sucking sap from their host plant. They are represented by nearly 8,000 species classified within 32 families (Kondo, Gullan, & Williams, 2008). Depending on their feeding strategy, they are categorised as feeding from parenchyma tissue or sucking phloem-sap. The phloem-sap feeders are well known for secreting honeydew and, consequently, as precursors of sooty mould fungi on host plants. This group, which includes families such as Pseudococcidae (mealybugs), Coccidae (soft scales) and Eriococcidae (felt scales), includes important crop pests (Hamon & Hodges, 2001; Howard et al., 2006).

Scales and their associated pathogens such as fungi and viruses are spread worldwide. They can produce important economic damage and reduce the socioeconomic value of natural resources. In North America, *Cryptococcus fagisuga* Lindinger (Eriococcidae) has been the principal protagonists of beech bark disease, affecting timber production and ecological resources (Cale et al., 2017). In Africa, *Phenacoccus manihoti* Matile-Ferrero (Pseudococcidae) has significantly reduced the production of cassava (*Manihot esculenta* Crantz) (Herren & Neuenschwander, 1991; Yonow, Kriticos, & Ota, 2017), a primary food resource (FAO, 2008). Among mealybugs, *Planacoccus ficus* Signoret (Pseudococcidae) is globally recognised as a major vector of grapevine (*Vitis vinifera* L.) viruses such as leafroll viral disease (GLRaV-3), which causes significant economic losses in the wine market (Jordan et al.,, Petersen, Morgan, & Segaran, 1993; Martelli, 1993; Mahfoudhi, Digiaro, & Dhouibi, 2009). *Saissetia oleae* Olivier (Coccidae) damages olive and citrus plants, reducing yields (Mansour et al., 2017). As a consequence, controlling scale species has become an important pest control activity around the world (Daane et al., 2012).

The application of treatments such as insecticides, Insect Growth Regulators (IGRs) or biological control, have effectively controlled and reduced scale pests (Mendel, Blumberg, & Ishaaya, 1991; Ben-Dov & Hodgson, 1997; Grafton-Cardwell et al., 2006). For example, the population of *S. oleae* and *P. ficus* has been reduced using the IGR buprofezin (Daane et al., 2006; Mansour et al., 2017). This and other treatments have been mainly focused on reducing young instars, when they are still free of cotton sac, mobile, and disperse easily. However, results have not always been successful. Uncontrolled scale pests have a direct effect on the morphology and physiology of the

host, for example, reduced number of leaves, plant height, root growth or dieback (Newbery, 1980a, 1980b; Vranjic & Gullan, 1990; Turner & Buss, 2005). Other plant features such as nectar and floral display size could also be affected, and subsequently, pollinator visitation as well (Grindeland, 2005; Fowler, 2016).

Pollinators such as honey bees (*Apis mellifera* Linnaeus; Apidae) are recognised worldwide as playing a crucial role in crop pollination (McGregor et al., 1976; Morse & Calderone, 2000; Smagghe, 2012). Honey bees visit flowers collecting pollen and nectar rewards (von Frisch, 1967; Kevan & Baker, 1983; Fowler, Rotheray, & Goulson, 2016). While the former is their protein source (Herbert, 1992), the latter is their main nutritional source (Herbert, 1992) and a key component to their visitation to flowers (Willmer, 2011); they are mostly driven by olfactory stimuli (Taylor & Whelan, 1988; Raguso, 2004; Renner, 2006; Giurfa, 2007; Armbruster, 2011). Studies have demonstrated the influence of nectar quality and quantity, particularly sugar content, on bee visitation (Vansell, 1944; Butler, 1945; Wykes, 1952; Corbet, Unwin, & Prŷs-Jones, 1979; Nicolson, Nepi, & Pacini, 2007). Several studies have shown the preference of honey bees for sucrose, followed by fructose and glucose (Wykes, 1952; Waller, 1972; Bachman & Waller, 1977). However, honey bee choice can be influenced not only by sugar composition, but also by nectar quantity (Gange & Smith, 2005). Although sucrose is the preferred sugar for honey bees, studies have shown a possible preference for a combination of sucrose, glucose, and fructose in comparison to a pure sucrose nectar (Wykes, 1952; Furgala, Gochnauer, & Holdaway, 1958; Waller, 1972; Afik et al., 2006). In addition, non-sugar components such as amino acids (AAs), minerals and phenolic compounds have been suggested as potential elements influencing honey bee visitation (Waller, 1972; Alm et al., 1990; Hagler & Buchmann, 1993; Adler, 2000).

Importantly, sugar and non-sugar compounds vary depending on their environmental context (e.g. flower phenotype, plant species, and environmental conditions), which affects the decision-making process of honey bees (Baker & Baker, 1983a, 1983b; Freeman & Wilken, 1987; Galetto & Bernardello, 2004; Baude et al., 2016). For example, Percival (1961) showed in her study that plant families characterised by tubular flowers appears to have flowers that produce sucrose-nectar rather than hexose-dominant nectar. In contrast, shallow flowers found in plant species like *Leptospermum scoparium* J.R. Forst. et G. Forst. (mānuka; Myrtaceae) frequently produce hexose-dominant nectar as

has been confirmed recently in this species (Williams et al., 2014; Nickless et al., 2016). These differences among flower phenotypes could affect the olfactory stimuli of honey bees (Percival, 1961; Gómez et al., 2008; Witt, Jürgens, & Gottsberger, 2013), and subsequently their preferences for gathering nectar (Wykes, 1952). Along with the olfactory stimuli, visual stimuli are also considered drivers of honey bee visitation (Goulson, 1999; Srinivasan, 2010). Among these, floral traits such as floral display size, colour, and additional pollinators can influence honey bee visitation (Comba, Corbet, Hunt, & Warren, 1999; Gronquist et al., 2010; Srinivasan, 2010). For instance, previous studies have found that the number of visits can decrease as floral display size increases (Robertson & Macnair, 1995; Ohashi & Yahara, 2002). Other pollinators can compete with honey bees and displace them. Bennik (2009), found a negative correlation between honey bee visits and fly visits on *L. scoparium*.

Leptospermum scoparium is a shrub native to New Zealand (Stephens et al., 2005). This species is not only ecologically valuable, but is also considered crucial for the New Zealand honey industry – a revenue of $1.2 billion is expected for the mānuka honey industry by 2028[1]. The uniqueness of this honey is determined by the nonperoxide antibacterial activity (NPA) developed mainly by dicarbonyl methylglyoxal (MGO) present in the honey (Molan, 2008, 2015). However, the key ingredient appears in the nectar of *L. scoparium*. This nectar contains mostly sugars, but it also contains the carbohydrate dihydroxyacetone (DHA), which is the precursor of MGO during the honey-making process and drives the biological activity of the honey (Adams et al., 2009).

Studies have revealed that plant genotype influences not only the DHA content of *L. scoparium* (Williams et al., 2014; Millner et al., 2016), but also the sugar content. Consequently, honey bee visitation may depend on the genotype. Different genotypes are characterised by different flower phenotypes, floral display size, and nectar quality and quantity (Millner et al., 2016; Clearwater et al., 2018). Due to these differences, honey bees are involved in a decision-making process based on reward and depletion. Although previous studies have evaluated the composition of mānuka honey (Adams et al., 2009; Stephen et al., 2017) and different nectar components of *L. scoparium* (Williams et al.,

[1]http://www.mpi.govt.nz/funding-and-programmes/primary-growth-partnership/primary-growth-partnership-programmes/high-performance-manuka-plantations/

2014; Nickless et al., 2016; Smallfield et al., 2018), little research has been carried out to determine the influence of the main floral traits of *L. scoparium* on honey bee visitation.

In this study, the relative attractiveness of six *L. scoparium* cultivars to honey bees and other insects was analysed using a common garden experimental design. Specifically, the main aims were: (1) to validate a method for estimating flower density per plant using a sub-sampling approach, so that visitation can be assessed relative to the number of available flowers; (2) to reduce scale insect numbers using an insect growth regulator, and evaluate its effects on plant growth and nectar content on sprayed and unsprayed plants; (3) to determine nectar quality and quantity per flower on sprayed and unsprayed plants, and (4) to evaluate honey bee preferences based on flower density per plant and nectar content on unsprayed plants.

3.2. Material and methods

3.2.1. Study area and plant material

The study was conducted on the Moginie block of the Pasture & Crop Research Unit (PCRU) at Massey University (Palmerston North, New Zealand). The *Leptospermum scoparium* (mānuka) plantation was established in 2011 and included multiple replicates clones of plants, propagated by cuttings as six maternal lines (Table 3.1). Plants were labelled and provided by Comvita Limited, who designated a colour code (B: blue, LG: lime green, MG: mint green, O: orange, P: pink, and Y: yellow) to each cultivar for convenience (Fig. 3.1). The plantation included four replicates with six clones of each cultivar in rows with plants spaced at 1.5 m × 1.5 m in each replicate, and a replicate block spacing of 2 m. A split-plot block was designed with two insecticide treatments; plants between these two treatments were considered as a buffer (Fig. 3.2). In each replicate, 12 plants were selected from sprayed and 12 plants from unsprayed treatments to evaluate scale insect densities, flower density, and nectar quality and quantity. During the study two plants within cultivar blue (B) died. Overall, a total of 94 plants were studied.

Of the 94 plants, only plants where insecticide treatment was not applied were used for evaluating honey bee visitation rates. A total of 48 plants were included for this study.

For estimating flower density and evaluating nectar content on different cultivars, floral data and nectar samples were collected from both sprayed and unsprayed plants during October and November in 2014, and 2015. Plant from both treatments were also used for evaluating the effects of the insecticide treatment on scale insects. To do so, plants were monitored monthly by collecting stem samples from May to October 2015.

To evaluate the relative attractiveness of different cultivars to honey bees, visit observations were recorded on unsprayed plants during October and November in 2014, and again in 2015.

Table 3.1. Features of six cultivars of *L. scoparium* (B, LG, MG, O, P, and Y) used during the study.

Cultivar	Parentage	Plant form	Flower size
B	*L. scoparium* var. *scoparium* selection x *L. scoparium* var *incanum* selection	shrub	small
LG	*L. scoparium* var. *scoparium* selection x *L. rotundifolium* cultivar	tree	large
MG	*L. scoparium* var. *incanum* selection x *L. scoparium* var *incanum* cultivar	shrub	small
O	*L. Nicolsonii* cultivar x *L. scoparium* var. *scoparium* selection	shrub	small
P	*L. scoparium* var. *scoparium* selection x *L. rotundifolium* cultivar	tree	large
Y	*L. scoparium* var. *scoparium* field selection	tree	large

Fig. 3.1. Flowers produced by plants of *L. scoparium* growing at the PCRU. In order from left to right cultivars were coded as B, LG, MG, O, P, and Y.

Fig. 3.2. Experimental design showing the split-plot design with four replicate blocks with plants from six *L. scoparium* cultivars (B, LG, MG, O, P, and Y) and with two treatments: solid (unsprayed) and dashed (sprayed) at PCRU. Plants excluded from the treatments: solid (unsprayed) and dashed (sprayed) at PCRU. Plants excluded from the treatments were considered as a buffer.

3.2.2. Estimation of flower density through basal area

An objective of this study was to determine how honey bee preferences depended on the nectar content per flower and flower density per *L. scoparium* plant. There are potentially thousands of flowers on each *L. scoparium* plant, meaning that determining the total number of flowers per plant would be a time-consuming process. Consequently, it was necessary to establish a feasible method for estimating flower density per plant using a sub-sampling approach. The method applied considered that flowering appeared to be spread relatively evenly across all the branches of studied plants and that different sections of plants had similar flower density. Furthermore, it was hypothesised that the proportion of flowers on stems could be reliably estimated by the proportion of total stem basal area that each stem represented. This approach would allow estimation of flower density per plant depending on the proportion of the basal area that each stem represented.

To test the reliability of the sub-sampling approach, two plants (one per aspect) from each cultivar were selected from the buffer plants (Fig. 3.2). The basal diameter and height of each plant were measured using a height pole and a digital calliper. The total number of flowers per plant was counted in each cultivar when they were at peak flowering (Appendix 3.1). First, one branch (second ramification) per aspect (N, S, E and W) was selected around the middle of the vertical axis of the plant, tagged, and the basal diameter and length measured. Subsequently, the remaining branches were tagged with different tag colours and the total numbers of flower buds, flowers and green woody-fruit valves were counted using a digital tally counter (Fig. 3.3). Flowers were distinguished from buds when it was possible to recognise the sex of the flower. Green woody-fruit valves were distinguished from flowers when ≤ two petals were left. The total number of flowers was calculated as the sum of all buds, flowers, and woody-fruit valves present at the time of sampling.

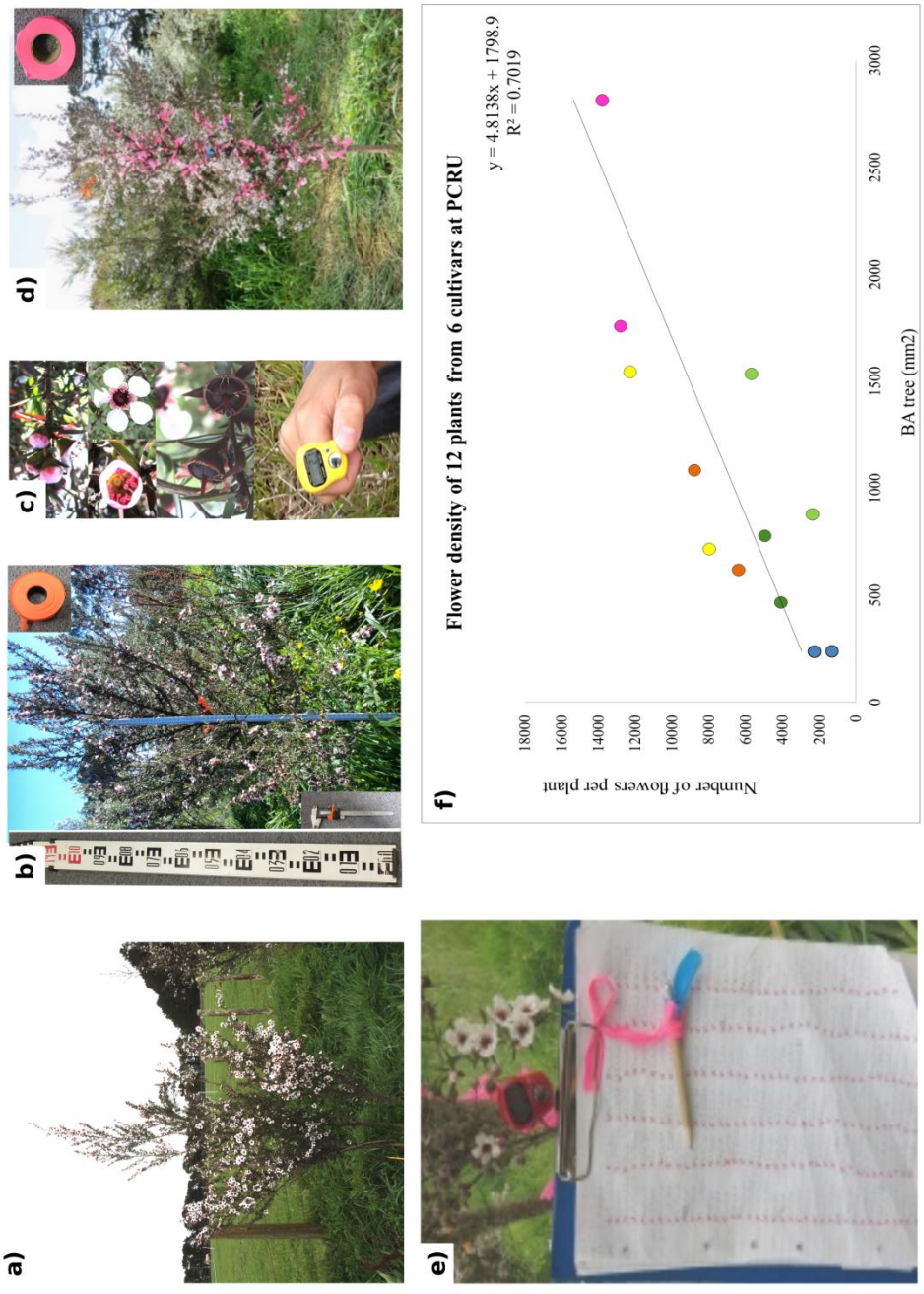


Fig. 3.3. Diagram showing main steps followed for counting all the flowers present on 12 plants classified within six *L. scoparium* cultivars (B, LG, MG, O, P, and Y). a) plant in its flowering peak; b) height pole, calliper an ruler used for growth measuring, tag used for selecting four second ramification branches within an aspect (N, S, E and W); c) from top to bottom: flower buds, flowers, green woody-fruit valves, ring tally counter; d) Plant from the orange cultivar where all flowers were counted (7981 flowers); e) list of all branches counted from a plant, pencil used for taking notes that included a tag for being attached to the finger. f) linear relation including the plant basal area and the number of total flowers counted (including flower buds, flowers and green woody-fruit valves).

To validate the method, the number of flowers per plant was predicted based on the proportion of stem basal area and a linear model was fitted between actual and predicted number of flowers (Fig. 3.4). A strong relationship (R^2 = 0.68) was found between these two variables. A Spearman's correlation test was performed between the number of flowers and the basal area for all the stems selected at each aspect (N, S, E and W). Results confirmed a positive correlation between these two parameters. A linear and a quadratic regression model were fitted, but the latter showed that the quadratic term did not significantly improve the goodness of fit of the model (Table 3.2).

Table 3.2. Coefficients obtained from the quadratic model fitted from the data basal area proportion and proportion of flowers.

Coefficients	Estimate ± SE	t-value	*P*
Linear regression			
Intercept	0.262 ± 0.09	2.88	**<0.0001**
Basal area	0.905 ± 0.08	11.34	**<0.0001**
Quadratic regression			
Intercept	0.99 ± 0.06	15.52	**<0.0001**
Poly (Basal area, 2) 1	5.05 ± 0.44	11.44	**<0.0001**
Poly (Basal area, 2) 2	–0.60 ± 0.44	–1.36	0.181

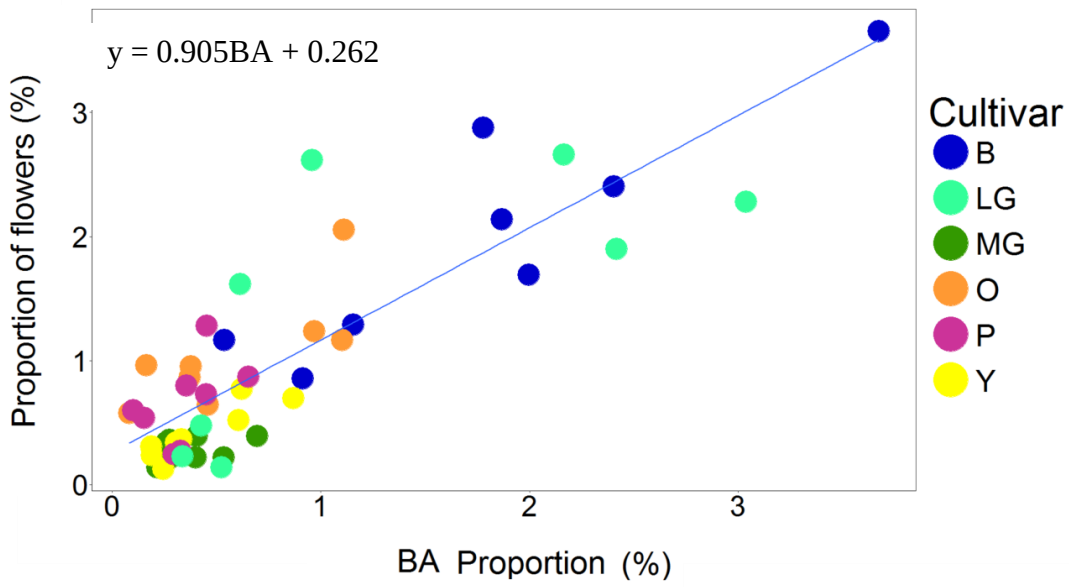


Fig. 3.4. Scatterplot showing the relationship between the proportion of basal area and the proportion of flowers from the basal area of 4 branches (N, S, E, W) of the twelve *L. scoparium* plants from six cultivars (B, LG, MG, O, P, and Y) where all the flowers were counted.

The sub-sampling approach for estimating flower density per plant was applied to 94 plants from the sprayed and unsprayed treatments. Basal diameter and height of each plant were measured in spring 2014 and 2015. Two branches (N and S aspect) were selected on each plant, tagged, and the branch diameter, length and its height above ground were measured. The total number of flower buds, flowers and green woody-fruit valves were counted. The number of male flowers were tallied separately from the hermaphrodite flowers, and noted to evaluate sex-ratio correlations. The total number of flowers was calculated as the sum of all flower buds, flowers and green woody-fruit valves present at the time of sampling. The branch basal area was expressed as a proportion of the plant basal area calculated. This proportion was used to estimate flower density per plant at the same ratio (1:1) between basal area proportion and flowers proportion.

3.2.3. Application of insecticide

Scale insects that had naturally infested the plants were used to evaluate the effectiveness of an insecticide treatment. The insecticide treatment started at the end of January 2015 when plants finished flowering. Sprayed plants were treated with the insect growth regulator (IGR) buprofezin (440 g/L of buprofezin, Mortar™) at the recommended rate for ornamentals (3 ml/10 L). Plants were sprayed until the foliage was completely dripping twice a month for 2 months. From April 2015, DC-Trom oil was used until the end of September 2015 to avoid IGR resistant problems in crawlers (first-instar nymph). The oil contained 839 g/l of mineral oil and was sprayed at the recommended rate of 10 ml/l.

To evaluate the influence of the treatment, the trial was monitored monthly by collecting a 20-cm length of stem from each plant from May to October 2015. Stem samples were sealed in ziplock-plastic bags for further analysis in the laboratory. A dissecting microscope was used to determine the number of scale insects (pupae, adult females and males) (Fig. 3.5) per stem. The presence or absence of crawlers (Fig. 3.5) and sooty mould were also recorded. As adult females are the main life stage for scale species identification, and produce large numbers of crawlers, responsible for dispersion, they were therefore considered the best indicators for evaluating the effectiveness of the treatment. Representative specimens at different life stages were preserved in 70% ethanol for species identification following the protocol described in Gullan (1984).

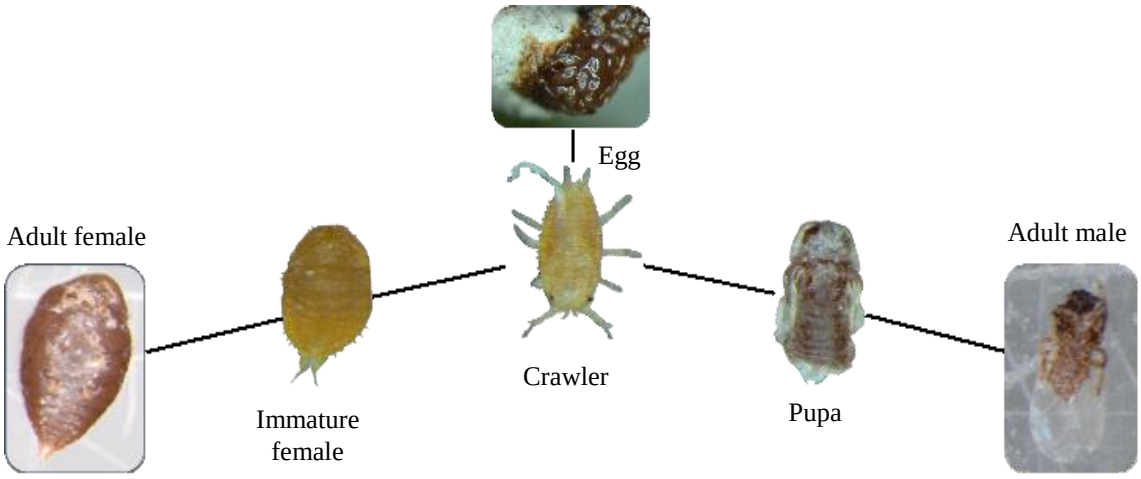


Fig. 3.5. Life stages of *Acanthococcus* spp.

3.2.4. Nectar quality and quantity

Nectar was collected during the flowering peak period (October, November) in 2014 and 2015. Plants were randomly selected from the unsprayed and sprayed plants. Nectar quantity was estimated following the protocol from Nickless et al. (2016) with some modifications. To prevent insect visitation and allow nectar to accumulate, mesh bags (40 cm × 80 cm) were placed over stems 24 hours before nectar collection (Appendix 3.2). Nectar was collected from 15 undamaged fully open flowers. Nectar was collected between approximately 10:00 am and 4:00 pm. To facilitate the collection of the nectar, 5 µl of distilled water was added to the hypanthium using a pipette. The nectar-water mix of each flower was sucked up with the pipette. The retrieved nectar-water mix from the 15 flowers was pooled in an Eppendorf tube (2 ml), placed on ice, and transferred to a freezer at –80°C for storage. Eppendorf tubes were weighed prior to nectar collection and again post-nectar collection. When reporting nectar volume the 75 µl (5 µl × 15 flowers) of distilled water added during sampling is excluded.

To analyse nectar sugar and nectar DHA, nectar samples were left at room temperature for 10 minutes to thaw before samples were prepared for nectar DHA and sugar analysis using High-Performance Liquid Chromatography (HPLC). To separate nectar components using HPLC analysis, reagent solutions (referred as mobile phases), standard samples and nectar samples were prepared as follows.

Nectar sample size differed among cultivars as the flowering period differed between them. A total of 124 and 104 nectar samples were collected in 2014 and 2015 respectively.

However, some samples were removed from the dataset, as HPLC chromatogram test results were unclear. In addition, three samples (B: 2; LG: 1) from 2014 and six samples (B: 4; LG: 1; P: 1) 2015 produced too little nectar to accurately test for sugar. Therefore, 187 nectar samples in total were used for the analysis.

3.2.4.1. Preparation of mobile phases

Two mobile phases (A and B) were prepared for nectar DHA analysis and one for nectar sugar analysis. For nectar DHA, Phase A was a solution of nanopure water with acetonitrile (ACN) at ratio of 70:30 (v/v). Phase B was 100% ACN. Reaction solutions were Hydroxyacetone (HA) (3.01 mg/ml) with *O*-(2, 3, 4, 5, 6-Pentafluorobenzyl) hydroxylamine (PFBHA) as a derivatising reagent (19.8 mg/ml in citrate buffer of 0.1 M adjusted to pH 4 with sodium hydroxide (NaOH) 4 M). For sugar analysis, the mobile phase solution was prepared by dissolving ethylenediaminetetraacetic acid (EDTA) (50 mg/L) in deionised MilliQ water and run using an isocratic elution system and a constant flow rate of 0.6 ml per minute.

3.2.4.2. Preparation and determination of DHA in nectar

Six DHA standard (100, 75, 60, 50, 25, 0 µl) aliquots were taken from a stock solution of DHA (3.88 mg/ml) made up to 10 ml. To analyse samples, 20 µl of nectar or standard sample were added to an Eppendorf tube (2 ml), 25 µl of HA was added and samples mixed and shaken on a rotatory table for one hour. For the derivatising agent, 40 µl of PFBHA was added and samples mixed for a further hour. Finally, 1.5 ml of ACN and 0.5 µl of deionised MilliQ water was added and the samples were then filtered (0.2 µm) before HPLC using a UV Diode array detector (λ = 263 nm, UVD340U) with a Synergi Fusion column (size 4.6 × 7.5 mm, 4 µm particle size). The column was kept at 30°C to ensure stable conditions.

3.2.4.3. Preparation and determination of fructose, glucose and sucrose in nectar

A 1% sugar stock solution was made up per each sugar (sucrose, glucose and fructose). Five standards including the three sugars at different concentrations were prepared as shown in Table 3.3. Sucrose concentrations were 0.0025%, 0.005%, 0.01%, 0.1%, and 0.2% in 2014 and 0.005%, 0.01%, 0.05%, 0.1%, and 0.2% in 2015. Glucose and fructose concentrations were prepared with the similar concentrations of 0.025%, 0.05%, 0.1%, 0.2%, and 0.4% in 2014 and 0.01%, 0.05%, 0.1%, 0.2%, and 0.4% in 2015. Standard

concentrations were modified from 2014 to 2015 after the results obtained from 2014 analysis. Nectar analysis followed a similar HPLC protocol used for nectar samples from other *L. scoparium* plantations from the High Performance-mānuka plantation programme (Millner et al., 2016) so, nectar results from the present study should be comparable with other studies from the High Performance-mānuka plantation programme.

To analyse samples, 20 µl of nectar or standard was diluted 50 times and filtered (0.2 µm). Sucrose, glucose and fructose were analysed using a RI detector (Shodex RI-101) with a Sugar-Pak I column (Waters, size 6.5 × 300 mm). The column was kept at 75°C.

Table 3.3. Sucrose, glucose and fructose concentrations used to prepare five standards for including in HPLC analysis in 2014 and 2015.

Standards	2014			2015		
	Sucrose	Glucose	Fructose	Sucrose	Glucose	Fructose
Standard 1	0.0025 %	0.025 %	0.025 %	0.005 %	0.01 %	0.01 %
Standard 2	0.005 %	0.05 %	0.05 %	0.01 %	0.05 %	0.1 %
Standard 3	0.01 %	0.1 %	0.1 %	0.05 %	0.1 %	0.05 %
Standard 4	0.1 %	0.2 %	0.2 %	0.1 %	0.2 %	0.2 %
Standard 5	0.2 %	0.4 %	0.4 %	0.2 %	0.4 %	0.4 %

3.2.4.4. Determination of normalised DHA

Nectar DHA levels were normalised to 80°BRIX, which indicates the potential level of DHA that can be expected in the resulting honey. Specifically, nectar DHA was transformed to mg/800 g of sugar, which is based on the average composition of sugar in honey (80%). This standardisation protocol was used as it is a protocol used by New Zealand honey companies, and it has been used previously by other studies from the High Performance-mānuka plantation (Nickless et al., 2014; 2016; Millner et al., 2016).

3.2.5. Recording honey bees' visitation

A behavioural observation sampling method (Lehner, 1991) was chosen to study the attraction of *L. scoparium* cultivars to honey bees on unsprayed plants during the flowering period in 2014 and 2015. The method consisted in recording all insects that landed in a plant at determined time intervals. This method was used to count visits

observed in the entire plant following a similar behavioural observation sampling approach used in previous studies (Comba et al., 1999a, 1999b; Memmott, 1999; Alarcón, Waser, & Ollerton, 2008). The observer approached each of the 48 plants (4 replicates × 6 cultivars × 2 plants) in the unsprayed plots during a standard walk passing around each plant to record the number of insects observed at first approach using a voice recorder. An insect was recorded if it landed on a flower. Insects were classified as honey bees, native bees, bumble bees, flies (calliphorids, tachinids and syrphids), wasps, moths, butterflies, dragonflies and katydids. Observations were conducted in calm and warm conditions; temperature and wind speed were measured using a digital handheld recorder. Depending on the weather conditions, observations were taken 2–3 times (10.30 am, 1.30 pm, 4.30 pm) per sampling day, three days a week. Due to the lack of visitations in 2015, a hive was placed approximately 75 meters from the plantation to increase the use of the flowers by honey bees.

Insect visitation was recorded for approximately one month in 2014 and 2015 in all cultivars. However, the flowering period differed slightly between cultivars and consequently nectar collection was only possible on some days for each cultivar. A total of 768 observations were deleted out of 2803 visits, as these were recorded from cultivars that were not in their main flowering period (Fig. 3.6).

To evaluate the effect of nectar on honey bee visitation, the number of honey bees recorded in the morning, afternoon or late in the afternoon when analysing the visitation rates were kept separate. The mean of nectar productivity per cultivar was calculated separately for samples collected during the morning (10–12 pm), afternoon (12–2 pm), and late in the afternoon (2–4 pm).

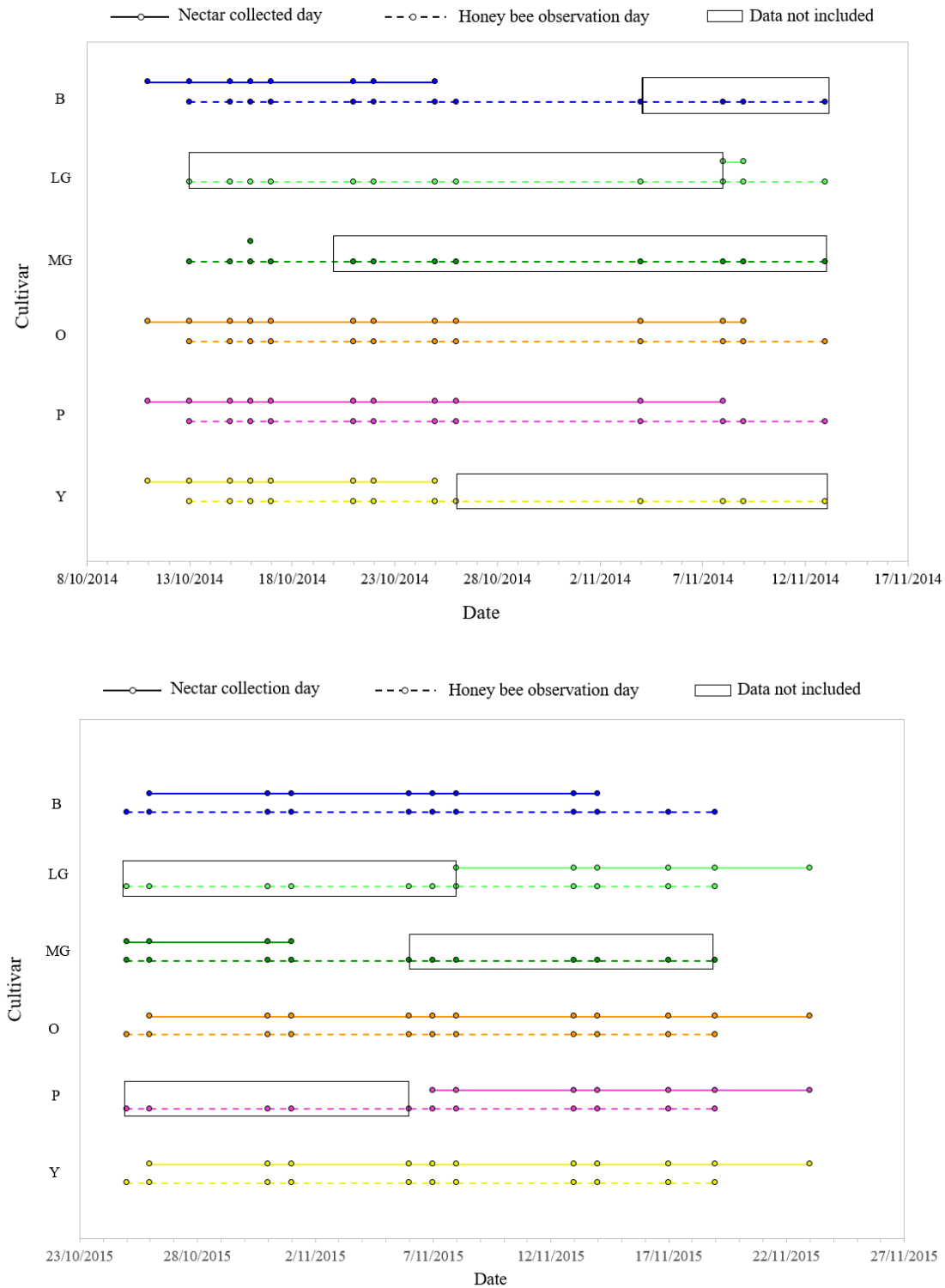


Fig. 3.6. Dates when nectar was collected and honey' bees visits were recorded for six *L. scoparium* cultivars (B, LG, MG, O, P, and Y). During the "Data not included" phases: honey bee visits were excluded from the final dataset as cultivars were not in their main flowering period.

3.2.6. Description of statistical analysis

Statistical analysis was performed using "nlme", "MASS", "car", "plyr", "multicompView" and ggplot2 in R 3.3.1 (R Core Team, 2016). Plant height, plant basal

area, flower density, nectar sugar, and DHA data were transformed to meet the assumption of a normal distribution in the residuals. However, transformation of the insecticide treatment and honey bee did not meet the assumption, so Generalized Linear Models were applied for their respective analysis. The R function "glm" was used to fit the binomial and Poisson models. The negative binomial was fitted using the function "glm.nb". The goodness of fit for selecting the most appropriate model was evaluated using Akaike criterion (AIC) values, likelihood ratio test and chi-square test based on the residual deviance. The R functions used were "AIC", lrtest", and "pchi" respectively. The functions "anova" and "summary" were used for obtaining ANOVA results and coefficients for the explanatory variables,

3.2.6.1. Plant morphology (height and basal area) and flower density among cultivars

An Analysis of Variance (ANOVA) among cultivars was performed for plant height, plant basal area and flower density data. To evaluate differences between cultivar means for these data a Tukey post-paired comparison test was performed in both years.

3.2.6.2. Insecticide treatment

A Generalized Linear Model (GLM) was chosen to determine the effects of the insecticide application on adult females and crawlers. To analyse the effects on number of adult females Poisson and negative binomial distributions were tested, since the number of adult females was a count variable. The final model was fitted using a negative binomial distribution with a logistic link function, as the data were over-dispersed. The model included the number of adult females as a response variable, and insecticide treatment, cultivar and month as explanatory variables. The interaction between explanatory variables was dropped from the final model where it was not significant ($P > 0.25$).

To evaluate the presence/absence of crawlers, a binomial distribution with a logit link function was selected including the same explanatory variables included for the negative binomial model. The interaction between explanatory variables was dropped from the final model where it was not significant ($P > 0.25$).

To evaluate differences between cultivars on sprayed and unsprayed plants, an ANOVA with Tukey post-pair test was used. Linear models were fitted for each response variable stem basal area, plant height, sugar content and DHA content, and cultivar as the explanatory variable.

3.2.6.3. Nectar quality and quantity

An ANOVA with a Tukey post-pair test was used to determine the significance of cultivar differences in nectar sugar, nectar DHA, normalised DHA, and nectar volume between sprayed and unsprayed plants. Nectar sugar (µg/flower) and nectar DHA (µg/flower) were fitted separately using a linear regression model against: radiation, humidity, temperature, cultivar and year. To evaluate the effect of the weather on nectar, the weather data included in the model were obtained from the National Institute of Water and Atmospheric Research Ltd. (NIWA[1]) and were classified by hour of observation. The interaction between explanatory variables was again dropped from the final model where it was not significant ($P > 0.25$).

3.2.6.4. Honey bee visitation

To evaluate honey bee visitation per flower, a GLM with a quasipoisson distribution was used as the data were over-dispersed. The explanatory variables included in the model were estimated flowers per plant, total sugar per flower, percentage glucose in the nectar, temperature and time of day, nectar DHA content, and fly visits per flower. A preliminary model also included the explanatory variable cultivar, but due to the collinear association with sugar content, the cultivar was removed from the final model.

[1] https://cliflo.niwa.co.nz/

3.3. Results

3.3.1. Flower density estimation through basal area

The cultivars differed significantly in basal area, height and estimated flower density (Fig. 3.7) (ANOVA results shown in Appendix 3.3). Two groups were apparent. The first, constituted plants belonging to cultivars Y and P, approximately 2 m in height with more than 10,000 flowers produced in each season. The second group, cultivars B and MG, was approximately 1.5 m tall and produced less than 5,000 flowers (Appendix 3.1).

All cultivars presented a higher proportion of hermaphrodite flowers than male flowers per branch sample (Fig. 3.8) (Appendix 3.4). The cultivar MG was found the cultivar with the highest proportion of males flowers in 2014 (42.52%) and 2015 (15.61%). Spearman's correlation test showed a significant correlation (r = –0.28, P < 0.0001) between number of male flowers and the height of the branch above ground. The number of male flowers was also correlated with the length of the branch (r = 0.12, P = 0.02). However, no significant correlation (r = –0.003, P = 0.95) was found between number of male flowers and hermaphrodite flowers.

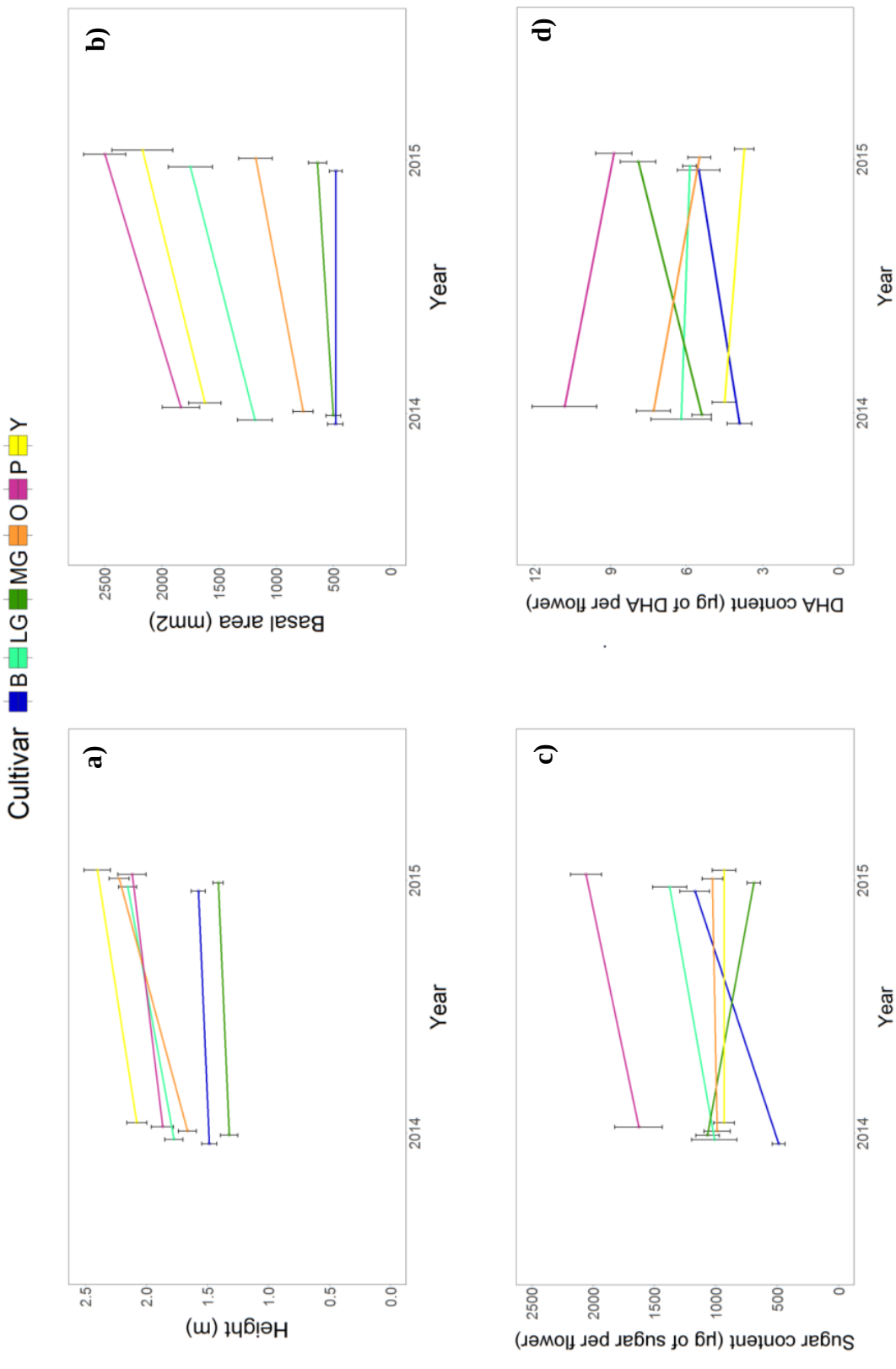


Fig. 3.7. Multi-paired plots showing the mean values determined for the variables a) height; b) basal area; c) sugars content; d) DHA content in six cultivars of *L. scoparium* (B, LG, MG, O, P, and Y) in 2014 and 2015. Error bars represent the standard errors of the mean.

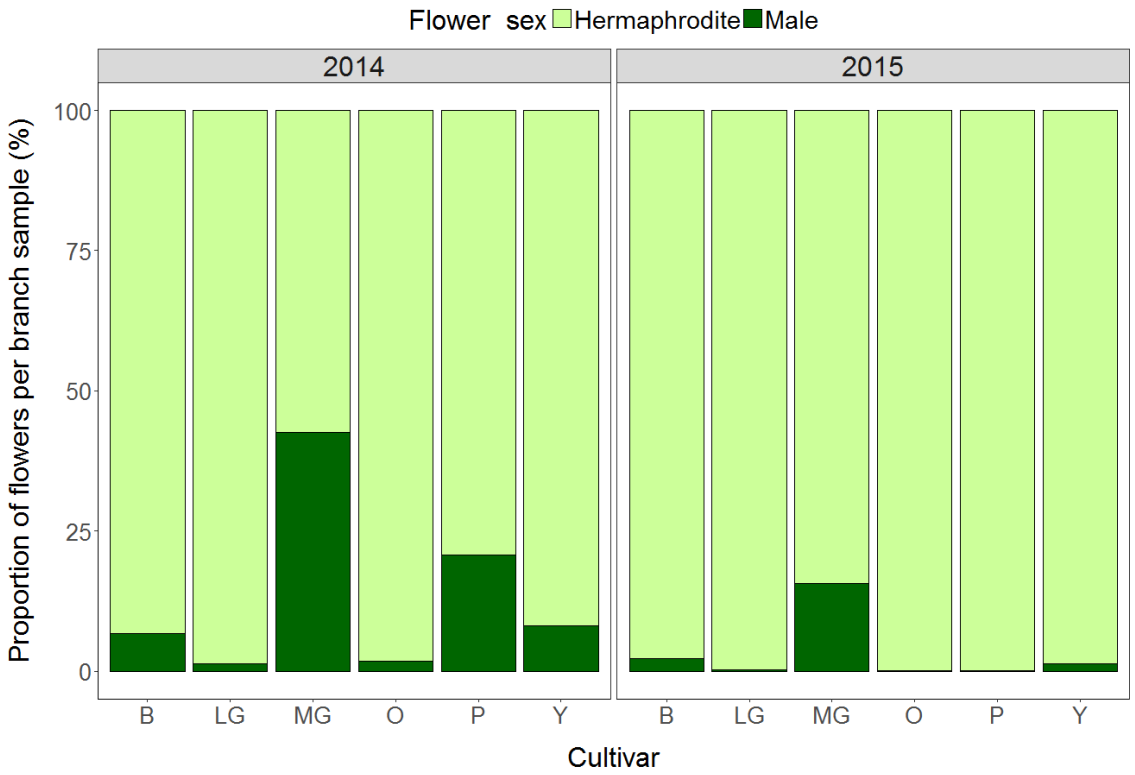


Fig. 3.8. Bar chart showing the average proportion of hermaphrodite and male flowers per branch sample on sprayed and unsprayed plants of six *L. scoparium* cultivars (B, LG, MG, O, P, and Y).

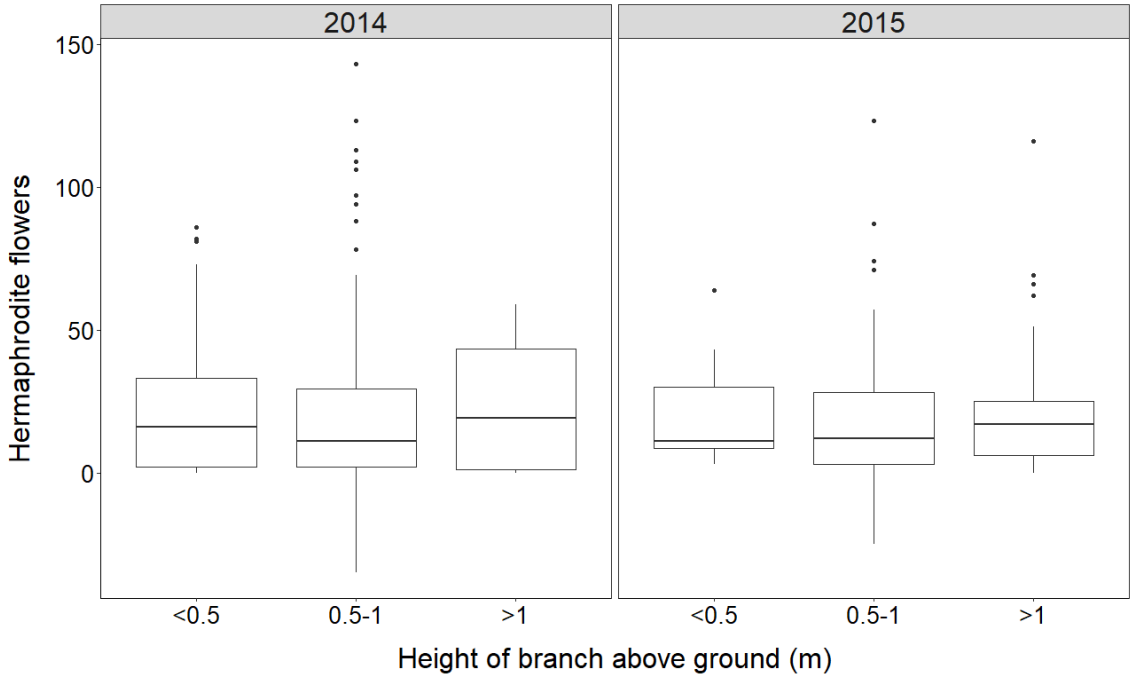


Fig. 3.9. Average number of hermaphrodite flowers per branch sample at different heights (<0.5 m, 0.5–1 m, and >1 m) on sprayed and unsprayed plants of six *L. scoparium* cultivars (B, LG, MG, O, P, and Y).

Male flowers were more abundant on branches located closer to the ground, while the number of hermaphrodite flowers increased as the height of the branch above ground increased (Fig. 3.9) (Appendix 3.5).

3.3.2. Influence of an insecticide treatment on scale insects

3.3.2.1. Influence of an insecticide treatment on adult female density

A total of 565 stem samples were collected from unsprayed and sprayed plants over a 6-month period. Two species of *Acanthococcus* were identified – *Acanthococcus campbelli* Hoy and *A. leptospermi* Maskell. However, no distinction was made between them for evaluating the effectiveness of the insecticide treatment. The insecticide treatment reduced the number of adult females on sprayed plants in all cultivars (Fig. 3.10). While the median was zero adult females per 20-cm stem on sprayed plants, the median was two adult females in unsprayed plants. Unsprayed LG plants had a median of five adult females per 20 cm of stem. A median of one to three adult females were found on remaining cultivars (Fig. 3.10).

The insecticide treatment reduced the number of adult females in all cultivars in all months (Fig. 3.11). May and October had the lowest adult female numbers in both treatments, with a median of zero adult females on sprayed plants and one adult female on unsprayed plants. Despite the treatment, adult females were mostly present on sprayed plants in cultivar Y every month during the study period. In the sprayed trial, the cultivar MG was characterised by a high infestation level, with a median of three adult females per 20-cm stem. Among cultivars and months, adult females were mostly absent in October on sprayed plants, apart from on cultivar P.

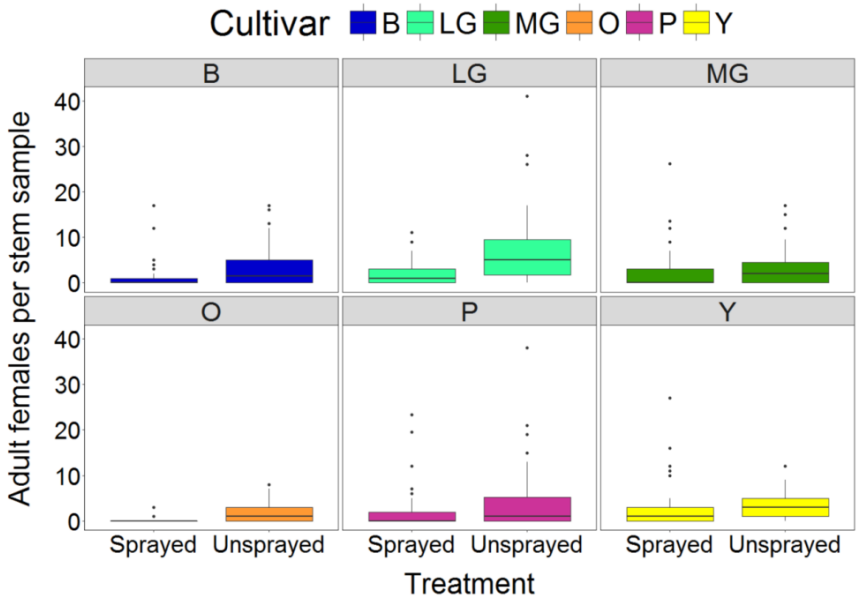


Fig. 3.10. Boxplots showing the median adult females found per 20-cm stem on sprayed and unsprayed plants of six *L. scoparium* cultivars (B, LG, MG, O, P, and Y) monitored for six months (May, June, July, August, September, and October) at PCRU.

Results from the GLM showed that insecticide treatment, cultivar and month significantly affected adult female numbers. In addition, the interaction between treatment and cultivar, and cultivar and month were also significant suggesting the effectiveness on the treatment varied among the cultivars (Table 3.4) (see Appendix 3.6 for the coefficients). Results showed that whereas cultivar O has response to the treatment, sprayed and unsprayed plants from the cultivar Y present almost the same level of infestation after they are sprayed (Appendix 3.6).

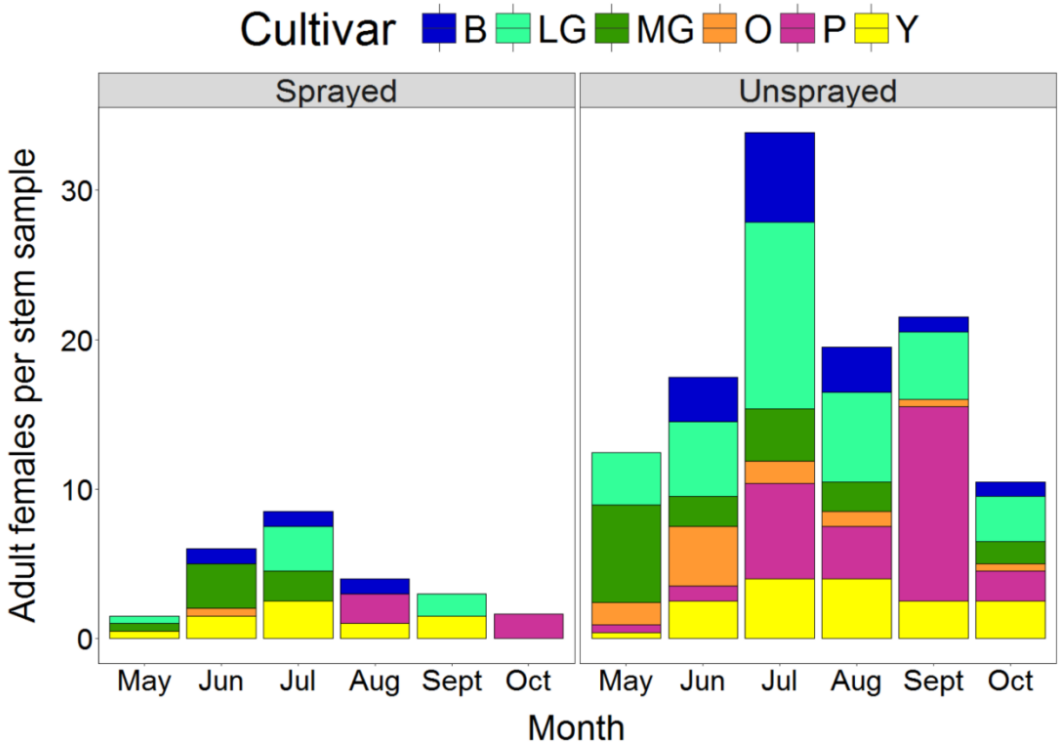


Fig. 3.11. Bar chart showing the median number of adult females per 20-cm stem on sprayed and unsprayed plants of six *L. scoparium* cultivars (B, LG, MG, O, P, and Y) monitored for six months (May, June, July, August, September, and October) at PCRU.

Table 3.4. Significance effects of the insecticide treatment on adult females on six *L. scoparium* cultivars (B, LG, MG, O, P, and Y). A GLM test with a negative binomial distribution and logit link function was chosen to evaluate the explanatory variables treatment, cultivar, month and the interaction between treatment and cultivar.

Explanatory variables	d.f.	*Deviance*	*Resid. df*	*Resid. Dev*	*P*
Treatment	1	55.81	562	781.11	**<0.0001**
Cultivar	5	60.05	557	721.06	**<0.0001**
Month	5	50.54	552	670.52	**<0.0001**
Treatment: Cultivar	5	20.01	547	650.51	**0.0012**
Cultivar: Month	25	83.11	522	567.40	**<0.0001**

3.3.2.2. Influence of the treatment on crawlers' presence

The presence of crawlers was strongly affected by insecticide treatment on stem and by month (Fig. 3.12). Cultivar had a weak effect on crawler presence (Table 3.5) (coefficients shown in Appendix 3.7).

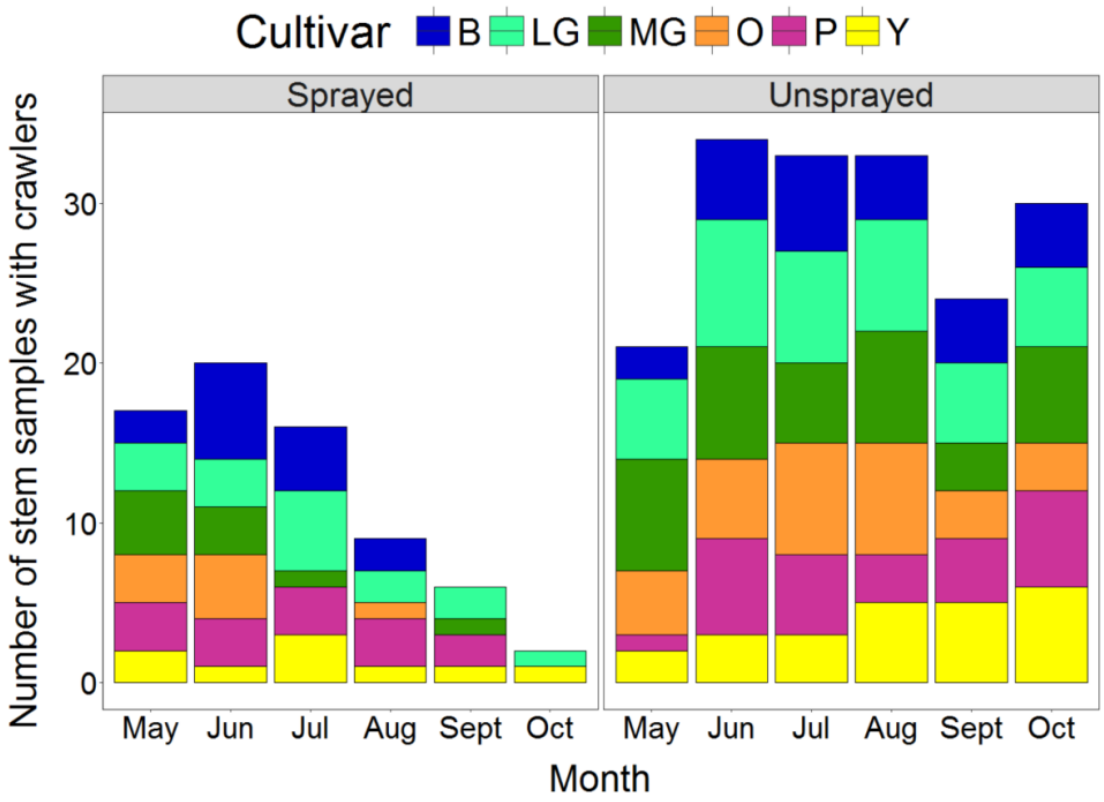


Fig. 3.12. Bar chart showing the total number of stems samples (20-cm stem length) recorded with crawler (first-instar nymph) presence on sprayed and unsprayed plants of six *L. scoparium* cultivars (B, LG, MG, O, P, and Y) monitored for six months (May, June, July, August, September, and October) at PCRU.

Table 3.5. Significance effects of the insecticide treatment on crawler's presence on six *L. scoparium* cultivars (B, LG, MG, O, P, and Y). A GLM test with a binomial distribution was chosen to evaluate the explanatory variables insecticide treatment, cultivar and month.

Explanatory variables	d.f.	*Deviance*	*Resid. df*	*Resid. Dev*	*P*
Treatment	1	80.91	562	690.43	**<0.0001**
Cultivar	5	11.99	557	678.26	**0.0350**
Month	5	23.10	552	655.05	**<0.0001**
Treatment: Cultivar	5	8.30	547	646.75	0.1405
Cultivar: Month	25	33.67	522	613.08	0.1150

3.3.2.3. Influence of the treatment on plant growth, flower density, and nectar content

Despite reducing scale insect density, the insecticide treatment had no effect on growth or nectar production, nor did it affect plant basal area, height, sugar content or DHA content (Table 3.6). The only variable marginally affected was flower density. However, all five variables were significantly influenced by cultivar. Flower density was also the only variable that was influenced by the interaction between treatment and cultivar (Appendix 3.8).

Table 3.6. ANOVA showing the influence of the insecticide treatment on plants of six *L. scoparium* cultivars (B, LG, MG, O, P, and Y) on stem basal area, plant height and flower density.

Response variable; explanatory variables	d.f.	Sum of squares	Mean squares	*F*	*P*
Basal area***					
Treatment	1	106.5	106.49	1.68	0.1988
Cultivar	5	9957.0	1991.39	31.38	**<0.0001**
Treatment: Cultivar	5	191.0	38.20	0.60	0.6985
Residuals	82	5203.3	63.45		
Height**					
Treatment	1	0.001	0.001	0.19	0.6653
Cultivar	5	0.650	0.130	27.01	**<0.0001**
Treatment: Cultivar	5	0.028	0.005	1.14	0.3438
Residuals	82	0.395	0.005		
Estimated flower density**					
Treatment	1	0.179	0.179	3.78	0.0553
Cultivar	5	6.568	1.314	27.72	**<0.0001**
Treatment: Cultivar	5	1.381	0.276	5.83	**0.0001**
Residuals	82	3.886	0.047		
Sugar content**					
Treatment	1	0.016	0.016	0.60	0.4383
Cultivar	5	2.463	0.492	18.27	**<0.0001**
Treatment: Cultivar	5	0.032	0.006	0.23	0.9459
Residuals	85	2.292	0.027		
DHA content**					
Treatment	1	0.002	0.002	0.06	0.8006
Cultivar	5	1.219	0.244	9.32	**<0.0001**
Treatment: Cultivar	5	0.064	0.0127	0.49	0.7857
Residuals	85	2.222	0.0261		

*Logarithm transformation was applied to plant height, nectar sugar and DHA data to fit a normal distribution

**Square roots transformation was applied to basal area data to fit a normal distribution

3.3.3. Nectar quality and quantity

3.3.3.1. Nectar quality and quantity among cultivars

Nectar sugar content per flower differed among cultivars (ANOVA results shown in Appendix 3.9). Among cultivars, Cultivar P showed a significant difference of sugar content compared with the other cultivars, producing the highest nectar sugar content in 2014 and 2015 (Table 3.7). Cultivar B produced the lowest nectar volume in 2014, and cultivar Y the lowest in 2015. Cultivar P) and cultivar LG produced the greatest volume in 2014 and 2015 (Table 3.8). Cultivar B produced the lowest nectar sugar content in 2014, and cultivar MG the lowest in 2015. Cultivar P produced the highest nectar DHA content in both years. The lowest nectar DHA in 2014 and 2015 were produced by cultivar B and cultivar Y in 2014 and 2015 respectively. Cultivar Y had the lowest DHA concentration in both years (Table 3.8) (see ANOVA results in Appendix 3.10). Cultivar O and P produced the highest DHA concentration in 2014, and MG the highest in 2015.

Fructose and glucose Molarity were generally higher in all cultivars in 2015 than in 2014 (Table 3.9). However, MG had higher concentrations of all sugars in 2014 than in 2015. Cultivar MG had the highest concentration of fructose (0.46 M) in 2014. In 2015, cultivar P had the highest concentration of fructose (1.08 M) and glucose (0.56 M). Sucrose concentrations were lower in 2015 than in 2014. Cultivar O showed the highest Molarity with 0.004 M in 2014 and 0.0010 M in 2015. As some samples presented a minimal concentration of sucrose and they were not detected by the HPLC; they were classified as below the detection limit (< LOD).

Table 3.7. Mean and standard error of sugar and DHA content (µg/flower) of six *L. scoparium* cultivars (B, LG, MG, O, P, and Y). Significance differences between cultivar means are indicated by letters (comparison Tukey-post pair test after ANOVA). Cultivars sharing a letter are not significantly different.

Cultivar	2014				2015			
	Sugars* ± SE (mg/flower)	DHA* ± SE (µg/flower)	Normalised DHA* ± SE (mg/kg 80°BRIX)	*n*	Sugars* ± SE (µg/flower)	DHA* ± SE (µg/flower)	Normalised DHA*± SE (mg/kg 80°BRIX)	*n*
B	0.49 ± 0.05^{c}	3.89 ± 0.49^{c}	6403.49 ± 474.25^{a}	12	1.17 ± 0.11^{b}	5.49 ± 0.84^{c}	3800.54 ± 385.35^{b}	18
LG	1.01 ± 0.18^{b}	6.20 ± 1.20^{bc}	4753.29 ± 403.27^{ab}	13	1.38 ± 0.17^{ab}	5.86 ± 0.27^{abc}	3554.99 ± 268.20^{b}	7
MG	1.07 ± 0.10^{ab}	5.39 ± 0.38^{bc}	4081.85 ± 301.23^{ab}	8	0.69 ± 0.05^{c}	7.90 ± 0.70^{ab}	9652.38 ± 834.22^{a}	17
O	0.99 ± 0.11^{b}	7.30 ± 0.68^{ab}	6115.58 ± 332.27^{a}	24	1.03 ± 0.084^{b}	5.48 ± 0.44^{bc}	4421.41 ± 282.72^{b}	18
P	1.63 ± 0.19^{a}	10.81 ± 1.28^{a}	5466.09 ± 556.29^{a}	17	2.00 ± 0.13^{a}	8.86 ± 0.72^{a}	3588.79 ± 313.44^{b}	20
Y	0.93 ± 0.08^{b}	4.48 ± 0.49^{c}	3931.41 ± 356.43^{b}	16	0.93 ± 0.10^{bc}	3.70 ± 0.38^{c}	3384.82 ± 337.67^{b}	17

*Logarithm transformation was applied to sugar content and DHA to fit a normal distribution for ANOVA test

Table 3.8. Mean and standard error of sugar and DHA concentration (mg/ml), and volume per flower on six cultivars of *L. scoparium* (B, LG, MG, O, P, and Y) sampled in 2014 and 2015.

Cultivar	2014				2015			
	Volume* ± SE (µl/flower)	Sugar* ± SE (mg/ml)	DHA* ± SE (mg/ml)	*n*	Volume* ± SE (µl/flower)	Sugar* ± SE (mg/ml)	DHA* ± SE (mg/ml)	*n*
B	1.23 ± 0.35^{c}	77.92 ± 6.98^{b}	0.61 ± 0.06^{bc}	12	0.79 ± 0.18^{b}	200.09 ± 17.60^{b}	0.92 ± 0.12^{c}	18
LG	4.49 ± 0.94^{b}	102.98 ± 11.93^{ab}	0.62 ± 0.08^{bc}	13	1.48 ± 0.36^{ab}	211.49 ± 17.26^{ab}	0.91 ± 0.04^{abc}	7
MG	3.40 ± 0.98^{bc}	139.92 ± 11.60^{a}	0.71 ± 0.05^{abc}	5	0.98 ± 0.12^{b}	113.68 ± 7.68^{c}	1.31 ± 0.10^{a}	17
O	2.95 ± 0.33^{b}	123.72 ± 10.43^{a}	0.91 ± 0.07^{a}	24	0.97 ± 0.10^{b}	172.03 ± 13.36^{b}	0.91 ± 0.07^{bc}	18
P	6.55 ± 0.78^{a}	139.99 ± 13.25^{a}	0.91 ± 0.08^{ab}	17	1.99 ± 0.24^{a}	297.01 ± 18.78^{a}	1.25 ± 0.08^{ab}	20
Y	3.15 ± 0.42^{b}	115.59 ± 9.20^{ab}	0.56 ± 0.05^{c}	16	0.66 ± 0.08^{b}	162.27 ± 14.96^{bc}	0.65 ± 0.06^{c}	17

*Logarithm transformation was applied to sugar content and DHA to fit a normal distribution for ANOVA test

Table 3.9. Mean and standard errors of the sugar concentrations (fructose, glucose and sucrose) expressed as Molarity of six *L. scoparium* cultivars (B, LG, MG, O, P, and Y) in 2014 and 2015. Numbers in brackets is the number of samples where sucrose was available. LOD: limit of detection.

Cultivar	2014				2015			
	Fructose ± SE (M)	Glucose ± SE (M)	Sucrose ± SE (M)	*n*	Fructose ± SE (M)	Glucose ± SE (M)	Sucrose ± SE (M)	*n*
B	0.25 ± 0.02	0.18 ± 0.02	$0.003 \pm 1.10\times10^{-4}$	12	0.79 ± 0.08	0.32 ± 0.05	< LOD	18
LG	0.33 ± 0.04	0.24 ± 0.03	$0.003 \pm 3.62\times10^{-5}$	13 (12)	0.82 ± 0.08	0.35 ± 0.03	< LOD	7
MG	0.46 ± 0.02	0.29 ± 0.04	$0.020 \pm 1.02\times10^{-2}$	8	0.38 ± 0.04	0.25 ± 0.02	$0.0004 \pm 1.75\times10^{-4}$	17
O	0.39 ± 0.03	0.28 ± 0.02	$0.004 \pm 6.45\times10^{-4}$	24	0.66 ± 0.06	0.29 ± 0.02	$0.0010 \pm 3.11\times10^{-4}$	18
P	0.44 ± 0.04	0.33 ± 0.03	$0.003 \pm 2.71\times10^{-4}$	17 (15)	1.08 ± 0.09	0.56 ± 0.04	$0.0002 \pm 1.49\times10^{-4}$	20
Y	0.37 ± 0.03	0.26 ± 0.02	$0.003 \pm 4.19\times10^{-4}$	16 (15)	0.61 ± 0.06	0.29 ± 0.02	< LOD	17

The ternary diagram (Fig. 3.13) indicates the predominance of fructose (61.8%) and glucose (37.6%) in the nectar of *L. scoparium* cultivars, with sucrose present in very low concentrations (0.68%).

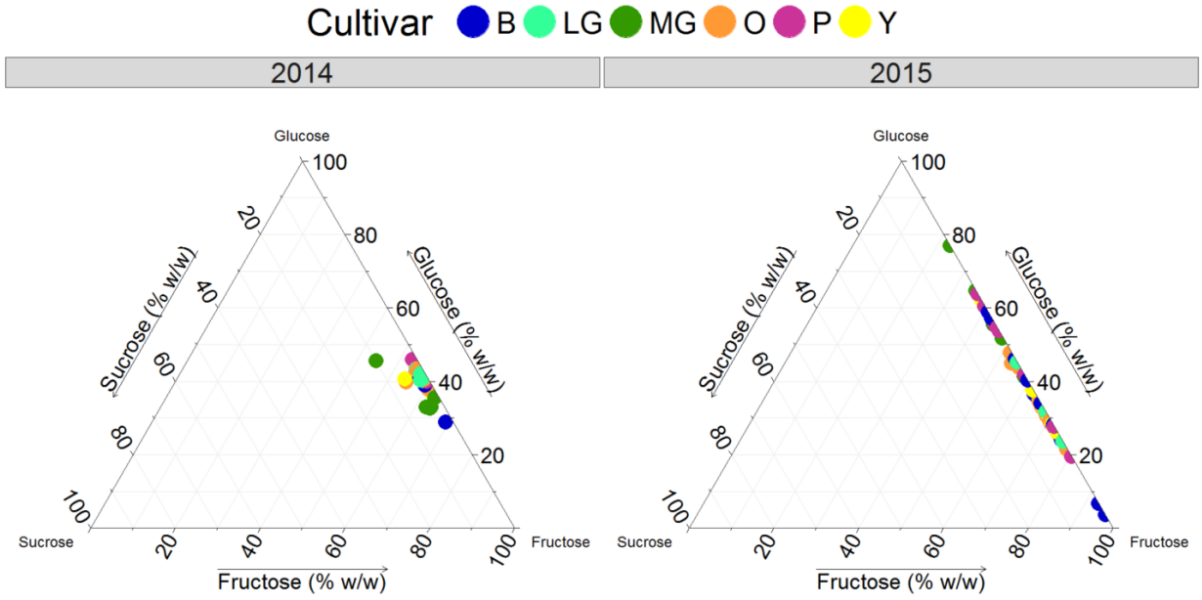


Fig. 3.13. Ternary diagram showing the sugar composition of nectar samples present from six *L. scoparium* cultivars (B, LG, MG, O, P, and Y) during 2014 and 2015. Each circle represents the percentage of each individual sugar (sucrose, glucose and fructose) in the nectar.

3.3.3.2. Normalised DHA

Cultivar MG had the highest normalised DHA levels (the predicted DHA content per gram in honey) (9652.38 mg/kg 80°BRIX) (Table 3.7) in 2015. Interestingly, this cultivar had one of the lowest levels of normalised DHA in 2014 (4081.85 mg/kg 80°BRIX), a result of high sugar content per flower and low DHA content that year. In contrast, cultivar Y presented the lowest level of normalised DHA both years as it produced relatively low DHA content in both seasons.

3.3.3.3. Influence of different variables on nectar quality and quantity

Spearman' correlation was positive between sugar content and DHA content ($r = 0.64$, $P < 0.0001$) (Fig. 3.14). DHA content was also positively correlated with normalised DHA ($r = 0.47$, $P < 0.0001$). In contrast, the correlation between sugar content and normalised DHA was negative ($r = –0.37$, $P < 0.0001$). Ratios between nectar DHA: sugar varied among cultivars (Table 3.10), which suggests that high DHA content might not translate to high normalised DHA.

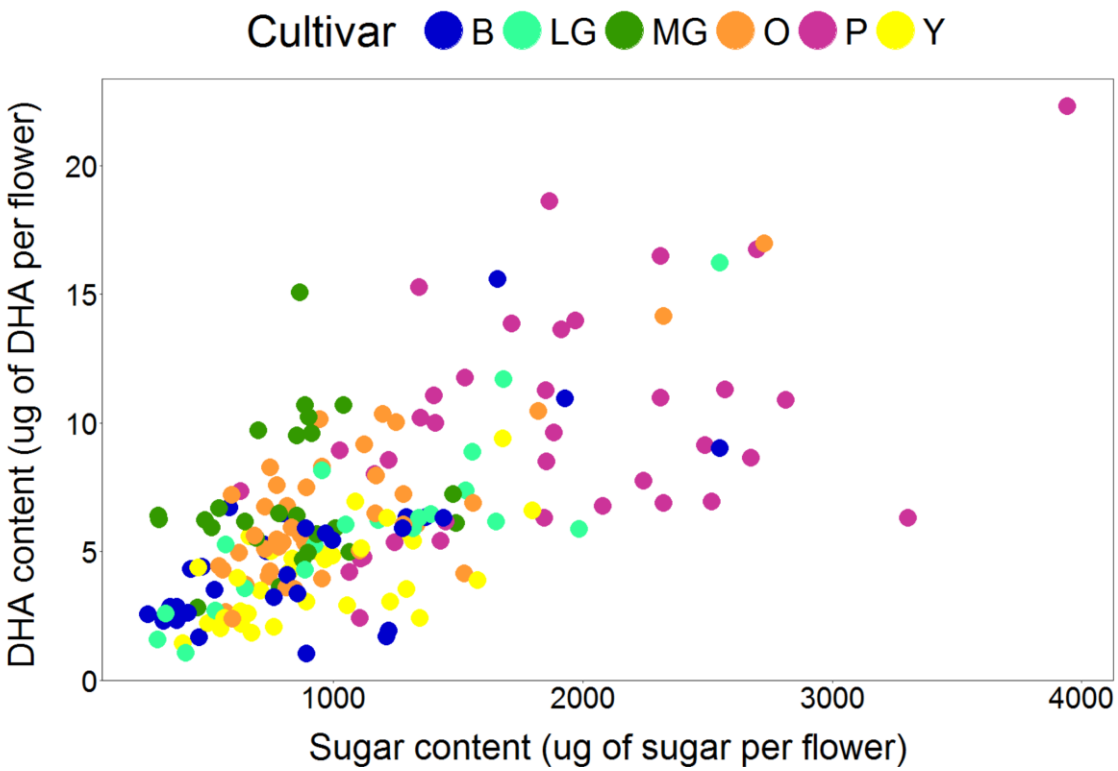


Fig. 3.14. Scatterplot showing the relationship between nectar sugar content (µg/flower) and DHA (µg/flower) in six *L. scoparium* cultivars (B, LG, MG, O, P, and Y) collected during 2014 and 2015.

Table 3.10. Mean values of the ratio DHA/total sugars from six cultivars of *L. scoparium* (B, LG, MG, O, P, and Y) sampled in 2014 and 2015. Significance differences between cultivar means are indicated by letters (comparison Tukey-post pair test after ANOVA). Cultivars sharing a letter are not significantly different.

Cultivar	2014		2015	
	DHA/Total sugars ± SE	*n*	DHA/Total sugars ± SE	*n*
B	0.008 ± 0.0006^{a}	12	0.005 ± 0.0005^{b}	18
LG	0.006 ± 0.0005^{ab}	13	0.004 ± 0.0003^{b}	7
MG	0.005 ± 0.0002^{b}	8	0.012 ± 0.0010^{a}	17
O	0.008 ± 0.0004^{a}	24	0.006 ± 0.0004^{b}	18
P	0.007 ± 0.0006^{ab}	17	0.004 ± 0.0004^{b}	20
Y	0.005 ± 0.0004^{b}	16	0.004 ± 0.0004^{b}	17

To evaluate the influence of the variables cultivar, humidity, radiation, temperature collected from NIWA, and year, a linear model was fitted for nectar DHA and sugar (Table 3.11) (coefficients shown in Appendix 3.11). Cultivar and temperature significantly influenced DHA and sugar content. Year also significantly influenced sugar

content. The variables humidity and radiation did not have a significant effect on any of the two nectar compounds, sugar and DHA.

Table 3.11. Significance of the explanatory variables: cultivar, temperature and year on nectar DHA and sugar content. A linear model was fitted to evaluate these explanatory variables on nectar sugar and DHA.

Response; explanatory variables	d.f.	Sum of squares	Mean squares	*F*	*P*
DHA content					
Cultivar	5	11.94	2.39	19.40	**<0.0001**
Humidity	1	0.01	0.01	0.04	0.8351
Radiation	1	0.04	0.04	0.34	0.5598
Temperature	1	2.46	2.46	19.99	**<0.0001**
Year	1	0.03	0.03	0.22	0.6422
Cultivar: Humidity	5	0.88	0.18	1.44	0.2143
Cultivar: Radiation	5	1.87	0.37	3.04	**0.0120**
Cultivar: Temperature	5	1.05	0.21	1.71	0.1355
Cultivar: Year	5	2.83	0.56	4.59	**0.0006**
Residuals	157	19.33	0.12		
Sugar content					
Cultivar	5	3.04	0.61	22.09	**<0.0001**
Humidity	1	0.12	0.12	4.36	**0.0383**
Radiation	1	0.04	0.04	1.59	0.2089
Temperature	1	0.60	0.60	21.91	**<0.0001**
Year	1	0.34	0.34	12.17	**0.0006**
Cultivar: Humidity	5	0.09	0.02	0.68	0.6394
Cultivar: Radiation	5	0.28	0.06	2.06	0.0730
Cultivar: Temperature	5	0.25	0.05	1.81	0.1137
Cultivar: Year	5	0.85	0.17	6.15	**<0.0001**
Residuals	157	4.32	0.03		

3.3.4. Relative visitation rates of honey bees and other insects

A total of 8908 visitations were recorded over 22 days during the flowering period in 2014 and 2015. Visitations were recorded on 58 occasions, with a total of 19.3 hours of observations. Visitors (Fig. 3.15) recorded belonged mostly to the families Apidae, Calliphoridae and Syrphidae (Appendix 3.12); 91.7% of the visitations were from honey bees, followed by bumble bees (4%) and all fly families (3.6%).

Fig. 3.15. Visitors on flowers of *L. scoparium* during the flowering season. (a) *Vanessa gonerilla*; (b) *Apis mellifera*; (c) *Calliphora stygia*.

Overall, cultivar P had the highest number of honey bee visits, with 66.6% of all visits in 2014 and 50.7% in 2015. Cultivar O was the second most visited cultivar, with 15.7% of visits in 2014 and 26.9% in 2015. Bumble bees and flies were mostly observed on cultivar Y and P, with 41.52% and 31.64% respectively.

Results from the GLM model showed that honey bee visitation was influenced by flower density per plant, sugar per flower, percentage glucose, temperature, time of day, fly visits per flower, and year. Sugar content per flower (Table 3.12), percentage glucose, and number of fly visits as very strong predictors of honey bee visitation. In addition, the flower density per plant also had a significant effect in 2014, with the number of honey bee visits per flower declining as the number of flowers increased (coefficients shown in Appendix 3.13).

Other variables such as temperature and time of the day also showed a moderate effect on honey bee visitation during 2014 and 2015. For instance, number of visits increased with the warmer temperatures (10.30 am and 1.30 pm) and they decreased at 4.30 pm, when temperatures were lower. DHA per flower was also included in the model, but it did not influence honey bee visitation in 2014, but its influence was weakly significant in 2015.

As fly visits influenced significantly with honey bee visitation, a Spearman's correlation was performed and results showed a positive correlation ($r = 0.18$, $P < 0.0001$).

Table 3.12. Significance effect on honey bee visits of the variable explanatory: estimated flower density per plant, sugar content per flower, percentage of glucose per flower, temperature, time of the day, DHA content per flower, flies per flower, and year on honey bee visits present on plants propagated as six cultivars (B, LG, MG, O, P, and Y) of *L. scoparium* during 2014 and 2015. A GLM test with a quasipoisson distribution was chosen to evaluate the explanatory variables on honey bee visits.

Year; explanatory variable	d.f.	χ^2	*P*
Estimated flower density/plant	1	27.402	**<0.0001**
μg of sugar per flower	1	77.810	**<0.0001**
Glucose per flower (% w/w)	1	73.096	**<0.0001**
Temperature	1	36.666	**<0.0001**
Time of day	2	43.878	**<0.0001**
μg of DHA per flower	1	0.393	0.5309
Fly visits per flower	1	97.092	**<0.0001**
Year	1	66.739	**<0.0001**

Table 3.13. Mean of honey bees' visits recorded per flower on plants propagated as six cultivars (B, LG, MG, O, P, and Y) of *L. scoparium* during 2014 and 2015 (approximately 38 seconds per plant recording visitations).

Cultivar	2014		2015	
	Visits flower^{-1} ± SE	*n*	Visits flower^{-1} ± SE	*n*
B	0.74 ± 0.39	144	1.27 ± 0.15	203
LG	5.06 ± 1.14	72	1.63 ± 0.12	152
MG	0.29 ± 0.12	96	2.63 ± 0.50	104
O	2.12 ± 0.19	240	3.84 ± 0.20	232
P	6.72 ± 0.36	240	9.58 ± 0.54	152
Y	0.46 ± 0.05	168	2.18 ± 0.16	232

Mean sugar content and estimated flower density differed between cultivars; the mean number of honey bee visits also differed among cultivars. Cultivar P had the highest number of visits per flower in 2014 and 2015 (Table 3.13). A clear preference of honey bees to visit flowers from cultivar P was revealed, as 6.7 and 9.6 honey bees per flower were estimated on cultivar P. In contrast, cultivar MG had the lowest number of visits in 2014 and cultivar B the lowest in 2015, with 0.2 and 1.3 honey bee visits per flower respectively.

The relative rate of honey bee visits per flower was evaluated daily and the results corroborated the strong preference of honey bees for cultivar P and the rejection of other cultivars (B) (Fig. 3.16) (Appendix 3.14). Some cultivars differed in flowering time with cultivar MG being the earliest flowering and cultivar LG the last to flower. Although the duration of the flowering period and nectar availability of each cultivar varied, cultivar P and O generally had the longest flowering period. Cultivar P also had the highest relative rate of visitation in 2014, with an average of 57% (10.30 am), 61% (1.30 pm), and 56% (4.30 pm) of visits. A similar pattern was observed in 2015 (Fig. 3.16) with 49% (10.30 am), 53% (1.30 pm), and 52% (4.30 pm) of the total visits. Nevertheless, because cultivar P started to flower slightly later in 2015, cultivar O was the most visited during the last week of October in 2015, while cultivar P had not begun to flower. Cultivar LG had the second highest visit numbers with 39% (10.30 am), 27% (1.30 pm), and 10% (4.30 pm) in 2014.

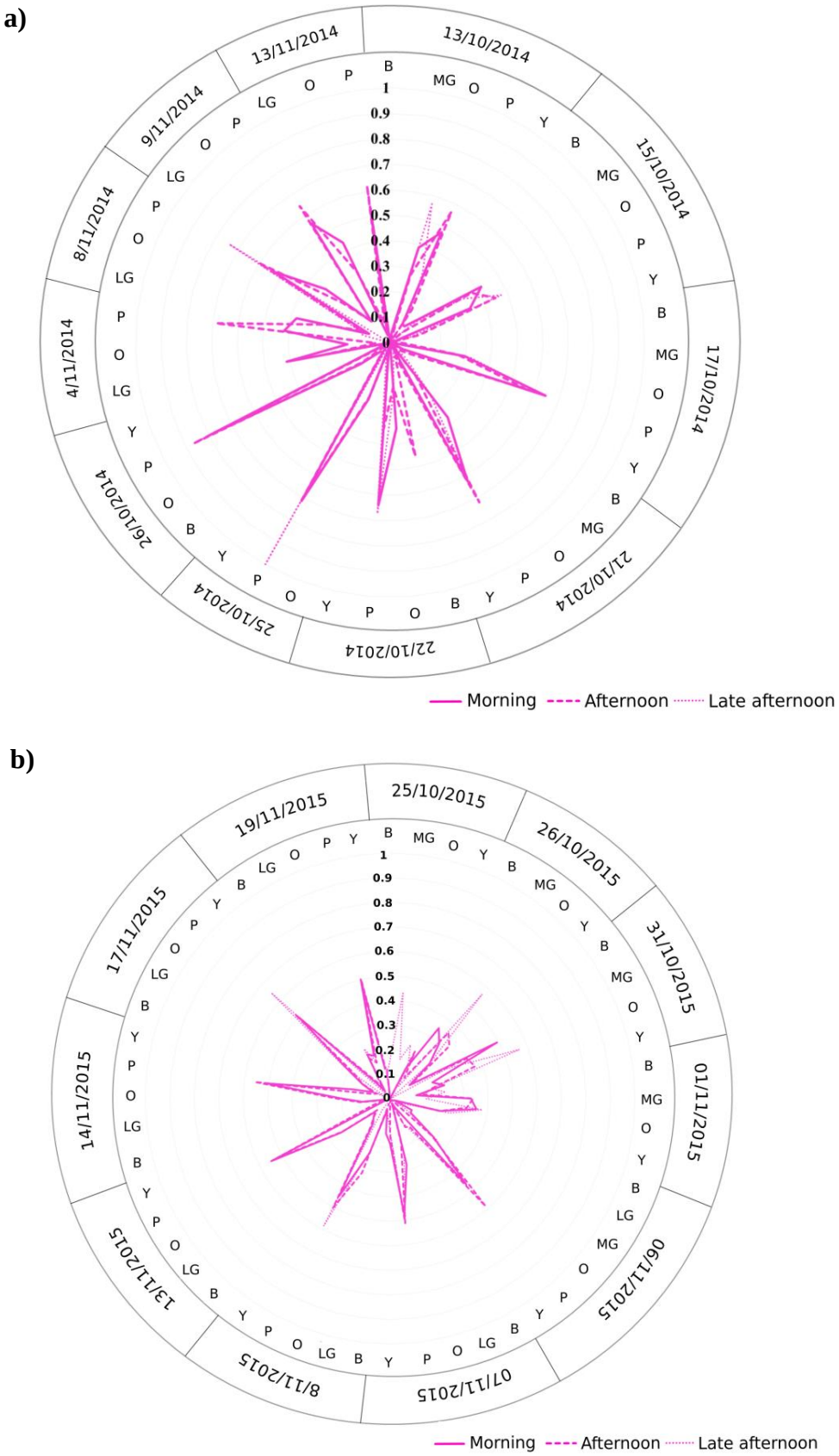


Fig. 3.16. Relative honey bee' visitation rate (mean honey bees per flower/mean total honey bees recorded at this time) on six *L. scoparium* cultivars (B, LG, MG, O, P, and Y) recorded on different days in a) 2014 and b) 2015. Radar graph only included clone lines in flower. The preference of honey bees is indicated by the relative length of these spokes in these graphs. If honey bees were not selective, these graphs would collapse to a circle.

A fitted model of honey bee visits per flower and total sugar per flower indicated the positive relationship between these two factors for 2014 (slope = 5.78×10^{-7}, $P < 0.0001$, $R^2 = 0.404$) and 2015 (slope = 5.38×10^{-7}, $P < 0.0001$, $R^2 = 0.39$). However, some cultivars were much more rewarding than others, and were visited more frequently (Fig. 3.17). The highly favoured cultivar P had the highest sugar per flower. Interestingly, even though all the cultivars produced more sugar in 2015 than in 2014, cultivars B, MG, and LG received less visits per flower in 2015 than in 2014.

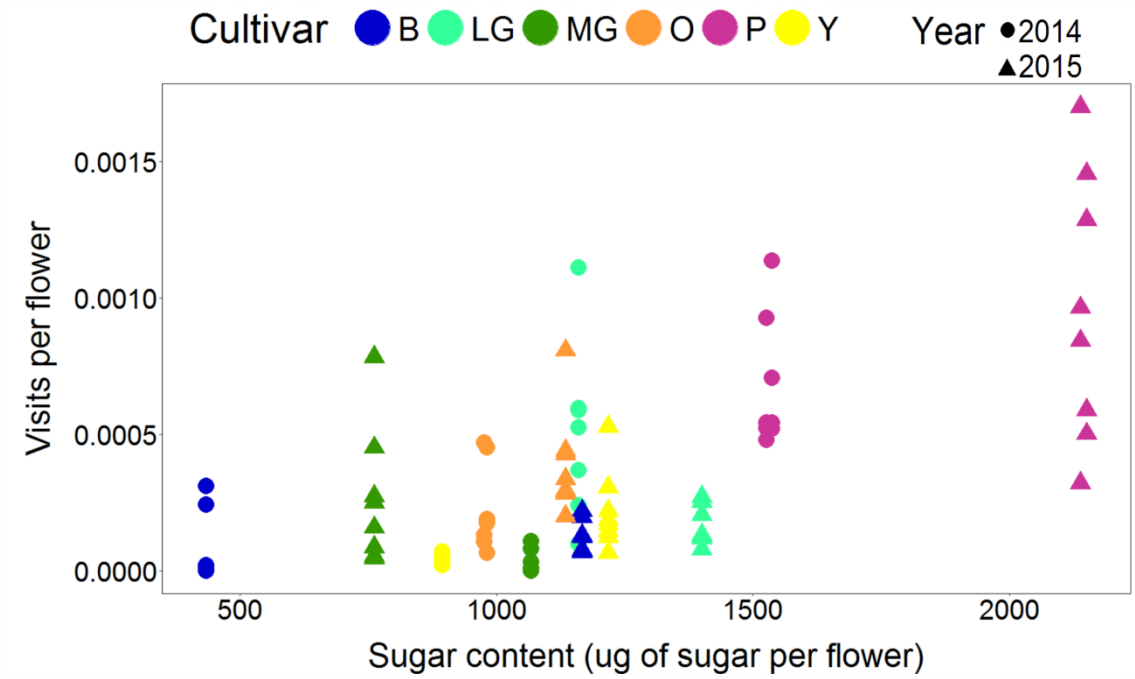


Fig. 3.17. Relationship between the mean of honey bee visits per flower and the mean of sugar content per flower present on six *L. scoparium* cultivars (B, LG, MG, O, P, and Y) during 2014 and 2015. (2014 slope = 5.78×10^{-7}, $P < 0.0001$, $R^2 = 0.404$); 2015 (2015 slope = 5.38×10^{-7}, $P < 0.0001$ $R^2 = 0.388$).

3.4. Discussion

This study evaluated scale insect presence, honey bee visitation and nectar quality and quantity in different cultivars of *L. scoparium*. Results showed significant variation in estimated flower density, nectar sugar content and DHA among cultivars. The insecticide treatment reduced but did not eliminate the scale insect population in all cultivars. Scale insects did not affect plant growth, nectar sugar production or DHA levels. Honey bees revealed a consistent preference for cultivar P, which contained the highest amount of nectar sugar. While time of day, temperature and number of fly visits also influenced on honey bee visits, DHA, which is a key ingredient in the nectar of *L. scoparium*, did not influence honey bee visits.

3.4.1. Influence of an insecticide treatment on scale insect

The application of IGR buprofezin and DC-Trom oil was partially effective: the populations of *Acanthococcus* spp. were reduced in all cultivars. Previous studies on scale insect control have used different treatments including neonicotinoids (Rebek & Sadof, 2003; Turner & Buss, 2005) and pyrethroids (Clarke, Negron, & Debarr, 1992). However, their negative impact on parasitoids (Hoddle et al., 2001) and honey bees has discouraged their use (Woodcock et al., 2017). On the other hand, the application of IGRs has previously demonstrated a lower level of toxicity and their potential effectiveness on scale pests (Hodgson, 1997; Cock & Degheele, 1998). The application of the IGR pyriproxyfen for controlling *Unaspis euonymi* Comstock (Diaspididae) on *Euonymus fortune* (Turcz.) Hand.-Maz. (Celastraceae) resulted in more effective control than neonicotinoids including imidacloprid and thiamethoxam (Rebek & Sadof, 2003). Even though IGRs have low toxicity risk, it has been demonstrated that they can be toxic to parasitoids and their level of toxicity differs among IGRs. For example, the kinoprene and pyriproxyfen IGRs are more toxic to parasitoids like *Bemisia argentifolii* Bellows and Perring (Aleyrodidae) than is buprofezin (Hoddle et al., 2001).

Buprofezin is recommended for reducing scale insect populations, being safe for bees, mites, and other parasites. In the present study, the application of buprofezin was partially effective against *Acanthococcus* spp. Similar results were found by Grafton-Cardwell et al. (2006); where buprofezin was applied to control *Aonidiella aurantii* Maskell (Diaspididae) on citrus plants, reducing *A. aurantii* only partially. An important factor to

consider for obtaining a good response to buprofezin is insect life stage (Mendel et al., 1991). Mendel et al. (1991) found higher vulnerability in crawlers (LC_{50}[1] = 0.0011%) compared with third-instar nymphs (LC_{50} = 0.042%) of *Icerya purchasi* Maskell (Monophlebidae), indicating the application of buprofezin can control crawlers and second-instar nymphs, as the juvenile cuticles are inhibited before the moulting process occurs (Reynolds, 1987; Grafton-Cardwell et al., 2006; Mansour et al., 2017). Mendel et al. (1991) also recommended the application of buprofezin on ovipositing females to reduce egg hatching (Yarom, Blumberg, & Ishaaya, 1988; Darvas & Varjas, 1990) and consequently, pest dispersion.

In the present study, the number of stem samples presenting crawlers and the number of adult females of *Acanthococcus* spp. were reduced after insecticide application, and the crawler population steadily declined on sprayed plants, while remaining high on unsprayed plants. These results suggest the effectiveness of the treatment for ovipositing females, and subsequently the reduction of crawlers. However, the treatment was only partially effective probably because of the presence of multiple generations (Zondag, 1977; Miller & Miller, 1992; Pellizzari, Germain et al., 2010). In his study, Zondag (1977) indicated that *A. orariensis* Hoy (Eriococcidae) could develop two to three overlapping generations a year on *L. scoparium* in New Zealand.

Importantly, geographical conditions, seasonal effects, and climate could affect the number of generations of *Acanthococcus* spp. (Zondag, 1977; Miller & Miller, 1992; Pellizzari et al., 2010). For example, *Acanthococcus azalea* Comstock (Eriococcidae) requires double the time to develop a generation in winter (250 days) than in summer (130 days) (Miller & Miller, 1992). Zondag (1977) found that while *A. orariensis* matured in summer in only 11 weeks, it needed 25 weeks in winter. However, the number of studies that referred to the generations of *Acanthococcus* spp. is limited. The life cycle of *A. campbelli* and *A. leptospermi* in the present study was assumed to be similar to that of *A. orariensis*. According to Hoy (1954), crawlers of *A. orariensis* appeared mainly from March to September, having a peak from April to mid-May. During this study, observations indicated that the presence of crawlers started to decrease in August on sprayed plants, as the number of stem samples presenting crawlers was very low in this

[1] LC_{50}: Lethal concentration where half of the population is eliminated

month. For adult females, Hoy indicated that their main activity occurred from June to February. In this study, the number of adult females increased in July, but reduced its density in August, September and October. July to September across all cultivars. Importantly, the species of *Acanthococcus* identified in this study were *A. campbelli* and *A. leptospermi*. This suggests the replacement of *A. orariensis* by these two species, which have been found throughout New Zealand (Chapter 2).

Plant growth was evaluated in the unsprayed and sprayed plants during the study. Results did not show any apparent effect on plant growth and nectar quality and quantity.

3.4.2. Nectar quality and quantity

In this study, nectar sugar was positively correlated with nectar DHA content but not closely, so the ratio varies with cultivars and among samples of the same cultivar, which leads to varying normalised DHA levels.

The carbohydrate DHA (the precursor of MGO) is one of the main factors influencing the Unique Manuka Factor (UMF) content of honey and, consequently, characterises the level of NPA assigned to honey indicating the biological activity of the honey (Williams et al., 2014; Molan, 2015). In the present study, normalised DHA differed greatly among cultivars in 2014 and 2015, which means that likely DHA levels present in honey should also vary depending on the cultivar and the year. While cultivar B presented the highest normalised DHA in 2014, cultivar MG showed the highest level in 2015. Previous studies have shown cultivar B as one of the cultivars of *L. scoparium* with the highest normalised DHA (Nickless, Holroyd, Stephens, Gordon, & Wargent, 2014; Nickless, Anderson, Hamilton, Stephens, & Wargent, 2016). However, the normalised DHA for MG in these studies ranged from 3266.70 mg/kg 80°BRIX (Nickless et al., 2016) to 5524.11 mg/kg 80°BRIX (Nickless et al., 2014), and 4081.85 80°BRIX in my study. A possible explanation for this variation could be that a different analysis method was used, sampling collection was in different years, their studies were carried out under glasshouse conditions, or the variability of sugar and/or DHA production. For the former study (Nickless et al., 2016), DHA was analysed by HPLC and for the later (Nickless et al., 2014) LC-MS was selected. During this study, DHA was analysed using the HPLC technique, and results showed that normalised DHA in the cultivar MG varied significantly between 2014 and 2015. A reason for the low value for MG in 2014 could

be that the sample size was limited as consequence of the early start to the flowering period for this cultivar, which resulted in unfavourable environmental conditions for nectar sampling. In contrast, consistent normalised DHA results were found in all other cultivars. For example, cultivar Y, which was propagated from wild plants of *L. scoparium* from New Zealand, showed the lowest normalised DHA in 2014 and 2015. Similar results were obtained in a previous study, where wild plants of *L. scoparium* showed the lowest normalised DHA (Millner et al., 2016). The consistency of these results confirms the potential for cultivated elite plants in comparison to wild plants of *L. scoparium*, in terms of DHA content in nectar, and subsequently, in honey.

An important factor to consider for evaluating nectar quality is temperature. The present study showed the influence of temperature on DHA content. In addition, other factors such as salinity and drought are suggested as drivers of DHA and MGO variability (Yadav et al., 2005).

Nectar sugar composition differed with year and was more variable in 2015. Temperature and year had a significant effect on sugar content and, consequently, could have affected the sugar composition (Percival, 1961; Corbet et al., 1979; Wyatt, Broyles, & Derda, 1992; Jakobsen & Kritjánsson, 1994). Previous studies have found higher variability in sugar composition in plants grown in the field and exposed to variable environmental conditions than in plants cultivated in a glasshouse (Freeman & Wilken, 1987; Canto et al., 2007). Another potential factor in this variability could be the nectaries; it has been shown previously that nectaries from flowers belonging to the same plant can differ in nectar secretion and composition (Herrera, Pérez, & Alonso, 2006). In this study, sucrose content was lower in 2015 than in 2014. In contrast, fructose and glucose content were higher. The enzymatic process of hydrolysis could be involved, transforming more sucrose into the monosaccharides, glucose and fructose (Nicolson et al., 2007). Results also showed that cultivar and temperature influenced nectar quality, but previous studies have demonstrated that nectar quantity can also be affected, for example, by the phenotype of the flower (Jakobsen & Kritjánsson, 1994; Galetto & Bernardello, 2004; Nicolson et al., 2007). For example, deep corolla-tubes could reduce the evaporation of water in nectar, resulting in more nectar in the flowers (Pleasants, 1983; Witt et al., 2013), which is likely to be more dilute as a result..

3.4.3. Relative visitation rates of honey bees and other insects

This study evaluated honeybee visitation to six cultivars of *L. scoparium* and demonstrated the relative attractiveness of cultivar P to honey bees, in both 2014 and 2015. As the flowering period differed slightly among cultivars, honey bees showed a preference for cultivar O when cultivar P was not flowering. This preference was shown, for example, at the end of October 2015, when the majority of the visits were received on cultivar O until cultivar P started flowering in early November. Cultivar P presented the highest sugar content in 2014 and 2015, which suggested the preference of cultivars with higher sugar content compared with cultivars with low sugar content (e.g. cultivar Y). Previous studies have also indicated sugar composition as a significant driver of honey bee visitation; specifically, the positive influence of the disaccharide sucrose compared with the monosaccharides fructose and glucose (Wykes, 1952; Baker & Baker, 1983b). However, though it has been found that sucrose-dominant nectars are more attractive than hexose-dominant nectars to honey bees (Wykes, 1952; Waller, 1972; Winston, 1991), their choice changed when nectar was pure sucrose (Wykes, 1952; Furgala et al., 1958). For example, Wykes found the preference of honey bees for a mixture of nectars composed by sucrose, fructose and glucose at ratio 1:1:1 compared to pure nectar or just one of these sugars. However, Waller (1972) found pure sucrose nectar was more attractive to honey bees than a nectar composed of an equal ratio of sucrose, fructose and glucose.

Along with sugar, other nectar components such as amino acids, phenolic compounds, minerals, and lipids are suggested as drivers of pollinator activity (Waller, 1972; Baker, 1977; Alm et al., 1990; Adler, 2000; Hendriksma et al., 2014; Fowler et al., 2016). For example, the species *Allium cepa* L. (Amaryllidaceae) and *Aloe littoralis* Baker (Liliaceae) have been reported as discouraging the visits of honeybees by a high potassium content (Waller, 1972) and phenolic compounds respectively (Frankie et al., 1982; Hagler & Buchmann, 1993). On the other hand, amino acids (AAs) have been shown to positively influence honey bee visitation (Inouye & Waller, 1984; Alm et al., 1990; Kim & Smith, 2000; Carter et al., 2006). A recent study demonstrated the preference of honey bees for nectars with low sucrose and high phagostimulant AAs (phenylalanine) compared with nectars with high sucrose and deterrent AAs (glycine) (Hendriksma et al., 2014). This finding suggests nectar with low sucrose but high levels of AAs such as phenylalanine could be used to attract honey bees. As AAs, other non-

sugar components could potentially play a role in honey bee visitation. In the present study, it was considered that DHA could act as a phagostimulant or deterrent to honey bee visitation; however, it was found that DHA content did not influence honey bee visits. Previous studies have evaluated honey bee visitation using an artificial context where nectar solutions are artificial, flowers are coloured, and environmental conditions (e.g. temperature, wind, precipitation, and radiation) are manipulated using glasshouses and laboratories (Wykes, 1952; Waller, 1972; Frankie et al., 1982; Alm et al., 1990; Afik et al., 2006). Adler (2000) highlighted the effect of non-sugar components present on nectar as a deterrent to bees under manipulated conditions. In the present study, DHA content was evaluated in a large sample size under natural conditions and the results suggested that DHA content did not affect honey bee visitation, but controlled condition studies might be useful. Environmental factors such as temperature, precipitation, and wind have been reported as limiting the foraging activity of honey bees, suggesting studies under manipulated conditions would be less affected by these factors (Arroyo, Primack, & Armesto, 1982; Primack, 1983; Arroyo, Armesto, & Primack, 1985; Totland, 1994). Among these environmental factors, in the present study temperature was found to significantly influence honey bee visitation. For example, honey bees were clearly more active between 10.30 am and 1.30 pm than at 4.30 pm (Appendix 3.15), indicating that foraging activity was higher at the warmest part of the day, in agreement with other studies (McCall & Primack, 1992; Totland, 1994).

In the present study, it was found that nectar content affected honey bee visitation, although previous studies have indicated that flower densities can also influence honey bee visitation (Bullock, Rio, & Ayala, 1989; Robertson, 1992; Ohashi & Yahara, 1998; Garbuzov, Samuelson, & Ratnieks, 2014). Flower density was therefore compared in this study. While cultivar Y was shown as presenting the highest estimated flower density, it also produced flowers with lowest sugar content and the lowest number of honey bee visits in 2014 and 2015. In contrast, cultivar P, the most visited cultivar, did not have high flower density. Similar results were found in previous studies, where the number of pollinator visits decreased as floral density increased (Bullock et al., 1989; Robertson & Macnair, 1995; Grindeland, Sletvold, & Ims, 2005; Lobo, de Lacerda Ramos, & Braga, 2016). Grindeland et al. (2005) showed the decrease of pollinator visits as the flower display size of *Digitalis purpurea* L. (Plantaginaceae) increased. Even though rewards were not evaluated in their study, they hypothesised that reward positively influenced

pollinator attractiveness. This suggests the achievement of ideal-free distribution (IFD) (Fretwell & Lucas, 1970) across flowers by pollinators. This achievement has been suggested several times by researchers. For example, Robertson & Macnair (1995) found that bee visitation was not proportional to the number of flowers of *Erythranthe guttatus* (Fisch. DC.) G.L.Nesom (Phrymaceae) and Ohashi & Yahara (2002) found the decrease of bumble bee visitation with larger floral displays.

The complexity for honey bees to make a decision regarding floral rewards not only involves olfactory stimuli, but also involves visual stimuli (Kay et al., 1984; Menzel, 1993; Giurfa, 2007). Flower factors such as size, age, sex, symmetry, or colour could help to drive honey bee activity (von Frisch, 1967; Gronquist et al., 2001; Kay et al., 1984; Campbell et al., 2010; Srinivasan, 2010; Muth, Papaj, & Leonard, 2016). Studies have shown that while flower size is directly correlated with nectar volume (Wunnachit, Jenner, & Sedgley, 1992; Blarer, Keasar, & Shmida, 2002; Krizek & Anderson, 2013), which influences honey bee visitation, it also influences the energetic cost of detecting the target flower (Spaethe, Tautz, & Chittka, 2001). In the present study, factors driving the olfactory and visual stimuli of honey bees were not examined. However, in the field it was noticed that flowers from cultivar P and cultivar LG were larger than flowers from cultivar B and MG (author's pers. obs.), and number of visits were higher in the former than in the latter. Although in this study sugar content influences honey bee visitation, it could be considered that flower size might have indirectly influenced honey bee visitation as well, but this factor was not investigated in the present study.

In addition to floral features, other insect visits could influence honey bee visitation (Murphy & Robertson, 2000; Bennik, 2009). For example, results from the present study showed a positive correlation between fly visitation rates and honey bee visitation rates. In contrast, previous studies found that native flies (Murphy & Robertson, 2000) and large flies (Bennik, 2009) were negatively correlated with honey bees. An explanation could be that fly visits are influenced by environmental conditions (e.g. temperature and wind) and habitat (Arroyo et al., 1982; Primack, 1983; Arroyo et al., 1985). A similar conclusion was adopted in a study where the effects of honey bees and bumble bees (*Bombus terrestris* L.) on native pollinators were evaluated in Tasmania (Goulson, Stout, & Kells, 2002). While honey bees influenced native bee visitation, bumble bees did not affect the diversity of native pollinators. In addition, they found a positive correlation between the

bumble bee and the native bee *Lasioglossum clelandi* (Halictidae), which Goulson et al. (2002) suggested could be due to both pollinators sharing a similar habitat. This suggests that habitat and floral traits influence pollinator attractiveness.

3.5. References

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3.6. Appendices

Appendix 3.1. Twelve plants where all the flowers were counted including four stems (N: North, S: South, E: East, and W: West) that were included for the correlation between basal area and number of flowers on six *L. scoparium* cultivars. *H*: plant height (m); *BA*: plant basal area (mm^2).

Plant	Characteristics	Aspect	BA	Flowers	Plant	Characteristics	Aspect	BA	Flowers
1B	*H*: 1.41	N	4.26	37	4B	*H*: 1.67	N	5.72	54
	BA: 239.98	S	1.29	15		*BA*: 238.21	S	4.75	38
	Flowers: 1285	E	8.81	47		*Flowers:* 2244	E	4.45	48
		W	2.19	11			W	2.75	29
1LG	*H:* 1.9	N	9.40	92	3LG	*H:* 2.5	N	8.40	62
	BA: 1535.79	S	8.04	8		*BA*: 878.77	S	21.24	45
	Flowers:5681	E	5.15	13		*Flowers:* 2369	E	26.70	54
		W	6.51	27			W	19.01	63
1MG	*H:* 1.85	N	2.03	17	3MG	*H:* 1.42	N	3.24	16
	BA: 778.57	S	1.67	7		*BA*:466.83	S	1.86	9
	Flowers:4951	E	2.18	11		*Flowers:* 4074	E	1.89	16
		W	2.13	18			W	2.49	9
2O	*H:* 2.18	N	2.84	41	3O	*H:* 2.33	N	7.89	93
	BA: 619.04	S	2.32	61		*BA*: 716.55	S	2.66	69
	Flowers: 6380	E	0.50	37		*Flowers:* 7981	E	7.94	164
		W	5.98	79			W	1.77	77
1P	*H:* 2.05	N	8.30	34	3P	*H:* 2.47	N	7.89	93
	BA: 2815.15	S	18.32	120		*BA*: 1759.26	S	2.66	69
	Flowers: 13,794	E	9.19	38		*Flowers:* 12,792	E	7.94	164
		W	9.95	110			W	1.77	77
2Y	*H:* 1.88	N	3.30	30	4Y	*H:* 2.79	N	9.57	95
	BA: 1084.68	S	3.60	32		*BA*: 1544.94	S	2.92	29
	Flowers: 8779	E	2.01	27		*Flowers:* 12,285	E	13.40	86
		W	2.66	11			W	9.35	64

Appendix 3.2. Mesh bags (40 cm × 80 cm) placed over stems 24 hours before nectar collection to prevent insect visitation.

Appendix 3.3. Significance of the explanatory variable cultivar on plant height, plant basal area and estimated flower density.

Year; response; explanatory variables	d.f.	Sum of squares	Mean squares	*F*	*P*
2014					
Height					
Cultivar	5	5.77	1.15	12.55	**<0.0001**
Residuals	88	8.10	0.09		
Plant basal area					
Cultivar	5	6290.60	1258.12	26.66	**<0.0001**
Residuals	88	4152.10	47.18		
Estimated flower density					
Cultivar	5	50.22	10043.80	14.88	**<0.0001**
Residuals	88	60.08	675.10		
2015					
Height					
Cultivar	5	12.04	2.41	21.48	**<0.0001**
Residuals	88	9.87	0.11		
Plant basal area					
Cultivar	5	9957.00	1991.39	31.56	**<0.0001**
Residuals	88	5500.70	62.51		
Estimated flower density					
Cultivar	5	6.56	1.31	21.23	**<0.0001**
Residuals	88	5.44	0.06		

Appendix 3.4. Average proportion of hermaphrodite and male flowers, buds, total number of flowers and developing fruits per branch sample.

Year; cultivar	Proportion of male flowers (%)	Proportion of hermaphrodite flowers (%)	Number of buds	Number of flowers	Developing fruits
2014					
B	6.69	93.31	75.67	20.40	21.13
LG	1.26	98.74	43.00	9.78	2.81
MG	42.52	57.48	7.72	7.66	28.29
O	1.79	98.21	45.38	50.78	25.22
P	20.76	79.24	68.84	11.94	9.69
Y	8.06	91.94	45.38	39.72	47.22
2015					
B	2.24	97.76	15.44	23.48	9.85
LG	0.23	99.77	40.28	5.19	0.28
MG	15.61	84.39	24.09	12.06	12.81
O	0.07	99.93	39.09	39.25	13.47
P	0.07	99.93	29.13	15.25	3.00
Y	1.27	98.73	25.66	25.63	10.94

Appendix 3.5. Average proportion of hermaphrodite and male flowers, buds, total number of flowers developing fruits per branch sample.

Branch feature	Proportion of male flowers (%)	Proportion of hermaphrodite flowers (%)	Number of buds	Number of flowers	Developing fruits
Height above ground (m)					
<0.5	17.12	82.88	42.84	29.09	14.87
0.5–1	10.17	89.83	40.04	21.34	17.21
>1	1.04	98.96	32.78	20.35	10.94
Branch length (cm)					
<40	7.25	92.75	23.36	15.48	10.92
>40	9.84	90.16	53.70	28.11	19.98

Appendix 3.6. Coefficients for the explanatory variables insecticide treatment, cultivar, month, and the interaction between insecticide and treatment: cultivar on number of eriococcid females.

Explanatory variable	Estimate ± SE	Z value	*P*
Unsprayed	0.88 ± 0.31	2.942	**0.0032**
LG	–0.29 ± 0.31	–0.580	0.5622
MG	–0.57 ± 0.31	–1.096	0.2733
O	–2.47 ± 0.40	–3.858	**0.0001**
P	3×10^{-4} ± 0.31	0.001	0.9995
Y	0.58 ± 0.31	1.168	0.2427
May	–1.97 ± 0.20	–3.387	**0.0007**
June	0.06 ± 0.20	0.125	0.9007
July	0.26 ± 0.21	0.558	0.5769
September	–0.97 ± 0.22	–1.929	0.0537
October	–1.45 ± 0.20	–2.733	**0.0063**
Unsprayed: LG	0.52 ± 0.40	1.302	0.1930
Unsprayed: MG	0.21 ± 0.40	–0.515	0.6063
Unsprayed: O	1.09 ± 0.49	2.217	**0.0266**
Unsprayed: P	–0.06 ± 0.41	–0.159	0.8738
Unsprayed: Y	–0.67 ± 0.40	–1.688	0.0914
LG: May	1.48 ± 0.74	2.000	**0.0455**
MG: May	2.67 ± 0.74	3.625	**0.0003**
O: May	2.60 ± 0.82	3.183	**0.0014**
P: May	–0.33 ± 0.83	–0.400	0.6888
Y: May	0.15 ± 0.77	0.191	0.8484
LG:June	–0.37 ± 0.65	–0.572	0.5675
MG: June	0.87 ± 0.65	1.338	0.1809
O: June	0.87 ± 0.73	1.189	0.2343
P: June	–1.56 ± 0.69	–2.270	**0.0232**
Y: June	–0.46 ± 0.64	–0.722	0.4702
LG: July	0.53 ± 0.63	0.845	0.3981
MG: July	0.74 ± 0.64	1.149	0.2506

O: July	0.09 ± 0.75	0.126	0.8996
P: July	–0.10 ± 0.63	–0.157	0.8756
Y: July	–0.34 ± 0.63	–0.547	0.5846
LG: September	1.18 ± 0.66	1.779	0.0752
MG: September	0.95 ± 0.69	1.385	0.1659
O: September	0.62 ± 0.81	0.776	0.4440
P: September	1.65 ± 0.66	2.508	**0.0121**
Y: September	0.78 ± 0.66	1.180	0.2382
LG: October	0.88 ± 0.70	1.261	0.2074
MG:October	0.66 ± 0.74	0.899	0.3685
O: October	0.90 ± 0.85	1.065	0.2871
P: October	1.18 ± 0.69	1.702	0.0888
Y: October	0.54 ± 0.70	0.775	0.4384
Null deviance: 836.92 on 563 degrees of freedom			
Residual deviance: 567.40 on 522 degrees of freedom			

Appendix 3.7. Coefficients for the explanatory variables insecticide treatment, cultivar and month on crawlers.

Explanatory variable	Estimate ± SE	Z value	*P*
Unsprayed	1.35 ± 0.53	2.57	**0.0101**
LG	0.26 ± 0.88	0.30	0.7647
MG	–0.91 ± 0.99	–0.92	0.3556
O	–0.16 ± 0.91	–0.18	0.8575
P	–0.05 ± 0.87	–0.06	0.9498
Y	–0.34 ± 0.89	–0.38	0.7038
May	–0.69 ± 0.84	–0.82	0.4099
June	1.75 ± 0.89	1.96	**0.0497**
July	1.34 ± 0.85	1.58	0.1146
September	–0.69 ± 0.84	–0.82	0.4099
October	–0.69 ± 0.84	–0.82	0.4099
Unsprayed: LG	0.76 ± 0.73	1.06	0.2912
Unsprayed: MG	1.63 ± 0.81	2.02	**0.0436**
Unsprayed: O	0.97 ± 0.75	1.28	0.1990
Unsprayed: P	–0.34 ± 0.69	–0.49	0.6256
Unsprayed: Y	0.15 ± 0.71	0.21	0.8302
LG: May	0.37 ± 1.17	0.31	0.7542
MG: May	2.35 ± 1.27	1.85	0.0642
O: May	0.35 ± 1.18	0.30	0.7679
P: May	0.07 ± 1.16	0.06	0.9488
Y: May	0.04 ± 1.17	0.03	0.9748
LG: June	–1.07 ± 1.22	–0.88	0.3804
MG: June	–0.51 ± 1.29	–0.39	0.6940
O: June	–1.41 ± 1.22	–1.15	0.2490
P: June	–0.94 ± 1.16	–0.81	0.4189
Y: June	–2.41 ± 1.21	–1.99	**0.0468**
LG: July	–0.28 ± 1.21	–0.23	0.8147
MG: July	–1.75 ± 1.24	–1.41	0.1596
O: July	–1.68 ± 1.19	–1.42	0.1567

P: July	–0.79 ± 1.13	–0.71	0.4811
Y: July	–1.34 ± 1.15	–1.15	0.2451
LG: September	0.04 ± 1.17	0.03	0.9740
MG: September	–0.55 ± 1.26	–0.44	0.6632
O: September	–1.18 ± 1.25	–0.94	0.3450
P: September	0.69 ± 1.13	0.62	0.5388
Y: September	0.69 ± 1.15	0.61	0.5449
LG: October	–0.30 ± 1.18	–0.25	0.8022
MG: October	0.28 ± 1.24	0.23	0.8193
O: October	–1.18 ± 1.25	–0.94	0.3450
P: October	0.69 ± 1.13	0.62	0.5388
Y: October	1.00 ± 1.14	0.87	0.3858

Null deviance: 772.13 on 563 degrees of freedom

Residual deviance: 613.08 on 522 degrees of freedom

Appendix 3.8. Coefficients for the explanatory variables insecticide treatment, cultivar, and the interaction between treatment and cultivar on flower density.

Explanatory variables	Estimate ± SE	t-value	*P*
Sprayed	0.05 ± 0.12	0.45	0.6543
LG	0.42 ± 0.11	3.71	0.0004
MG	–0.22 ± 0.11	–1.91	0.0595
O	0.70 ± 0.11	6.18	**<0.0001**
P	0.68 ± 0.11	6.08	**<0.0001**
Y	0.72 ± 0.11	6.41	**<0.0001**
Spray: LG	–0.03 ± 0.16	–0.19	0.8508
Spray: MG	0.24 ± 0.16	1.52	0.1325
Spray: O	–0.51 ± 0.16	–3.19	**0.0020**
Spray: P	–0.21 ± 0.16	–1.35	0.1819
Spray: Y	–0.31 ± 0.16	–1.94	0.0555

Appendix 3.9. Significance of the explanatory variable cultivar on normalised DHA, DHA content and sugar content.

Year; response; explanatory variables	d.f.	Sum of squares	Mean squares	*F*	*P*
2014					
Sugar content					
Cultivar	5	9.88	1.98	10.32	**<0.0001**
Residuals	84	16.09	0.19		
DHA content					
Cultivar	5	7.14	1.43	8.17	**<0.0001**
Residuals	84	14.69	0.17		
Normalised DHA					
Cultivar	5	3.02	0.60	5.95	**<0.0001**
Residuals	84	8.52	0.10		
2015					
Sugar content					
Cultivar	5	13.11	2.62	19.40	**<0.0001**
Residuals	91	12.30	0.14		
DHA content					
Cultivar	5	12.85	2.57	9.59	**<0.0001**
Residuals	91	24.38	0.27		
Normalised DHA					
Cultivar	5	13.76	2.75	18.05	**<0.0001**
Residuals	91	13.88	0.15		

Appendix 3.10. Significance of the explanatory variable cultivar on nectar volume, sugar concentration and DHA concentration.

Year; response; explanatory variables	d.f.	Sum of squares	Mean squares	*F*	*P*
2014					
Nectar volume					
Cultivar	5	11.67	2.33	10.35	**<0.0001**
Residuals	84	18.93	0.22		
Sugar concentration					
Cultivar	5	2.95	0.59	5.01	**<0.0001**
Residuals	84	9.88	0.12		
DHA concentration					
Cultivar	5	0.66	0.13	5.30	**<0.0001**
Residuals	84	2.10	0.02		
2015					
Nectar volume					
Cultivar	5	3.88	0.78	8.17	**<0.0001**
Residuals	91	8.66	0.10		
Sugar concentration					
Cultivar	5	9.26	1.85	16.66	**<0.0001**
Residuals	91	10.11	0.11		
DHA concentration					
Cultivar	5	1.34	0.27	8.22	**<0.0001**
Residuals	91	2.98	0.03		

Appendix 3.11. Coefficients for the explanatory variables cultivar, temperature, and year on nectar DHA and sugar content

Response; explanatory variable	Estimate ± S.E.	t-value	*P*
DHA content			
LG	914.1 ± 673.8	1.36	0.1769
MG	172.6 ± 103.7	0.17	0.8680
O	719.5 ± 520.7	1.38	0.1690
P	391.1 ± 477.0	0.82	0.4136
Y	371.7 ± 521.9	0.71	0.4775
Humidity	–0.013 ± 0.008	–1.53	0.1280
Radiation	0.100 ± 0.185	0.54	0.5905
Temperature	0.095 ± 0.068	1.39	0.1659
Year	0.129 ± 0.201	0.64	0.5237
LG: Year	–0.451 ± 0.333	–1.35	0.1783
MG: Year	–0.086 ± 0.513	–0.17	0.8678
O: Year	–0.357 ± 0.258	–1.38	0.1687
P: Year	–0.194 ± 0.236	–0.82	0.4131
Y: Year	–0.185 ± 0.259	–0.71	0.4772
LG: Humidity	0.033 ± 0.020	1.69	0.0931
MG: Humidity	0.039 ± 0.024	1.66	0.0999
O: Humidity	0.023 ± 0.012	1.90	0.0600
P: Humidity	0.010 ± 0.012	0.90	0.3684
Y: Humidity	0.005 ± 0.0125	0.40	0.6930
LG: Radiation	0.426 ± 0.287	1.48	0.1402
MG: Radiation	0.402 ± 0.290	1.38	0.1681
O: Radiation	0.007 ± 0.228	0.03	0.9745
P: Radiation	–0.309 ± 0.217	–1.43	0.1559
Y: Radiation	–0.242 ± 0.249	–0.97	0.3337
LG: Temperature	–0.501 ± 0.163	–3.07	**0.0025**
MG: Temperature	–0.213 ± 0.167	–1.28	0.2041
O: Temperature	–0.0430 ± 0.085	–0.51	0.6141
P: Temperature	0.076 ± 0.0873	0.87	0.3848

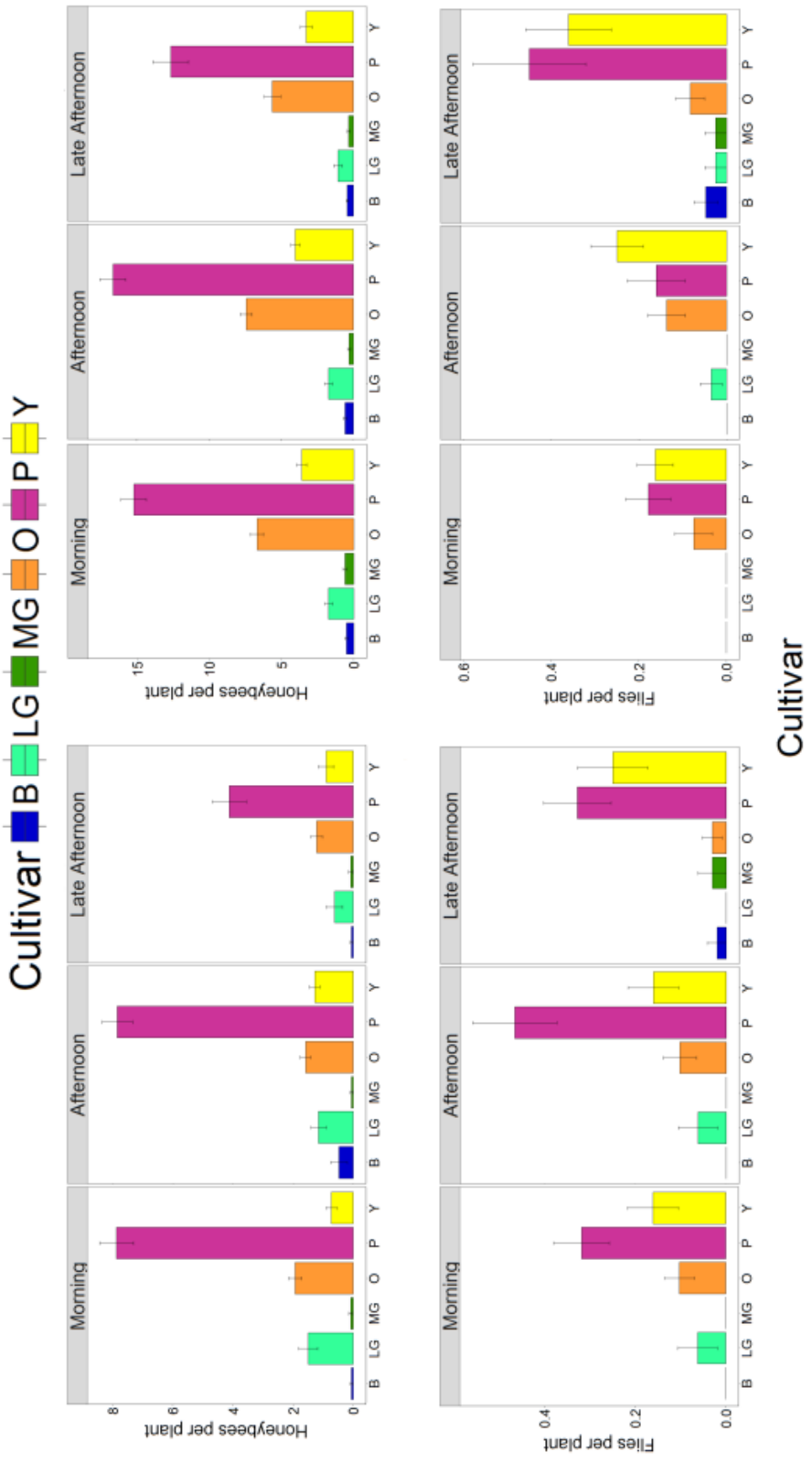
Y: Temperature	0.036 ± 0.0861	0.42	0.6783
Sugar content			
LG	843.8 ± 318.8	2.64	**0.0089**
MG	111.5 ± 490.4	2.27	**0.0244**
O	560.1 ± 246.3	2.27	**0.0243**
P	380.0 ± 225.7	1.68	0.0943
Y	747.1 ± 246.9	3.03	**0.0029**
Humidity	–0.004 ± 399.0	–0.96	0.3373
Radiation	0.026 ± 875.1	0.29	0.7682
Temperature	0.020 ± 323.4	0.61	**<0.0001**
Year	0.349 ± 951.8	3.66	**<0.0001**
LG: Year	–0.417 ± 157.9	–2.64	**0.0090**
MG: Year	–0.554 ± 242.9	–2.28	**0.0240**
O: Year	–0.278 ± 122.3	–2.28	**0.0243**
P: Year	–0.188 ± 112.0	–1.68	0.0948
Y: Year	–0.371 ± 122.6	–3.03	**0.0029**
LG: Humidity	0.005 ± 934.3	0.53	0.5994
MG: Humidity	0.009 ± 112.5	0.86	0.3884
O: Humidity	0.003 ± 564.5	0.47	0.6361
P: Humidity	–0.004 ± 546.6	–0.74	0.4580
Y: Humidity	0.004 ± 590.4	0.65	0.5182
LG: Radiation	0.211 ± 135.9	1.55	0.1230
MG: Radiation	0.112 ± 137.1	0.82	0.4157
O: Radiation	–0.006 ± 107.9	–0.06	0.9532
P: Radiation	–0.146 ± 102.6	–1.42	0.1569
Y: Radiation	–0.006 ± 118.0	–0.05	0.9608
LG: Temperature	–0.228 ± 772.0	–2.96	**0.0036**
MG: Temperature	–0.033 ± 791.7	–0.42	0.6736
O: Temperature	0.013 ± 402.3	0.33	0.7415
P: Temperature	0.025 ± 413.2	0.60	0.5465
Y: Temperature	0.013 ± 407.5	0.33	0.7436

Appendix 3.12. Insect visits recorded on six *L. scoparium* cultivars during the flowering period in 2014 and 2015 at PCRU.

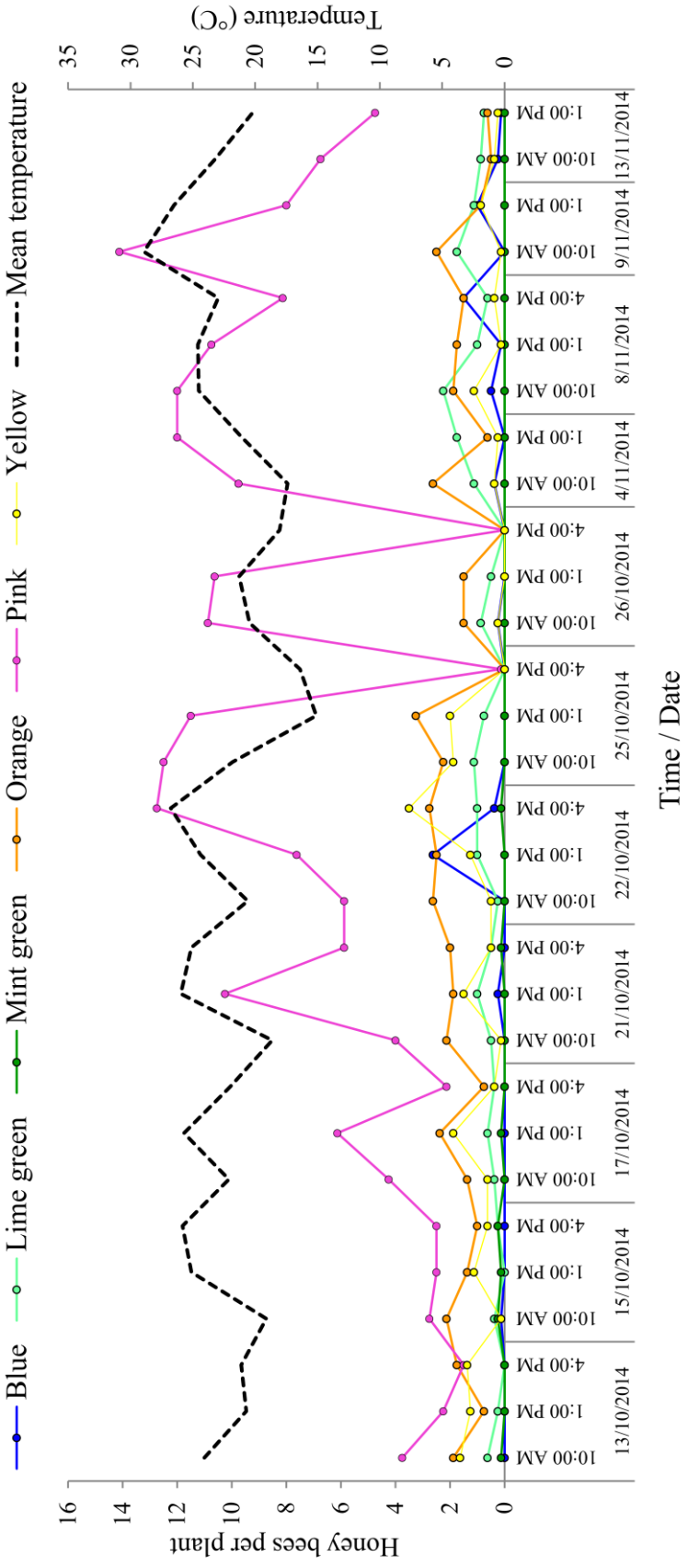
Cultivar	Honeybees	Bumblebees	Native bees	Flies	Wasps	Butterflies	Moths	Dragonflies	Katydid
2014									
B	60	4	0	6	2	0	0	0	0
LG	173	27	0	9	1	1	0	0	0
MG	9	6	0	1	3	0	0	0	0
O	389	18	1	20	6	0	0	0	0
P	1648	72	1	90	3	1	1	1	0
Y	195	97	0	60	7	2	0	0	0
2015									
B	105	4	0	3	2	0	0	0	0
LG	240	14	0	3	1	0	0	0	0
MG	81	2	0	2	3	0	0	0	0
O	1535	20	0	23	0	0	0	1	1
P	2886	40	1	49	5	1	0	3	1
Y	846	50	0	59	4	1	1	9	0

Appendix 3.13. Coefficients for the explanatory variables estimated flower density per plant, sugar content per flower, percentage of glucose per flower, temperature, time of the day, DHA content per flower, and fly visits per flower on honey bee visits present on plants propagated as six *L. scoparium* cultivars (B, LG, MG, O, P, and Y) during 2014 and 2015.

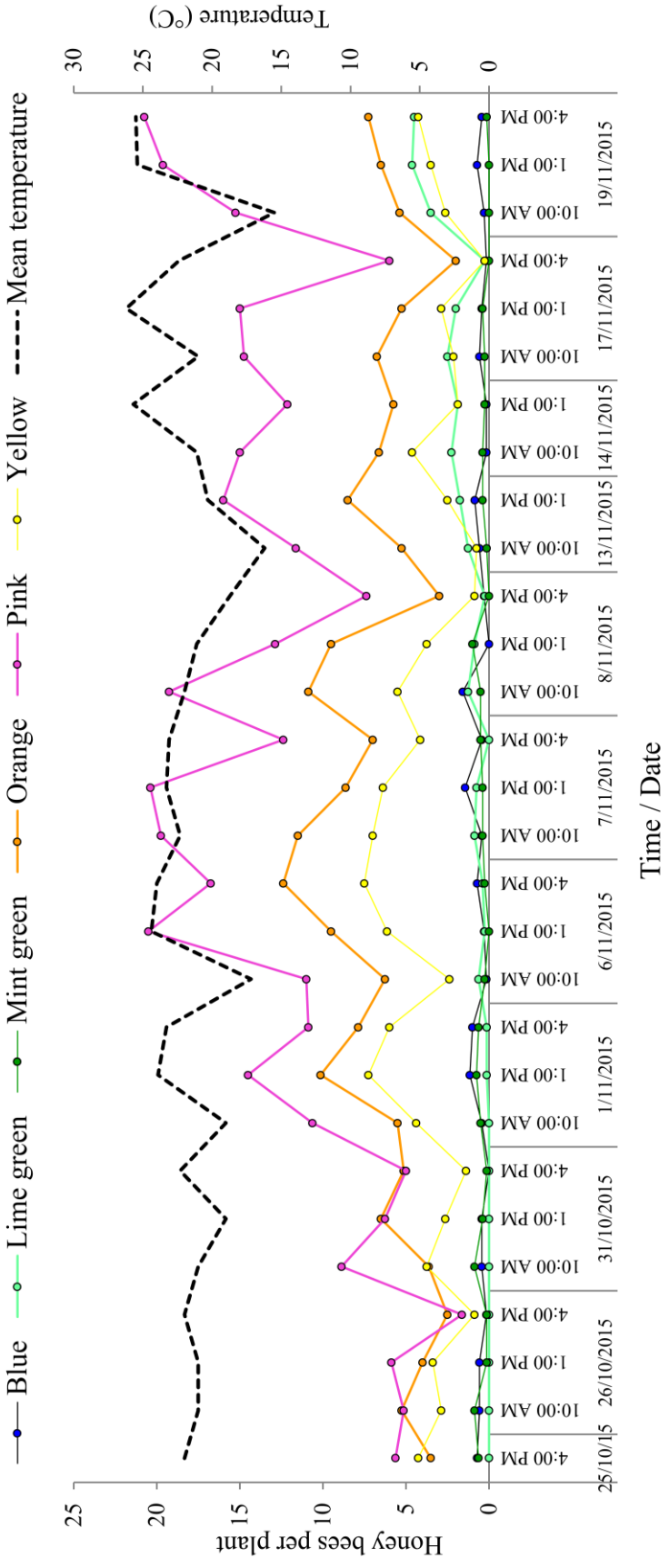
Year; explanatory variable	Estimate ± SE	t-value	*P*
Estimated flower density/plant	$-1.69\times10^{-5} \pm 3.24\times10^{-6}$	–5.07	**<0.0001**
μg of sugar per flower	$6.83\times10^{-4} \pm 1.66\times10^{-4}$	8.92	**<0.0001**
Glucose per flower (% w/w)	$4.18\times10^{-1} \pm 3.38\times10^{-2}$	8.91	**<0.0001**
Temperature	$5.00\times10^{-2} \pm 1.38\times10^{-2}$	6.03	**<0.0001**
Record time 10.30 am	$-1.02\times10^{-1} \pm 1.07\times10^{-1}$	–1.85	0.0648
Record time 4.30 pm	$-6.77\times10^{-1} \pm 1.42\times10^{-1}$	–4.93	**<0.0001**
μg of DHA per flower	$-5.77\times10^{-2} \pm 2.09\times10^{-2}$	–0.62	0.5321
Fly visits per flower	$2.10\times10^{+3} \pm 1.53\times10^{+2}$	13.76	**<0.0001**
Year 2015	$5.99\times10^{-1} \pm 7.20\times10^{-2}$	8.33	**<0.0001**
Null deviance: 1.13 on 2034 degrees of freedom			
Residual deviance: 0.89 on 2025 degrees of freedom			



Appendix 3.14. Mean of honey bees and flies observed per plant on six *L. scoparium* cultivars (B, LG, MG, O, P, and Y) at 10.30 am, 1.30 pm and 4.30 pm. a) honey bees per plant in 2014; b) honey bees per plant in 2015; c) flies per plant in 2014; d) flies per plant in 2015.



Appendix 3.15. Diagram showing the mean of honeybees observed per plant on six *L. scoparium* cultivars (B, LG, MG, O, P, and Y) at 10.30 am, 1.30 pm and 4.30 pm in 2014 and 2015. Diagram includes the temperature mean of the day (dash line).


Temperature (°C)
30
25
20
15
10
5
0
Blue
Lime green
Mint green
Orange
Pink
Yellow
Mean temperature
Honey bees per plant
25
20
15
10
5
0
Time / Date
4:00 PM
25/10/15
10:00 AM
1:00 PM
4:00 PM
26/10/2015
10:00 AM
1:00 PM
4:00 PM
31/10/2015
10:00 AM
1:00 PM
4:00 PM
1/11/2015
10:00 AM
1:00 PM
4:00 PM
6/11/2015
10:00 AM
1:00 PM
4:00 PM
7/11/2015
10:00 AM
1:00 PM
4:00 PM
8/11/2015
10:00 AM
1:00 PM
4:00 PM
13/11/2015
10:00 AM
1:00 PM
14/11/2015
10:00 AM
1:00 PM
17/11/2015
10:00 AM
1:00 PM
19/11/2015

Chapter 4.

Mycorrhizal fungal communities associated with *Leptospermum scoparium*

"The study of plants without their mycorrhizas is the study of artefacts. The majority of plants, strictly speaking, do not have roots; they have mycorrhizas"

BEG-Committee, 1993

4.1. Introduction

Plants interact with a diversity of organisms, including lichens, bacteria and fungi that can play a crucial role in ecosystems (Grube & Berg, 2009; Gaiero et al., 2013). Specifically, fungal species can be ecologically decisive, depending on the symbiotic relationship with their host. For example, symbionts such as mycorrhizal fungi play a crucial role in the uptake and transport of water and nutrients such as phosphorus and nitrogen (Smith & Read, 2008; Courty et al., 2010), subsequently they stimulate plant growth (Suparno et al., 2015) and improve seedling establishment (Lodge & Wentworth, 1990). They can contribute to the richness of soil microbial communities (Wagg et al., 2014), promote plant diversity (Wagg et al., 2014; Heijden et al., 2015), and improve pathogen resistance (Sikes, Cottenie, & Klironomos, 2009) and drought tolerance (Michelsen & Rosendahl, 1990; Smith & Read, 2008).

Approximately 40,000 to 50,000 mycorrhizal fungal species are associated with nearly 250,000 host plants. Among these, arbuscular mycorrhizal (AM) fungi are associated with around 74% of plant species (Smith & Read, 2008; Brundrett, 2009) and ectomycorrhizal fungi (EcM) with around 2% of plant species (Brundrett, 2009). Interestingly, despite the number of plants using the respective groups, the number of EcM fungal species (20,000) outnumbers the AM fungal species (300–1600) (Heijden et al., 2015), though it is suggested that there are many unknown AM fungal species (Kõljalg et al., 2013; Öpik et al., 2013).

In addition to abundance differences, functional and morphological differences occur between EcM and AM fungi. On the one hand, ecosystems dominated by EcM are capable of acquiring more carbon than ecosystems dominated by AM fungi (Phillips, Brzostek, & Midgley, 2013; Averill, Turner, & Finzi, 2014; Heijden et al., 2015; Sulman et al., 2017). On the other hand, AM fungi can be faster colonisers than EcM, dominating initial stages and being replaced in successional stages by EcM fungi (Chilvers, Lapeyrie, & Horan, 1987; Chen, Brundrett, & Dell, 2000; Lodge, 2000; Santos et al., 2001). Morphologically, while EcM fungi can be recognised macroscopically by fungal structures such as the fungal mantle (Brundrett, 2004), microscopy techniques are necessary to detect AM fungal structures like arbuscules (Peterson, Massicotte, & Melville, 2004). These structures and others such as spores have been widely studied using staining and microscopy (Krüger et al., 2012; Redecker, Hijri, & Wiemken, 2003;

Schüßler & Walker, 2010). However, the difficulty of identifying some EcM fungi at species level (Kim et al., 2003) and cultivating AM fungi (Schubler, 1999), and the availability of molecular biology techniques has prompted researchers to use DNA for a better understanding of fungal communities (Tedersoo, May, & Smith, 2010; Krüger et al., 2012; Tedersoo & Smith, 2013).

In 1992, DNA from AM fungi was first amplified from the Small Subunit (SSU) rRNA. Subsequently, this led to the development of PCR primers that were specific for AM fungi (Simon, Lalonde, & Bruns, 1992; Redecker, 2000). At that time, the AM fungi were included in the phylum Zygomycota, but, due to their molecular distinctiveness, were recognised as a monophyletic group in 2001 (Schüßler, Schwarzott, & Walker, 2001). Consequently, a new phylum named Glomeromycota (now subphylum Glomeromycotina; phylum Mucoromycota (Spatafora et al., 2016)) was established to include all the species identified as AM fungi. Four years later, next generation sequencing (NGS) platforms were introduced (Margulies et al., 2005). The pioneer sequencing platform was 454-pyrosequencing, which was used for examining AM fungal communities in the boreo-nemoral forest in Estonia (Öpik et al., 2009). Öpik's findings revealed a similar number of AM fungal taxa (51) and host plant species (57) within this forest, and indicated that recovered AM fungi were distributed worldwide. While this study evaluated AM fungal communities at a local scale, a recent study has demonstrated the low host specificity of AM fungi at global scale (Davison, 2015). Despite the AM fungi being characterised by low host specificity, different factors at local and regional scale contribute to the differences among the diversity of AM fungal communities. For example, soil depth (Oehl et al., 2005; Pickles & Pither, 2014; Bahram, Peay, & Tedersoo, 2015), host plant age (Weijtmans et al., 2007), host plant species (Tedersoo et al., 2008; Davis et al., 2013), land use intensity (Oehl et al., 2010; Davison et al., 2015), and vegetation type (i.e. grassland, forest) (Moyersoen & Fitter, 1999; Moyersoen & Beever, 2004; Rodríguez-Echeverría et al., 2017) all influence a typical fungal community.

New Zealand provides unique habitats such as geothermal areas, a distinctive habitat type in which specialised thermally adapted elements like *Leptospermum scoparium* J.R.Forst. & G.Forst. coexist (Chiarucci et al., 2008; Hreggvidsson et al., 2017). Apart from *L. scoparium*, only two other woody plant genera, *Nothofagus* Blume and *Kunzea* Rchb., are known to form EcM fungal associations in New Zealand (Moyersoen & Fitter, 1999;

Orlovich & Cairney, 2004). Unusually, *Leptospermum scoparium* and *Kunzea* spp. are able to form both EcM fungal and AM fungal associations (Moyersoen & Fitter, 1999), which is a dual mycorrhizal association that is globally uncommon (Harley & Harley, 1987; Brundrett & Abbott, 1991; Chen et al., 2000; Lodge, 2000). Studies have shown an overall high diversity of fungal species (ca 400) on these host plants (Orlovich & Cairney, 2004; McKenzie, Johnston, & Buchanan, 2006), and suggested that the EcM fungal group are more diverse than the AM fungal group on *L. scoparium* (Davis et al., 2013). However, most studies have been focussed on studying the diversity of EcM fungal species rather than AM fungi in *L. scoparium (*Moyersoen & Fitter, 1999; Orlovich & Cairney, 2004; McKenzie et al., 2006; Johnston et al., 2010). Of the approximately 400 fungal species recorded on *Leptospermum*, only two species are classified as AM fungi (*Acaulospora laevis* Gerd. & Trappe and *Glomus pallidum* I.R. Hall) (Hall, 1977; McKenzie et al., 2006), as *Glomus tenue* (Greenall) I.R. Hall is currently classified as a fine endophyte (Orchard et al., 2017). Moreover, previous studies have mainly evaluated the diversity of EcM fungi or AM fungi on *L. scoparium* using morphological features, which makes the identification of AM fungi difficult. However, the implementation of molecular biological techniques such as NGS platforms provides an opportunity to study the diversity and distribution of both EcM and AM fungal communities on *L. scoparium*.

Using NGS platforms such as Illumina MiSeq along with different target regions can give a broader resolution of fungal communities, including mycorrhizal fungi. Specifically, the examination of the ITS region is important, as ITS is considered the universal fungal barcode due to its high success in PCR amplification and its variability (Stockinger, Krüger, & Schüßler, 2010; Schoch et al., 2012). However, the ITS region has two limitations: 1) poor resolution for certain fungal lineages (Tedersoo et al., 2015), and 2) high variability within lineages, which complicates phylogenetic approaches for species identification (Stockinger, Walker, & Schüßler, 2009; Schoch et al., 2012; Halwachs et al., 2017). For example, the spacer ITS1, which can be amplified using the primer pair ITS1F/ITS2, is recognised for recovering mycorrhizal fungi and other fungal lineages (Li et al., 2016; Nguyen et al., 2016; Zhang et al., 2016; Ding et al., 2017; Fernandez et al., 2017), but has poor resolution for Glomeromycotina (Tedersoo et al., 2015). Several studies used a different set of primers (ITS1F/ITS4) (Branco, Bruns, & Singleton, 2013; Johansen et al., 2017) instead of ITS1F/ITS2. However, the use of ITS1F/ITS4 is not recommended since it may generate longer size amplicons (i.e. > 400 bp) using Illumina

MiSeq Chemistry. Among these two primer pairs, ITS1F/ITS2 and ITS1F/ITS4, the former amplifies the ITS1 spacer and the latter amplifies both spacers. On one hand, ITS1F/ITS2 is regarded as the more variable region so it can facilitate the identification of species (Nilsson et al., 2008; Teasdale et al., 2013; Nilsson et al., 2015). On the other hand, ITS1F/ITS4 is characterised by longer reads, which contain more information but risk increasing biases and the number of chimeras (Lindahl et al., 2013). Therefore, while Lindahl et al. (2013) suggested that studies on fungal community diversity should use either ITS1 or ITS2, there is as yet no consensus criterion for the choice of an ITS spacer (Bazzicalupo, Bálint, & Schmitt, 2013; Blaalid et al., 2013; Lindahl et al., 2013; Tedersoo et al., 2015).

For phylogenetic studies and taxon classification, additional amplification of the Large Subunit (LSU) rDNA and Small Subunit (SSU) rDNA regions is recommended as well as that of the ITS region (Krüger et al., 2009; Kohout et al., 2014; Taylor, Helgason, & Öpik, 2017). Specifically, the SSU region has been widely used for studying AM fungi, which has produced the majority of data present on public databases. This region is more conserved than ITS, which facilitates further phylogenetic studies (e.g. species level) and potentially the discovering of novel species (Öpik, Davison, et al., 2013). Within the SSU region, the primer pair NS31/AML2 has been commonly used, since AML2 is considered specific for AM fungi (Lee, Lee, & Young, 2008; Kohout et al., 2014). The SSU region with NS31/AML2 as primers is commonly used for studying AM fungal communities using the sequencing platforms 454-pyrosequencing and Ion semiconductor sequencing (Taylor et al., 2017). However, studies of AM fungi using Illumina MiSeq platform are scarce, although this approach could give a better resolution of the AM fungal communities. Therefore, the aim of the present study is to combine different ITS and SSU with Illumina MiSeq platform to lead to a better understanding of the assembly of mycorrhizal fungi and other associated fungi on the host *L. scoparium*.

This study aims to evaluate the diversity of EcM fungi, AM fungi and other associated fungi on cultivated and wild plants of *L. scoparium* at three sites in the North Island. It is hypothesised that fungal communities would differ among cultivated and wild plants, and potentially among sites. Illumina MiSeq was used to compare the fungal diversity from roots of *L. scoparium* using the ITS and the SSU region.

4.2. Material and methods

4.2.1. Study area

The three sites that were studied in Chapter 2, located in Wanganui and Hawkes's Bay regions (Fig. 4.1), were selected for root collection. Each site included plantation and wild plants of *L. scoparium*. According to the providers of plant material, no mycorrhizal fungi were intentionally introduced before planting the cultivars. Rangitatau (Fig. 4.1 a), previously in pasture, comprised blocks planted with three different cultivars of *L. scoparium* (CVT1, CVT3 and CVT4) in 2011 and scattered wild plants. Ruatiti (Fig. 4.1 b) included CVT1 and CVT4, planted in 2008 and 2012 respectively, and scattered wild plants. CVT1 was planted on a site previously in *Pinus radiata* D.Don located next to a stand of *Kunzea ericoides* (A.Rich.) Joy Thomps., while CVT4 is planted on a site previously in pasture. Tutira (Fig. 4.1 c) included three cultivars (CVT2, CVT3 and CVT4) planted in 2013 and scattered wild plants that were previously in pasture.

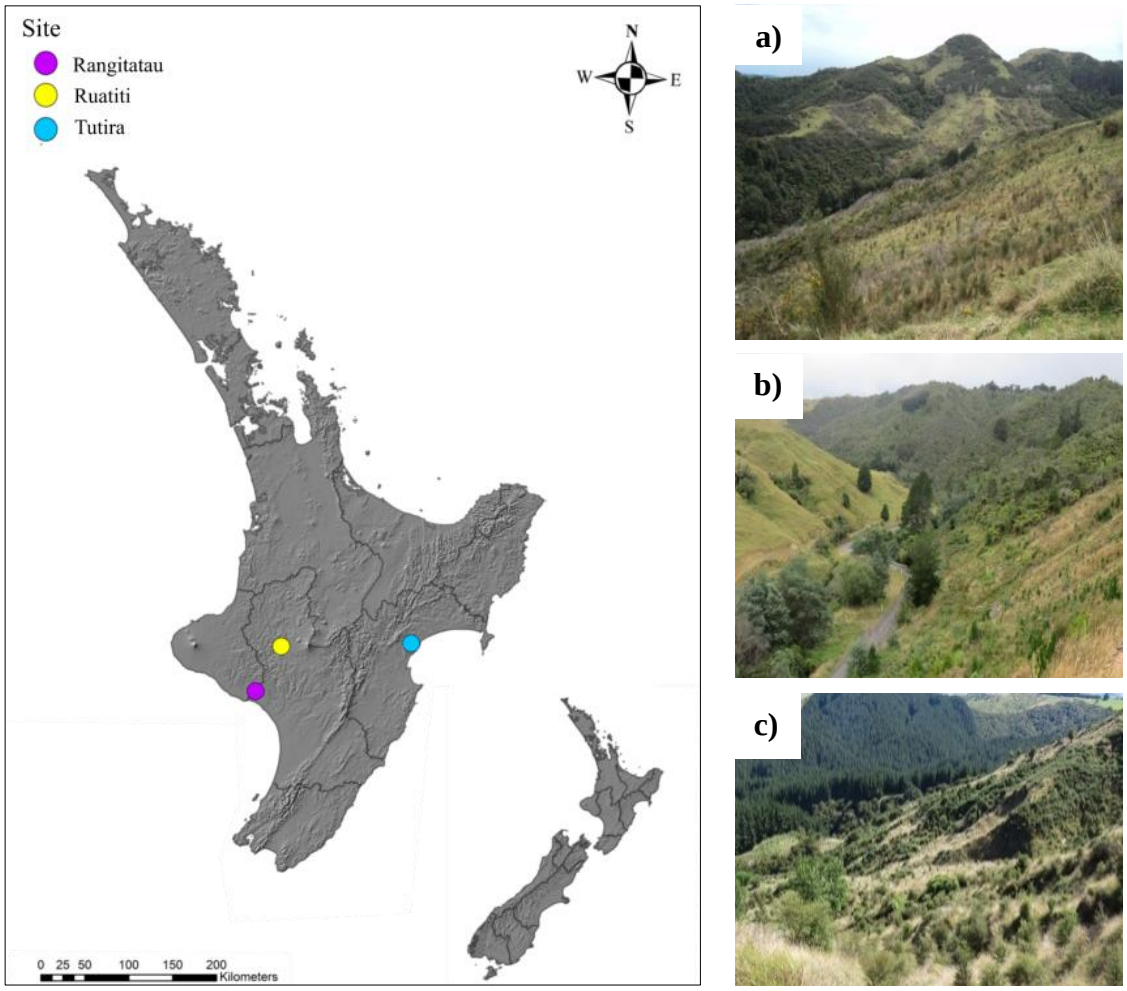


Fig. 4.1. Map showing the study sites: a) Rangitatau; b) Ruatiti; c) Tutira.

4.2.2. Sample collection

Root samples were collected over 3 days during summer 2016 (February and March). At each site, nine plants from cultivated and wild plants were selected for sampling. A earlier stratified sample collection scheme was designed using a map and selecting sampling points in each cultivar plantation. Each cultivar was divided into nine grids and a sampling point was established in the middle of each grid, being the sampling points equally distributed in each plantation. The GPS coordinates were recorded for each sampling point. For wild plants, sampling points were directly selected in the field, as they were scattered and established naturally in the field. Wild plants were sampled from all over the site to get an overall representation depending on their distribution and their presence within each cultivar plantation.

In the field, a GPS was used to locate the position of the selected sampling points and the closest healthy plant to each point was sampled. The root system of each plant was excavated until the fine roots were visible. Fine roots were haphazardly selected and traced back to the main stem to confirm that they belonged to *L. scoparium*. Roots were placed in ziplock plastic bags, placed in ice for transportation and kept at 4°C for 2 days in the lab. Samples were washed vigorously using a 2-mm sieve, preserved in 50% ethanol, and kept at 4°C. For DNA extraction, samples were used during the first 48 hours. For root staining, samples were preserved for a longer period at 4°C.

Roots were collected from 36 plants (CVT1: 9; CVT3: 9; CVT4: 9; wild: 9) at Rangitatau, 27 plants (CVT1: 9; CVT4: 9; wild: 9) at Ruatiti and 36 plants (CVT2: 9; CVT3: 9; CVT4: 9; wild: 9) at Tutira. A total of 99 plants were sampled.

4.2.3. Root staining

Roots were stained and observed under a microscope to confirm the presence of fungi. Thin roots (<1 mm) were subsampled and washed three times with nanopure water. Roots were stained according to the method for clearing and staining mycorrhizal roots described by Brundrett & Bougher (1996), though 5% KOH was used in this case rather than 10% KOH. First, roots were placed in flask bottles and covered with 5% KOH. Roots were cleared following the protocol defined by Dalpé & Séguin (2013). Flask bottles were heated using a sequence of 20 sec medium, 20 sec medium, 10 sec low, 10 sec low and

10 sec low in an 1150 watt microwave. If roots were not sufficiently clear, they were heated for a longer period. Samples were rinsed in water and acidified in 5% HCl for 10 minutes. Roots were subsequently stained with trypan blue solution (0.05% w/v trypan blue in lactoglycerol (1:1:1 lactic acid, glycerol, water) in a water bath for 10 minutes at 65°C, and placed in petri-dishes with de-stain solution (1:1:1 lactic acid: glycerol: water) for root inspection. External root tissues were visualised under a dissecting microscope and samples colonised by hyphae were selected. These root tissues were subsampled and small pieces were placed on microscope slides with a small drop of de-stain solution to ascertain the presence of fungal structures. Slides were examined under a compound microscope to verify the presence of fungal structures such as hyphae, spores, vesicles or arbuscules.

4.2.4. DNA extraction

Root samples were washed three times with MilliQ water and the thinnest roots were selected for DNA extraction. Roots were cut into small pieces (1–3 mm) and 0.2 g of fresh roots were collected per sample and placed in a 2-ml Eppendorf tube. Samples were dried using a speed vacuum centrifuge concentrator (Savant Instruments) for 5 hours and placed in a 1.5-ml tube with two tungsten carbide beads (Riedel, Germany). The roots were then ground in two consecutive sessions of 30 seconds at 4000 rpm using a beater (MagNA Lyser, Roche). DNA extraction was conducted using the E.Z.N.A® HP Plant DNA Mini Kit (Omega bio-tek) following the manufacturer's protocol. DNA was quantified using the Qubit Fluorometric Quantification kit (Life Technologies, Thermo Fisher Scientific Inc.). The DNA samples were frozen at –80°C until analysed. DNA samples were diluted 1:10 with MilliQ water and amplicons from the nuclear ribosomal regions ITS and SSU were generated by PCR.

4.2.4.1. DNA amplification of the ITS region

The ITS1 region was chosen for PCR amplification. Using the primer pair ITS1F and ITS2 (Table 4.1), DNA samples were shipped to a sequencing facility (Australian Genome Research Facility, AGRF). AGRF conducted the preparatory procedure (DNA amplification, index and barcoded PCRs, and PCR purification). Amplicons were sequenced on the Illumina MiSeq platform in a 2×300 bp paired-end run by AGRF.

4.2.4.2. DNA amplification of the SSU region

For the SSU region, I performed the first two rounds of PCR and the last PCR (adding barcodes) was performed by New Zealand Genomics Limited (NZGL) (Palmerston North, New Zealand). The first PCR step involved DNA amplification using the universal eukaryotic primer NS31 combined with the AM fungal specific primer AML2. The first PCR reaction (10 µl) contained 0.5 µl of NS31 primer (5 µM), 0.5 µl of AML2 primer (5 µM), 5 µl of Emerald ® GT PCR Master Mix, 3.5 µl of molecular biology grade H_2O and 0.5 µl of diluted DNA. The PCR reaction was run on a thermal cycler under the following conditions: initial heating to 94 °C for 3 min; 35 cycles at 94 °C for 30 sec; 50 °C for 1 min; 72 °C for 1 min; 72 °C for 5 min. The first PCR product was purified using SAP/EXO kit (Life Technologies, Thermo Fisher Scientific Inc.) following the instructions of the manufacturer.

The second PCR step, used the primers NS31-A and AML2-A to add the Illumina adapters to the amplicon. However, this PCR was unsuccessful and by using combinations of NS31-A/AML2 and NS31/AML2-A it was determined that the problem was with the reverse primer AML2-A. Thus, the alternative reverse primer AML2-2 (see below) was designed using Primer 3 from the National Centre for Biotechnology Information (NCBI[1]).

The second PCR was performed using the forward primer NS31-A and the designed reverse primer AML2-2-A. For the second PCR reaction, the cocktail (20 µl) included 1 µl of NS31-A (10 µM), 1 µl of AML2-2-A (10 µM), 10 µl of Emerald® GT PCR Master Mix, 7 µl of molecular biology grade H_2O, and 1 µl of purified PCR product. PCR was run using the thermal-cycling conditions described previously. PCR products were purified with the ZYMO DNA Clean and Concentrator kit (Ngaio Diagnostics), quantified using the Qubit Fluorometric Quantification kit (Life Technologies, Thermo Fisher Scientific Inc.) and normalised to an equal concentration (40 ng/ml) for all products. Samples were submitted to NZGL for the last PCR step (adding barcodes) and then pooled for library preparation. Amplicons were sequenced on Illumina MiSeq platform in a 2×250 bp paired-end run by New Zealand Genomics Limited.

[1] https://blast.ncbi.nlm.nih.gov/Blast.cgi

Table 4.1. Primers used during the study. Illumina adapters are indicated in blue. F: forward direction. R: reverse direction.

Primer	Target region	Direction	Sequence	Reference
ITS1F	ITS	F	5´-CTTGGTCATTTAGAGGAAGTAA-3´	Gardes & Bruns, 1993
ITS2	ITS	R	5´-GCTGCGTTCTTCATCGATGC-3´	White, Bruns, Lee, Taylor, & others, 1990
NS31	SSU	R	5´-TTGGAGGGCAAGTCTGGTGCC-3´	Simon, Lalonde, & Bruns, 1992b
NS31-A	SSU	F	5´-TCGTCGGCAGCGTCAGATGTGTATAA GAGACAGTTGGAGGGCAAGTCTGGTGCC-3´	
AML2	SSU	R	5´-GAACCCAAACACTTTGGTTTCC-3´	Lee, Lee, & Young, 2008
AML2-A	SSU	R	5´-GTCTCGTGGGCTCGGAGATGTGTATAA GAGACAG GAACCCAAACACTTTGGTTTCC-3´	
AML2-2	SSU	R	5´-ATGGTTAAGACTACGACGGTATCTG-3´	This study
AML2-2-A	SSU	R	5´-GTCTCGTGGGCTCGGAGATGTGTATAA GAGACAGATGGTTAAGACTACGACGGTATCTG-3´	

4.2.4.2.1. Design of the reverse primer AML2-2 for the SSU rDNA region

The primer pair NS31/AML2 was considered in the first instance as the best choice for evaluating AM fungal species. This primer pair is widely used in AM fungal studies and the reverse primer AML2 is specifically designed for capturing AM fungi. Thus, there are abundance of sequences to compare from this region, on the database, for downstream bioinformatics analysis.

In the present study, the primer pair (NS31/AML2) amplified products of the expected size in a first round of PCR (using genomic DNA as template). However, when adapters (specified by the Illumina library protocol) were added to each primer, and a second round of PCR was conducted using the PCR product as template, the amplification was not successful. Different PCRs were conducted using combinations of the primers with and without adapters ((NS31/AML2 (product), NS31/AML2-A (no product), NS31-A/AML2 (product), NS31-A/AML2-A (no product)), and it was determined that the AML2-A primer was causing the problem. To overcome this, a new reverse primer (AML2-A, AML2-2-A) was designed. The new primer lies just inside the AM-specific primer and is

not AM-specific itself (this was concluded from looking at an alignment of different AM and non-AM fungi across this region. Therefore, a first round of PCR using the primers NS31/AML2 (and genomic DNA as template) needed to be done to capture only the AM fungi, followed by a second round using NS31-A/AML2-2-A (with the first round PCR products as template) to add the adapters for MiSeq library preparation. In order to design such primer (AML2-2) sequences from AM fungal and non-AM fungal species present on the public databases (i.e. NCBI, Maarj*AM*, and SILVA) were selected and downloaded for alignment (Table 4.2).

Sequences were aligned using CLUSTALX 2.1 (Larkin et al., 2007) and Sequencher® 5.4.6 (Gene Codes Corp., Ann Arbor, Michigan) (Appendix 4.1). The designed primer AML2-2 included in the alignment amplified both AM fungal and non-AM fungal species. Thus, for the first PCR the primers NS31/AML2 were selected to capture only AM fungal species and the second PCR used NS31-A/AML2-2-A to add the Illumina adapters necessary for sequencing.

Table 4.2. Organisms selected for designing the reverse primer AML2-2 using sequences present on the public databases NCBI, SILVA and Maarj*AM*.

Organism	Accession number
Acaulospora sp	EF033119
Ambispora leptoticha (N.C. Schenck & G.S. Sm.) C. Walker, Vestberg & A. Schüssler	AB015052
Ambispora leptoticha (N.C. Schenck & G.S. Sm.) C. Walker, Vestberg & A. Schüssler	AJ006466
Endogone sp.	KC708391
Gigaspora albida N.C. Schenck & G.S. Sm.	AJ852600
Gigaspora gigantea (T.H. Nicolson & Gerd.) Gerd. & Trappe	AJ852601
Claroideoglomus etunicatum (W.N. Becker & Gerd.) C. Walker & A. Schüßler	AJ852598
Rhizophagus intraradices (N.C. Schenck & G.S. Sm.) C. Walker & A. Schuessler	AJ852526
Glomus macrocarpum Tul. & C. Tul	FR750376
Glomus sinuosum (Gerd. & B.K. Bakshi) R.T. Almeida & N.C. Schenck	AJ133706
Diversispora trimurales (Koske & Halvorson) C. Walker & A. Schüßler	FR686957
Scutellospora heterogama (T.H. Nicolson & Gerd.) C. Walker & F.E. Sanders	AJ852609
Urnula craterium (Schwein.) Fr.	AF104347
Urnula hiemalis Nannf.	Z49754
Zygomycete sp.	EU428766
Zygomycete sp.	EU428769

A PCR was conducted using the primers NS31-A/AML2-2-A to confirm the successful amplification with DNA extracted from roots of *L. scoparium* (Fig. 4.2).

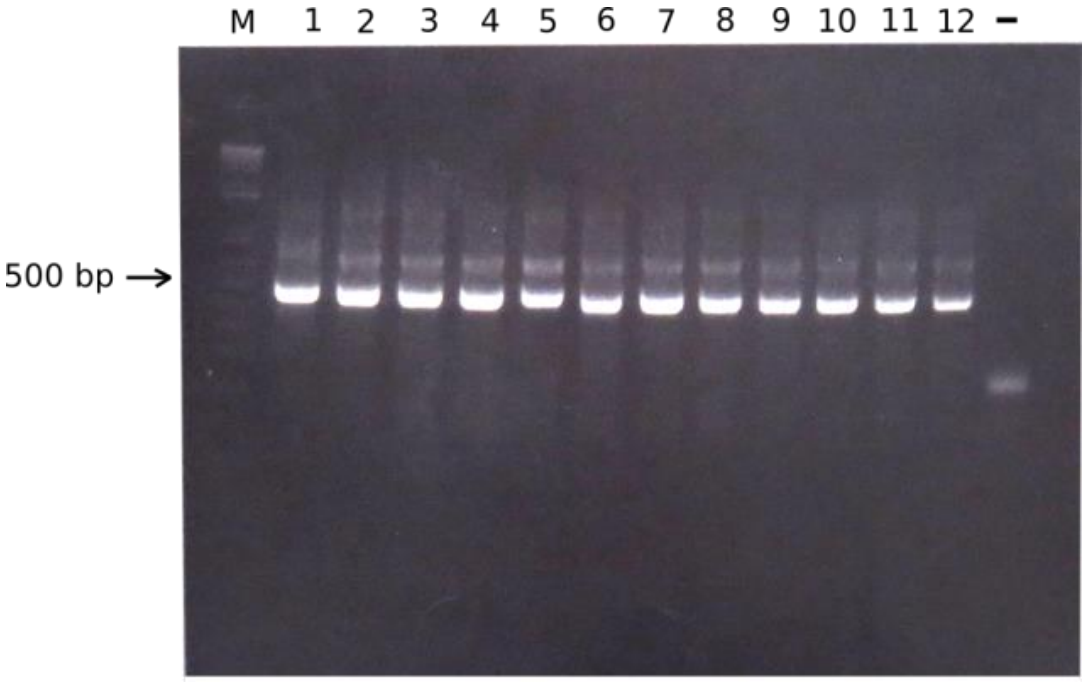


Fig. 4.2. Agarose electrophoresis showing the results of PCR amplification of DNA extracted from roots of *L. scoparium* and amplified with the primer set NS31-A/AML2-2-A, which included Illumina adapters. Lane 1–6: DNA at 1:1 µl concentration amplified from roots collected at Ruatiti (lane 1–4) and Tutira (lane 5–6). Lane 7–12: DNA diluted at 1:10 µl amplified from roots collected at Ruatiti (lane 7–10) and Tutira (lane 11–12).. M: molecular weight marker 1kb; -: negative control.

4.2.5. Bioinformatics analysis

Reads were mapped and labelled using Python 2.7.12. Cutadapt 1.14 was used to trim forward and reverse primers with wildcard match parameter enabled. Sequences where both the forward and reverse primers were not detected were removed. PEAR 0.9.10 (Zhang, Kobert, Flouri, & Stamatakis, 2014) was used to merge pair ends with a minimum assembly length of 50 and low quality sequences were discarded with a max expected error of 0.5 through vsearch v2.4.3. Sequences were dereplicated and singletons removed. Further analysis was performed using QIIME 1.9.1 (Caporaso et al., 2010) and USEARCH 8.1 (Edgar, 2010). OTUs were clustered with *de novo* at 97% similarity with chimera filtering. Chimeras present on sequences from the ITS region were checked against the UNITE reference database[1] of the ITS1 region (Nilsson et al., 2015) with the minimum score to report chimeras of 0.5. For the SSU region, chimeras from the

[1] uchime_reference_dataset_01.12.2016

sequences of the SSU region were checked against the Maarj*AM* database (Öpik et al., 2010).

To assign taxonomy, OTUs recovered from the ITS region were blasted against the UNITE 7.1. dynamic reference database and identified to a species hypothesis (SH). Species hypotheses were mapped to the EcM lineage generated by Tedersoo et al. (2010) and to the list of interacting data using UNITE (https://unite.ut.ee/analysis.php). Species hypothesis that were unclassified or classified as non-ectomycorrhizal were removed. OTUs assigned to an EcM lineage were blasted against NCBI. Sequences that indicated a break in the blast alignment or a chimera assembly were removed from the dataset following the guidelines of Nilsson et al. (2012). Only certain OTUs assigned to an EcM fungal lineage that showed a BLAST match of a regular sequence, as recommended by Nilsson et al. (2012), were used for comparison among provenances and sites. For evaluating closely related species, the 20 most abundant OTUs were matched to the top listed species at NCBI.

For the SSU region, OTUs retained after clustering were blasted against the Maarj*AM* and NCBI databases. As for ITS region, only certain OTUs with a regular alignment were retained (Nilsson et al., 2012). The OTUs retained that belonged to Glomeromycotina were used for evaluating the taxonomic classification and the AM fungal community among provenances and sites. The NCBI top match at species level was assigned to each OTU for determining closely related species.

As a phylogenetic study, OTUs that belonged to the Glomeromycotina that showed a query score[1] of 100% and ≥ 97% identity were mapped to a reference sequence (*Paraglomus majaweskii*) to create an alignment with default settings using Geneious 10.1.3. Alignments were checked by eye looking for inconsistency. A Bayesian phylogenetic tree was generated using the aligned sequences with 1,000,000 interactions and a burn-in of 10,000 using a GTR invgamma model in Mr Bayes 3.2 (Huelsenbeck & Ronquist, 2001). Nine representative sequences within the clade Glomeromycota (*Paraglomus majewskii*, *Claroideoglomus* GIBb1.2, *Glomus viscosum*, *Glomus macrocarpum*, *Acaulospora laevis*, *Diversispora celata*, *Scutellospora nodosa*, *Ambispora fennica*, and *Geogiphon pyriformis*) and two outgroup species (*Umbellopsis*

[1] Quality score: % of OTU sequence aligned to a NCBI sequence

ramanianna and *Mortierella verticillata*) were added into the trees to give indicative clades for the OTUs obtained. Trees were visualised using the software FigTree 1.4.3[1].

4.2.6. Statistical analysis

Venn diagrams[2] were generated for the ITS and SSU region to evaluate unique and shared OTUs and virtual taxa[3] (VT) among sites and among provenances (Öpik, Davison, Moora, & Zobel, 2013). For the ITS region, the EcM fungal communities were evaluated according to the EcM fungal lineages defined by Tedersoo et al. (2010). For the SSU region, the virtual taxa identified as AM fungi were selected. Data was transformed and a matrix was calculated using the Bray-Curtis method. Results were used to perform Nonmetric Multidimensional Scaling (NMDS) and plots were generated for visualising the results using the package ggplot in R 3.3.1 (R Core Team, 2016). A PermANOVA test was performed using the vegan package in R to evaluate fungal communities among cultivated (CVT1, CVT2, CVT3 and CVT4) and wild plants and among sites (Rangitatau, Ruatiti and Tutira).

[1] http://tree.bio.ed.ac.uk/software/figtree/
[2] http://bioinformatics.psb.ugent.be/software/details/Venn-Diagrams
[3] Virtual taxon (VT): Virtual taxon (VT) defined as "a phylogenetically defined group of closely related SSU rDNA gene sequences with sequence identity equal or higher than 97%." (Maarja Öpik, Davison, Moora, & Zobel, 2013).

4.3. Results

4.3.1. Root staining

Root staining confirmed the association of AM fungal and other fungal species with cultivated and wild plants of *L. scoparium*. Figure 4.3 shows the presence of arbuscules, spores and hyphae on wild plants of *L. scoparium* in Rangitatau and Ruatiti.

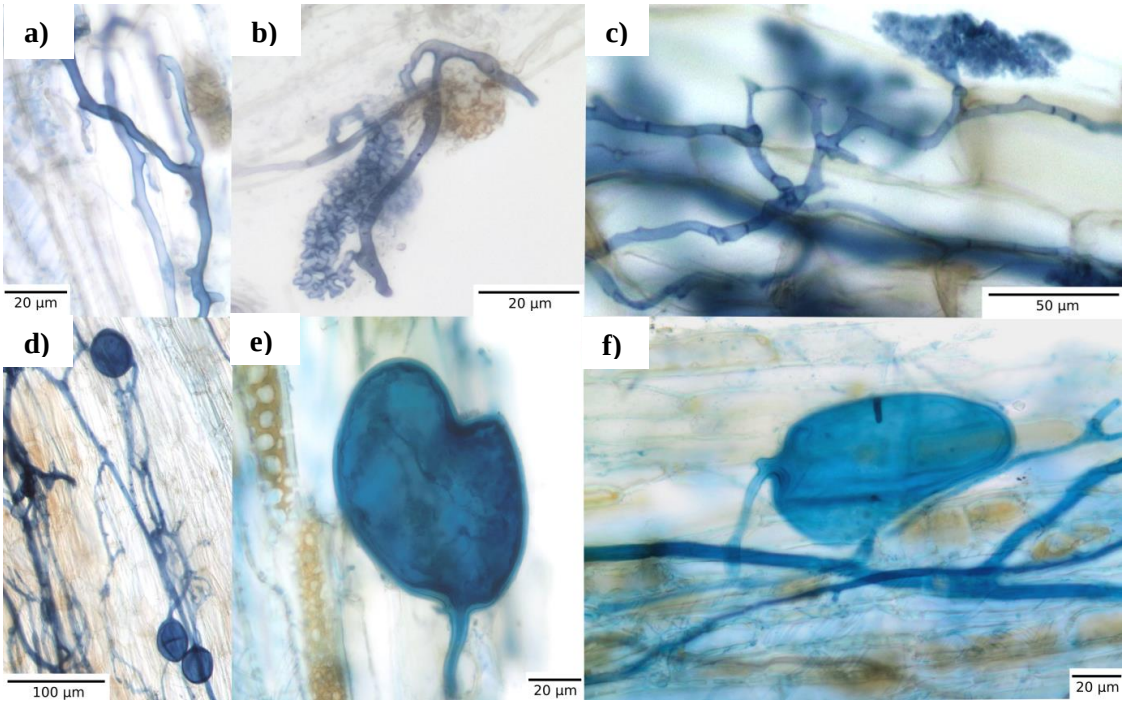


Fig. 4.3. Fungal structures found in roots of wild plants of *L. scoparium* collected at Rangitatau and Ruatiti. a) hyphae; b), c) arbuscules; d), e), and f) vesicles.

4.3.2. ITS region

4.3.2.1. Next generation sequencing results

Although DNA was successfully extracted from 99 samples from three different field locations, only 96 samples that showed high DNA yield were including for sequencing. For the ITS region, 9,245,994 paired reads were obtained from Illumina MiSeq 2×300 bp, but 8,290,279 reads were retained after low quality sequences were discarded. The average length of the reads was 271.6 bp. A total of 3349 operational taxonomic units (OTUs) were recovered after the OTU picking process was conducted. After chimera control, 3318 OTUs were retained for further taxonomic classification. Another sample from a CVT2 plant from Tutira was removed from the biological observation matrix (BIOM) table due to the low number of sequences generated (1106). Overall, Rangitatau

showed the highest number of OTUs with 2033, followed by Tutira with 2023 and Ruatiti with 1653. Tutira contained more unique OTUs (637) than Rangitatau (577) and Ruatiti (419) (Fig. 4.4 a). In comparison to the cultivars, wild plants of *L. scoparium* showed the highest number of unique OTUs at Ruatiti (448) (Fig. 4.4 c) and at Rangitatau (322) (Fig. 4.4 b). At Tutira, the cultivar CVT4 presented the highest number of unique OTUs (281) followed by wild plants (269) (Fig. 4.4 d).

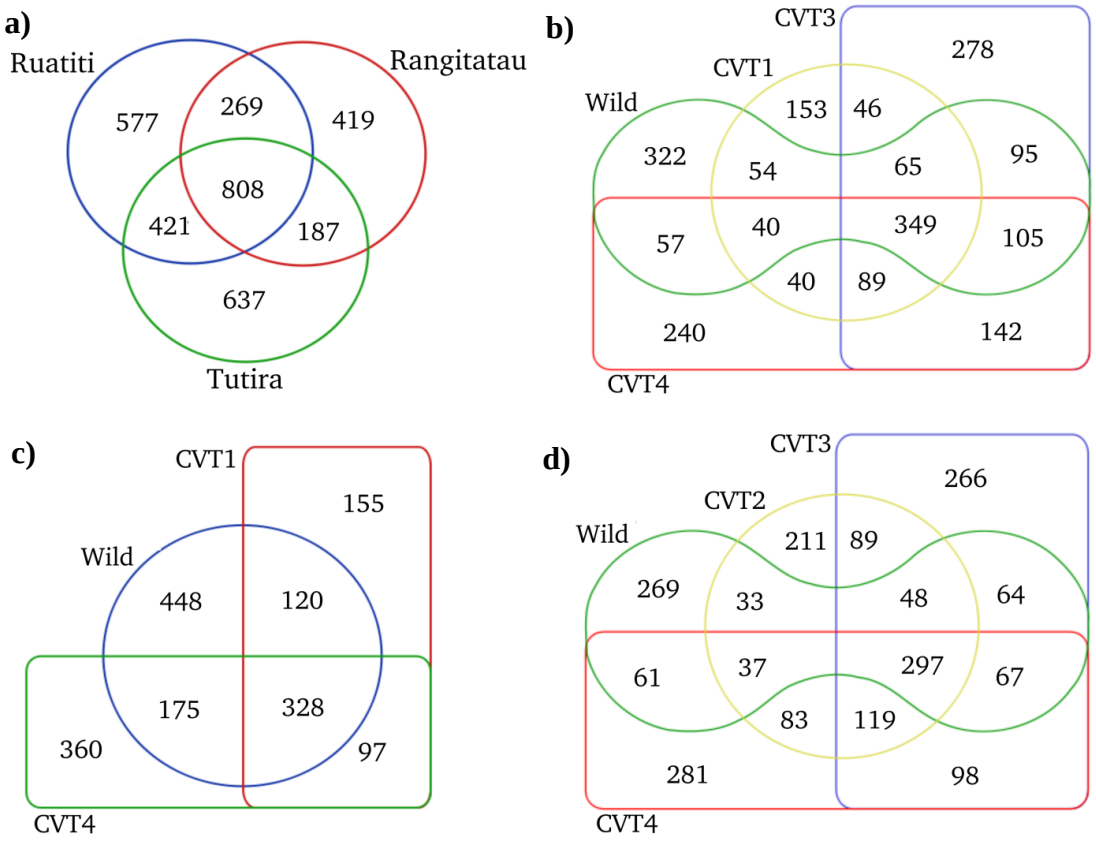


Fig. 4.4. Venn diagrams showing the number of shared and unique fungal OTUs retained from roots of *L. scoparium* per site of cultivated (CVT1, CVT2, CVT3, and CVT4) and wild plants of *L. scoparium*. a) overall and per site; b) Rangitatau; c) Ruatiti; d) Tutira.

Table 4.3. Total number of sequences, OTUs and unique OTUs, considered belonging to the Fungi kingdom, recovered from roots of cultivated (CVT1, CVT2, CVT3 and CVT4) and wild plants of *L. scoparium* at Rangitatau, Ruatiti, and Tutira classified as fungi.

Site; provenance	n	Total sequences	Total OTUs	Total unique OTUs
Rangitatau				
CVT1	9	484,043	820	149 (15.34%)
CVT3	9	805,113	1153	276 (28.42%)
CVT4	9	900,647	1039	231 (23.79%)
Wild	9	728,354	1063	315 (32.44%)
Ruatiti				
CVT1	9	570,886	686	150 (15.89%)
CVT4	8	632,462	947	355 (37.61%)
Wild	9	650,084	1051	439 (46.50%)
Tutira				
CVT2	8	580,381	917	211 (20.54%)
CVT3	9	598,086	1048	266 (25.90%)
CVT4	8	534,390	1043	281 (27.36%)
Wild	8	1,712,218	876	269 (26.19%)

Wild plants of *L. scoparium* showed the largest number of fungal sequences at both Ruatiti and Tutira with 650,084 and 1,712,218 sequences respectively. At Rangitatau, CVT4 presented more sequences compared with the other provenances, with 900,647 sequences (Table 4.3). Although CVT4 showed the highest number of sequences at Rangitatau, the total number of unique OTUs retained was higher from wild plants (32.91%) than from CVT4 (23.79%). Similar results were found at Ruatiti, where 46.50% of the unique OTUs belonged to wild plants and 37.61% to the cultivar CVT4.

4.3.2.2. A summary of fungal communities associated with Leptospermum scoparium

A total of 7,643,310 sequences were classified within five phyla and 25 classes (Table 4.4). Among phyla, the phylum Basidiomycota was represented by 5,570,686 sequences and Ascomycota by 2,206,897 sequences. Wild plants at Tutira were highlighted by presenting the largest number of sequences within the phylum Ascomycota and Basidiomycota, with 517,743 and 1,160,981. At Rangitatau the Mucoromycota was mainly found within the cultivar CVT3 and CVT4, presenting 13,618 and 10,839 sequences respectively. Within phylum Ascomycota, the class Leotiomycetes (882,315 sequences) and Sordariomycetes (490,448 sequences) were the most abundant. In

addition, the class Pezizomycetes (101,942 sequences) was predominant in CVT4 at Ruatiti. Compared with the phylum Ascomycota, the majority of sequences retained within the phylum Basidiomycota were included within the Agaricomycetes (5,495,122 sequences). Among sites, Ruatiti showed the highest number sequences unidentified within a class in wild plants (98,278 sequences). Wild plants (67,542) at Tutira also showed the highest number of sequences unidentified compared to the cultivars CVT2, CVT3 and CVT4. In contrast, the cultivar CVT4 indicated the highest number of unidentified sequences within a class at Rangitatau.

Table 4.4. Total number of sequences retained per fungal class using the ITS region from roots of cultivated (CVT1, CVT2, CVT3 and CVT4) and wild plants of *L. scoparium* at Rangitatau (Ra), Ruatiti (Ru) and Tutira (Tut). The size of the blue bar indicates the relative number of sequences within a phylum. The proportion is relative to the provenance that contains the highest number of sequences. Cells filled with the green colour scale indicate the proportion of sequences within a fungal class. The proportion is relative to the provenance that contains the highest number of sequences.

Phylum; Class \ Sit; Prov	Ra_CVT1	Rut_CVT1	Tut_CVT2	Ra_CVT3	Tut_CVT3	Ra_CVT4	Rut_CVT4	Tut_CVT4	Ra_wild	Rut_wild	Tut_wild
Ascomycota	**134,061**	**33,493**	**93,008**	**198,977**	**146,011**	**293,221**	**232,802**	**155,743**	**138,358**	**263,480**	**517,743**
Archaeorhizomycetes	6,044	1,574	624	12,673	5,932	8,899	3,149	23,248	1,713	1,608	20,175
Dothideomycetes	5,743	449	25,070	15,119	37,116	16,377	23,951	13,021	9,087	68,306	82,165
Eurotiomycetes	3,179	2,282	7,037	10,316	32,963	15,467	5,453	14,309	5,849	10,273	24,322
Geoglossomycetes	57	1	75	467	2	101	2	798	3	4	126
Lecanoromycetes	1,196	51	28		986	1,527	63	32	1,160	233	519
Leotiomycetes	90,426	10,881	18,262	82,452	31,909	162,620	63,108	35,288	42,252	83,266	261,851
Orbiliomycetes	31	45	441	175	1,397	560	85	2,174	148	286	1,099
Pezizomycetes	3,409	2,012	144	749	531	2,370	101,942	930	11,668	9,205	7,815
Saccharomycetes	18	2	86	417	19	373	61	100	57	0	15
Sordariomycetes	16,823	13,905	35,645	60,530	22,036	61,076	28,437	58,094	50,472	61,462	81,968
Taphrinomycetes	0	0	0	0	4	0	15	2	0	0	40
Basidiomycota	**334,635**	**480,145**	**470,895**	**550,809**	**438,704**	**534,259**	**358,997**	**365,839**	**563,366**	**312,056**	**1,160,981**
Agaricomycetes	328,133	479,101	469,609	539,783	431,107	522,274	358,051	338,007	562,089	307,359	1,159,609
Agaricostilbomycetes	5	0	0	28	28	0	0	7	2	0	361
Atractiellomycetes	187	9	54	1,756	4,861	5,850	8	3,113	84	3	8
Cystobasidiomycetes	0	0	25	26	72	39	4	0	0	1	1
Entorrhizomycetes	0	0	0	0	0	2	0	1	0	200	0
Microbotryomycetes	55	156	56	65	33	122	3	66	311	195	32
Pucciniomycetes	0	22	0	172	0	1	0	0	4	91	0
Tremellomycetes	5,416	837	1,047	8,223	1,530	5,681	872	724	754	2,568	525
Tritirachiomycetes	0	5	14	7	1	0	0	4	1	0	9
Wallemiomycetes	0	0	0	0	0	0	0	0	0	0	54
Chytridiomycota	**24**	**32**	**4,552**	**516**	**246**	**226**	**123**	**1,665**	**4,108**	**472**	**393**
Blastocladiomycetes	0	0	8	0	0	0	0	0	0	0	0
Chytridiomycetes	14	9	4,500	472	181	77	121	1,604	4,025	204	378
Monoblepharidomycetes	5	0	0	0	0	0	0	1	0	61	0
Mucoromycota	**1,316**	**257**	**1,495**	**13,618**	**2,325**	**10,839**	**1,208**	**5,310**	**2,278**	**6,536**	**3,628**
Glomeromycetes	1,291	220	1,310	13,529	2,205	10,756	1,168	5,247	2,216	6,481	3,600
Fungi_Class_Incertae_sedis	**22,009**	**59,309**	**16,344**	**56,946**	**25,171**	**86,460**	**45,954**	**37,620**	**36,434**	**98,278**	**67,542**

For taxonomic classification 59 OTUs unidentified or classified within the kingdom Protista were removed from the dataset and 3259 OTUs were retained as fungi. OTUs were classified predominantly within the phylum Ascomycota (61.7%), Basidiomycota (17.4%), Mucoromycota (5.6%) and Zygomycota (2.3%). Of the 3259 OTUs, 311 OTUS were unclassified. Among the 25 classes, 17 classes retained more than 10 OTUs. The dominant classes were Dothideomycetes (559 OTUs), Sordariomycetes (485 OTUs), Agaricomycetes (439 OTUs), Leotiomycetes (208 OTUs), Eurotiomycetes (187 OTUS) and Glomeromycetes (175 OTUs). Among sites, the class Sordariomycetes was represented at Tutira by 341 OTUs, at Rangitatau by 329 OTUs and in Ruatiti by 246 OTUs. Similar pattern among sites was almost found for the classes Leotiomycetes, Eurotiomycetes and Glomeromycetes, where the number of OTUS ranged from 101 to 138 (Fig. 4.5).

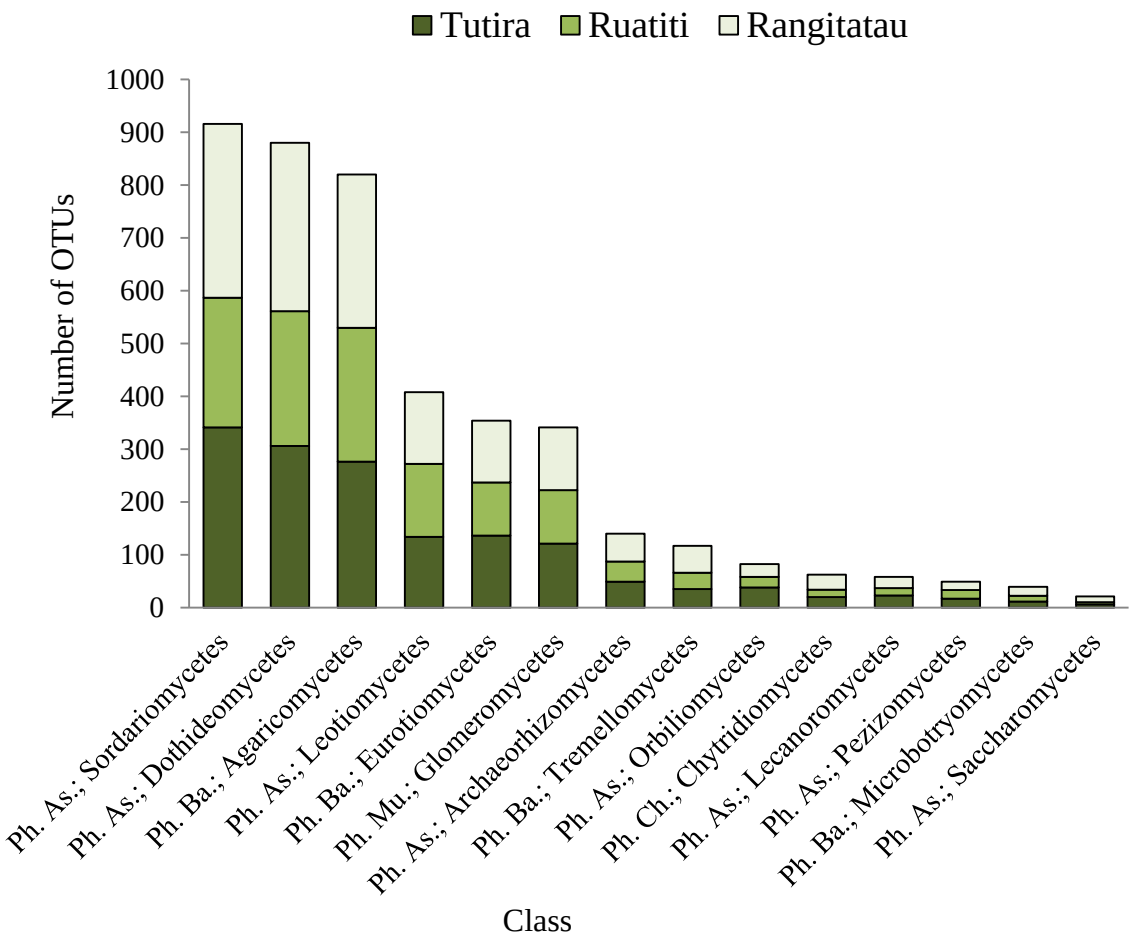


Fig. 4.5. Number of OTUs retained per classes of fungi from roots of cultivated (CVT1, CVT2, CVT3 and CVT4) and wild plants of *L. scoparium* at Rangitatau, Ruatiti and Tutira. The classes included in the bar chart presented overall more than 10 OTUs within a class. Classes were classified within the phylum Ascomycota (Ph. As.), Basidiomycota (Ph. Ba.), Chytrydiomycota (Ph. Ch.) and Mucoromycota (Ph. Mu.).

Of the 3259 OTUs, a total of 113 OTUs recovered from the BLAST against UNITE database matched an EcM fungal lineage according to Tedersoo (2010). For AM fungi, 149 OTUs matched within an AM fungal family after BLAST against UNITE database was performed. Figure 4.6 indicates that sequences were ascribed to EcM fungal lineages as well as to AM fungal families. All CVT provenances retained approximately 250,000 to 500,000 sequences of putatively mycorrhizal fungal sequences. However, whereas wild plants from Tutira retained 958,989 sequences, CVT4 only retained 169,189 sequences.

Figure 4.6 shows the limited use of the ITS region and UNITE reference database for AM fungi. Thus, further studies using ITS sequences involved only the study of EcM fungal lineages. For the study of AM fungal community, a different approach was selected, using the SSU rDNA and Illumina MiSeq 2×250 bp (see section 3.3).

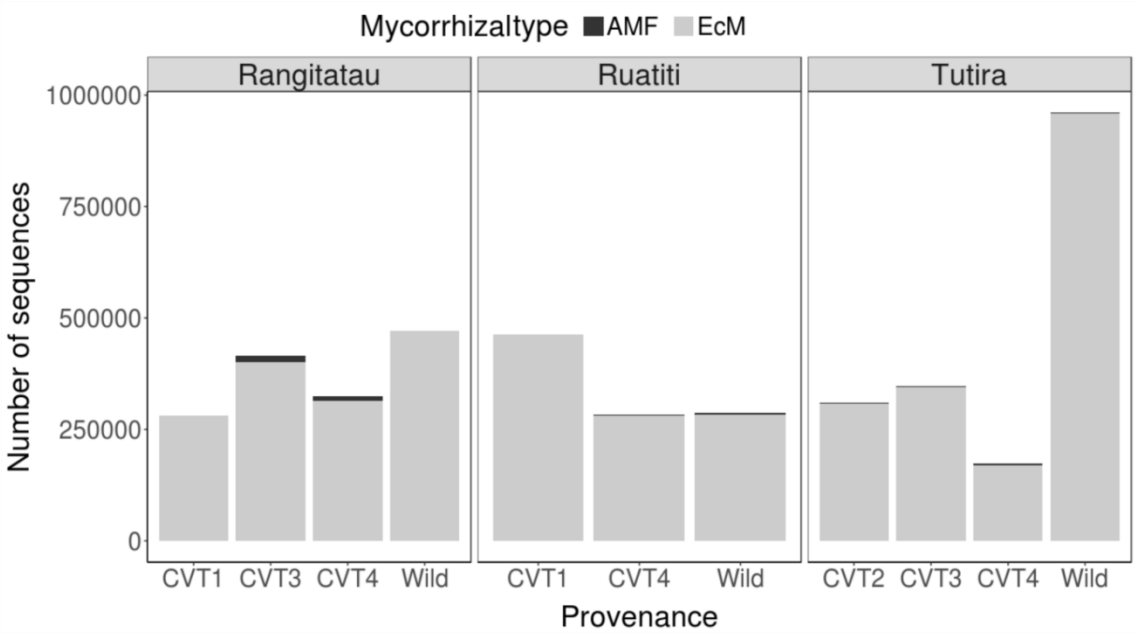


Fig. 4.6. Total number of sequences classified to an EcM fungal lineage or AM fungal family obtained from roots of cultivated (CVT1, CVT2, CVT3 and CVT4) and wild plants of *L. scoparium* at Rangitatau, Ruatiti, and Tutira. The ver7_dynamic_20.11.2016 reference database from UNITE and the EcM fungal lineage from Tedersoo (2010) were used for classification.

4.3.2.3. EcM fungal lineages associated with Leptospermum scoparium

Of the 113 OTUs, 86 OTUs (Appendix 4.2) were kept after 27 OTUs (98,825 sequences) (Appendix 4.3) were discarded for showing a break alignment or chimera assembly in the BLAST results (Nilsson et al., 2012). The 86 OTUs were represented by 4,176,887 sequences, which indicated that ca 50% of the total number of sequences were retained

as an EcM fungal lineage after the bioinformatics analysis. Overall, 16 EcM lineages were recovered among the three studied sites (Table 4.5). Of the 16 EcM lineages, five were considerably more common than the others. The EcM lineage /tomentella-thelephora was the most dominant as it represented 46.8% of the total number of EcM fungal sequences. The lineages /cortinarius, /laccaria, /clavulina and /russula-lactarius were also well represented, retaining 14%, 13.9%, 12.7% and 7.5% respectively. A total of 10 EcM fungal lineages (Appendix 4.4 a) represented by 43 OTUs were shared among sites (Appendix 4.4 b). Rangitatau contained 14 EcM fungal lineages represented by 66 OTUs, Ruatiti also contained 14 EcM fungal lineages that were represented by 63 OTUs, while Tutira was represented by 12 EcM fungal lineages and 61 OTUs.

Table 4.5. Shared and unique EcM fungal lineages with the respective number of sequences and OTUs recovered from roots of cultivated (CVT1, CVT2, CVT3 and CVT4) and wild plants of *L. scoparium* at Rangitatau, Ruatiti, and Tutira. X was assigned to an EcM fungal lineage that were recovered from only one site.

Phylum	Class	Order	EcM fungal lineage	Total sequences	Total OTUs
Basiomycota	Agaricomycetes	Thelephorales	/tomentella-thelephora	1,956,033	30
Basiomycota	Agaricomycetes	Agaricales	/cortinarius	584,222	17
Basiomycota	Agaricomycetes	Agaricales	/laccaria	581,209	10
Basiomycota	Agaricomycetes	Cantharellales	/clavulina	532,033	5
Basiomycota	Agaricomycetes	Russulales	/russula-lactarius	313,210	4
Basiomycota	Agaricomycetes	Sebacinales	/sebacina	165,309	3
Basiomycota	Agaricomycetes	Agaricales	/descolea	29,976	3
Basiomycota	Agaricomycetes	Agaricales	/inocybe	6,591	4
Basiomycota	Pezizomycetes	Pezizales	/terfezia-peziza depressa	2803	1
Basiomycota	Agaricomycetes	Agaricales	/amanita	1653	1
Ascomycota	Pezizomycetes	Pezizales	/tuber-helvella	1,483	1
Basiomycota	Agaricomycetes	Boletales	/boletus	1391	1
Basiomycota	Agaricomycetes	Agaricales	/hebeloma-alnicola	876	2
Basiomycota	Agaricomycetes	Atheliaceae	/amphinema-tylospora	68	2
Ascomycota	Dothideomycetes	Mytilinidiales	/cenococcum	22	1
Basiomycota	Agaricomycetes	Agaricales	/tricholoma	8	1

The EcM fungal lineages identified as /clavulina, /cortinarius, /descolea, /laccaria, /russula-lactarius and /tomentella-thelephora were shared between all the cultivars and wild plants at the three sites (Fig. 4.7). Rangitatau was the only site where wild plants were associated with EcM fungal lineages (/hebeloma-alnicola, /sebacina and /tuber-

helvella) that were not also present in cultivated plants. Ruatiti was the site where the most lineages were shared among provenances (10). In comparison, only six lineages were shared among provenances at both Rangitatau and Tutira. The /cenococcum lineage was only found at Ruatiti and the /tricholoma lineage was only recovered in Tutira. The lineage /cenococcum was represented by 22 sequences in Ruatiti and /thricoloma was represented by 8 sequences in Tutira.

The /tomentella-thelephora lineage was dominant at Rangitatau and Tutira, representing 927,617 sequences (23 OTUs) and 708,408 sequences (21 OTUs) respectively. In contrast, Ruatiti only contained 320,008 sequences (20 OTUs) within this lineage. This site was dominated by the /cortinarius lineage with 382,164 sequences (15 OTUs). At Tutira, the /clavulina lineage was also well represented with 423,788 sequences (5 OTUs). However, the /laccaria lineage was most common at Ruatiti with 198,980 (8 OTUs) and Rangitatau with 175,666 (7 OTUs) (Table 4.6). Ruatiti was the site where the majority of sequences identified within the /amanita lineage were found. Of the 1653 sequences of this lineage recovered from the study, 1644 sequences were found in CVT1 at Ruatiti.

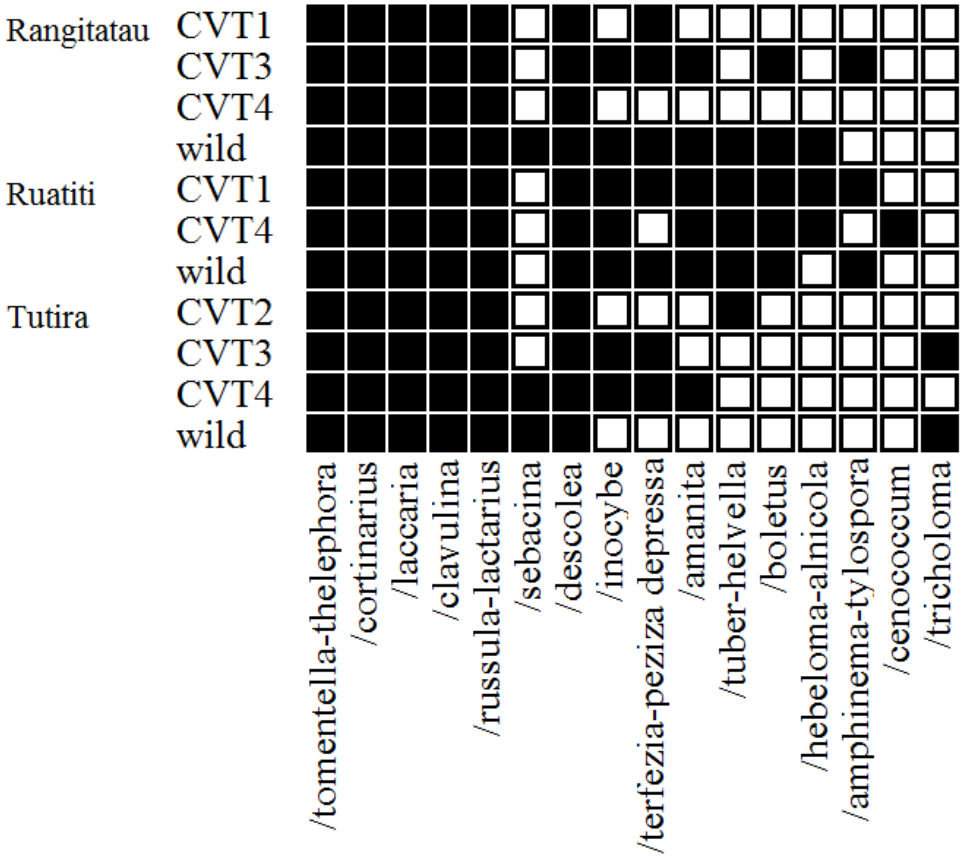


Fig. 4.7. Presence or absence of EcM fungal lineages found on roots of cultivated (CVT1, CVT2, CVT3 and CVT4) and wild plants of *L. scoparium* at Rangitatau, Ruatiti, and Tutira. Filled squares indicate the presence of an EcM fungal lineage within a provenance and site.

Table 4.6. Shared EcM fungal lineages recovered from roots of cultivated (CVT1, CVT2, CVT3 and CVT4) and wild plants of *L. scoparium* at Rangitatau, Ruatiti, and Tutira.

Site ; EcM fungal lineage	*n*	Total sequences	Total OTUs
Rangitatau	36		
/clavulina		103,122	4
/cortinarius		111,406	15
/descolea		25,690	3
/laccaria		175,666	7
/russula-lactarius		41,787	4
/tomentella-thelephora		927,617	23
Ruatiti	27		
/amanita		1646	1
/boletus		5	1
/clavulina		5123	3
/cortinarius		382,164	15
/descolea		681	3
/inocybe		182	3
/laccaria		198,980	8
/russula-lactarius		109,648	3
/tomentella-thelephora		320,008	20
/tuber-helvella		1473	1
Tutira	32		
/clavulina		423,788	5
/cortinarius		90,652	14
/descolea		3,605	2
/laccaria		206,563	7
/russula-lactarius		161,775	3
/tomentella-thelephora		708,408	21

4.3.2.4. Diversity and distribution of EcM fungal lineages

The /tomentella-thelephora lineage was strongly abundant in all provenances at Rangitatau (Fig. 4.8). CVT4 was mainly colonised by this EcM fungal lineage (84.8%) along with the /laccaria lineage (14.4%). The /laccaria lineage was also common in CVT1. In contrast, CVT3 and wild plants showed a higher variety of EcM fungal lineages. For example, the /clavulina lineage represented 15.1% of the sequences found in wild plants while /cortinarius lineage represented 20.7% of CVT3 sequences. Compared with Rangitatau, Ruatiti showed more EcM fungal diversity and a decrease in the importance of the /tomentella-thelephora lineage. Even though all three provenances hosted /cortinarius, this lineage was most strongly represented in CVT1 (55.8%) at Ruatiti. This cultivar also contained the majority of the sequences identified within the /amanita lineage (93.07%).

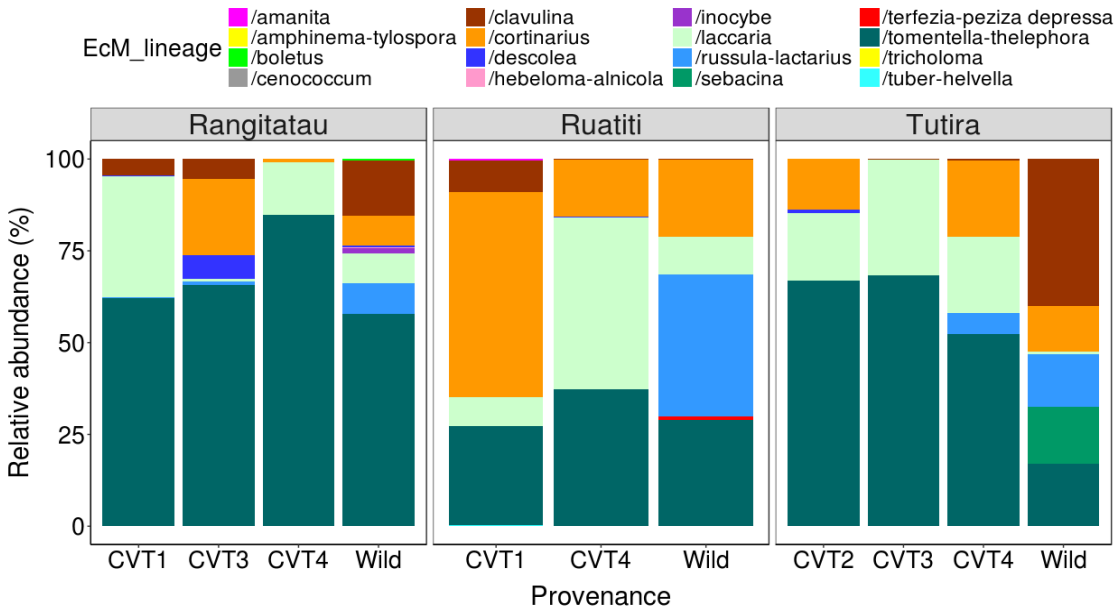


Fig. 4.8. Relative abundance of EcM fungal lineages recovered from roots of cultivated (CVT1, CVT2, CVT3 and CVT4**)** and wild plants of *L. scoparium* at Rangitatau, Ruatiti, and Tutira.

The indices of Shannon's diversity per provenance were mostly similar among provenances, only CVT1 showing a higher mean Shannon index (0.67). The indices for CVT2, CVT3, CVT4 and wild plants were 0.19, 0.23, 0.25, and 0.36 respectively. However, overall, wild plants were slightly associated with a wider EcM community than cultivated plants (Appendix 4.5).

Cultivar CVT4 was predominantly colonised by the /laccaria lineage (46.7%) and wild plants by the /russula-lactarius lineage (38.6%). As with Rangitatau, Tutira showed a predominance of the /tomentella-thelephora lineage, but only in cultivated plants. However, wild plants presented more diversity and included the /clavulina lineage (39.9%). Interestingly, the cultivars contained the /laccaria lineage (20–30%), while this lineage was almost absent in wild plants.

Of the 86 OTUs, the most abundant 20 OTUs were classified within the EcM fungal lineages /tomentella-thelephora (6), /cortinarius (6), /laccaria (3), /russula-lactarius (2) /clavulina (1), /descolea (1), and /sebacina (1). These 20 OTUs were identified within the NCBI top match at species level according to their EcM fungal lineage (Table 4.7). The second most abundant OTU (OTU_3), which was represented by more than 480,000 sequences, was matched to *Laccaria glabripes* with 100% of identity. Although with a lower representation, the OTU_35 also matched at 100% of identity within the species

Descolea maculata. Along with these two species, *Lactifluus subclarkeae* was almost identical to the 150,219 sequences described here as OTU_8.

Overall, among the most abundant 20 OTUs, the species closest matched differed among provenances. While wild plants at Tutira recovered the majority of sequences within a species closely related to *Clavulina subrugosa* (97% identity), wild plants at Ruatiti contained a majority of sequences with an OTU that matched with *Lactifluus subclarkeae* at 99% of identity. However, the closest match to *Laccaria glabripes* (100% identity) was mostly found in cultivated plants, especially in CTV4 (129,109 sequences) at Ruatiti, CVT1 (87,773 sequences) at Rangitatau, and CVT3 (81,674 sequences) at Tutira.

Among sites, the most abundant 10 OTUs per site were matched to the closest related species showed in NCBI (Table 4.8). At Ruatiti, *Laccaria glabripes* was dominant in cultivar CVT4 and a species closely related to *Cortinarius waiporianus* mainly colonised CVT1. In contrast, wild plants were mostly colonised by a species closely related to *Russula chloroides*. At Rangitatau, *Tomentella* was abundant in all the provenances and *L. glabripes* was mainly present in cultivar CVT1. Importantly, wild plants at Tutira were dominated by a species closely related to *Clavulina subrugosa* and predominated by a species closely related to *Lactifluus subclarkeae*.

Table 4.7. The top NCBI match at species level within the respective EcM fungal lineage for the most abundant 20 OTUs recovered from roots of cultivated and wild plants of *L. scoparium* at Rangitatau, Ruatiti and Tutira. OTUs were classified according the EcM fungal lineage (Tedersoo, 2010), Species Hypothesis (UNITE) and NCBI accession number (acc. no.). All matches indicated a query cover of 100%, except for *Cortinarius waiporianus*, which showed a query cover of 96%. The taxonomic classification at species level is shown according to Mycobank[1].* Landcare Auckland NZ (Johnston P.R. and Park, D., unpublished; 2010, 2016). ** Otago University (Orlovich, D.A. and Anderson, T.P., unpublished; 2016). *** On soil under *Leptospermum* (Matheny, P.B., Swenie, R.A. and Cooper, J., unpublished; 2017). **** Australia (Uehling, Henkel, Aime, Vilgalys, & Smith; published; 2012).

OTU ID	Order	EcM fungal lineage	NCBI match	Species hypothesis	NCBI acc. no	Total sequences	NCBI identity (%)
20	Agaricales	/cortinarius	*Cortinarius cystidiocatenatus*	SH222726.07FU_UDB002773_reps	AY669651.1	63,063	96%
76	Agaricales	/cortinarius	*Cortinarius elaiops*	SH033501.07FU_UDB004055_reps_singleton	KT875181.1*	14,005	95%
12	Agaricales	/cortinarius	*Cortinarius waiporianus*	SH222467.07FU_UDB004058_reps	KT875191.1*	159,090	98%
3158	Agaricales	/cortinarius	*Cortinarius waiporianus*	SH222467.07FU_UDB004058_reps	KT875191.1*	104,104	97%
2560	Agaricales	/cortinarius	*Cortinarius waiporianus*	SH222467.07FU_UDB004058_reps	KT875191.1*	42,111	98%
10	Agaricales	/cortinarius	*Dermocybe indotata*	SH223047.07FU_UDB002760_reps	KJ421110.1	179,109	97%
35	Agaricales	/descolea	*Descolea maculata*	SH220031.07FU_DQ192181_refs	KU523936.1**	26,092	100%
672	Agaricales	/laccaria	*Laccaria bicolor*	SH220982.07FU_JX270706_reps	KM067839.1	59,045	99%
449	Agaricales	/laccaria	*Laccaria fraterna*	SH179273.07FU_JX270719_reps	MF461608.1***	36,340	99%
3	Agaricales	/laccaria	*Laccaria glabripes*	SH179271.07FU_JX270697_reps	HQ533019.1*	483,560	100%
7	Auriculariales	/sebacina	*Sebacina epigaea*	SH199504.07FU_HM146868_refs	KF000417.1	161,751	89%
4	Cantharellales	/clavulina	*Clavulina subrugosa*	SH220240.07FU_JN228221_reps	JN228221.1****	515,540	97%
8	Russulales	/russula-lactarius	*Lactifluus subclarkeae*	SH190181.07FU_HQ318283_reps	KR364095.1	150,219	99%
11	Russulales	/russula-lactarius	*Russula chloroides*	SH220525.07FU_UDB016008_reps	FJ845429.1	159,919	95%
1	Thelephorales	/tomentella-thelephora	*Tomentella badia*	SH177979.07FU_UDB016695_reps	KJ140664.1	660,060	90%
21	Thelephorales	/tomentella-thelephora	*Tomentella badia*	SH184543.07FU_AB211278_reps	JQ711882.1	77,165	92%
28	Thelephorales	/tomentella-thelephora	*Tomentella coerulea*	SH177941.07FU_FJ897224_reps	KR019826.1	69,674	91%

[1] http://www.mycobank.org/

14	Thelephorales	/tomentella-thelephora	*Thelephora penicillata*	SH184599.07FU_JF960837_reps	U83484.1	99,555	90%
25	Thelephorales	/tomentella-thelephora	*Tomentella stuposa*	SH028889.07FU_UDB013306_reps_singleton	EU819523.1	36,092	94%
2	Thelephorales	/tomentella-thelephora	*Tomentella sublilacina*	SH198956.07FU_KC222657_reps	KY693713.1	965,402	92%

Table 4.8. Total number of sequences retained within the closest NCBI match at species level for the most abundant 10 OTUs per cultivated (CVT1, CVT2, CVT3 and CVT4) and wild plants of *L. scoparium* at Rangitatau, Ruatiti, and Tutira. The bar indicates the relative proportion of sequences within a closest match. . Cell colour indicates the study site (Green: Ruatiti, blue: Rangitatau and Red: Tutira).

Site	OTU_ID	Closest Genbanck match (% identity)	CVT1	CVT2	CVT3	CVT4	Wild
Rangitatau	2	*Tomentella sublilacina* (92%)	98,341	-	152,792	175,596	102,842
	1	*Tomentella badia* (90%)	32,458	-	38,126	87,286	141,268
	3	*Laccaria glabripes* (100%)	87,773	-	2,419	36,838	18,989
	4	*Clavulina subrugosa* (97%)	11,029	-	15,706	113	69,031
	10	*Cortinarius indotatus* (97%)	98	-	69,980	1,684	1,032
	21	*Tomentella badia* (92%)	16,691	-	34,555	2,264	190
	11	*Russula chloroides* (95%)	129	-	210	98	37,820
	14	*Thelephora penicillata* (90%)	22,609	-	486	75	2,203
	20	*Cortinarius cystidiocatenatus* (96%)	63	-	389	75	23,967
	35	*Descolea maculata* (100%)	15	-	22,371	50	1
Ruatiti	3	*Laccaria glabripes* (100%)	40,120	-	-	129,109	28,283
	2	*Tomentella sublilacina* (92%)	4,194	-	-	96,253	56,424
	12	*Cortinarius waiporianus* (98%)	120,783	-	-	1,149	18,130
	11	*Russula chloroides* (95%)	159	-	-	315	109,075
	3158	*Cortinarius waiporianus* (97%)	100,014	-	-	205	2,045
	1	*Tomentella badia* (90%)	69,534	-	-	982	8,225
	28	*Tomentella coerulea* (91%)	52,637	-	-	97	140
	2560	*Cortinarius waiporianus* (98%)	251	-	-	41,683	0
	20	*Cortinarius cystidiocatenatus* (96%)	1,450	-	-	83	36,914
	10	*Cortinarius indotatus* (97%)	29,122	-	-	52	107
Tutira	4	*Clavulina subrugosa* (97%)	-	81	209	502	418,143
	1	*Tomentella badia* (90%)	-	149,281	73,167	30,696	29,037
	2	*Tomentella sublilacina* (92%)	-	56,460	158,119	33,172	31,209
	7	*Sebacina epigaea* (89%)	-	0	0	1	161,749
	8	*Lactifluus subclarkeae* (99%)	-	54	34	9,694	139,875
	3	*Laccaria glabripes* (100%)	-	29,013	81,674	22,539	6,803
	10	*Cortinarius indotatus* (97%)	-	31,682	89	34,727	10,536
	14	*Thelephora penicillata* (90%)	-	64	49	22,732	50,419
	672	*Laccaria bicolor* (99%)	-	27,538	12,510	43	13
	25	*Tomentella stuposa* (94%)	-	47	25	163	35,221

4.3.2.5. Community analysis of EcM fungal lineages on cultivated and wild plants

PermANOVA test revealed more significant differences among provenances at Ruatiti than at other sites (Table 4.9). This is shown in the Nonmetric Multidimensional Scaling (NMDS) analysis (Fig. 4.9b), where a clear distinction among plants from CVT1 and CVT4 was found for diversity of EcM fungal lineages. Whereas CVT1 was mainly represented by the /cortinarius lineage, CVT4 was dominated by the /laccaria lineage. Compared to cultivated plants, EcM fungal lineages were more diverse in wild plants, which were mostly dominated by the /russula-lactarius lineage.

The PermANOVA test showed that the significance of provenance was moderate at Tutira and non-significant at Rangitatau. At Tutira, the NMDS analysis illustrated the cluster of CVT3 against CVT2, CVT4 and wild plants (Fig. 4.9c). The cultivar CVT3 was dominated by /laccaria and /tomentella-thelephora lineage, but CVT2, CVT4 and wild plants were more diverse. In wild plants there was an extended diversity of EcM fungal lineages, although /clavulina lineage was the clear dominant lineage.

However, Rangitatau presented a somewhat different pattern compared with CVT3 and wild plants, where the pattern of EcM fungal lineages were similar (Fig.4.9a). Cultivar CVT3 showed a moderate increase of the /cortinarius, and /descolea lineage, while wild plants showed a diverse range of lineages, including the /inocybe and /hebeloma-alnicola lineage.

Table 4.9. Results of PermANOVA test showing the effect of the variable provenance (CVT1, CVT2, CVT3, CVT4 and wild) on EcM fungal lineages at Rangitatau, Ruatiti, and Tutira.

Site; provenance	d.f.	Sum of squares	Mean squares	*F*	*P*
Rangitatau					
Provenance	3	1.19	0.40	1.38	0.139
Residuals	32	9.20	0.29		
Total	35	10.39			
Ruatiti					
Provenance	2	1.40	0.70	2.56	**0.006**
Residuals	23	6.27	0.27		
Total	25	7.66			
Tutira					
Provenance	3	1.58	0.57	1.70	**0.050**
Residuals	28	8.65	0.31		
Total	31	10.23			

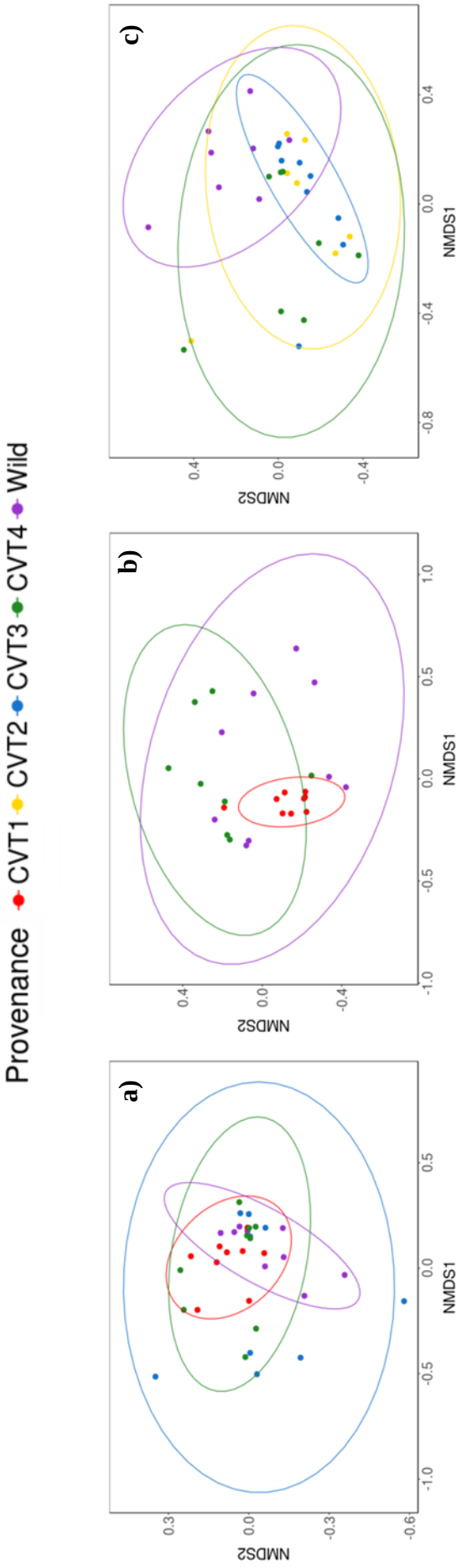


Fig. 4.9. Nonmetric Multidimensional Scaling (NMDS) plots on the Bray-Curtis matrix calculated from the number of sequences recovered from the EcM fungal lineages from roots of cultivated (CVT1, CVT2, CVT3 and CVT4) and wild plants of *L. scoparium*. a) Rangitatau (stress value 0.154); b) Ruatiti (stress value 0.218); c) Tutira (stress value 0.088).

4.3.3. SSU region

4.3.3.1. Next generation sequencing results

Although DNA was extracted from 99 root samples, only the 95 amplicons with better yield quality were sequenced through Illumina MiSeq. A total of 9,213,876 sequences were obtained with Illumina MiSeq 2×250 bp. After a cutadapt was performed and fastq files were filtered for both reads, 5,427,931 sequences were kept. Pair ends had an average length of 434.3 bp. A total of 779 OTUs were selected and they were checked against Maarj*AM* and NCBI databases. Of these, only 208 OTUs (1,547,214 sequences) definitely belonged to the phylum Mucoromycota, being predominantly represented by the subphylum Glomeromycotina (203 OTUs; 1,546,875 sequences) and the rest of them were within the subphylum Mucoromycotina (5 OTUs; 339 sequences). Among sites, Tutira showed the highest number of OTUs (164) compared with Ruatiti (132) and Rangitatau (122). Although Ruatiti showed higher numbers of OTUs than Rangitatau, Rangitatau was characterised by a higher number of sequences (425,649) than Ruatiti (157,853) (Table 4.10). Wild plants of *L. scoparium* showed the highest number of OTUs at Ruatiti (107). Interestingly, wild plants from this location presented the largest number of unique OTUs (36) compared to Rangitatau and Tutira with 58 and 74 OTUs respectively.

Table 4.10. Total number of sequences, OTUs and unique OTUs, retained from the SSU region belonging to the phylum Mucoromycota recovered from roots of cultivated (CVT1, CVT2, CVT3 and CVT4) and wild plants of *L. scoparium* at Rangitatau, Ruatiti, and Tutira.

Site; provenance	***n***	Total sequences	Total OTUs	Total unique OTUs
Rangitatau				
CVT1	8	44,277	75	7
CVT3	9	188,855	94	12
CVT4	9	126,611	77	6
Wild	6	65,906	58	10
Ruatiti				
CVT1	9	16,472	67	9
CVT4	9	49,980	78	12
Wild	9	91,401	107	36
Tutira				
CVT2	9	121,011	104	8
CVT3	9	206,664	113	14
CVT4	9	414,001	117	21
Wild	9	221,697	74	4

4.3.3.2. Diversity and distribution of AM fungal families on Leptospermum scoparium

Eight AM fungal families were found during this study. The family Glomeraceae showed the highest relative number of sequences mostly within cultivated and wild plants at all three sites. However, wild plants showed a predominance of Claroideoglomeraceae (49.7%) over Glomeraceae (42.2%). While Claroideoglomeraceae was the second most common family at all sites for most provenances, Archaeosporaceae was more abundant in CVT1 and CVT4 at Rangitatau (Fig. 4.10).

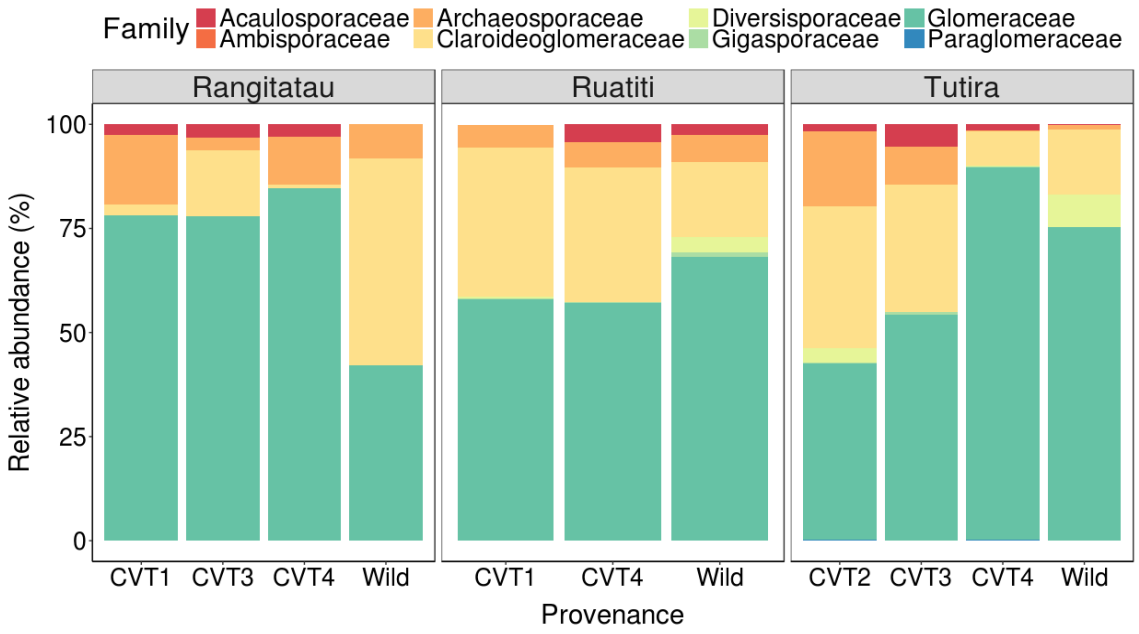


Fig. 4.10. Relative number of sequences classified within an AM fungal family that were recovered from roots of cultivated (CVT1, CVT2, CVT3 and CVT4) and wild plants of *L. scoparium* at Rangitatau, Ruatiti, and Tutira.

Of the 203 OTUs identified within an AM fungal family, 113 OTUs matched with the family Glomeraceae, followed by the families Claroideoglomeraceae (43) and Archaeosporaceae (23). Although the families Acaulosporaceae (10), Ambisporaceae (1), Diversisporaceae (6), Gigasporaceae (4) and Paraglomeraceae (3) were identified, the number of OTUs retained for these families was small.

OTUs were identified according to the virtual taxon (VT^3) in the Maarj*AM* database. A total of 85 VT were recovered; Glomeraceae represented by 51 VT, Claroideoglomeraceae by 10 VT, Archaeosporaceae by 5 VT, Acaulosporaceae by 8 VT, Diversisporaceae by 5 VT, Gigasporaceae by 2 VT, Paraglomeraceae by 3 VT and Acaulosporaceae by 1 VT. The number of VT in different provenances and sites was compared. Tutira, Ruatiti and Rangitatau recovered 59, 57 and 50 VT respectively. A majority of VT (41) were shared among provenances. However, a few unique VT were found at just one site. Ruatiti showed 10 unique VT, Tutira 4 VT and Rangitatau only 1 unique VT (Fig. 4.11 a). The taxonomic classification of each unique VT associated with site and provenance indicated the predominance of Glomeraceae (42.4%) and Acaulosporaceae (24.2%) (Fig. 4.12) (Appendix 4.6).

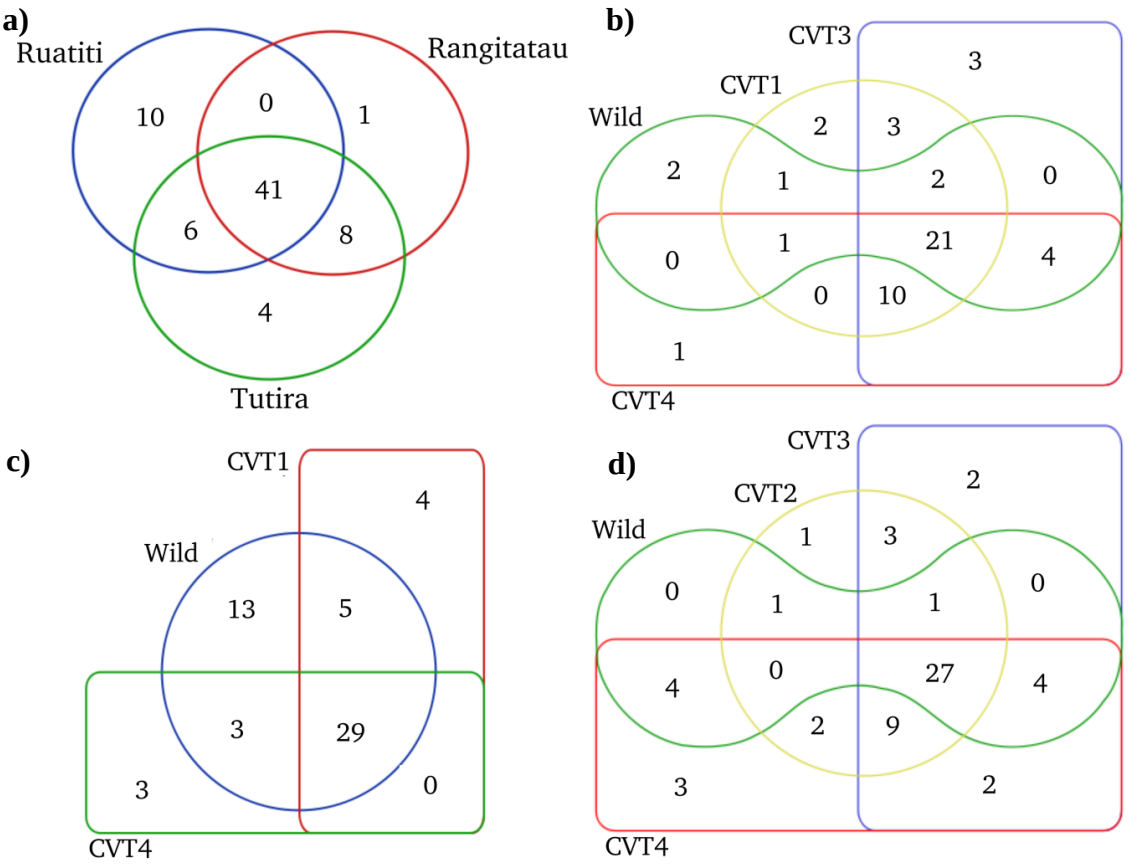


Fig. 4.11. Venn diagram showing shared and unique VT[1] identified as AM fungal retained from roots of cultivated (CVT1, CVT2, CVT3 and CVT4) and wild plants of *L. scoparium* a) overall and per site: b) Rangitatau, c) Ruatiti and d) Tutira.

The family Glomeraceae dominated the AM fungal diversity, followed by the family Claroideoglomeraceae (Fig. 4.12). Wild plants from Ruatiti (61) showed the highest number of VT, followed by Tutira with 47 and Rangitatau with 33. A range of other families were also present. Paraglomeraceae was represented by a limited number of VT at Ruatiti (4 VT) and at Tutira (5 VT), but was absent at Rangitatau. Similar results were found for the family Gigasporaceae, which was absent at Rangitatau and in the wild plants at Tutira. Whereas no taxa within the family Gigasporaceae were found in wild plants at Tutira, four VT were recovered from wild plants at Ruatiti. In addition, wild plants at Ruatiti were the only plants that recovered a VT identified as belonging to Ambisporaceae. These plants at Ruatiti (5 VT) also contained more taxa classified within the Diversisporaceae family than Tutira (3 VT) and Rangitatau (1 VT). Among sites, the family Acaulosporaceae and Archaeosporaceae were common to all the provenances. While the family Acaulosporaceae presented VT that ranged between two to six VT per provenance, the family Archaeosporaceae ranged from three to fifteen VT per provenance. Rangitatau and Ruatiti were more even, with the number of VT found per

provenance within the family Archaeosporaceae. However, Tutira showed more variable range among VT, presenting CVT3 15 VT and CVT4 only 3VT.

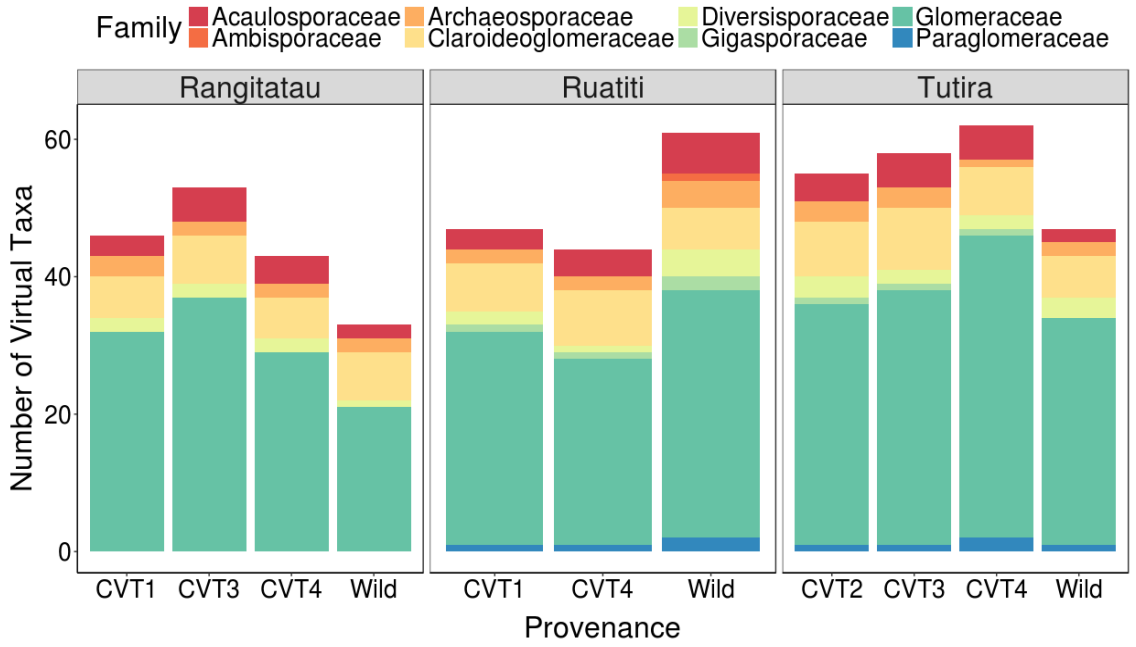


Fig. 4.12. Number of different VT[3] recovered from roots of cultivated (CVT1, CVT2, CVT3 and CVT4) and wild plants of *L. scoparium* at Rangitatau, Ruatiti, and Tutira.

4.3.3.3. Phylogenetic analysis

Of the 203 OTUs, 76 OTUs were retained after a filter (query coverage 100%, max identity > 97%) was applied (Appendix 4.7) and a phylogenetic tree generated (Fig. 4.13). Eight AM fungal genera were identified, though *Glomus* was the most abundant genus represented by 72.9% of the sequences, followed by *Claroideoglomus* (17.5%), *Archaeospora* (5.2%), *Acaulospora* (2.3%), and *Diversispora* (1.7%). Although the genera, *Scutellospora*, *Paraglomus* and *Ambispora* were present, they were represented by less than 1% of the sequences. *Scutellospora* was represented by four OTUs at both Ruatiti and Tutira. However, this genus was absent at Rangitatau.

The family Glomeraceae was most commonly represented by OTU_2 and OTU_3, which were classified within the genus *Glomus.* These two OTUs were dominant in cultivar CVT4 and wild plants at Tutira, and higher in CVT4 than in the wild plants. Whereas OTU_2 retained 226,066 sequences in CVT4 and 99,358 sequences in wild plants, OTU_3 retained 74,951 sequences and 49,123 sequences respectively (Appendix 4.8). For the family Claroideoglomeraceae, the majority of the sequences were retained within the OTU_10, OTU_6 and OTU_55. These OTUs, classified within the genus

Claroideoglomus, were present in all the provenances at all the sites. While OTU_10 was abundant in CVT2 (24,173 sequences) and CVT3 (30,929 sequences) at Tutira, OTU_6 was mainly present in wild plants (24,007 sequences) at Tutira and CVT3 (22,813 sequences) at Rangitatau. At Rangitatau, OTU_55 was most commonly found in wild plants (24,079 sequences).

Among the OTUs classified within the family Acaulosporaceae, OTU_18 and OTU_34 were predominant. Compared with wild plants at Rangitatau (0 sequences) and Tutira (248 sequences), the majority of sequences were OTU_18 (1304 sequences) at Ruatiti. Furthermore, wild plants at Ruatiti were the only plants that contained OTU_99 (177 sequences), a taxon that appears to be closely related to *Archaeospora schenckii* (Archaeosporaceae). However, the family Archaeosporaceae was mainly represented by OTU_7, which was represented by 10,000 sequences in the cultivars CVT2 and CVT3 at Tutira, and in CVT4 at Rangitatau.

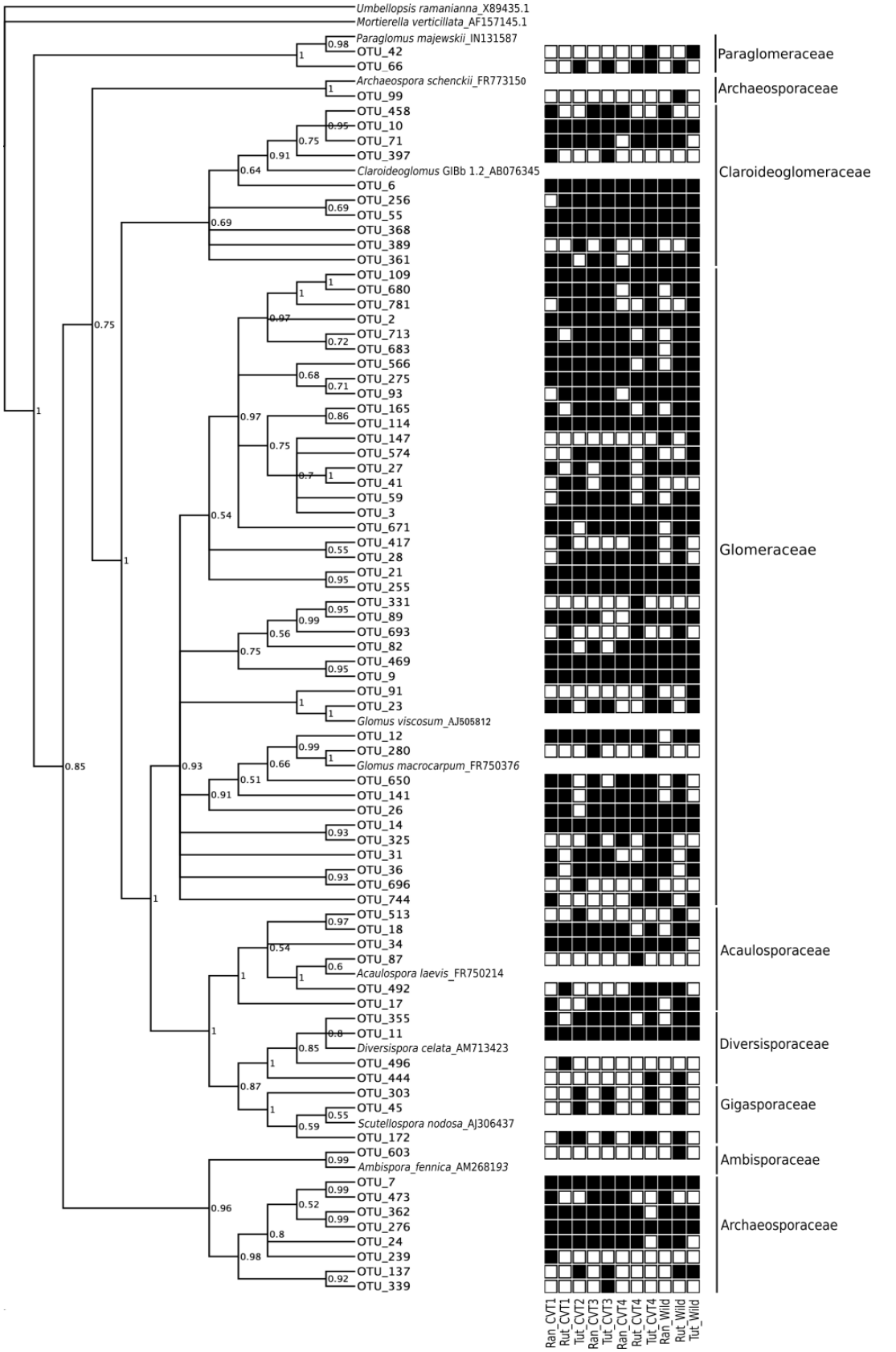


Fig. 4.13. Phylogenetic tree from Mr Bayes showing the phylogenetic relationship of the AM fungal sequences with a query cover of 100% and at least 97% of identity with representative sequences from Maarj*AM* database. The values next to the branches indicate Bay posterior probabilities. Presence of the OTU in roots of cultivated (CVT1, CVT2, CVT3 and CVT4) and wild plants of *L. scoparium* at Rangitatau, Ruatiti, and Tutira is shown by filled squares.

Among the 76 OTUs of AM fungi found (Appendix 4.9), a total of 16 OTUs were represented by more than 10,000 sequences (Table 4.11). These sequence OTUs were matched against NCBI and the top listed species results indicated ten different AM fungal species classified into six different genera (*Acaulospora*, *Claroideoglomus*, *Diversispora*, *Geosiphon*, *Glomus* and *Rhizophagus*). The three most abundant closely related species were *Rhizophagus irregularis* with 100% identity (592,218 sequences), *Rhizophagus proliferus* with 97% (188,050 sequences), and *Claroideoglomus lamellosum* with 98% (110,321 sequences) (Fig. 4.14). At all sites, these three (or at least closely related species) and the species closely related to *Geosiphon pyriforme* were dominant. The species closely related to *R. irregularis* was the most abundant at all sites. In contrast, the species closely related to *R. proliferus* colonised plants mostly at Tutira (10.32%), moderately at Rangitatau (6.94%), and it was almost absent at Ruatiti (0.1%). At Ruatiti, the species closely related to *C. lamellosum* (25.5%) was more abundant than at Tutira (14.99%) and Rangitatau (9.14%). Rangitatau showed the highest abundance of *G. pyriformis* (7.29%) compared with Ruatiti (6.62%) and Tutira (3.37%).

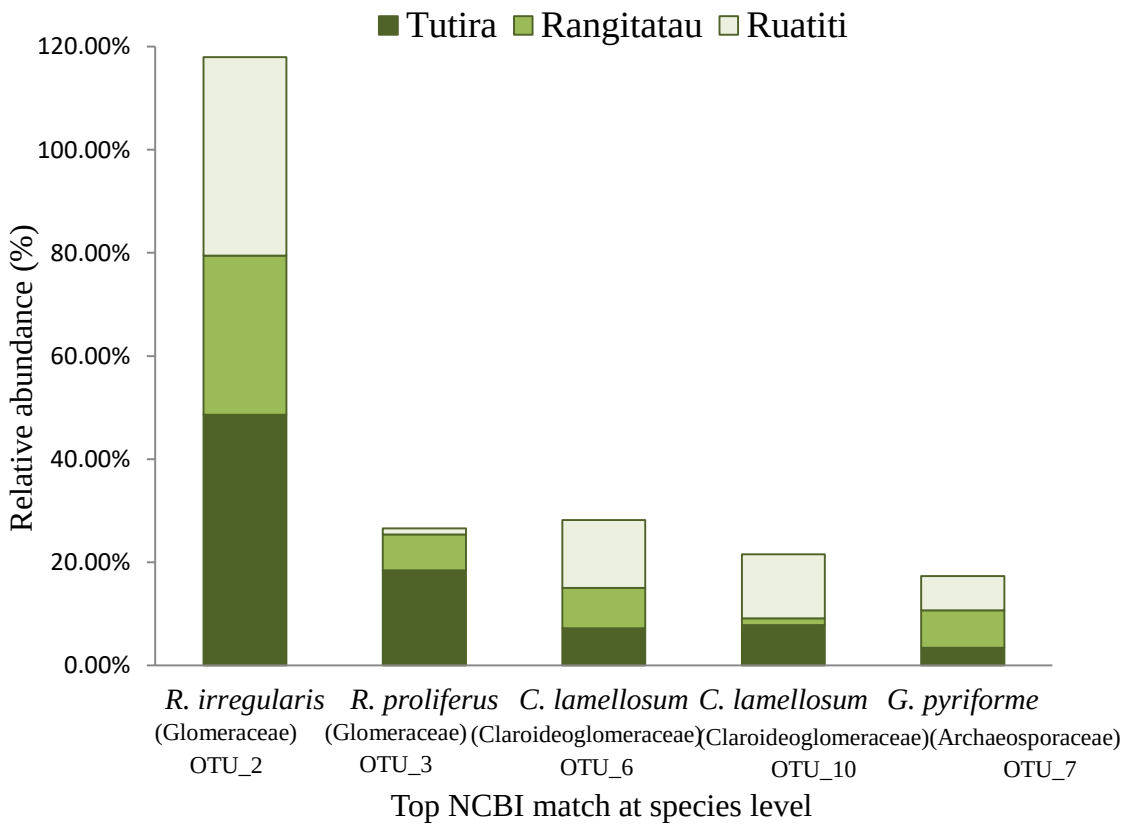


Fig. 4.14. Relative abundance of the top NCBI matches at species level for the most abundant five OTUs that retained more than 10,000 sequences in roots of cultivated (CVT1, CVT2, CVT3 and CVT4) and wild plants of *L. scoparium* at Rangitatau, Ruatiti, and Tutira.

Table 4.11. The top NCBI matches at species level and the top match from Maarj*AM* database for OTUs represented by more than 10,000 sequences. The NCBI accession number, Maarj*AM* virtual taxon, identity values from NCBI and Maarj*AM*, and total number of sequences recovered from the study that matched to the defined OTUs are indicated. All matches indicated a query cover of 100%. The taxonomic classification at species level is shown according to Mycobank.

OTU ID	Family	NCBI match	NCBI acc. no.	NCBI identity (%)	Maarj*AM* match	Maarj*AM* Virtual taxon	Maarj*AM* identity (%)	Total sequences
17	Acaulosporaceae	*Acaulospora brasiliensis*	KU359227.1	96%	*Acaulospora*	VTX00010	98.6%	15,114
18	Acaulosporaceae	*Acaulospora brasiliensis*	FN825899.1	99%	*Acaulospora*	VTX00228	99.8%	11,376
6	Claroideoglomeraceae	*Claroideoglomus lamellosum*	KU136434.1	98%	*Claroideoglomus*	VTX00057	100%	110,321
10	Claroideoglomeraceae	*Claroideoglomus lamellosum*	KX879068.1	94%	*Claroideoglomus*	VTX00056	100%	89,488
55	Claroideoglomeraceae	*Claroideoglomus lamellosum*	KX879068.1	98%	*Claroideoglomus*	VTX00193	98.9%	51,408
11	Diversisporaceae	*Diversispora aurentia*	KU136395.1	99%	*Diversispora*	VTX00054	99.8%	20,427
7	Geosiphonaceae	*Geosiphon pyriforme*	AJ276074.2	92%	*Archaeospora*	VTX00005	99.8%	66,327
114	Glomeraceae	*Glomus fasciculatum*	AY919853.1	96%	*Glomus*	VTX00223	98.2%	62,075
12	Glomeraceae	*Glomus macrocarpum*	FR772325.1	96%	*Glomus*	VTX00191	100%	24,555
93	Glomeraceae	*Rhizophagus clarus*	KP144311.1	97%	*Glomus*	VTX00345	99.5%	13,032
9	Glomeraceae	*Rhizophagus iranicus*	KX879064.1	98%	*Glomus*	VTX00315	100%	61,973
14	Glomeraceae	*Rhizophagus iranicus*	HM153423.1	97%	*Glomus*	VTX00153	100%	39,579
26	Glomeraceae	*Rhizophagus iranicus*	HM153424.1	96%	*Glomus*	-	99.8%	22,656
2	Glomeraceae	*Rhizophagus irregularis*	KX879067.1	100%	*Glomus*	VTX00113	100%	592,218
21	Glomeraceae	*Rhizophagus irregularis*	KX879063.1	97%	*Glomus*	VTX00219	100%	18,921
3	Glomeraceae	*Rhizophagus proliferus*	KM114365.1	97%	*Glomus*	VTX00074	100%	188,050

Among sites, the most abundant 10 OTUs were determined and compared among provenances (Table 4.12). Overall, the most abundant OTUs matched with species closely related to the genera *Rhizophagus* and *Claroideoglomus*. The species closely related to *Rhizophagus irregularis* was the most common across the sites and within cultivars CVT3 and CVT4 at Rangitatau. In Tutira it was also abundant in CVT4 and wild plants, while at Ruatiti it was mostly present in wild plants.

Table 4.12. Total number of sequences retained within the closest NCBI match at species level for the most abundant 10 OTUs per cultivated (CVT1, CVT2, CVT3 and CVT4) and wild plants of *L. scoparium* at Rangitatau, Ruatiti, and Tutira. Cell colour indicates the study site (Green: Ruatiti, blue: Rangitatau and Red: Tutira).

Site	OTU_ID	Closest Genbanck match (% identity)	CVT1	CVT2	CVT3	CVT4	Wild
Rangitatau	2	*Rhizophagus irregularis* (100%)	15,347	-	55,910	44,572	3,328
	114	*Glomus fasciculatum* (96%)	10,198	-	33,899	14,877	118
	14	*Rhizophagus iranicus* (97%)	1,627	-	12,039	18,310	1,486
	6	*Claroideoglomus lamellosum* (98%)	86	-	22,813	299	7,058
	7	*Geosiphon pyriforme* (92%)	5,547	-	4,633	12,907	5,042
	3	*Rhizophagus proliferus* (97%)	331	-	19,200	7,219	19
	9	*Rhizophagus iranicus* (96%)	187	-	3,577	2,708	19,378
	55	*Claroideoglomus lamellosum* (98%)	6	-	4	162	24,079
	12	*Glomus macrocarpum* (96%)	683	-	8,264	1,079	0
	59	*Rhizophagus clarus* (97%)	0	-	3	7,837	0
Ruatiti	2	*Rhizophagus irregularis* (100%)	5,533	-	-	10,359	36,410
	6	*Claroideoglomus lamellosum* (98%)	2,963	-	-	12,182	2,685
	10	*Claroideoglomus lamellosum* (94%)	2,684	-	-	1,978	12,224
	26	*Rhizophagus iranicus* (96%)	377	-	-	6,467	7,098
	7	*Geosiphon pyriforme* (92%)	699	-	-	2,994	5,301
	12	*Glomus macrocarpum* (96%)	1	-	-	1,438	4,413
	9	*Rhizophagus iranicus (* 96%)	301	-	-	1,544	3,859
	89	*Rhizophagus iranicus* (99%)	44	-	-	3,501	435
	11	*Diversispora aurentia* (99%)	22	-	-	1	2,874
	141	*Glomus macrocarpum* (95%)	111	-	-	1,502	1,278
Tutira	2	*Rhizophagus irregularis* (100%)	-	20,065	75,270	226,066	99,358
	3	*Rhizophagus proliferus* (97%)	-	21,013	14,627	74,951	49,123
	10	*Claroideoglomus lamellosum* (94%)	-	24,173	30,929	8,307	4,195
	6	*Claroideoglomus lamellosum* (98%)	-	7,201	13,650	17,377	24,007
	9	*Rhizophagus iranicus* (96%)	-	2,626	5,936	16,840	5,017
	7	*Geosiphon pyriforme* (92%)	-	13,375	14,844	971	14
	55	*Claroideoglomus lamellosum* (98%)	-	7,585	12,821	1,631	2,944
	11	*Diversispora aurentia* (99%)	-	2,879	7	151	14,487
	93	*Rhizophagus clarus* (97%)	-	143	490	11,114	607
	21	*Rhizophagus irregularis* (97%)	-	3,282	2,862	2,114	713

4.3.3.4. Community analysis of AM fungal communities on cultivated and wild plants

Results from the PermANOVA test indicated the significant influence of the provenance on the AM fungal community at all three sites (Table 4.13). Nonmetric Multidimensional Scaling showed differences in AM fungal pattern between wild plants and cultivated plants at Tutira (Fig. 4.15). At Tutira, the cultivars CVT2 and CVT3 were clustered since they presented a similar AM fungal community, with an abundance of the family Claroideoglomeraceae and Glomeraceae (Fig. 4.15c). However, plants within CVT4 and wild plants were mostly dominated by Glomeraceae. The NMDS analysis showed that CVT1, CVT3, and CVT4 were clustered against wild plants (Fig. 4.15a). The main difference was that cultivated plants were mainly dominated by the family Glomeraceae, while wild plants were heavily represented by the family Claroideoglomeraceae. Results from the NMDS revealed that the AM communities among cultivated and wild plants were more similar, as no clear cluster distinction was found among provenances.

Table 4.13. Results of PermANOVA test showing the significance of the variable provenance (CVT1, CVT2, CVT3, CVT4 and wild) on AM fungal families at Rangitatau, Ruatiti, and Tutira.

Site; provenance	d.f.	Sum of squares	Mean squares	*F*	*P*
Rangitatau					
Provenance	3	1.91	0.64	2.71	**0.002**
Residuals	28	6.57	0.23		
Total	31	8.48			
Ruatiti					
Provenance	2	1.17	0.58	1.59	**0.031**
Residuals	24	8.79	0.37		
Total	26	9.96			
Tutira					
Provenance	3	2.19	0.73	4.26	**0.001**
Residuals	32	5.49	0.17		
Total	35	7.68			

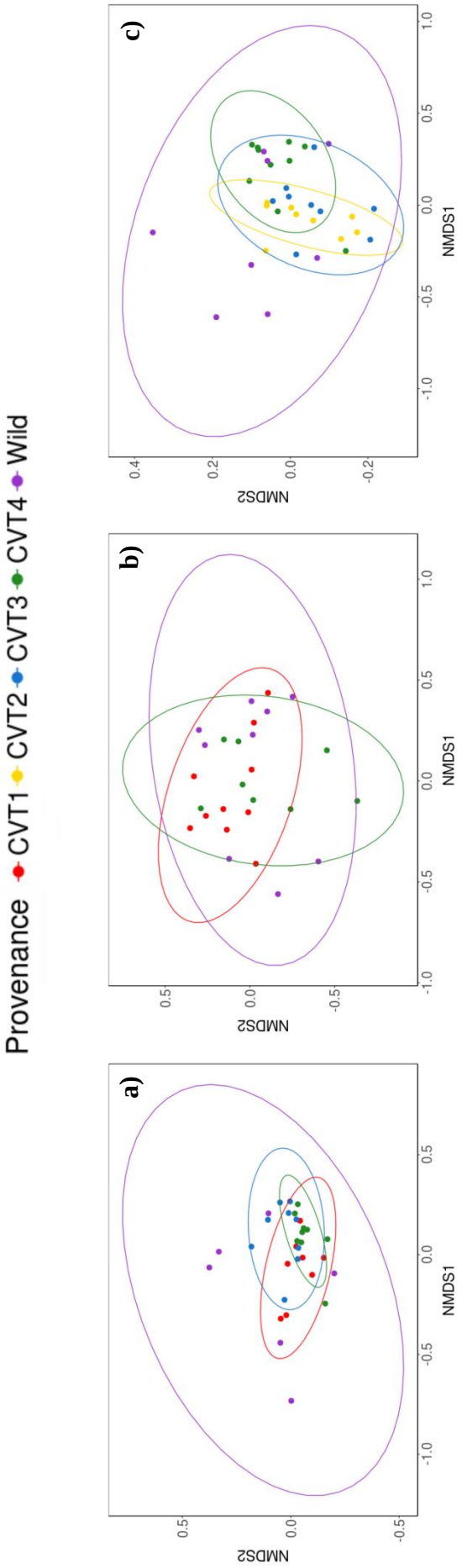


Fig. 4.15. Nonmetric Multidimensional Scaling (NMDS) plots on the Bray-Curtis matrix calculated from the number of sequences recovered from each AM fungal family from roots of cultivated and wild plants of *L. scoparium* at a) Rangitatau (stress value 0.126); b) Ruatiti (stress value 0.202) and c) Tutira (stress value 0.091).

4.4. Discussion

This study examined the diversity and distribution of mycorrhizal fungi and other associated fungi among different *L. scoparium* provenances and sites. The results demonstrated that *L. scoparium* is associated with a diverse fungal community. Specifically, this host is associated with at least sixteen EcM fungal lineages and eight AM fungal families. Despite the majority of lineages being present in all provenances, wild plants showed a more diverse mycorrhizal fungal community than cultivated plants, but cultivated plants still showed considerable diversity despite the relative youth of the plantations.

4.4.1. Fungal communities associated with Leptospermum scoparium

This study revealed the association of *L. scoparium* with a widely diverse fungal community within the phyla Ascomycota, Basidiomycota and Mucoromycota. Ascomycota and Basidiomycota were the two major groups associated with *L. scoparium*, but this is not surprising, as most fungal species belong to these two fungal phyla (Buée et al., 2009). Among both phyla, Ascomycota was mostly represented by the classes Sordariomycetes and Dothideomycetes, which are also globally defined as the dominant classes (Kirk et al., 2008). Previous studies have shown they can be dominant in different contexts. For instance, whereas Ding et al. (2017) found these two classes dominant in farmland Black soils, Johansen et al. (2016) showed their dominance on *Spinifex sericeus* R.Br. (Poaceae) in New Zealand dunes. As with Johansen's study, in this study Agaricomycetes was revealed as the main class within the phylum Basidiomycota. This class, dominant in temperate forests (Buée et al., 2009), also had the majority of EcM fungal lineages associated with *L. scoparium*. Among the EcM lineages, the genera *Tomentella* Pers. ex Pat., *Clavulina* J.Schröt., *Laccaria* Berk. & Broome, *Cortinarius* (Pers.) Gray and *Russula* Pers. were dominant. In New Zealand, these five genera have been previously associated with *L. scoparium* and *Nothofagus* Blume (Nothofagaceae) (McKenzie, Buchanan, & Johnston, 2000; Orlovich & Cairney, 2004; McKenzie et al., 2006; Johnston et al., 2010). However, the molecular identification of these fungal associations has been limited within *L. scoparium* to date. In contrast, the use of molecular techniques has revealed the dominance of *Cortinarius*, *Laccaria* and *Russula* in *Nothofagus* rainforests (Dickie, Richardson, & Wiser, 2009). Dickie et al. (2009) indicated the similarity of EcM fungal communities within *Nothofagus* in New Zealand

and Chile, which supports previous studies, where it was suggested that Australasia and South America are characterised by similar EcM fungal communities (McNabb, 1969; Horak, 1983; Tedersoo et al., 2010). Among EcM lineages, Dickie et al. (2009) also reported the presence of the /tomentella-thelephora lineage, which is globally distributed (Tedersoo et al., 2010). However, compared with other lineages, /tomentella-thelephora showed a low species-richness in Dickie's study (2009). Interestingly, this lineage was dominant within *L. scoparium*, but the identity match was between 90% and 94%, and species identification was limited. Previous studies have also found it challenging to identify species within this lineage in New Zealand (Dickie et al., 2009; Walbert et al., 2010; Dickie et al., 2010), which suggests that potentially novel species could be recovered within this lineage.

In contrast to /tomentella-thelephora, the lineages /russula-lactarius and /cortinarius have shown better identity matches in New Zealand studies (Dickie et al., 2009, 2010; Walbert et al., 2010). These two global genera are commonly associated with *Leptospermum* and *Nothofagus* (Taylor, 1970; McNabb, 1971; Orlovich & Cairney, 2004; Peintner, Moncalvo, & Vilgalys, 2004). Specifically, the genus *Cortinarius* is widely diverse in Australia, New Zealand and the South of South America (Dickie et al., 2010; Tedersoo et al., 2010). In New Zealand, this genus is considered one of the largest EcM fungal lineages, being represented in *Nothofagus* by 176 species and in *L. scoparium* by 30 species (Teasdale et al., 2013). In Tasmania, a study found that *Cortinarius* retained the highest number of OTUs and the highest diversity within *Eucalyptus delegatensis* R.T.Baker (Myrtaceae) (Horton et al., 2017). However, even though *Cortinarius* is well represented in Australia and New Zealand, studies have shown their limited presence on exotic host plants such as *Pinus radiata* D. Don (Pinaceae), *Alnus glutinosa* (Betulaceae) and *Salix fragilis* (Salicaceae) (Walbert et al., 2010; Bogar, Dickie, & Kennedy, 2015). Along with *Cortinarius*, *Descolea* is recognised for being common in the Southern Hemisphere (Horak, E., 1971; Tedersoo et al., 2010; Kuhar et al., 2017). Furthermore, this genus is suggested as having originated in the Southern Hemisphere (Horak, 1983; Bougher & Malajczuk, 1985) and potentially to have co-evolved with *Nothofagus* (Horak, E., 1971; Horak, 1983). However, although it co-evolved with *Nothofagus*, the species richness of *Descolea* is apparently limited in New Zealand *Nothofagus* forests (Dickie et al., 2009) and its abundance was apparently limited within the *L. scoparium* in the present

study. This result is not surprising, as *Descolea* is also recognised for colonising mainly *Nothofagus* (Kuhar et al., 2017).

As with the EcM fungal communities, the AM fungal communities associated with *L. scoparium* were also diverse in this study overall. This is not surprising, as arbuscular mycorrhizal fungal species have been found to be more widespread around the world that first thought (Davison et al., 2015). They can be found in a wide range of habitats, which includes from tropical forests to grasslands (Souza, 2015). Among the AM families present, the family Glomeraceae was dominant, which is the most dominant family within Glomeromycota (Schüßler & Walker, 2010; Krüger et al., 2012) and its abundance in roots (Varela-Cervero et al., 2015; Alguacil et al., 2016; Xu et al., 2017).

In New Zealand, this family has been previously found associated with *Agathis australis* (D.Don) Loudon (Araucariaceae) (Padamsee et al., 2016), *Spinifex sericeus*, and *Leymus mollis* (Trin.) Pilg. (Poaceae) (Johansen et al., 2015, 2016). All these hosts have also formed associations with other families such as Acaulosporaceae, Claroideoglomeraceae and Diversisporaceae (Hall, 1977; Johnson, 1977; Johansen et al., 2015, 2016; Padamsee et al., 2016). However, *A. australis*, *S. sericeus* and *L. mollis* have shown no associations with the families Ambisporaceae and Paraglomeraceae. Among these host plants, the associations within Claroideoglomeraceae were interesting, as this family was the second most abundant with *L. scoparium* and was mostly absent in previous New Zealand studies (Johansen et al., 2015, 2016; Padamsee et al., 2016). An explanation for why this study recover a wider AM community could be the genetic target region selected, the sequencing platform, and the host plant.

The target region is crucial for recovering fungal communities. For example, the SSU region has been widely used for studying AM fungal communities, because the majority of AM data available in public databases is generated from targeting the SSU region (Oehl et al., 2010; Lindahl et al., 2013; Hart et al., 2015; Taylor, Helgason, & Öpik, 2017). Furthermore, as SSU is considered a more conserved region than the ITS region, it facilitates phylogenetic studies (Stockinger et al., 2010; Johansen et al., 2017). For example, Johansen et al. (2017), using the primer pair ITS1F/ITS4 in their study, recovered only a limited number of sequences that appeared to belong to AM fungi, suggesting their primer pair choice was perhaps not optimal for examining AM fungal communities. Another recent study used the primer pair fITS7/ITS4 to evaluate the AM

fungal communities associated with *Thismia* Griff. (Thismiaceae) in Australia and New Zealand (Gomes et al., 2017). The majority of OTUs recovered from this study were classified within Glomales, while there were a limited number of OTUs from other lineages such as Archaesporales and Diversisporales. These findings suggest that, compared to the SSU region, the ITS region is currently a limiting factor for recovering AM fungal communities. Along with target region, the sequencing platform used is also indicated as an important factor for recovering more information and increasing DNA yield (Lindahl et al., 2013; Taylor et al., 2017). While fungal communities have been widely studied using the combination approach of ITS region and Illumina platform such as MiSeq, studies of AM fungal communities using this approach have been limited (Taylor et al., 2017). Further studies, therefore, should considerate the advantages of using this latter approach.

4.4.2. Diversity and distribution of fungal communities among Leptospermum scoparium provenances

The mycorrhizal fungal community found on *L. scoparium* was very diverse. While several mycorrhizal lineages were shared among provenances, there were few lineages present only in specific provenances like wild plants. Statistical analysis indicated provenance as an important driver on mycorrhizal fungal communities, which supports previous studies where host plant was a major factor driving fungal communities rather than soil conditions (Nguyen et al., 2016; Kolaříková et al., 2017; Krüger et al., 2017). However, the provenance effects, although significant, were quite small and other factors probably play an important role. Soil conditions (e.g. nutrients, pH, humidity) have been shown to affect mycorrhizal fungal communities (Long et al., 2010; Alguacil et al., 2016; Johansen et al., 2016; Casazza et al., 2017). Pollution level in soils impact on AM fungal communities, particularly on fungal taxa unrelated to the family Glomeraceae (Oehl et al., 2010). Specifically, highly-polluted soils are characterised by Glomeraceae species like *Rhizophagus irregularis* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schuessler, and low-polluted soils present species associated to families such as Claroideoglomeraceae (Hassan et al., 2014).

Along with soil conditions, host age and successional stage have also been shown to drive mycorrhizal fungal communities (Martínez-García, Richardson, Tylianakis, Peltzer, & Dickie, 2015; Kolaříková et al., 2017; Krüger et al., 2017b). Kolaříková et al.,'study

(2017) showed that whereas Pezizales was the dominant lineage in 12 year old mine spoil site, Thelephorales and Russulales dominated sites older than 20 years. Similar results were found in the present study, where /russula-lactarius lineage was more abundant in wild plants, which were naturally established in the field and probably older on average than cultivated plants. Contrary to /russula-lactarius, which is considered a late-stage lineage (Fernandez et al., 2017), /laccaria and /cortinarius are recognised as EcM lineages generally present in earlier stages (Last, Dighton, & Mason, 1987; Davey et al., 2015). Davey et al. (2015) also indicated that /cortinarius could be present in later stages, which supports the results obtained from Ruatiti in the present study. At Ruatiti, the three provenances could be assumed to differ in age, CVT4 being the youngest host and wild plants the oldest hosts. Results showed CVT4 dominated by /laccaria, CVT1 dominated by /cortinarius, and wild plants by /russula-lactarius lineage, which supports previous findings. However, the study of mycorrhizal fungal communities is complex as variables can influence each case study differently. In addition, different approaches are usually used for evaluating the fungal communities, including sample preservation, sequencing platforms, and bioinformatics analysis. Caution should therefore be taken when comparing results from different studies.

4.4.3. Mycorrhizal fungal species associated with Leptospermum scoparium

Results revealed the association of *L. scoparium* with species closely related to EcM fungal species endemic to New Zealand that are commonly associated with *Leptospermum* and *Nothofagus* (McNabb, 1972; Horak, 1988; Petersen, 1988; Soop, 2013) (Appendix 4.10). Specifically, species closely related to *Clavulina subrugosa* (Cleland) Corner, *Cortinarius waiporianus* Soop, *Laccaria glabripes* McNabb, and *Dermocybe indotata* E. Horak were included among the most abundant 20 EcM OTUs. Among these OTUs, the sequence OTU_3 matched the representative sequence of *L. glabripes* (HQ533019.1) with 100% identity, highly supporting this species match (Johnston & Park; unpublished). Therefore, it can be considered that *L. glabripes* colonised mostly cultivated plants in the present study, which is not surprising, as this fungus is commonly associated with nursery-raised plants (P. Johnston, pers. comm.). In particular, *L. glabripes* was abundant in cultivars CVT4 (Ruatiti), CVT1 (Rangitatau), and CVT3 (Tutira) in the present study. Interestingly, the representative sequence of *L. glabripes* (HQ533019.1) has been recently classified as a new lineage within the genus

Laccaria in Australia (Sheedy et al., 2013), which suggests the potential for finding new EcM fungal lineages from the data generated in the present study.

Along with *L. glabripes*, a species closely related to *C. waiporianus* was dominant on cultivated plants, particularly on the cultivar CVT1 at Ruatiti. The respective sequence OTU matched at 97–98% identity with a representative sequence of *C. waiporianus* generated from *Leptospermum* in New Zealand (KT875191.1) (Johnston & Park; unpublished). *Cortinarius waiporianus* was defined recently by Soop (2012), who indicated this species as endemic to New Zealand. This species belongs to the Cortinariaceae lineage, which is known for colonising *Nothofagus*, *Leptospermum*, and *Kunzea* in New Zealand (McKenzie et al., 2000, 2006; Tedersoo et al., 2010). However, as Teasdale et al. (2013) indicated, the information about *Cortinarius* and its associated EcM hosts in New Zealand is limited. In their study, Teasdale et al. (2013) compiled 26 *Cortinarius* species and four *Dermocyb*e species associated with *L. scoparium* in New Zealand that were reported in previous studies (McKenzie et al., 2000; Segedin & Pennycook, 2001; Soop, 2002; McKenzie et al., 2006; Gasparini & Soop, 2008; Soop, 2012). After Teasdale's study, another 24 *Cortinarius* species have been described in New Zealand (Soop, 2014, 2016), which indicates that *Cortinarius* is a very species-rich genus in New Zealand. Given the diversity within this genus, it is possible the sequence found in this study, which is similar to *C. waiporianus* but not an exact match, could represent a possible undescribed species. Besides *C. waiporianus*, another Cortinariaceae species closely related to *D. indotata* was found in this study. *Dermocybe indotata* was described approximately three decades ago (Horak, 1988) and has been recorded as associated with the hosts *L. scoparium* and *Nothofagus* in New Zealand (McKenzie et al., 2000, 2006). However, the identity match of the sequence OTU_10 with the representative sequence of *D. indotata* (KJ42110.1) was 97%, which suggests that OTU_10 could again represent another undescribed species related to *D. indotata*. This closely related species, like the species closely related to *C. waiporianus*, was abundant in cultivated plants in the present study. It was particularly abundant in the cultivar CVT3 at Rangitatau and the cultivars CVT1 and CVT4 at Tutira.

In comparison with cultivated plants, wild plants showed a broader EcM fungal community among sites. Importantly, the sequence OTU_4 was particularly abundant at Tutira. This OTU closely matched (97% identity) a representative sequence of *Clavulina*

subrugosa (JN228221.1) recovered from a New Zealand sporocarp[1] (Uehling et al., 2012). In addition, *C. subrugosa* has been previously associated with *L. scoparium* and *K. ericoides* in New Zealand[2], which suggests that OTU_4 does indeed represent *C. subrugosa*. Another consideration is that this could represent a known or unknown species closely related to *C. subrugosa*. For instance, a recent study has described a new species within the genus *Clavulina* from the South of China (He, Chen, & Yan, 2016). This species, *Clavulina livida* Shu Z. Yan, G. He & Shuang L. Chen, is phylogenetically very closely related to *Clavulina amethystine* (Bull.) Donk, *Clavulina cinerea* (Bull.) J. Schröt., *Clavulina coralloides* (L.) J. Schröt., *Clavulina samuelsii* R.H. Petersen, and *Clavulina subrugosa* (Cleland) Corner (He et al., 2016). Therefore, other unknown *Clavulina* species could be associated with wild plants of *L. scoparium*.

In addition to these endemic EcM fungal species (*C. subrugosa*, *C. waiporianus*, *L. glabripes*, and *D. indotata*), a species closely related to *Lactifluus subclarkeae* (Grgur.) Verbeken previously known from Australia was abundant in wild plants at Tutira. While the sequence OTU_8 matched (99%) with a representative sequence of *L. subclarkeae* (KR364095.1) endemic to Australia, it appears that *L. subclarkeae* has not been previously reported in New Zealand. This species was first described from Australia and currently its phylogenetic position is unclear within the genus *Lactifluus* (Pers.) Roussel, which is a genus commonly associated with *Eucalyptus* and *Leptospermum* (Grgurinovic et al., 1997; De Crop et al., 2017). Although *L. subclarkeae* was not previously recorded in New Zealand, other species like *Lactarius clarkeae* Cleland (= *Lactifluus clarkeae*) have been found associated with *L. scoparium* in New Zealand[3]. However, the association of *L. clarkeae* with *L. scoparium* was first reported as an unknown species (McKenzie et al., 2006). This suggests that even though there are no previous records of *L. subclarkeae* in New Zealand, this EcM fungus could have a long association with *L. scoparium*.

Another Australian species, *Descolea maculata* Bougher (KU523936.1), was matched to OTU_35 at 100% identity. This representative sequence was generated from Otago University in New Zealand (Orlovich & Anderson; unpublished), which suggests that OTU_35 is *D. maculata*. This species was firstly reported with *Eucalyptus diversicolor*

[1] https://unite.ut.ee/bl_forw.php?id=261855
[2] https://scd.landcareresearch.co.nz/Search

F.Muell. and *Eucalyptus marginata* Donn ex Sm. in Australia (Bougher & Malajczuk, 1985). In New Zealand, using a molecular approach, this species was reported as sharing habitat with *Leptospermum*. However, the morphological approach suggested that the *Descolea* species sharing habitat with *Leptospermum* is actually *Descolea gunnii* (Massee) E. Horak or another species[1]. Therefore, it cannot be concluded that *D. maculata* was previously recorded with *Leptospermum*, but a species closely related to *D. maculata* was present in cultivar CVT3 at Rangitatau in the present study.

As was the case for the EcM fungal species, a few AM fungal species revealed their dominance among provenances; particularly, species closely related to *Rhizophagus irregularis* and *Claroideoglomus lamellosum* (Dalpé, Koske & Tews) C. Walker & A. Schüßler. The former is known for being a generalist AM fungi usually found in grassland and it has been widely used as inoculum (Ceballos et al., 2013).

However, the presence of these species and their respective genera in New Zealand is limited and not well understood. *Claroideoglomus lamellosum* has recently been used along with *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler and *Scutellospora calospora* (T.H. Nicolson & Gerd.) C. Walker & F.E. Sanders as mycorrhizal inoculum (Monk et al., 2014). These three AM fungal species have been isolated from New Zealand farm soil collected in the Canterbury region (Monk et al., 2014). *Rhizophagus irregularis* has been also used as inoculum with *Trifolium subterraneum* L. (Fabaceae) in New Zealand soils (Orchard et al., 2017).

In contrast, other AM fungal genera have been previously recorded in New Zealand soils. Mosse & Bowen (1968) showed the presence of different Endogonaceae spores collected from Australian and New Zealand soils. However, as Hall (1977) indicated, spore details and taxonomic classification were limited in Mosse's study. In their study, Mosse & Bowen (1968) found that wide-neck spores presented a similar colonisation pattern to *Glomus tenue* (Greenall) I.R. Hall spores. In 1977, Hall confirmed the association of this fungus, and *Acaulospora laevis* Gerd. & Trappe and *Glomus pallidum* I.R. Hall with *L. scoparium*. *Glomus tenue* and *G. pallidum* were not found in this study. A species closely related to *Glomus macrocarpum* Tul. & C. Tul. , which has a possible but not yet clearly established association with *L. scoparium* (Hall, 1977; McKenzie et al., 2006), was

[1] https://scd.landcareresearch.co.nz/Search

recovered in the present study. Importantly, *G. macrocarpum* has been found associated with other native hosts such as *Coprosma robusta* Raoul (Rubiaceae) and *Microlaena avenacea* (Raoul.) Hook.f. (Poaceae) (Hall, 1977; Johnson, 1977), which could promote the dispersal of this AM fungus. In the present study it was found sequences that were closely related to *G. macrocarpum* and the placement of the representative OTUs close to this species were very well supported (1) in the phylogenetic tree. Thus, results suggest that *G. macrocarpum* or an unknown closely related species is associated with *L. scoparium*. As Öpik, Zobel, et al. (2013) suggested, it is expected that there will be a discovery of a large number of AM fungal species within Glomeromycota in Oceania. Therefore, a novel *Glomus* species could be associated with *L. scoparium*.

Another OTU matched with a representative sequence of *A. laevis* at 100% identity. This species was previously reported for being associated with *L. scoparium* (Hall, 1977), but the number of sequences recovered from *L. scoparium* was small in the present study. On the other hand, a species closely related to *A. brasiliensis* presented a higher number of sequences. A recent study has also reported a species closely related to *A. brasiliensis* in the root nodules of *A. australis* in New Zealand (NS31_OTU_185) (Padamsee et al., 2016). Results from the present study therefore suggest that *A. brasiliensis* is potentially present in New Zealand soils. Another potential AM fungal species is *Diversispora aurentia* (Blaszk., Blanke, Renker & Buscot) C. Walker & A. Schüßler, which matched 99% identity with the OTU_11 in this study. This closely related species was represented by more than 20,000 sequences and was most common in wild plants at Tutira, though it was also found at Ruatiti. This result suggests that specific mycorrhizal fungal species or lineages might occur only on wild plants, and not in cultivated plants.

This study showed that *L. scoparium* is colonised by a widely diverse fungal community that includes both EcM and AM mycorrhizal fungi. While it has been suggested that EcM fungal presence can inhibit the growth of AM fungi (Chilvers et al., 1987), this study revealed the coexistence among both EcM and AM fungal groups, which were both present as a diverse mycorrhizal community on *L. scoparium*. Furthermore, while it was expected that cultivated plants would present a reduced fungal community compared with wild plants, it seems that both cultivated and wild plants share a diverse range of fungal lineages. As results indicated, cultivars established in the field for just a few years are already naturally colonised by mycorrhizal fungi, and an additional mycorrhizal fungal

inoculum is not required. However, specific mycorrhizal fungal lineages could be only associated with wild plants and be absent on cultivated plants. Subsequently, wild plants could receive some benefits from these fungi that would be absent on cultivated plants.

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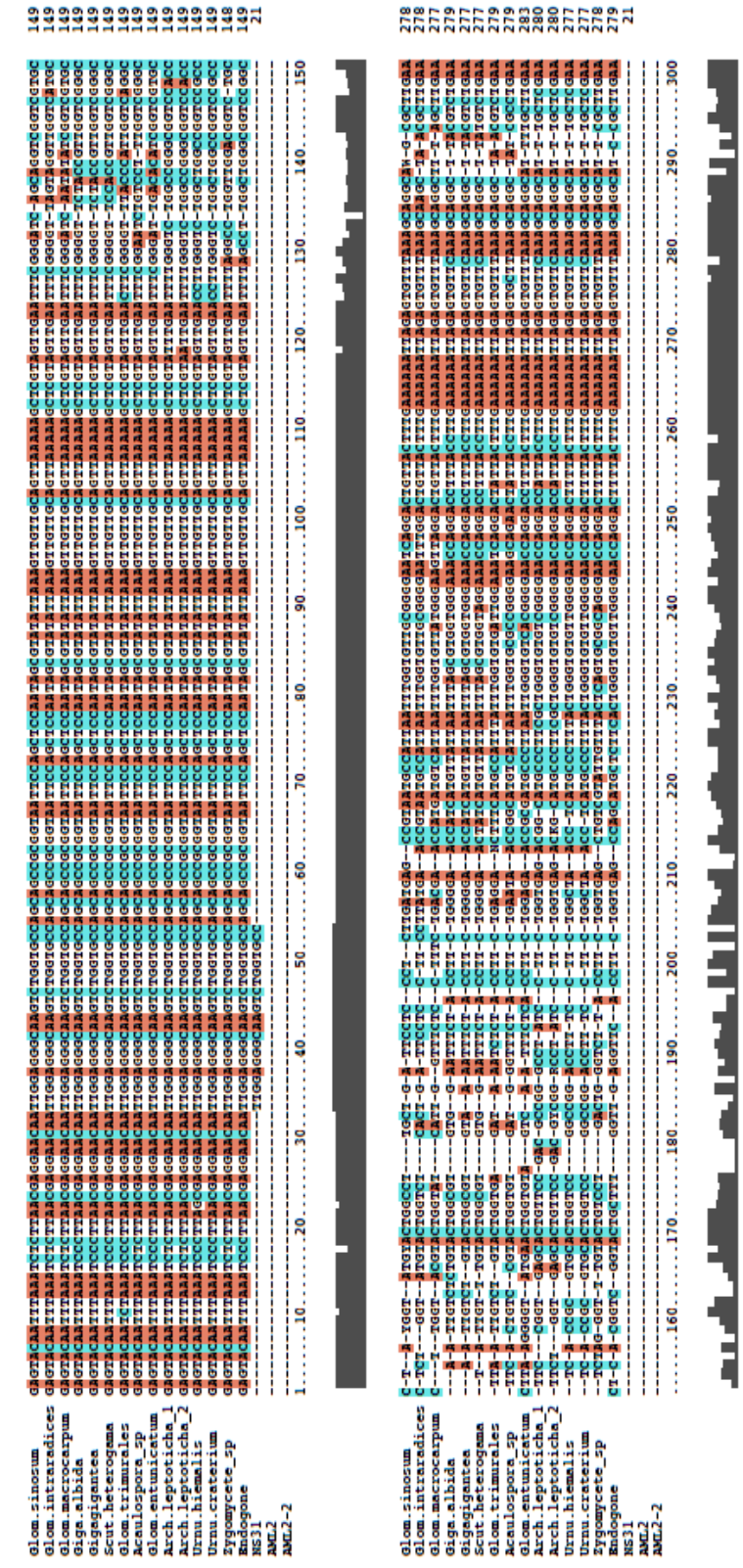
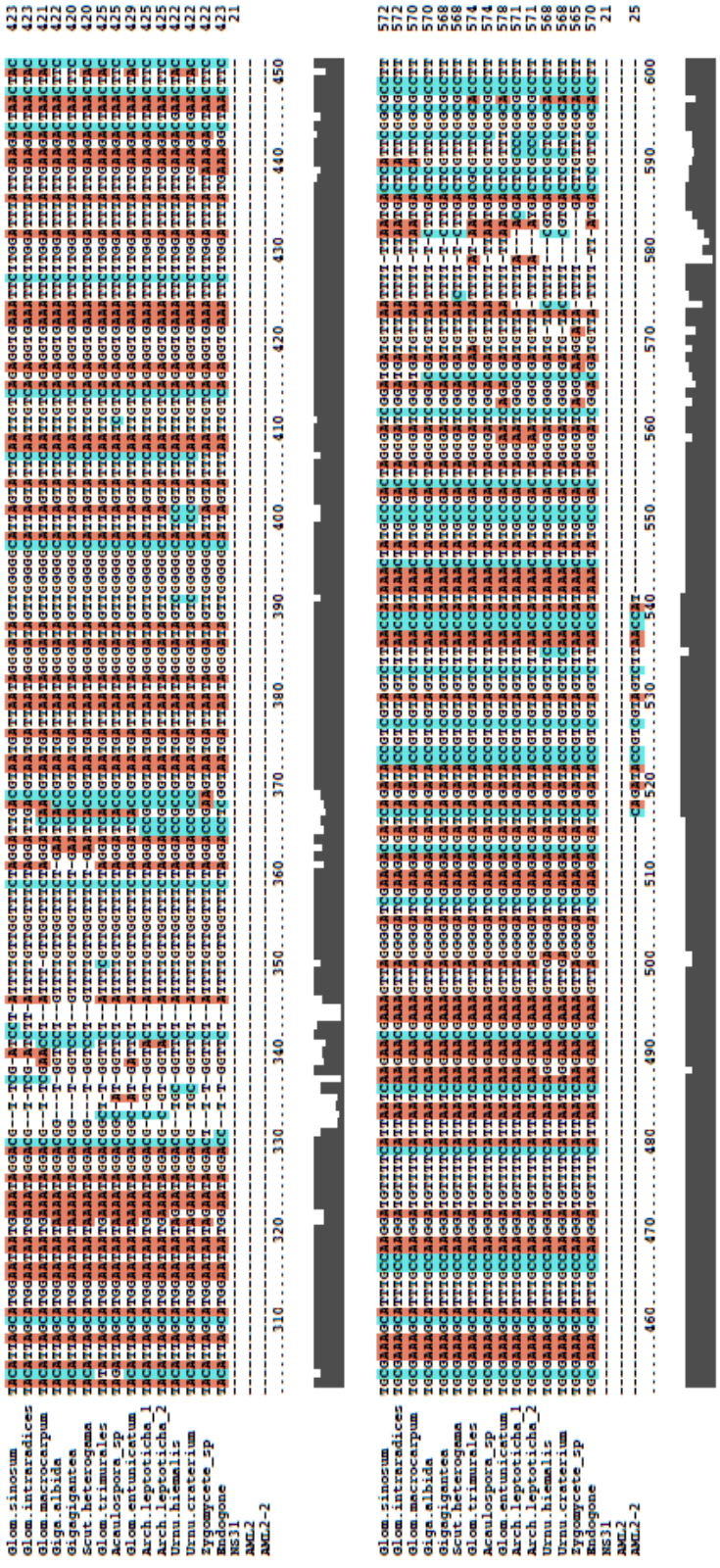
4.6. Appendices



Appendix 4.1. Multiple sequence alignment (CLUSTAL) from the 16 sequences of AM fungi and non-AM fungi and the universal eukaryotic primer NS31, the AM fungal specific primer AML2 and the newly designed reverse primer AML2-2. Sequences from AM fungal and non-AM fungal species were selected from public databases (i.e. NCBI, Maarj*AM*, and SILVA).



Glom.sinosum
Glom.intraradices
Glom.macrocarpum
Giga.albida
Gigagigantea
Scut.heterogama
Glom.trimurales
Acaulospora_sp
Glom.entunicatum
Arch.leptoticha_1
Arch.leptoticha_2
Urnu.hiemalis
Urnu.craterium
Zygomycete_sp
Endogone
NS31
AML2
AML2-2
610
620
630
640
650
660

Appendix 4.2. The top NCBI match for the OTUs classified as an EcM fungal lineage (Tedersoo, 2010) associated with cultivated and wild plants of *L. scoparium* at Rangitatau, Ruatiti and Tutira. OTUs were classified according the EcM fungal lineage (Tedersoo, 2010), species hypothesis (UNITE) and NCBI.

OTU ID	Order	EcM lineage	NCBI match	UNITE reference database	Species hypothesis	Number of sequences	NCBI identity (%)
301	Agaricales	/amanita	*Amanita muscaria*	*Amanita muscaria*	SH028892.07FU_JX316282_reps_singleton	1653	99%
2243	Agaricales	/cortinarius	*Cortinarius australis*	*Cortinarius australis*	SH178000.07FU_JQ346863_reps	11	97%
1953	Agaricales	/cortinarius	*Cortinarius cretax*	*Cortinarius* sp.	SH189367.07FU_FJ553568_reps	12	98%
2436	Agaricales	/cortinarius	*Cortinarius cystidiocatenatus*	*Cortinarius* sp.	SH192265.07FU_KF007260_reps	2397	100%
10	Agaricales	/cortinarius	*Cortinarius indotatus*	*Cortinarius* sp.	SH223047.07FU_UDB002760_reps	179,109	97%
228	Agaricales	/cortinarius	*Cortinarius junghuhnii*	*Cortinarius* sp.	SH193728.07FU_JX414197_reps	2112	99%
12	Agaricales	/cortinarius	*Cortinarius* sp.	*Cortinarius waiporianus*	SH222467.07FU_UDB004058_reps	159,090	99%
3158	Agaricales	/cortinarius	*Cortinarius* sp.	*Cortinarius waiporianus*	SH089647.07FU_HQ204746_refs_singleton	104,104	97%
20	Agaricales	/cortinarius	*Scleroderma* sp.	*Cortinarius* sp.	SH179808.07FU_UDB013841_refs	63,063	100%
2560	Agaricales	/cortinarius	*Cortinarius* sp.	*Cortinarius waiporianus*	SH002707.07FU_JF714649_reps_singleton	42,111	95%
3241	Agaricales	/cortinarius	*Cortinarius* sp.	*Cortinarius waiporianus*	SH184599.07FU_JF960837_reps	10,626	91%
2221	Agaricales	/cortinarius	*Cortinarius* sp.	*Cortinarius waiporianus*	SH031509.07FU_DQ328069_reps_singleton	3711	98%
3102	Agaricales	/cortinarius	*Cortinarius* sp.	*Cortinarius waiporianus*	SH222405.07FU_UDB014313_reps	2646	91%
2860	Agaricales	/cortinarius	*Cortinarius* sp.	*Cortinarius waiporianus*	SH028332.07FU_JF960683_reps_singleton	117	92%
1720	Agaricales	/cortinarius	*Cortinarius* sp.	*Cortinariaceae* sp.	SH176299.07FU_UDB015836_reps	38	92%
1773	Agaricales	/cortinarius	*Cortinarius* sp.	*Cortinariaceae* sp.	SH192852.07FU_HQ625475_reps	22	99%
348	Agaricales	/cortinarius	*Descomyces* sp.	*Cortinarius* sp.	SH178629.07FU_UDB008937_reps	1048	99%
76	Agaricales	/cortinarius	*Thaxterogaster* sp.	*Cortinariaceae* sp.	SH191996.07FU_HQ533015_refs	14,005	99%

35	Agaricales	/descolea	*Descolea maculata*	*Descolea maculata*	SH177941.07FU_FJ897224_reps	26,092	92%
1304	Agaricales	/descolea	*Descomyces* sp.	*Descomyces albus*	SH028889.07FU_UDB013306_reps_singleton	3813	90%
1093	Agaricales	/descolea	*Descomyces* sp.	*Descomyces* sp.	SH188456.07FU_JF419510_reps	71	96%
482	Agaricales	/hebeloma-alnicola	*Naucoria salicis*	*Agaricales* sp.	SH216303.07FU_FJ348397_reps	749	100%
801	Agaricales	/hebeloma-alnicola	*Naucoria salicis*	*Agaricales* sp.	SH220980.07FU_JQ824881_reps	127	100%
117	Agaricales	/inocybe	*Inocybe semifulva*	*Inocybe semifulva*	SH002708.07FU_GQ267491_reps_singleton	6275	99%
931	Agaricales	/inocybe	*Inocybe lacera*	*Inocybe lacera*	SH205568.07FU_UDB004621_reps	149	95%
879	Agaricales	/inocybe	*Inocybe fuscidula*	*Agaricales* sp.	SH033605.07FU_DQ328160_reps_singleton	147	97%
1850	Agaricales	/inocybe	*Inocybe* sp.	*Agaricales* sp.	SH184855.07FU_UDB016194_refs	20	94%
672	Agaricales	/laccaria	*Laccaria bicolor*	*Laccaria* sp.	SH184627.07FU_EF507257_refs	59,045	94%
315	Agaricales	/laccaria	*Laccaria bicolor*	*Laccaria laccata*	SH214503.07FU_UDB005535_refs	959	86%
2973	Agaricales	/laccaria	*Laccaria bicolor*	*Agaricales* sp.	SH029725.07FU_UDB016709_reps_singleton	13	91%
2497	Agaricales	/laccaria	*Laccaria canaliculata*	*Agaricales* sp.	SH220025.07FU_DQ328068_reps	488	99%
3	Agaricales	/laccaria	*Laccaria glabripes*	*Agaricales* sp.	SH179271.07FU_JX270697_reps	483,560	100%
784	Agaricales	/laccaria	*Laccaria glabripes*	*Agaricales* sp.	SH220227.07FU_JF960646_reps	152	96%
449	Agaricales	/laccaria	*Laccaria lateritia*	*Laccaria lateritia*	SH032037.07FU_GU222258_reps_singleton	36,340	100%
2887	Agaricales	/laccaria	*Laccaria lateritia*	*Laccaria lateritia*	SH220525.07FU_UDB016008_reps	433	93%
1215	Agaricales	/laccaria	*Laccaria* sp.	*Laccaria laccata*	SH203429.07FU_AF461655_reps	58	99%
743	Agaricales	/laccaria	*Laccaria tortilis*	*Agaricales* sp.	SH179028.07FU_UDB015859_refs	161	99%
7	Sebacinales	/sebacina	*Sebacinaceae* sp.	*Sebacina* sp.	SH199504.07FU_HM146868_refs	161,751	93%
3228	Sebacinales	/sebacina	*Sebacinaceae* sp.	*Sebacina* sp.	SH222467.07FU_UDB004058_reps	2	97%
189	Sebacinales	/sebacina	*Sebacina* sp.	*Sebacina* sp.	SH178661.07FU_HQ222006_refs	3556	99%
2603	Agaricales	/tricholoma	*Tricholoma terreum*	*Tricholoma terreum*	SH220240.07FU_JN228221_reps	8	98%
1259	Atheliaceae	/amphinema-tylospora	*Atheliaceae* sp.	*Tylospora* sp.	SH220982.07FU_JX270706_reps	66	99%
3232	Atheliaceae	/amphinema-tylospora	*Atheliaceae* sp.	*Tylospora* sp.	SH222576.07FU_KJ635233_reps	2	99%

260	Boletales	/boletus	*Xerocomellus riparielus*	*Xerocomellus riparielus*	SH021206.07FU_HQ829326_reps_singleton	1391	89%
4	Cantharellales	/clavulina	*Clavulina* sp.	*Clavulina subrugosa*	SH220240.07FU_JN228221_reps	515,540	98%
103	Cantharellales	/clavulina	*Clavulina* sp.	*Clavulina* sp.	SH213994.07FU_UDB004030_reps	8282	86%
1413	Cantharellales	/clavulina	*Clavulina* sp.	*Clavulina subrugosa*	SH179272.07FU_GU969249_reps	5625	100%
309	Cantharellales	/clavulina	*Clavulina* sp.	*Clavulina* sp.	SH222662.07FU_UDB013118_reps	2567	95%
2888	Cantharellales	/clavulina	*Clavulina* sp.	*Clavulina* sp.	SH179824.07FU_GQ267476_reps	19	100%
1715	Mytilinidiales	/cenococcum	*Cenococcum geophilum*	*Cenococcum* sp.	SH188456.07FU_JF419510_reps	22	93%
178	Pezizales	/terfezia-peziza depressa	*Peziza* sp.	*Peziza* sp.	SH220227.07FU_JF960646_reps	2803	97%
249	Pezizales	/tuber-helvella	*Tuber* sp.	*Tuber* sp.	SH180021.07FU_AJ875375_refs	1483	93%
8	Russulales	/russula-lactarius	*Lactifluus subclarkeae*	*Lactifluus clarkeae*	SH190181.07FU_HQ318283_reps	150,219	99%
11	Russulales	/russula-lactarius	*Russula brevipes*	*Russula brevipes*	SH220525.07FU_UDB016008_reps	159,919	95%
245	Russulales	/russula-lactarius	*Russula vinaceocuticulata*	*Russula vinaceocuticulata*	SH179624.07FU_JF495172_reps	3066	83%
2872	Russulales	/russula-lactarius	*Russula vinaceocuticulata*	*Russula vinaceocuticulata*	SH177836.07FU_JX316401_reps	6	94%
1922	Thelephorales	/tomentella-thelephora	*Tomentella badia*	*Tomentella* sp.	SH179810.07FU_KJ676521_refs	410	99%
2958	Thelephorales	/tomentella-thelephora	*Tomentella badia*	*Thelephoraceae* sp.	SH021752.07FU_JX258832_reps_singleton	5	94%
262	Thelephorales	/tomentella-thelephora	*Tomentella botryoides*	*Tomentella agbassaensis*	SH198956.07FU_KC222657_reps	1420	91%
297	Thelephorales	/tomentella-thelephora	*Tomentella coerulea*	*Tomentella* sp.	SH007255.07FU_JF908235_reps_singleton	1065	81%
100	Thelephorales	/tomentella-thelephora	*Tomentella ellisii*	*Thelephoraceae* sp.	SH033501.07FU_UDB004055_reps_singleton	7438	97%
214	Thelephorales	/tomentella-thelephora	*Tomentella lateritia*	*Thelephoraceae* sp.	SH184581.07FU_FJ816768_reps	2029	98%
14	Thelephorales	/tomentella-thelephora	*Tomentella* sp.	*Thelephoraceae* sp.	SH184599.07FU_JF960837_reps	99,555	91%
21	Thelephorales	/tomentella-thelephora	*Tomentella* sp.	*Tomentella* sp.	SH222726.07FU_UDB002773_reps	77,165	96%
25	Thelephorales	/tomentella-thelephora	*Tomentella* sp.	*Tomentella* sp.	SH184543.07FU_AB211278_reps	36,092	92%
99	Thelephorales	/tomentella-thelephora	*Tomentella* sp.	*Tomentella* sp.	SH177836.07FU_JX316401_reps	6573	95%
154	Thelephorales	/tomentella-thelephora	*Tomentella* sp.	*Tomentella sublilacina*	SH189391.07FU_HM849637_reps	5940	98%
237	Thelephorales	/tomentella-thelephora	*Tomentella* sp.	*Tomentella* sp.	SH027714.07FU_UDB002742_reps_singleton	3112	94%

2612	Thelephorales	/tomentella-thelephora	*Tomentella* sp.	*Tomentella* sp.	SH205568.07FU_UDB004621_reps	376	97%
2845	Thelephorales	/tomentella-thelephora	*Tomentella* sp.	*Tomentella sublilacina*	SH028332.07FU_JF960683_reps_singleton	88	95%
1330	Thelephorales	/tomentella-thelephora	*Tomentella* sp.	*Tomentella* sp.	SH210648.07FU_JQ085928_refs	37	90%
28	Thelephorales	/tomentella-thelephora	*Tomentella stuposa*	*Tomentella* sp.	SH028889.07FU_UDB013306_reps_singleton	69,674	94%
692	Thelephorales	/tomentella-thelephora	*Tomentella stuposa*	*Tomentella* sp.	SH177909.07FU_UDB008894_reps	337	97%
3125	Thelephorales	/tomentella-thelephora	*Tomentella stuposa*	*Tomentella stuposa*	SH216915.07FU_JN943179_reps	2	84%
1196	Thelephorales	/tomentella-thelephora	*Tomentella subtestacea*	*Thelephoraceae* sp.	SH177836.07FU_JX316401_reps	59	94%
2	Thelephorales	/tomentella-thelephora	*Thelephoraceae* sp.	*Thelephoraceae* sp.	SH198956.07FU_KC222657_reps	965,402	92%
1	Thelephorales	/tomentella-thelephora	*Thelephoraceae* sp.	*Tomentella* sp.	SH177979.07FU_UDB016695_reps	660,060	90%
63	Thelephorales	/tomentella-thelephora	*Thelephoraceae* sp.	*Tomentella* sp.	SH220031.07FU_DQ192181_refs	11,597	100%
1566	Thelephorales	/tomentella-thelephora	*Thelephoraceae* sp.	*Tomentella ellisii*	SH179271.07FU_JX270697_reps	6464	96%
559	Thelephorales	/tomentella-thelephora	*Thelephoraceae* sp.	*Tomentella* sp.	SH209621.07FU_UDB000484_refs	446	99%
1076	Thelephorales	/tomentella-thelephora	*Thelephoraceae* sp.	*Tomentella* sp.	SH179273.07FU_JX270719_reps	70	99%
1814	Thelephorales	/tomentella-thelephora	*Thelephoraceae* sp.	*Tomentella* sp.	SH201232.07FU_AM882815_refs	21	99%
2743	Thelephorales	/tomentella-thelephora	*Thelephoraceae* sp.	*Thelephoraceae* sp.	SH178438.07FU_FJ176728_refs	16	81%
2815	Thelephorales	/tomentella-thelephora	*Thelephoraceae* sp.	*Thelephoraceae* sp.	SH214461.07FU_AB839377_reps	15	99%
2737	Thelephorales	/tomentella-thelephora	*Thelephoraceae* sp.	*Thelephoraceae* sp.	SH189381.07FU_UDB008534_reps	4	99%
2391	Thelephorales	/tomentella-thelephora	*Thelephoroid* sp.	*Thelephoraceae* sp.	SH220990.07FU_JX504172_reps	561	100%

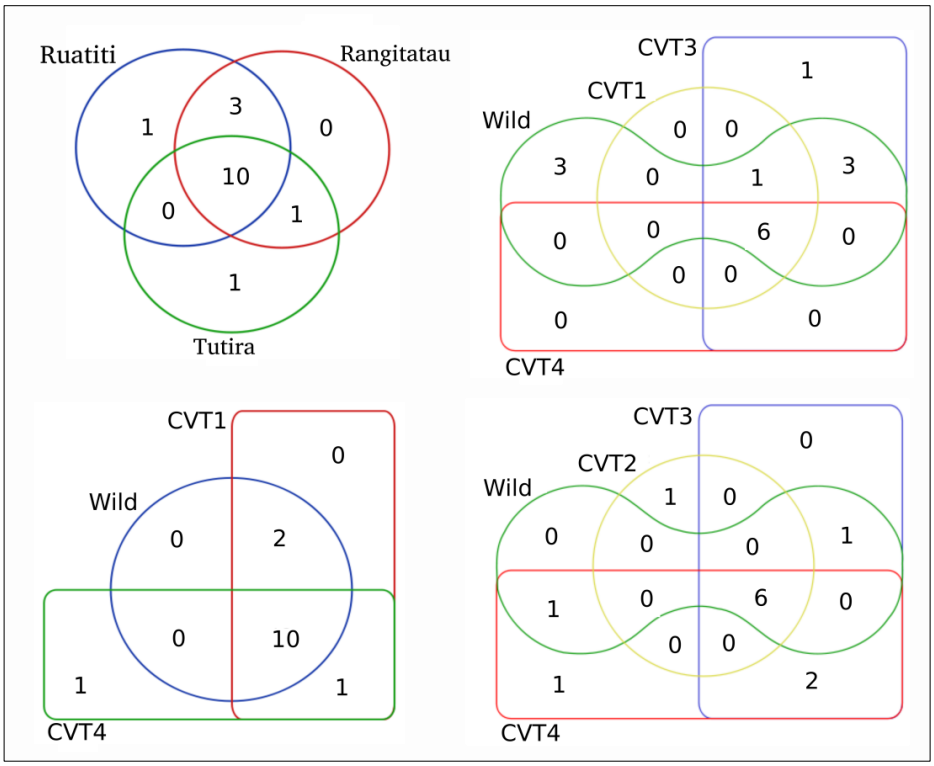
Appendix 4.3. OTUs discarded after they were blasted against NCBI and did not show a regular alignment according to (Nilsson et al., 2012). Initially, these OTUs were classified within an EcM fungal lineage (Tedersoo, 2010). OTUs were classified according the EcM fungal lineage (Tedersoo, 2010), species hypothesis (UNITE) and NCBI.

OTU ID	Order	EcM lineage	UNITE reference database	Species hypothesis	Total sequences	Query cover (%)	NCBI identity (%)
1654	Agaricales	/amanita	*Amanita rimosa*	SH178438.07FU_FJ176728_refs	20	49%	81%
2090	Agaricales	/amanita	*Amanita* sp.	SH216915.07FU_JN943179_reps	14	100%	84%
2405	Agaricales	/amanita	*Amanita* sp.	SH029277.07FU_UDB017826_reps_singleton	7	100%	85%
606	Agaricales	/inocybe	*Inocybaceae* sp.	SH203429.07FU_AF461655_reps	396	100%	99%
729	Agaricales	/inocybe	*Inocybe fulvilubrica*	SH210648.07FU_JQ085928_refs	345	100%	90%
203	Agaricales	/inocybe	*Inocybe* sp.	SH007255.07FU_JF908235_reps_singleton	2031	100%	81%
1921	Agaricales	/inocybe	*Inocybe* sp.	SH021752.07FU_JX258832_reps_singleton	14	100%	94%
1070	Boletales	/pisolithus-scleroderma	*Scleroderma albidum*	SH179810.07FU_KJ676521_refs	85	100%	99%
19	Boletales	/pisolithus-scleroderma	*Scleroderma* sp.	SH179808.07FU_UDB013841_refs	57,539	100%	100%
2813	Cantharellales	/cantharellus	*Hydnum albomagnum*	SH091380.07FU_DQ218305_refs_singleton	3	21%	96%
909	Cantharellales	/cantharellus	*Sistotrema* sp.	SH192852.07FU_HQ625475_reps	103	100%	99%
3107	Cantharellales	/clavulina	Clavulinaceae sp.	SH485098.07FU_UDB014486_reps_singleton	3	27%	100%
190	Pezizales	/genea-humaria	*Humaria* sp.	SH179624.07FU_JF495172_reps	3758	100%	83%
198	Pezizales	/terfezia-peziza depressa	*Pezizaceae* sp.	SH021206.07FU_HQ829326_reps_singleton	2228	100%	89%
392	Pezizales	/terfezia-peziza depressa	*Pezizaceae* sp.	SH205568.07FU_UDB004621_reps	637	100%	95%
1460	Pezizales	/terfezia-peziza depressa	*Pezizaceae* sp.	SH205568.07FU_UDB004621_reps	34	100%	97%
2432	Pezizales	/terfezia-peziza depressa	*Pezizaceae* sp.	SH205568.07FU_UDB004621_reps	6	98%	92%
1830	Russulales	/russula-lactarius	*Russula brevipes*	SH220525.07FU_UDB016008_reps	225	100%	93%

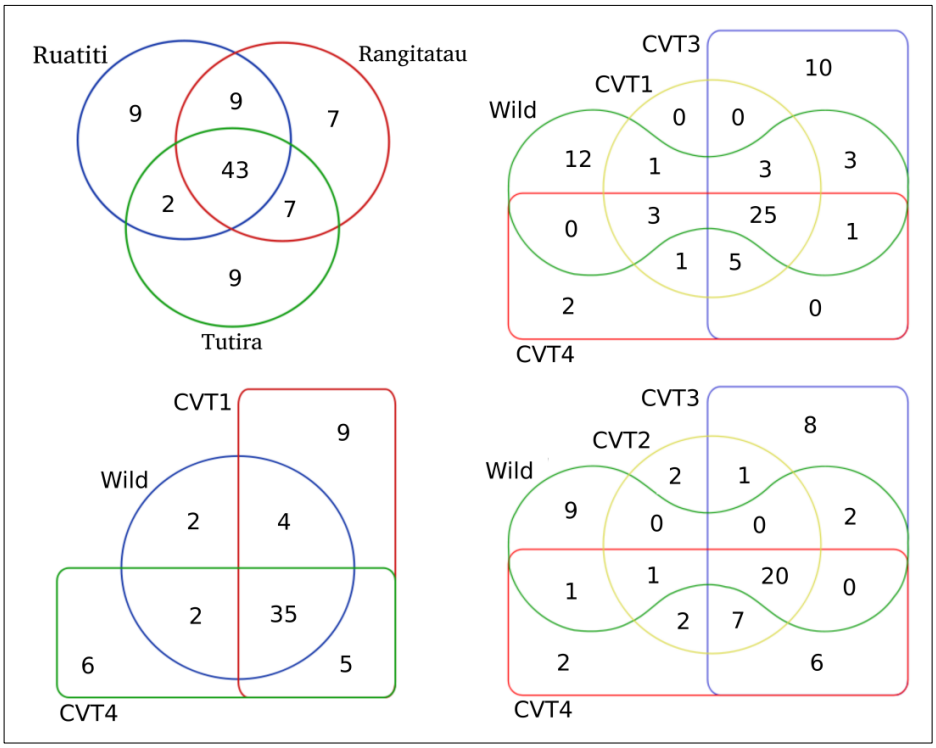
235	Sebacinales	/sebacina	*Sebacina* sp.	SH214503.07FU_UDB005535_refs	1517	100%	86%
191	Sebacinales	/serendipita	*Sebacinales* sp.	SH180021.07FU_AJ875375_refs	2453	100%	93%
93	Sordariales	/sordariales	*Sordariales* sp.	SH213994.07FU_UDB004030_reps	7211	100%	86%
200	Thelephorales	/tomentella-thelephora	*Thelephoraceae* sp.	SH198956.07FU_KC222657_reps	2164	100%	91%
2725	Thelephorales	/tomentella-thelephora	*Thelephoraceae* sp.	SH010111.07FU_AJ633587_reps_singleton	162	100%	80%
46	Thelephorales	/tomentellopsis	*Tomentellopsis* sp.	SH191996.07FU_HQ533015_refs	17,842	100%	99%
1053	Thelephorales	/tomentellopsis	*Tomentellopsis bresadolana*	SH184855.07FU_UDB016194_refs	112	99%	94%
2147	Thelephorales	/tomentellopsis	*Tomentellopsis* sp.	SH089647.07FU_HQ204746_refs_singleton	9	100%	97%
2644	Thelephorales	/tomentellopsis	*Tomentellopsis echinospora*	SH184856.07FU_UDB018590_refs	7	100%	81%

Appendix 4.4. Venn diagram showing shared and unique a) EcM lineages, and b) OTUS retained within and EcM fungal lineage, recovered from roots of cultivated (CVT1, CVT2, CVT3 and CVT4) and wild plants of *L. scoparium* at Rangitatau, Ruatiti, and Tutira.

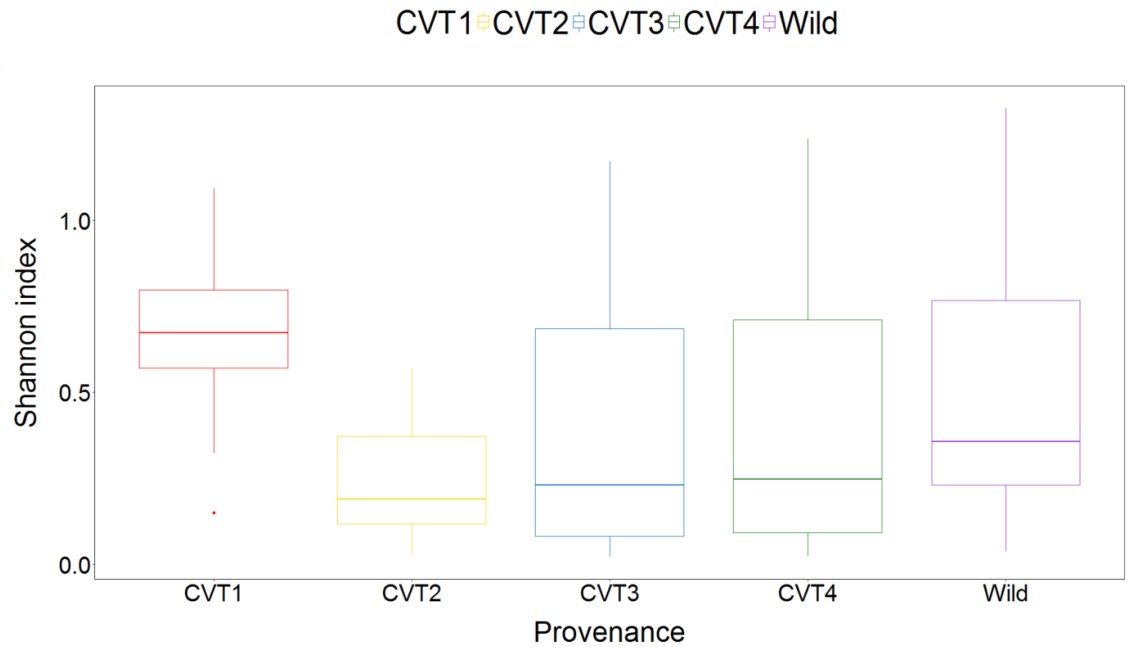
a)



b)



Appendix 4.5. Boxplot showing the mean Shannon's diversity index for each *L. scoparium* provenance (CVT1, CVT2, CVT3, CVT4 and wild plants).

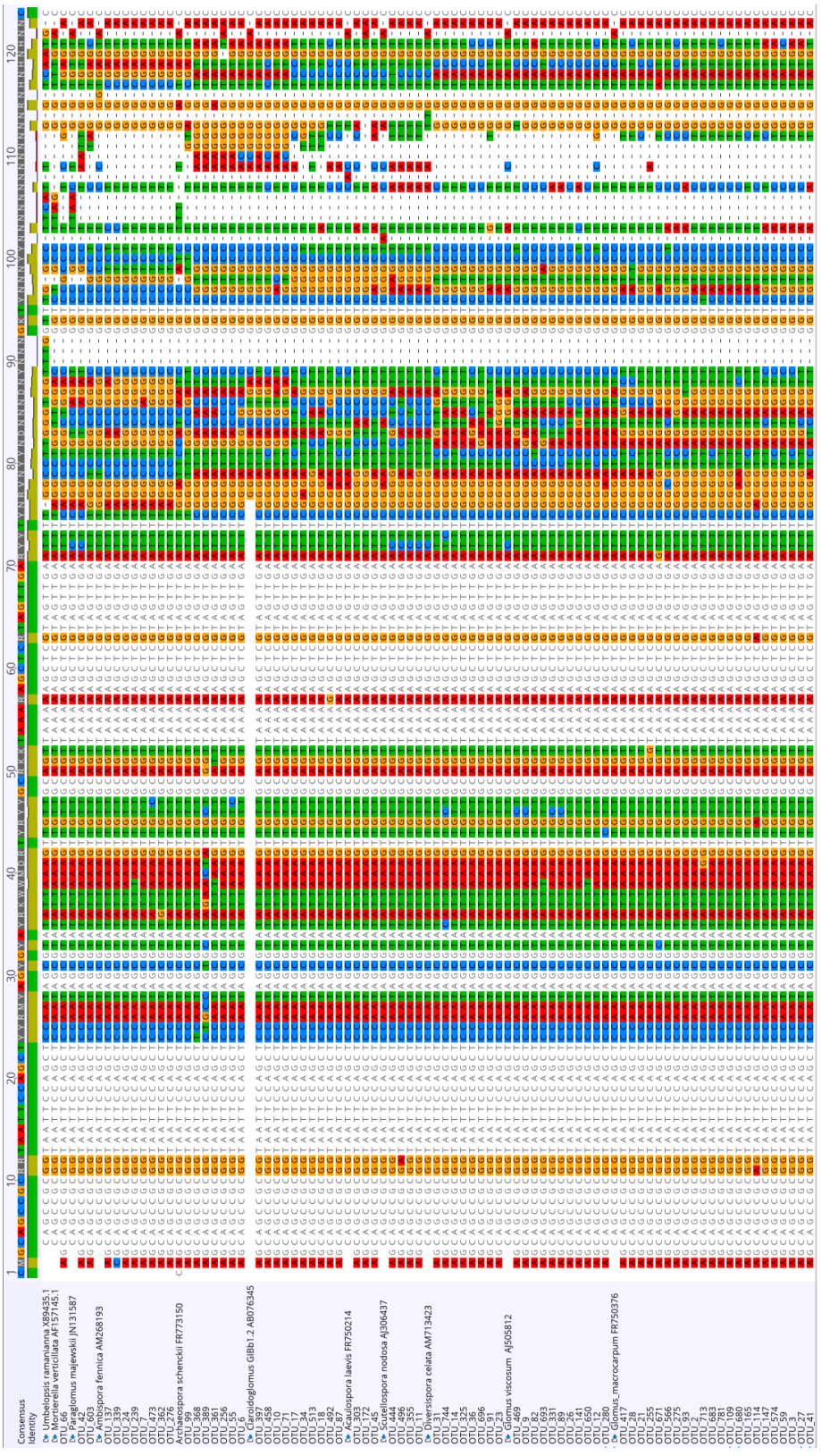


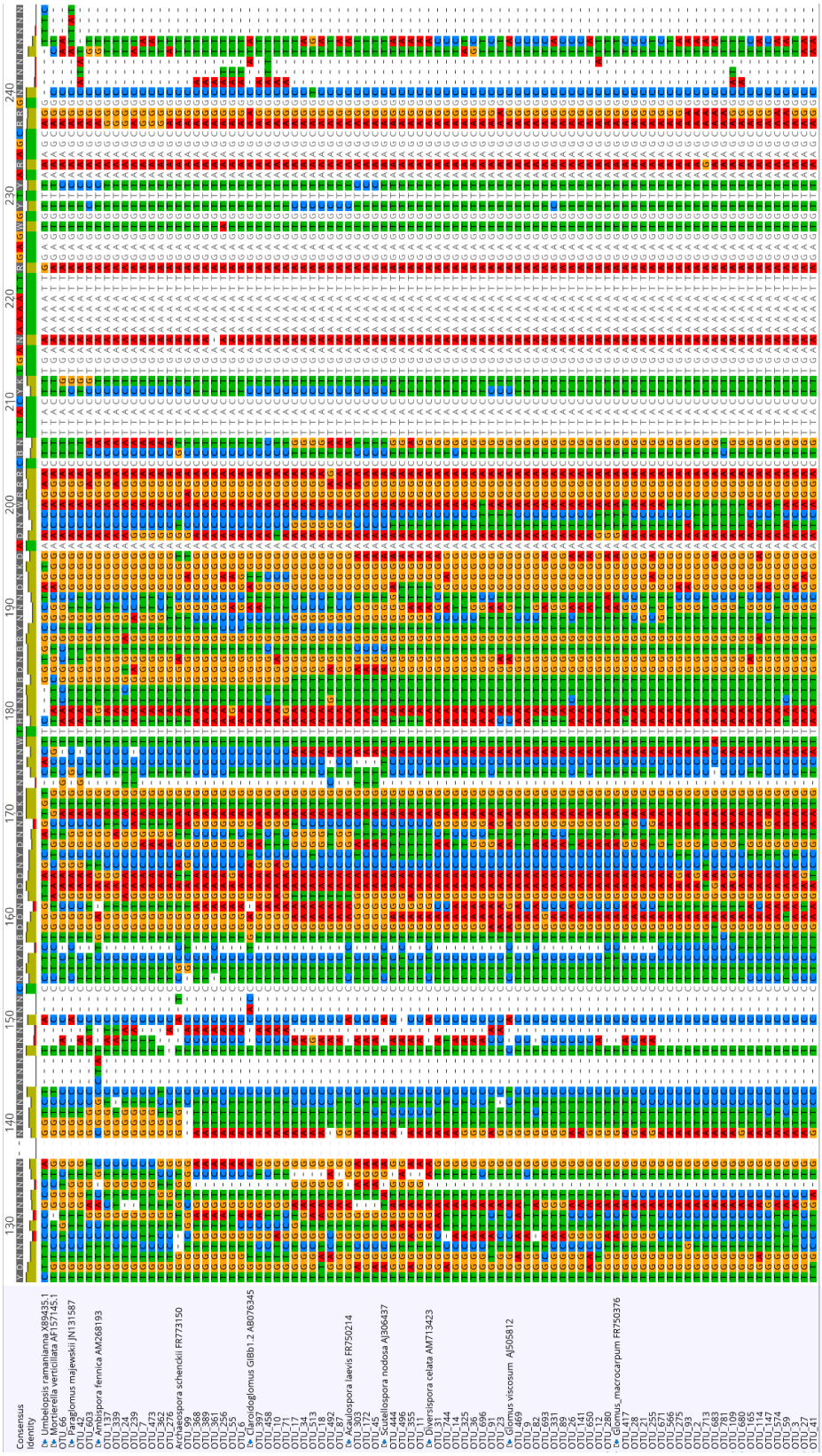
Appendix 4.6. Unique virtual taxa (VT) recovered from roots of cultivated (CVT1, CVT2, CVT3 and CVT4) and wild plants of *L. scoparium* at Rangitatau, Ruatiti, and Tutira.

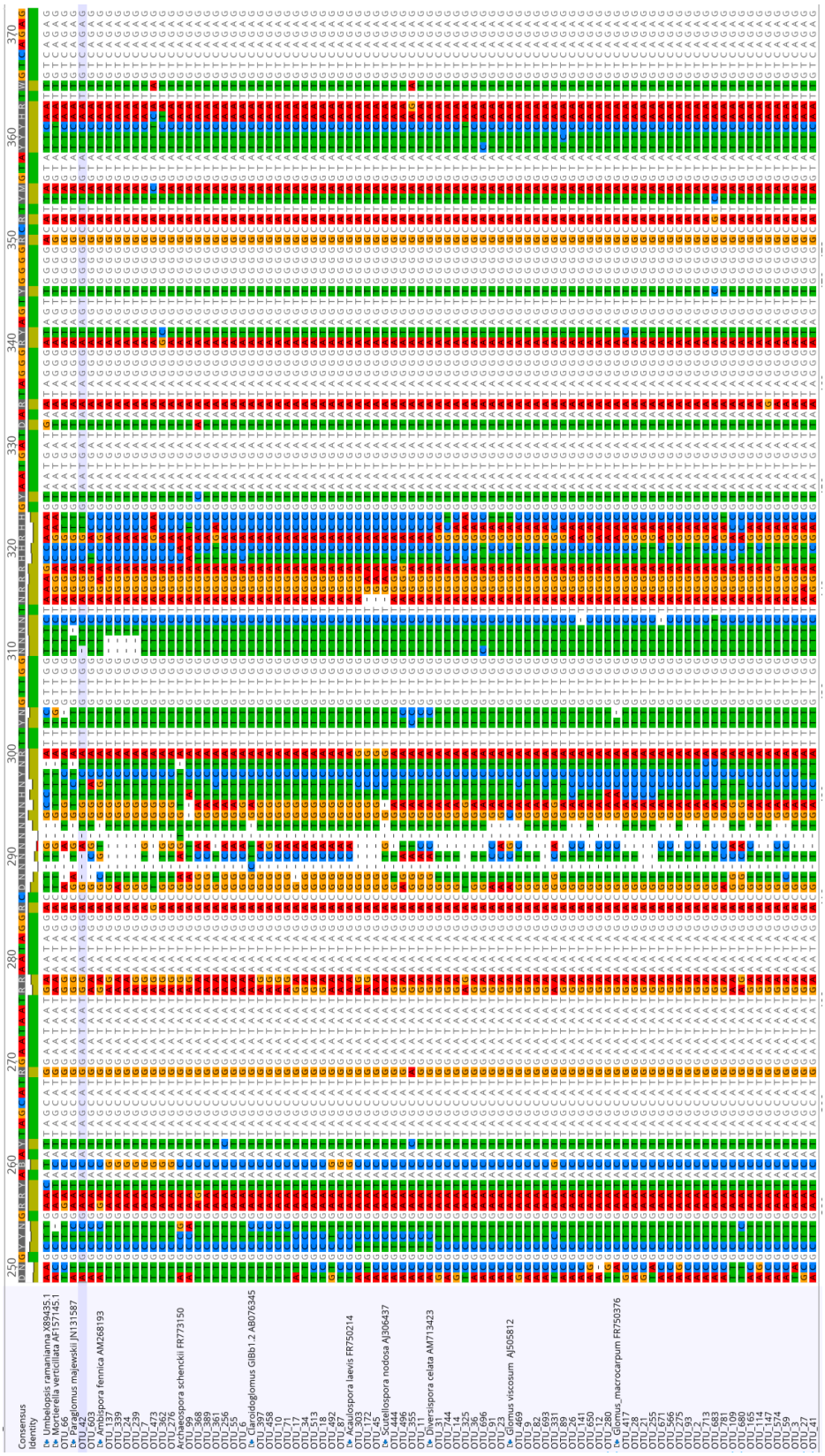
Site; provenance; VT	Family	Genus
Rangitatau		
CVT1		
VTX00006	Archaeosporaceae	*Archaeospora* sp.
VTX00276	Claroideoglomeraceae	*Claroideoglomus* sp.
CVT3		
VTX00379	Acaulosporaceae	*Acaulospora* sp.
VTX00199	Glomeraceae	*Glomus* sp.
VTX00399	Glomeraceae	*Glomus* sp.
CVT4		
VTX00310	Glomeraceae	*Glomus* sp.
Wild		
VTX00030	Acaulosporaceae	*Acaulospora* sp.
VTX00357	Claroideoglomeraceae	*Claroideoglomus* sp.
Ruatiti		
CVT1		
VTX00061	Diversisporaceae	*Diversispora* sp.
VTX00064	Glomeraceae	*Glomus* sp.
VTX00072	Glomeraceae	*Glomus* sp.
VTX00310		
CVT4		
VTX00023	Acaulosporaceae	*Acaulospora* sp.
VTX00143	Glomeraceae	*Glomus* sp.
VTX00159	Glomeraceae	*Glomus* sp.
Wild		
VTX00004	Archaeosporaceae	*Archaeospora* sp.
VTX00013	Acaulosporaceae	*Acaulospora* sp.
VTX00230	Acaulosporaceae	*Acaulospora* sp.
VTX00283	Ambisporaceae	*Ambispora* sp.
VTX00338	Archaeosporaceae	*Archaeospora* sp.
VTX00052	Gigasporaceae	*Scutellospora* sp.
VTX00060	Diversisporaceae	*Diversispora* sp.
VTX00380	Diversisporaceae	*Diversispora* sp.
VTX00069	Glomeraceae	*Glomus* sp.
VTX00078	Glomeraceae	*Glomus* sp.
VTX00093	Glomeraceae	*Glomus* sp.
VTX00165	Glomeraceae	*Glomus* sp.
VTX00327	Glomeraceae	*Glomus* sp.

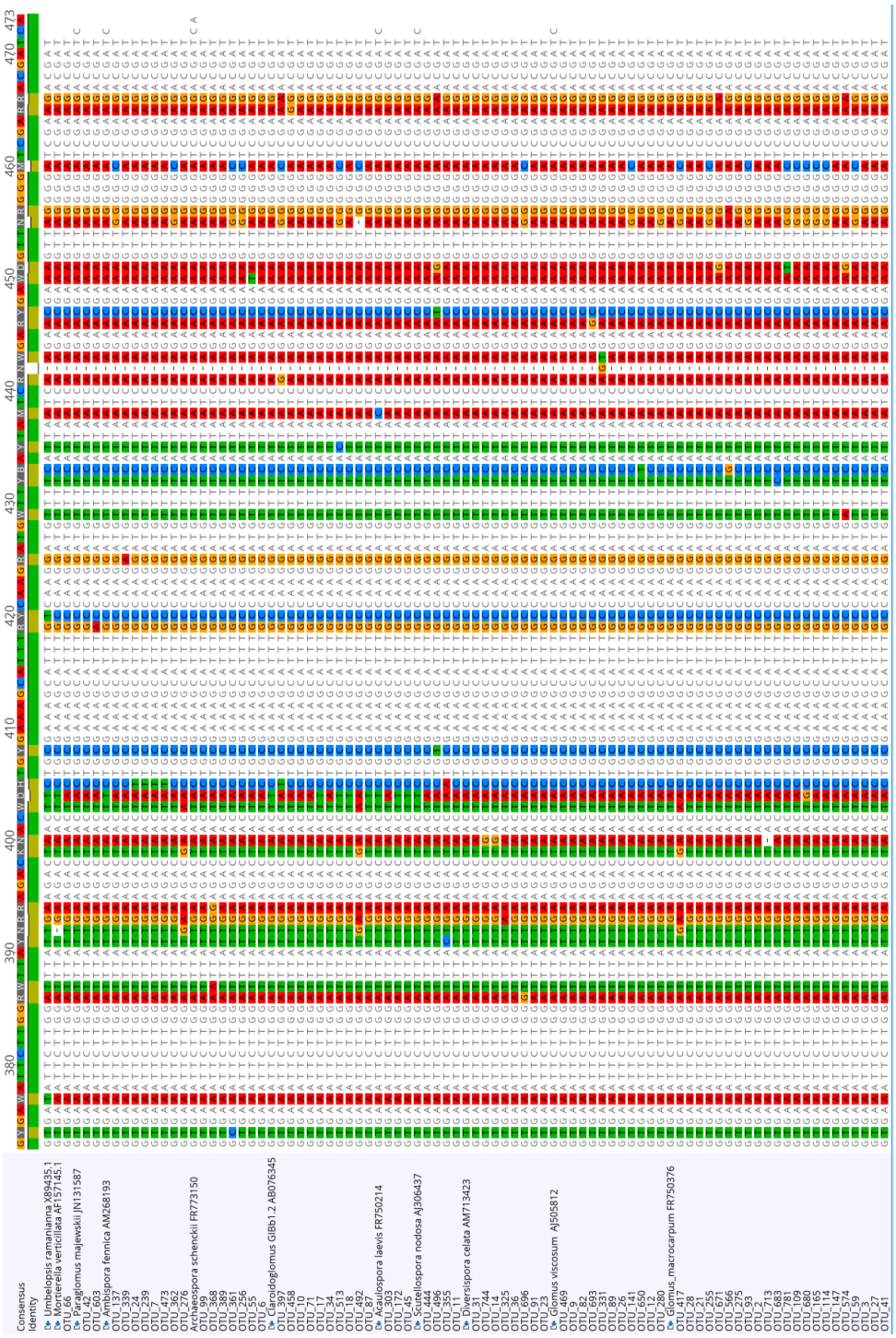
Tutira		
CVT2		
VTX00230	Acaulosporaceae	*Acaulospora* sp.
CVT3		
VTX00379	Acaulosporaceae	*Acaulospora* sp.
VTX00276	Claroideoglomeraceae	*Claroideoglomus* sp.
CVT4		
VTX00030	Acaulosporaceae	*Acaulospora* sp.
VTX00199	Glomeraceae	*Glomus* sp.
VTX00372	Glomeraceae	*Glomus* sp.

Appendix 4.7. Multiple sequence alignment including the 76 OTUs and 10 AM and non-AM fungal species included in Mr Bayes phylogenetic tree.

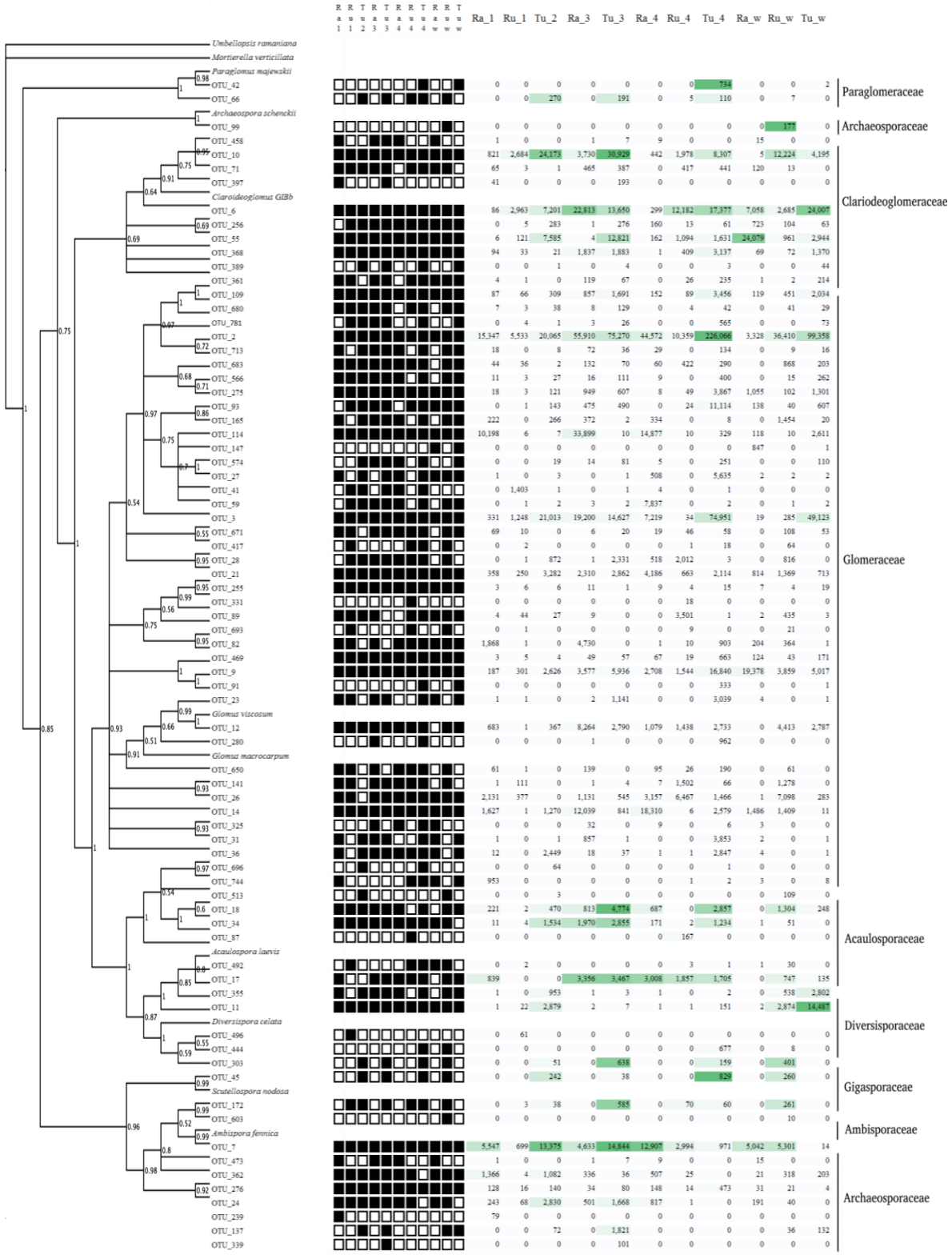








Appendix 4.8. Phylogenetic tree from Mr Bayes showing the phylogenetic relationship of the AM fungal sequences with a query cover of 100% and at least 97% of identity with representative sequences from Maarj*AM* database after poorly aligned positions were removed. The values next to the branches indicate Bay posterior probability.



Appendix 4.9. The top NCBI match and the top match from Maarj*AM* database at species level for the 76 OTUs included in Mr Bayes phylogenetic tree. The NCBI accession number, Maarj*AM* virtual taxon, identity values from NCBI and Maarj*AM*, and total number of sequences recovered from the study that matched to the defined OTUs are indicated. All matches indicated a query cover of 100%. The taxonomic classification at species level is shown according to Mycobank.

OTU ID	Family	NCBI match	NCBI acc. no.	NCBI identity (%)	Maarj*AM* match	Maarj*AM* Virtual taxon	Maarj*AM* identity (%)	Total sequences
34	Acaulosporaceae	*Acaulospora brasiliensis*	KU359227.1	98%	*Acaulospora* sp.	VTX00231	99.50%	7833
513	Acaulosporaceae	*Acaulospora brasiliensis*	FN825900.1	98%	*Acaulospora* sp.	VTX00230	97.90%	112
492	Acaulosporaceae	*Acaulospora cavernata*	KU900343.1	97%	*Acaulospora* sp.	VTX00030	97.50%	37
24	Archaeosporaceae	*Archaeospora gerdemannii*	AM400227.1	91%	*Archaeospora* sp.	VTX00009	100.00%	6359
339	Archaeosporaceae	*Archaeospora gerdemannii*	AM400227.1	92%	*Archaeospora* sp.	VTX00004	98.40%	101
603	Archaeosporaceae	*Archaeospora gerdemannii*	AM400227.1	95%	*Ambispora* sp.	VTX00283	97.00%	10
87	Acaulosporaceae	*Acaulospora laevis*	AF131041.1	100%	*Acaulospora* sp.	VTX00023	100.00%	167
137	Archaeosporaceae	*Archaeospora leptoticha*	AB047309.1	92%	*Archaeospora* sp.	VTX00004	99.50%	2061
239	Archaeosporaceae	*Archaeospora leptoticha*	AJ301861.1	91%	*Archaeospora* sp.	VTX00009	99.80%	79
99	Archaeosporaceae	*Acaulospora trappei*	KP144303.1	92%	*Archaeospora* sp.	VTX00338	99.30%	177
458	Claroideoglomeraceae	*Claroideoglomus claroideum*	Y17636.2	93%	*Claroideoglomus* sp.	VTX00225	99.10%	80
397	Claroideoglomeraceae	*Claroideoglomus lamellosum*	KX879068.1	94%	*Claroideoglomus* sp.	VTX00276	98.40%	397
389	Claroideoglomeraceae	*Claroideoglomus lamellosum*	AJ276087.2	97%	*Claroideoglomus* sp.	VTX00193	97.10%	389

368	Claroideoglomeraceae	*Claroideoglomus lamellosum*	KX811533.1	99%	*Claroideoglomus* sp.	TX00193	98.60%	368
361	Claroideoglomeraceae	*Claroideoglomus lamellosum*	AJ276087.2	97%	*Claroideoglomus* sp.	VTX00193	97.30%	361
256	Claroideoglomeraceae	*Claroideoglomus lamellosum*	AJ276087.2	98%	*Claroideoglomus* sp.	VTX00193	98.40%	256
71	Claroideoglomeraceae	*Claroideoglomus lamellosum*	AJ276083.3	95%	*Claroideoglomus* sp.	VTX00225	100.00%	71
355	Diversisporaceae	*Diversispora aurantia*	KU136395.1	97%	*Diversispora sp.*	VTX00380	97.50%	4301
496	Diversisporaceae	*Diversispora epigaea*	X86687.3	98%	*Diversispora sp.*	VTX00061	97.90%	61
444	Diversisporaceae	*Diversispora spurca*	LN890624.1	97%	*Diversispora sp.*	VTX00380	97.20%	685
473	Geosiphonaceae	*Geosiphon pyriforme*	AJ276074.2	90%	*Archaeospora* sp.	VTX00005	97.20%	33
362	Geosiphonaceae	*Geosiphon pyriforme*	AJ276074.2	91%	*Archaeospora* sp.	VTX00005	98.20%	3898
276	Geosiphonaceae	*Geosiphon pyriforme*	AJ276074.2	91%	*Archaeospora* sp.	VTX00005	98.60%	1089
303	Gigasporaceae	*Scutellospora aurigloba*	AJ276092.2	98%	*Scutellospora* sp.	VTX00052	98.40%	1249
45	Gigasporaceae	*Scutellospora calospora*	KU136415.1	99%	*Scutellospora* sp.	VTX00052	99.50%	1369
172	Gigasporaceae	*Cetraspora pellucida*	KX879059.1	99%	*Scutellospora* sp.	-	99.50%	1017
27	Glomeraceae	*Funneliformis mosseae*	FR751295.1	97%	*Glomus* sp.	VTX00069	99.50%	6154
91	Glomeraceae	*Funneliformis mosseae*	KU136407.1	100%	*Glomus* sp.	VTX00067	100.00%	334
141	Glomeraceae	*Glomus macrocarpum*	FR750376.1	95%	*Glomus* sp.	VTX00233	99.50%	2970
280	Glomeraceae	*Glomus macrocarpum*	FR772325.1	99%	*Glomus* sp.	VTX00199	99.30%	963
650	Glomeraceae	*Glomus macrocarpum*	KX879069.1	96%	*Glomus* sp.	-	97.90%	573
41	Glomeraceae	*Glomus sinuosum*	AJ133706.1	96%	*Glomus* sp.	VTX00310	99.50%	1410
59	Glomeraceae	*Rhizophagus clarus*	MF196920.1	97%	*Glomus* sp.	VTX00360	99.50%	7850
165	Glomeraceae	*Rhizophagus clarus*	KP144311.1	97%	*Glomus* sp.	VTX00327	99.30%	2678
275	Glomeraceae	*Rhizophagus intraradices*	FR750209.1	97%	*Glomus* sp.	VTX00108	98.90%	8080
683	Glomeraceae	*Rhizophagus intraradices*	KU136414.1	97%	*Glomus* sp.	VTX00114	97.20%	2127

147	Glomeraceae	*Rhizophagus intraradices*	FR750206.1	96%	*Glomus* sp.	-	99.80%	848
781	Glomeraceae	*Rhizophagus intraradices*	KU136414.1	97%	*Glomus* sp.	VTX00113	97.20%	672
671	Glomeraceae	*Rhizophagus intraradices*	FJ009604.1	97%	*Glomus* sp.	VTX00247	97.20%	389
82	Glomeraceae	*Rhizophagus iranicus*	KX879064.1	97%	*Glomus* sp.	VTX00163	99.30%	8082
89	Glomeraceae	*Rhizophagus iranicus*	KX879064.1	99%	*Glomus* sp.		99.80%	4026
36	Glomeraceae	*Rhizophagus iranicus*	KU136399.1	97%	*Glomus* sp.	VTX00143	100.00%	5370
31	Glomeraceae	*Rhizophagus iranicus*	HM122275.1	96%	*Glomus* sp.	VTX00135	99.80%	4716
469	Glomeraceae	*Rhizophagus iranicus*	HM153423.1	99%	*Glomus* sp.	VTX00155	99.10%	1205
744	Glomeraceae	*Rhizophagus iranicus*	HM153423.1	95%	*Glomus* sp.	VTX00159	97.90%	967
255	Glomeraceae	*Rhizophagus iranicus*	KU136399.1	95%	*Glomus* sp.	VTX00219	97.20%	85
696	Glomeraceae	*Rhizophagus iranicus*	KU136399.1	94%	*Glomus* sp.	VTX00143	97.70%	65
325	Glomeraceae	*Rhizophagus iranicus*	KU136399.1	96%	*Glomus* sp.	VTX00153	97.50%	50
693	Glomeraceae	*Rhizophagus iranicus*	KX879064.1	97%	*Glomus* sp.	VTX00166	97.50%	31
331	Glomeraceae	*Rhizophagus iranicus*	KX879064.1	96%	*Glomus* sp.	-	97.00%	18
28	Glomeraceae	*Rhizophagus irregularis*	KX879063.1	98%	*Glomus* sp.	VTX00212	99.80%	6554
566	Glomeraceae	*Rhizophagus irregularis*	FJ009612.1	97%	*Glomus* sp.	VTX00115	97.50%	854
109	Glomeraceae	*Rhizophagus irregularis*	KX879067.1	96%	*Glomus* sp.	VTX00344	98.20%	109
680	Glomeraceae	*Rhizophagus irregularis*	FJ009612.1	94%	*Glomus* sp.	VTX00344	97.00%	301
417	Glomeraceae	*Rhizophagus irregularis*	KX879063.1	95%	*Glomus* sp.	VTX00213	97.70%	85
574	Glomeraceae	*Rhizophagus vesiculifer*	FR750374.1	96%	*Glomus* sp.	VTX00074	97.90%	480
713	Glomeraceae	*Rhizophagus vesiculifer*	FR750374.1	98%	*Glomus* sp.	VTX00115	97.90%	322
23	Glomeraceae	*Septoglomus constrictum*	KU136423.1	100%	*Glomus* sp.	VTX00064	100.00%	4189
42	Paraglomeraceae	*Paraglomus majewskii*	JN131599.1	99%	*Paraglomus* sp.	VTX00335	99.80%	736
66	Paraglomeraceae	*Paraglomus laccatum*	AM295493.1	99%	*Paraglomus* sp.	VTX00281	100.00%	583

Appendix. 4.10. Ectomycorrhizal fungal species closely related to previously found endemic OTUs from New Zealand. Table includes the New Zealand host status, biostatus and main references associated to these EcM fungal species.

Ectomycorrhizal fungal species	Main New Zealand hosts	Biostatus	Reference
Clavulina subrugosa (Cleland) Corner (97%)	Myrtaceae	Present. Indigenous, endemic	Petersen, R.H. (1988). The clavarioid fungi of New Zealand. New Zealand Department of Scientific and Industrial Research, Bulletin 236: 170 pp.
Cortinarius waiporianus Scoop (97% – 98%)	Myrtaceae, Nothofagaceae, Pinaceae	Present. Indigenous, endemic	Soop, K. 2013: A contribution to the study of the cortinarioid mycoflora of New Zealand, V. Australasian Mycologist 31: 1-9.
Dermocybe indotata E. Horak (97%)	Myrtaceae, Nothofagaceae	Present. Indigenous, endemic	Horak, E. 1988: New species of *Dermocybe* (Agaricales) from New Zealand. Sydowia 40: 81-112.
Descolea maculata Bougher (100%)	Myrtaceae	Uncertain	Bougher, N.L.; Malajczuk, N. 1985. A new species of *Descolea* (Agaricales) from Western Australia, and aspects of its ectomycorrhizal status. Australian Journal of Botany. 33(6):619-627.
Laccaria bicolor (Maire) P.D. Orton (99%)	No records New Zealand	-	Orton, P.D. 1960. New check list of British Agarics and Boleti, part III (keys to *Crepidotus*, *Deconica*, *Flocculina*, *Hygrophorus*, *Naucoria*, *Pluteus* and *Volvaria*). Transactions of the British Mycological Society. 43(2):159-439.
Laccaria fraterna (Sacc.) Pegler (100%)	Myrtaceae, Nothofagaceae, Pinaceae, Salicaceae	Indigenous. Non endemic	McNabb, R.F.R. 1972: The Tricholomataceae of New Zealand. 1. *Laccaria* Berk. & Br. New Zealand Journal of Botany 10(3): 461-484.
Laccaria glabripes McNabb (100%)	Myrtaceae, Nothofagaceae, Pinaceae, Salicaceae	Present. Indigenous, endemic	McNabb, R.F.R. 1972: The Tricholomataceae of New Zealand. 1. *Laccaria* Berk. & Br. New Zealand Journal of Botany 10(3): 461-484.
Lactifluus subclarkeae (Grgur.) Verbeken (99%)	No records New Zealand	-	Verbeken, A.; Van de Putte, K.; De Crop, E. 2012. New combinations in *Lactifluus*. 3. L. subgenera *Lactifluus* and *Piperati*. Mycotaxon. 120:443-450. Grgurinovic CA (1997) Larger fungi of South Australia. Botanical Gardens of Adelaide and State Herbarium and The Flora and Fauna of South Australia Handbooks Committee: Adelaide.

Grgurinovic CA (1997) Larger fungi of South Australia. Botanical Gardens of Adelaide and State Herbarium and The Flora and Fauna of South Australia Handbooks Committee: Adelaide.

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Chapter 5.

Soil inoculation trial on *Leptospermum scoparium* seedlings

"Relatively few studies have investigated how changes in mycorrhizal communities in the field alter plant growth and ecosystem functioning, and most glasshouse studies compare the effects of mycorrhiza with a nonmycorrhizal control, which is a very rare situation in nature"

Heijden, Martin, Selosse, & Sanders, 2015

Chapter 5 – Soil inoculation trial on *Leptospermum scoparium* seedlings

5.1. Introduction

Leptospermum scoparium is characterised by its association with both arbuscular mycorrhizal (AM) and ectomycorrhizal (EcM) fungi (Hall, 1977; McKenzie, Johnston, & Buchanan, 2006); a dual association globally present on a limited number of host plant genera and species (Harley & Harley, 1987; Brundrett & Abbott, 1991; Brundrett & Bougher, 1996; Lodge, 2000). These dual associations can stimulate the growth of *L. scoparium* (Baylis, 1971; Hall, 1975; Cooper, 1976), and subsequently, could influence nectar quantity and quality (Gange & Smith, 2005; Kiers et al., 2010). Previous studies have shown the yield improvements of crops (Qiang-Sheng et al., 2016) when mycorrhizal fungal inoculum has been applied in species such as cassava (*Manihot esculenta* Crantz) (Ceballos et al., 2013), pepper (*Capsicum annuum* L. var. *longum*) (Hernádi et al., 2012), and cocoa (*Theobroma cacao* L.) and coffee (*Coffea arabica* L.) (Suparno et al., 2015). Therefore, it will be important to examine the potential effects of mycorrhizal fungal inoculum on *L. scoparium*, and its evaluation on nectar quality and quantity, and mānuka honey yield.

In New Zealand, a previous study has shown the positive response of *L. scoparium* when soil, which was collected from *Nothofagus* Blume and *Pinus* L. habitat associated with mycorrhizal fungi, is used as inoculant on *L. scoparium* plants (Cooper, 1976). However, mycorrhizal colonisation levels can differed depending on mycorrhizal fungal type and the location where soil is collected (Cooper, 1976). For example, Cooper showed 66% of colonisation within *L. scoparium* by AM fungus type 1 when soil was collected from Leith Valley (Dunedin), while only 18% of colonisation by this fungus was found when the soil was collected from Pine Hill (Dunedin). Even though both locations were characterised by the presence of *Pinus radiata* D.Don, the level of EcM fungi colonisation also differed, being double when soil was collected from Pine Hill (20%) instead of Leith Valley (10%) (Cooper, 1976). As in Cooper's study (1976), another recent study of mānuka has again shown that soil inoculation effects can vary depending on the location (Davis et al., 2013). Davis et al.'s study (2013) also suggested the need of further research to evaluate if the application of inoculum on *L. scoparium* in grassland would be limited by the optimal mycorrhizal fungi.

In this thesis (Chapter 4), it was found that mycorrhizal fungal communities, mainly EcM fungi, vary among study sites and it was also found some variability between cultivated

and wild plants. Therefore, different inoculation effects could be expected depending where soil would be collected. It is possible that inoculating seedlings stock with high quality fungal partners at planting or in the nursery might help the establishment and performance of plantation *L. scoparium*, particularly in harsh sites where mortality is often high and growth rates poor.

To investigate the potential of inoculation, a pilot study was conducted at Massey University. To do so, *L. scoparium* seedlings from different nurseries were established on a benign field site. Field soil was collected from *L. scoparium* habitat colonised by mycorrhizal fungi and added as soil inoculant to the seedlings. Plant growth rate was evaluated to determine the potential effects of this inoculation.

5.2. Material and methods

Seedlings of *L. scoparium* were used from four nurseries based in the Manawatū (2 nurseries), Hawke's Bay (1 nursery), and Taupō regions (1 nursery) (North Island, New Zealand). From each nursery, ten seedlings were selected and DNA extracted following the protocol described in Chapter 4. From these, eight were randomly selected for staining roots as described in Chapter 4. In addition, the root system of another ten seedlings was frozen at -80°C for future studies.

For the inoculation trial, soil samples were collected on 22nd August 2016 at Rangitatau (Wanganui region). Wild plants of *L. scoparium* were selected and a soil corer (Ø 2.5 cm) was used to collect samples underneath of those plants. A total of 5 kg of soil was collected and placed on ice until arrival at the lab. The soil was sieved through a 5-mm sieve to homogenise and remove thicker roots.

In addition, a total of 90 seedlings from three different nurseries (Bare rooted seedlings: Manawatū; CVT6 seedlings: Hawke's Bay; Taupō seedlings: Taupō) were planted on 26th August 2016 at the Moginie block of the Pasture & Crop Research Unit (PCRU) at Massey University (Palmerston North, New Zealand). The seedlings were planted in a randomised block design with two factors: inoculation treatment and nursery. The experimental design included three replicates per inoculation treatment (inoculated and non-inoculated). Each replicate included 5 seedlings from each nursery. In each replicate, seedlings from the same nursery were separated by a distance of 0.5 m. A distance of 5 m was established between seedlings from different nurseries. Replicate blocks were spaced 5 m apart (Fig. 5.1.).

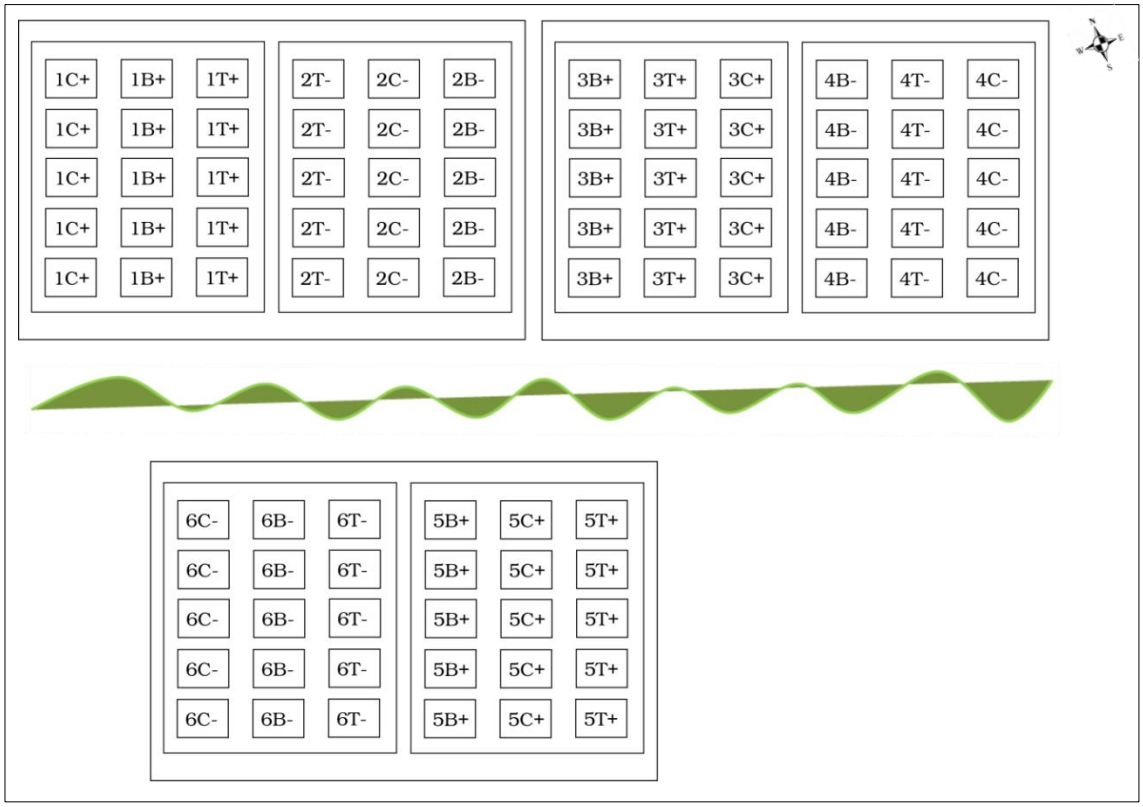

Fig. 5.1. Randomized block design with two treatments (inoculated (+), non-inoculated (-)) applied to seedlings of *L. scoparium* from three nursery with 15 replicates of each at PCRU (Massey University). B: bare roots (Manawatū nursery); C: CVT6 (Hawke's Bay nursery); T: Taupō (Taupō nursery).

Each root system of inoculated seedlings received 100 grams of soil inoculum during planting and was covered with normal soil. Seedlings were protected from browsing hares with a net sleeve during the early stage (Fig. 5.2 a). A post-planting release was conducted 4 weeks later to facilitate the establishment of the seedlings (Fig. 5.2 b). To do so, a solution including Gallant® (2.5 ml/L; haloxyfop) and Versatill® (5 ml/L; clopyralid) was applied to the trial following the basic backpacking/spot spraying protocol.

To evaluate seedling growth, the stem basal diameter and height of all seedlings were measured using a measurement tape and a digital calliper. Measurements were taken at the initial stage of the plantation (August 2016), at 3 months (December 2016), at 6 months (February 2017), at 9 months (May 2017) (Appendix 5.1), and at 1 year and 1 month (September 2017).

To evaluate the influence of soil inoculum on *L. scoparium* seedlings, statistical analysis was performed using R 3.3.1 (R Core Team, 2016). Data were log-transformed to help height and basal area to fit a normal distribution. The explanatory variables treatment, nursery and aspect of the facing slope, and the interaction of treatment: nursery, and

treatment: aspect were included in the model. An ANOVA test was performed to determine the significance of the explanatory variables on the height and basal area of *L. scoparium* seedlings.

Fig. 5.2. Inoculation trial including 90 seedlings from three nurseries at PCRU showing a) their protection with a net sleeve in the early stages of establishment; b) post-planting release effect.

5.3. Results

Root staining

Different fungal structures were found among nursery seedlings. Bare roots from the Manawatū nursery were characterised by presenting more arbuscules than other nurseries (Fig. 5.3 a, b). Seedlings from the other Manawatū nursery also showed the presence of arbuscules. CVT6 seedlings from Hawke's Bay nursery showed abundance of dark septate endophyte (DSE)-like structures (Fig. 5.3 e, f, g).

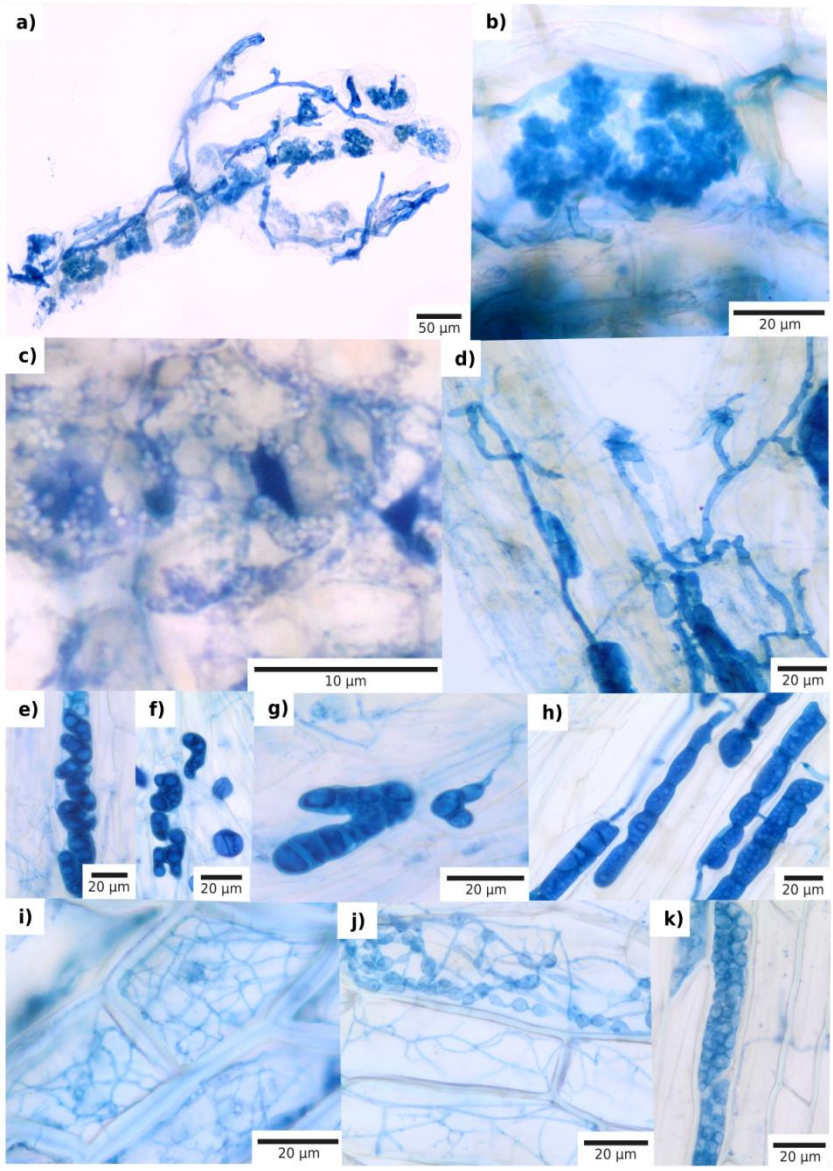

Fig. 5.3. Some fungal structures found in stained roots from *L. scoparium* seedlings from four nurseries. a), b) arbuscules found in bare roots; c) Hartig net-like structure found in seedlings from Manawatū nursery; d) hyphae; e), f), g) dark septate endophyte (DSE)-like structures present found in CVT6 seedlings from Hawke's Bay nursery; h) DSE-like found in seedlings from Manawatū nursery; i) fine endophytes found in seedlings from Taupō nursery j) fine endophytes found in CVT6 seedlings from Hawke's Bay nursery; k) microsclerotium found in seedlings from Manawatū nursery.

Seedling growth

Figure 5.4 and 5.5 show that the growth of seedlings planted on the north-facing slope exceeded that of seedlings planted on the south-facing slope (Appendix 5.2, 5.3).

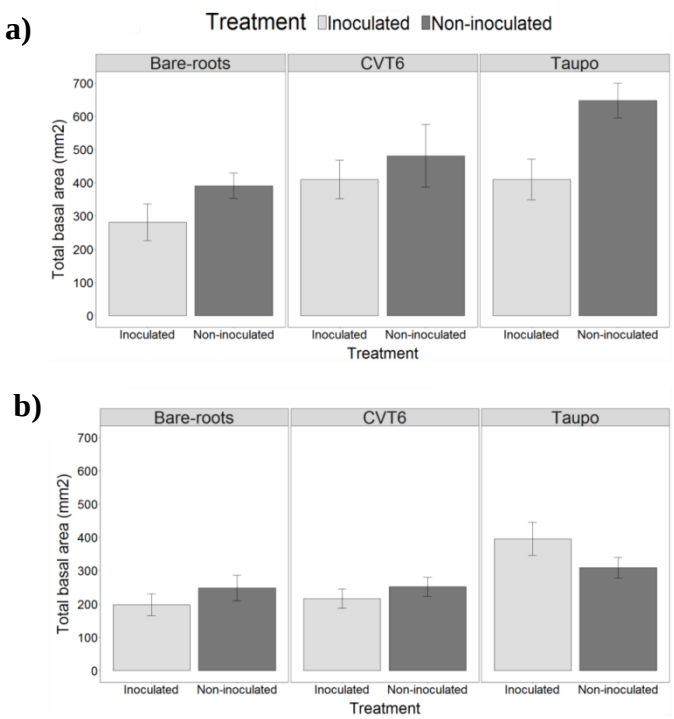

Fig. 5.4. Bar diagram showing height and basal area mean from inoculated and non-inoculated *L. scoparium* seedlings from three nurseries (Manawatū, Hawke's Bay and Taupō region) planted in a) north and b) south facing slopes.

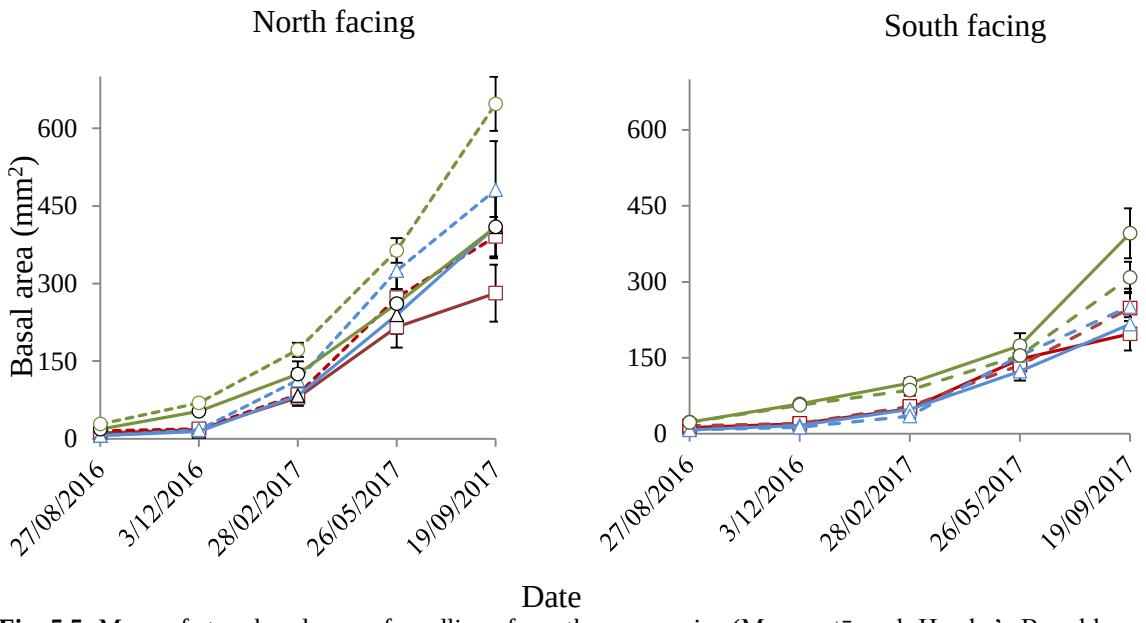

Fig. 5.5. Mean of stem basal area of seedlings from three nurseries (Manawatū: red; Hawke's Bay: blue; and Taupō: green) that were non-inoculated (– – –) or inoculated (———) with soil collected beneath wild plants.

The inoculating treatment did not show any effect on seedling growth, but this needs to be studied over a long-term. However, aspect significantly influenced stem basal area and seedling height (Table 5.1, 5.2).

Among inoculation treatments, inoculated seedlings from the Taupō nursery planted on south-facing slope were the only seedlings that showed a greater stem basal area than non-inoculated seedlings, but the interaction of treatment and nursery was non-significant.

Table 5.1. Significance of the explanatory variables treatment, aspect, nursery, interaction between treatment and aspect, and between treatment and nursery, on stem basal area and seedling height.

Response; explanatory variables	d.f.	Sum of squares	Mean squares	*F*	*P*
Basal area					
Treatment	1	0.079	0.089	2.29	0.1343
Facing	1	0.930	0.930	26.96	**<0.0001**
Nursery	2	0.732	0.366	10.61	**<0.0001**
Treatment: aspect	1	0.089	0.089	2.59	0.1115
Treatment: nursery	2	0.039	0.019	0.56	0.5727
Residuals	78	2.691	0.034		
Height					
Treatment	1	0.001	0.001	0.21	0.6477
Facing	1	0.141	0.141	41.25	**<0.0001**
Nursery	2	0.126	0.063	18.45	**<0.0001**
Treatment: aspect	1	0.001	0.001	0.22	0.6360
Treatment: nursery	2	0.011	0.005	1.57	0.2138
Residuals	78	0.267	0.003		

Table. 5.2. Coefficients for the explanatory variables inoculation treatment, facing slope, nursery, and the interaction of inoculation treatment: facing slope and the interaction inoculation treatment: nursery.

Response; explanatory variable	Estimate ± S.E.	t-value	*P*
Basal area			
Non-inoculated	0.20 ± 0.09	2.31	**0.0238**
Facing south	–0.15 ± 0.06	–2.51	**0.0141**
CVT6	0.09 ± 0.07	1.32	0.1889
Taupō	0.27 ± 0.07	3.90	**0.0002**
Non-inoculated: facing south	–0.14 ± 0.08	–1.63	0.1078
Non-inoculated: CVT6	–0.03 ± 0.10	–0.36	0.7194
Non-inoculated: Taupō	–0.10 ± 0.10	–1.04	0.2996
Height			
Non-inoculated	0.04 ± 0.03	1.61	0.1101
Facing south	–0.08 ± 0.02	–4.13	**<0.0001**
CVT6	0.02 ± 0.02	0.77	0.4434
Taupō	0.10 ± 0.02	4.31	**<0.0001**
Non-inoculated: facing south	–0.01 ± 0.03	–0.43	0.6670
Non-inoculated: CVT6	–0.05 ± 0.03	–1.73	0.0871
Non-inoculated: Taupō	–0.04 ± 0.03	–1.19	0.2382

5.4. Discussion

This pilot study shows that seedlings inoculated with mycorrhizal fungi did not receive benefits within 13 months. However, seedlings should be monitored for a longer period to confirm that seedling growth is not improved by mycorrhizal fungal inoculum.

In addition to time, the benign site where seedlings were established and the location where soil was collected could be a limiting factor for improving plant growth (Cooper, 1976; Davis, 2013). In the previous study of this thesis (Chapter 4), it was found some variability of mycorrhizal fungal communities among sites. For example, whereas wild plants were predominated by the family Claroideoglomeraceae at Rangitatau, Glomeraceae was predominant at Ruatiti and Tutira (Chapter 4). This difference could have influenced the success on this inoculation experiment, since different AM fungi could have different colonisation strategies (Johnson, 1976). Johnson showed that the level of mycorrhizal fungal colonisation was higher on *L. scoparium* when it was inoculated by *Acaulospora laevis* Gerd. & Trappe rather than by *Planticonsortium tenue* (Greenall) C. Walker & D. Redecker. In contrast, plant phosphorus content was higher when plants were colonised by *P. tenue*, rather than by *A. laevis* (Johnson, 1976). Therefore, it may be that species within the family Claroideoglomeraceae, and species closely related with *Claroideoglomus lamellosum*, could have a lower success colonising roots than other AM fungal species. However, further studies (histological or molecular) would be necessary to corroborate the main mycorrhizal fungal communities associated with the inoculated seedlings.

As was the case for AM, the dominance of EcM fungal communities differed among sites (Chapter 4). While Rangitatau was predominated by /tomentella-thelephora lineage at Rangitatau, the /russula-lactarius and /clavulina lineage dominated wild plants at Ruatiti and Tutira respectively (Chapter 4). Furthermore, EcM fungi may appear in successional colonisation stages previously colonised by AM fungi (Chilvers, Lapeyrie, & Horan, 1987; Lodge & Wentworth, 1990; Santos et al., 2001). Therefore, the short timeframe for evaluation could have limited the opportunity of observing some EcM fungal effects. Although, some field observations showed the presence of fruiting bodies (Appendix

5.6.1 c). Davis et al.'s study (2013) also revealed the mainly absence of EcM fungi on *L. scoparium* seedlings, but the authors encouraged further studies.

In summary, this experiment is a pilot study established in a benign site. Using harsher sites rather than benign sites could improve seedling growth and establishment after mycorrhizal fungal inoculum application. Furthermore, this pilot study is a long-term process that could reveal successful results later on, but there is the need of monitoring seedlings and using histological or molecular techniques for revealing the presence or absence of mycorrhizal communities and their identities. Finally, site could be a factor that has limited the successful colonisation of mycorrhizal fungi, and subsequently, plant growth stimulus. This brings a new question: what would have happened if seedlings would have been inoculated with soil collected from Ruatiti or Tutira?

5.5. References

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5.6. Appendices

Appendix. 5.1. Inoculation trial showing a) seedlings nine months' old (May 2017); b) root tips; c) ectomycorrhizal fungal fruit body.

Appendix. 5.2. Mean of stem basal area of seedlings from three nurseries (Bare roots: Manawatū, CVT6: Hawke's Bay and Taupō: Taupō) that were non-inoculated (-) or inoculated (+) with soil collected beneath wild plants, which showed previously that are widely colonized by mycorrhizal fungi. Means were calculated independently for North- and South- facing slopes. Seedlings were measured every 3 months (2016: August, December; 2017: February, May, and September).

Month	Stem basal area (mm^2)							
	North facing				South facing			
	+	*n*	-	*n*	+	*n*	-	*n*
Bare roots: Manawatū								
Aug 2016	11.35 ± 2.54	5	15.15 ± 3.42	5	12.12 ± 2.09	10	15.89 ± 1.02	10
Dec 2016	18.07 ± 2.52	5	19.15 ± 4.26	5	19.86 ± 3.79	9	20.42 ± 2.72	10
Feb 2017	79.89 ± 16.13	5	85.45 ± 5.74	5	49.72 ± 7.26	9	53.96 ± 7.25	10
May 2017	216.03 ± 39.88	5	272.82 ± 44.28	5	147.80 ± 18.34	9	135.80 ± 18.88	10
Sept 2017	281.45 ± 55.23	5	390.77 ± 38.03	5	197.53 ± 32.85	9	247.98 ± 38.84	10
CVT6: Hawke's Bay								
Aug 2016	6.12 ± 0.77	5	6.11 ± 0.48	5	8.01 ± 0.68	10	7.89 ± 0.65	10
Dec 2016	14.41 ± 2.06	5	17.06 ± 2.25	5	16.55 ± 1.18	10	12.68 ± 1.05	10
Feb 2017	83.51 ± 14.83	5	113.30 ± 22.20	5	48.52 ± 6.32	10	35.09 ± 4.66	10
May 2017	239.36 ± 36.38	5	324.63 ± 35.26	4	123.40 ± 18.00	10	154.66 ± 20.09	10
Sept 2017	409.64 ± 58.36	5	481.08 ± 94.15	4	216.40 ± 29.20	10	251.48 ± 28.62	10
Taupō: Taupō								
Aug 2016	18.73 ± 0.90	5	28.24 ± 2.49	5	22.80 ± 1.53	10	22.71 ± 1.08	10
Dec 2016	53.34 ± 6.79	5	69.37 ± 1.46	10	59.31 ± 3.94	5	56.52 ± 4.99	9
Feb 2017	125.09 ± 24.47	5	172.11 ± 13.69	5	100.43 ± 9.34	9	86.04 ± 10.73	9
May 2017	261.25 ± 52.42	5	363.94 ± 23.62	5	174.21 ± 24.83	9	154.61 ± 12.80	9
Sept 2017	409.59 ± 61.02	5	647.38 ± 52.14	5	395.65 ± 49.29	9	308.69 ± 30.40	9

Appendix. 5.3. Mean of height of seedlings from three nurseries (Manawatū, Hawke's Bay and Taupō region) that were non-inoculated (-) or inoculated (+) with soil collected beneath wild plants, which showed previously that are widely colonized by mycorrhizal fungi. Means were calculated independently for North- and South- facing slope. Seedlings were measured every 3 months (2016: August, December; 2017: February, May, and September).

Month	Mean height (cm)							
	North facing				South facing			
	+	n	-	n	+	n	-	n
Bare roots: Manawatū								
Aug 2016	38.78 ± 1.22	5	40.94 ± 1.96	5	38.25 ± 1.29	10	39.85 ± 0.79	10
Dec 2016	51.88 ± 4.13	5	47.54 ± 4.52	5	42.86 ± 1.51	9	46.64 ± 2.42	10
Feb 2017	92.34 ± 5.26	5	102.34 ± 3.44	5	78.22 ± 4.01	9	82.29 ± 4.34	10
May 2017	116.76 ± 6.60	5	130.20 ± 4.46	5	98.89 ± 5.03	9	104.12 ± 3.48	10
Sept 2017	120.20 ± 7.70	5	136.8 ± 2.82	5	97.78 ± 5.10	9	103.5 ± 3.19	10
CVT6: Hawke's Bay								
Aug 2016	41.82 ± 2.68	5	39.82 ± 2.21	5	40.01 ± 2.34	10	41.22 ± 2.39	10
Dec 2016	60.80 ± 4.35	5	57.44 ± 3.15	5	52.43 ± 2.50	10	51.22 ± 2.08	10
Feb 2017	99.26 ± 6.83	5	96.18 ± 10.91	5	83.60 ± 3.89	10	83.67 ± 1.64	10
May 2017	113.50 ± 8.88	5	116.75 ± 6.54	4	100.36 ± 4.60	10	86.19 ±10.14	10
Sept 2017	123.2 ± 9.41	5	118.75 ± 8.11	4	102.50 ± 4.51	10	98.2 ± 3.79	10
Taupō: Taupō								
Aug 2016	68.56 ± 0.88	5	72.38 ± 2.46	5	67.59 ± 0.96	10	65.96 ± 0.78	10
Dec 2016	88.66 ± 2.05	5	89.92 ± 5.43	10	89.03 ± 1.71	5	81.71 ± 3.38	9
Feb 2017	121.80 ± 5.99	5	126.60 ± 7.14	5	105.08 ± 3.43	9	99.56 ± 3.40	9
May 2017	134.20 ± 1.43	5	136.80 ± 5.46	5	117.77 ± 5.75	9	114.60 ± 4.22	9
Sept 2017	143.2 ± 2.03	5	143.40 ±6.65	5	125.44 ± 8.40	9	125.11 ±6.21	9

Chapter 6.

General discussion

“La paciencia es la madre de la ciencia”

Spanish proverb

6.1. Synthesis

Findings from this thesis show the relevance of the nectar content produced by *Leptospermum scoparium* flowers and the diversity of mycorrhizal fungal communities, which might play a major role in *L. scoparium* as well as in New Zealand ecosystems. Ecosystems are driven by biotic elements that interact and sustain food webs (Ings et al., 2009). These multitrophic interactions, linking below to above ground biota, imply that effects on a biotic element below ground could indirectly be reflected on other biotic elements above ground (Barber & Gorden, 2014). Understanding the influence of biotic elements through a community approach (Gehring & Bennett, 2009) could facilitate a greater understanding of the ecological implications in ecosystems (Strauss & Irwin, 2004), but also potential economic implications. Therefore, it is important to research multitrophic interactions (Strauss & Irwin, 2004), as most studies have mainly focused on examining interactions between limited number of species or on an unique food web level (Stanton, 2003). This limits the potential to understand the overall implications from an ecological and economic perspective.

Below ground, mycorrhizal fungi have been highlighted for their ability to improve seedling establishment (Lodge & Wentworth, 1990), plant growth (Suparno et al., 2015), drought tolerance (Smith & Read, 2008) and pathogen resistance (Sikes, Cottenie, & Klironomos, 2009). However, limited studies have indicated the implications of mycorrhizal fungi on above biotic elements such as herbivores (Bennett & Bever, 2007; Gehring & Bennett, 2009) and pollinators (Barber & Gorden, 2014), which could indirectly have an economic and social effect.

In New Zealand, *L. scoparium* is involved in multiple symbiotic relationships, including the association with mycorrhizal fungi (Hall, 1977; McKenzie et al., 2006), scale insects (Hoy, 1961) and honey bees (Bennik, 2009) (Fig. 6.1). These associations could also be interconnected. For example, previous studies have shown that nectar (Gange & Smith, 2005; Kiers et al., 2010) and pollinator visits (Wolfe et al., 2005; Barber et al., 2013) can be affected when plants are inoculated with arbuscular mycorrhizal (AM) fungi. This suggests that the presence of AM fungi on *L. scoparium* could have an indirect effect on honey bee visits (Fig. 6.1), mānuka honey yield, and subsequently, on the New Zealand honey industry.

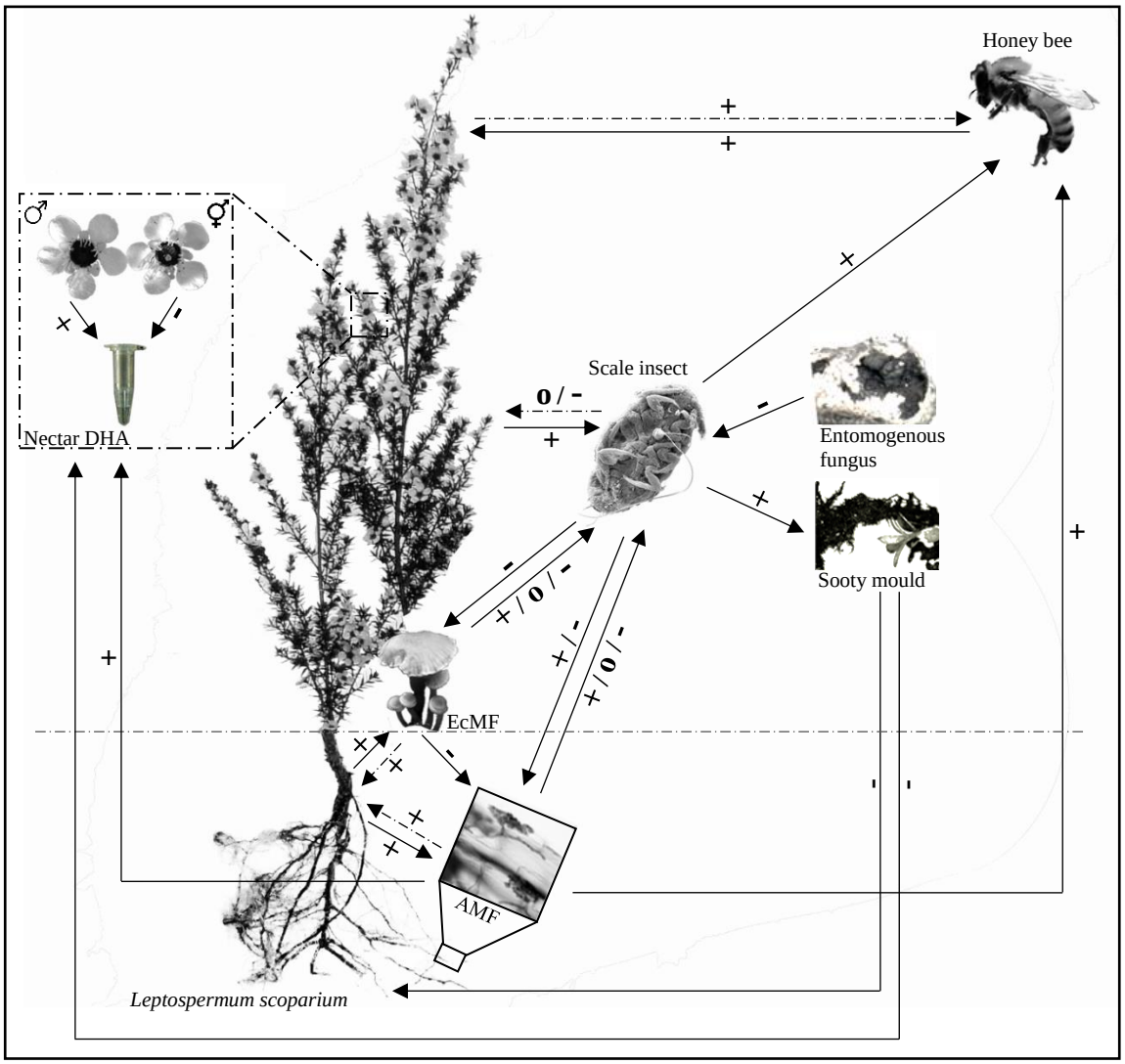

Fig. 6.1. Diagram illustrating the interactions between arbuscular mycorrhizal (AM) fungi, ectomycorrhizal (EcM) fungi, the entomogenous fungus (previously identified as *Angatia thwaitesii*), honey bees, *Leptospermum scoparium*, scale insects, and sooty mould (previously identified as *Capnodium walteri*). Flowers enclosed in the square represent a male and a hermaphrodite flower, and their effects on nectar dihydroxyacetone (DHA). Arrows in dashes indicate that those biotic elements have been researched during this study. Symbols (**+:** positive; **o:** neutral; **-:** negative) indicate the potential direct or indirect effect that a biotic element has on another element or nectar.

In contrast to AM fungi, the potential implications of ectomycorrhizal (EcM) fungi on pollinator visits seems to be unknown (Barber & Soper Gorden, 2014). However, it is known that the presence of EcM fungi could have effects on other biotic elements such as insect herbivores (Gehring & Bennett, 2009), although effects would vary depending on factors like insect, mycorrhizal fungal and host plant species (Gange & Smith, 2005; Gange, et al., 2005; Gehring & Bennett, 2009). An example is the decrease in numbers of adult leaf chewing insects (*Anomala cupripes*; Scarabaeidae) on *Eucalyptus urophylla* S.T.Blake (Myrtaceae) plants inoculated with the EcM fungal species *Laccaria laccata* (Scop. ex. Fr.) Berk., while the adults were not affected on plants inoculated with AM

fungi (Gange & Smith, 2005). In my study, another species from the same genus, *Laccaria glabripes*, was one of the dominant closely related EcM fungal species present, and may have the potential to reduce scale insect density on *L. scoparium*. However, further research is required to evaluate the effects of specific EcM fungal species on scale insects.

Leptospermum scoparium is one of the few plants worldwide able to form both AM and EcM fungal associations (Harley & Harley, 1987; Brundrett & Abbott, 1991; Chen et al., 2000; Lodge, 2000). This could imply that specialist scale insects present on *L. scoparium* could be naturally controlled by EcM fungi. A double benefit could be recovered from this dual mycorrhizal association, plant growth improvement (Suparno et al., 2015), and subsequently, nectar quality and quantity improvement (Gange & Smith, 2005; Kiers et al., 2010). In my study, it was found that uncontrolled scale insect presence did not affect significantly plant growth or nectar quality. This apparent host resistance could be due to different factors, including mycorrhizal fungal species identity or eriococcids species identity. *Acanthococcus campbelli* and *A. leptospermi* were the main species found in this study rather than *A. orariensis*, which was the main causative agent of *L. scoparium* mortality in the past (Hoy, 1961). *Acanthococcus campbelli* was found to damage *L. scoparium* more significantly than *A. orariensis* in Australia, as well as being present in more *Leptospermum* species than *A. orariensis* (Hoy, 1959). However, the effects of *A. campbelli* on *L. scoparium* appears to be mild in New Zealand according to findings from the present study. It would be important to evaluate its effects independently from *A. leptospermi*, since I could not disentangle the effects of both species in this study. An explanation could be that the presence of EcM fungal species is conferring this resistance. Consequently, the influence of *A. campbelli* on plant health could be supressed, and subsequently, nectar and honey production keep intact.

Even though scale insects have previously been shown to have a negative impact on *L. scoparium* (Mulcock, 1954; Hoy, 1961), their honeydew can play an important role as a food source for other insects (Markwell et al., 1993) and lizards (Evans et al., 2015). In addition, it has been questioned whether the key nectar ingredient dihydroxyacetone (DHA) is also present in this honeydew. This finding could provide potential economic value to the honeydew excreted from scale insects present on *L. scoparium*. However, only one study examining the presence of DHA in honeydew from *L. scoparium*, found

DHA was absent (Smallfield et al., 2018). Smallfield et al.'s (2018) findings are interesting, but it would be important to specify scale insect species and life cycle stage, as honeydew composition can differ depending on these factors (Fischer & Shingleton, 2001; Dhami et al., 2011). For example, Coelostomidiidae species are characterised for excreting larger amounts of honeydew than Eriococcidae species (Hoy, 1961). Among Coelostomidiidae species, immature adult females of *Coelostomidia wairoensis* excrete large amounts of honeydew, while adult females and immature males are characterised by the lack of mouthparts (Hoy, 1961). This morphological feature absent on adult females and immature males limits their opportunity for sucking sap. Knowing the origin of the honeydew analysed from Smallfield et al.'s study (2018) could indicate the potential value of the honeydew excreted by the scale insect species found in the present study. Furthermore, it would suggest if further studies should be carried out for analysing honeydew from species such as *A. campbelli*, *A. leptospermi* and *C. wairoensis*. Currently, *L. scoparium* is still associated with sooty mould due to scale insect presence (Fig. 6.1), but it appears that the nectar from plants covered by sooty mould is not affected (Williams et al., 2014), since no correlation was found between the ratio of DHA/total sugar and the presence of sooty mould (Williams et al., 2014). Finding scale insect species that do not affect plant development, but excrete honeydew characterised by DHA could bring the opportunity of a new potential value for *L. scoparium*.

The importance of the component DHA is well recognised, since it is the precursor of methylglyoxal (MGO) in mānuka honey (Adams et al., 2008; Mavric, Wittmann et al., 2008); and subsequently, of its antibacterial properties (Adams et al., 2009). Consequently, there is an interest for selecting cultivars with high level of DHA, as DHA content varies depending on the *L. scoparium* cultivar (Williams et al., 2014; Millner et al., 2016; Clearwater et al., 2018). Along with DHA, sugar content varies among cultivars (Chapter 3). As sugar is one of the driver on the attractiveness of honey bees to flowers (Wykes, 1952; Corbet et al., 1979; Nicolson et al., 2007), this key ingredient needs to also be considered when breeding programs are carried out. For example, selecting cultivars such as "Y", which was characterised by low sugar content and low nectar volume in this study would limit the number of honey bee visits that a flower receives. In contrast, having cultivars such as "P", which was characterised by higher sugar content and nectar volume would increase the probability of receiving more honey bee visits. Importantly, the composition of the nectar sugar needs to be regarded if the main goal is to establish

L. scoparium plantations for increasing the production of mānuka honey. *Leptospermum scoparium* flowers produce hexose-dominant nectar (Williams et al., 2014; Nickless et al., 2016), which is known for being less attractive to honey bees that sucrose-dominant nectar (Wykes, 1952; Waller, 1972). Therefore, selecting cultivars with the highest levels of sucrose and avoiding the establishment of *L. scoparium* plantations with neighbouring vegetation characterised by sucrose-dominant nectar could increase the number of visits to *L. scoparium* flowers.

Along with *L. scoparium* cultivar, flower sex can influence nectar content (Fig. 6.1). This was shown in Williams et al.'s study (2014), where male flowers presented higher DHA levels than hermaphrodite flowers. This finding could suggests that having a higher proportion of male flowers could increase nectar quality, and subsequently, mānuka honey quality. In the present study, hermaphrodite flowers were predominant (91.5%). In addition, it was found that flower sex ratio was correlated with the branch height above ground, increasing the proportion of hermaphrodite flowers as branch height increased. Similar results were found in the past (Primack & Lloyd, 1980), but the proportion of male flowers were dominant in comparison to hermaphrodite flowers. It is possible that some of the male flowers had not yet opened, since it appears that male flower peaks occur later (Primack & Lloyd, 1980).

Even though floral features such as flower sex can affect nectar quality and quantity (Primack & Lloyd, 1980), biotic elements associated with this plant could also affect the nectar quality and quantity (Gange & Smith, 2005; Kiers et al., 2010) (Fig. 6.1). For example, the AM fungal species *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßlerand and *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler have shown to increase nectar sugar content and nectar yield of *Tagetes erecta* L. (Asteraceae) (Gange & Smith, 2005). Same AM fungal species have been used for inoculating other two plant species, but these species have not shown any effect on nectar sugar content (Gange & Smith, 2005), which suggests that same mycorrhizal fungal species can influence differently on different plant species. Therefore, it would be important to understand the effects of the main mycorrhizal fungal species found on cultivated and wild plants during this study. For example, the AM fungal species that were most abundant in this study (*Claroideoglomus lamellosum*, *Rhizophagus irregularis* and *Rhizophagus proliferus*) could be selected for inoculating different *L.*

scoparium cultivars. Cultivars such as "P" and "Y", which ranged from the highest to the lowest nectar quality and quantity, could be used for evaluating the effects on inoculated and non-inoculated plants. Beside nectar, honey bee visitation could be also examined, since AM fungi have shown previously indirect effects on honey bee visitation (Gange & Smith, 2005) (Fig. 6.1). Importantly, the potential benefits of AM fungi for the host might be limited when EcM fungi are also present (Chen et al., 2000) (Fig. 6.1), since EcM fungi might cover roots and inhibit the penetration of AM fungi (Chilvers et al., 1987). However, it has been suggested that both types of mycorrhizal could might differ in colonisation stage (Chilvers et al., 1987). Whereas AM fungi might establish within the host in a first colonisation stage (Chen et al., 2000), EcM fungi would colonise in a successional stage the host. Consequently, a dual mycorrhizal benefit would be conferred to the host plant, seedling establishment and plant growth stimulation (Chilvers et al., 1987; Chen et al., 2000; Lodge, 2000).

In this study, both mycorrhizal fungal associations were found on cultivated and wild plants of *L. scoparium*. This finding suggests that mycorrhizal fungi are naturally established in cultivated plants once they are in the field. Most importantly, some EcM fungal species were closely related to endemic EcM fungal species from New Zealand (e.g. *Clavulina subrugosa* and *Cortinarius waiporianus*). This indicates that endemic mycorrhizal fungal species are more likely to be associated with host plants established in their native habitat than with exotic hosts, as was found in previous studies (Dickie et al., 2010; Walbert et al., 2010). However, the emblematic New Zealand flora could still be influenced indirectly by the presence of invasive EcM fungi and their invasive host plants. For example, *Amanita muscaria* (L.) Lam., which is known to be associated with the invasive host *Pinus* (Sawyer et al., 2001; Dickie et al., 2010), facilitates the establishment, plant growth, and dispersion of its host. In the present study, species closely related to exotic EcM fungal species such as *Amanita muscaria* were not commonly found. Specifically, only 1653 sequences matched with this EcM fungal species, being the majority of sequences recovered from the cultivar CVT1 at Ruatiti (1644), and only five sequences were recovered from wild plants. The cultivar CVT1 was planted on a site where *Pinus radiata* was previously established. In a previous study, this exotic EcM fungus was able to persist for at least 36 years in Australian *P. radiata* plantation forests (Sawyer et al., 2001). This suggests that *A. muscaria* may have spread via the previous exotic host *P. radiata* at Ruatiti.

Amanita muscaria is able to take up a large proportion of available nutrients when present in *Nothofagus* forests, reducing the availability of nutrients for native EcM fungal species (Dickie et al., 2008). Consequently, the establishment and dispersion of *Nothofagus* could be limited by this invasion and *Nothofagus* forest, which dominates 60% of New Zealand forests (Dickie et al., 2008), less able to colonise new soil surfaces. Along with *Nothofagus*, *Leptospermum* and *Kunzea*, which are the only other woody plant genera that form EcM associations in New Zealand (Moyersoen & Fitter, 1999; Orlovich & Cairney, 2004), could be also similarly limited by *A. muscaria*. Studies have shown that the presence of one of these EcM fungal hosts can facilitate the establishment and dispersion of the other EcM fungal host – a mutual benefit (Cooper, 1976; Wardle, 1980; Davis et al., 2013). If the establishment of one of this host is limited, the others could be also affected, and their ecosystems with them. However, the presence of *A. muscaria* was limited on wild plants of *L. scoparium* during this study, which suggests that it is unlikely that this host is threatened by *A. muscaria*, at least in the three studied sites.

Overall, this study shows that *L. scoparium* is a complex system driven by many symbiotic relationships (Fig. 6.1), which might influence the benefits obtained from an ecological and economic perspective. The potential of *L. scoparium* resides in the availability for forming dual mycorrhizal associations and producing flowers characterised by nectar DHA. Revealing that cultivated plants are naturally colonised by mycorrhizal fungi implies that seedlings are likely to receive mycorrhizal fungal benefits when they have been established in the field. Furthemore, both AM and EcM fungal associations might be the main drivers of *L. scoparium* resistance in extreme conditions and against scale insects. However, it would be important to understand the role of specific mycorrhizal fungal species, especially, endemic New Zealand species. Findings from different mycorrhizal fungal species could bring the opportunity of getting the optimal mycorrhizal inoculum. Consequently, the likelihood of obtaining economic benefits could be maximised as result of the increment on plant resistance, plant growth, nectar quality and honey bee visitation.

6.2. Challenges and limitations

This thesis aims to understand ecological interactions on *Leptospermum scoparium* through the broad study of three biotic elements: scale insect, honey bee and mycorrhizal fungus. The aim of this thesis is ambitious and it has involved the use of a range of techniques. Subsequently, it has been challenging to understand and compile findings together. Along with this challenge, limitations emerged during the experimental studies. These limitations are outlined below.

Plant material provided

Chapter 2 and 4 aimed to study plant material provided by funders. Four different cultivars (CVT1, CVT2, CVT3, and CVT4) were aimed to be studied at three different study sites (Rangitatau, Ruatiti and Tutira). However, the experimental design of the established plant material was unbalanced, since all cultivars were not present at the three study sites. This unbalance design implies that both factors (cultivar and site) are partially confounded, which limited the interpretation of the potential effects that cultivar and site may have had on scale insect, sooty mould, and mycorrhizal fungal diversity.

Insecticide treatment effectiveness

The insecticide treatment applied on different cultivars (Chapter 3), which were provided by funders, was limited to the non-flowering period, avoiding the presence of honey bees and any potential impact on them. As a consequence, the effectiveness of the treatment could be restricted by this limited application.

Differences in flowering time among cultivars

Chapter 3 studied cultivars characterised by flowering early in the season, and among cultivars, some cultivars flowered earlier than others. Flowering early in the season implies that cultivars like “MG” flowered previous honey bees were active during the experimental study. Therefore, the number of observations on this cultivar was limited. However, the preference of honey bees for this cultivar could be influenced by its establishment in a site characterised by a colder weather, where flowering period would occur later.

DNA amplification

Unsuccessful results were obtained when the adapter (specific to Illumina library protocol) was added to the reverse primer AML2, specifically designed for capturing AM fungi (Chapter 4). Different PCRs were conducted with different primers combinations, and it was determined that this reverse primer was causing the problem. Therefore, a new reverse primer (AML2-2) was designed.

Mycorrhizal fungal identification

Several mycorrhizal fungal studies and public databases are based on operational taxonomic unit (OTU) rather than on mycorrhizal fungal clades. This limitation implies that some OTUs recovered from this study could not be classified within a mycorrhizal fungal species, and they could belong to novel species still unknown, which could be globally dispersed, or specific to New Zealand.

6.3. Future research

Visual stimuli are defined as important drivers for honey bee flower visits (Goulson, 1999; Srinivasan, 2010). The influence of floral features such as hypanthium and UV-patterns on visitation rated to *L. scoparium* flowers are suggested as areas for future research. These factors have been shown to be implicated in the complex honey bee decision-making process along with olfactory stimulus factors (Comba, Corbet, Hunt, & Warren, 1999; Gronquist et al., 2010). Within olfactory stimulus factors, future research evaluating olfactory floral traits, such as amino acids (e.g. phenylalanine) present on *L. scoparium* flowers could help identify a sucrose replacement reward, as sucrose is a major driver of honey bee visits (Wykes, 1952; Waller, 1972), but is minimal in the nectar of *L. scoparium* flowers (Nickless et al. 2016; Millner et al., 2016).

The phylogenetic study of the data generated from the mycorrhizal fungal study could lead to a better understanding of the fungal species currently associated with *L. scoparium* in New Zealand. Furthermore, novel mycorrhizal and non-mycorrhizal fungal species or lineages could be discovered with these data. Previous studies have already indicated there is limited available information about fungi in New Zealand, specifically, about the diversity and distribution of mycorrhizal fungi (Tedersoo, 2010; Teasdale et al., 2013). Therefore, further research to identify these mycorrhizal fungal species is suggested.

Different mycorrhizal fungal species could have different implications for *L. scoparium* growth. Selecting specific EcM fungal species (e.g. *Laccaria* spp.), that could improve plant growth (Thomson et al., 1991), and AM fungal species (e.g. *Acaulospora morrowea*, *Rhizophagus irregularis*), which could improve drought tolerance (Augé, 2001; Ruiz-Lozano et al., 2015), could improve the quality of *L. scoparium* seedlings, increasing their survival and their establishment in stressful conditions. To select specific mycorrhizal fungal species, mycorrhizal fungal species should be characterised by: 1) a high colonisation rate; 2) fast colonisation; and 3) the ability to produce great number of natural inoculum like spores. Along with these considerations, selecting mycorrhizal species that colonise their host at different successional stages could be important.

There are limited host plants able to form the dual AM and EcM association, but *L. scoparium* is one of them (Mcnabb, 1968; McKenzie et al., 2006). This provides the opportunity for a better understanding of the potential effect of EcM fungi on AM fungi,

as EcM fungi could limit the symbiotic benefits the host plant could receive from AM fungi.

6.4. References

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