Survival of white clover rhizobium isolate S11N9 in different delivery systems: assessment of potential for commercialisation

Shengjing SHI[1*], Laura VILLAMIZAR[1], Elissa BARRETT[2], John CARADUS[3], and Maureen O'CALLAGHAN[1]

[1]*AgResearch Ltd, Christchurch, New Zealand*
[2]*New Edge Microbials, Albury, NSW, Australia*
[3]*Grasslanz Technology, Palmerston North, New Zealand*
*Corresponding author: shengjing.shi@agresearch.co.nz

Abstract

New Zealand pastoral farmers benefit from N_2 fixed by the white clover-rhizobium symbiosis, but rhizobia isolates vary widely in their N-fixation ability. *Rhizobium leguminosarum* S11N9, isolated in NZ, outperforms the current commercial isolate TA1 in laboratory, glasshouse, and field trials. This study investigated production and shelf-life of S11N9 to establish its feasibility as a potential new rhizobium inoculant for white clover. Freeze dried and peat inoculants were prepared for both the S11N9 and TA1 rhizobia. Peat inoculants were subsequently formulated into granules and seed coatings using AgResearch technologies. Both isolates produced similar fermentation yields. S11N9 stored as freeze-dried powder at 4 ºC survived longer than TA1 (12 vs. 10 months, respectively). Similarly, S11N9 peat inoculant had a longer shelf-life than TA1 when stored at 4 °C (44.7 vs. 21.7 months, respectively) and 20 °C (17.2 vs 9.1 months, respectively). Seeds coated with S11N9 had higher initial loadings than TA1 (10^7 vs 10^6 rhizobia/g seeds, respectively) but both declined on seeds stored at 20 °C at a similar rate. Both isolates were stable in peat granules for two months at 20 °C, but TA1 dropped below target specifications after three months while S11N9 maintained above the threshold. Results suggest that isolate S11N9 is a promising alternative to TA1 and has high potential to be developed as a commercial inoculant for white clover.

Keywords: peat inoculant, seed coating, granule, N_2-fixing efficiency, shelf-life

Introduction

Forage legumes, in particular white clover, have always been a key component in New Zealand (NZ) pasture systems where they improve feed quality and provide nitrogen (N) through their interaction with their symbiotic partner *Rhizobium leguminosarum* bv. *trifolii* (*Rlt*) (Caradus et al. 1996). With both the global drive for more sustainable farming practices and the increased cost of synthetic fertilisers, there is growing interest in utilising biologically fixed N_2 to replace, at least partially, fertiliser N.

NZ seed companies have been using a commercial isolate *Rlt* TA1 (isolated from Tasmania in 1950s) for white clover. Recent studies have shown that some NZ-sourced rhizobia isolates fixed more N_2 than TA1 with modern white clover cultivars (e.g., Tribute) in laboratory, glasshouse and field trials (Shi et al. 2019; Shi et al. 2023). In particular, isolate S11N9 had a symbiotic potential of 168 % that of the current commercial isolate TA1 in laboratory bioassays (Shi et al. 2023) and was more competitive in terms of nodule occupation than TA1 in an *in vitro* assay (Shi et al. 2019). In addition, the consistent performance of S11N9 has been demonstrated in three field trials resulting in the inoculation of S11N9 significantly increasing clover biomass as compared to uninoculated control, while the performance of TA1 was not consistent and did not show any inoculation benefits over the uninoculated control in two trials (Shi et al. 2023). S11N9 has potential to be further developed as a commercial inoculant for white clover. However, while efficient N_2 fixation is a key prerequisite for successful inoculants, the development of stable formulations that allow their commercialization and use through improved shelf life is also essential (Bashan et al. 2014; Baena-Aristizábal et al. 2019).

Rhizobium inoculants are commonly marketed as wettable powders (e.g., peat inoculants and freeze-dried biomass), concentrate suspensions (liquid formulations) and granules (Rice et al. 2000; Albareda et al. 2008; Zabaleta-Verde & Soriano 2020). The ability of rhizobia to survive as commercial inoculants or on pre-inoculated seeds depends on many factors including the carriers, formulation excipients, moisture content, presence of contaminant microorganisms and storage temperature, as well as the intrinsic traits of the rhizobia isolates (Baena-Aristizábal et al. 2019). Liquid formulations offer several advantages including better stabilisation of the microorganisms during production, packaging, distribution, and storage, and ease of mixing with the seeds (Elsakhawy et al.

ISSN 2463-2872 (Print) ISSN 2463-2880 (Online) https://doi.org/10.33584/jnzg.2024.86.3705

2021). However, most commercially available rhizobia inoculants are solid products in which the rhizobia are grown and maintained on a solid carrier. Peat is the most commonly used carrier, although many other substrates have been used (Albareda et al. 2008). Solid rhizobia inoculants generally provide several months shelf-life when stored under refrigeration (Khavazi et al. 2007) and in some cases at room temperature (Ruíz-Valdiviezo et al. 2015). There is often limited success in coating seeds with rhizobia because it is difficult to maintain living bacterial cells through various stresses including desiccation and exposure to toxic substances used in the coating process or produced by the seeds during storage (Zhou et al. 2017).

This study aimed to evaluate yield, symbiotic effectiveness, and stability in different delivery systems of NZ isolate *Rlt* S11N9, providing insights as to the prospects of the strain as an alternative white clover inoculant.

Materials and Methods

Rhizobium isolates, production, and quantification

Rlt S11N9 was isolated from a white clover nodule during a previous study (van Ham et al. 2016) and TA1 was originally sourced from the Australian Legume Inoculant Research Unit. Both isolates were stored at −80 °C using the "Protect" microorganism preservation system in the AgResearch culture collection (AgResearch, Lincoln). Each isolate was produced in a 160 L fermenter and the bulk liquid ferments were used as either an inoculum source for the peat-based product or processed further to create the freeze-dried powder. For the peat-based inoculant, under aseptic conditions, 100 mL of the liquid culture were added to 150 g of sterile peat contained in a sterile plastic bag (15 cm x 20 cm). The inoculated peat was incubated for 14 days at 28 °C. For the freeze-dried powder, one 160 L was centrifuged to create a paste. A volume of 15 mL was dispensed into glass vials, freeze-dried and sealed with rubber stoppers.

Quantification of *Rlt* for this study was by serial dilution plating (Baena-Aristizábal et al. 2019). Briefly, 1 mL or gram of the sample was added to 9 ml of phosphate buffer solution (pH 7.0), mixed thoroughly and a dilution series was prepared. Three dilutions were plated in duplicate onto yeast mannitol agar (YMA) plates, which were incubated at 28 °C for 4 days when the colony forming units (CFU) were counted.

Formulation of granules and coated seeds

Coated seeds: a new technology developed by AgResearch Ltd. that incorporates a novel biopolymer and drying material was used to coat white clover seeds. Peat inoculant (two months old) of each isolate (S11N9 or TA1) was mixed with the biopolymer at a rate of 25 g peat/100 mL. The peat slurry was then manually inoculated onto white clover seeds (0.1 mL/g seeds, 20 g seeds/batch), and drying material was added at 0.24 g/g seed. No extra drying process was used for coating treatment (Shi et al. 2019). Samples of coated seeds (10g) were packaged in heat sealed plastic bags (5cm x 5cm).

Granules: a new formulation developed by AgResearch Ltd. was used to produce granules for both isolates (S11N9 and TA1). Briefly, the peat inoculants were mixed with other excipients (drying protectants, carriers and disintegrants) to make a dough that was then extruded through a 2 mm diameter extruder. Granules (approximately 2 mm long) were collected and dried for 16 hours at 25 °C. For the storage trial, samples of dry granules (10 g) were packaged in heat sealed aluminium sachets (5 cm x 5 cm).

Shelf-life studies

Viable numbers of rhizobia as freeze-dried paste, peat, or granules and coated on seeds were assessed at time 'zero' of producing (i.e., initial counts before storage) and at monthly intervals during storage according to the following conditions: Freeze-dried powder - 12 months of storage at 4 °C; Peat inoculants - 12 months of storage at 4 °C and 20 °C; Coated seeds - storage at 20 °C until numbers dropped below the threshold of 500 CFU/seed or 5 x 10^5 CFU/g seed (Drew et al. 2012) and peat granules - storage at 20 °C until numbers dropped below the threshold of 10^7 CFU/g granule. Two replicated samples in individual packages were used for assessing the rhizobium loadings at each sampling time.

Symbiotic effectiveness of rhizobium isolates

The N-fixing efficiency of peat inoculants of S11N9 and TA1 with white clover cv. Tribute was tested in a standard laboratory assay (Shi et al. 2019; Shi et al. 2023). Briefly, white clover seeds were surface sterilised and pre-germinated before planting into a 70-ml pottle containing sterile vermiculite saturated with an 40 ml of McKnight's solution containing trace N (0.1 mM NH_4NO_3) (Wakelin et al. 2018). A suspension of each *Rlt* peat inoculant containing approximately 10^7 CFU of *Rlt* was added to each pottle containing a 4-day-old seedling. In addition, similar amount of TA1 cells grown in YMA plates were also added to seedlings as a control, together with an uninoculated treatment and a positive mineral N control (1 mL of 10 mM NH_4NO_3 solution added weekly). This assessment was set up in a randomised complete block design in a growth room (16 h light at 22 ºC; 8 h dark at ambient temperature), with 12 replicate pottles per treatment. Plants were harvested 6 weeks after inoculation and shoot dry weight (SDW) measured after drying at 60 ºC. Nodules in clover root systems were visually assessed.

Statistical analyses

Rhizobia counts in various formulations were $\log_{10}$ transformed for analysis, and shelf-lives were predicted using the least squares fit and the threshold established for each type of product/formulation. Significant differences in viable number of rhizobia on stored inoculants, formulations and coated seeds were compared at each storage time by ANOVA and Fisher's Least Significant Difference (LSD) Test (95 %) using Statistix 8.1. To compare the clover dry weight biomass, ANOVA was performed, with Fisher's least significant difference (LSD) method for pair-wise comparisons, using Genstat 23rd (VSN International Ltd).

Results

Fermentation yield

Two ferments were conducted for both S11N9 and TA1, with yield ranging from 1.6 x 10^9 to 3.0 x 10^9 CFU/mL. The average yields for S11N9 and TA1 were 2.0 x 10^9 CFU/ml and 2.5 x 10^9 CFU/mL, respectively. The result indicates S11N9 fermented similarly to the current commercial isolate TA1 under an industrial production setting.

Storage stability of *Rhizobium* isolates in freeze-dried powder

The initial loading for both S11N9 and TA1 isolates were well above the product specification of 5 x 10^{10} CFU/g powder. Both isolates started with similar loadings (10^{12} CFU/g powder), but loadings of S11N9 were significantly higher ($p<0.05$) compared to TA1 at all three storage timepoints over the 12-months of the trial (Figure 1). Extrapolation of viable CFU/g in freeze dried powders stored at 4 ºC using a first-order kinetic model suggests it would take 12 months for S11N9 in freeze dried powder to drop below 5 x 10^{10} CFU/g vs 10 months for TA1.

Storage stability of *Rhizobium* isolates in peat

When peat inoculants were stored at 4 ºC, the loading of both S11N9 and TA1 remained above 10^8 CFU/g peat for the twelve months of the storage trial, although the decline of TA1 was faster than S11N9 (Figure 2a). The estimated shelf-life was 44.7 months for S11N9 and 21.7 months for TA1, based on a first-order kinetic model.

Similar patterns were observed when peat inoculants were stored at 20 ºC, with rhizobia loadings declining more rapidly than in peat stored at 4 ºC, as expected. Although both isolates started at similar loadings (1.45 x 10^9 CFU/g peat for S11N9 and 1.48 x 10^9 CFU/g peat for TA1), viable counts were significantly higher ($p<0.05$) for S11N9 than for TA1 at all post-storage timepoints over the12-months storage period (Figure 2b). Using a first-order kinetic model, the estimated shelf-life for S11N9 was 17.2 months and for TA1 peat inoculant was 9.1 months when using 10^8 CFU/g peat as the product specification.

Stability of *Rhizobium* isolates on coated white clover seeds

The peat inoculants of S11N9 and TA1 were applied to white clover seeds using the formulation developed by AgResearch, as described above. The loading for

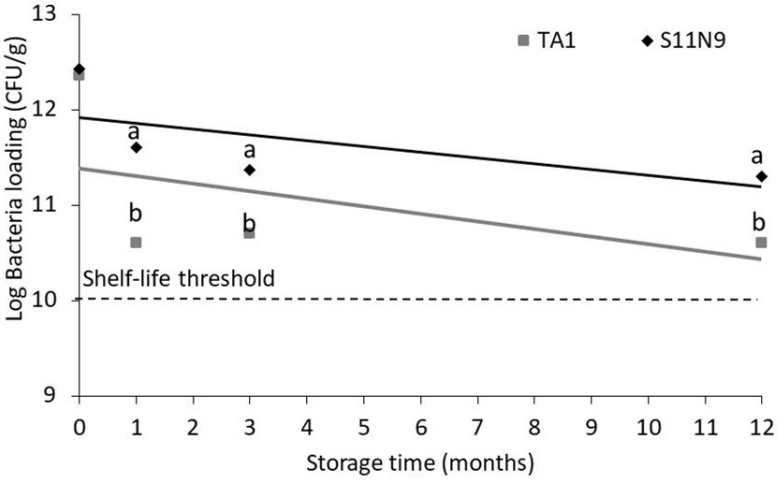

Figure 1 Stability of isolates S11N9 and TA1 in freeze-dried powder stored at 4 °C. Solid lines for treatments correspond to trendlines calculated using the method of least squares. The specification for shelf-life is 10 which corresponds to a bacterial loading of 1.0 x 10^{10} CFU/g peat (dotted line). Treatments with different letters are significantly different according to Fisher's Least Significant Difference (LSD) Test (95 %) used to compare data at each time point.

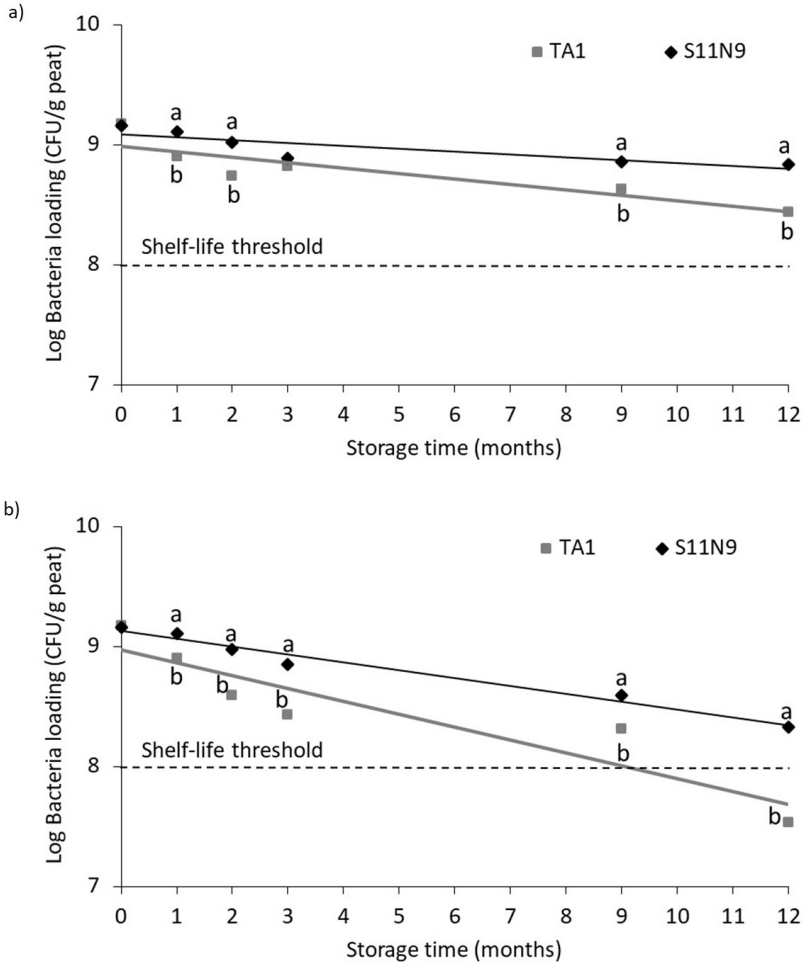

Figure 2 Stability of isolates S11N9 and TA1 on peat inoculants when stored at a) 4 °C and b) 20 °C. Solid lines for treatments correspond to trendlines calculated using the method of least squares. The specification for shelf-life is 8 which corresponds to a bacterium loading of 1.0 x 10^8 CFU/g peat (dotted line). Treatments with different letters are significantly different according to Fisher's Least Significant Difference (LSD) Test (95 %) used to compare data at each time point.

S11N9 was 10^7 CFU/g seeds, while significantly lower loading ($p<0.05$) of 10^6 CFU/g seeds was determined for TA1, immediately after coating of seeds. After 1-month storage, the loadings of both isolates had decreased but were maintained above the specification of 5 x10^5 CFU/g seeds (NSW Department of Primary Industries 2017). Rhizobium loadings of both isolates continued to decline and after two months of storage, both isolates were below the threshold (Figure 3). The estimated shelf-lives (first-order kinetic model) were 1.16 months for S11N9 and 0.87 months for TA1 when stored at 20 °C using the threshold of 10^5 CFU/g seeds.

Storage stability of rhizobium in peat granules

Rhizobium peat inoculants were also formulated as granules using AgResearch technology. Immediately after formulation, granules containing both rhizobia isolates presented similar loading ($p>0.05$) of about 10^8 CFU/g granule, which then declined at similar rates during storage at 20 °C (Figure 4). For TA1, the loading was below the AgResearch Ltd. specification of 10^7 CFU/g granule at 3-month sampling timepoint, while the loading of S11N9 was still above the threshold. The estimated shelf-lives (first-order kinetic model) were 3.0 months for S11N9 and 2.3 months for TA1 when stored at 20°C using the threshold of 10^7 CFU/g granule.

Symbiotic effectiveness of rhizobium inoculant

The nitrogen fixation efficiency of S11N9 and TA1 peat inoculants were also tested using the standard laboratory bioassay with white clover cultivar Tribute.

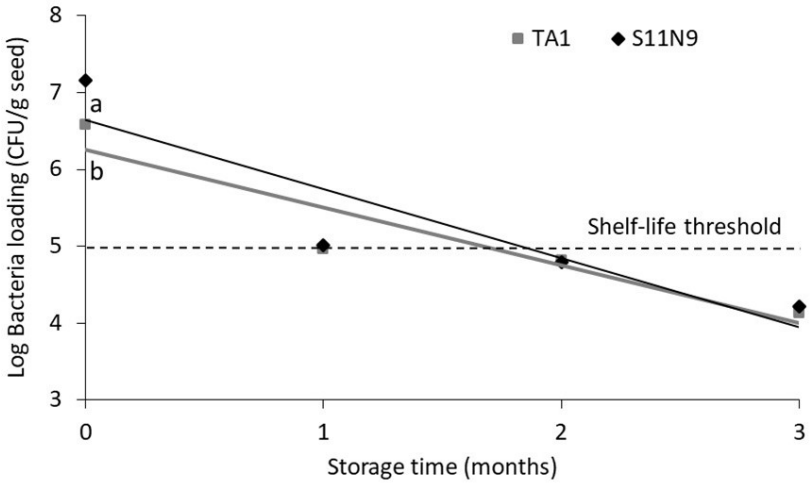

Figure 3 Stability of isolates S11N9 and TA1 on coated white clover seeds stored at 20 °C**.** Solid lines for treatments correspond to trendlines calculated using the method of least squares. The specification for shelf-life is 5 which corresponds to a bacterium loading of 5.0 x 10^5 CFU/g seed (dotted line). Treatments with different letters are significantly different according to Fisher's Least Significant Difference (LSD) Test (95 %) used to compare data at each time point.

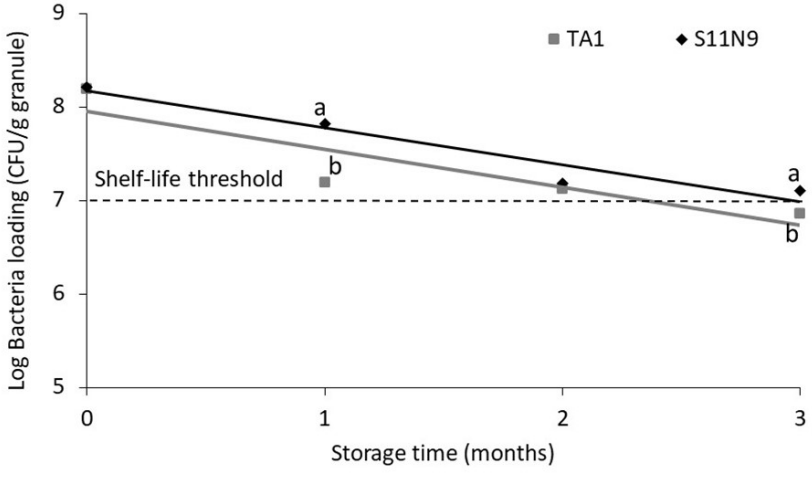

Figure 4 Stability of isolates S11N9 and TA1 on peat granules when stored at 20 ºC. Dotted line indicates the targeted loading of 10^7 CFU/g granule. Solid lines for treatments correspond to trendlines calculated using the method of least squares. The specification for shelf-life is 7 which corresponds to a bacterium loading of 1.0 x 10^7 CFU/g peat (dotted line). Treatments with different letters are significantly different according to Fisher's Least Significant Difference (LSD) Test (95 %) used to compare data at each time point.

The clover SDW was 6.5 mg per plant in the control treatment where no rhizobia were inoculated (Figure 5). The inoculation of TA1 in cell culture increased the clover SDW by 186 % compared to control. Increased production of SDW (17.3 mg per plant) was also observed when TA1 peat inoculant was added to clover seedlings as compared to control. The highest clover SDW (23.6 mg per plant, 275 % increase as compared to control) was observed following inoculation with S11N9 peat, which was significantly higher than the treatment inoculated with TA1 peat by 36.4 % (Figure 5). Pink nodules were found in clover roots when rhizobium was added (S11N9, TA1, TA1 culture), but not present in N positive and negative control treatments.

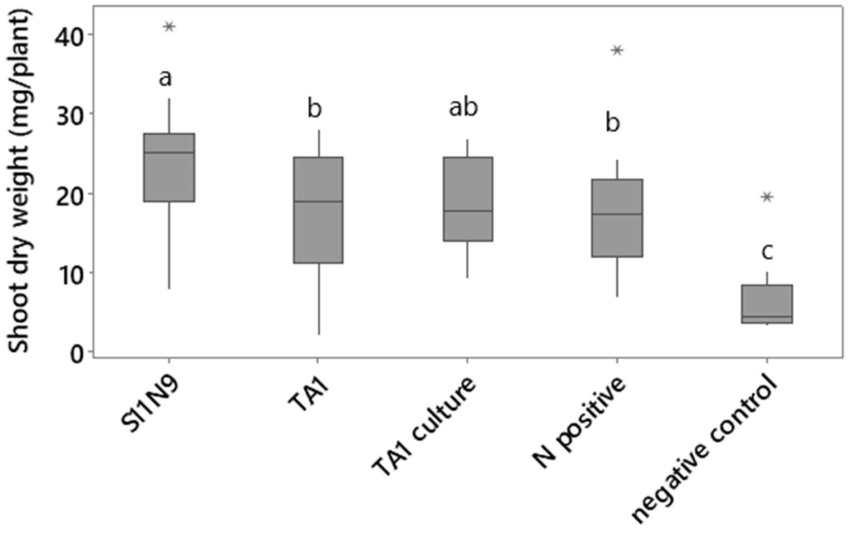

Figure 5 N-fixation efficiency of S11N9 and TA1 peat inoculant on white clover cv. Tribute as shown clover shoot dry weight. Data shown as box plots with the third quartile (Q3) and first quartile (Q1) range of the data, with the median in the middle. The length of the whiskers is from Q3 or Q1 to the distance of 1.5 times the interquartile range. Data outside this distance are outliers as indicated by stars. Different letters indicate significant difference ($P<0.05$) in clover shoot dry weight across treatments.

Discussion

To develop an effective N_2-fixing isolate into a commercial bioinoculant product, the isolate needs to be amenable to large scale production, and tolerant of stresses imposed during formulation processes and during storage. Here the performance of the new isolate of *R. leguminosarum* bv. *trifolii* S11N9, a potential inoculant for white clover, was compared against the current commercial isolate TA1. In addition to its improved N fixing capacity over TA1, S11N9 compared well against TA1, both in terms of mass production and formulation/product storage stability. In particular, the peat inoculant and freeze-dried paste of S11N9 had longer shelf-lives when compared to TA1 at both 4 °C and 20 ºC.

Commercial peat inoculants based on TA1 should normally be stored at between 4 ºC and 10 ºC to ensure its shelf-life of at least 12 months after manufacture. Using the same specification (>10^8 CFU/g peat), peat inoculant based on S11N9 produced under the same commercial system had a shelf-life longer than one year even at room temperature (17 months at 20 ºC and 44 months at 4 ºC). This long shelf-life without refrigeration suggests S11N9 may have significant advantages in terms of technical production and economic feasibility as a commercial product. Shelf life of 1–2 years without refrigeration conditions for peat inoculants is highly desirable (Bashan et al. 2014). Although it is common, storage under refrigeration is not ideal due to increased cost and distribution can be limited by need for refrigeration during distribution (Aloo et al. 2021; Roy 2021).

Although the initial loading in the peat granules was similar for the two isolates, the viability of TA1 decreased significantly faster compared to S11N9, resulting in a shorter shelf life. This result is consistent with the results obtained in the storage stability study of peat inoculants, indicating that the isolate S11N9 may be more tolerant to some stress conditions than TA1. Granular inoculants are widely used in the USA and Canada, while in New Zealand the preferred rhizobium delivery system is by seed coating. However, granular inoculants have several advantages through their ability to maintain high rhizobia populations over time in storage and in the field, as well as the ease of application with appropriate seeding equipment, that allows placement of the granules below or adjacent to seeds to optimise nodulation and influence the distribution of nodules on legume roots (Stephens & Rask 2000).

Solid formulations are typically problematic for non-sporulating bacteria such as rhizobia because desiccation damages cell membranes, leading to cell death and loss of viability during rehydration, which can have a significant negative impact on the commercialisation of the product (Berninger et al. 2018). However, the granular formulation evaluated in the present study based on the isolate S11N9, had a shelf life of 3 months at room temperature, almost double the shelf life of this isolate on white clover coated seeds. These results are promising, especially

considering that this prototype granular formulation can be further optimized to extend its shelf life through adjustments in the formulation and the development of a suitable packaging system. Further research to establish application rates and methods, evaluate field efficacy, and scale up the manufacturing process is required to develop this granular formulation into a commercial product.

Seed coating remains the most common way to deliver *Rlt* on farm in NZ, although there are well-known issues with rhizobia survival on seeds. Survival on seeds is affected by many factors such as the initial condition of the cells in the inoculant (moisture status, age, purity), the initial loading in the inoculant, the isolate, the type of inoculant and polymeric adhesives, and other additives such as dyes or pigments, plant nutrients or pesticides for seed protection, desiccation stress and toxic water-soluble legume-seed exudates (Temprano et al. 2002; Lowther & Kerr 2011; Deaker et al. 2012). This study demonstrated S11N9 performed similarly when coated onto seeds as TA1 when using the AgResearch seed coating technology at laboratory scale. It is expected the performance of S11N9 would be similar to TA1 in industrial seed coating settings, as S11N9 was compatible with the coating ingredients commonly used in the industry.

Higher N-fixation efficiency, similar production yield and longer stability of S11N9 in both freeze-dried paste and peat suggests this new isolate could be readily adopted by the industry to replace the current commercial strain TA1. No modification of fermentation conditions, manufacturing process or facilities is required to commercially produce S11N9 broth, freeze-dried paste, or peat inoculants. With sufficient market demand, the production of the new inoculant could be relatively easily achieved. For farmers, the application process of *Rlt* inoculants, both as freeze-dried powder and peat inoculant, would remain the same. Further evaluation of field performance under a range of different environmental conditions would encourage development and adoption of an improved inoculant for white clover in NZ.

Conclusions

Rlt isolate S11N9 not only showed higher N-fixing symbiosis with white clover as compared to the current commercial isolate TA1 in laboratory, glasshouse and field trials (Shi et al. 2019; Shi et al. 2023), but also performed well in terms of culturing and shelf-life properties for various delivery systems. We suggest that further research and development of S11N9 as a potential replacement for TA1 is warranted.

ACKNOWLEDGEMENTS

We would like to thank Emily Gerard, David Wright, Sandra Young, Caitlin Hyde, Jill Gibson, and Mary Ann Tuboltsev (AgResearch) for technical support and useful discussions through the whole research programme. We also thank Chikako van Koten for statistical support for this study. Special thanks go to New Edge Microbials for their strong support for this work. We thank the Pre-seed Kiwinet for funding.

REFERENCES

Albareda M, Rodríguez-Navarro DN, Camacho M, Temprano FJ. 2008. Alternatives to peat as a carrier for rhizobia inoculants: Solid and liquid formulations. *Soil Biology and Biochemistry 40*: 2771-2779. https://doi.org/10.1016/j.soilbio.2008.07.021

Aloo BN, Mbega ER, Tumuhairwe JB, Makumba BA. 2021. Advancement and practical applications of rhizobacterial biofertilizers for sustainable crop production in sub-Saharan Africa. *Agriculture & Food Security 10*: 57. https://doi.org/10.1186/s40066-021-00333-6

Baena-Aristizábal CM, Foxwell M, Wright D, Villamizar-Rivero L. 2019. Microencapsulation of Rhizobium leguminosarum bv. trifolii with guar gum: Preliminary approach using spray drying. *Journal of Biotechnology 302*: 32-41. https://doi.org/10.1016/j.jbiotec.2019.06.007

Bashan Y, de-Bashan LE, Prabhu SR, Hernandez J-P. 2014. Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). *Plant and Soil 378*: 1-33. https://doi.org/10.1007/s11104-013-1956-x

Berninger T, González López Ó, Bejarano A, Preininger C, Sessitsch A. 2018. Maintenance and assessment of cell viability in formulation of non-sporulating bacterial inoculants. *Microbial Biotechnology 11*:277-301. https://doi.org/10.1111%2F1751-7915.12880

Caradus JR, Woodfield DR, Stewart AV. 1996. Overview and vision for white clover. *Agronomy Society of New Zealand Special Publication No 11/ Grassland Research and Practice Series 6*: 1-6. https://doi.org/10.33584/rps.6.1995.3368

Deaker R, Hartley E, Gemell G. 2012. Conditions affecting shelf-life of inoculated legume seed. *Agriculture 2*: 38-51. https://doi.org/10.3390/agriculture2010038

Drew EA, Herridge D, Ballard R, O'Hara G, Deaker R, Denton M, Yates R, Gemell G, Hartley E, Phillips L. 2012. Inoculating legumes: a practical guide. *GRDC*.

Elsakhawy T, Ghazi A, Abdel-Rahman MA. 2021. Developing Liquid Rhizobium Inoculants with

Enhanced Long-Term Survival, Storage Stability, and Plant Growth Promotion Using Ectoine Additive. *Current Microbiology 78*: 282-291. https://doi.org/10.1007/s00284-020-02265-z

Khavazi K, Rejali F, Seguin P, Miransari M. 2007. Effects of carrier, sterilisation method, and incubation on survival of Bradyrhizobium japonicum in soybean (Glycine max L.) inoculants. *Enzyme and Microbial Technology 41*: 780-784. https://doi.org/10.1016/j.enzmictec.2007.06.011

Lowther WL, Kerr GA. 2011. White clover seed inoculation and coating in New Zealand. *Proceedings of the New Zealand Grasslands Association 73*: 93-102. https://doi.org/10.33584/jnzg.2011.73.2841

NSW Department of Primary Industries. 2017. *Inoculating and pelleting pasture legume seed*: Primefact 1537.

Rice WA, Clayton GW, Olsen PE, Lupwayi NZ. 2000. Rhizobial inoculant formulations and soil pH influence field pea nodulation and nitrogen fixation. *Canadian Journal of Soil Science 80*: 395-400. https://doi.org/10.4141/S99-059

Roy A. 2021. Biofertilizers for agricultural sustainability: Current status and future challenges. In: Ajar Nath Yadav JS, Chatarpal Singh, Neelam Yadav Ed. *Current Trends in Microbial Biotechnology for Sustainable Agriculture*, pp. 525-553. Singapore: Springer. http://dx.doi.org/10.1007/978-981-15-6949-4_21

Ruíz-Valdiviezo VM, Canseco LM, Suárez LA, Gutiérrez-Miceli FA, Dendooven L, Rincón-Rosales R. 2015. Symbiotic potential and survival of native rhizobia kept on different carriers. *Braz J Microbiol 46*: 735-742. https://doi.org/10.1590%2FS1517-838246320140541

Shi S, Villamizar L, Gerard E, Ronson CW, Wakelin SA, Ballard RA, Caradus JR, O'Callaghan M. 2019. Increasing biological nitrogen fixation by white clover-rhizobia symbiosis. *Journal of New Zealand Grasslands 81*: 231-234. https://doi.org/10.33584/jnzg.2019.81.380

Shi S, Wakelin S, Gerard E, Young S, van Koten C, Caradus J, Griffiths AG, Ballard RA, O'Callaghan M. 2023. Screening and field evaluation of white clover rhizobia for New Zealand pastures. *Crop and Pasture Science 74*: 1258-1271. https://doi.org/10.1071/CP22405

Stephens JHG, Rask HM. 2000. Inoculant production and formulation. *Field Crops Research 65*: 249-258. https://doi.org/10.1016/S0378-4290(99)00090-8

Temprano FJ, Albareda M, Camacho M, Daza A, Santamaría C, Rodríguez-Navarro DN. 2002. Survival of several Rhizobium/Bradyrhizobium strains on different inoculant formulations and inoculated seeds. *International Microbiology 5*: 81-86. https://doi.org/10.1007/s10123-002-0067-y

van Ham R, O'Callaghan M, Geurts R, Ridgway HJ, Ballard R, Noble A, Macara G, Wakelin SA. 2016. Soil moisture deficit selects for desiccation tolerant Rhizobium leguminosarum bv. trifolii. *Applied Soil Ecology 108*: 371-380. https://doi.org/10.1016/j.apsoil.2016.09.016

Wakelin S, Tillard G, van Ham R, Ballard R, Farquharson E, Gerard E, Geurts R, Brown M, Ridgway H, O'Callaghan M. 2018. High spatial variation in population size and symbiotic performance of *Rhizobium leguminosarum* bv. trifolii with white clover in New Zealand pasture soils. *PLOS ONE 13*: e0192607. https://doi.org/10.1371/journal.pone.0192607

Zabaleta-Verde D, Soriano B. 2020. Inoculante constituido por Rhizobium leguminosarum, Burkholderia ubonensis y Trichoderma harzianum incrementa el número de sitios activos para la nodulación de Pisum sativum. *Manglar 17*: 95-99. http://dx.doi.org/10.17268/manglar.2020.015

Zhou J, Deng B, Zhang Y, Cobb AB, Zhang Z. 2017. Molybdate in Rhizobial Seed-Coat Formulations Improves the Production and Nodulation of Alfalfa. *PLOS ONE 12*: e0170179. https://doi.org/10.1371/journal.pone.0170179