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Topic 1.5 — Relationship with endophyte populations

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Plant & Food Research: [1]Lincoln, [2]Te Puke; [3]Scion, Rotorua

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Topic 1.5 — Relationship with endophyte populations

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Contents

Executive summary **2**

1 Introduction **3**

2 Methodology **3**

2.1 Sample collection 3
2.2 Recovery and identification of live cultures 4
2.3 Amplicon sequencing 4
2.4 Bioassay 5

3 Results **5**

3.1 Culture collection 5
3.1 Amplicon sequencing 7
3.2 Bioassay 8

4 Discussion **9**

5 Conclusions **10**

6 References **10**

Executive summary

Plant resistance to disease is known to be modulated by the microbial endophytes, collectively termed the endomicrobiome, that are resident within their tissues. For the New Zealand Myrtaceae it was unknown whether microbial community-related fitness phenotypes existed. In other countries it has been shown that new season's growth is significantly less resistant to infection by the pandemic biotype of *Austropuccinia psidii*. Thus, the hypothesis tested in this work was that last season's growth would have a different endomicrobiome when compared with new season's growth and that endophytes found in last season's growth would be antagonistic towards rust spores.

The research focused on characterising the foliar endomicrobiome of *Lophomyrtus bullata* (ramarama), *Leptospermum scoparium* (mānuka) and *Metrosideros excelsa* (pōhutukawa), Myrtaceae that are infected by *A. psidii*. A live collection of 867 microbial endophytes was created of which 71% were fungi and 29% were bacteria. Of these 53%, 41% and 6% were from mānuka, pōhutukawa and ramarama, respectively. As some endophytes are not able to be cultured outside of a live host this work was complemented by amplicon sequencing to characterise both the unculturable and culturable endophytes. The results from both approaches showed that host, tissue type and tissue age were major drivers of the community structure. This is the first study to characterise the endomicrobiome of pōhutukawa, the first to compare two Myrtaceae and the first to show that the foliar endomicrobiome of new foliage is dynamic, changing within a season.

An *in vitro* assay was developed to assess the ability of microbial endophytes to inhibit the germination of rust spores in comparison to a fungicide control. The results showed that the endomicrobiome of mānuka and pōhutukawa contained bacteria that were antagonistic towards germination by rust spores, to an equivalent level as the fungicide control. This provides a potential mechanism by which older foliar tissues are less susceptible to myrtle rust infection. However, this assay was tested on the proxy poplar rust and further validation on *A. psidii* is required.

Collectively, based on the observation that new growth and young plants are worst affected by the *A. psidii* infection, the findings that there are differences in the endomicrobiome according to tissue age, and the fact that there are bacterial endophytes antagonistic to rusts in last season's growth, support the possibility that there is a role for the microbial community in tolerance to *A. psidii*.

Recommendations

1. The ability of isolates within the curated collection to inhibit myrtle rust is fully tested
2. That the microbial community within those plant genotypes with greater resistance to myrtle rust are characterised
3. That the taxa vertically transmitted by the seed of resistant plant genotypes is determined along with their survival during seed banking.

1 Introduction

Plants are metaorganisms, forming associations with a large number of microorganisms. Those microorganisms that live within the plant are termed endophytes and collectively they form the plant endomicrobiome. The plant endomicrobiome has been formed during a process of joint evolution and selective pressure (Hassani et al. 2018) and it is known to have a significant role in plant growth, physiology and health (Porras-Alfaro & Bayman 2011). Research has demonstrated that key members of the plant endomicrobiome can help the plant defend itself against pathogens.

Recent work has demonstrated a functional role for endophytes in the myrtaceous species mānuka (Wicaksono et al. 2017a, 2017b) and demonstrated that deliberate inoculation with key taxa can modify plant growth, metabolite profile and disease resistance. For mānuka, endophyte communities are strongly driven by tissue type and vary between seedlots. Thus, the microbiome imparted to the seedlings through vertical or horizontal transmission may correlate with the resistance status of plants. Work done internationally has shown that endophytes can mediate resistance against poplar (Busby et al. 2016) and pine blister rust (Ganley et al. 2008), providing support for endophytes to play a long-term role in the effective management of myrtle rust.

The goal of this work was to characterise the complete (both culturable and unculturable) microbial community profiles of pōhutukawa, ramarama and mānuka. In parallel with microbial community analysis, a curated collection of culturable microbial taxa was generated from the foliage of the tolerant last season's growth and the susceptible new season's growth. The antifungal potential of key taxa was determined through *in vitro* assays as a first selection criteria. This information, combined with new understanding of plant genetics, provides knowledge to help protect Myrtaceous species from myrtle rust.

2 Methodology

2.1 Sample collection

The project team selected three Myrtaceous species that are hosts of the myrtle rust disease as the subjects for research to discover the diversity of their endophytic microbial communities. These were *Lophomyrtus bullata* (ramarama), *Metrosideros excels*a (pōhutakawa) and *Leptospermum scoparium* (mānuka). The rationale for this choice was that they represented a highly susceptible species, a species that produced an iconic floral display and one that is an emerging economic strength for New Zealand, respectively. Connection was made with mana whenua in four sites to facilitate collection of material. We sincerely acknowledge and appreciate the help from Hone Ropata, Aleise Puketapu and Alby Marsh who undertook this.

Plant material was collected from Auckland, Bay of Plenty, Taranaki and Canterbury. At each site twigs from healthy specimens of each species (where available) were sampled. Each twig contained previous and current season's growth. Fewer samples of ramarama were obtained because of the overwhelming infection in the North Island observed in the spring. Samples of stems and leaves were collected for isolation of live organisms and for characterising the total microbiome by molecular methods (Figure 1).

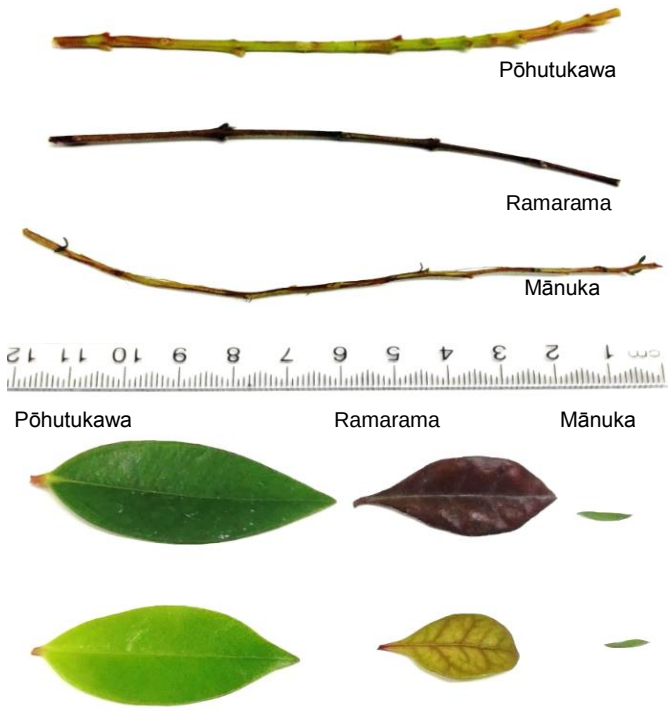

Figure 1. Examples of the leaves and stems of *Metrosideros excelsa* (pōhutakawa), *Lophomyrtus bullata* (ramarama) and *Leptospermum scoparium* (mānuka) from which microbial endophytes were recovered.

2.2 Recovery and identification of live cultures

Culturable microorganisms recovered from the leaves and stems can be used to undertake bioassays to understand whether they can contribute to the ability of the host plant to resist myrtle rust. Thus, a curated collection of culturable microorganisms, representative of the diversity in the host plants, was established.

Twigs were collected to have leaves and stems from different life groups (old and new growth). Leaves and stems of old (last season) and new (current season) growth for each plant at each site were surface sterilised, bisected across the mid rib and plated onto semi-selective media to recover a range of endophytic fungi and bacteria. To ensure a similar frequency of recovered microorganisms from the leaves, which varied substantially in size (Figure 1), different numbers of leaves were plated per species. Per twig these were a single leaf for pōhutakawa, two for ramarama and nine for mānuka. Emerging cultures were purified and identified by sequencing a portion of the ribosomal DNA. Isolation of endophytic microorganisms was done within a week of receiving the samples.

2.3 Amplicon sequencing

Only a small proportion of endophytic microorganisms can be recovered into live culture. Thus, the live curated collection represented only a small portion of the total community of microorganisms living inside the target host plants. To characterise the complete community of microorganisms resident within the foliage, amplicon sequencing was used. Because of the difficulties in obtaining sufficient material from ramarama, only pōhutakawa and mānuka were used for amplicon sequencing.

DNA was extracted from surface sterilised leaves and stems and enriched for endophyte DNA. Fungal and bacterial sequences were amplified from these tissues using universal primers to

generate amplicon libraries that were representative of the total microbial community. These amplicon libraries were sequenced by Massey University using the Illumina MiSeq v2 platform. Positive, negative and known ratio controls were included. In total, 384 samples were sequenced.

2.4 Bioassay

Endophytes that live internally within the foliage are likely to represent a key component of the plant defence system. In New Zealand, there is little knowledge of these communities and their role in plants. From the curated collection, selected isolates were grown in liquid culture to produce both cells and filtrates for testing. A spore germination assay was developed and tested using poplar rust as a proxy for myrtle rust at the Lincoln Research Centre, as myrtle rust is not yet confirmed in the Canterbury region.

Sterilised agar slides covered with a thin film of 2% agar-water were sprayed by airbrush with rust spores suspended in oil and an aliquot of either culture supernatant or cell suspension was spread on the slide. The ability of 20 of the recovered bacterial endophytes to inhibit germination of rust spores was determined by observation under the microscope. Those that inhibited spore germination were identified by ANOVA using GenStat 17th Edition.

3 Results

3.1 Culture collection

All types of foliar tissues from all hosts contained live, endophytic microorganisms and representatives of these were recovered into live culture (Figure 2).

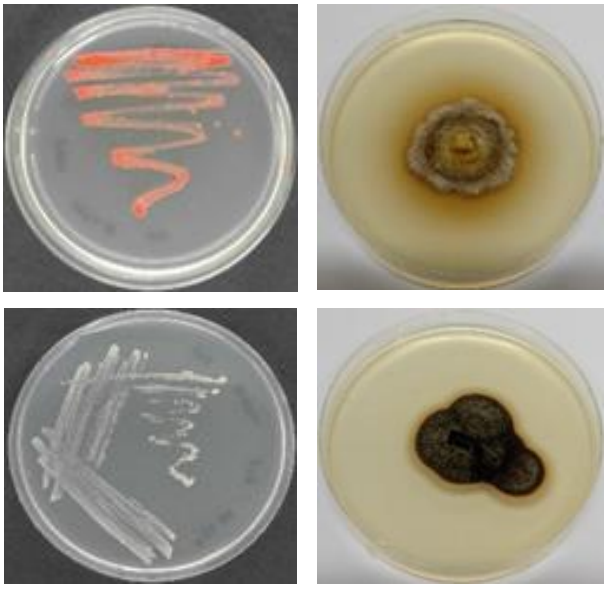

Figure 2. Examples of bacteria (left-hand plates) grown on R2A agar and fungi (right-hand plates) grown on potato dextrose agar isolated from *Leptospermum scoparium* (mānuka).

Isolates of both bacteria (including Actinobacteria) and fungi were obtained; however, no colonies grew on the CRNH *Phytophthora* selective media. In total, 867 microorganisms were isolated, of which 461, 356 and 50 were from mānuka, pōhutukawa and ramarama, respectively. There were 239, 219, 293 and 116 recovered from host plants from the Auckland, Taranaki, Bay of Plenty and Canterbury regions, respectively (Figure 3).

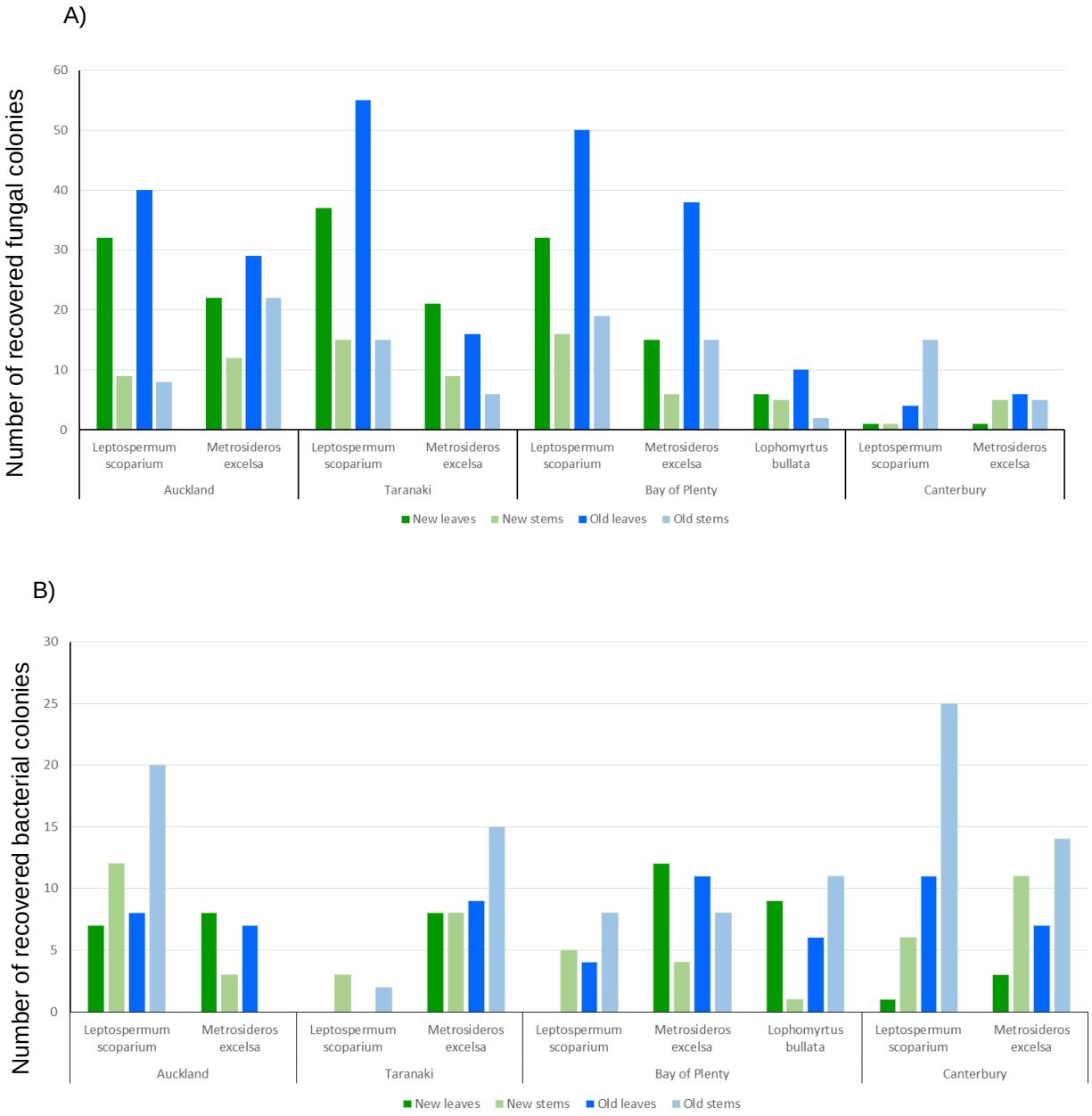

Figure 3. Number of isolates of A) fungi and B) bacteria recovered from four tissues collected from three Myrtaceous species (*Leptospermum scoparium* (mānuka)*, Metrosideros excelsa* (pōhutukawa)*, Lophomyrtus bullata* (ramarama)) growing in four regions of New Zealand.

Overall, 615 fungi were recovered from leaves and stems for all species and sites. The greatest number of colonies were recovered from the old leaves (n=250; 40%) compared with young leaves (27%), old stems (18%) and young stems (15%). Cultures that were representative of the different morphotypes were retained and curated. DNA from 275 curated samples was extracted for identification by DNA sequencing of the ribosomal DNA. Within these 275 sequenced fungi, 59 individual genera were represented. When the data were combined for

both host plants, there were 34, 29, 22 and 18 genera in old leaves, young leaves, old stems and young stems, respectively. Of the 34 genera found in old leaves, 14 were unique, 18 were shared with young leaves and two were shared with stems (Figure 4). Only 16 (27%) of taxa were shared between stem ad leaves.

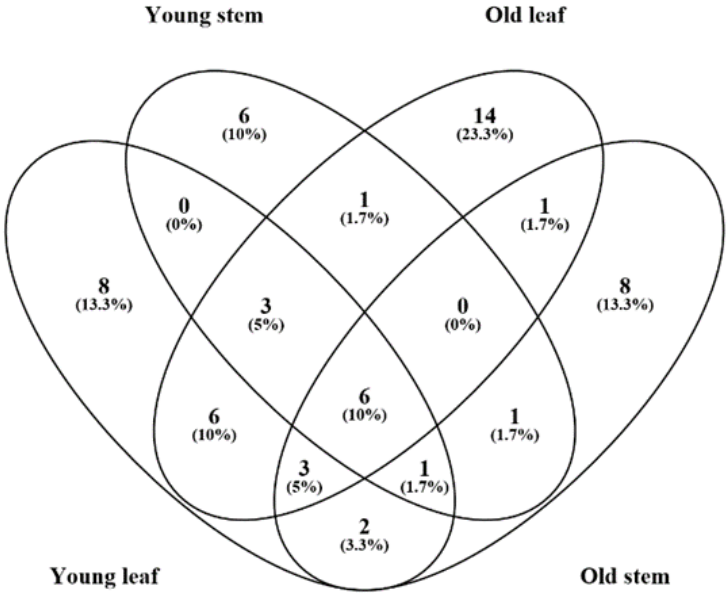

Figure 4. Venn diagram of the number (percentage) of taxa in each tissue type combined for *Leptospermum scoparium* (mānuka) and *Metrosideros excelsa* (pōhutukawa). Overlap between tissue types is indicated by the overlap of ellipses.

Because of the lack of significant morphological features, the bacterial colonies were not morphotyped. Of the 267 bacteria recovered, 220 (82%) were identified by sequencing the 16S region of the ribosomal DNA. The four individual tissues of mānuka and pōhutukawa were similar in the diversity of bacterial genera present, ranging from 6–9 in mānuka and 8–11 in pōhutukawa. For ramarama there was a lower diversity of bacteria in young stems (n=1) but this is the result of the overall small number of colonies recovered (n=4). The remaining three tissue types for ramarama had 5–8 genera present, which is similar to the other two host plants. Overall, the dominant genus was *Bacillus*, representing 96 (44%) of the sequenced colonies.

3.1 Amplicon sequencing

The amplicon library generated for fungi, from DNA extracted from the four tissues sampled from mānuka and pōhutukawa across four regions of New Zealand, generated a total of 3.9 M reads. These were divided into 690 distinct operational taxonomic units (OTUs).

Analysis of the resemblance matrix generated from the OTU table using PERMANOVA showed that the total community of microbial endophytes found in different sites did not differ from each other (PERMANOVA p=0.818), indicating that most fungal taxa were present at all sites. However, the endophyte communities recruited by the plant hosts at each site were different (PERMANOVA p=0.019) (Figure 5) and that these fungal communities differed between tissues and according to the age of tissues (PERMANOVA p=0.003, p=0.001, respectively). Mānuka tissues contained more OTUs grouped as *Capnodiales* sp., Amphisphaeriaceae and *Phaeothecoidea* sp. In contrast, pōhutukawa contained relatively higher proportions of grouped in the *Sporomiaceae*, *Alternaria* sp. and Malasseziales. There were differences in the relative

abundance of taxa between tissues. For example, old stems had relatively more *Amphisphaeriaceae*, whereas young stems had a greater abundance of *Capnodiales* sp. (sooty mould fungi). Old leaves contained relatively more *Phyllosticta* sp. and young leaves had more *Phaeomoniella* sp.

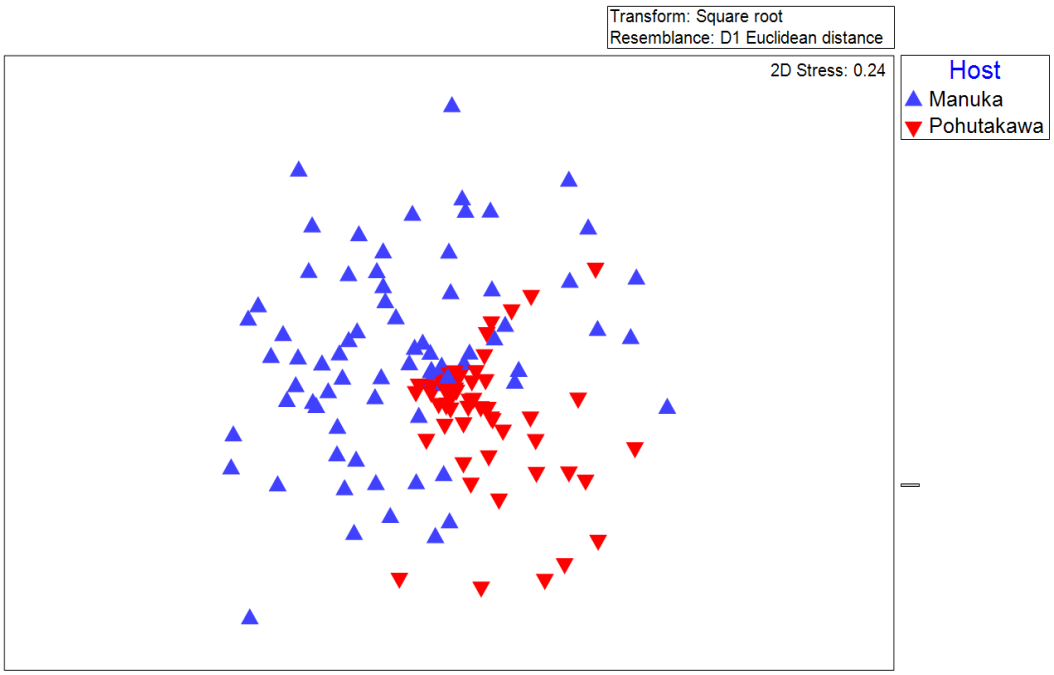

Figure 5. Nonmetric multidimensional scaling (MDS) plot showing fungal operational taxonomic units (OTUs) derived from the leaves and stems of *Leptospermum scoparium* (mānuka) (▲) and *Metrosideros excelsa* (pōhutakawa) (▼). Each triangle is an individual tree (genotype).

The bacterial amplicon libraries produced substantially fewer reads than expected and should be repeated to confirm the relative abundance of different taxa. The OTUs generated were placed into eight families, of which the Acetobacteraceae and Methylocystaceae were the most abundant.

3.2 Bioassay

A bioassay was developed and optimised to measure the effect of live cultures and filtrates from endophytic bacteria on the germination of rust spores. Poplar rust was used as a proxy for *A. psidii* because of the absence of this pathogen in the Canterbury region. This *in vitro* assay was validated on 20 bacteria encompassing five genera (*Pseudomonas, Pantoea, Bacillus, Burkholderia* and *Rahnella*). Of these, the majority (n=15) had been isolated from last season's growth with even representation from each of the two host plants. There was a significant difference between the ability of the bacteria to inhibit rust spore germination (p<0.001; LSD = 0.05) with seven bacteria inhibiting spore germination to a level similar to that of the fungicide control (100 ppm/mL strobilurin). The species able to inhibit spore germination were from the genera *Bacillus, Burkholderia, Pantoea* and *Pseudomonas*. They were able to reduce spore germination from 86% in the control to 16–24%.

4 Discussion

Research on endophytes of native New Zealand plants is scarce and this is the first study to characterise the complete endomicrobiome of pōhutukawa and to compare it to that of mānuka. This work also contributes to the growing body of global literature on the structure, diversity and function of plant microbiomes and provides an important knowledge baseline from which to build an understanding of the role microbial endophytes play in plant resistance to myrtle rust.

This research confirmed the hypothesis that all three plant hosts, mānuka, pōhutukawa and ramarama, contained communities of fungi and bacteria within their foliage. This was demonstrated by both the recovery of >800 live organisms and by the amplicon sequencing. Thus, there is a resource of microbial communities from which those that improve tolerance to myrtle rust can be selected. Foliar endophytes have previously been shown to modulate disease severity in poplar rust (Busby et al. 2016), pine blister rust (Ganley et al. 2008), and to confer disease resistance in endangered plants (Zahn & Amend 2017) and tropical trees (Arnold et al. 2003). For mānuka, previous work has demonstrated that the foliage contains microorganisms that are antagonistic towards bacterial and fungal plant pathogens (Wicaksono et al. 2016). Further work should also investigate the proportion of taxa that are vertically transmitted by seed to identify those that can be introduced into the seedbank of Myrtaceous species.

This work is the first to compare the microbial communities of two native species and showed that the microbiomes differed between the two host plants. This result was in agreement with the current body of literature that identifies host as a major driver of microbial community structure. This is likely because of the independent evolution of these plant species and their associated microorganisms in response to variable selection pressures (Hassani et al. 2018). The demonstrated variation in susceptibility to myrtle rust by some genotypes of myrtaceous species, including mānuka and pōhutukawa (Grant Smith, per comm.), provides the opportunity to test whether microbial community-related fitness phenotypes exist in some Myrtaceae. Extrapolation of the current dataset to include plant genotypes of known susceptibility should be the subject of future work.

Field observations and deliberate inoculations of many hosts in a number of countries have shown that the pandemic biotype causes more severe symptoms on young stem and leaf tissue (Makinson 2018). The results from both the culture recovery and the amplicon sequencing showed that the microbial community structure within mānuka and pōhutukawa differed with tissue type (leaf or stem) and also according to the age of the material (current or last season's growth). To the best of our knowledge this is the first study, globally, to demonstrate that the microbial community resident in new season's growth is dynamic and changes over the season. Accordingly, representatives of the taxa abundant in older tissues that had been recovered into culture were given greater priority in bioassays.

The bioassay showed that seven bacteria, from four genera, that had been recovered from the three host plants were able to inhibit the germination of rust spores to levels equivalent to that of the fungicide control. Of these, six strains had been isolated from last season's growth. Several of these taxa have been reported as inhibitory towards rusts previously. *Bacillus graminis* and *Bacillus lentimorbus* control powdery mildew of wheat caused by *Blumeria graminis* f. sp. *tritici* (Gao et al. 2015) and coffee rust caused by *Hemileia vastatrix* (Shiomi et al. 2006), respectively. *Pantoea agglomerans* has previously been shown to inhibit bean rust caused by *Uromyces appendiculatus* and was the most promising bacterial isolate out of 120 tested (Yuen et al. 2001). *Pseudomonas fluorescens* suppressed rust of groundnuts caused by *Puccinia arachidis.*

5 Conclusions

Overall, the research has demonstrated that:

i. Fungal and bacterial endophytes are found in all foliar tissues on mānuka, pōhutukawa and ramarama

ii. Endophyte composition between plant species is significantly different

iii. Age and tissue type influences the composition of the endophyte community

iv. Host and tissue type rather than region in which the plants are grown are major drivers of microbial community structure

v. Endophytes with the ability to inhibit the germination of rust spores are present in mānuka and pōhutukawa.

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