Increasing biological nitrogen fixation by white clover–rhizobia symbiosis

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Abstract
Biological nitrogen fixation (BNF) is the process of converting atmospheric nitrogen to ammonia through legume–rhizobia symbiosis. The nitrogen fixed by rhizobia in root nodules is available for plant use. This process can be harnessed to improve N fertility on farm. Field surveys across New Zealand (NZ), within a farm and within paddocks, have revealed large spatial variability of rhizobial population size and symbiotic effectiveness with white clover. These results indicate that naturalised rhizobia may not be supporting optimal BNF. Over 500 strains of clover-nodulating rhizobia were isolated from NZ pasture soils, with more than 90 demonstrating greater N-fixation capacity with white clover than the commercial inoculant strain TA1. Seven NZ isolates were tested for nodule occupancy and all seven had significantly higher occupancy rates than TA1 in an *in vitro* assay, indicating increased competitiveness of those strains. In addition, novel seed-coating technology improved the survival of TA1 and isolate S10N9 from 1 month to more than 4 months compared with a standard coating formulation. There is potential to increase the symbiotic capacity of white clover in pastures through use of more effective and competitive rhizobial strains, along with their improved survival on seed provided by a new coating technology.

Keywords: Spatial variability, symbiotic effectiveness, competitiveness, seed coating, delivery efficiency

Introduction
White clover has historically been a significant component of New Zealand (NZ) pastures, improving feed quality and enhancing soil fertility by providing biologically fixed nitrogen (N) through its symbiotic partnership with rhizobia bacteria (Caradus et al. 1996). As rhizobia capable of nodulating white clover roots (*Rhizobium leguminosarum* bv. *trifolii* (*Rlt*)) were not native to NZ (Greenwood 1965), inoculation of white clover seeds became standard practice from the 1960s. Only a few rhizobial strains have been used as commercial inoculants in NZ. These were mostly sourced from Australia, with the current strain TA1 isolated from annual clover in Tasmania in the 1950s (Lowther & Kerr 2011). Following decades of seed inoculation, *Rlt* populations are widespread in NZ's pasture soils, leading to the assumption that routine inoculation of white clover is now generally of little benefit, except under certain conditions, such as after >10 years maize cropping (Lowther & Kerr 2011).

Recent work has shown that soil populations of *Rlt* are indeed widespread in pasture soils but their abundance and N-fixation capacity (effectiveness) varies widely. In a broad survey across 26 sites, their abundance ranged from <100 to 10^8 rhizobia per g soil (Wakelin et al. 2018). Perhaps more importantly, the N-fixation ability of the soil rhizobial populations varied from 14 to 143% compared to the commercially available strain TA1. The study found no significant correlation between population size and N-fixation ability of the rhizobia, but significant spatial variability in the effectiveness of soil rhizobia within farms and within paddocks (e.g. 4–135% of TA1 across 0.8 ha). This localised patchiness in population size and effectiveness suggests that naturalised rhizobia are unlikely to support optimal performance of clover symbiosis at field-level.

Another key challenge to improving the symbiotic performance of white clover is the delivery of effective rhizobia that can achieve a high level of nodule occupancy by out-competing rhizobia already present in soil (Lowther & Kerr 2011). We report progress on improving the performance of the white clover–rhizobia symbiosis by: (1) seeking elite rhizobial strains that have greater symbiotic effectiveness with modern white clover cultivars; (2) determining if these elite strains can outcompete naturalised rhizobia already in the soil; and (3) improving seed coating technology to deliver a high loading of viable rhizobia on farm.

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Materials and Methods

Isolation of rhizobia from NZ soils

Soil samples were collected from 26 pastures across NZ as described in detail by Wakelin et al. (2018). Over 500 isolates of *Rlt* were sourced from the nodules of white clover (*Trifolium repens* L. cv. Tribute) plants grown in sterile vermiculite mixed with soil samples as described earlier (van Ham et al. 2016).

Symbiotic effectiveness of rhizobial isolates

Symbiotic effectiveness of the rhizobial isolates was determined by measuring the growth of *T. repens* cv. Tribute inoculated with an isolate and grown in a 70-mL plastic container containing sterile vermiculite saturated with an N-deficient nutrient solution (Wakelin et al. 2018). Commercially available *Rlt* strain TA1 (supplied by Australian Inoculants Research Group) was used for comparison. A suspension of each rhizobial culture containing approximately 10^6 CFU (colony-forming units) was added to each pot containing a 4-day-old seedling. An uninoculated treatment and a positive N control (weekly added 1 mL of 10 mM NH_4NO_3 solution) were also included. This assessment was set up in a randomised complete block design in a growth room (16 h light at 22ºC; 8 h dark at ambient temperature), with eight replicated pots per treatment. Plants were harvested 6 weeks after inoculation and shoot dry weight (SDW) measured after drying at 60ºC. The symbiotic effectiveness of each isolate was calculated relative to TA1 as described previously (Wakelin et al. 2018).

Competitiveness assay

Seven of the rhizobial isolates obtained (Table 1) were assayed for their competitiveness against TA1. They were selected because of their high symbiotic effectiveness with white clover plants. To assess their competitive ability, a rapid *in vitro* pair-wise comparison method adapted to *Rlt* was developed (Sánchez-Cañizares & Palacios 2013, Ferguson 2018). For the pair-wise competitiveness assay, *celB* and *gusA* genes were introduced into two strains of interest, respectively (e.g. *celB* to strain A and *gusA* to strain B). *CelB* codes for a heat-tolerant β-galactosidase and *gusA* for β-glucuronidase (Ferguson 2018). To account for any potential fitness disadvantage by introducing the marker genes, the assays were repeated by reverse matching of marker genes (i.e. *gusA* to strain A and *celB* to strain B). Pairs of strains marked with *gusA* or *celB* were inoculated together onto clover seedlings grown in water-agar. Seedlings were inoculated with ~ 500 cells of each strain (1:1 ratio) with 4 replicates per treatment. Twenty-four days post inoculation, clover roots were harvested and stained. 5-Bromo-6-chloro-3-indolyl-β-D-glucuronide was used to stain the *gusA*/strain A nodules pink and 5-bromo-4-chloro-3-indolyl-β- D-galactopyranoside was used to stain the *celB*/strain B nodules blue (Ferguson 2018). Nodules with different colours (pink or blue) were counted and the proportion of nodules occupied by each strain was calculated.

Seed-coating

To improve the survival of rhizobia after seed coating, a new technology (referred to as AgRF) with novel biopolymer and drying material was developed. To simulate the current commercial coating treatment, commercially used biopolymer methyl cellulose (Methocel™ A4C, Dupont) and milled limestone (drying material) were used. Strain TA1 and *Rlt* isolate S10N9 were tested. Peat cultures of two strains were produced as described by Deaker et al. (2012), and mixed with biopolymer at a rate of 25 g peat/100 mL. The peat slurries were then manually inoculated onto white clover seeds (0.1 mL/g seeds, 20 g seeds/batch) and drying material added at 0.24 g/g seed. No extra drying process was used for both treatments. Viable numbers of rhizobia were measured immediately after coating (i.e. before storage) and monthly during storage at 20°C until numbers dropped below the threshold of 10^5 CFU/g seed (Deaker et al. 2012).

Statistical analyses

To compare the symbiotic effectiveness of NZ rhizobia isolates with TA1, one-way analysis of variance (ANOVA) was performed, with Bonferroni's correction for multiple comparisons, using Genstat 17 (VSN International Ltd). Nodule occupancy by each strain with different marker genes was averaged to account for any fitness issue that might have resulted from introducing a marker gene into the rhizobia. Competitive ability of each strain was compared to TA1 using a one-sample t-test. All t-tests were carried out with Minitab (version 16). Significant differences in viable number of rhizobia on seeds coated with different formulations were compared by ANOVA and Tukey test (95%) using Genstat 17 software (VSN International, UK).

Results

Symbiotic effectiveness of NZ isolates

More than 500 isolates were retrieved from NZ pasture soils and the majority of these were confirmed to be *Rhizobium leguminosarum* using 16S rRNA sequencing (van Ham et al. 2016). Over 250 isolates were tested for symbiotic effectiveness with white clover and the effectiveness varied greatly, ranging from 1–227% when compared with TA1. Approximately 51% of isolates had symbiotic potential less than 80% of TA1, and more than 90 isolates had a greater symbiotic potential than TA1 (data not shown). A selection of 11 of these rhizobia are shown in Figure 1.

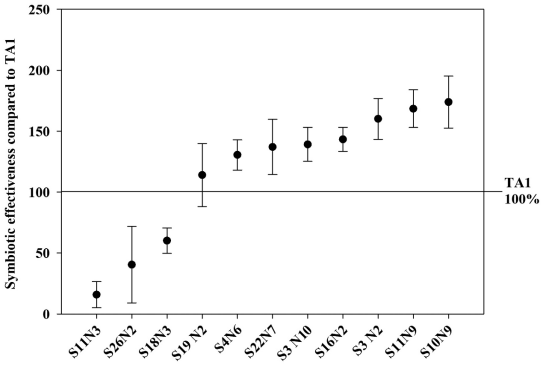

Figure 1 Symbiotic effectiveness of selected NZ *Rlt* isolates on white clover cultivar 'Tribute' in comparison to the current commercial strain TA1 (set at 100%). Data are mean ± standard error (n=8).

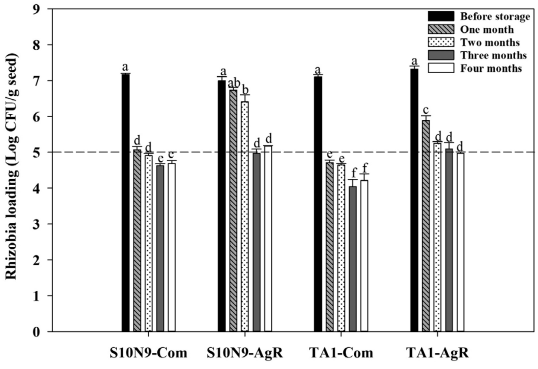

Figure 2 Survival of TA1 and *Rlt* strain S10N9 on coated white clover seeds stored at 20°C. Data are shown as mean ± SD. Different letters indicate significant difference (P<0.05) in rhizobia loading on seeds. Dotted line indicates the targeted loading of 105 CFU/g seed.

Competitiveness assay

Across all seven pair-wise comparisons when TA1 was supplied with together individual NZ isolates at the same initial ratio, clover root nodule occupancies for TA1 were between 20-34% (Table 1). All seven strains tested had significantly higher nodule occupancies than TA1, suggesting they may be more competitive than TA1. In particular, strain S4N6 occupied 80% of clover nodules.

Survival of rhizobia coated onto white clover seeds

The initial rhizobial loading for both TA1 and S10N9 was > 10^7 CFU/g seed using either the commercial or the AgRF seed-coating treatments (Figure 2). The viable rhizobia number on coated seeds decreased following storage at 20ºC but the shelf-life was significantly longer (P<0.05) for both TA1 and S10N9 coated with the AgRF treatment compared with the commercial treatment (≥4 months vs. 1 month). Interestingly, strain S10N9 survived longer on seeds than TA1, indicating this strain may be more stress tolerant than TA1.

Table 1 Nodule occupancies of seven NZ rhizobial strains versus TA1 when supplied to white clover at 1:1 ratio. Numbers reported are the mean % of nodules that contained TA1 ± standard error (n=4).

Pair-wise comparison	TA1 nodule occupancy (%)	P-value
S4N6 vs. TA1	19.9 ± 2.8	<0.001
S11N9 vs. TA1	20.9 ± 3.0	<0.001
S16N2 vs. TA1	34.4 ± 4.6	0.011
S19N2 vs. TA1	24.6 ± 3.3	<0.001
S22N7 vs. TA1	27.0 ± 2.9	<0.001
S3N2 vs. TA1	29.3 ± 4.3	0.002
S3N10 vs. TA1	32.8 ± 6.1	0.026

Discussion

A field survey (Wakelin et al. 2018) confirmed that white clover rhizobia have widely colonised pasture soils in NZ. However, the large spatial variability of size and effectiveness of naturalised rhizobia populations is likely to be limiting the symbiotic potential of white clover in many pastures. Considering the extent of variability of rhizobia effectiveness in soils and the importance of BNF in pasture farming systems (Caradus et al. 1996), it would be prudent to challenge the assumption that routine inoculation of white clover is of little benefit. The suboptimal effectiveness and reduced competitiveness of the commercially available *Rlt* strain TA1 compared to some of the NZ *Rlt* isolates tested here may explain why responses to inoculation with TA1 have not been observed in some previous studies.

Using the rhizobial strains identified in this study that have greater N fixation capacity and are more competitive, should increase the potential for inoculation responses in the field. If significant inoculation benefits are able to be demonstrated using the new strains, the replacement of TA1 for the inoculation of white clover in NZ would be well justified. In addition, it may be possible to select strains of rhizobia that are better suited to contemporary clover cultivars. Future white-clover breeding programmes should consider this opportunity.

Inoculated rhizobia must be able to outcompete the background rhizobial populations in order to form nodules on clover roots. This competitive ability varies among strains (Ames-Gottfred & Christie 1989) and in this study, all seven strains tested were significantly more competitive than TA1. While TA1 has been reported to result in low nodule occupancy (Denton et al. 2003; Lowther & Kerr 2011), most studies attribute the low competitiveness of TA1 to the poor survival

on seeds prior to and after sowing. However, our study directly supplied the rhizobia to the clover seedlings grown in an agar-based system ruling out survival as an issue. This assay provides a direct measurement of strain competitiveness and indicates that TA1 may not be an ideal inoculant candidate, especially in soils with large rhizobial populations. We are now validating these results in soil-plant assays.

Without an effective delivery system, the benefits of elite rhizobia will be limited. The most common way of delivering rhizobia to soils in NZ is *via* seed coating. As highlighted by Lowther and Kerr (2011), inoculation of white clover seed can be extremely challenging as rhizobia can die rapidly due to desiccation stress, antibacterial compounds on seed coats, and other factors. The trial seed-coating technique developed in this study improved the shelf-life of rhizobia-coated seeds from a few weeks to 4 months. When used with selected NZ isolates, the shelf life may be able to be further extended.

Granules are an alternative delivery system commonly used in Australia and USA to deliver rhizobia for white clover and other legumes (Denton et al. 2009; Denton et al. 2017). Granules can be stored separately from white clover seed and therefore avoid contact with the toxin released by seed and desiccation stresses encountered in the coating process. In addition, granular inoculants can be delivered with the seed when sown, or can be added to an established clover field by broadcasting or as liquid suspension. This alternative delivery system has some advantages over seed coating and is worth exploring.

Conclusions

With large differences in rhizobial symbiotic effectiveness from field surveys (Wakelin et al. 2018) and amongst rhizobia isolated from NZ soils (this study), it is time to challenge the assumption that inoculation is not worthwhile when sowing white clover. Strains of rhizobia have been identified that are superior to the current commercial strain TA1 in terms of N-fixing efficiency and competitive ability. These newly identified strains have the potential to be used to improve the performance of white clover on NZ farms. Further improvements in performance are possible through using new seed coating technology.

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