Is kanuka and manuka establishment in grassland constrained by mycorrhizal abundance?

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Abstract: Two indigenous small tree and shrub species, kanuka (*Kunzea ericoides*) and manuka (*Leptospermum scoparium*), have potential as reforestation species in New Zealand as they are forest pioneer species that can invade grassland naturally from present seed sources. The aim of this study was to determine if establishment of kanuka and manuka from seed in grassland distant from stands of these species might be constrained by lack of appropriate mycorrhizal fungi. Both species were grown in an unsterilised grassland soil from a low-productivity montane site assumed to be devoid of appropriate mycorrhizal fungi and inoculated with sterilised or unsterilised O-horizon or mineral soil from beneath three kanuka and three manuka communities expected to contain such fungi. Inoculation with unsterilised O-horizon soil improved kanuka biomass by 36–92%, depending on the source of the inoculant. Inoculation did not improve manuka biomass. No ectomycorrhizal infection was observed on either kanuka or manuka in samples examined under binocular microscope. The biomass response by kanuka to inoculation may be due to introduction of more effective arbuscular mycorrhizal fungi from kanuka communities or possibly to the introduction of soil microorganisms. Testing of inoculation under field conditions will be essential to determine whether establishment of either species in grassland soil by seeding is seriously constrained by lack of appropriate mycorrhizal fungi or soil microorganisms.

Keywords: arbuscular mycorrhiza; ectomycorrhiza; *Leptospermum*; reforestation; seedling establishment; soil inoculation

Introduction

Indigenous forest once covered about 75% of New Zealand (Newsome 1987), but much of the forest has been removed and the land converted to agricultural use, particularly to grassland for pastoral farming. Increasingly, there is interest in re-establishing indigenous forests for carbon sequestration or other environmental benefit on land that is marginal for pastoral use because of isolation, steepness or low productivity. Two indigenous small tree and shrub species, kānuka (*Kunzea ericoides* (A.Rich.) Joy Thomps.) and mānuka (*Leptospermum scoparium* J.R.Forst. & G. Forst.), have potential as reforestation species as they are forest pioneer species that can invade grassland naturally from present seed sources (Wardle 1991). For land areas that lack adjacent seed sources, these species can be established by planting and possibly by seeding (Stevenson & Smale 2005; Dodd & Power 2007; Ledgard et al. 2008), which is potentially a lower cost option than planting. Once established, kānuka or mānuka should facilitate forest succession by providing ectomycorrhizal inoculum (Dickie et al. 2012) or ameliorating microsite conditions (Wardle 1970; Davis et al. 2013).

Successful restoration of indigenous vegetation may require restoration of components of the microbial community (Williams et al. 2011). The root systems of all indigenous forest tree and shrub species are infected by mycorrhizal fungi that form symbioses with the host plant and play an essential role in the nutrition and water uptake of the host. Many of New Zealand's native woody flora form mycorrhizas exclusively with one or other of two types of mycorrhizal fungi – arbuscular mycorrhizal fungi (AMF) or ectomycorrhizal fungi (EMF). Arbuscular mycorrhizal fungi produce arbuscles, hyphae and vesicles within root cortex cells whereas EMF form a mantle around roots and a Hartig net between root cells (Brundrett et al. 1996). The mycorrhizas also differ functionally, with EMF generally having a greater ability to obtain nitrogen and phosphorus from organic polymers than AMF (Read & Perez-Moreno 2003). Kānuka and mānuka are unusual among New Zealand's flora in that they are colonised by both AMF and EMF (Orlovich & Cairney 2004; McKenzie et al. 2006).

In agricultural and forestry systems, plant performance is frequently constrained by lack of appropriate mycorrhizal fungi (Smith & Read 1997; Lekberg & Koide 2005). The extent to which the invasion of kānuka and mānuka into grassland communities is constrained by abundance of appropriate mycorrhizal fungi is unknown. Grassland communities are dominated by species infected by AMF and it is possible that some of these fungi will also infect kānuka and mānuka and facilitate the establishment of these species. Growth and competitiveness of kānuka and mānuka may be improved, however, by introduction of AMF from kānuka or mānuka communities as has been shown for *Podocarpus cunninghamii* in New Zealand (Williams et al. 2011). This would also be the case for EMF, which are unlikely to be present in grassland communities (Dickie et al. 2012). To test whether kānuka and mānuka establishment in grassland soil may be constrained by lack of appropriate mycorrhizal fungi, both species were grown in kānuka and mānuka free grassland soil inoculated or not inoculated with O-horizon and mineral soil collected from beneath extant stands of the respective species. We

hypothesised that, if establishment of kānuka and mānuka in grassland soil was constrained by lack of appropriate mycorrhizal fungi, inoculation with soil from extant stands would provide functionally appropriate fungal species and improve growth of the host species.

Methods

Two soil inoculation trials, one with kānuka and the other with mānuka, were conducted simultaneously in a greenhouse. A soil mapped as Acheron stony loam (Soil Bureau 1968), collected from kānuka- and mānuka-free grassland near Mt Barker (Rakaia Valley, Canterbury) was used as the growth medium for both trials. No attempt was made to remove the existing bacteria and fungi by sterilisation. The nearest kānuka and mānuka stands were 300 m and more than 3000 m distant respectively from the site where the soil was collected. Vegetation at the site was of low productivity and dominated by the native tussock-grass *Festuca novae-zelandiae* (Hack.) Cockayne and the adventive herb *Pilosella officinarum* Vaill. Analysis showed the soil to be moderately acidic (pH 5.3), have medium carbon (6.4%) and nitrogen (0.47%) levels and C:N ratio (13.6), and a low level of bicarbonate (Olsen) extractable phosphorus (6.9 mg kg^{-1}). Exchangeable cation levels were 1.9, 6.4 and 0.48 $cmol^{+}\ kg^{-1}$ for potassium, calcium and magnesium respectively. The top 5 cm of mineral soil was removed and discarded before the underlying soil was collected, to reduce the potential of inclusion of ectomycorrhizal fungal spores that might infect kānuka or mānuka. The soil was potted to a depth of 75 mm, in pots 80 × 80 × 100 mm deep, and covered by a 5-mm-deep layer of inoculum (see below), which was in turn covered by a further 10-mm layer of grassland soil to reduce the potential of cross contamination of inocula between pots.

Two types of soil inoculum were collected from beneath each of three kānuka and three mānuka stands (Table 1) for testing the effect of inoculation on the respective species. The first consisted of organic horizon (O-horizon) material overlying the mineral soil and the second consisted of the upper (0–50 mm) layer of mineral soil. The inocula were passed through a 5-mm sieve to remove coarse material, separately mixed and stored in a refrigerator before being used in the trials. A quantity of each inoculant sufficient to inoculate the required number of pots was steam-sterilised under pressure at 121°C for 15 min while the remainder was left unsterilised. A randomised complete block design with eight blocks (replicates) was used, with the three inoculation treatments, namely site of inoculant collection, inoculant type (O-horizon or mineral soil), and sterilisation (unsterilised or sterilised), being applied in factorial combination. An uninoculated control treatment, which had 5 mm of grassland soil in place of an inoculant layer, was included. The two trials were conducted simultaneously but separately in the same glasshouse on adjacent benches.

Seeds of each species were germinated on sterilised seed testing paper in Petri dishes and seedlings were transplanted into pots (four per pot) when they were large enough (c. 5 mm) to handle. Kānuka seedlings were thinned to two per pot after 8 weeks' growth. The trials were planted in mid-winter (June) and harvested after 5 months. Watering was by an automated overhead misting system. The pots were re-randomised twice during the trial period. Prior to harvest the pots were soaked in water for 1–2 h and soil was gently freed from the root systems under water. After cleaning, root systems were removed from shoots and stored moist to allow examination for mycorrhizas. Roots and shoots were oven-dried at 65°C and weighed.

All roots of the first replicate pot of each treatment harvested were examined for presence of EMF infection by counting a minimum of 200 fine root tips and recording whether or not each tip was ectomycorrhizal. For most seedlings, 50% of the root system was examined by displaying roots over a clear plastic grid and examining alternate rows under a stereo microscope (4× to 50×). Where there was any doubt, root tips were mounted on glass slides and examined with compound microscopy to confirm mycorrhizal assessments (presence of mantle, Hartig net, and/or extraradical hyphae; 40× to 1000×). It was assumed the plants would develop arbuscular mycorrhizas from propagules present in the grassland soil, hence no attempt was made to quantify AMF infection. After examination, roots were oven-dried and weighed as above.

The biomass response of kānuka and mānuka plants was analysed using SAS Version 9.2. Normality and homogeneity of the data were tested to ensure the underlying assumptions of the analysis were met. Assessment of the fixed treatment effects of site of inoculant collection, type of inoculant (organic or mineral soil), and sterilisation of inoculant (sterilised or unsterilised) on plant biomass components was then undertaken by analysis of variance (ANOVA) using the Mixed procedure in SAS. The ANOVA included terms for replicate (random effect), and the fixed treatment effects and their interactions nested within the inoculation treatment as experimental factors. Comparisons of pair-wise least-square means were made according to the Tukey–Kramer method (Kramer 1956).

Table 1. Location of the soils collected for inoculating kānuka (*Kunzea ericoides*) and mānuka (*Leptospermum scoparium*).

Species	Site name	Location	NZMG Easting	NZMG Northing	Altitude (m)
Kānuka	Acheron	Rakaia catchment	2399062	5761425	650
	Okuti	Banks Peninsula	2496536	5712654	230
	Taupo	Lake Taupo catchment	2744885	6274359	558
Mānuka	Bealey	Waimakiriri catchment	2397296	5797167	630
	Craigieburn	Waimakiriri catchment	2407544	5784310	830
	Okuku Pass	North Canterbury foothills	2464979	5788623	600

Results

Seedling biomass

Seedlings of both species in the uninoculated control treatments appeared healthy and grew satisfactorily. Mean heights after 5 months' growth were 11 cm and 8 cm for kānuka and mānuka respectively. However, soil inoculation with unsterilised O-horizon soil increased shoot, root and whole-plant dry weight of kānuka over that of the uninoculated control treatment by 54%, 70% and 57% respectively ($P < 0.01$, < 0.05 and < 0.01 respectively; data for whole-plant dry weight only shown in Fig. 1). Inoculation with sterilised O-horizon soil or sterilised or unsterilised mineral soil did not significantly increase kānuka shoot, root or whole-plant weight above that of the control (Fig. 1). The inoculation effect was site dependent (Table 2). Inoculation with unsterilised O-horizon soil from the Taupo site increased whole-plant biomass over that of the control by 92% ($P < 0.001$), whereas inoculation with unsterilised organic material from the Acheron and Okuti sites resulted in smaller (36–43%) and non-significant increases in biomass (Table 3). Soil inoculation marginally increased the root:shoot ratio of kānuka, irrespective of type, origin or sterilisation treatment of the inoculant (Table 2; Fig. 2).

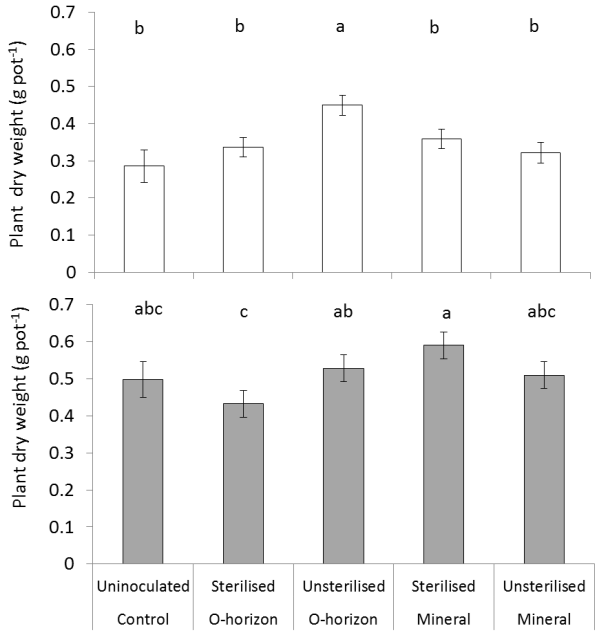

Figure 1. Whole-plant dry weight of kānuka (*Kunzea ericoides*) (upper, open) and mānuka (*Leptospermum scoparium*) (lower, shaded) in a grassland soil inoculated with sterilised or unsterilised O-horizon or mineral soil collected from stands of the respective species. Values show the mean effect for three collection sites. Treatments without a letter in common are significantly different ($P < 0.05$). Bars show standard errors.

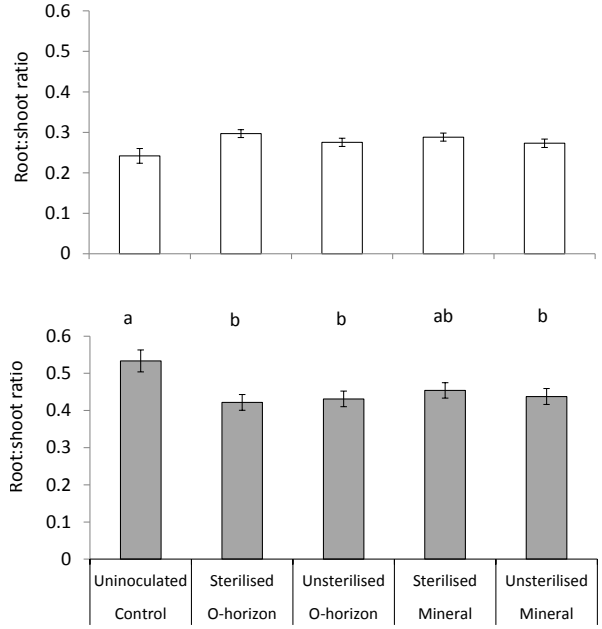

Figure 2. Root:shoot ratios of kānuka (*Kunzea ericoides*) (upper, open) and mānuka (*Leptospermum scoparium*) (below, shaded) in a grassland soil inoculated with sterilised or unsterilised O-horizon or mineral soil collected from stands of the respective species. Values show the mean effect for three collection sites. For mānuka, treatments without a letter in common are significantly different ($P < 0.05$). There were no significant differences between kānuka treatments. Bars show standard errors.

Table 2. *F*-values from mixed-model analysis of variance for shoot, root and whole-plant dry weight as well as shoot:root ratio for kānuka (*Kunzea ericoides*) and mānuka (*Leptospermum scoparium*) with site of inoculant collection, type of inoculant (organic or mineral soil) and sterilisation of inoculant (sterilised or unsterilised) as fixed effects and replication as a random effect ($n = 8$).

Treatment	Kānuka d.f.	Shoot weight	Root weight	Total weight	Root:shoot ratio	Mānuka d.f.	Shoot weight	Root weight	Total weight	Root:shoot ratio
Inoculation	$F_{1, 79}$	3.46	4.89*	3.64	4.79*	$F_{1, 80}$	1.47	1.19	0.17	13.15***
Site of inoculant collection	$F_{2, 79}$	3.54*	2.30	3.02	0.63	$F_{2, 80}$	3.60*	3.29*	3.68*	0.16
Type of soil inoculant	$F_{1, 79}$	6.09*	5.32*	5.61*	0.30	$F_{1, 80}$	8.51**	7.96**	8.83**	1.62
Site × type	$F_{2, 79}$	5.99**	1.48	4.31*	1.65	$F_{2, 80}$	2.21	4.69*	3.12*	1.24
Sterilisation of inoculant	$F_{1, 79}$	4.16*	0.62	2.81	3.32	$F_{1, 80}$	0.14	0.06	0.11	0.05
Site × sterilisation	$F_{2, 79}$	4.97**	4.85*	4.70*	0.53	$F_{2, 80}$	1.17	0.29	0.49	1.74
Soil × sterilisation	$F_{1, 79}$	12.91***	8.00**	10.99**	0.10	$F_{1 ,80}$	14.66***	11.21**	14.23***	0.73
Site × soil × sterilisation	$F_{2, 79}$	3.68*	1.59	3.2*	0.24	$F_{2, 80}$	0.54	0.45	0.37	1.20

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3. Whole-plant dry weight (g pot^{-1}) of kānuka (*Kunzea ericoides*) in grassland soil not inoculated or inoculated with sterilised or unsterilised O-horizon or mineral soil collected from kānuka stands at three sites. Values are means of eight replicates. Values without a letter in common are significantly different ($P < 0.05$).

Site	Inoculant type	Sterilisation	Mean (SE)	Significance
Control	Uninoculated	-	0.29 (0.043)	bc
Acheron	Organic	Sterilised	0.45 (0.041)	ab
Acheron	Organic	Unsterilised	0.41 (0.041)	abc
Acheron	Mineral	Sterilised	0.41 (0.041)	abc
Acheron	Mineral	Unsterilised	0.34 (0.043)	bc
Okuti	Organic	Sterilised	0.26 (0.041)	c
Okuti	Organic	Unsterilised	0.39 (0.043)	abc
Okuti	Mineral	Sterilised	0.35 (0.041)	bc
Okuti	Mineral	Unsterilised	0.36 (0.043)	bc
Taupo	Organic	Sterilised	0.30 (0.041)	bc
Taupo	Organic	Unsterilised	0.55 (0.041)	a
Taupo	Mineral	Sterilised	0.32 (0.041)	bc
Taupo	Mineral	Unsterilised	0.26 (0.043)	bc

Table 4. Whole-plant dry weight (g pot^{-1}) of mānuka (*Leptospermum scoparium*) in grassland soil not inoculated or inoculated with sterilised or unsterilised O-horizon or mineral soil collected from mānuka stands at three sites. Values are means of eight replicates. Values without a letter in common are significantly different ($P < 0.05$).

Site	Inoculant type	Sterilisation	Mean (SE)	Significance
Control	Uninoculated	-	0.50 (0.048)	ab
Bealey	Organic	Sterilised	0.41 (0.051)	b
Bealey	Organic	Unsterilised	0.56 (0.048)	ab
Bealey	Mineral	Sterilised	0.67 (0.048)	a
Bealey	Mineral	Unsterilised	0.60 (0.051)	ab
Craigieburn	Organic	Sterilised	0.47 (0.048)	b
Craigieburn	Organic	Unsterilised	0.51 (0.051)	ab
Craigieburn	Mineral	Sterilised	0.54 (0.048)	ab
Craigieburn	Mineral	Unsterilised	0.45 (0.048)	b
Okuku	Organic	Sterilised	0.42 (0.048)	b
Okuku	Organic	Unsterilised	0.51 (0.048)	ab
Okuku	Mineral	Sterilised	0.56 (0.048)	ab
Okuku	Mineral	Unsterilised	0.48 (0.051)	ab

Mānuka biomass was not significantly improved by inoculation. Mānuka biomass was significantly lower when soil was inoculated with sterilised O-horizon soil than when inoculated with either unsterilised O-horizon soil or sterilised mineral soil, but these treatments did not differ significantly from the uninoculated control soil (Fig. 1). Similarly, although mānuka biomass was significantly greater when soil was inoculated with sterilised mineral soil from Bealey than some organic or mineral soils from other locations, biomass in this treatment was not significantly greater than the control (Table 4). For mānuka biomass, the three-way interaction between type, origin and sterilisation of inoculant was not significant (Table 2). In contrast to kānuka, soil inoculation decreased the root:shoot ratio of mānuka, irrespective of type, origin or sterilisation treatment of the inoculant (Table 2; Fig. 2).

Mycorrhizal formation

No infection by EMF was observed in either kānuka or mānuka in the 200 root-tip samples examined under the binocular microscope. A single root tip with distinct mantle, observed under compound microscopy, was observed in a mānuka short root inoculated with unsterilised organic soil from Okuku; however, this was not part of the 200-root-tip sample. Fungal hyphae that were consistent in appearance with AMF were occasionally observed during compound microscopy.

Discussion

The biomass of kānuka growing in grassland soil was substantially improved by inoculation with unsterilised O-horizon soil from the kānuka stand from the Taupo site. As no response was obtained from inoculation with sterilised O-horizon soil, it can be concluded that the improvement was due to mycorrhizal or microbial activity rather than to nutrients contained in the inoculating soil. Our hypothesis was therefore supported for kānuka, though not for mānuka, which showed no enhancement of biomass in response to inoculation. No development of ectomycorrhizas was observed on kānuka roots so enhancement due to EMF infection can be ruled out. The observed biomass response may be due to infection of kānuka roots by more functionally appropriate AMF than were present in the grassland soil. The response is consistent with results of many studies of plants in unsterilised soil that have shown responses to inoculation with AMF (Lekberg & Koide 2005). Soil microorganisms, including plant-growth-promoting bacteria (PGPB) and microbivorous invertebrates, contained in O-horizon soil may also have contributed to the growth enhancement of kānuka. PGPB may improve plant growth by suppression of pathogens (e.g. Hebbar et al. 1991), production of phytohormones such as indole acetic acid (e.g. Vonderwell et al. 2000) or improving formation of arbuscular mycorrhizas (Artursson et al. 2006), whereas microbivorous invertebrates may improve plant growth by accelerating nutrient release from microbes (Colinas et al. 1994).

All unsterilised O-horizon soils improved kānuka biomass to some extent, but soil from the Taupo site caused the greatest improvement in biomass. AMF spore numbers can vary greatly in different natural habitats (Brundrett et al. 1996) as well as seasonally (Smith & Read 1997). Similar variation might be expected to occur with PGPB or other soil microorganisms. Additionally, much of the functional diversity within AMF occurs at the isolate level rather than the species level (Brundrett et al. 1996). Site-by-site variation in soil inoculum level and effectiveness could therefore explain the differential response of kānuka to inoculation with O-horizon soils from the different sites. Such site-by-site variation in soil inoculum level may also have contributed to the failure of mānuka to show a response to inoculation. Williams et al. (2011) found growth of *Podocarpus cunninghamii* was enhanced when inoculated with forest AMF and grown in competition with the grass *Agrostis capillaris*, but was not enhanced when grown in the absence of competition. Testing of inoculation under field conditions will be essential to determine whether establishment of kānuka or mānuka in grassland soil by seeding

is seriously constrained by lack of appropriate mycorrhiza or other soil microorganisms.

Examination of roots for ectomycorrhizas showed inoculation with soil collected from extant stands of kānuka and mānuka did not promote formation of ectomycorrhizas. This finding was unexpected as both kānuka and mānuka form ectomycorrhizas as well as arbuscular mycorrhizas (Orlovich & Cairney 2004; Moyersoen & Fitter 1999). In the field, colonisation of mānuka with AMF and EMF appears to vary with soil and other habitat conditions. Moyersoen and Fitter (1999) found mānuka plants collected from South Island West Coast coastal communities had ectomycorrhizas principally in areas with ectomycorrhizal tree species (*Nothofagus*) in the vicinity, but infection by EMF was scarce in areas dominated by arbuscular mycorrhizal (*Podocarpus*) species and absent on ultramafic soils. They considered that mānuka was unable to maintain ectomycorrhizas in the absence of ectomycorrhizal hosts. In a montane environment (Rakaia catchment, Canterbury), however, mānuka was found to have generally much higher colonisation by EMF than by AMF (Weijtmans et al. 2007), and ectomycorrhizal plants occurred in grassland distant from other indigenous ectomycorrhizal species. It was suggested that the montane environment with low nitrogen and phosphorus mineralisation rates and availability would favour colonisation by EMF rather than by AMF because of the greater ability of ectomycorrhizas to access these nutrients directly from organic sources (e.g. Read & Perez- Moreno 2003). Although a montane soil with low field vegetation productivity was used as the potting medium in the present study, nitrogen and phosphorus availability in a pot experiment would have been less constraining for plant growth because of enhanced organic matter mineralisation from soil disturbance as well as the glasshouse environment.

A succession from arbuscular mycorrhizas to ectomycorrhizas may occur in species with dual mycorrhizas. In *Eucalptus demosa* grown in sterilised soil inoculated with surface soil from under *E. demosa* trees, seedlings were largely infected with AMF after 2 months and ectomycorrhizas were scarce, but after 5 months ectomycorrhizas had become abundant and appeared to be replacing arbuscular mycorrhizas (Lapeyrie & Chilvers 1985). A similar successional process was reported in *Helianthemum chamaecistus* (Read et al. 1977). The use of unsterilised grassland soil with presumably high AMF inoculation potential as the potting medium in the present study would have reinforced any early dominance of AMF species should similar succesional processes occur in kānuka and mānuka. The almost complete lack of ectomycorrhizal formation after 5 months in the present study remains surprising, however, and further study of factors influencing ectomycorrhizal formation in kānuka and mānuka seedlings is warranted.

In unamended soil the root:shoot ratio of mānuka was nearly twice that of kānuka. Root:shoot ratios can change with plant age and size, but the plants in the unamended soil in this study were of similar age and dry weight (kānuka 0.14 g, mānuka 0.12 g). Addition of soil inoculum consistently decreased the root:shoot ratio of mānuka, irrespective of the source, type or sterilisation treatment of the soil inoculum. Plant root:shoot ratios almost always decline when conditions for growth improve as a result of increased nutrient or soil moisture availability (Wilson 1988; Marschener 1995). This suggests that soil inoculum addition improved growth conditions for mānuka; however, the reduction in root:shoot ratio in mānuka occurred in the absence of a corresponding change in plant biomass. In contrast to mānuka, the root:shoot ratio of kānuka increased with addition of soil inoculum, but the change, although significant, was small in comparison to that for mānuka.

In conclusion, this study has shown that establishment of kānuka by seeding in grassland distant from neighbouring kānuka stands may be constrained by lack of native AMF or microorganisms, but field testing is required to determine whether this might critically influence the establishment of kānuka. In contrast, there was no indication that establishment of mānuka was similarly constrained. Further work is necessary to elucidate the causative agent(s) of the response by kānuka to soil inoculation and determine how they may be influenced by environmental factors, to enable development of practical inoculation techniques to aid kānuka establishment.

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