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REGULAR ARTICLE

Dry matter yield, root traits, and nodule occupancy of lucerne and Caucasian clover when grown in acidic soil with high aluminium concentrations

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Abstract
Aims and methods Lucerne and Caucasian clover dry matter were measured in response to recommended lime and capital P inputs for six years in an acidic soil in the New Zealand high country. The initial three years of the field experiment indicated successful establishment and persistence of both legumes. Lucerne dry matter (DM) yield was up to 4 t/ha/yr in this period and higher than Caucasian clover yields. However, a lack of persistence of lucerne was apparent from this point forward compared with Caucasian clover which produced 7.7 t DM/ha in Year 6. An experiment using tubes of soil was used to investigate whether differences in root traits, nodulation and nodule occupancy were responsible for the differences observed in field persistence over time.
Results These showed that when rhizobia inoculant was added, the fine root length of Caucasian clover was unaffected (R^2 = 0.14) by aluminium (Al) content of the soil. In contrast, fine root growth of lucerne was suppressed (R^2 = 0.79) by the soil Al content. Nodulation of Caucasian clover was unaffected by soil pH or Al when the rhizobia inoculant was provided which suggests the viability of the commercial genotype ICC148 in this soil with a pH of 5.5 and Al ca. 7 mg/kg soil. For lucerne, the maximum nodulation score of 7.3 occurred with 2 t/ha of lime added (soil pH ca.6, Al ca. 0.3 mg/kg) plus inoculant.
Conclusions This suggests an Al toxic threshold of <1.0 mg Al/kg soil for effective lucerne nodulation. From the lucerne nodules, eight naturalized strains of *Ensifer meliloti* were identified. In contrast, only one *R. leguminosarum* strain was detected in the Caucasian clover nodules. The competition between those rhizobia genotypes may negatively affect the efficiency of biological nitrogen fixation in lucerne. Therefore, the lack of genetic diversity of *R. leguminosarum* bv. *trifolii* in New Zealand soils might be an advantage especially if the commercial strain is acid soil tolerant.

Keywords Alfalfa · Aluminium · *Ensifer meliloti* · *Medicago sativa* L. · Nodulation · *R. leguminosarum* bv. *Trifolii* · *Trifolium ambiguum* Bieb.

Introduction

Several studies (Edmeades et al. 1991; Moir and Moot 2010; Mullen et al. 2006; Munns 1965; Rechcigl et al. 1988) have indicated low persistence of legume species, and in particular lucerne (*Medicago sativa* L.), in soils of low pH (pH <5.8) and high Al (Soil exchangeable Al > 1.0 me/100 g). Those studies suggested amelioration either through soil amendment by lime (Black and Cameron 1984; Wheeler and Edmeades 1995; Moir and Moot 2010) and/or P application (Giller 2001; Lekberg and Koide 2005), or the use of more acid tolerant legume

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species. Caradus et al. (2001) monitored the growth of 15 forage legume species for three years on three different Otago upland soils with moderate to high soil Al levels and reported promising performance of Caucasian clover (*Trifolium ambiguum* Bieb.). Scott (1998) indicated the slow establishment of Caucasian clover, but reportedly it increased to become the dominant and the most persisted species after a decade of his field experiment.

A factor that contributes to the poor performance of lucerne plants in moderately acid soils is the effect of low pH on the establishment of the symbiosis between the legume and its nitrogen-fixing symbiont *Ensifer* (syn. *Sinorhizobium*) *meliloti* (Cooper et al. 1983; Charman et al. 2008; Khan et al. 2010). *E. meliloti* is reported to be the most acid-sensitive species of rhizobia (Graham and Vance 2000; Tiwari et al. 1992) with its occurrence reduced below pH 6 (Graham 1992) in the absence of calcium. Several reports have indicated *Ensifer* grew best in pH range 7–8 (Garau et al. 2005; Wang et al. 2002) that could give them a competitive advantage over other legume symbionts in alkaline soils/desserts (Sankhla et al. 2017). To obtain optimal nodulation, Punenbor et al. (1991) reported the soil pH had to be neutralized with lime around the seed and at least 10^5 cells of *E. meliloti* were required for successful nodulation. Mullen et al. (2006), suggested that the benefit of liming on lucerne is driven by its impact on improving nodulation through reducing Al toxicity and provide a Ca response for nodulation. In addition, the physical damage to root tips by Al will decrease the root depth and functionality leading to poor persistence of lucerne. Soto et al. (2004), described the effects of pH and Ca on different aspects of lucerne nodulation with *E. meliloti* and the acid-tolerant *Rhizobium sp*. strain LPU83. They suggested that reduced attachment of *E. meliloti* cells to lucerne roots is the main factor that limits the *E. meliloti*-lucerne symbiosis at low pH. Low productivity shown by lucerne plants in moderately acid soils is influenced by the impairment in the establishment of symbiotic associations with effective *E. meliloti* strains and the presence of highly competitive acid-tolerant, lucerne-nodulating bacteria with poor nitrogen-fixing ability. These bacteria are able to compete with effective *E. meliloti* strains for the formation of nodules at low pH, contributing to a reduction in crop yield (Lascano et al. 2001).

In many areas of New Zealand, particularly the high country, a lack of legume growth restricts pasture production and animal growth. Traditionally, white clover (*Trifolium repens* L.) has been drilled or oversown into this environment with limited success. But there are other legume options available. Previous success with Caucasian clover (Caradus et al. 2001; Scott 1998) in low pH soils, and lucerne in a high country site (Anderson et al. 2014) with naturally high pH that increases with depth (5.8 to 7.3) (Avery et al. 2008), has highlighted their importance. To assess the potential of a range of legume species, their relative performance was assessed over a six-year field experiment in the Lees Valley located in North Canterbury, New Zealand. The aim was to monitor the production and persistence of perennial legume species in response to the recommended lime and capital P inputs in an acidic high country soil. Full results of their production are reported in Berenji et al. (2017). This showed a contrast in the production and persistence of lucerne and Caucasian clover. Therefore, a further tube experiment was undertaken with these two species to investigate the mechanisms responsible for these differences and potential agronomic solutions to overcome them. Specifically, the tube experiment examined root traits, and nodule occupancy of lucerne and Caucasian clover in response to soil pH and Al content.

Materials and methods

Full details of the field experiment are reported in Berenji et al. 2017. Here an outline is given of information relevant to this paper.

Field experiment

The field experiment was established in 2006 at Mt. Pember station (high country marginal site, 43°, 08′, 25″ S and, 172°, 11′, 20″ E) in the Lees Valley, North Canterbury, New Zealand. The soil is a high country yellow brown shallow stony soil (NZ classification: Orthic brown soil, Hewitt and Whenua (1998); USDA: Dystrochrept, Soil Survey Staff). The texture of the top-soil is a silt loam. The maximum top-soil depth was 0.3 m, below which layers of stones were dominant down the soil profile. The top-soil pH was 5.3. Therefore, 5 t/ha of lime (Agrilime) was surface applied and incorporated through conventional cultivation in April 2005. The Olsen P (Olsen et al. 1954) was 9 mg/L prior to the start of the experiment. Therefore, 400 kg/ha of single superphosphate and 300 kg/ha of diammonium phosphate fertilisers were also incorporated during cultivation. A further 750 kg/ha of single superphosphate

was applied after sowing in October 2007 and 300 kg/ha in September 2008. The experiment was sown as a randomised complete block (RCB) design. There were four perennial legume species sown in four blocks (replicates) with a Duncan seed drill. Lucerne ('Kaituna', 10 kg/ha), red clover (*Trifolium pratense* L.) 'Pawera' (5 kg/ha), and white clover ('Demand', 4 kg/ha) were autumn sown in February 2006, but the known slow establishment of Caucasian clover (Scott 1998; Black et al. 2014) meant it ('Endura', 8 kg/ha) was sown the following spring in November 2006. For lucerne, 'Superstrike®' coated seed (with rhizobia, molybdenum, and lime) was used. Caucasian clover seeds were inoculated with commercial peat inoculant ('CC238b'; *Rhizobium leguminosarum bv. trifolii,* 'Nodulaid'; Becker Underwood Ltd., Australia) before sowing. The agronomic data of the botanical composition and seasonal growth of those four legume species in response to soil moisture content have been reported (Berenji et al. 2017). In this paper, the focus is on explaining the differences seen in the field for total DM of lucerne and Caucasian clover.

Dry matter yield

Above ground dry matter (shoot) yield was measured prior to each grazing and in each regrowth cycle over six years in all plots (Berenji et al. 2017). The accumulated yields (t DM/ha/yr) for sown legumes were analysed as an RCB by analysis of variance (ANOVA) using Genstat version 12 statistical software. The mean values were compared using Fisher's protected LSD (5%).

Pot experiment

Lucerne and Caucasian clover were grown from seed in soil taken from the Lees Valley. Top soil was collected in late September 2010 to a depth of 0.3 m. The soil had a similar management history to the field experiment site at Mt. Pember Station before it had been fertilised. This meant the selected paddock had no known farming background or fertiliser application in the last 60 years. Soil analysis (Table 1) showed phosphorus and calcium deficits (12 mg/L, and 1.9 me/100 g soil, respectively), low pH of 5.2 (1:2 (*v*/v) soil: water), and high aluminium levels (15.1 mg/kg soil). The anaerobic mineralisable nitrogen content of the soil in 0.15 m of soil profile was 113 mg/g in Sep. 2009.

Table 1 The soil properties of a high country soil sampled from the Lees Valley, North Canterbury, used for the tube experiment

Soil properties	Lees valley*
pH_{water}	5.2
Olsen Phosphorus (mg/L)	12
Sulphate Sulphur (mg/kg)	9
Potassium (me/100 g)	0.57
Calcium (me/100 g)	1.9
Magnesium (me/100 g)	0.93
Sodium (me/100 g)	0.05
CEC (me/100 g)	15
Total Base Saturation (%)	22
Aluminium**(mg/kg)	15.1
Total Molybdenum (mg/kg)	0.3
Organic Matter (%)	5.7
Total Carbon (%)	3.3

*Soil was collected from an uncultivated bare-ground ca. 2 km east of the Mt. Pember station field experiment

**$CaCl_2$ extractable

To allow roots to be excavated and nodulation quantified, soil was packed into PVC tubes to obtain a bulk density of 0.96 g/cm^3. Plants were grown outside at the Lincoln University Nursery (43°, 38′, 41″ S and, 172°, 27′, 42″ E). Tubes were cut uniformly from the polyvinylchloride (P.V.C) pipe. Each tube was 0.15 m in diameter and 0.8 m long, with a wall thickness of 4 mm. A plastic pot (0.2 × 0.15 m) was placed in the bottom of each tube to hold the soil in the tube and allow drainage.

Experiment design

The experiment consisted of 240 tubes arranged in a split-split plot design. Nitrogen source was the main plot with three treatments (N1 - Control; N2–50 kg N/ha; and N3-rhizobia inoculation). Phosphorus treatment was the first order sub-plot with two levels (P1–0, and P2–250 mg P/kg soil applied as [Ca $(H_2PO_4)_2.H_2O$] containing 24.6% P (*w*/w)). Lime rates were the sub-sub-plots with 5 treatments using laboratory grade $CaCO_3$ (equivalent to 0, 0.5, 1, 2 and 4 t lime/ha). The lime was added to soil and mixed 28 days before sowing the seeds. Phosphorus was added to the soil one day before sowing.

The tubes were filled with 0.3 m of stone (5 mm ≤ diameter of stones ≤100 mm) obtained from a commercial source, and the amended soil from the Lees Valley (5.1 kg soil provided as lime and P treatments) in the

top 0.3 m above, to mimic the Lees Valley soil profile. Commercial peat-based inoculants (*Ensifer meliloti* strain RRI128 for lucerne and *R. leguminosarum* bv. *Trifolii* strain ICC148 for Caucasian clover) were used to inoculate lucerne and Caucasian clover seeds, respectively, two hours before sowing on 29 March 2011. The sowing rate for lucerne and Caucasian clover was equivalent to 14 and 10 kg seed/ha, respectively. After 15 days, seedlings were thinned to 20 plants per tube. The water-holding capacity of the soil in the tubes was measured (30% w water/w soil) prior the start of the experiment. Soil moisture was maintained to be above 50% of field capacity. The tubes were rain-fed except on three occasions when the soil moisture was below 50% of field capacity according to the soil moisture readings by a portable TDR (Trace system, Soil Moisture Equipment, Santa Barbara, CA, USA). The number of lucerne and Caucasian clover seedlings in each tube, as a measure of plant survival, was recorded eight times during the 228-day growth period.

Harvest and nodulation assessment

Plants from each tube were excavated 228 days after sowing on 17 November 2011, when approximately 30% of lucerne plants were mature and at the flowering stage. The soil and stones were separated carefully from the roots. Plants from each tube were gently washed in the root washing facility at Lincoln University. Nodulation score was assessed by evaluating nodule colour, number, position, and size according to modified criteria from Rice et al. (1977). The total score was achieved by accumulated numerical values given to the respective criteria and ranged from 0 to 10.

Acetylene reduction assay (ARA)

Nitrogenase activity of nodules was tested using the acetylene reduction assay (ARA) via gas chromatography (Cummings et al. 2009). Although not recommended for quantifying nitrogen fixation owing to a fundamental error in the assay (Minchin et al. 1983) ARA can be a highly sensitive indicator of the presence of nitrogenase activity. Nodules from two randomly selected tubes per species per rep (16 tubes) of the inoculated plants were used for ARA. Ten percent (*v*/v) acetylene gas was injected into tightly sealed 12 ml vials containing a small fraction of root samples with 10 nodules attached to the roots. The vials were left to incubate for one hour at room temperature before a 5 ml sample of gas was extracted and analysed for ethylene production by standard flame ionisation gas chromatography (SRI 8610) standardised with pure ethylene. Root samples without nodules were used as the control. Every vial which had ethylene production (μmol C_2H_4 /h) of at least one order of magnitude greater than its control, was considered to be fixing atmospheric N_2.

Bacteria isolation and genotyping

Three nodules from each tube were randomly selected for rhizobia isolation. A total of 60 nodules were taken from each species. Bacteria were cultured and isolated from the nodules on YMA media (Vincent 1970), followed by DNA extraction using a PUREGENE™ (Gentra Systems, USA) DNA extraction kit. Cultures of rhizobium were identified using their major morphological characteristics (Nangul et al. 2013). The genotyping of rhizobium isolates used ERIC-PCR based on Versalovic et al. (1991). Genotypes were visualised by electrophoresis on a 1% agarose gel. The gel image from each sample was used to group the bacteria into different genotypes. A ~ 1300 bp fragment of the 16S rRNA was amplified for nine representative genotypes. Sequences were compared with those of known origin using BLAST from the National Centre for Biotechnological Information (NCBI).

Dry matter and fine root length assessment

The roots were subsequently separated from shoots and stored in the refrigerator at 4 °C for root analysis. Shoot samples were weighed after drying in an oven at 60 °C for 48 h. The commercial software package WinRHIZO 4.1 (Regent Instruments Inc., Quebec, Canada, 2000) was used to measure fine root length (<0.5 mm in diameter). After assessment, the root samples were dried at 70 °C for 48 h and then weighed. The soil from each lucerne tube was thoroughly mixed and a sample taken to determine final phosphorus and aluminium content.

Treatment effects were compared by analysis of variance (ANOVA) using GenStat version 12 statistical software using the full split-split plot analysis. The mean values were compared using Fisher's protected LSD (5%). Regression analysis and curve fitting were undertaken using SigmaPlot 11.0 and GenStat 12 software.

Results

Annual yield of field grown lucerne and Caucasian clover

The dry matter yield of Caucasian clover was low at 1.7 t/ha in Year 1, but reached 4.0 t/ha in Year 2 (2007/2008), and 7.7 t DM/ha in Year 6 (Fig. 1). There was a constant decline in lucerne DM production over time beginning at 4.2 t/ha in Year 1 and declining to 1.0 t/ha in Year 4. The total DM of both species was unable to be assessed in Year 5 due to an unscheduled on-farm spring grazing event. Therefore, the recorded yields underestimate production for that year. In Year 6, the total DM of lucerne was only 1.0 t/ha.

Seedling survival in P.V.C tubes

There was a rapid decline in the number of lucerne seedlings in the unamended soil control treatment during the 228 days with only 15% survival. Lime rates improved ($P<0.001$) survival (Fig. 2a) to 75% in the 4 t/ha of lime treatment, at the final measurement in November 2011. In contrast, at the final measurement, Caucasian clover seedlings had 60% plant survival in the control and this improved ($P<0.001$) to 85% with the addition of 4 t/ha of lime application (Fig. 2b).

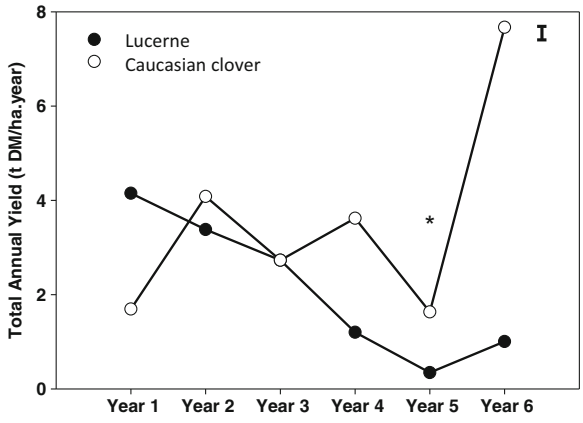

Fig. 1 Total annual dry matter yield (t/ha.yr) of lucerne and Caucasian clover grown on farm in the Lees Valley, Canterbury, New Zealand. Plots were sown as an RCB design in 2006. Years 1 to 6 refers to 2006/2007 to 2011/2012 growth seasons, respectively. The error bars indicate the maximum standard error of the mean (SEM = 0.24, LSD = 0.73). *Missing data from Dec 2010 to Jun 2011

Lucerne plant survival was affected ($P < 0.01$) by the interaction of nitrogen source and lime (Fig. 3a) at the end of the 228 days growth period. On average only six of 20 lucerne seedlings survived in the control tubes. These remained at the cotyledon stage and had not developed any trifoliate leaves by the end of the experiment. Use of 50 kg N/ha and rhizobia inoculant doubled the number of surviving plants to 13. Seventeen (out of 20) seedlings survived with the application of the 0.5 t lime/ha to the inoculated lucerne tubes. The addition of P resulted in ($P < 0.01$) the survival of one more lucerne seedling (14 seedlings with P2 application compared with 13 in P1). There was no effect ($P = 0.19$) of nitrogen source or P application on the survival of Caucasian clover seedlings.

Soil test results showed the available Al levels were associated ($R^2 = 0.67$) with soil pH. Soil available Al

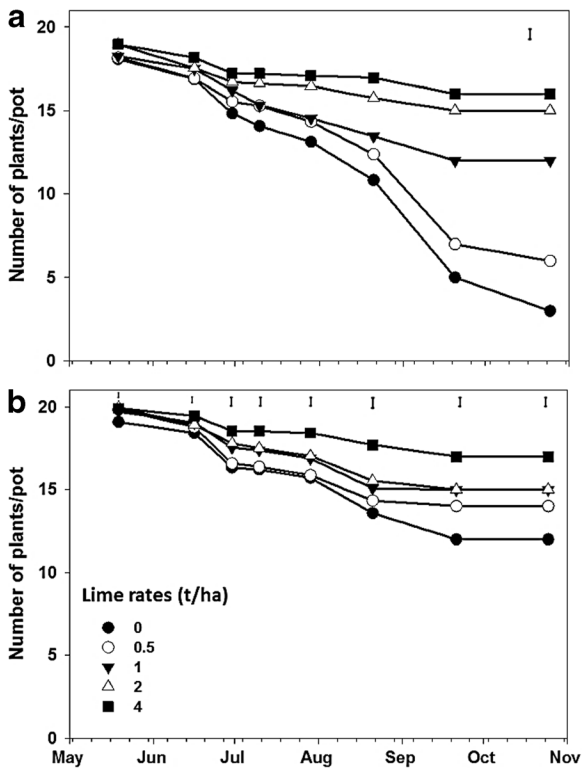

Fig. 2 Plant survival of lucerne (**a**) and Caucasian clover (**b**) in response to lime over a 228 day growth period, when grown in P.V.C tubes that contained an acidic high country soil from the Lees Valley, North Canterbury, New Zealand. The error bars indicate the maximum standard error of the mean (SEM = 0.53 for lucerne and 0.47 for Caucasian clover)

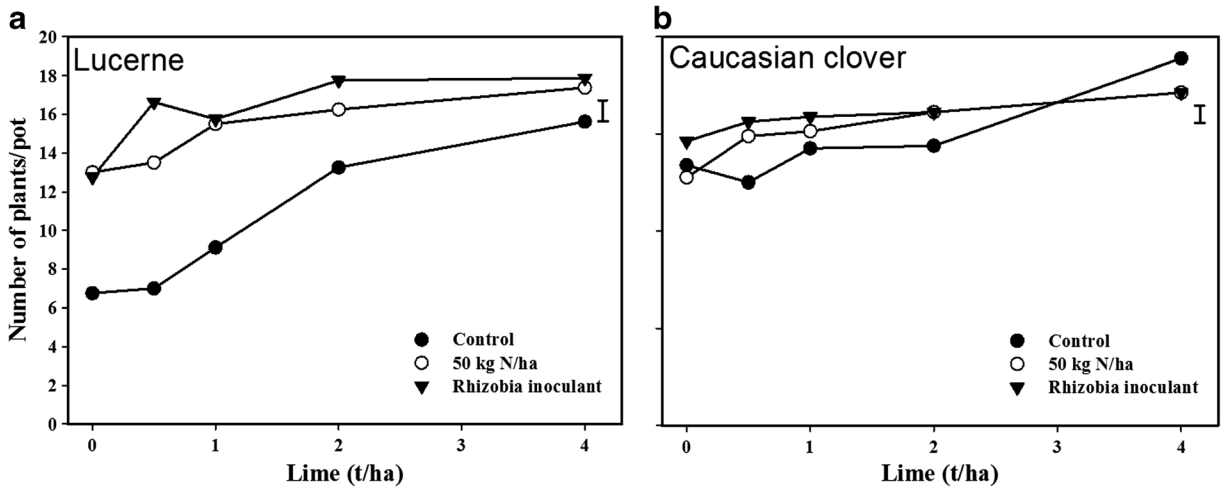

Fig. 3 Number of lucerne (**a**) and Caucasian clover (**b**) plants after 228 days when grown in P.V.C tubes at the Lincoln University nursery and supplied with 50 kg N/ha or rhizobia inoculant across five rates of lime. The error bars indicate the maximum standard error of the mean (for lucerne; SEM = 1, LSD = 2.85 and for Caucasian clover; SEM = 0.84, LSD = 2.4)

was low within the soil pH range of 5.6–6.7. Below this range, Al levels increased sharply to 7 mg/kg soil at a soil pH of 5.4 in the control tubes (Fig. 4). The soil pH was increased ($P<0.001$) quadratically from 5.5 to 6.5 by the addition of 0.5 to 4 t/ha of lime applied (Fig. 5).

Dry matter yield in P.V.C tubes

Lucerne dry matter yield increased ($P < 0.01$) from 127 g/m^2 in N1 (no nitrogen treatment) to 180 g/m^2 with the 50 kg N/ha applied (N2), and to 464 g/m^2 when

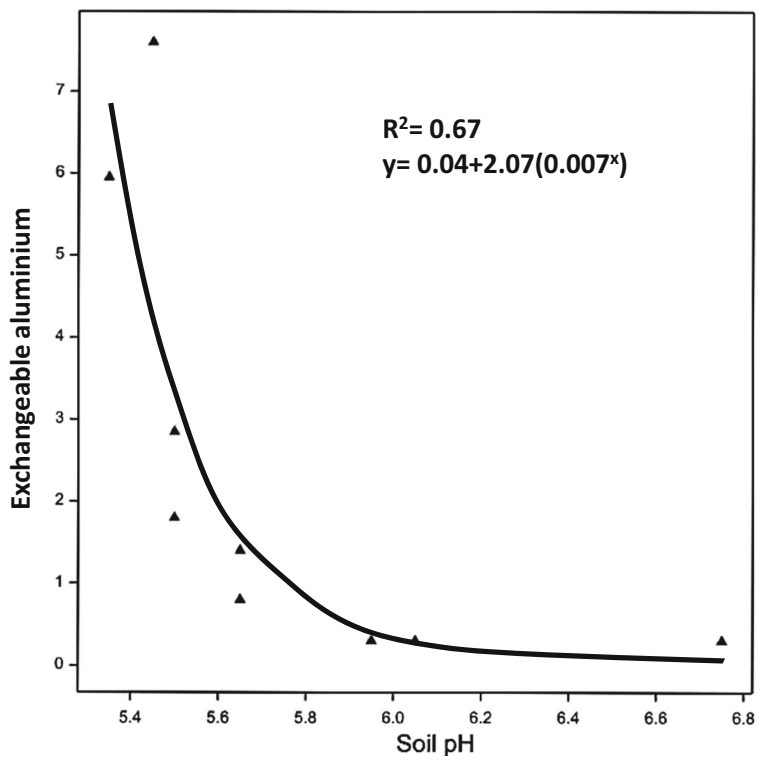

Fig. 4 The relationship between exchangeable aluminium (mg/kg soil) and soil pH of the Lees Valley

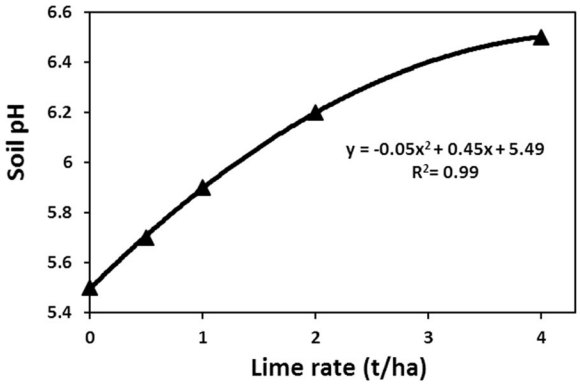

Fig. 5 Soil pH of the Lees Valley in response to applied lime, measured after excavating plants from P.V.C tubes

plants were inoculated with rhizobium (N3) (Fig. 6a). Lime rates increased ($P < 0.001$) the shoot DM from 27 g/m^2 in 0 lime where only a few small seedlings survived, to 280 g/m^2 by adding 2 t lime/ha (Fig. 6b). Shoot DM was 202 g/m^2 in P1 (no-P treatment), compared with ($P \leq 0.064$) 319 g/m^2 when P2 (250 mg/kg soil) was applied.

Lucerne root dry weight was affected ($P<0.05$) by the three-way interaction of lime, phosphorus, and nitrogen. It increased from 0 in the control (N1P1 and no lime) to 1170 g/m^2 in the 2 t/ha lime + rhizobia inoculant +P treatment (Fig. 7a). In the 0 (N1P1 and N1P2), and 50 kg N/ha treatments (N2P1 and N2P2), root DM of lucerne was less than 130 g/m^2 and only increased, up to 850 g/m^2 when 4 t/ha of lime plus P was added. In the rhizobia treatment (N3P1) lucerne root DM increased from 2 g/m^2 to 260 g/m^2 with 1 t/ha of lime, and then to 880 g/m^2 with 2 t/ha of lime. There was also a rapid increase from 80 g/m^2 to 800 g/m^2 with only 0.5 t/ha of lime application plus 250 mg P/kg in the inoculated tubes (N3P2). The root DM was at its maximum (1170 g/m^2) with the 2 t/ha of lime application plus P. The application of the equivalent of half a tonne of lime with 250 mg P/kg, reduced Al from 7.6 mg/kg to 1.8 mg/kg soil (Fig. 7b). The measured Olsen P from P1 treatment tubes showed no response to lime rates.

Shoot dry weight of Caucasian clover was affected ($P \leq 0.05$) by the interaction of nitrogen source and lime rates. It ranged from 6 g/m^2 in the N1 no lime treatment to 462 g/m^2 in the 4 t lime/ha when the rhizobia inoculant was added (Fig. 8a). Shoot dry weight increased from 160 g/m^2 to 350 g/m^2 with the addition of 1 t lime/ha in the rhizobia inoculant treatment, but there was no response in the N1 or 50 kg N/ha treatments. The interaction was due to the response between the 0.5 and 1 t lime/ha treatments. This level did not affect shoot dry weight in the control pots (N1) or the 50 kg N/ha treatment but increased shoot dry weight of Caucasian clover from 180 g/m^2 to 350 g/m^2 in the inoculant treatment (Fig. 8a). Root dry weight of Caucasian clover was increased ($P \leq 0.05$) by the nitrogen source, from 77 g/m^2 in N1 to 318 g/m^2 in the rhizobia inoculant (N3), and by lime rates ($P \leq 0.01$) from 68 g/m^2 in 0 lime, to 338 g/m^2 in the 4 t lime/ha application (data not shown).

Nodulation assessment

The nodulation score of lucerne was affected ($P \leq 0.01$) by the interaction of N source and lime rates (Fig. 9a).

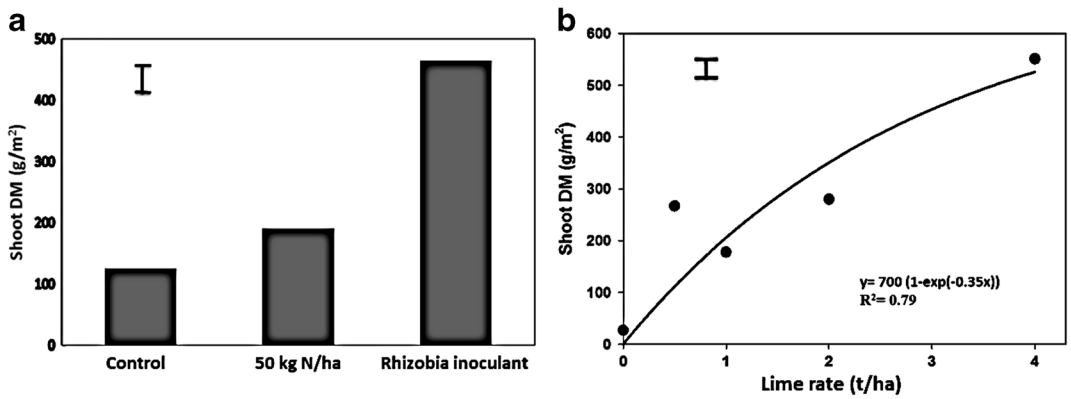

Fig. 6 Shoot dry matter (DM) (g/m^2) of lucerne, after 228 days of growing in P.V.C tubes, in response to nitrogen source (**a**), and lime rates (**b**). The error bar indicates the maximum standard error of the mean. For (a); SEM = 54, LSD = 185, and for (b); SEM = 64.2, LSD = 181

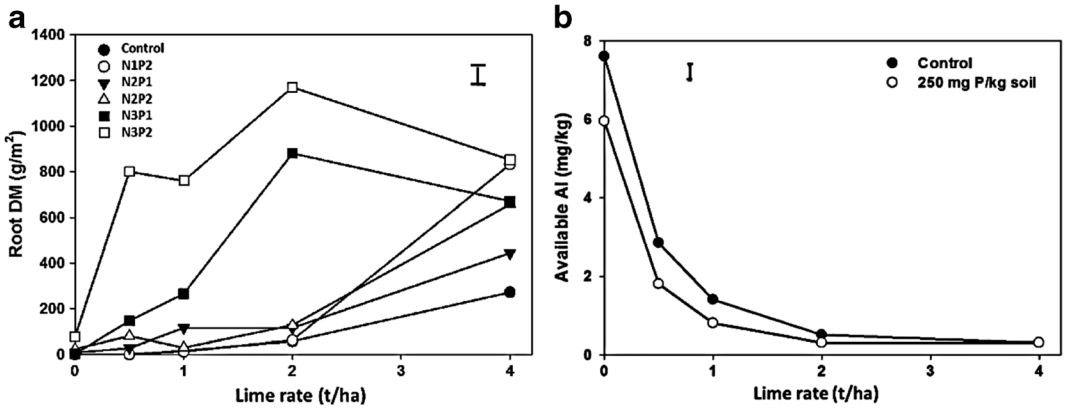

Fig. 7 **a** Lucerne root dry matter (DM) (g/m^2) in response to nitrogen source × P × lime rate. Lucerne plants were grown for 228 days in P.V.C tubes that contained the soil from the Lees Valley. N1 (Control), N2 (50 kg N/ha), N3 (Rhizobia inoculant), P1 (Control), P2 (250 mg P/kg soil). The error bar indicates the standard error of the mean for the interaction effect (SEM = 102, $LSD_{5\%}$ = 290). **b** Soil available aluminium (mg/kg) in response to lime (t/ha) and P (mg/kg soil) application after 228 days of growing lucerne plants in P.V.C tubes (SEM = 0.18, LSD = 0.56)

With no rhizobium inoculant added, the score increased ($P \leq 0.01$) from 0 in the 0 lime, to 5.7 with 4 t lime/ha. In contrast, for inoculated plants, the nodule score was 4.8 in the 0 lime and increased to 7.3 with the addition of 2 and 4 t/ha of lime. The nodulation score of Caucasian clover plants was also affected ($P \leq 0.001$) by the interaction of the N source and lime rates (Fig. 9b). With no rhizobium inoculant added, it increased from 0 in the 0 lime to 6 with the incorporation of 4 t/ha of lime (Fig. 9b). The inoculated treatment (N3) started with a nodule score of 5.5 with 0 lime and increased to 7.3 with 4 t/ha of lime (Fig. 9b). There was no effect of phosphorus treatment on the nodulation of either lucerne or Caucasian clover.

Fine root assessment

Root length/plant for both species was negatively associated with the level of available Al in the control (N1) and the 50 kg N/ha (N2) treatments (Fig. 10). When rhizobia inoculant (N3) was added, the fine root length of Caucasian clover was unaffected ($R^2 = 0.14$) by Al content of the soil. In contrast, the fine root length of lucerne decreased ($R^2 = 0.79$) when soil Al content increased.

Nitrogenase activity and rhizobium strain identification

The ARA confirmed the nitrogenase activity of the nodulated lucerne and Caucasian clover in inoculated

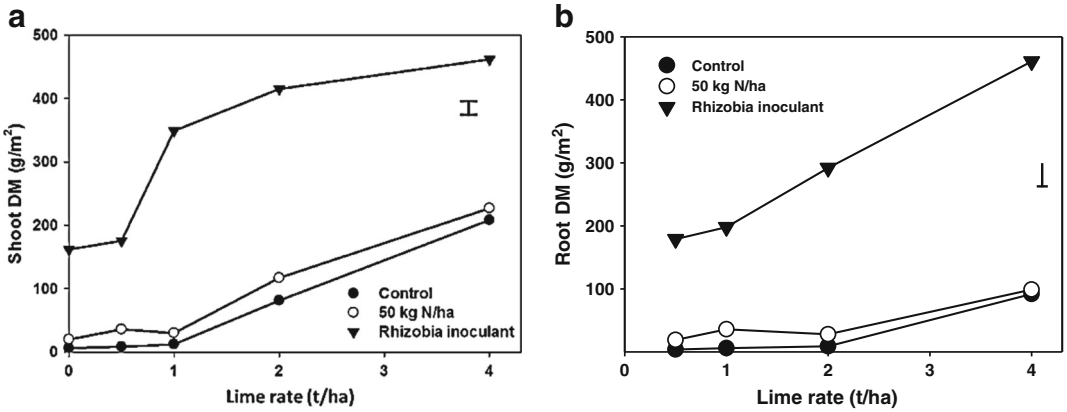

Fig. 8 (a) Shoot dry matter (DM) of Caucasian clover in response to nitrogen source and lime rates, after 228 days of growing in P.V.C tubes. The error bar indicates the standard error of the mean for the interaction effect (SEM = 45.8, LSD (5%) = 138). (b) Root DM of Caucasian clover in response to nitrogen source and lime rates (SEM = 57.5, LSD = 169)

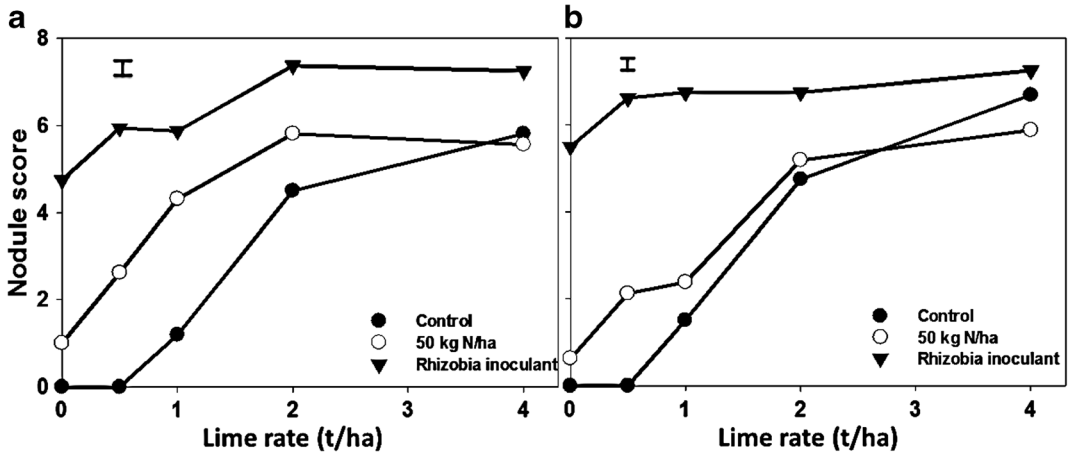

Fig. 9 Nodule score of lucerne (**a**) and Caucasian clover (**b**) after 228 days of growing in P.V.C tubes, in response to nitrogen source and lime rates. The error bar indicates the standard error of the mean for the interaction effect. For (a); SEM = 0.63, LSD (5%) = 1.78, and for (b); SEM = 0.51, LSD (5%) = 1.48

(N3) plants compared to ($P < 0.001$) un-nodulated plants. The mean ethylene level produced by legume nodules was 261.5 μL C_2H_4/L per hour (Fig. 11) and was not affected ($P = 0.15$) by the host plant.

Out of 60 nodules for each legume species, 54 isolates from lucerne and 42 isolates from Caucasian clover nodules had the morphological characteristic of rhizobia. The list of rhizobia genotypes recovered from lucerne and Caucasian clover nodules are given in Table 2. From the 60 selected nodules of Caucasian clover 42 rhizobia isolates were recovered from both bare and inoculated seed sown legumes. All of them were the same genotype of *R. leguminosarum bv. trifolii strain* ICC148 (AC). The 54 rhizobia isolates (27 from each bare and inoculated seed) recovered from 60 lucerne nodules consisted of nine different genotypes. The commercial strain RRI128 was present in 17 nodules selected from inoculated lucerne. This isolate was not present in any of the nodules on plants that had not been inoculated (Fig. 12).

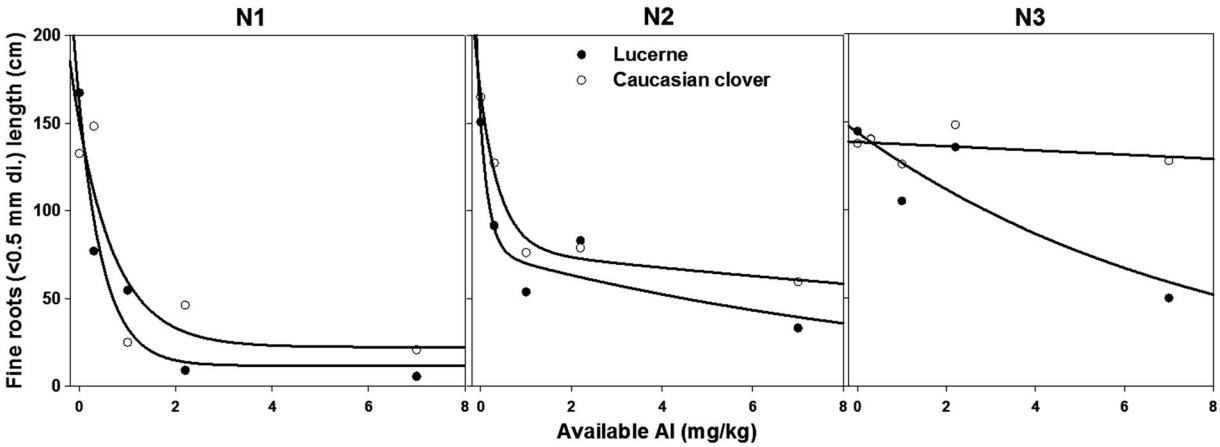

Fig. 10 Relationship between fine root length per plant of the sown legumes and the soil available Al (mg/kg). Lucerne and Caucasian clover plants were grown for 228 days in P.V.C tubes that contained the Lees Valley soil. N1, N2, and N3 = control, 50 kg N/ha and rhizobia inoculant, respectively. N1 (control): For lucerne; y = 11.5+ 149 × exp. (−1.94x), $R^2 = 0.95$, SEE (standard error of estimate) = 21. For Caucasian clover; y = 22.2+ 127 × exp. (−1.23x), $R^2 = 0.79$, SEE = 39. N2 (50 kg N/ha): For lucerne; y = 74.3 × exp. (−5.2x) + 76.5 × exp. (−0.1x), $R^2 = 0.91$, SEE = 27. For Caucasian clover; y = 88.6 × exp. (−2.3x) + 78.1 × exp. (−0.04x), $R^2 = 0.98$, SEE = 11.6. N3 (rhizobia inoculant): For lucerne; y = 71.2 × exp. (−0.13x) + 73 × exp. (−0.13x), $R^2 = 0.79$, SEE = 35. For Caucasian Clover; y = −138.6-3.6x, $R^2 = 0.14$, SEE = 10

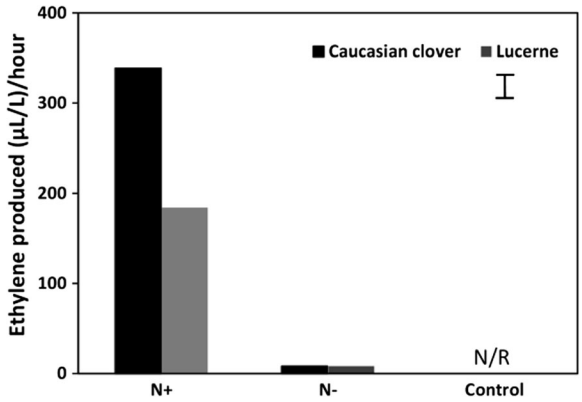

Fig. 11 Ethylene produced (µL C_2H_4/L) per hour by lucerne and Caucasian clover nodules recovered from the P.V.C tubes that contained soil from the Lees Valley. (N+; Nodulated root samples, N-; roots without nodules). The error bar indicates the standard error of the mean (SEM = 50, LSD = 160)

Discussion

The first three years of the field experiment indicated successful establishment and persistence of both legumes (Fig. 1) with the incorporation of 5 t/ha of lime and adequate P fertiliser to remediate soil nutrient levels. Lucerne yielded up to 4 t/ha over these first 3 years. In contrast, the establishment of Caucasian clover was slow, as expected (Scott 1998; Black et al. 2014) but by year 6 it was capable of producing 7.7 t DM/ha (Fig. 1). A visual observation of the site was also made on 5 July 2016, nine years after sowing. New leaves of Caucasian clover were abundant throughout the sown plots. Sampling indicated nodulated roots along with a large network of rhizomes. In contrast, there were only a few scattered lucerne plants in the sown plots and these had no nodules. The potential and therefore importance of Caucasian clover as a persistent and productive legume in these high Al soils was confirmed (Caradus et al. 2001). These field observations led to the pot experiment to try and isolate the cause of differences in the agronomic performance of these two species.

Seedling survival of lucerne was dependent on the N source and application of lime (Figs. 2 and 3). Seedling survival of 85% was achieved when as little as 0.5 t lime/ha was provided to plants inoculated with rhizobium. However, at the highest lime rate (4 t/ha) but the absence of inoculant, fewer than 75% of lucerne plants survived (Fig. 3a). This indicates that an ability to fix N was more important than the absolute soil pH for lucerne survival. Despite this seedling survival of lucerne was more responsive than Caucasian clover to the amount of lime applied (Fig. 2a). Caucasian clover seedling survival, in contrast, was unaffected by N source or P application. This result explains the initial difference between lucerne and Caucasian growth. It suggests N and P deficiencies impacted more on lucerne seedling survival compared with Caucasian clover. Therefore, in the field, the incorporated lime and P fertiliser can be expected to have initially benefitted lucerne more than Caucasian clover and therefore explains the initial high survival and productivity of lucerne.

Soil pH was significantly increased by lime addition (Fig. 5) as observed in the previous studies (Black and Cameron 1984; Wheeler and Edmeades 1995; Moir and Moot 2010) on similar soils. However, the magnitude of response in pH change per unit of added lime was higher

Table 2 Sequence similarity of 16S rRNA gene (Sequenced with primers F27, F485, and R 1494) of nine representative rhizobia genotypes recovered from lucerne and Caucasian clover nodules grown in P.V.C tubes. The sequences were compared with those of known origin using BLAST from the National Centre for Biotechnological Information (NCBI)

Lane no.	Isolate name	Genotype code	Species	Host plant	Accession No.	Coverage (%)	Identity (%)	Product size (bp)
2	RRI128	AL	*E. meliloti*	Lu	JX292367.1	100	100	1344
3	Lu 32	AL2	*E. meliloti*	Lu	CP004140.1	100	100	1361
4	Lu 50	AL5	*E. meliloti*	Lu	CP004140.1	100	99	1342
5	Lu 62	IL	*E. meliloti*	Lu	CP004140.1	100	100	1361
6	Lu 76	AL3	*E. meliloti*	Lu	CP004140.1	100	99	1344
7	Lu 77	CL	*E. meliloti*	Lu	CP004140.1	99	99	1342
8	Lu 66	BL	*E. meliloti*	Lu	CP004140.1	100	100	1343
9	Cc45	AC	*R. leguminosarum* bv. *Trifolii*	Cc	JF810503.1	100	100	1342
10	Lu 59	TL	*E. meliloti*	Lu	CP004140.1	100	100	1324

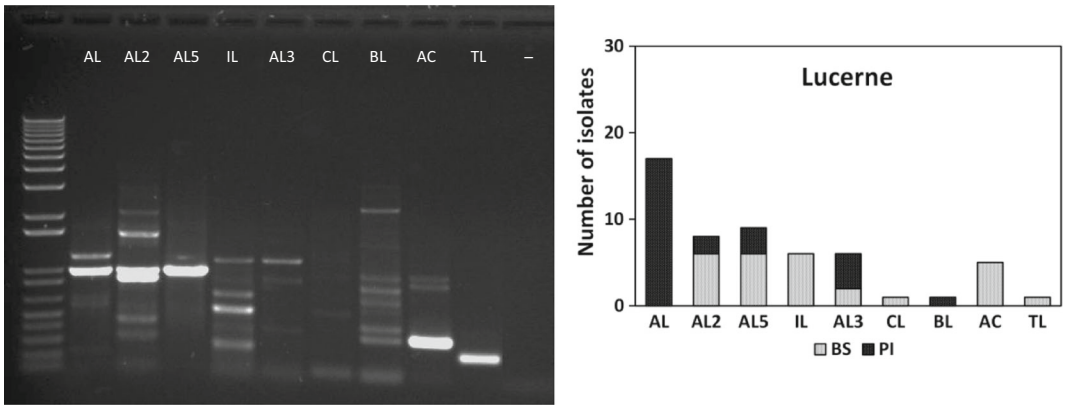

Fig. 12 Agarose gel (1%) of nine representative ERIC-PCR fingerprints from bacteria recovered from lucerne and Caucasian clover grown in P.V.C tubes that contained acidic soils with high Al. The code for each fingerprint and the negative control (−) is shown at the top of the gel. The first lane is 1 kb plus DNA ladder (Invitrogen, Australia). The frequency of isolates in bare seed (BS) or peat inoculated (PI) lucerne nodules are given in the histogram. Caucasian clover nodules only contained the commercial genotype

than expected, compared with previous reports from the same soil when agricultural lime was applied (Moir and Moot 2010). This higher response in pH change could be due to the evenness of lime-soil mixture in the tube experiment compared with similar rates of surface lime application in the field. The levels of plant available aluminium were strongly related ($R^2 = 0.67$) to soil pH (Fig. 4). A similar relationship between exchangeable soil aluminium and soil pH ($R^2 = 0.73$) was reported for the same soil by Moir and Moot (2010). This available Al plays an important role in increasing the soil acidity, by releasing further H^+ from water ($Al^{3+} + 3H_2O \rightarrow Al(OH)_3 + 3H^+$) to soil, and the magnitude of the pH decrease depends on the Al concentrations in the solution (Sparks 2003; Gardiner and Miller 2008). This means the Al^{3+} content was an important source of acidification in the Lees Valley soil solution. The aluminium content decreased to 0.3 mg/kg soil with the addition of 2 t/ha of lime (Fig. 4) and the soil pH increased to 6.1. At this soil pH, the lucerne growth was unlikely to have been affected by aluminium toxicity (Moir and Moot 2010).

Greatest improvements in plant growth in the tube experiment were due mostly to the ability of plants to fix nitrogen. Shoot and root growth of both species were higher ($P \leq 0.05$) when rhizobia inoculant was successful, compared with bare seed sown legumes or the 50 kg N/ha treatment (Figs. 6a, 7a, and 8). The dry matter production of inoculated lucerne was four times higher than those sown with bare seed (Fig. 6a). The nitrogen content of the original soil was low (113 kg/ha) and shoot DM response to 50 kg N/ha application, as a starter nitrogen treatment, was not significant (Fig. 6a). Therefore, it is important that lucerne is effectively nodualted when sown into soils comparable to that of the Lees Valley soil.

Nodulation of both legumes in the tube experiment were affected by soil Al content. No nodules were found when lucerne and Caucasian clover were grown in the original Lees Valley soil (control). When the inoculant was added (N3) both lucerne and Caucasian clover were nodulated and fixed nitrogen, as confirmed by ARA (Fig. 11), by their respective commercial strains, without any lime application. This indicates the survival and capability of both added commercial strains in the Less Valley soil. However, the nodulation score response to the lime application was different between lucerne and Caucasian clover when the inoculant was added. The nodulation score of lucerne was responsive to added lime up to 2 t/ha. In contrast, there was no effect of lime on the nodulation score of Caucasian clover. It should be noted that these results are from legumes grown for 228 days in tubes. In the field experiment with lime and inoculant application lucerne persisted during the first three years due to the top-soil pH amended by the surface lime application. Caucasian was established slowly and produced rhizomes as well as tap roots. However, when lucerne roots reached to the sub-soil layer that had low pH and high Al content both the nodulation score and fine root length would have been suppressed as shown in the tube experiment. Caucasian clover, in contrast, persisted in the field experiment as

the nodulation score and fine root length were not affected by soil Al content.

The root DM of lucerne was affected by the three-way interaction of lime, phosphorus and nitrogen treatments (Fig. 7a). The highest root DM was achieved by adding 2 t lime/ha + 250 mg P/kg soil + rhizobia inoculant (N3P2 in 2 t lime/ha). This indicates lucerne root ideally would grow in this soil if pH was increased to 6 and Al content decreased below 1 mg/kg soil. This suggests the Al toxic threshold of <1.0 mg/kg soil for lucerne nodulation and root growth. Still, the yield response to lime was greater than phosphorus in the aluminium-saturated soil of the Lees Valley. This result agrees with Mugwira and Haque (1993) who showed that in an acid soil with toxic aluminium levels, yield response to lime was greater than those for phosphorus. Consequently, the lime response for lucerne is most likely to have come through improved nodulation as found by Munns (1965). This suggests selection of rhizobia that are tolerant of higher levels of aluminium than the current commercial inoculant would aid lucerne establishment in similar soils.

There was no difference in the growth parameters of Caucasian clover in response to added P compared to no-phosphorus treatment (P1). This suggests lower phosphorus requirements for Caucasian clover than lucerne, or more importantly higher acid-tolerance of Caucasian clover's fine roots that could have resulted in an improved P uptake once roots penetrated below the soil surface compared with lucerne. This advantage for Caucasian clover may have contributed to its superior long-term persistence in the field conditions of the Lees Valley. The amount of P fertilizer applied to the Lees Valley field experiment should have addressed the P deficiency. However, lucerne was not able to utilise phosphorus due to the suppressed fine root growth in the sub-soil of the Lees Valley caused by high Al levels present in low pH soils.

The fine root growth of Caucasian clover was unaffected by Al when the rhizobia inoculant was added compared to N1. This suggests the nitrogen was available for Caucasian clover plants (as shown by nodulation assessment and ARA) and therefore, seedlings were able to extend their fine roots. The added mineral N at 50 kg/ha probably was not enough to address the plant's demand for nitrogen. However, if the higher level of nitrogen had been used then similar effect as the inoculant would be expected. For lucerne, in contrast, the nitrogen (via BNF) improved root growth when 0.5 t lime/ha plus 250 mg P/kg soil were added (Fig. 7a). This meant the soil pH of 5.7 (Fig. 5) and Al < 2 mg/kg soil (Fig. 7b). In this soil condition, the higher mineral N would have been effective. But in the original soil of the Lees Valley even if the mineral N was provided, lucerne could not be able to utilise it due to the suppressed root growth by high Al content of the soil (Fig. 10). The negative effect of Al on legume species root growth has been reported previously (Andrew et al. 1973; Kim et al. 1985; Brady et al. 1990; Hecht-Buchholz et al. 1990). Most of the nodules were found on fine roots of lucerne and Caucasian clover which agrees with previous reports (Viands et al. 1982; Gault et al. 1995; Vaughan et al. 2002). Therefore, selection for an increase in fibrous or lateral roots, and hence the number of sites for nodulation, is important to increase nodule mass, particularly for lucerne (Humphries and Auricht 2001) at low soil pH levels.

ERIC-PCR fingerprint analysis showed high specificity of Caucasian clover for rhizobia symbiont to nodulate the roots. Genotype AC (commercial strain ICC148) was the only *R. leguminosarum* genotype that recovered from Caucasian clover nodules. This indicates the lack of strain diversity for *R. leguminosarum* bv. *trifolii* in the Lees Valley soil. This agrees with Patrick and Lowther (1995) who reported the absence of indigenous rhizobia capable of forming effective nodules on Caucasian clover in New Zealand soils. Seguin et al. (2001) also reported the lack of genetic diversity of *R. leguminosarum* genotypes occupying Caucasian clover nodules among the North American isolates. Black et al. (2014), indicated the importance of delivery of suitable rhizobia inoculants to the seed for the establishment of Caucasian clover in high country grasslands in New Zealand. Inoculation is therefore essential for Caucasian clover because few soils outside its centre of origin in the Caucasus contain effective indigenous rhizobia (Elliot et al. 1998). Despite the lack of genetic diversity of *R. leguminosarum* bv. *trifolii* in the Lees Valley soil, our results confirmed the existence of strain ICC148 in the bare-seed sown Caucasian clover nodules. This suggests this strain can survive in the acidic high Al soil of the Lees Valley. Strain ICC148 was isolated in 1993 from a Caucasian clover nodule at the Mt. John Research Station, Tekapo (Scott and Mason 1992). Seeds were inoculated in 1975 (at sowing) and further rhizobia applied in 1983, but further detail is not available (Pryor et al. 1998). However, this background indicates the survival of strain ICC148 for

more than 10 years in the acidic high country soil of Mt. John Research Station.

The survival of rhizobia in the soil cannot be deduced from the results in this study. However, low nodulation of lucerne in the soil pH below 6 can be attributed to an inadequate number of rhizobium in the soil. Moreover, the difference in nodulation between plants treated by inoculant compared with control (no nitrogen) and the 50 kg N/ha application, is an indirect evidence of a suppressed number of rhizobia cells in the soil. This meant the low pH and Al toxicity associated with poor rhizobia survival in the original soil of the Lees Valley. As a consequence, the rhizobium community of the soil was insufficient for effective nodulation of sown legumes. *E. meliloti* is reported to be the most acid-sensitive species of rhizobia (Vincent 1981; Tiwari et al. 1992). Brockwell et al. (1991), reported that the number of *E. meliloti*/g of soil decreased sharply in soil with a pH below 6. Their results showed that the mean number of *E. meliloti* at 16 sites with soil pH > 7.0 was 89,000/g soil, while the mean number at 37 sites of pH ≤ 6 was only 37/g.

Eight naturalized strains of *E. meliloti* were identified from lucerne nodules. The identification of indigenous or naturalized rhizobium strains can lead to the selection for increased nitrogen fixation, competitive ability, or environmental stress tolerance (Thies et al. 1991, 1992). However, the competition between these rhizobia genotypes derived from this high frequency may negatively affect the efficiency of BNF in lucerne. Clear differences in nitrogen fixation efficiency (up to 10-fold of host legume growth) have been reported, with different rhizobium isolates (Burdon et al. 1999). Rhizobium strains of sub-optimal effectiveness have been isolated from fields worldwide and are often cited as a factor contributing to poor agronomic yields (Akçay and Roughgarden 2007; Denison and Kiers 2004; Kiers et al. 2007). Our previous study showed the replacement of the commercial inoculant RRI128 with other rhizobia strains when the soil pH was elevated by lime application (Berenji et al. 2014). Nodulation by mixed populations of rhizobia has been shown to limit symbiotic N_2 fixation (Pryor et al. 2004) via successful competition with the commercial inoculant for nodule sites (Meade et al. 1985). In cases where indigenous rhizobia capable of nodulating the host were already present in soil but not very efficient, the capacity of the inoculant to outcompete those strains is important (Cheng et al. 2004). Therefore, the lack of genetic diversity of *R. leguminosarum* bv. *trifolii* in New Zealand soils might be an advantage in the introduction of commercial strains particularly if an acid tolerant commercial strain could be developed.

Conclusion

This investigation has provided quantified evidence to explain production and persistence differences between lucerne and Caucasian clover in an acidic soil with high Al content. The main factor that limited lucerne was nitrogen deficiency due to the limited nodulation and reduction in fine roots, which was caused by Al toxicity. Caucasian clover, in contrast, showed Al tolerance for both nodulation and fine root growth which supports its use as a persistent legume option for low input farm systems with similar soil constraints.

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