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Arbuscular mycorrhizal fungi associated with *Leptospermum scoparium* (mānuka): effects on plant growth and essential oil content

Wisnu Adi Wicaksono[1] · **Catherine E. Sansom**[2] · **E. Eirian Jones**[1] · **Nigel B. Perry**[2] · **Jana Monk**[1] · **Hayley J. Ridgway**[1]

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Abstract *Leptospermum scoparium* or mānuka is a New Zealand native medicinal plant that produces essential oils with antimicrobial properties. This study investigated the arbuscular mycorrhizal fungi (AMF) community in mānuka by culture dependent (trap culture) and independent (denaturing gradient gel electrophoresis) approaches. Furthermore, to assess whether mycorrhizal inoculation could alter growth and essential oil composition of mānuka, plants of a single regional chemotype were grown in unsterilized soil and inoculated with five AMF isolates. Leaf essential oil compositions and yields were determined by microscale solvent extraction and gas chromatography-mass spectrometry (GC-MS) analysis. AMF inoculation significantly increased growth compared to uninoculated plants. Qualitative i.e. different relative proportions of compounds, which are distinctive in chemotypes and quantitative (i.e. absolute concentrations of compounds, expressed as mg/g of dry leaf or equivalent) effects of AMF inoculation on mānuka essential oil composition depended on the isolate. AMF inoculation modified the Gammaproteobacterial community on roots and this may have contributed to changes in essential oil composition. Overall, these results demonstrated that AMF can improve the growth of mānuka and affect plant secondary metabolites in leaves, which would be valuable in commercial essential oil production from plantation-grown mānuka.

Keywords *Leptospermum scoparium* · Arbuscular mycorrhizal fungi · GC-MS analysis · Essential oils · Denaturing gradient gel electrophoresis · Gammaproteobacteria

1 Introduction

Mānuka, *Leptospermum scoparium* J. R. et G. Forst. (Myrtaceae), is a New Zealand indigenous shrub. Essential oils are produced from foliage of this plant commercially for their antimicrobial properties (Perry et al. 1997; Lis-Balchin et al. 2000). Mānuka from different regions of New Zealand have qualitatively distinct essential oil compositions, i.e. chemotypes which differ greatly in the relative proportions of different compounds (Perry et al. 1997; Porter and Wilkins 1990). A study of mānuka from 87 locations across New Zealand proposed ten chemotypes (Douglas et al. 2004). In particular, essential oils from the East Cape showed a higher antimicrobial triketone content compared to other areas of New Zealand. Different chemotypes in medicinal plants, including mānuka, are likely to be controlled by plant genetic factors (McGimpsey et al. 1996; Perry et al. 1997; Medina-Holguín et al. 2008). However, arbuscular mycorrhizal fungi (AMF) have been shown to affect essential oil production in other plants, by improvement of P nutrition (Rydlová et al. 2016) and by increased peltate gland numbers (Copetta et al. 2006).

AMF have been shown to form associations with mānuka (Moyersoen and Fitter 1999; McKenzie et al. 2006; Weijtmans et al. 2007) but their effects on the essential oil composition were not examined. We now report the AMF

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✉ Hayley J. Ridgway
Hayley.Ridgway@lincoln.ac.nz

[1] Faculty of Agriculture and Life Sciences, Lincoln University, Lincoln, New Zealand

[2] The New Zealand Institute for Plant & Food Research Limited, Department of Chemistry, University of Otago, P.O. Box 56, Dunedin, New Zealand

communities associated with mānuka collected from nine sites across New Zealand. Selected AMF were inoculated onto one regional chemotype of mānuka to test their effects on plant growth and/or essential oil contents. We also investigated the impact of AMF inoculations on associated microbial communities, which might allow AMF to indirectly influence mānuka essential oil composition.

2 Materials and methods

2.1 Plant sampling

Mānuka samples were collected between July and August 2013 from three sites in the South Island of New Zealand and a second sampling was done between August 2014 and January 2015 from six sites across New Zealand (Table 1). Mature plants (> 20 years old) were chosen for this study by estimating age from local knowledge of historic forest fires/planting dates and/or by the size/trunk girth of the plants. Five lateral roots (approximately 1 mm in diameter and 3–5 cm long) were sampled from each mānuka tree and stored at 4 °C for up to 1 week prior to processing.

2.2 Recovery of arbuscular mycorrhizal fungi using trap cultures

2.2.1 Trap culture establishment

A silica sand:pumice medium (1:1 *v*/v), sterilized in an autoclave (1 h, 121 °C, 1.5 atm) prior to inoculation, was used for trap culture establishment. Root samples (3–5 g fresh weight) were washed, cut into 2–3 cm pieces and used to inoculate the medium in 500 mL pots (1 sample per pot). Seeds from a single stand of mānuka from Travis Wetland heritage and restoration site, Christchurch, and those of "Grasslands Lancelot" plantain (*Plantago lanceolata* L.), were used as hosts for trap culture. Pots with no added root samples were used as uninoculated controls to check for any contamination during the growth phase. After 2 weeks, 20 mL phosphate free Hoagland solution (Elias and Safir 1987) was added every month and plants were allowed to grow for 4–5 months (approximately 20 cm in height). Pots were left to dry at ambient temperature (average between 13 °C and 22 °C) for 1–2 weeks and watered daily as needed. No supplemental light was provided during the experiment.

AMF spores were isolated using a modified wet sieving method (INVAM, http://invam.caf.wvu.edu). Approximately 10 g of culture media were mixed with 100 mL of tap water and allowed to stand after mixing in room temperature for 30 min to sediment the heavier fraction. The supernatant was wet sieved using three stacked sieves of decreasing pore size 500, 150 and 50 μm. The washing suspensions (± 10 mL) from the 150 and 50 μm sieves were transferred to a 50 mL tube and centrifuged at 2000×*g* for 3 min and the supernatant was discarded. Forty-five mL of 50% *w*/*v* sucrose was added into the pellet and the suspension centrifuged again at 2000 x *g* for 40 s. The supernatant was sieved using the 50 μm sieve and washed with tap water to remove excess sucrose. The spores were collected on filter paper and grouped based on colour and shape. The spores were stored at 4 °C for further analysis.

2.2.2 Identification of AMF based on 18S rRNA gene

DNA from five to ten spores from individual morphotypes were extracted using the REDExtract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich) according to manufacturer's instructions, except that the volumes of extraction and dilution solutions were both reduced to 10 μL. Spores were crushed using sterile pipette tips under a stereo microscope (Nikon Instruments Inc., USA). A portion of the 18S rRNA gene was amplified using primers AML1 and AML2 as described by Lee et al. (2008) except that annealing was at 50 °C. The PCR products were sequenced at the Lincoln University Sequencing Facility, then viewed and manually trimmed using Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) to remove ambiguous sequences. Sequences were then compared with

Table 1 *Leptospermum scoparium* sampling sites used in this study

Sampling site	Latitude	Longitude	Region	No. of plants
Travis Wetland (TWL)	−43.48424	172.6902	Central South Island	3
Craigeburn Forest Park (CR)	−43.15275	171.7314	Central South Island	3
Island Hill Station (IHS)	−42.74402	172.5617	Northern South Island	6
West Coast (WC)	−41.93865	171.4259	Northern South Island	5
Aoraki/Mt. Cook National Park (AMC)	−43.73295	170.0959	Southern South Island	3
Mount Aspiring National Park (MA)	−44.71971	168.2820	Southern South Island	3
Taihape Scenic Reserve (THP)	−39.67635	175.8056	Central North Island	2
Tongariro National Park (TNP)	−39.02237	175.7181	Central North Island	2
Kaimanawa Forest Park (KFP)	−38.94721	175.9437	Central North Island	2

those of know origin using NCBI (http://www.ncbi.nlm.nih.gov) and MaarjAM (Öpik et al. 2010) database, and were deposited in the NCBI database under accession numbers KX811527-KX811533.

2.3 Identification of arbuscular mycorrhizal fungi using denaturing gradient gel electrophoresis (DGGE)

2.3.1 DNA extraction

The root samples were surface sterilized and treated with propidium monoazide (PMA, Biotium Inc., USA) prior to DNA extraction, to exclude any residual surface DNA from PCR as described by Wicaksono et al. (2016). Roots were ground to a fine powder with liquid nitrogen, prior to total DNA extraction using the PowerPlant™ DNA isolation kit (MoBio Laboratories, Carlsbad, USA) following the manufacturer's instructions.

2.3.2 PCR and DGGE analysis

A specific region of 18S rRNA gene was amplified using a nested PCR. The first PCR was done using a primer pair AML1 and AML2 as described previously. The PCR product was diluted 1/10 and used as template for a second PCR. The second PCR was done using primer pair Glo-1 and NS-31GC as previously described by Cornejo et al. (2004).

DGGE were performed with Cipher DGGE Electrophoresis System (CBS Scientific) with a 30 to 45% linear denaturing gradient as described by Wicaksono et al. (2016). The gels were run in 0.5 × TAE buffer for 16 h at 90 V and 60 °C. The DGGE staining using silver stain was done as described by Wicaksono et al. (2016). Prominent DGGE bands representative of diversity at each site were excised (at least four bands per sites), suspended in 50 μL of sterile water, crushed using 200 μL tips (Axygen, USA) and subsequently heated at 65 °C for 40 min using a thermal cycler (Applied Biosystem Veriti) to elute the DNA. Bands were reamplified using primer pair Glo-1 and NS-31GC as described previously. The PCR-amplified excised DGGE bands were Sanger sequenced at the Lincoln University Sequencing Facility. Ambiguous sequences manually trimmed using Bioedit. Sequences with more than 97% similarity with known AMF sequences were deposited in the NCBI database under accession numbers KY010202-KY010225.

2.3.3 Phylogenetic analysis

Known AMF sequences from the GenBank database, together with those obtained from spores generated in trap cultures and those from excised DGGE bands with sequence length ± 200 bp, were aligned using CLUSTALW and the distance matrices and phylogenetic trees were calculated by maximum likelihood algorithms with 1000 bootstrap replication using MEGA 6 software (Molecular Evolutionary Genetic Analysis; Tamura et al. 2013). *Smittium culisetae* (JN940701) and *Batrachochytrium dendrobatidis* (AH009052) sequences were used as outgroup taxa.

2.4 Spore propagation

Spore morphotypes (n = 2) of *Acaulospora* spp. M1 and M4 were propagated. Other morphotypes (n = 5) were excluded due to low numbers of spores (<5 spores/10 g) recovered from spore trap culture or similar identity based on 18S rRNA sequence compared to other morphotypes. Spore propagation was done as described previously in the trap culture establishment Section by replacing root samples with 100 healthy and uniform spores from each morphotype into wide, 3 cm deep holes in a 500 mL pots (three pots for each morphotype) containing sterile silica sand: pumice medium (1:1 *v*/v). Holes were then filled with the medium. Seeds of plantain and from a single stand of mānuka from the Travis Wetland were used as hosts for culture purification and bulk production. Three other AMF isolates were also propagated and applied as described above: *Acaulospora* sp. MPC47, isolated from macrocarpa (*Cupressus macrocarpa*) in Canterbury, *Funneliformis* sp. MPC8, isolated from apple in Canterbury, and *Scutellospora* sp. MPC13, isolated from apple in Canterbury, obtained from the Plant Microbiology Group culture collection, Lincoln University. Pots to which no spores were added were used as uninoculated controls to check for contamination during the growth phase. All fungal cultures were maintained and harvested as previously described.

2.5 Glass house experiment

Seeds from a single stand of mānuka from the Travis Wetland were sown in a potting mix medium composed of 20% pumice, 80% composted bark, 2 kg/m^3 Osmocote® Extract Standard 3–4 months gradual release fertiliser (16:3.5:10; N:P:K, respectively, plus trace elements), 1 kg/m^3 agricultural lime, 500 g/m^3 Hydraflo® 2 (granular wetting agent, Scott Product New Zealand, Ltd) in July 2015. Seeds were left to germinate for two months in a greenhouse at ambient temperature and with natural light.

Non-sterilized soil from Lincoln University was used: a permanent pasture soil with mottled pallic soil and low phosphate availability (pH 5.8, Olsen phosphorus 14 mg/L, organic matter 3.8%, total carbon 2.2%, and total nitrogen 0.17%). The soil was air-dried and then passed through to a 2 mm sieve to remove stones and plant root debris.

Forty grams of sterile sand:pumice medium containing approximately 100 spores from selected AMF strains (n = 5) were inoculated into 5 cm deep holes in 1.2 L pots filled with unsterilized soil. Mānuka seedlings with similar height

(approximately 5 cm) and form were placed on top of the AMF inoculum and the roots then covered with the soil. The pots were arranged in a complete randomized design with 9 replicates per treatment and grown in a glasshouse at ambient temperature (average between 13 °C and 22 °C), natural light and watered daily. The plants were grown from September 2015 until February 2016, i.e. spring to summer.

2.6 Plant growth and arbuscular mycorrhizal fungal colonization

Shoot heights were measured from the soil line to to the tallest outstretched leaf, and internodes were counted. Shoot and washed root portions were weighed after oven drying at 60 °C for 2 days.

Roots were washed in tap water and stained as described by Phillips and Hayman (1970). Four subsamples per replicate were mounted on slides in lactoglycerol solution and examined under a compound microscope (Nikon Instruments Inc., USA) at 200× magnification. Arbuscular mycorrhizal fungal colonization from 120 intersections per plant was determined as described by McGonigle et al. (1990). The criteria for an arbuscular mycorrhizal fungal colonization was if the vertical crosshair intersected either a hyphae, an arbuscule, or a vesicle (Miller and Sharit 2000).

2.7 Essential oil analysis

Leaves of four randomly selected plants from each treatment in the glass house experiment were collected for essential oil extraction. Each sample (approximately 15 mg) was ground in liquid nitrogen and a 10 mg subsample weighed into a 4 mL vial, then 0.5 mL of dichloromethane containing internal standard (eicosane, 0.2 mg/mL) was added. After mixing on an orbital shaker overnight, each extract was filtered (0.45 μm PTFE).

GC-MS analyses were performed on an Agilent 7890A gas chromatograph (Agilent Technologies, Australia) with a CTC Analytics PAL system auto-sampler and an Agilent 5975C inert XL MSD with triple axis detector under the control of Enhanced Masshunter software. Extracts (1 μL) were injected splitless (260 °C) onto a 30 m Agilent HP-5 ms column with a 0.25 mm ID and a 0.25 μm film. The carrier gas was H_2 with a flow of 1.5 mL/min. The oven was heated from 50 °C to 175 °C at 5 °C/min then to 300 °C at 20 °C/min and held for 8.75 min. Detection was done by mass spectrometry (MS) and FID. The flow was split between MSD (0.5 m L × 0.1 mm ID) and FID (2 m L × 0.18 mm ID) using deactivated silica columns. The MS transfer line was held at 300 °C, the MS source was held at 230 °C and the MS quadrupole held at 150 °C, *m/z* 35–350. Electron ionisation MS energy was 70 eV. Selected ion monitoring data was collected for m/z 93 (3–14 min), 161 (14–19.5 min) and 85.1, 223, 237 and 266 (19.5–25 min) and 85.1, 300 and 314 (25–40 min) to aid peak identification. Peak areas were expressed as percentage of total FID peak areas. Compounds were identified by comparing their retention time and mass spectra with specialized literature (Adams 2007) and spectral databases (MS libraries NIST 8th edition).

Qualitative profiles used uncorrected FID peak areas as % of total FID peak areas. Quantitative estimates for the major components used FID peak area relative to the peak area of the internal standard eicosane (individual response factors not measured) to estimate concentration per dry weight of the leaf material (% *w*/w).

2.8 Microbial community structure analysis using denaturing gradient gel electrophoresis (DGGE)

2.8.1 Sample collection

To investigate effects of AMF inoculation on microbial community structure, root and soil samples of four randomly selected plants from the glass house experiment were collected for DNA extraction (Online Resource 1). Fine roots with length approximately 3–5 cm were collected from four different parts of the root systems (top, middle top, middle bottom and bottom) then transferred to 50 mL tubes (Axygen, USA), then 45 mL Millipore sterile water was added. Tubes were shaken (rotary, Chiltern Scientific) for 10 min then left undisturbed for 10 min. The supernatants from the soil samples were removed by centrifuging the sample at 13,000×*g* for 15 min. Roots and soil were removed and transferred to new 1.7 mL tubes (Axygen, USA). The soil and root samples were stored at 4 °C for up to one-week prior to DNA extraction.

2.8.2 Total DNA extraction from soil and root samples

Roots were surface sterilized and treated with PMA prior to DNA extraction as previously described. Total DNA from roots and soils were extracted using the PowerPlant™ and PowerSoil™ DNA isolation kits (MoBio Laboratories, Carlsbad, USA) respectively, following the manufacturer's instructions.

2.8.3 PCR and DGGE analysis

The 16S rRNA genes from the total bacterial and Gammaproteobacterial communities were amplified as described by Muyzer et al. (1993) and Mühling et al. (2008). The 18S rRNA gene from the total fungal community was amplified using nested PCR with primer pairs AU2 and AU4 for the first PCR and FF390 and FR1-GC for the second PCR as described by Vandenkoornhuyse et al. (2002) and Vainio and Hantula (2000), respectively. The PCR protocol for the fungal second PCR was modified as follows: initial

denaturation at 95 °C for 3 min, followed by 8 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s (touchdown 1 °C per cycle), extension at 72 °C for 1 min, and followed by 27 cycles of denaturation at 95 °C for 30 s, annealing at 47 °C for 30 s, extension at 72 °C for 1 min and with final extension at 72 °C for 7.5 min. DGGE were performed with Cipher DGGE Electrophoresis System (CBS Scientific) with 30 to 65% linear denaturing gradient for total bacteria, 25–55% for fungi and 40–60% for Gammaproteobacteria. The DGGE staining process was done as described by Wicaksono et al. (2016).

2.9 Statistical analysis

Data on plant growth parameters and major essential oil compounds were analysed by one-way analysis of variance (ANOVA) to determine the significance of treatment effects, followed by orthogonal contrast to assess differences between means using GenStat (VSN International Ltd., UK). The data were square root or arcsine transformed when needed to fulfil the ANOVA assumption but means and standard errors (S.E) shown in tables are for untransformed data.

To obtain a better clustering of essential oil composition of each treatment, compounds with relative contents below 0.5% were excluded from the statistical analyses. Data were square-root transformed before the analysis. Resemblance matrices for essential oil composition were built by calculating similarities between samples using Euclidean distances after normalizing the data using Primer 7 (Primer-E Ltd., Plymouth Marine Laboratory, UK) multivariate software package. Main and pair-wise PERMANOVA tests were used to test the statistical difference of essential composition between treatments.

Analyses of the microbial communities were performed using Phoretix 1D Pro Gel Analysis (Totallab, UK). Resemblance matrices for community profiles were built by calculating similarities between each pair of samples by Jaccard coefficient (Clarke and Warwick 1994) using Primer 7. Main and pair-wise PERMANOVA tests were used to test the statistical difference of microbial communities between treatments. The number of bands per sample was used as a diversity indicator of the microbial taxa richness. The microbial richness was analysed with ANOVA to determine the significance of treatment factors and followed by Tukey's ad-hoc analysis at $P < 0.05$ using Minitab 17 (Lead Technologies, Australia).

The correlations between essential oil composition and microbial community structure (different microbial community groups in different samples types – rhizosphere soils and roots) were estimated by Mantel test (Mantel 1967) using Euclidean (essential oil composition) and Jaccard (microbial community structure) similarity distance matrices. The analysis was performed using "XLSTAT" V2015 package (Addinsoft Inc., New York, USA).

3 Results

3.1 Identification of arbuscular mycorrhizal fungi using trap culture

Seven spore morphotypes with globose shape were isolated from root samples of mānuka from nine sites across New Zealand (Table 2, Online Resource 2). The identified morphotypes belong to *Acaulospora*, *Rhizophagus* and *Claroideoglomus* spp. with 99–100% similarity based on 18S rRNA sequences in the NCBI database (Table 2). *Rhizophagus* sp. morphotype M6 was isolated from four of the nine sites. Sites from which spores were trapped successfully were on the North Island except the Craigieburn Forest Park. Five morphotypes (M1, M2, M3, M4 and M5) were consistent with *Acaulospora* spp. *Claroideoglomus* sp.

Table 2 Origin, morphotype and identity of the representative arbuscular mycorrhiza fungal spores isolated from *Leptospermum scoparium* using trap culture

Type	Plant location*	Spore morphotype		Closest match (Accession No.)Ψ	Identity
		Colour	Size range (μM)		
M1	TWL	Dark brown	180–200	*Acaulospora* sp. (KC708363)	100%
M2	TWL	Black	200–220	*Acaulospora* sp. (KC708363)	100%
M3	TWL, IHS	Pale brown	130–150	*Acaulospora* sp. (KC708363)	100%
M4	WC	Brown	110–130	*Acaulospora* sp. (EU332727)	100%
M5	WC	White	150–180	*Acaulospora* sp. (EU332727)	100%
M6	CR, THP, TNP, KFP	Pale brown	130–150	*Rhizophagus intraradices* (KU136414)	100%
M7	AMC, MA, KFP	Pale cream	130–150	*Claroideoglomus lamellosum* (FR750221)	99%

* *TWL*, Travis Wetland; *IHS*, Island Hill Station; *WC*, West Coast; *CR*, Craigeburn Forest Park; *THP*, Taihape Scenic Reserve; *TNP*, Tongariro National Park; *KFP*, Kaimanawa Forest Park; *AMC*, Aoraki/Mt. Cook National Park; *MA*, Mount Aspiring National Park

Ψ Accession No. based on NCBI database

morphotype M7 which was recovered from two South Island sites and one on the North Island (Table 2).

3.2 Identification of arbuscular mycorrhizal fungi using denaturing gradient gel electrophoresis (DGGE)

Twenty-nine percent (55 of 194) of the DGGE bands were excised, re-amplified and sequenced. Thirty-eight of these DGGE bands were assigned to AMF, whereas, 17 resulted in multiple sequence signals (Online Resource 3). The phylogenetic tree indicated that 18 AMF sequences from DGGE belong to *Rhizophagus*, *Glomus* and *Acaulospora* spp. (Fig. 1). Ten sequences formed a clade that matched to "uncultured *Glomeraceae* environmental samples", whereas 10 sequences matched an "uncultured *Paraglomus* environmental sample". The similarity of some of the sequences was below 97%, especially sequences that aligned with "uncultured *Paraglomus* environmental sample" (Online Resource 3).

Different AMF communities were identified by the two approaches in the samples from some sites (Table 2, Fig.1). For instance, *Glomus* spp. were detected in the West Coast sample and *Rhizophagus* spp. were detected from Island Hill Station using DGGE but neither were detected by trap culture. On the other hand, the *Acaulospora* sp. recovered from Island Hill Station and West Coast using trap culture, and the *Claroideoglomus* sp. recovered from Aoraki/Mt. Cook, Mount Aspiring and Tongariro National Park using trap culture, were not detected using DGGE.

Nonmetric Multidimensional Scaling (MDS) showed that samples were separated based on location (region) (Online Resource 4). Pairwise comparison analysis using PERMANOVA showed that each region had different AMF communities ($P < 0.05$) except the central South Island which had a similar AMF community to northern South Island ($P = 0.105$). There was no significant difference in AMF richness between the locations ($P = 0.068$).

3.3 Plant growth and arbuscular mycorrhizal fungi colonization

The inoculated seedlings showed a higher growth compared to the uninoculated seedlings ($P < 0.001$, Table 3). At the species level, the seedlings inoculated with different AMF species did not differ in plant growth parameter ($P > 0.05$). Higher AMF colonization % was observed in the seedlings with AMF inoculation compared to the uninoculated seedlings ($P < 0.001$). There was no difference in AMF colonization between the seedlings inoculated with different AMF species ($P > 0.05$).

3.4 Essential oil analysis

Forty-one GC-MS peaks were detected from the inoculated Travis Wetland mānuka essential oil extracts with relative contents more than 0.5% (Online Resources 5 and 6). Seven major compounds (relative content >3.5%) dominated these essential oils: maltol, β-elemene, α–selinene, β–selinene, *trans*-calamenene, grandiflorone and flavonoid 298, which contributed on average 43.3% of total GC-MS peak area (Online Resource 6). Other major compounds (mean > 2% of total GC-MS peak area) were RI ester 1305, flavonoid 284, α-cubebene, β-caryophyllene and Unknown 12.

Inoculation with different AMF isolates affected essential oil composition (PERMANOVA, $P = 0.049$), with *Acaulospora* sp. M4 or *Scutellospora* sp. MPC13 inoculated seedlings oils statistically different compared to uninoculated seedlings oils.

Pearson correlation test showed that six compounds made strong contribution to the differences in essential oil composition associated with different AMF inoculation (Online Resource 7). The relative contents of α-selinene and β-selinene were higher in seedlings inoculated with either *Acaulospora* sp. M1 or uninoculated, compared to seedlings inoculated with other AMF isolates (Online Resource 6). The relative contents of α-cubebene, *trans*-calamenene and *trans*-cadina-1,4-diene showed the opposite effect.

In quantitative terms, i.e. peak area per unit mass of dry leaf, there was no difference in total essential oil between uninoculated and inoculated seedling ($P > 0.005$, Table 4). However, in terms of major essential oil compounds, the inoculated seedling showed significantly decreased α-selinene and β-selinene concentration compared to the uninoculated seedlings ($P = 0.012$ and 0.015, respectively, Table 4) whereas they did not differ on flavonoid 298 concentration. No significant differences were observed in terms of the concentrations of maltol, β-elemene, *trans*-calamenene or grandiflorone between the treatments ($P > 0.05$, data not shown). At species level, *Acaulospora* inoculation showed a higher α-selinene concentration compared to *Scutellospora* sp. MPC13 inoculation whereas *Scutellospora* sp. MPC13 inoculation showed a higher flavonoid 298 compared to other species ($P = 0.015$ and $P = 0.003$, Table 4). However, no different were observed on β-selinene concentration at species level ($P > 0.05$, Table 4).

Fig. 1 Phylogenetic tree based on alignment of partial 18S rRNA ▶ sequences of arbuscular mycorrhizal fungi associated with *Leptospermum scoparium* obtained from trap culture ($n = 7$) (○) and DGGE ($n = 38$) (●). Arbuscular mycorrhizal fungi isolated from non-*Leptospermum scoparium* that were used for the pot experiment (n = 3) (□)

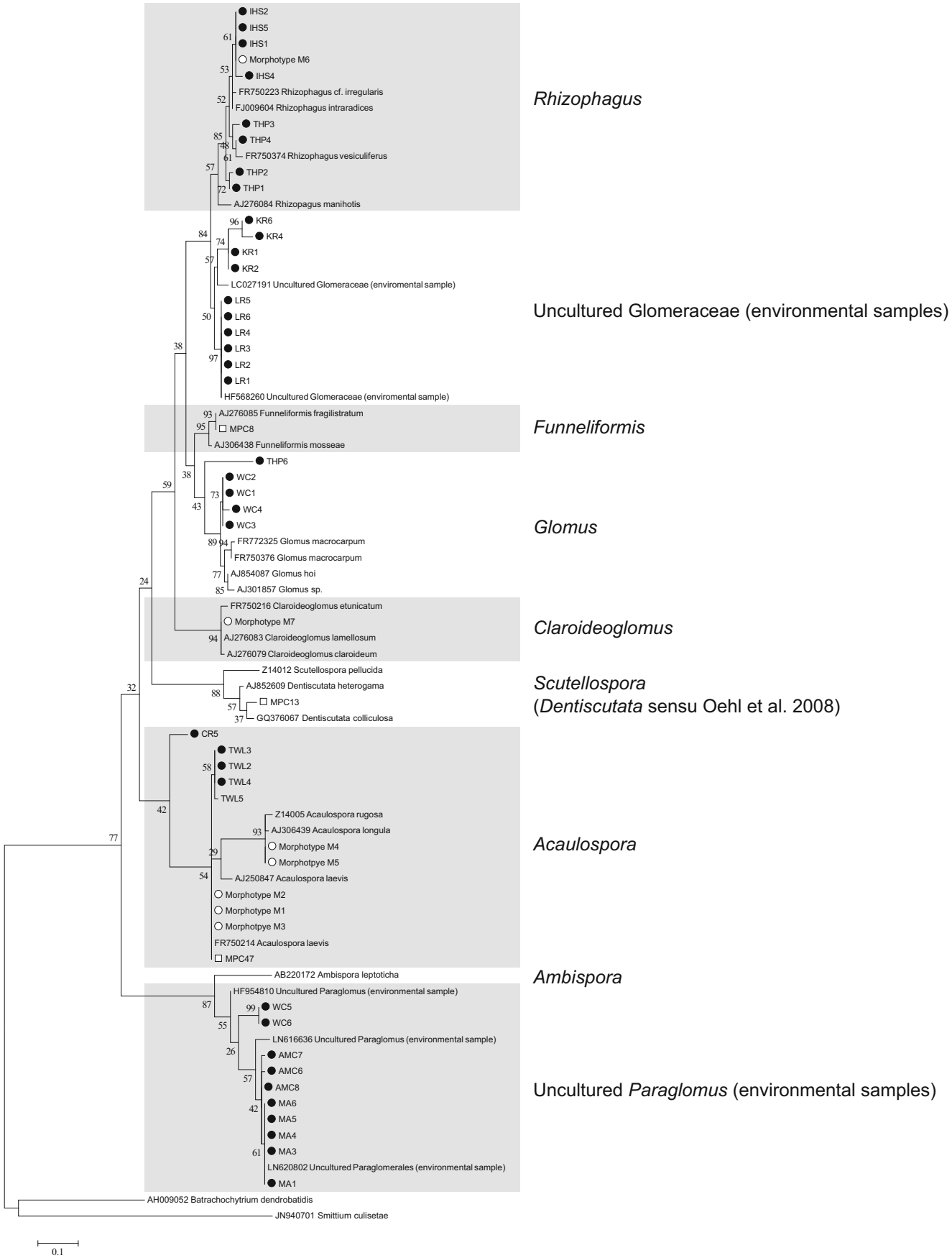
IHS2
IHS5
IHS1
Morphotype M6
IHS4
FR750223 Rhizophagus cf. irregularis
FJ009604 Rhizophagus intraradices
THP3
THP4
FR750374 Rhizophagus vesiculiferus
THP2
THP1
AJ276084 Rhizopagus manihotis
KR6
KR4
KR1
KR2
LC027191 Uncultured Glomeraceae (enviromental sample)
LR5
LR6
LR4
LR3
LR2
LR1
HF568260 Uncultured Glomeraceae (enviromental sample)
AJ276085 Funneliformis fragilistratum
MPC8
AJ306438 Funneliformis mosseae
THP6
WC2
WC1
WC4
WC3
FR772325 Glomus macrocarpum
FR750376 Glomus macrocarpum
AJ854087 Glomus hoi
AJ301857 Glomus sp.
FR750216 Claroideoglomus etunicatum
Morphotype M7
AJ276083 Claroideoglomus lamellosum
AJ276079 Claroideoglomus claroideum
Z14012 Scutellospora pellucida
AJ852609 Dentiscutata heterogama
MPC13
GQ376067 Dentiscutata colliculosa
CR5
TWL3
TWL2
TWL4
TWL5
Z14005 Acaulospora rugosa
AJ306439 Acaulospora longula
Morphotype M4
Morphotpye M5
AJ250847 Acaulospora laevis
Morphotype M2
Morphotype M1
Morphotpye M3
FR750214 Acaulospora laevis
MPC47
AB220172 Ambispora leptoticha
HF954810 Uncultured Paraglomus (environmental sample)
WC5
WC6
LN616636 Uncultured Paraglomus (environmental sample)
AMC7
AMC6
AMC8
MA6
MA5
MA4
MA3
LN620802 Uncultured Paraglomerales (environmental sample)
MA1
AH009052 Batrachochytrium dendrobatidis
JN940701 Smittium culisetae
0.1
Rhizophagus
Uncultured Glomeraceae (environmental samples)
Funneliformis
Glomus
Claroideoglomus
Scutellospora
(*Dentiscutata* sensu Oehl et al. 2008)
Acaulospora
Ambispora
Uncultured *Paraglomus* (environmental samples)

Table 3 Growth response and AMF colonization of *Leptospermum scoparium* seedlings towards five treatments of arbuscular mycorrhizal fungal species *Acaulospora* (*Acaulospora* sp. M1, *Acaulospora* sp. M4, *Acaulospora* sp. MCP47), *Funneliformis* sp. MPC8 and *Scutellospora* sp. MPC13 after five months growth

Treatment	Shoot height (cm)*	Number of internodes (n)*	Shoot dry weight (g)*	Root dry weight (g)*	AMF colonization (%)*
Acaulospora sp. M1	40.5 ± 2.3	25.5 ± 1.3	1.54 ± 0.13	0.34 ± 0.04	42 ± 1.6
Acaulospora sp. M4	40.0 ± 1.6	26.0 ± 1.2	1.44 ± 0.11	0.32 ± 0.02	48 ± 2.2
Acaulospora sp. MPC47	41.1 ± 2.2	27.5 ± 1.8	1.51 ± 0.07	0.31 ± 0.02	45 ± 6.2
Funneliformis sp. MPC8	42.0 ± 1.8	26.4 ± 0.9	1.58 ± 0.06	0.34 ± 0.03	42 ± 4.3
Scutellospora sp. MPC13	39.5 ± 2.5	24.5 ± 1.8	1.61 ± 0.05	0.29 ± 0.03	40 ± 2.9
Control	31.7 ± 2.1	20.6 ± 1.5	1.12 ± 0.10	0.20 ± 0.02	23 ± 5.0
Treatments compared (*P* value of linear orthogonal contrast)					
C vs I	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Aca vs Fun	0.552	0.962	0.476	0.559	0.527
Aca vs Scu	0.692	0.249	0.323	0.259	0.270
Fun vs Scu	0.419	0.326	0.820	0.164	0.691

C, uninoculated seedling; *I*, inoculated seedling; *Aca*, *Acaulospora* inoculated seedling; *Funneliformis* inoculated seedling; *Scutellospora* inoculated seedling

* Value are means ± S.E of nine replicates for each treatment; bold indicates statistically significant value ($P < 0.05$)

3.5 Microbial community structure analysis using denaturing gradient gel electrophoresis (DGGE)

3.5.1 Total bacteria

AMF inoculation modified the total bacterial community composition in the rhizosphere soil (PERMANOVA, $P = 0.008$, Table 5). Total bacterial communities in rhizosphere soil from seedlings inoculated with *Acaulospora* sp. M1 were different to uninoculated seedlings ($P \leq 0.05$). Different total bacterial communities were also observed between the different AMF isolate inoculations in rhizosphere soil ($P \leq 0.05$). For instance, seedlings inoculated with *Acaulospora* sp. M1 had a different community to seedlings inoculated with *Scutellospora* sp. MPC13. No different were observed on total bacterial communities in the roots ($P = 213$, Table 5).

Table 4 Quantitative composition of essential oils in the foliage of *Leptospermum scoparium* seedling: total essential oil yield and selected major component concentrations (relative concentration > 3.5%) towards five treatments of arbuscular mycorrhizal fungal species *Acaulospora* (*Acaulospora* sp. M1, *Acaulospora* sp. M4, *Acaulospora* sp. MCP47), *Funneliformis* sp. MPC8 and *Scutellospora* sp. MPC13 after five months plant growth

Treatment	Total essential oil*	Major essential oil compounds*		
		α-selinene	β-selinene	flavanoid 298
Acaulospora sp. M1	141.25 ± 7.79	18.05 ± 2.34	17.48 ± 2.39	4.20 ± 0.49
Acaulospora sp. M4	146.92 ± 20.07	11.43 ± 1.93	10.98 ± 1.96	5.40 ± 0.80
Acaulospora sp. MPC47	152.98 ± 14.83	16.30 ± 1.66	15.50 ± 1.65	6.83 ± 1.13
Funneliformis sp. MPC8	135.70 ± 29.03	11.93 ± 4.83	10.78 ± 4.30	3.93 ± 0.73
Scutellospora sp. MPC13	125.20 ± 22.62	8.95 ± 1.77	7.83 ± 1.76	9.03 ± 2.22
Control	175.97 ± 14.67	22.10 ± 3.36	21.18 ± 3.10	4.78 ± 0.20
C vs M	0.124	**0.012**	**0.015**	0.43
Aca vs Fun	0.618	0.134	0.237	0.156
Aca vs Scu	0.342	**0.027**	0.067	**0.015**
Fun vs Scu	0.706	0.5	0.56	**0.003**

C, uninoculated seedling; *I*, inoculated seedling; *Aca*, *Acaulospora* sp. M1, *Acaulospora* sp. M4 and *Acaulospora* sp. MPC47 inoculated seedling; *Fun*, *Funneliformis* sp. MPC8 inoculated seedling; *Scu*, *Scutellospora* sp. MPC13 inoculated seedling

* Response compared to internal standard-eicosane, mg/g. Value are means ± S.E of nine replicates for each treatment; bold indicates statistically significant value ($P < 0.05$)

Table 5 Summary of results from statistical analyses: effects of arbuscular mycorrhizal fungi inoculations on soil and root microbial composition and richness

	Total bacteria		Total fungi		γ proteobacteria	
	Soil	Root	Soil	Root	Soil	Root
Microbial composition$^{\Psi}$	$P = 0.008$	$P = 0.213$	$P = 0.053$	$P = 0.001$	$P = 0.001$	$P = 0.007$
Microbial richness*	$P = 0.003$	$P = 0.902$	$P = 0.428$	$P = 0.854$	$P = 0.315$	$P = 0.193$

*Result from ANOVA analysis by calculating number DGGE band in each sample

$^{\Psi}$ Result from PERMANOVA analysis with the resemblance matrices which were built by calculating similarities between each pair of samples using Jaccard coefficient

There was a significant difference in total bacterial richness among treatments in soils ($P = 0.002$, Table 5). Seedlings inoculated with *Acaulospora* sp. M1 had a significantly more bands ($n = 66.2$) than uninoculated seedlings ($n = 46.2$), *Acaulospora* sp. M1 ($n = 49.7$) or *Scutellospora* sp. MPC13 ($n = 51.0$) ($P < 0.05$). There were no significant differences in total bacterial richness between treatments in roots ($P = 0.902$, Table 5).

3.5.2 Total fungi

AMF inoculation modified total fungal community composition in roots but not in the rhizosphere soils (PERMANOVA, $P = 0.001$ and 0.053, respectively, Table 5). Roots of seedlings inoculated with AMF isolates, except *Scutellospora* sp. MPC13, had different total fungal communities compared to uninoculated seedlings ($P \leq 0.05$). There were no significant differences in total fungal richness between treatments in rhizosphere soils and roots ($P = 0.428$ and 0.854 respectively, Table 5).

3.5.3 Gammaproteobacteria

AMF inoculation significantly influenced Gammaproteobacterial communities in both the rhizosphere soils and roots (PERMANOVA, $P = 0.001$ and 0.007, respectively, Table 5). All seedlings inoculated with *Acaulospora* had different Gammaproteobacterial communities in rhizosphere soil and roots when compared to uninoculated seedlings ($P \leq 0.05$). Seedlings inoculated with *Scutellospora* sp. MPC13 had different Gammaproteobacterial communities in roots compared to uninoculated seedlings. Different Gammaproteobacterial communities in rhizosphere soil between different AMF isolate inoculations were also observed ($P \leq 0.05$). There was no significant difference in Gammaproteobacterial richness among treatments in rhizosphere soils and roots ($P = 0.315$ and 0.193, respectively, Table 5).

3.6 Correlation between essential oil and microbial community structure

A Mantel test did not find any significant correlations of essential oil composition with total bacterial and fungal community in roots ($P = 0.179$ and 0.570 respectively), or with Gammaproteobacterial community in rhizosphere soils ($P = 0.840$). There were significant correlations between essential oil composition with total bacterial and fungal community in soil ($P = 0.007$ and 0.011). A highly significant correlation was observed between essential oil composition and Gammaproteobacterial community in roots ($P < 0.0001$).

4 Discussion

This is the first study to demonstrate that AMF communities of mānuka can modulate essential oil composition *in planta*. Despite their key importance to the native plants of New Zealand, AMF communities have not been the subject of many studies, and mostly prior to modern molecular tools (Baylis et al. 1963; Cooper 1976; Hall 1977; Johnson 1977). *Acaulospora*, *Rhizophagus* and *Glomus* associations with mānuka were known (Hall 1977; McKenzie et al. 2006). This study reports two new AMF associations on mānuka. *Claroideoglomus* was previously classified as *Glomus* (Krüger et al. 2012) and may not have been identified before due to overlapping morphological features with *Glomus*. Sequences that correlated with an uncultured *Paraglomus* sp. might also be a novel association with mānuka but the size of sequences (± 200 bp) were insufficient to confirm identity to the species levels (<97%).

In this study two techniques (trap culture and DGGE) were used to characterize AMF communities and identified a greater number of species than either alone. The use of trap cultures to isolate AMF can fail to capture some species due to their poor colonization and sporulation (Kowalchuk et al. 2002; Redecker et al. 2003; Ryszka et al. 2010). An absence of some AMF taxa in DGGE bands was also observed when compared to trap cultures. This can be explained by i) a minor AMF population being excluded from DGGE due to preferential amplification of the major population (>1% of total population) (Muyzer et al. 1993), ii) a smaller amount of root sample being used for DGGE analysis compared to that used for trap culture initiation (approximately 60–100× more), iii) some excised DGGE bands representing >1 sequence and thus, some AMF taxa being masked by co-migrating taxa, and iv) not all bands were excised and sequenced from the DGGE gels. Therefore, the combination of trap culture and DGGE

complemented each other to characterise the AMF community in mānuka.

Plant location influenced the AMF community in mānuka and this has also been reported by Weijtmans et al. (2007). As the AMF community in natural ecosystems is fairly constant during the year (Davison et al. 2012) the different sampling times are likely to have had little influence on the AMF community detected. Plant location consists of a complex range of environmental and abiotic factors that influence microbial communities (Sessitsch et al. 2002; Costa et al. 2006; Öpik et al. 2006; Rodríguez-Echeverría and Freitas 2006). Thus, the differences in AMF communities between sites with different geographic location and environment were expected in this study.

This study used unsterilized soil to assess the effect of AMF inoculation. This soil included a resident microbial community, including AMF. Thus, the AMF colonization that observed in the control plants was expected in this study. Some studies that investigate AMF effects are performed in sterile soil (Rodriguez and Sanders 2015). However, using unsterilized soil that is representative of field conditions, provides more practical relevance because the introduced AMF must compete with other microbes (Rydlová et al. 2016; Ordoñez et al. 2016; Hristozkova et al. 2016). In this study mycorrhizal colonization was lower in the uninoculated compared to the inoculated seedlings. In addition the AMF inoculant was added directly to the root zone of the seedlings. Thus, the comparative effect of the resident AMF was likely to be small in this study. AMF inoculation increased mānuka plant biomass. In Myrtaceae species 45–60% colonization of the root length by mycorrhiza has been reported, which is similar to the results of this study (Moyersoen and Fitter 1999; Chen et al. 2000; Janos et al. 2013).

The key finding of this research was that inoculation with AMF species altered the essential oil composition of mānuka foliage. Inoculation with *Acaulospora* sp. M4 and *Scutellospora* sp. MPC13 qualitatively changed the essential oil composition and indicated a functional diversity between AMF species. However, the other treatments did not qualitatively change essential oils indicating that this is not a general response to inoculation and was independent of growth promotion. A similar result was reported by Karagiannidis et al. (2011). In quantitative terms, AMF inoculation decreased the major essential compounds α-selinene and β-selinene. In other work AMF inoculation has been demonstrated to both increase and decrease major essential oil compounds in sweet basil, fennel and common sage (Kapoor et al. 2004; Copetta et al. 2006; Geneva et al. 2010).

Several direct mechanisms by which AMF modify essential oils have been described in previous studies. Although not investigated here, AMF can alter the number of peltate glands and glandular trichomes and regulate plant secondary metabolite gene expression (Harrison and Dixon 1993; Taylor and Harrier 2003; Copetta et al. 2006; Chaudhary et al. 2008). In mānuka, essential oil compounds are produced in oil glands within leaves, and the densities and sizes of these oil glands vary between regional chemotypes (Killeen et al. 2015, 2016). Examination of the oil glands or the expression of the genes responsible for essential oil production may help to determine the mechanism by which essential oil contents are modified in mānuka.

In this work an indirect mechanism by which AMF could alter essential oils was demonstrated. AMF inoculation changed the microbial community in rhizosphere soil and roots. A significant correlation between the Gammaproteobactera community in root and essential oil composition was observed. Gammaproteobacteria are an important group of bioactive bacteria in mānuka and a key component of the mānuka microbiome (Wicaksono et al. 2016). Gammaproteobacteria are considered to have a synergistic relationship with AMF (Artursson et al. 2006; Toljander et al. 2007) and the species *Pantoea*, *Pseudomonas*, and *Acinetobacter* can modify essential oil composition in other plant species (Del Giudice et al. 2008; Dharni et al. 2014; Wang et al. 2015; Zhou et al. 2016). Therefore, modification of the Gammaproteobacteria in the roots by AMF is likely to influence essential oil composition in mānuka.

From the work presented here it is clear that inoculation with AMF would enhance seedling establishment and growth. Plant growth improvement might enhance economic returns by increasing foliage yields for essential oil production from mānuka in cultivation as has been reported from other medicinal plant studies (Kapoor et al. 2004; Khaosaad et al. 2006). This work is also important in the context of the mānuka industry in New Zealand which relies on establishing mānuka plantations that produce high quality essential oils rich in triketones. Currently the composition of essential oils in plants of different chemotypes is highly variable (Thompson et al. 2003; Douglas et al. 2004; Viljoen et al. 2005). Achieving a greater understanding of the microbial influence on plant chemotype may ultimately improve the consistency of essential oils produced through deliberate inoculation with micro-organisms.

In conclusion, we confirmed that *Acaulospora*, *Rhizophagus*, *Glomus, Paraglomus* and *Claroideoglomus* genera were associated with mānuka. Inoculation of seedlings with these AMF increased growth and modified essential oil composition in leaves. The AMF inoculation also modified the Gammaproteobacterial community in the roots and this was implicated as an indirect mechanism of essential oil modification by AMF.

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