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Phytoremediation of microbial contamination in soil by New Zealand native plants

Maria Jesus Gutierrez-Gines [a,*], Hossein Alizadeh [b], Elizabeth Alderton [a], Vikki Ambrose [a,1], Alexandra Meister [c], Brett H. Robinson [c], Sky Halford [a,2], Jennifer A. Prosser [a,3], Jacqui Horswell [a,4]

[a] Institute of Environmental Science and Research (ESR) Ltd., 34 Kenepuru Drive, Kenepuru, Porirua 5022, New Zealand
[b] Bio-Protection Research Centre, PO Box 85084, Lincoln University, 7647 Canterbury, New Zealand
[c] School of Physical and Chemical Sciences, University of Canterbury, 20 Kirkwood Ave, Christchurch 8041, New Zealand

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ABSTRACT

Novel research has demonstrated that the roots of some bioactive plants - called pathogen phytoremediation plants - enhance die-off of pathogenic organisms in the soil. Strategic establishment of pathogen phytoremediation plants may reduce the transport of human pathogens to water sources. Such plantings could be used in riparian margins, as buffer strips to protect drinking water supplies, or block planting in 'critical source areas' of microbial contamination, such as grazing paddocks, organic waste – including sewage sludge - amended land, animal feedlots and housing facilities, and manure storage areas. This work aimed to investigate the antimicrobial activity of a range of New Zealand native plants known for their antimicrobial potential from previous research or through indigenous knowledge, and to assess if any of them could potentially be used for pathogen phytoremediation. Two laboratory screening experiments demonstrated the antimicrobial activity of *Leptospermum scoparium*, including the local variety swamp mānuka, *Kunzea ericoides*, *Pseudowintera colorata*, and *Metrosideros robusta* against three human pathogens and two indicator organisms. A greenhouse experiment showed a 90% reduction of *Escherichia coli* numbers in dairy shed effluent irrigated pots after 14 days in soils under swamp *L. scoparium* and *M. robusta*, compared with 45 days in soil under *Lolium perenne*. The pH in the soil under swamp *L. scoparium* and *M. robusta* was significantly lower than under L. *perenne*, which could partially explain the faster *E. coli* reduction.

1. Introduction

Medicinal use of plants is as old as humanity, with the first written records of plant medicinal use dating from 2600 BC in Mesopotamia (Gurib-Fakim, 2006). Medicinal plants were also the first source of pharmaceuticals; Aspirin, produced from salicin extracted from bark of *Salix alba*, was one of the first examples of a plant derived drug (McRae et al., 2007). Bioprospecting, the process of discovery and commercialisation of new products from biological resources, has become an important discipline, above all in the search for new drugs to treat human diseases and/or conditions (McRae et al., 2007). Currently, the development of pathogens with antibiotic resistance is pushing researchers to find new antibiotic products and compounds (Gorlenko et al., 2020).

Recent novel research has investigated the use of medicinal plants to inhibit or kill human pathogens in the soil. Yossa et al. (2010) demonstrated the efficacy of treating soil with essential oils from different plant species to reduce contamination of food by *Escherichia coli* (*E. coli*) O157:H7 in organic agriculture. Prosser et al. (2016) demonstrated a faster die-off of *E. coli* under soil containing bioactive plants compared with soil planted with pasture only, and suggested the release of antimicrobial compounds as a probable mechanism. These authors were the first

* Corresponding author.
E-mail address: maria.gutierrez-gines@esr.cri.nz (M.J. Gutierrez-Gines).
[1] Present address: Nelson City Council, 110 Trafalgar Street, Nelson 7010, New Zealand.
[2] Present address: School of Physical and Chemical Sciences, University of Canterbury, 20 Kirkwood Ave, Christchurch 8041, New Zealand.
[3] Present address: Lowe Environmental Impact, 441 Church Street, Palmerston North 4410, New Zealand
[4] Present address: Ministry for Primary Industries, PO Box 2526, Wellington 6140, New Zealand.

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ones to refer to this research as phytoremediation of microbial contamination.

Contamination of soil by pathogenic microorganisms can occur via many pathways including: excrements deposited by grazing livestock, land application of organic waste – including manure or sewage effluent, the use of animal products as soil conditioners, animal feedlots and housing facilities, and manure storage areas (Unc and Goss, 2004; Nicholson et al., 2005; Mosaddeghi et al., 2009; Semenov et al., 2009; Horswell et al., 2010; Ongeng et al., 2015). Horswell et al. (2010) demonstrated that the presence of organic matter increases the survival period of pathogens in the soil. This suggests that pathways of microbial contamination that include high concentration of organic matter - such as manures, organic waste, or dairy effluent - might be high risk settings for sources of pathogens to other systems, like waterways.

The transport of microbial contaminants to water sources significantly impacts water quality and is a major public health concern worldwide (WHO, 2015). Ingestion of contaminated water, or food irrigated with contaminated water can cause a variety of infectious water-borne diseases (Cooley et al., 2007; Pachepsky et al., 2011). Diarrhoeal disease caused by food and water contamination causes over 1.8 million deaths per year (WHO, 2015). In 2016 in New Zealand, the contamination of a drinking-water supply with *Campylobacter jejuni* by run-off from an adjacent sheep farm infected over 8000 people (Gilpin et al., 2020). Strategic establishment of plants with antimicrobial properties in the root systems (pathogen phytoremediation plants) may reduce the transport of human pathogens from contaminated soils to water sources through their use in riparian margins, block planting in 'critical source areas' of microbial contamination, or even as buffer strips to protect drinking water supplies.

The Myrtaceae plant species from New Zealand (*Leptospermum scoparium* and *Kunzea* spp.) has demonstrated enhanced *E. coli* die-off in soil (Prosser et al., 2016) and are well known for their antimicrobial properties through use in wound care products and as topical agents. These species have a high economic value, producing honey, oil, and cosmetics which are sold widely throughout the world (Perry et al., 1997; Weston et al., 2000; Stephens et al., 2005; Adams et al., 2008). Potential economic return provides an extra incentive for their planting. Different plant species have antimicrobial activity against different types of pathogenic organisms (Calder et al., 1986). If plantations for pathogen phytoremediation were to be used, a high biodiversity in such plantations would likely increase the spectrum of targeted organisms. In addition, higher biodiversity would better represent native ecosystems and diversify the root system structure, thus increasing the infiltration of run-off (Bharati et al., 2002) or even provide a wider range of ecosystem services (Gilvear et al., 2013).

Due to the high rate of endemism in New Zealand, 80% of the plant species are not found anywhere else in the world (DOC, 2020). This provides an opportunity for investigating new plant species with antimicrobial properties, which will not be found in other countries. Some New Zealand plant species from different taxonomic families, commonly used in traditional Māori medicine (rongoā), are being bioprospected for the discovery, isolation and use of bioactive plant compounds, such as species from Myrtaceae (Wollenweber et al., 2000; Owens et al., 2013; Killeen et al., 2016; Lawrence et al., 2019), Fabaceae (McDougal et al., 2018) and Winteraceae families (Wayman et al., 2010). However, little is known about their microbial phytoremediation potential in the soil. To the best of our knowledge, Prosser et al. (2016) are the only authors worldwide who proposed and investigated the potential use of bioactive plants to remediate human pathogens in soils. This work provides new results in a totally unexplored area of phytoremediation research.

The objective of this work was to investigate a) the antimicrobial properties of water soluble extracts from a variety of New Zealand native plants with a potential to be used in microbial phytoremediation plots, and b) if their antimicrobial properties would be also effective in soil.

2. Materials and methods

2.1. Screening plants for antimicrobial potential

2.1.1. Plant selection and plant extract preparation

The criteria considered to select the plants (Table 1) for this study

Table 1
List of plants tested for potential antimicrobial activity. Names and families of plant species were checked October 2020 in www.nzflora.info.

Scientific name	Vernacular name	Family	Reasons for selection
Phormium tenax J. R.Forst. & G. Forst	Harakeke, flax	Asphodelaceae	Multiple medicinal uses, many of them against infections and used as rongoā by Māori (Jones, 2007; MWLR, 2020).
Olearia paniculata (J.R.Forst. & G. Forst.) Druce	Akiraho, golden akeake	Compositae	Some *Olearia* species are poisonous (Crowe, 2004).
Kunzea ericoides (A.Rich.) Joy Thomps.	Kānuka, tea tree	Myrtaceae	Multiple medicinal uses, many of them against infections (MWLR, 2020).
Leptospermum scoparium J.R. Forst. & G. Forst.	Mānuka, tea tree	Myrtaceae	Multiple medicinal uses, many of them against infections and used as rongoā by Māori (Jones, 2007; MWLR, 2020).
Leptospermum scoparium J.R. Forst. & G. Forst.	Swamp mānuka	Myrtaceae	Local variety of *L. scoparium* that grows in swamp areas in the Lower Waikato region and it is recognized by local indigenous tribes to have healing properties, probably even stronger than other varieties (personal communication from Ngā Muka Development Trust and Matahuru Marae).
Metrosideros robusta A.Cunn	Rātā, northern rata	Myrtaceae	Multiple medicinal uses, many of them against infections and used as rongoā by Māori (Jones, 2007; MWLR, 2020).
Piper excelsum G. Forst.	Kawakawa	Piperaceae	Multiple medicinal uses, many of them against infections and used as rongoā by Māori (Jones, 2007; MWLR, 2020).
Veronica stricta Banks & Sol. ex Benth	Koromiko	Plantaginaceae	Multiple medicinal uses, many of them against infections and used as rongoā by Māori (Jones, 2007; MWLR, 2020).
Myoporum laetum G.Forst.	Ngaio	Scrophulariaceae	Multiple medicinal uses, many of them against infections and healing wounds. Leaves are also poisonous if ingested (Crowe, 2004; MWLR, 2020).
Aciphylla aurea W. R.B.Oliv.	Golden spaniard	Umbelliferae	Not known as medicinal plant, but used for the strong scent (MWLR, 2020).
Aciphylla subflabellata W. R.B.Oliv.	Spaniard	Umbelliferae	Not known as medicinal plant, but used for the strong scent (MWLR, 2020).
Pseudowintera colorata (Raoul) Dandy	Horopito, pepper tree	Winteraceae	Leaves used to heal wounds as rongoā by Māori (MWLR, 2020).

were: a) readily available commercially, so they can easily be planted in microbial phytoremediation plots, and b) known or suspected to possess antimicrobial properties according to existence of a scientific evidence, or Rongoā Māori (traditional Māori healing), having strong scent, and/or being poisonous. *Lolium perenne* L. was chosen as a negative control plant, since it has negligible antimicrobial properties (Prosser et al., 2014).

Seedlings (approximately 20 cm high) of each species were purchased from commercial nurseries. When possible, plants of the same species were purchased from the North and South Island to compare results based on location, since there could be strong differences in the chemical composition of plants in between locations, as shown for *L. scoparium* (Douglas et al., 2004). As explained by these authors, the different *L. scoparium* chemotypes may be the evolutionary result of the local populations adaptation to their environment. *Lolium perenne* leaves were cut from the Lincoln University research field area. Given that previous research showed higher antimicrobial activity in leaves compared to roots (Prosser et al., 2014), and creating root extracts is very labour intensive, requiring thorough washing of considerable amounts of plant material, only the leaves of all species were used for the first screening. Root extracts were only tested for those species selected for the pot experiment. Leaf and root samples were ground using a mortar and pestle after flash-freezing in liquid nitrogen, and the extracts prepared in water at a ratio of 1:3 to 1:4 depending on the texture of the mixture. The mixture was shaken at 320 rpm for an hour, and then centrifuged at 6000 rpm for 10 min before they were filtered through 0.22 μm syringe filters. Extracts were kept at −20 °C until required.

2.1.2. Acute toxicity lux bioassay

A rapid first screen for antimicrobial activity of all leaf extracts was undertaken using the *E. coli* lux biosensor (Horswell and Dickson, 2003). This bacterial bioluminescence-based bioassay is an acute toxicity test (results are checked after 30 min) adapted from Horswell et al. (2006), and was performed as described by Prosser et al. (2014). The biosensor (supplied by the University of Aberdeen, UK) produces luminescence when exposed to favourable conditions, and exhibits reduced luminescence in less favourable conditions, such as presence of toxic compounds or changes in pH. This test was carried out with 10% diluted leaf extracts.

The results of Lux bioassay were expressed as percentage of the luminescence of the extract when compared to water. For quality control, the Lux - *E. coli* were exposed to test solutions containing serial dilutions of TCP™ antiseptic liquid (Pfizer Ltd., UK) as described in Redshaw et al. (2007).

2.1.3. Microplate sensitivity assay

Leaf extracts that showed toxicity in the acute toxicity Lux bioassay were chosen for further investigation to determine their antimicrobial potential against four microorganisms: *E. coli*, *Staphylococcus aureus (S. aureus)*, *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Burkholderia cepacia (B. cepacia)*. *E. coli* was selected because it is the most common bacterial indicator for faecal contamination in water and soil. *S. aureus*, *P. aeruginosa* and *B. cepacia* were selected to represent both gram-positive and gram-negative pathogenic organisms in which antibiotic resistance is common (Pray, 2008; Rhodes and Schweizer, 2016). This would represent a worst-case scenario of higher risk bacterial contamination. Bacterial strains were sourced from the New Zealand Reference Culture Collection (ESR, Porirua, New Zealand): *E. coli* 2718, *S. aureus* 87, *P. aeruginosa* 3007, *B. cepacia* 2768 as freeze-dried cultures. The freeze-dried bacterial strains were resuscitated in Tryptic Soy Broth (TSB) at 37 °C, and 180 rpm for 24 h. Cultures were then centrifuged at 2000 rpm for 1 h. The pellet was then washed twice with Ringer's solution (Oxoid TM), re-suspended in 5 mL of Ringer's solution to form a seed stock, and stored at 4 °C until required. The seed stock was refreshed every three weeks. The day before an experiment, 100 μL of the seed stock was transferred to 20 mL of TSB and incubated overnight at 37 °C, at 180 rpm. The bacterial suspension was then diluted 1/100 in TSB, and this final suspension was immediately used in the experiments. This dilution factor was optimized previously to create a full growth curve visible over a 24 h incubation period.

The plant extracts were tested against the four bacterial strains using a spectrophotometric bioassay described by Prosser et al. (2014), which has been previously used to determine microbial sensitivity to *L. scoparium* leaf extracts. Tests were performed in 96 well plates laid out as shown in Fig. 1, with individual total volume of 200 μL, which consisted of 100 μL bacterial suspension, and 100 μL of plant extract. Maximum extract concentration tested for *O. paniculata* and *V. stricta* was 50%. However, when plant extracts of *L. scoparium*, swamp *L. scoparium*, *K. ericoides*, *M. robusta* and *P. colorata* extracts were added to bacterial suspensions, the suspension was highly turbid which made it difficult to read by spectrophotometry. In these cases, the maximum concentration of extract was reduced to 20%, with the tests consisting of 160 μL bacterial suspension, and 40 μL of plant extract (Fig. 1). *L. scoparium* and *K. ericoides* extracts were not tested against *E. coli* because their effect against this bacterium was previously demonstrated (Prosser et al., 2014; Prosser et al., 2016).

Additionally, leaf and root extracts of the species selected for the glasshouse experiment were tested for their activity against an environmental strain of *E. coli* that was previously isolated from dairy shed effluent (DSE *E. coli*).

The 96-well plates were incubated in a UV–Vis Spectrophotometer (FLUOstar Optima, BMG Labtech) at 37 °C overnight. Optical density (OD) was measured (595 nm), after shaking the plate for 15 s, every 30 min for 12 to 20 h, depending on the growth rate of each bacterium, which had been determined prior to the experiments.

Blank ODs were subtracted from each extract dilution OD at each measuring time to obtain the final OD result.

When OD results were not conclusive, mainly due to strong colour development with some extracts, the final culture of the test was plated to determine colony forming units (CFU), 200 μL of bacterial and extract suspension were transferred from the 96-well plate to a 2 mL Eppendorf tube and centrifuged at 3000 rpm for 20 min. The supernatant was discarded, and the pellet was re-suspended in 1 mL of Ringer's solution. A 10-fold serial dilution was carried out in Ringer's solution, and 10 μL of those dilutions were plated in triplicate on Tryptic Soy Agar plates, and incubated for 24 h at 37 °C. CFU were counted in the dilutions where CFU were >3 and <30 across the three replicates. If no growth was observed in these plates, it would indicate a bacteriostatic effect of the plant extract.

2.2. Investigating the antimicrobial properties of plants in the soil

2.2.1. Experimental design and setup

From all the plants tested, swamp *L. scoparium*, *P. colorata*, and *M. robusta* showed antimicrobial properties, and were therefore selected for the glasshouse experiment to test the antimicrobial properties in soil. *L. perenne* was included as control. The topsoil (0–20 cm) of a Perch-Gley Pallic soil (LandcareResearch, 2018) was collected from an experimental sheep farm in Massey University, Palmerston North (40° 23′ 30″ S, 175° 36′ 22″ E). This soil had a pH of 5.75, total C of 4.32% and total N of 0.45% (Prosser, 2011). Pots were layered with 2 cm of gravel at the bottom for drainage and filled with 1.3 kg (dry weight equivalent) of fresh sieved soil (5 mm). 24 seedlings per species (total 96 pots) were planted after removal of potting mix from the roots by gently washing with tap water. Seedlings were grown in a glasshouse for one year until the roots had visibly colonised the whole pot. During that time, pots were set up randomly in the glasshouse, and their positions were exchanged every week to avoid location biases. The pots were irrigated to field capacity with tap water every two-three days in winter and twice per day in summer.

Before *E. coli* application, two pots of every plant species were destructively harvested to demonstrate no presence of background

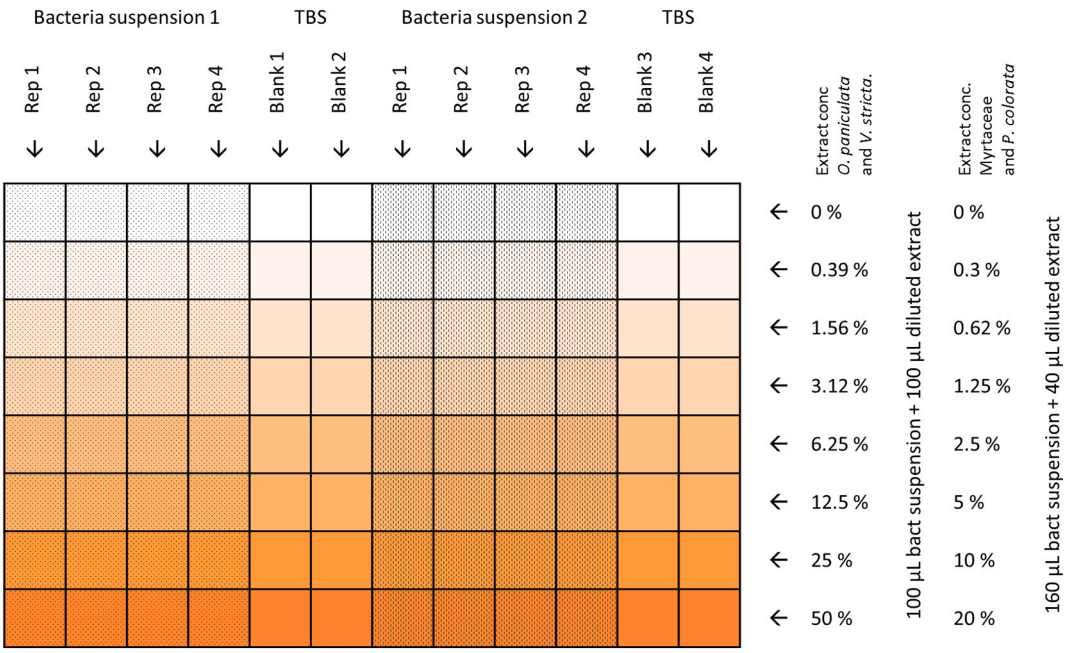

Fig. 1. Layout of the experiments in the 96-well plates.

E. coli. Due to the death of some *P. colorata* plants, only one pot was harvested for this plant species for the background sampling.

2.2.2. *E. coli application*

The bacterium *E. coli* was used as an indicator organism. Once the roots had colonised the whole pot, the pots were returned to the laboratory and placed over ethanol sterilized containers to collect leachates. To avoid edge flow in the pots, space between the soil and the pot was filled with petroleum jelly (Cameron et al., 1992). *E. coli* application was similar to Prosser et al. (2016), but with spiking of the DSE with additional *E. coli* to represent the practice of irrigation with high nutrient content organic waste, as worst-case scenario of long survival of bacteria in the soil (Horswell et al., 2010). DSE was collected fresh from a dairy farm milking shed the day before the experiment, *E. coli* was enumerated, and the DSE was stored at 4 °C overnight. The following day, it was spiked with the DSE *E. coli* strain (as used in the Microplate Assays) to ensure the presence of *E. coli* at an approximate concentration of 10^6 cfu/mL, which is consistent with the average values found in DSE (Donnison et al., 2011).

The *E. coli* spiked DSE was added with a pipette to each pot at a rate of 100 mL of DSE over 2.5-h period. This was equivalent to a 7 mm irrigation, or a one-month irrigation of an annual 200 kg N/ha, standard practice in New Zealand (Houlbrooke et al., 2004; Wallace and Johnstone, 2010). Shortly after, a rain fall event was simulated by addition of 50 mL of water to the surface of each pot (equivalent 3.5 mm) via spray bottles over a 1.5-h period. The pots were weighed and kept at the same moisture level throughout the experiment by daily watering with a spray bottle. When present, leachates were collected daily with volume recorded and *E. coli* enumerated as mentioned in follows.

2.2.3. *Harvest and E. coli enumeration in leachates and soil*

Bacteria enumeration in soil occurred on days 1, 3, 7, 14, and 21 after the DSE application and the rainfall simulation event. The soil was separated from the roots, sieved (4 mm), homogenized, and a 20 g subsample was collected for *E. coli* enumeration and soil moisture determination. Four pots per plant species were harvested per day. *P. colorata* pots were only sampled until day 7 due to plant death. For the same reason, only three *L. scoparium* pots could be harvested on day 21.

E. coli was enumerated in the soil by the five-tube Most Probable Number (MPN) method described by Horswell et al. (2007). Enumeration was completed by comparing the results with a MPN table and calculated per gram of dry soil. *E. coli* was enumerated in the leachates using the Colilert method (IDEXX Laboratories, Inc.).

2.2.4. *Soil pH and moisture content determination*

Sieved fresh soil was oven-dried at 104 °C for 24 h to determine soil moisture. Another subsample was dried at 40 °C for four days, sieved through a 2 mm sieve, and pH was analysed in a 1:2.5 water extract with a HACH Multi-Parameter Meter HQ440d with pH probe PHC735.

2.3. *Data analysis*

The results of luminescence from the Lux-bioassay for each plant extract were calculated as percentage of the luminescence produced by control tests with just water. The results were analysed with an analysis of variance (ANOVA) analysis, and differences between plant extracts were analysed using Tukey's test.

Data from the Microplate Sensitivity Assay was used to obtain extinction curves by plotting the percentage of OD of each treatment compared to the Control (0% extract) at the stationary phase of the growth curve, against the concentration of extract in the treatment, as described by Prosser et al. (2014). These graphs were used to calculate the effective plant extract concentration that produced a 50% reduction in OD (EC50) by interpolating the results with the best fitted model.

Differences in soil moisture and soil pH between plant species were analysed with an ANOVA and differences between treatments were analysed using Tukey's test, after homoscedasticity and normality of the data were confirmed. *E. coli* numbers in the soil and leachates were compared between plant species for each sampling day, using a non-parametric Kruskall-Wallis. Calculations were performed with Statgraphics Centurion.

Decimal reduction time (DRT) indicates the days required to reduce *E. coli* numbers in the soil 90% or one Log_{10} from initial numbers (Horswell et al., 2010). Horswell et al. (2010) calculated DRT based on an exponential decay curve. However, the best fit model was used for these results to best explain the variance of our data. For this, Log_{10}(MPN) results for each plant species were plotted against time, and models were fitted with Statgraphics Centurion. Only medians are

represented in Fig. 4, although the tests and models were performed with all the replicates (as shown in Supplementary materials).

3. Results

3.1. Screening plants for antimicrobial potential

In the Lux Bioassay, all plant extracts - except *M. laetum* from North Island, and *P. excelsum* from both North and South Island - presented significant differences in the bioluminescence reduction compared with L. *perenne*. The greatest reduction in luminescence was observed in *P. colorata* and *M. robusta* extracts, followed by *L. scoparium*, *K. ericoides*, *O. paniculata*, and swamp *L. scoparium* (Fig. 2). No other species produced a reduction in luminescence of >50% when compared with water (EC50).

A summary of the results (EC50) of the Microplate sensitivity assays is shown in Table 2. Graphs of the growth curves for each test are presented in Supplementary Material. *O. paniculata* and *V. stricta* leaf extracts slowed down the growth of *P. aeruginosa*, *B. cepacia* and *S. aureus*. However, in the stationary phase the optical density (OD) was similar, or not lower than 50% compared to the control, even if the maximum extract concentration tested for these plant species was 50% (=125 mg/mL fresh weight).

Extracts of *L. scoparium* inhibited the growth of all tested bacteria, although the EC50 was relatively high compared with the effect of swamp *L. scoparium* on *S. aureus*, with an EC50 of 3.3 mg/mL. In this case the highest tested concentration of the extract (32 mg/mL) produced a four Log_{10} reduction of CFU compared with the control (Table 3). *K. ericoides* inhibited the growth of *B. cepacia*, *S. aureus*, and *P. aeruginosa* in that order. Extracts of both *M. robusta* and *P. colorata* showed the strongest inhibition against most of the bacteria. Leaf extracts of *P. colorata* were the most effective against *E. coli*, with an EC50 of 43.5 mg/mL fresh leaves, although the highest concentration tested (50 mg/mL) produced less than one Log_{10} reduction in CFU (Table 3) compared with the control. *P. colorata* leaf extracts reduced the growth of *S. aureus*, *P. aeruginosa* and *B. cepacia* (Table 2). *M. robusta* and swamp *L. scoparium* showed the highest growth inhibition against *E. coli* present in DSE. However, when the bacterial cultures exposed to these plant extracts were plated, the CFU were no lower than one Log_{10} compared with water-control (Table 3). *M. robusta* leaf extracts also decreased the growth of *P. aeruginosa*, *S. aureus* and *B. cepacia*, and in the last two cases, no CFU were recovered after exposure to the highest extract concentrations (Table 3). Root extracts of swamp *L. scoparium*, *M. robusta* and *P. colorata* showed less antimicrobial effect against all five strains when compared to leaf extracts. Only *M. robusta* and swamp *L. scoparium* root extracts showed some inhibitory effect against *P. aeruginosa* and *B. cepacia*.

3.2. Antimicrobial properties of plants in the soil

The fresh DSE contained 2.8×10^5 MPN *E. coli*/mL. Based on the cfu present in the seed stock of DSE *E. coli*, inoculated DSE was calculated to contain *E. coli* at 2.4×10^6 cfu/mL. Results from the Colilert® test of spiked DSE on the day of the irrigation confirmed a concentration of 2.15×10^6 MPN/mL, which corresponds to 2.15×10^8 MPN/pot.

Despite each pot receiving the same amount of DSE and rainfall (100 mL of DSE (7 mm equivalent over 2.5 h); and 50 mL of irrigation (3.5 mm over 1.5 h)), there was large variability between and within species for leaching and surface ponding. Some pots started to leach immediately, and some pots presented surface ponding after application of DSE and rainfall water. Leachates collected the day after irrigation, as well as total *E. coli* leached per pot, were also highly variable and not related to the plant type (Fig. 3).

Despite large amounts of *E. coli* leached from most of the pots (between 10^6 and 10^7 MPN/pot, Fig. 3), *E. coli* remaining in the soil was over 90% of the applied *E. coli*, as shown by the *E. coli* enumeration in soil in Day 1, which was similar for all treatments (Fig. 4). Adjusting the irrigation level to accommodate moisture loss, resulted in very occasional leachate production, and the total amount of *E. coli* leached was always lower than the levels leached on Day 1.

The results of *E. coli* numbers in the soil are represented in Fig. 4. The total numbers of *E. coli* in the soil on day 1, (median of 10^7 MPN/pot) were similar across all plant types. Similarly for leachate results, *E. coli* numbers in the soil were highly variable (see Supplementary Material). Despite that, there were statistically significant differences in the survival of *E. coli* between L. *perenne*, swamp *L. scoparium* and *M. robusta* on day 14 (p-value = 0.06) and 21 (p-value = 0.03). *P. colorata* plants did not grow under the experimental conditions, and only 14 survived. *E. coli* could only be analysed in the soil under *P. colorata* on days 1, 3 and 7, and there was no evidence of a reduction in *E. coli* (see Supplementary Material).

There was a significant negative correlation between *E. coli* numbers

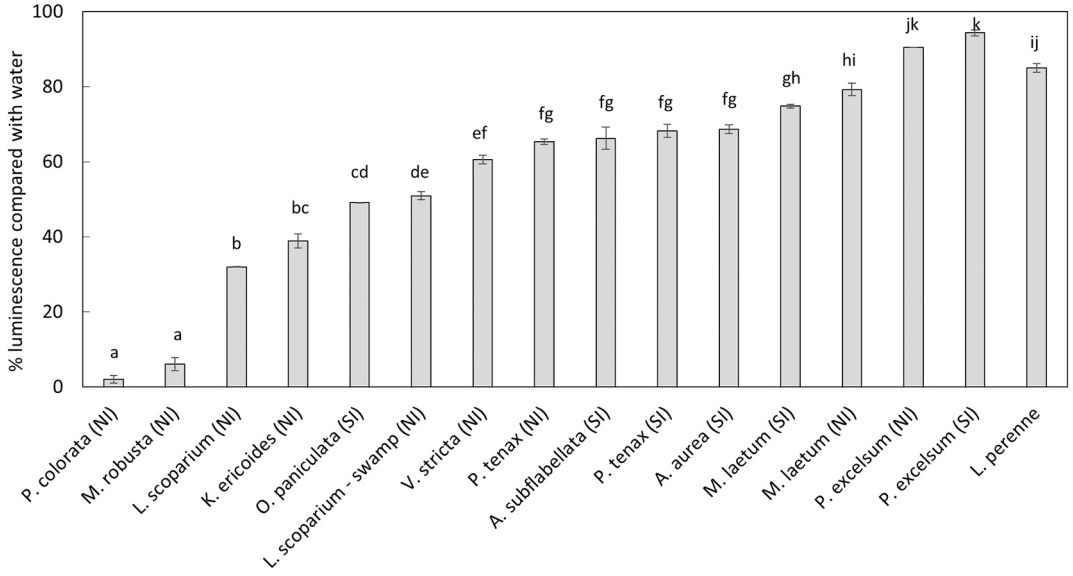

Fig. 2. Percentage of luminescence of Lux *E. coli* in plant extracts compared with water. Results indicate average and standard error ($n = 3$). SI: South Island, NI: North Island. Different letters indicate significant differences between plant species ($P < 0.05$).

Table 2
Concentration of plant extract (mg/mL fresh weight) that reduced OD by 50% (EC50).

Species	Plant part	***E. coli***	***S. aureus***	***P. aeruginosa***	***B. cepacia***	DSE E. coli
O. paniculata	Leaf	>125	>125	>125	>125	–
K. ericoides	Leaf	–	42.0	70.0[a]	5.0	–
L. scoparium	Leaf	–	35.9	48.3	27.7	–
Swamp *L. scoparium*	Leaf	>50	3.3	>50	32.8	29.7
	Root	>64.6	>64.6	78.4[a]	63.5	>64.6
M. robusta	Leaf	70.1[a]	12.5	2.4	1.6	29.1
	Root	>63.8	>63.8	23.4	30.3	>63.8
V. stricta	Leaf	>125	>125	>125	>125	–
P. colorata	Leaf	43.5	34.6	21.1	34.1	>54.6
	Root	>43.8	>43.8	>43.8	>43.8	>43.8

– Test not performed, as explained in the methods section.
[a] Result is an extrapolation.

Table 3
Average Log_{10} reduction of CFU on the most concentrated extract test compared with the water control. NR: not recovered, there was no growth in the plate. Blank cells indicate that the test was not performed.

Species	Plant part	*E. coli*	*S. aureus*	*P. aeruginosa*	*B. cepacia*	DSE E. coli
Swamp *L. scoparium*	Leaf		4.2		0.85	<0.5
M. robusta	Leaf	<0.5	NR	1.5	NR	0.72
P. colorata	Leaf	0.7	5.1			<0.5

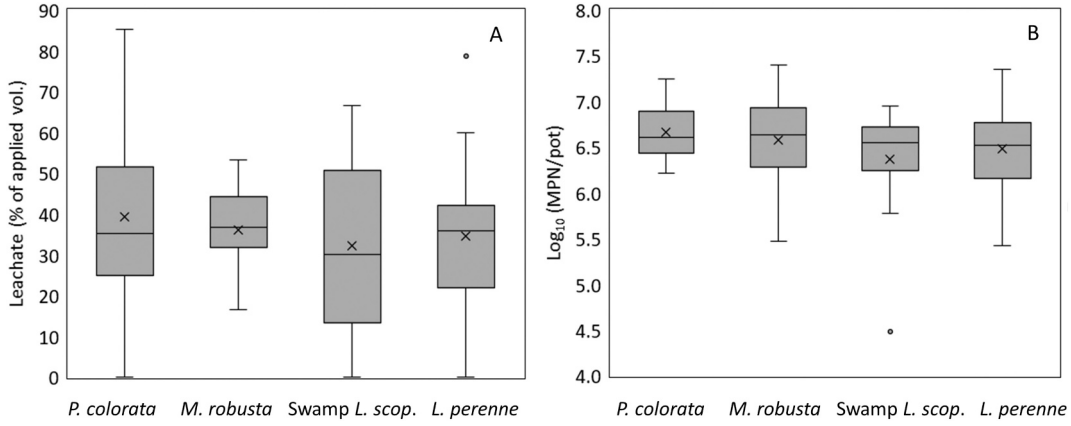

Fig. 3. (A) Percentage of the volume applied on day 0 that leached on day 1 in each of the treatments. (B) Total amount of *E. coli* leached on day 1 in each of the treatments ($n = 24$ expect for *P. colorata* $n = 14$ and Swamp *L. scoparium* $n = 21$).

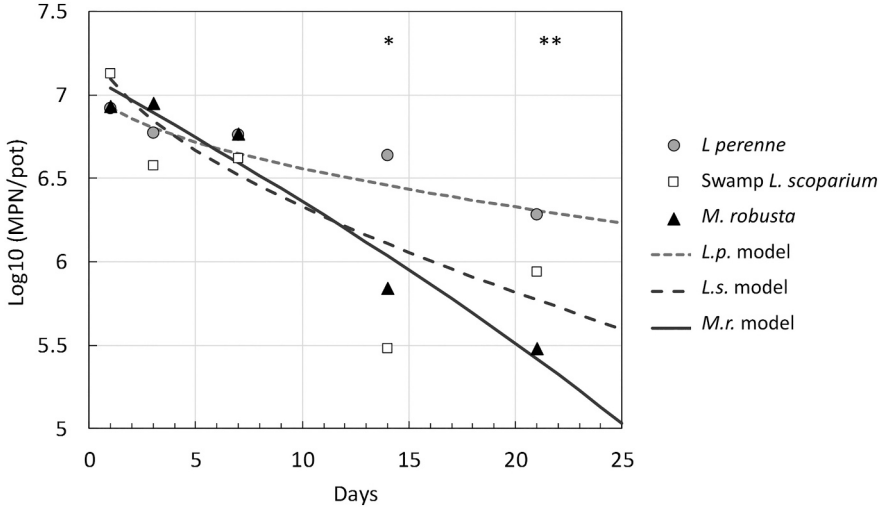

Fig. 4. Median and best fitted model of *E. coli* recovered from soil over time for each plant type. *Kruskal-Wallis test significance higher than 90%, **Kruskal-Wallis test significance higher than 95%.

in soil over time (after DSE application) for the three types of plant species tested (Table 4). Results showed that *M. robusta* had the strongest negative correlation, and the fitted model explained over 80% of the variance. Interpolation with the models for *M. robusta* and swamp *L. scoparium* indicated that a reduction of 90% *E. coli* in the soil is likely to be reached by day 14. Extrapolation from L. *perenne* data indicated that this reduction would happen by day 45.

Soil pH was significantly different depending on the plant that was growing on it, and decreased in the order L. *perenne* > *P. colorata* > swamp *L. scoparium* > *M. robusta* (Fig. 5A). Soil moisture, on the other hand, was similar in the soil under all plant species (Fig. 5B).

4. Discussion

The results of plant screening for antimicrobial potential partially support previous research that reported the antimicrobial capacity of *L. scoparium*, *Kunzea* sp., *P. colorata* and *M. robusta*. The work by Calder et al. (1986) is the largest screening of NZ vegetation with antimicrobial activity currently published. However, Calder et al. (1986) used methanol to prepare leaf extracts, and inhibition tests were based on the diameter of the inhibition zone. High activity of *P. colorata*, *L. scoparium*, *Kunzea* sp. (called *Leptospermum ericoides* at the time of that publication), and *M. robusta* against *S. aureus* growth was reported. However, in that work, only *P. colorata* presented growth inhibitory activity against *E. coli* and *P. aeruginosa*, which was not detected in other Myrtaceae species (Calder et al., 1986). Interestingly, our work did show a strong activity of the extracts of Myrtaceae species tested against gram-negative bacteria, which is not commonly reported in the literature, as it was also highlighted by Prosser et al. (2014).

The strong inhibition of luminescence by *P. colorata* and *M. robusta* leaf extracts could be explained in part by the low pH of those extracts, pH 4.15 and 3.93 respectively, as the optimal pH range for this toxicity test is approx. 5.5–7 (Horswell and Dickson, 2003). However, when those extracts were mixed with TSB, it resulted in optimal pH conditions for bacterial growth even in the highest concentration of the leaf extracts (pH 6.9–7.1). For this reason, we suggest the compounds within the extract, likely with an acidic nature, might be the main reason for the antimicrobial activity of these extracts; rather than free H^+ that would be buffered by TSB.

The chemistry of essential oils, and secondary metabolites with antimicrobial activity from *P. colorata*, *K. ericoides* and *L. scoparium* have been extensively described (Perry et al., 1997; Christoph et al., 2000; Wyatt et al., 2005; Perry and Gould, 2010; Van Vuuren et al., 2014; Killeen et al., 2015; Killeen et al., 2016). The main compounds described with antimicrobial activity are β-triketones such as leptosperomone, isoleptosperomone, and grandiflorone, in *L. scoparium* (Douglas et al., 2004; Killeen et al., 2015; Killeen et al., 2016), a high variety of mono- and sesquiterpenes in *Kunzea* spp. (Wyatt et al., 2005; Van Vuuren et al., 2014), and *P. colorata*, in this case specially polygodial, (Perry and Gould, 2010; Wayman et al., 2010). Phytochemical composition of *M. robusta* is largely unknown (Wollenweber et al., 2000), and the compounds responsible for its antimicrobial activity have not yet been described. For *P. colorata*, *K. robusta* and *L. scoparium*, all described compounds with antimicrobial activity are lipophilic. It is highly unlikely that any of those compounds were present in the water extracts in enough concentration to produce the inhibition shown in Tables 2 and 3. Our research, as well as previous work by Prosser et al. (2014), suggests that compounds in the water extracts are hydrophilic, and probably of acidic nature. Future research should determine whether any other compounds, apart from the ones already described, are responsible for the antimicrobial activity of these plants.

The results of the greenhouse experiment (Fig. 4) were similar to those of Prosser et al. (2016), who found a faster *E. coli* reduction with *Kunzea robusta* and *L. scoparium* than L. *perenne*. However, the calculated period for reaching 90% reduction of *E. coli* in soil was much faster in Prosser's experiment (5 and 8 days respectively) than in this experiment. The soil C and N content of this experiment were higher than those of Prosser et al. (2016) and might explain the difference. In addition to that, application of DSE further increased the presence of organic matter and nutrients. Both factors (organic matter and nutrients) have shown to promote the survival, or even favour the regrowth of some enteric bacteria in the soil (Jamieson et al., 2002; Horswell et al., 2010).

Changes in soil induced by roots of *M. robusta* and swamp *L. scoparium*, are likely to explain the enhanced die-off of *E. coli* compared with L. *perenne*. These changes in the soil differ from one plant species to another due to production of root exudates (with or without antimicrobial properties), water and nutrient uptake, and/or colonisation by associate microorganisms (Philippot et al., 2013; Zhang et al., 2017). In this experiment, the significant differences found in soil pH under the different plant species (Fig. 5A) can partially explain the quicker die-off of *E. coli* in soil under *M. robusta* > swamp *L. scoparium* > *L. perenne*. *E. coli* survival is the highest at neutral to alkaline pH and it decreases in acidic pH in the same soil type (Jamieson et al., 2002). Apart from temperature (Underthun et al., 2017), soil moisture is usually recognized as an important factor affecting the survival of *E. coli* or other pathogenic organisms in the soil (Jamieson et al., 2002; Horswell et al., 2007). However, soil moisture was not significantly different irrespective of plant type (Fig. 5B), which indicates that in this case soil moisture does not contribute to the different survival rates of *E. coli* in the soil.

Excretion of root exudates could be one of the reasons for the changes in soil pH, and for enhanced die-off of *E. coli* under *M. robusta* and swamp *L. scoparium* compared with *L. perenne*, as was also hypothesised by Prosser et al. (2016). Stress factors, such as changes in temperature, presence of plant pathogens or herbivores, nutrient, water, or light shortages can induce secondary metabolite production (Isah, 2019), many of which (i.e. terpenes, flavonoids or phenolic compounds) are described to have antimicrobial properties.

Finally, although not part of this experiment, the diverse range of organisms associated with roots of *L. scoparium* (McKenzie et al., 2006) and probably *M. robusta*, could also be antagonistic to *E. coli*, facilitating its die-off compared with L. *perenne*. Jamieson et al. (2002); Jiang et al. (2002); Erickson et al. (2014) demonstrated the presence of competing organisms limits the survival of enteric bacteria, *Salmonella* spp., and *E. coli* respectively, in soil or manure. In addition, Wicaksono et al. (2017) showed that some endophytes associated with *L. scoparium* present antibacterial activity against plant pathogens.

Despite the potential of these Myrtaceae species to enhance the die-off of *E. coli* in the soil, the leaching of this bacterium on the first day after irrigation with DSE was very high and not dependent on plant type. This indicated that under high irrigation regimes, when the soil is already saturated (in this case by the application of DSE), *E. coli* move quickly through the soil through by-pass flow, as was also demonstrated by Mishra (2018).

The results from this work, together with the lacuna of knowledge in this area of the phytoremediation, suggest the need for future research to

Table 4
Best fitted models for MPN correlation over time for each plant species, and the calculated days needed to show a reduction in *E. coli* by 90% from day 1 (Log red.).

Plant species	Best fitted model	Correlation coefficient	P-value	R^2	Log red. (days)
L. perenne	$Log_{10}(MPN) = (50.2–2.3 \times day^{1/2})^{1/2}$	−0.61	0.0000	36.7%	45
Swamp *L. scoparium*	$Log_{10}(MPN) = (55.1–4.7 \times day^{1/2})^{1/2}$	−0.72	0.0005	51.8%	14.5
M. robusta	$Log_{10}(MPN) = (50.5–1.0 \times day)^{1/2}$	−0.90	0.0000	80.7%	14

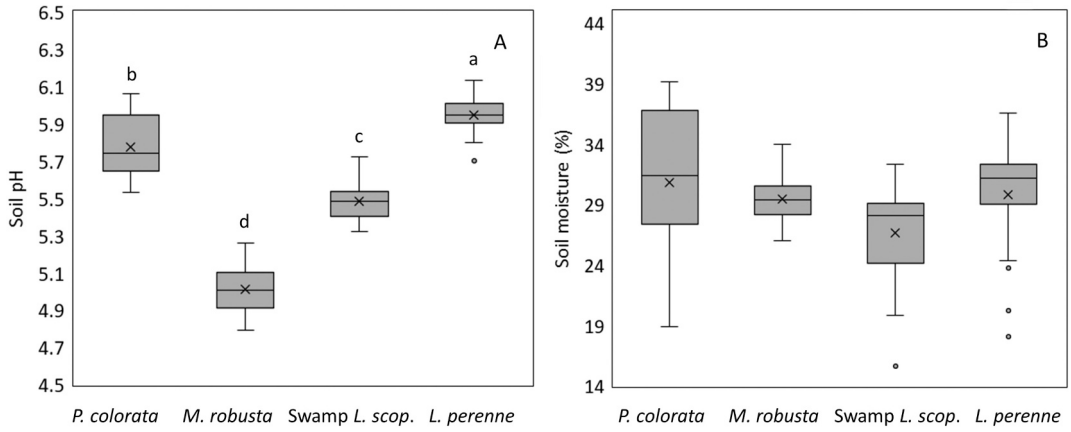

Fig. 5. (A) Soil pH for all soils collected during the experiment. (B) Soil moisture (%) for all soils collected during the experiment (n = 24 except for *P. colorata* n = 14 and Swamp *L. scoparium* n = 21).

a) better understand the benefits and limitations of plantings with bioactive species to prevent the contamination of freshwater resources, and b) find new applications of bioactive plants in different settings, such as biofilters (Galbraith et al., 2019).

5. Conclusions

This work demonstrated that water extracts of three species of Myrtaceae family, and one to Winteraceae family (out of 11 plant species tested from the North and South Island of New Zealand) showed antimicrobial activity against one or more of the five human pathogens or indicator organisms used in these experiments. Leaf extracts from L. *scoparim*, swamp *L. scoparium*, *K. ericoides*, *P. colorata*, and *M. robusta* demonstrated a bacteriostatic effect. In addition, leaf extracts of *M. robusta* and *P. colorata* showed bactericidal effect against *S. aureus* and *B. cepacia*. The acidic water soluble leaf extracts of those plants indicated that it is unlikely that the previously described lipophilic antimicrobial compounds are responsible for the antimicrobial potential demonstrated in these experiments. *M. robusta* and the local variety of *L. scoparium*, called swamp mānuka, enhanced the die-off of *E. coli* in soil compared with L. *perenne* after irrigation with *E. coli* spiked dairy shed effluent. A 90% *E. coli* reduction was reached on day 14 for the two Myrtaceae species compared with 45 days calculated for L. *perenne*. Soil pH was significantly different for those plant species, whereby order of pH decrease was matched with *E. coli* reduction capability (*M. robusta* > swamp *L. scoparium* > *L. perenne*). This is a potential explanation for the differences in *E. coli* survival in soil. Despite these promising results, caution is needed, since under almost saturated soil conditions, irrigation with DSE created an initial flush of *E. coli* in the leachates after one day, which was not different between plant species. We demonstrated that under certain management conditions to avoid bypass flow, phytoremediation of microbially contaminated soils with bioactive plants is possible. Future research in field conditions would show the potential and/or limitations of bioactive plantings for preventing faecal or microbial contamination of freshwater resources from contaminated soil.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2021.104040.

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