The Influence of Naturalised Rhizobium leguminosarum bv. trifolii Populations on the Nodulation of Alternative Clovers (Trifolium spp.) in Alkaline Soils

MATT DENTON
B.Sc. (Hons). Adelaide University

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Agricultural and Natural Resource Sciences at Adelaide University.

Department of Agronomy and Farming Systems
Roseworthy Campus
Adelaide University

October 2000
Table of Contents

Abbreviations ........................................................................................................ vii
Summary ................................................................................................................ viii
Declaration ............................................................................................................ xi
Acknowledgements ............................................................................................. xii

Chapter 1: Introduction ....................................................................................... 1
  1.1 Australian Pastures ...................................................................................... 1
     1.1.1 Annual Medic Pastures ......................................................................... 1
     1.1.2 The Need for Pasture Diversity .............................................................. 2
     1.1.3 Rhizobial Requirements for Alternative Clovers ................................. 3
     1.1.4 The Influence of Edaphic Factors and Resident Soil Rhizobia on the Introduction of Rhizobial Inoculants .............................................................. 4
  1.2 Aims and Structure of this Thesis ................................................................. 4
     1.2.1 Aims ...................................................................................................... 4
     1.2.2 Structure of this Thesis ......................................................................... 5

Chapter 2: Literature Review ............................................................................. 7
  2.1 Introduction ................................................................................................... 7
  2.2 Establishment of Symbiotic Nitrogen Fixation in Legumes ................. 7
     2.2.1 Introduction ........................................................................................ 7
     2.2.2 Legumes ............................................................................................. 7
     2.2.3 Rhizobia ............................................................................................. 8
     2.2.4 Nodule Formation ................................................................................ 9
     2.2.5 Nitrogen Fixation ................................................................................ 10
     2.2.6 Genetics of Nitrogen Fixation ............................................................... 13
  2.3 Biological Nitrogen Fixation in Farming Systems .................................... 16
     2.3.1 Introduction ........................................................................................ 16
     2.3.2 Quantities of Nitrogen Fixed ................................................................. 16
     2.3.3 Benefits of Nitrogen Fixation ............................................................... 17
     2.3.4 The Fate of Fixed Nitrogen .................................................................. 20
     2.3.5 Techniques to Increase Biological Nitrogen Fixation ...................... 20
  2.4 Competition for Nodulation in the Field ................................................ 21
     2.4.1 Introduction ........................................................................................ 21
     2.4.2 Saprophytic Competence .................................................................... 22
     2.4.3 Motility of Rhizobia ............................................................................ 23
     2.4.4 Speed of Infection ............................................................................... 23
     2.4.5 Legume Interactions with Rhizobia ...................................................... 24
     2.4.6 The Effect of Combined Nitrogen on Rhizobial Competition and \(N_2\) Fixation .......................................................... 25
     2.4.7 Introducing Rhizobia into Fields with a Naturalised Rhizobial Population .......................................................... 26
     2.4.8 Response of Legumes and Rhizobia to Edaphic Constraints ............ 35
     2.4.9 Other Factors Influencing Rhizobial Competition ............................. 42
  2.5 Recently Developed Techniques Aiding the Study of Rhizobial Ecology .... 42
     2.5.1 Introduction ....................................................................................... 42
     2.5.2 Identification of Rhizobin Using Polymerase Chain Reaction Techniques .............................................................. 43
     2.5.3 The Use of GUS Marker Genes in Rhizobial Ecology ...................... 46
2.6 Conclusion and Thesis Aims ......................................................... 49

Chapter 3: Distribution, Abundance and Symbiotic Effectiveness of *Rhizobium leguminosarum* bv. *trifolii* from Alkaline Pasture Soils in South Australia ...... 51

3.1 Introduction ......................................................................................... 51

3.2 Methods .............................................................................................. 52
3.2.1 Survey Details ................................................................................. 52
3.2.2 Enumeration of Rhizobial Populations ............................................. 53
3.2.3 Symbiotic Effectiveness of Rhizobial Populations .......................... 53
3.2.4 Soil Factors ..................................................................................... 55
3.2.5 Statistical Analysis ......................................................................... 55

3.3 Results .................................................................................................. 56
3.3.1 Rhizobial Populations ................................................................. 56
3.3.2 Influence of Clover Presence Upon the Rhizobial Populations ....... 57
3.3.3 Symbiotic Effectiveness of Rhizobial Populations ........................ 58
3.3.4 Environmental Factors ................................................................. 60
3.3.5 Variables Correlated to Rhizobial Population Size ....................... 63

3.4 Discussion ........................................................................................... 64
3.4.1 Rhizobial Populations from Alkaline Soils of South Australia ........ 64
3.4.2 Influence of Clover Presence upon Rhizobial Numbers ................. 66
3.4.3 Implications of Rhizobial Populations and Effectiveness ............... 66
3.4.4 Influence of Environmental Variables Upon Rhizobial Populations ... 69

Chapter 4: The Influence of Inoculation on the Nodulation, Growth and N₂ Fixation of Alternative Clovers in Alkaline Field Soils ................................. 71

4.1 Introduction .......................................................................................... 71

4.2 Methods .............................................................................................. 72
4.2.1 Introduction .................................................................................... 72
4.2.2 Soil Descriptions ........................................................................... 72
4.2.3 Soil Nitrogen ................................................................................. 72
4.2.4 Experimental Design .................................................................... 73
4.2.5 Most Probable Number of Rhizobia ................................................ 73
4.2.6 Rhizobia ....................................................................................... 74
4.2.7 Site Maintenance 1997 ................................................................. 75
4.2.8 Site Maintenance 1998 ................................................................. 76
4.2.9 Experimental Sampling ............................................................... 76
4.2.10 Rhizosphere Populations of Rhizobium ......................................... 76
4.2.11 Nodulation .................................................................................. 77
4.2.12 Shoot and Seed Biomass ............................................................... 77
4.2.13 Measurement of Biological Nitrogen Fixation .............................. 77
4.2.14 Statistical Analysis ........................................................................ 79

4.3 Results .................................................................................................. 79
4.3.1 Site Description .............................................................................. 79
4.3.2 Precipitation .................................................................................. 79
4.3.3 Soil Nitrogen .................................................................................. 81
4.3.4 Naturalised and Inoculant Rhizobia ................................................ 81
4.3.5 Plant Establishment ....................................................................... 83
4.3.6 Rhizosphere Populations of Rhizobium ......................................... 84
4.3.7 Nodulation ..................................................................................... 86
4.3.8 Pasture Growth ............................................................................. 88
4.3.9 Seed Production ............................................................................ 89
4.3.10 Nitrogen Accumulation ............................................................... 89

4.4 Discussion ........................................................................................... 98
Chapter 5: Competition for Nodule Occupancy between Inoculant and Naturalised Rhizobia in a Field Environment

5.1 Introduction

5.2 Methods

5.2.1 General

5.2.2 Collection of Rhizobia from Nodules

5.2.3 Authentication

5.2.4 DNA Extraction

5.2.5 Polymerase Chain Reaction Methods

5.2.6 Optimization

5.2.7 Controls

5.2.8 Determination of Multiple Occupancy in Nodules

5.2.9 Gel Products

5.3 Results

5.3.1 Characterization of Inoculant Strains

5.3.2 Determination of Multiple Occupancy of Nodules

5.3.3 Nodule Occupancy

5.3.4 Confirmation of Nodule Isolates Using ERIC Primers

5.3.5 Common Field Isolates

5.4 Discussion

5.4.1 Nodule Occupancy

5.4.2 Implications of Nodule Occupancy

5.4.3 Multiple Occupancies

5.4.4 Field Dominant Strains

Chapter 6: Competition between Commercial and Naturalised Soil Rhizobia for Nodule Occupancy

6.1 Introduction

6.2 Methods

6.2.1 Strains, Plasmids and Growth Conditions

6.2.2 Minitransposon Mutagenesis of R. leguminosarum bv. trifolii

6.2.3 Plant Growth Conditions

6.2.4 Experiment 1: Effectiveness of Mutant Strains

6.2.5 Experiment 2: The Ability of Mutant Strains to Compete Against Wild Types for Nodule Occupancy

6.2.6 Experiment 3: Competitive Abilities of Commercial and Naturalised Rhizobia

6.2.7 Experiment 4: Competitive Abilities of a Soil-Applied Mutant Strain Against a Seed-Applied Commercial Strain in Forming Nodules

6.2.8 Determination of Nodule Occupancy

6.2.9 Assessment of Competitiveness for Nodulation

6.2.10 Statistical Analysis

6.3 Results

6.3.1 Mini transposon Mutagenesis of R. leguminosarum bv. trifolii

6.3.2 Experiment 1: Effectiveness of Mutant Strains

6.3.3 Experiment 2: The Ability of Mutant Strains to Compete Against Wild Types for Nodule Occupancy

6.3.4 Experiment 3: Competitive Abilities of Commercial and Naturalised Rhizobia
Summary

The majority of southern Australian farming systems rely upon biological N₂ fixation of legumes for inexpensive N inputs. Low rainfall, alkaline soil environments typically support annual medic (Medicago spp.) pastures, but there is growing recognition that other legume genera need to be explored to improve pastures in these environments. A number of alternative clover (Trifolium spp.) species adapted to alkaline soil environments are being evaluated to increase the legume pasture diversity on the low rainfall, alkaline soils of southern Australia. The investigation of alternative clover production necessitates that the ecology of their root-nodule bacteria (Rhizobium) is well understood, to ensure that biological N₂ fixation is not compromised due to sub-optimal symbioses. In particular, naturalised rhizobial populations can have a significant influence upon the legume responses to inoculation. For this reason, an investigation into the influence of naturalised R. leguminosarum bv. trifolii on the nodulation of alternative clovers on alkaline soils was undertaken.

A survey of 61 sites was conducted across South Australia to determine the size, distribution and effectiveness of Rhizobium leguminosarum bv. trifolii (clover rhizobia) populations resident in these low rainfall, alkaline soil environments. Clover rhizobia were detected at 56 sites, with a median density of 230 to 920 rhizobia/g soil. The majority of rhizobial populations were poor in their capacity to fix N₂. Rhizobial populations from fields provided 11 to 89% and 10 to 85% of the shoot biomass of commercial reference strains when inoculated onto host legumes T. purpureum (purple clover) and T. resupinatum (Persian clover), respectively. Rhizobial population size was correlated negatively to pH and percent of CaCO₃ in the soil, and was significantly increased in the rhizospheres of naturalised clover, found at 17 sites.
The ability for inoculation with commercial rhizobia to increase the nodulation, growth and N\textsubscript{2} fixation of alternative clovers (\textit{T. alexandrinum}, \textit{T. purpureum} and \textit{T. resupinatum}) was tested on alkaline soils. Addition of commercial rhizobia or 200\text{kgN/ha} failed to increase biomass above uninoculated treatments. While \textit{T. purpureum} produced the greatest biomass, it had low shoot N concentration (2.06-2.24\%). \textit{T. resupinatum} had a higher shoot N concentration (2.52-2.66\%), and fixed a greater amount of N\textsubscript{2} than \textit{T. purpureum}, (77\text{kg fixedN/ha} cf. 56\text{kg fixedN/ha}, Roseworthy; \textsuperscript{15}N natural abundance technique) nodulating well with the naturalised populations of rhizobia. All clovers fixed N\textsubscript{2} best with their recommended commercial strain.

The ability for commercial strains to compete with naturalised rhizobia and occupy nodules in the field study was determined using the polymerase chain reaction (PCR) to identify nodule isolates. \textit{R. leguminosarum} bv. \textit{trifolii} were accurately identified at the strain level using the primer RP01, which is directed towards the \textit{nif} promoter region and ERIC primers, which amplify intergenic repeat units. Analysis of nodule contents indicated that strain TA1 had poor occupancy of nodules across both field sites, with the exception that it nodulated \textit{T. alexandrinum} up to 39\% at the Roseworthy site in the first year. Strains CC2483g (commercial strain for \textit{T. resupinatum}) and WSM409 (commercial strain for \textit{T. purpureum}) showed reasonable colonization of nodules in the first year of inoculation and persisted into the second year at both field sites. A number of isolates with similar banding patterns, distinct from commercial strains, were consistently observed. These isolates cross-inoculated all 3 clover species and occurred in both years of the field trial with each isolate occurring in 5 to 19\% of the total nodules at a field site.

Two dominant field isolates were assessed for their ability to compete against commercial strain TA1. A number of mutant strains were constructed from field dominant strains by insertion of the \textit{gusA} gene.
The subsequent *gusA*-marked mutant strains did not differ in effectiveness of N\(_2\) fixation or in competitiveness for nodulation, compared to parental strains. Subsequently, the competitive ability of mutant strains was tested against commercial strain TA1. One field isolate was shown to be as effective in N\(_2\) fixation as *T. alexandrinum* inoculated with TA1; the other was ineffective. However, both field strains competed poorly against TA1 to form nodules. Competition was also tested by applying TA1 as seed inoculation in three rates (0, normal and 1/10 of normal) and by applying *gusA*-marked strains to soil in quantities of 0, 10\(^2\), 10\(^4\) and 10\(^6\)/g soil. Increasing both density of rhizobia in the soil and reducing seed inoculant reduced the number of nodules formed by strain TA1 in the top 5cm of root. The proportion of *gusA*-labelled:TA1 strains in the top 5cm of root varied enormously. It was concluded that the ability of the dominant field strains to colonize nodules was most likely to be due to poor inoculant survival, leading to greater numbers of naturalised rhizobia in the soil, compared to inoculant rhizobia.

The results of this study are pertinent to the evaluation of alternative clovers for low rainfall, alkaline soil environments. The results are discussed in terms of the influence that naturalised populations of rhizobia will have upon the nodulation, growth and N\(_2\) fixation of alternative clovers in alkaline soils.