Summary

Australian crop and pasture legumes are routinely inoculated with commercially available strains of nitrogen (N) fixing root nodule bacteria (rhizobia). Rhizobia provide a source of N for legume growth and contribute to the soil N economy of cropping systems through the breakdown of legume residues. Two key requirements for commercial rhizobial strains are that they:

1) Remain genetically stable in both the inoculant preparation and the field (i.e. they retain the capacity to nodulate and fix N with the host legume), and

2) Are persistent in the soil (so that nodulation is effected by the inoculant strain).

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Conclusions

1. The molecular fingerprinting protocols developed in this project have been used effectively for quality control within the Australian inoculant industry, for characterising medic rhizobial inoculant strains and field isolates, and for identification of bacterial isolates from several other GRDC projects.

2. The inoculant strains for annual medics (WSM688) and for perennial medics including lucerne (WSM826) were poorly persistent in most eastern states' pastures and field trials. Given the highly diverse background populations of medic rhizobia, it is unlikely that any introduced inoculant strain will persist in significant numbers beyond the year of introduction. Inoculation is, therefore, only likely to be beneficial at sites where medics have either never been grown or have not been grown for an extended period (i.e. where background populations of medic rhizobia are small).

3. South Australian (SA) soils contain background populations of rhizobia that are effective on *Medicago littoralis*. These strains may also be effective on other perennial medic species. *M. littoralis* may only benefit from inoculation at sites where perennial medics have not been grown or where background populations of associated rhizobia are small.

4. Strains recovered from background populations of annual medic rhizobia were less effective than the inoculant strain WSM688. Given the high proportion of background strains in field populations, it is possible that medic pastures are sub-optimally effective in their N fixing capacity in many eastern states' locations. This would need to be confirmed in field trials with added N fertiliser controls.

5. The capacity to identify colony variants in rhizobial inoculant strains can be affected by the composition of the culture media on which the rhizobia are grown. This probably accounts for differences in observed colony variation in strains WSM688 and WSM826 between Western Australian (WA) and the eastern states' laboratories. Colony variation in culture cannot always be detected using molecular fingerprinting, as observed for colony variants of WSM688. Observed colony and genetic variation was shown to be linked to reduced symbiotic effectiveness in strain WSM826.

Recommendations

1. The molecular fingerprinting protocols developed in this project should continue to be used by the Australian Legume Inoculants Research Unit (ALIRU) for quality control of commercial rhizobial inoculants on an annual basis. This will ensure that the correct strain type is issued to manufacturers and will improve the probability of detecting genetic variation in inoculant strains over time.

2. Because of the diverse background populations of medic rhizobia in Queensland (QLD), New South Wales (NSW) and Victoria (VIC) (which are potentially suboptimal in their N fixing capacity), extended periods between medic phases and use of alternative pasture legumes in cropping rotations to reduce the size of background populations and enhance...
nodule occupancy by the applied inoculant strain are advised.

3. In SA soils, *M. littoralis* (and possibly other perennial medics) are only likely to require inoculation where they have not been grown before or have not been grown for an extended period. The background populations are sufficiently dominant and effective to provide high rates of N fixation.

4. Rhizobial strains isolated from perennial medics in SA were more effective than the commercial inoculant strains WSM826 and WSM1115 and will be provided to Jo Slattery (Natural Resources and Environment (NRE) VIC), Ross Ballard (South Australian Research and Development Institute (SARDI)), John Howieson (Murdoch University) and ALIRU for inclusion in future strain selection programs.

5. Future selection programs for commercial rhizobial inoculant strains should avoid cultures with dual colony types or other evidence of genetic variation because they may have reduced N fixing capacity. Where selection of alternative inoculant strains is unlikely to be supported by funding bodies or research institutions (e.g. strains CB3126, CB3060 and CB1717), colony variants should be routinely tested for loss of nodulation and/or N fixation capacity in glasshouse effectiveness tests.

6. Potential commercial inoculant strains (in advanced stages of strain selection programs) should be grown on a broad range of growth media to increase the chance of identifying strains that produce colony variants prior to commercialisation.

Outcomes

**Expected Outcome (benefits)**

**Economic Outcomes**

1. A colony variant of the commercial inoculant strain for perennial medics (WSM826) was shown to be reduced in its N fixing capacity. This finding contributed to the decision to change the recommended strain to RRI128 in 2000. The strain change maximises the probability that inoculated lucerne and other perennial species will achieve high rates of N fixation and their potential yields in the field.

2. Molecular fingerprinting of the rhizobial mother cultures supplied to inoculant manufacturers by ALIRU showed that the commercial inoculant strain CB82 for *Stylosanthes* species had been substituted with strain CB756 (presumed laboratory error at ALIRU prior to 1997). Provision of an authentic culture by CSIRO Brisbane should substantially improve N fixation by inoculated *Stylosanthes* in the northern region.

3. The effective and dominant background strains of perennial medic rhizobia identified in SA soil populations have potential as inoculant strains and should be included in future strain selection programs. Adoption of these strains as inoculants could lead to improved N fixation rates and increased yield for perennial medics.

**Achievements/Benefits**

**Overview of Project Achievements**

A number of Australian commercial inoculant strains exhibit genetic instability (WSM826, WSM688, SU303, CB3126, CB3060 and CB1717), as evidenced by different colony types in culture. This may impact on legume productivity if either the nodulation or N fixing capacity of the genetic variants is reduced relative to the original inoculant strain. Genetic variation in rhizobial inoculant strains also creates a quality control issue for inoculant manufacturers, who must ensure that retail products are symbiotically effective. The nodulation and N fixing capacity of colony variants of the Australian commercial inoculant strains is mostly unknown. This project was initiated to investigate the mechanisms underlying genetic variation in commercial rhizobial inoculant strains and the consequences of this variation for nodulation and N fixation with host legumes. The aims of the project were to:

1. Develop molecular fingerprinting tools for characterising rhizobial isolates at the strain level. This included characterising all rhizobia currently used in Australian inoculants (for quality control purposes) and development of a genetic database of polymerase chain reaction (PCR) fingerprints (for evaluating strain genetic stability over time).

2. Assess the persistence, genetic variability and field performance of inoculant strains for medics across several states.

3. Explore the genetic basis for colony dimorphism in the commercial inoculant strains for annual medics (WSM688) and for lucerne (WSM826) (PhD scholarship component).
Four protocols were developed for molecular fingerprinting of rhizobia at the strain level (repetitive element (REP), BOXAIR, random amplified polymorphic DNA (RAPD), PCR and intergenic spacer (IGS)) between July 1997 and July 2001. The project commenced with the installation and testing of all molecular laboratory equipment purchased under the GRDC strategic initiative US258. Early stages in protocol development were facilitated by a University of Western Sydney (UWS)-sponsored visiting research fellowship for Dr Janice Thies to Dr Clive Ronson’s laboratory at the University of Otago (NZ). During this visit, Dr Thies received training in REP PCR fingerprinting and assessed the usefulness of this technique against established fingerprinting methods (RFLP analysis with radiolabelled probes and amplified fragment length polymorphism (AFLP)). Major protocol development was carried out by Dr Anne-Marie Vachot (Research Associate) from Feb 1999 to June 2000, with minor optimisation continuing until July 2001. One publication has arisen from this work to date (see Additional information).

This work was presented by Dr Vachot at the 17th North American Conference on Symbiotic Nitrogen Fixation, Quebec, Canada.

Within this project, optimised fingerprinting protocols have been used for:

- Quality control fingerprinting of the rhizobial mother cultures supplied to Australian commercial inoculant manufacturers in 1998, 1999 and 2000. Fingerprinting of the mother cultures for the ALIRU - GRDC Project DAN362SR revealed that two commercial inoculant strains (CB82 and CB756) were identical. An authentic culture of CB82 was obtained by ALIRU from Alison McInnes at CSIRO Brisbane and supplied to inoculant manufacturers for inoculant production in 2000. In 2001, ALIRU staff were trained by Dr Vachot in the fingerprinting protocols so that subsequent quality control can be performed at ALIRU (NSW Agriculture, Gosford).
- Fingerprinting of medic rhizobia isolates.

Optimised fingerprinting protocols have also been used for:

- Fingerprinting Pseudomonas isolates for Dr Steven Simpfendorfer (GRDC project DAN316).
- Fingerprinting chick pea and faba bean rhizobia (GRDC project UWS19).
- Fingerprinting pea and chick pea rhizobia for Ross Ballard (SARDI - GRDC National Rhizobium Program) and Kevin McCosker (QDPI - GRDC Northern Pulse Development Program) through the UWS Molecular Analysis Service (GRDC UWS26).

A broad assessment of the field persistence of medic inoculant strains, including an assessment of the extent of naturally occurring strain diversity by June 2002.

Medic rhizobia were isolated from inoculated annual and perennial medic pastures from QLD (Nindigully - 78 isolates from *M. scutellata* and *M. truncatula*), western NSW (217 isolates from *M. polymorpha* and *M. minima*), Cryon, NSW (126 isolates from *M. sativa* and *M. truncatula*), VIC (120 isolates from *M. truncatula*) and SA (27 isolates from *M. littoralis*, *M. rigidula* and *M. rigiduloides*). Isolate collections were used to determine whether the inoculant strains WSM688 (for annual medicus) and WSM826 (for lucerne, *M. littoralis*, *M. rigidula* and *M. rigiduloides*) were persistent in the field environment. The persistence of the new inoculant strain for lucerne (RRI128 - released in 2000) was also determined at one VIC site (59 isolates recovered). Approximately 40% of the 627 isolates were tested for their capacity to nodulate *M. truncatula* variety Sephi in plant infection tests. All medic isolates were fingerprinted using two protocols between July 2001 and June 2002. Fingerprints were analysed to determine whether nodule isolates were inoculant strains or background strains. Selected background strains from QLD, SA, VIC and NSW were tested for their N fixing capacity on either *M. truncatula* Sephi or *M. littoralis* Herald in glasshouse effectiveness tests (July-Oct 2002).

The inoculant strains for annual medic and lucerne were recovered in the highest numbers from VIC sites, with 33% of isolates from *M. truncatula* identified as WSM688 and 95% of isolates from lucerne identified as RRI128. At Nindigully (QLD), 12% of isolates recovered from annual medic trials were WSM688 and 3% of isolates recovered from Cryon (NSW) were WSM688. Strain WSM688 was absent from western NSW sites. Strain WSM826 was not recovered from either Cryon or SA (where lucerne and other perennial medics had been sown) or at any other site. The background strains recovered from field populations were genetically diverse and, with the exception of SA, few strains showed a capacity to dominate field populations. Even the most common background strain types represented less than 10% of the isolates recovered from QLD, NSW and VIC. The majority of SA isolates (85%) were closely related strains.

Glasshouse effectiveness tests of four background strains isolated from three *Medicago* species at two sites in SA showed that three strains were either as effective, or more effective, than the commercial inoculant strains WSM826 and RRI128. This
indicates that the two background perennial medic populations in SA were dominated by strains that were effective on M. littoralis, and that there may be no need to inoculate this species in SA, except where background populations of rhizobia are small. These highly effective strains have potential as inoculant strains and should be included in future strain selection programs. Conversely, strains from background populations of annual medic rhizobia from NSW, VIC and QLD were often less effective than either WSM688, or the new inoculant strain for annual medics (WSM1115 - released commercially in 2002). This suggests that eastern states' annual medic pastures may have sub-optimal rates of N fixation, although field trials with added N fertiliser controls would need to be conducted to confirm this. Given the complexity of the background populations, it is probable that the new inoculant strain WSM1115 will also become a minor component of background rhizobia populations in eastern states' medic pastures. It may be advisable to have an extended break between medic phases in crop rotations, and/or to intersperse medics with other forage legume species, to reduce the size of sub-optimally effective background populations of medic rhizobia before sowing. Two field reisolates of WSM688 from VIC (strains 1A56 and 2A6), which showed altered genetic fingerprints with both protocols, were as effective as WSM688 in their N fixing capacity. This indicates that genetic change in the inoculant strain had not led to reduced N fixation capacity for these two strains.

Understanding of the underlying basis for colony variation in medic inoculant strains and strategies to maintain strain stability in culture by June 2002.

Colony variants of the medic inoculant strains WSM688 and WSM826 were investigated as part of a PhD Scholarship commenced by Mrs Gaye Wingett on 22 February 1999. Mrs Wingett obtained colony variants of both strains from several sources and found that the expression of ‘dry’ and ‘gummy’ colonies (that vary in their exopolysaccharide production) was affected by phosphorus (P), ammonium (NH\textsubscript{4}+) and calcium (Ca) concentrations in growth media. This may partially account for differences in the expression of colony types observed in different Australian laboratories for these two strains. Both inoculant strains have been characterised by four fingerprinting protocols. Dry and gummy colonies of WSM688 produced identical fingerprints in all instances, but WSM826 dry and gummy colonies showed significant genetic variation. In a glasshouse effectiveness test, a gummy variant of WSM826 was altered in its capacity to fix N with M. truncatula. Although WSM826 is not the inoculant strain for M. truncatula, this result indicates that the symbiotic capacity of colony variants can be affected and this needs to be taken into account by inoculant manufacturers and rhizobial researchers during the selection of new inoculant strains. The outcomes from this research contributed to the strain change for lucerne from WSM826 to RRI128 in 2000. It is suggested that single colonies from cultures exhibiting colony variation are tested regularly for alteration in symbiotic performance and that future selection programs avoid strains that produce dual colony types. These results are currently being written up for a special edition of the Australian Journal of Experimental Agriculture, which features outcomes from the first round of the GRDC National Rhizobium Program (1997-2002).

Project UWS20 represents one component of a larger GRDC project (TMP70), which originally involved UWS, Sydney University and NSW Agriculture. The project was split into individual projects for each institution (UWS20, SU259 and DAN362SR) following the first Progress Report (1997-1998).

**Additional information**

**Publications**


A fourth McInnes and Thies publication is anticipated for field studies and effectiveness tests from project UWS19 and UWS20.