Lucerne dry matter and N-fixation, when sown with or without lime and inoculant

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Abstract

This paper quantifies the effect of inoculation and lime on lucerne growth and nitrogen fixation over the establishment and following season. The field experiment at Ashley Dene, in Canterbury, had an initial soil pH of 5.2 and moderate exchangeable aluminium content (4.2 mg/kg). The ^{15}N natural abundance ($\delta^{15}N$) method was used to quantify nitrogen fixation. The pH of the top-soil was increased to ca. 5.5 and the Al content was decreased to 2.0 and 1.3 mg/kg soil by the application of 1 and 2 t lime/ha, respectively. In Year 0 the dry matter yield increased from 3 to 4 t DM/ha with the addition of 2 t lime/ha. The nitrogen content of lucerne plants was 2.2% (w/w), and was unaffected by inoculant or lime in Year 0. Similarly, the $\delta^{15}N$ was 0.81‰ and unaffected by inoculant or lime. In Year 1, the N percentage of inoculated lucerne plants was 4.2% (w/w) compared with 3.6% in uninoculated plants. The $\delta^{15}N$ value was 0.81‰ in uninoculated plants compared with -0.23‰ in inoculated lucerne plants. The inoculated lucerne yielded 7.8 t DM/ha compared with 2.4 t DM/ha when uninoculated. The calculated proportion of legume N derived from atmospheric N_2 (%Ndfa) estimated that 70% of the nitrogen content in the inoculated lucerne shoots was derived from nitrogen-fixation in Year 1. There was no effect of lime on lucerne DM, or $\delta^{15}N$ values in Year 1. This suggests lucerne rhizobia tolerated moderate levels of Al in acidic soils. The application of lime and inoculant are therefore recommended for lucerne, particularly in areas where there is no history of lucerne. The available soil N was sufficient to meet crop demand in the establishing year. Lucerne was then reliant on biological nitrogen fixation for yield in Year 1 which suggests lucerne preferentially used soil available N in Year 0, before commencing N fixation.

Keywords: Aluminium, lucerne, lime, *Medicago sativa* L., nitrogen fixation.

Introduction

The ability of legumes to fix atmospheric nitrogen (N) by a symbiotic relationship with rhizobia supports legume growth and improves the nitrogen status of many pasture soils (Peoples & Baldock 2001). Legumes' biological nitrogen fixation (BNF) is environmentally and economically important to pastoral farming in New Zealand. Certified lucerne inoculants have been available in New Zealand since 1955 (Wynn-Williams 1982), but recently the need to inoculate perennial legumes in New Zealand has been challenged (Lowther & Kerr 2011). At the same time, there has been a sharp increase in fertiliser N inputs to grazed pastures, and this increase is expected to continue (Moot *et al.* 2010; Saggar 2004). This means New Zealand's traditional reliance on BNF in preference to inorganic fertilizer N is being challenged.

Successful nodulation of agriculturally important legumes usually requires high quality inoculant application, particularly when introducing a legume to an area for the first time. Biological and edaphic constraints have been reported to decrease N fixation. These include rhizobia survival in the soils with high acidity and aluminium (Mullen *et al.* 2006), high soil N (Ledgard & Steele 1992), low available phosphorus (P), and drought conditions (Giller 2001). Therefore, strategies to improve N fixation include lime and P application for acidic soils, and addition of selected strains of rhizobia inoculants.

Previous studies in New Zealand estimated on average 45–142 kg N fixed/ha/year from white clover (*Trifolium repens*), and 41–106 kg N fixed/ha/year from subterranean clover (*Trifolium subterraneum*), (Edmeades & Goh 1978; Lucas *et al.* 2010). However, there are fewer reports on lucerne nitrogen fixation in New Zealand (Goh *et al.* 1996), particularly in acidic soils. In contrast, symbiotic N_2 fixation by lucerne has been quantified in several field experiments overseas which have reported 25–82% of nitrogen derived from the atmosphere (Ndfa) for lucerne shoots, equivalent to 13–284 kg N fixed/ha/year (Brockwell *et al.* 1995a, b; Gault *et al.* 1995; Heichel *et al.* 1984; Kelner *et al.* 1997; McCallum *et al.* 1999; Peoples *et al.* 2001).

The aim of this study was to quantify the efficiency of rhizobia inoculant in a low pH and moderate Al soil with low lime inputs at Ashley Dene in Canterbury. To do this, lucerne production, nodulation and N fixation were measured during establishment and the following year.

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Methods

Site Location and soil properties

The experimental site was at Ashley Dene (Canterbury plains 43˚39′,15″S, 172˚,19′31″E, 30 m a.s.l), Lincoln University's dryland research farm. The paddock H2 was selected because it had no history of lucerne cultivation recorded in the previous 40 years. The soil in this paddock is a Lismore silt loam (Typic Dystrustept, USDA Soil Taxonomy), classified as Typic Orthic Gley soil in the New Zealand Soil Classification system (Cox 1978; Webb 2003). Twenty soil samples from each soil depth were taken randomly from paddock H2, to 75, 150 and 300 mm depth in September 2011 prior to the start of the field experiment. Samples were bulked and subsamples sent for analysis. The soil pH was low (5.2) in the surface layer (0–75 mm), but increased slightly to 5.4 at 75–150 mm of depth, and then to 5.5 at 150–300 mm of soil depth. The aluminium content, in contrast, decreased from 4.2 in the 0–75 mm layer to 3.3 at 150–300 mm (Table 1). Anaerobic mineralisable N was 126 kg/ha prior to the start of the experiment in September 2011.

Rainfall (mm), air temperature (°C), and Penman potential evapotranspiration (PET) were recorded at Broadfields Meteorological Station (National Institute of Water and Atmosphere Research, New Zealand), from 1 July 2011 to 30 July 2013 (Table 2). The rainfall for the period of 1 July to end of Dec 2011 was 341 mm, which followed the long term means. The annual rainfall for 2012 was 604 mm which was 30 mm lower than the long term mean. Total monthly PET increased from 19 mm in June to reach a maximum of 134 mm in January, before decreasing again. In the first season (2011/2012), PET exceeded rainfall from November to May. In the second season (2012/2013) PET exceeded rainfall from September 2012 to April 2013 (Table 2).

Agronomic management, measurements and statistical analysis

Buster® herbicide at 5 litres/ha (200 g a.i./litre glufosinate ammonium) was applied on 21 September 2011 and again on 5 October 2011. The plots were then sprayed with Roundup Transorb® (250 g a.i./litre) at 2.0 litres/ha on 18 October 2011. Lime (standard AgLime) was surface applied at 0, 1 or 2 t/ha on 4 November 2011, 4 days before sowing. Commercial peat-based inoculant, *Ensifer meliloti* strain RRI128 was used to inoculate lucerne seeds, 6 hours before sowing, for "rhizobia inoculated" plots. Lucerne 'Force 4' was sown at 10 kg/ha (based on bare seed weight), on 8 November 2011 followed by Cambridge rolling. Plots were also heavy rolled with a Flexi-seeder roller 1 day after sowing. A split-plot within a randomised complete block (RCB) design was used. The main plots were ±rhizobia and sub-plots (2.1×10 m) were the lime rates, replicated three times.

Lucerne dry matter yield was based on destructive quadrat cuts. Two 0.2 m^2 quadrats per plot were cut at 20–30 mm above ground level, systematically placed to avoid re-cutting previously sampled areas. There were three cuts in Year 0, and four cuts in Year 1. Plots were mown after cutting on 30 April 2012, 30 August 2012, 26 October 2012, and 14 December 2012. Herbage residuals were then removed from plots after each mowing. Samples were dried in a forced air oven at 60°C to a constant weight. Ten lucerne plants were excavated randomly from each plot on 7 February 2012, 90 days after sowing. Nodulation was assessed by evaluating nodule colour, number, position and size according to a modified scale from Rice *et al.* (1977). The nodulation assessment was repeated on 21 December 2012 (Year 1).

The ^{15}N natural abundance ($\delta^{15}N$) technique was

Table 1 Soil properties of the paddock H2 at Ashley Dene, Canterbury in September 2011, prior to the start of the field experiment.

Soil properties	Soil depth (mm)			Optimum level**
	0-75	75-150	150-300	
pH_{water}	5.2	5.4	5.5	5.8-6.3
Olsen phosphorus (mg/litre)	34	30	11	20-30
Sulphate sulphur (mg/kg)	19	8	4	7-15
Potassium (me/100g)	0.2	0.3	0.3	0.5-0.7
Calcium (me/100g)	7.8	6.9	5.9	6.0-12.0
Magnesium (me/100g)	1.4	1.3	1.1	1.0-3.0
Sodium (me/100g)	0.2	0.2	0.3	0.2-0.4
CEC (me/100g)	18	18	14	12-25
Total base saturation (%)	53	50	53	55-75
Aluminium*(mg/kg)	4.2	3.3	3.3	0.0

*$CaCl_2$ extractable. **Optimum range for legumes.

used to provide estimates of the proportion of legume N derived from atmospheric N_2 (%Ndfa) by comparing the $\delta^{15}N$ (‰) of inoculated lucerne shoots to the $\delta^{15}N$ of non-N_2-fixing, uninoculated lucerne reference plants grown at the same site (Unkovich *et al*. 2008). The $\delta^{15}N$ of a legume would be expected to be similar to that of atmospheric N_2 (0‰) if that legume plant was totally dependent on symbiotic N_2 fixation. However, shoots usually have the $\delta^{15}N$ of less than zero. Conversely, if the legume was totally reliant on soil mineral N, its $\delta^{15}N$ should be similar to that of the soil mineral N taken up (Unkovich *et al.* 2008). The criteria for the harvest date selection, to assess the $\delta^{15}N$ of lucerne samples, were based on lucerne dry matter results. Therefore, two harvest dates were chosen in Year 0 (8 March 2012 and 30 April 2012) when lucerne DM was not affected by rhizobia inoculant, and one harvest date in Year 1 (24 October 2012) when DM was affected by inoculant. Samples from each plot from the selected harvest dates were ground and thoroughly mixed. Subsamples were then finely ground again. The $\delta^{15}N$ (‰) and total N percentage (w/w) of lucerne samples were measured by mass spectrometry at Lincoln University. The percentage of N derived from the atmosphere (%Ndfa) was then calculated according to Equation 1 (Unkovich *et al.* 2008).

$$\%Ndfa = \frac{\delta^{15}N \text{ of reference plant} - \delta^{15}N \text{ of } N_2 \text{ fixing legume}}{\delta^{15}N \text{ of reference plant} - B} \times \frac{100}{1} \quad (1)$$

Where B value is the $\delta^{15}N$ of lucerne shoots that are fully dependent on N_2 fixation. The average B value of -0.68 was used according to Unkovich *et al.* (2008). Lucerne dry matter yields (t/ha) on the same day of sampling for N fixation measurements were used to quantify and express nitrogen fixation as kg Ndfa/ha. Amounts of N_2 fixed were calculated from %Ndfa estimates (Equation 2). The amount of lucerne N was calculated based on dry matter and tissue N content (Equation 3).

Amount of N fixed (kg N/ha) = lucerne N (kg N/ha) x (%Ndfa/100) (2)

Lucerne N (kg N/ha) = lucerne DM (kg/ha) x (%N/100) (3)

Ten nodules were selected per lime treatment (0, 1 or 2 t lime/ha) from lucerne excavated from inoculated plots in Nov 2012 to confirm the existence of the applied rhizobia strain. Bacteria were isolated on YMA media (Vincent 1970), followed by DNA extraction using PUREGENE™ (Gentra Systems, USA) DNA extraction kit. The strains were genotyped using 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 ca. 1300 bp fragment of the 16S rRNA was amplified for six representative genotypes. Sequences were compared with those of known origin using BLAST from the National Centre for Biotechnological Information (NCBI).

Lucerne shoot DM, number of nodules and nodule score, shoot N percentage and $\delta^{15}N$ response to rhizobia inoculant, and lime rates were analysed by split-plot ANOVA using Genstat 14 software. The mean values were compared using Fisher's protected LSD (5%).

Table 2 Monthly rainfall (mm), and Penman potential evapotranspiration (PET) for Ashley Dene in Canterbury, New Zealand from 1 July 2011 to 30 July 2013. Data were recorded at Broadfields Meteorological Station (National Institute of Water and Atmosphere Research, New Zealand, http://cliflo.niwa.co.nz/).

Year	Month	T_{max} (°C)	T_{min} (°C)	T_{mean} (°C)	Rainfall (mm)	PET (mm)
2011	Jul	11.4	0.9	6.2	15.8	27.5
	Aug	12.8	1.5	7.2	66.2	33.9
	Sep	14.8	3.5	9.2	25.7	57.8
	Oct	15.6	7.0	11.3	105	80.1
	Nov	18.7	7.7	13.2	54.3	121
	Dec	20.0	11.0	15.5	74.1	123
2012	Jan	21.6	10.2	15.9	36.2	134
	Feb	19.8	11.3	15.6	74.0	90.2
	Mar	18.9	8.2	13.6	37.0	77.6
	Apr	18.5	6.5	12.5	34.1	47.3
	May	14.4	3.8	9.1	8.4	28.5
	Jun	10.6	1.0	5.8	61.5	19.5
	Jul	11.8	1.6	6.7	48.0	18.9
	Aug	12.5	5.2	8.9	110	30.0
	Sep	15.5	5.0	10.3	26.5	67.0
	Oct	17.1	5.8	11.5	74.4	96.3
	Nov	16.8	6.7	11.8	60.5	105
	Dec	22.4	11.3	16.9	34.3	146
2013	Jan	23.9	11.3	17.6	39.6	165
	Feb	22.4	10.9	16.7	22.8	119
	Mar	21.5	11.0	16.3	36.3	81.5
	Apr	17.4	7.8	12.6	63.6	42.7
	May	14.0	5.2	9.6	95.2	26.6
	Jun	10.8	2.6	6.7	222	13.6
	Jul	13.5	2.8	8.2	38.0	23.0

Results

The soil pH and Al in response to surface lime application

Lime application increased ($P<0.01$) the pH of the top-soil (0–75 mm), from 5.2 in the control to 5.5 and

5.6 in the 1 and 2 t lime/ha treatments respectively, 5 months after application. The Al content of the top-soil (0–75mm) decreased (P<0.001) from 4.2 mg/kg in the control to 2.0 and 1.3 mg/kg in the 1 and 2 t lime/ha treatments, respectively. Neither the soil pH nor the Al content at 150–300 mm were affected by liming (P=0.34 and 0.58 respectively). Anaerobic mineralisable N was 187 kg/ha at the end of this field experiment in June 2013.

Lucerne dry matter (DM) in the establishment and following year

Lucerne yield (t DM/ha/year) was affected (P<0.05) by lime rates but unaffected (P=0.11) by rhizobia treatment in Year 0. Yield increased (P<0.05) from 3 to 3.5 t DM/ha with 1 t/ha lime and to 4 t DM/ha with 2 t/ha lime applied. In contrast, yield was unaffected (P=0.10) by lime rate in Year 1, but increased (P<0.05) from 2.4 to 7.8 t DM/ha with rhizobia inoculation (Figure 1). Yield in the inoculated plots exceeded the bare seed sown plots from September 2012 and this difference remained constant until the end of growth season in June 2013.

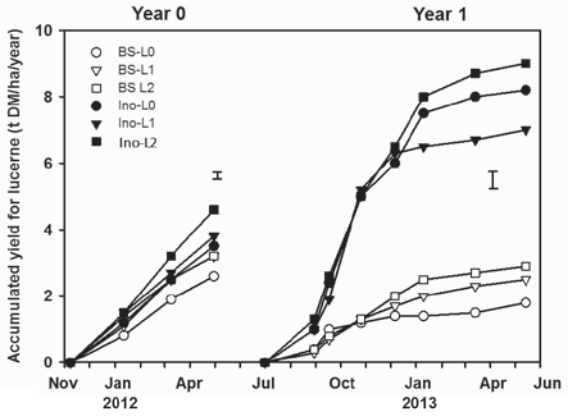

Figure 1 Accumulated dry matter yield (t DM/ha/year) for lucerne in response to lime rates (0, 1, and 2 t/ha) and rhizobia inoculant in Year 0 and Year 1. The error bars indicate the maximum standard error of means for lime treatment in Year 0 (SEM_{lime} = 0.14, LSD_{lime}= 0.45), and for rhizobia treatment in year 1 ($SEM_{inoculant}$ = 0.64, $LSD_{inoculant}$= 1.7). BS= bare seed sown, Ino= inoculated with rhizobia, L= lime rates (t/ha).

Nodulation assessment

In Year 0, 85% of inoculated seedlings were nodulated 90 days after sowing but no nodules (P≤0.001) were found on the bare seed lucerne roots. In Year 1, the inoculated lucerne roots were 100% nodulated, with 20–25 nodules per plant. The nodule score was 7.9 out of 10, and unaffected (P=0.37) by lime treatment. No nodules (P≤0.001) were found on the bare seed lucerne roots.

N percentage and $\delta^{15}N$ values of lucerne plants

In Year 0 (2011/2012) the N percentage and $\delta^{15}N$ values of lucerne plants were unaffected by inoculant or lime application. Four months after sowing (8 March 2012), the nitrogen content of lucerne plants was 2.5% (w/w), and unaffected by inoculant (P=0.22) or lime (P=0.32). Similarly, the $\delta^{15}N$ was 1.3‰ and unaffected by inoculant (P=0.92) or lime (P=0.38). Six months after sowing (30 April 2012), the nitrogen content of lucerne plants was 2.2% (w/w), and unaffected by inoculant (P=0.21) or lime (P=0.67). Similarly, the $\delta^{15}N$ was 0.81‰ and unaffected by inoculant (P=0.23) or lime (P=0.91). In Year 1 (2012/2013), the nitrogen content of inoculated plants was 4.2% (w/w) compared with 3.6% (P<0.05) in uninoculated lucerne plants. The $\delta^{15}N$ value was 0.81‰ in uninoculated plants compared with -0.23‰ (P<0.05) in inoculated lucerne plants. There was a trend (P=0.07) of increased herbage N percentage with lime from 3.7% in the control to 4.1% (w/w) with 1 t lime/ha applied. In contrast, there was no effect of lime on $\delta^{15}N$ values (P=0.18).

Estimation of N_2 fixation rates of lucerne plants in Year 1

The calculated %Ndfa estimated that 70% of the nitrogen content in inoculated lucerne shoots was derived from nitrogen fixation in Year 1. The inoculated lucerne plants harvested on 24 October 2012, yielded 4.14 t DM/ha compared with 1.31 t DM/ha in bare seed sown plants. This suggests 122 kg N/ha from inoculated lucerne was attributed to biological nitrogen fixation within 4 months, from the first of July to the end of October 2012 in Year 1. Molecular studies on the isolated bacteria from lucerne nodules confirmed the existence of the applied inoculant rhizobia (*Ensifer meliloti* strain RRI128) in 80% of the nodules (24/30 nodules).

Discussion

Rhizobia inoculation yielded a three-fold increase in lucerne dry matter at Ashley Dene in the second growing season compared with the bare seed sown crops. Data from this study demonstrated the advantage of inoculation to enable BNF for lucerne in these low pH and moderate aluminium soil conditions at Ashley Dene. This supports previous work (Black & Moot 2013) that showed a need for inoculation of lucerne in soils with no previous history of lucerne growth. In this study, no nodules were found on the roots of bare seed sown lucerne in either year. In contrast, the roots of inoculated lucerne were 100% nodulated, with 20–25 nodules per plant. The molecular study confirmed the existence of the applied inoculant. The

rhizobia capable of effective symbiosis with lucerne did not exist in New Zealand soils prior to European settlement. They have been introduced to the soil in many regions, but whether adequate numbers of rhizobia are sustained in the soil in the years following inoculation is questionable, especially in a hostile soil environment, for example low pH and high Al levels. The high responses to inoculation (Figure 1) are most likely to be observed when soil nitrate is low and when resident rhizobia are absent (Brockwell *et al.* 1995a). It should be noted that the paddock used in this experiment had no history of lucerne for the past 40 years. However, 85% of inoculated seedlings were nodulated 90 days after sowing. Despite this, the %Ndfa was not detectible within the first year of establishment. This suggested lucerne had formed nodules but the nitrate available in the soil was used in preference to the energy-consuming process of BNF. The 2.4% N (w/w) of lucerne dry matter in the first six months of establishment at Ashley Dene meant ca. 96 kg N/ha was utilised from soil. This indicated the measured soil N of 126 kg/ha of mineralisable N was adequate for lucerne growth in Year 0, and the plants preferentially used that soil N ahead of Ndfa.

The amount of N_2 fixation measured in this study (122 kg N/ha) was based on lucerne standing dry matter at the time of sampling (October 2012). However, it assumed total N fixed in shoots would be roughly 200 kg N/ha for the total annual yield of lucerne in Year 1 (7.8 t/ha), or ca. 25 kg N/t above-ground dry matter. This excludes root accumulated N that can account for up to 50% of total legume N (Unkovich *et al.* 2010). The lucerne dependence on N_2 fixation was calculated as 70% for Year 1. This %Ndfa was comparable to 65% reported by Yang *et al.* (2011) for irrigated lucerne. The other 30% of lucerne N from soil indicated lucerne also utilised ca. 60 kg N/ha from soil. Anaerobic mineralisable N was 187 kg/ha at the end of this field experiment in June 2013. This N is unlikely to cause a leaching problem, because lucerne has been shown to be effective in scavenging soil nitrate from depth (Russelle *et al.* 2001). Goh *et al.* (1996) reported substantial amounts of BNF (71 to 230 kg N/ha.year) with lucerne. They calculated lucerne derived 72% of its N from the atmosphere during the spring/summer period compared with 83–97% with clovers in New Zealand. They concluded the net N demand from the soil was higher for lucerne than the clover species. Their results indicate a low risk of N leaching and support the preferential uptake of soil mineral N by lucerne in this study. The perennial nature of lucerne growth usually ensures that most of the possible mineralised N is re-assimilated by the lucerne itself. As a consequence, the concentrations of soil mineral N remain low (Dear *et al.* 1999).

Lime application improved the establishment of lucerne at Ashley Dene. The lucerne DM increased ($P<0.05$) from 3 to 3.5 t DM/ha with the 1 t/ha lime, and to 4 t DM/ha with the 2 t/ha lime in Year 0. This was probably due to the soil pH increase, and decrease in Al content of the top soil. The strong relationships between soil pH and exchangeable plant-available Al has been reported in previous studies (Moir & Moot 2010, 2014). As a consequence lucerne seedlings could establish faster and yield more than the control (Figure 1). The release of phytotoxic Al in acidic soils is responsible for significant losses of lucerne production. Therefore, application of lime is required to obtain successful establishment and persistence of lucerne (Edmeades *et al.* 1983; Mullen *et al.* 2006). As the subsoil (150–300 mm) pH was higher than the top soil at Ashley Dene (Table 1), when the lucerne roots reached this deeper horizon in the following season, there was less growth limitation by low pH and high Al compared with the establishment season. Therefore, the lime application appears to have been less effective in Year 1 than in Year 0.

Conclusions

Inputs of lime (2 t/ha) and inoculation with rhizobia improved %Ndfa and establishment of lucerne in this soil where the initial pH was 5.2 and Al ca. 4 mg/kg soil. These strategies ensured an adequate number of effective rhizobia to nodulate lucerne, and increased lucerne DM production.

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