Changes in rhizobia population over time in inoculated and uninoculated lucerne plants

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Abstract
Successful establishment of lucerne is dependent on the formation of effective nodules by the bacterium *Sinorhizobium meliloti*. Recently there has been some debate on whether addition of commercial inoculants is needed if the site has previously been sown in lucerne. The genotype of bacteria in the nodules of lucerne plants inoculated with the commercial inoculant and an uninoculated bare seed control was investigated over a three year period. In 2011, two months after sowing and again in 2014, three years after sowing, bacteria were recovered from the nodules of lucerne plants grown at a site with a previous lucerne history. In 2011, the 194 isolates collected across the inoculated and uninoculated treatments, produced 17 unique genotypes using ERIC-PCR. Genotypes A (n=37; 19%) and B (n=47; 24%) were the most common. Genotype B was the inoculant strain *S. meliloti* RRI128 and DNA sequencing identified Genotype A as *Rhizobium sp*. Plants which had not received the commercial inoculant were also successfully nodulated. Genotype A was most common isolate in the nodules from these plants (n=14; 28%). In 2014, the 180 isolates collected produced 35 unique genotypes. Genotype B was the most common (n=63; 35%). An isolate not recovered in 2011, genotype 1M, was the second most common (n=14; 8%). Genotype 1M was also the most common isolate in the nodules of the uninoculated plants (n=11; 18%). Genotype A was not found. This paper reports on the survival of the commercial inoculant over time and changes in the naturalised rhizobia occupying the nodules of inoculated and uninoculated plants. Greater understanding of factors that influence nodule occupancy should aid selection of persistent strains.

Key words
Alfalfa, ALOSCA®, bare seed, coated seed, peat seed

Introduction

Nitrogen fixation occurs when legumes, such as lucerne, are nodulated by effective strains of rhizobia. In New Zealand this is ensured by applying commercially available inoculants to the seed. The use of commercial inoculant can increase dry matter (DM). Thus, historically there has been little debate on the need for lucerne inoculants on most of agricultural soils worldwide.

Lucerne has been grown in New Zealand pastures for over 80 years. The widespread practice of commercial inoculation means that, paddocks that were historically sown in lucerne may have been inoculated with commercial preparations of the lucerne specific rhizobia *Sinorhizobium meliloti.* Recent studies have demonstrated successful establishment of lucerne crops in New Zealand without inoculation (Wigley 2011; Khumalo 2012). However, no differences in DM production was found between inoculated and uninoculated lucerne. Effective nodules containing *Rhizobium* sp. were formed on the roots of uninoculated plants (Khumalo 2012; Wigley 2011). This work suggests soils into which lucerne is sown frequently contain adequate levels of effective rhizobia capable of nodulating lucerne. Jensen (1941) also reported that rhizobia are able to survive in the soil for 40 years providing temperature and pH are optimal. However, this has yet to be extensively studied. This paper reports on the survival of the commercial inoculant over time and investigates the change in naturalised rhizobia occupying the nodules of inoculated and uninoculated plants.

Materials and Methods

Experimental site

The experiment was located at the Lincoln University Field Research Centre (43°38□S and 172°28□E) on a Wakanui silt loam (*Udic Ustochrept*, USDA Soil Taxonomy) soil with a pH (H_20) of 6.0. The site had previously been grown Lucerne continuously from 2004 – 2007. Prior to the sowing of this experiment the site was in brassicas followed by short rotation annual ryegrass. No fertilizer was applied during the experiment

and plots were grazed as required. The experiment was a randomised complete block design. Plants were excavated from plots sown on the 4th November 2010. Three inoculation treatments (peat, coated and ALOSCA®) plus the bare seed control were the subplots.

Inoculation and sowing
'Stamina 5' lucerne was used for all treatments at a standard bare seed rate of 10.5 kg/ha which equated to 16 kg/ha of coated seed. All inoculant was freshly delivered and applied to reflect standard commercial practice. Peat slurry was prepared as recommended by the manufacturer. The peat inoculant was mixed with water. Immediately prior to sowing, the seed was then coated in the slurry. At sowing, ALOSCA® granules were mixed in the drill with bare seed at the recommended rate of 10.5 kg/ha. The commercially sourced coated seed reportedly contained the inoculant, a contact fungicide against *Pythium* spp., molybdenum and lime. It was stated that all inoculant treatments (peat, coated and ALOSCA®) contained *S. meliloti* strain RRI128. 14 row plots of 4.2 x 7 m with 0.5 m gaps between plots were sown with an Øyjoord cone seeder. The drill hoppers were pressure cleaned with air after each seed treatment and sown in the order of bare seed followed by ALOSCA® mix, coated seed and peat slurry mix.

Nodule collection
In January 2011, two months after sowing, 20 plants were randomly selected from duplicate plots for each of the four inoculation treatments. This was repeated three years after sowing (January 2014) for each inoculation treatment except ALOSCA®, except this time only 6-7 plants were randomly selected as the root systems were substantially bigger and more nodules were present per plant. The number of plants selected was determined by the number of nodules on each tap root. Plants from the edges of the plots were avoided. From each plant, 1 – 10 pink nodules were selected either directly from or within 10 – 20 mm of the main tap root, and were preferentially selected from the upper 10 cm of the root system. In 2011, the number of nodules per plant was often fewer than five whereas in 2014 it was often more than five. Nodules were taken from the peripheral root or lower down the root when no nodules were present on or close to the tap root. A maximum of 10 nodules per plant were collected and at least 50 nodules per inoculation treatment were plated.

Rhizobia were recovered from the nodules and DNA extraction and ERIC PCR of bacterial DNA was carried out as described in Wigley et al. (2015).

Amplification of 16S ribosomal DNA for isolate identification
The 16S rRNA gene of the most common genotypes at each site were amplified for DNA sequencing using primers F27 (5'-AGAGTTTGATC(A/C)TGGCTCAG-3') and R1494 (5' CTACGG(T/C)TACCT TGTTACGAC-3') (Weisburg *et al.* 1991) The PCR products were sequenced at the Lincoln University Sequencing Facility. The sequences obtained were viewed using Chromas Lite 2.1 (Technelysium Pty Ltd, Australia) and manually trimmed using DNAMAN 4.0 (Lynnon Biosoft, Canada) to remove ambiguous sequence. The sequences were then compared with those of known origin on the nucleotide database GenBank (www.ncbi.nlm.nih.gov/genbank/) using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1990).

Assessment of symbiotic potential
Seeds were surface sterilised by soaking them in 15% commercial bleach (0.25 g/L sodium hypochlorite; 10 min) and rinsed with sterile water. Seeds were then planted in 40 mL of vermiculite and McKnights nutrient solution. Plants were grown in a growth chamber with a 16 h photoperiod at a constant 22°C. 7 days after sowing, seedlings were inoculated by adding 51 mL of one of the selected strains (approximately 1 x 10^6 cfu) in 0.85% saline onto the plant. Uninoculated plants were supplied with saline only and used as controls. There were 10 replicate pottles per treatment. After 49 days dry matter production was be measured and effective nodules were counted.

Statistical analysis
Variables were analysed using Pearson's Chi-square test of independence at $\alpha = 0.05$ to determine any differences between the frequencies of each genotype found in each treatment. The symbiotic potential experiment was analysed using Genstat 16th edition (VSN International). A one way ANOVA was carried

out and Fisher's protected least significant difference (LSD) test was used to separate means for each factor when ANOVA gave a P value < 0.05.

Results and Discussion

In 2011, 194 strains of bacteria were recovered from the nodules of field grown plants, approximately 50 nodules from each treatment. The 194 isolates produced 17 unique genotypes using ERIC-PCR. Genotypes A (n=37; 19%) and B (n=47; 24%) were the most common. Genotypes F (n=23; 11%), G (n=20; 10%) and C (n=14; 7%) were also commonly found. In 2014, 180 strains of bacteria were recovered from the nodules of field grown plants. Of these 61, 59 and 60 were from the bare, lime and peat treated seed, respectively. The 180 isolates produced 35 unique genotypes using ERIC-PCR. Genotypes B (n=63; 35%) and 1M (n=14; 8%) were the most common. Genotype C (n=12; 7%) was also commonly found. All other genotypes occurred in < 10 nodules in total across all treatments. Genotypes A, F and G were not found in any of the nodules in 2014. This work demonstrated that in 2011 and 2014 lucerne nodules were occupied by a wide range of genotypically distinct bacteria even when inoculated with commercial preparations of *S. meliloti* RRI128 and the dominant genotypes changed over time. Jensen (1941) reported that rhizobia are able to survive in the soil for 40 years providing temperature and pH are optimal. It is likely that rhizobia was present in the soil and able to nodulate lucerne as the current experimental site had previously been sown in lucerne. It has also been found that worldwide, plants such as lucerne are nodulated by many strains and species of rhizobia (Burton 1972). However, some of these may be less effective at nitrogen fixation than the commercial inoculants which have been chosen for their ability to fix atmospheric nitrogen at high rates.

Wigley et al. (2015) showed that genotype B is identical to that of the commercial strain RRI128 obtained from the Australian Inoculant Research Group (AIRG). DNA sequencing of one strain from each inoculant produced 850 bp of sequence that had 100% coverage and was identical to strains of *S. meliloti*.

Seed treatment affected (P<0.001) the population of strains recovered (Table 1). Most of this variation was attributable to differences in genotypes A and B between inoculation treatments. In 2011, Genotype B was dominant and recovered from 40% of the nodules plants grown from coated seed (n=20). The lime, molybdenum and fungicide added to the seed coat may have produced favourable conditions for survival of the rhizobia and thus successful nodulation (Lowther and Kerr 2011). In 2014, genotype B was also most common in the nodules from peat and coated seed plants at 64% (n=34) and 48% (n=29). Genotype B occupancy of nodules had increased in both the peat (22%; n=11 vs. 64%; n=34) and coated (40%; n=20 vs. 48%; n=29) seed treatments from 2011 to 2014. In this study only 16% of nodules from plants inoculated with ALOSCA® contained *S. meliloti* strain RRI128 in 2011. Due to the low numbers of the commercial inoculant and genotype A in the nodules of the ALOSCA® plants this treatment was not sampled again in 2014.

Table 1. Frequency/count of the five most common genotypes observed in isolates recovered from the nodules of lucerne plants treated with ALOSCA®, lime coat, peat inoculant, or left as a bare seed control in soils from Lincoln University in 2011.

		Treatment[1]			
		Bare Seed	ALOSCA®	Coated Seed	Peat Seed
2011					
Genotype	**Species**				
A	*Rhizobium sp.*	14	8	0	15
B	*S. meliloti*	8	8	20	11
C	*Rhizobium sp.*	4	7	3	0
F	*Variovorax sp.*	5	7	3	8
G	*Rhizobium sp.*	6	7	7	0
2014					
B	*S. meliloti*	0		29	34
1M	*S. meliloti*	11		3	0
C	*Rhizobium sp.*	9		0	3

[1] Significant at P ≤ 0.001 using Pearson Chi – square test.

Naturalised rhizobia were found in the nodules of plants grown from uninoculated seed (bare seed control). *Rhizobium* sp. (genotype A) was the most common and found in 28% of nodules from plants grown from bare seed at Lincoln University in 2011. Burton (1972) stated that inoculation is essential for lucerne establishment and growth worldwide. In contrast, the results of this study suggested that an inoculant treatment is not always necessary for nodulation. In 2014, a new genotype, 1M was the most common isolated from bare seed plants with 19% (n=11) of nodules containing this genotype. Genotype A was not found. The driver for the change in dominant genotype from genotype A to genotype 1M is unknown but it could be due to the change in land use. Prior to sowing and the first sampling in 2011 the site had been in brassicas in 2008 followed by short – rotation ryegrass from 2009 – 2010. At the time of the second sampling the lucerne had been established at the site for 3 years. This is likely to have influenced the naturalised rhizobia population but more research is required to confirm this.

DNA sequencing of the 16S rRNA gene of the most common genotypes (A, 1M and B) identified them as *Rhizobium* sp. (genotype A) and *S. meliloti* (genotype 1M and B). Genotype C and G were also identified as *Rhizobium* sp. and genotype F was identified as *Variovorax* sp.

Symbiotic potential of the naturalised strains was measured. Shoot dry weight of genotype 1M was not different from shoot dry weight of the commercial strain for lucerne with an average shoot dry weight of 0.0321 g/plant and 0.0413 g/plant. These two strains produced more ($P < 0.001$) shoot dry matter then genotype A (0.0112 g/plant) and the minimal N control (0.0057 g/plant). Plants inoculated with genotype A and genotype 1M all had effective nodules.

Conclusion

The commercial inoculant was dominant in the nodules of lucerne plants grown from peat and coated seed three years after sowing. Plants from all seed treatments, including uninoculated plants were nodulated by naturalised strains of rhizobia. The dominant strain in the nodules of the bare seed plants changed over time from genotype A to genotype 1M. This is possibly due to a change in land use. Genotype 1M produced more dry matter than genotype A and both genotypes formed functional nodules. The effectiveness of these dominant naturalised strains to fix N requires further research.

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