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

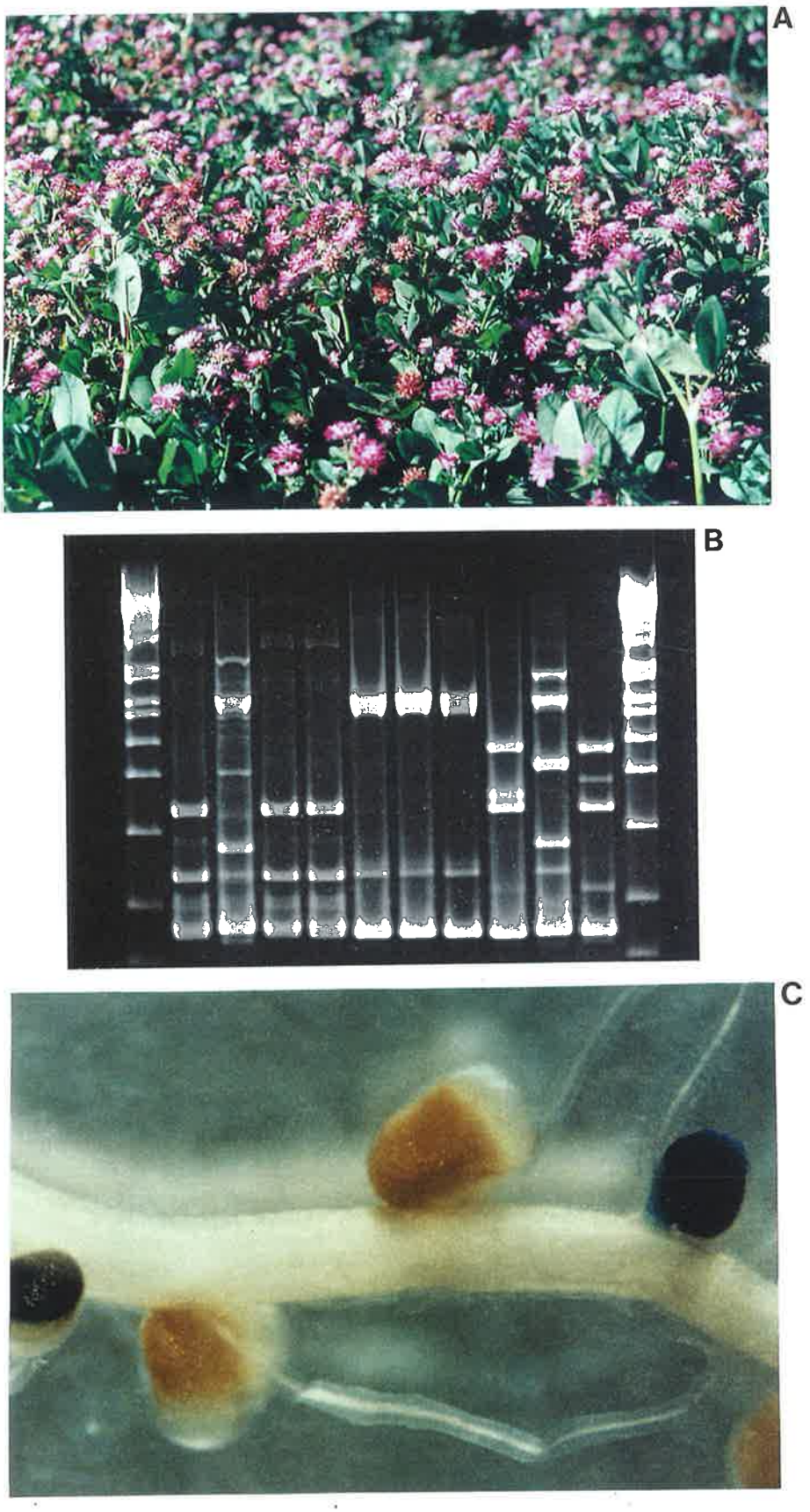

Fig. I (A) Persian clover (*Trifolium resupinatum* cv. Nitro) growing at the Roseworthy field site (Chapter 4); (B) amplification of *R. leguminosarum* bv. *trifolii* DNA from commercial strains and from nodule isolates at the Roseworthy field site using the RP01 primer (see Chapter 5 for details); (C) Berseem clover (*Trifolium alexandrinum* cv. Elite II) nodules occupied by commercial strain TA1 (pink) and blue nodules formed by rhizobia that contain the *gusA* gene, indicating the presence of β-glucuronidase (Chapter 6).

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 Rhizobia 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 N_2 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 Rhizobia 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 Rhizobia 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

4.4.1 The Influence of Inoculation and Nitrogen Addition on Pasture Growth and Nodulation 98
4.4.2 The Influence of Inoculation and Nitrogen Addition on N Accumulation 99
4.4.3 Nitrogen Fixation by Naturalised Populations of Rhizobia 101
4.4.4 Measurements of N_2 Fixation by ^{15}N Natural Abundance 102
4.5 Conclusion *103*

Chapter 5: Competition for Nodule Occupancy between Inoculant and Naturalised Rhizobia in a Field Environment 105

5.1 Introduction *105*
5.2 Methods *106*
5.2.1 General 106
5.2.2 Collection of Rhizobia from Nodules 106
5.2.3 Authentication 107
5.2.4 DNA Extraction 107
5.2.5 Polymerase Chain Reaction Methods 107
5.2.6 Optimization 108
5.2.7 Controls 109
5.2.8 Determination of Multiple Occupancy in Nodules 110
5.2.9 Gel Products 110
5.3 Results 111
5.3.1 Characterization of Inoculant Strains 111
5.3.2 Determination of Multiple Occupancy of Nodules 112
5.3.3 Nodule Occupancy 113
5.3.4 Confirmation of Nodule Isolates Using ERIC Primers 116
5.3.5 Common Field Isolates 117
5.4 Discussion *120*
5.4.1 Nodule Occupancy 120
5.4.2 Implications of Nodule Occupancy 123
5.4.3 Multiple Occupancies 126
5.4.4 Field Dominant Strains 127

Chapter 6: Competition between Commercial and Naturalised Soil Rhizobia for Nodule Occupancy. 130

6.1 Introduction *130*
6.2 Methods *132*
6.2.1 Strains, Plasmids and Growth Conditions 132
6.2.2 Minitransposon Mutagenesis of R. leguminosarum bv. trifolii 132
6.2.3 Plant Growth Conditions 134
6.2.4 Experiment 1: Effectiveness of Mutant Strains. 134
6.2.5 Experiment 2: The Ability of Mutant Strains to Compete Against Wild Types for Nodule Occupancy 135
6.2.6 Experiment 3: Competitive Abilities of Commercial and Naturalised Rhizobia 136
6.2.7 Experiment 4: Competitiveness of a Soil-Applied Mutant Strain Against a Seed-Applied Commercial Strain in Forming Nodules 137
6.2.8 Determination of Nodule Occupancy 138
6.2.9 Assessment of Competitiveness for Nodulation 140
6.2.10 Statistical Analysis 140
6.3 Results 141
6.3.1 Minitransposon Mutagenesis of R. leguminosarum bv. trifolii 141
6.3.2 Experiment 1: Effectiveness of Mutant Strains 143
6.3.3 Experiment 2: The Ability of Mutant Strains to Compete Against Wild Types for Nodule Occupancy 150
6.3.4 Experiment 3: Competitive Abilities of Commercial and Naturalised Rhizobia 155

6.3.5 Experiment 4: Competitiveness of a Soil-Applied Mutant Strain Against a Seed-Applied Commercial Strain in Forming Nodules 159
6.4 Discussion *167*
6.4.1 Competition Using gusA-Marked Strains 167
6.4.2 Nodule Occupancies 167
6.4.3 Implications of these Nodule Occupancy Studies 169
6.4.4 Assessment of Nodulation Competitiveness 174
6.4.5 Future Use of GUS in Rhizobial Ecology 176

Chapter 7: General Discussion 177

7.1 Discussion of Results 177
7.2 The Influence of Naturalised Populations of R. leguminosarum bv. trifolii in Alkaline Soils of Southern Australia 179
7.2.1 Introduction 179
7.2.2 Naturalised Populations of R. leguminosarum bv. trifolii 180
7.2.3 Inoculation of Alternative Clovers with Commercial R. leguminosarum bv. trifolii in the Field 181
7.2.4 Recommendations for the Production of Alternative Clovers 182

References 186
Appendix A 210
Appendix B 215
Appendix C 217
Appendix D 218

Abbreviations Used in Text

A	Absorbance
AAR	Average annual rainfall
bp	Base pairs
DAS	Days after sowing
dff	Days until first flower
DM	Dry matter
DMSO	Dimethyl sulphoxide
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylenediamine tetracetic acid
EtBr	Ethidium bromide
g	Gram
GUS	β–glucuronidase
gusA	The gene that expresses β–glucuronidase
kb	Kilobase
L	Litre
M	Molar
mg	Milligram
mL	Millilitre
MPN	Most Probable Number
OD	Optical density
PCR	Polymerase chain reaction
SDDW	Sterile double distilled water
Tris	Tris (hydroxymethyl) aminomethane
v/v	Volume per volume
XGlcA	5-bromo-4-chloro-3-indolyl β-D-glucuronide, cyclohexylammonium salt

Summary

The majority of southern Australian farming systems rely upon biological N_2 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 N_2 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 N_2. 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 $CaCO_3$ 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_2 fixation of alternative clovers (*T. alexandrinum*, *T. purpureum* and *T. resupinatum*) was tested on alkaline soils. Addition of commercial rhizobia or 200kgN/ha failed to increase biomass above uninoculated treatments. While *T. purpureum* produced the greatest biomass, it had low shoot N concentration (2.06-2.24%). *T. resupinatum* had a higher shoot N concentration (2.52-2.66%), and fixed a greater amount of N_2 than *T. purpureum*, (77kg fixedN/ha cf. 56kg fixedN/ha, Roseworthy; ^{15}N natural abundance technique) nodulating well with the naturalised populations of rhizobia. All clovers fixed N_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. *R. leguminosarum* bv. *trifolii* were accurately identified at the strain level using the primer RP01, which is directed towards the *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 *T. alexandrinum* up to 39% at the Roseworthy site in the first year. Strains CC2483g (commercial strain for *T. resupinatum*) and WSM409 (commercial strain for *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 *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 pertinant 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.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying.

Matt Denton.

October 2000.

Acknowledgements

I would like to thank my supervisors Prof. David Coventry, Dr. Bill Bellotti and A/Prof. John Howieson for providing insightful direction, continual support, friendship and encouragement through the course of the project. In particular, I am grateful to David for providing much wisdom and encouragement to enable me to complete this project with confidence. Bill provided the initial interest in pasture research and provided encouragement from near and far (while on sabbatical).

I am especially grateful to John for allowing me to work in his laboratory at the Centre for *Rhizobium* Studies, Murdoch University for two months to learn techniques of rhizobiology and PCR analysis. I am thankful to Bart McGann and Monika Zaklouta for input into that learning process.

I am also very grateful to Dr. Peter Murphy, in the Department of Applied and Molecular Ecology, for allowing the use of his laboratory, and facilities for the PCR work and GUS competition work for the project. Furthermore, I thank John DeMajnik, Keith Heinrich, Oka Karyanto, Aaron Mitchell, Chike Ouwama and Reza Zareie and others in the Department of Applied and Molecular Ecology for helpful discussions and friendship.

I would like to make a special note of appreciation to Dr. Wayne Reeve, Centre for *Rhizobium* Studies, Murdoch University, who provided great encouragement and expertise in the development and methodology for Chapter 6.

I would like to acknowledge the input that I have received from other nitrogen fixation researchers in Australia: Dr. Jeff Baldock, Mr. Ross Ballard, Mr. John Brockwell, Mr. Nigel Charman, Dr. Alan Richardson, Ms. Jo Slattery, Dr. Janice Thies and Dr. Murray Unkovich, for providing

helpful advice along the way.

I am indebted to many people for assistance: David Mathew helped in many financial aspects of the project, Colin Rivers (Soil Science) assisted with soil analysis and Marta Kasper and Caroline Versteeg provided technical assistance. Trevor Hancock and Michelle Lorimer gave useful statistical input for the project and Ursula Langridge provided maintenance of the PC2 glasshouse at the Waite Institute. Maurice Amato is thanked for mass spectroscopy analysis of ^{15}N isotopes. Annette Anderson, Ali Farhoodi and other postgraduates and staff at Agronomy and Farming Systems provided encouragement and a great working environment.

I am indebted to the Grains Research and Development Corporation who funded the project and, in addition, provided funds for travel to the 12th International Congress on Nitrogen Fixation in Brazil, 1999. I am also thankful to the A. W. Howard Memorial Trust for providing further funds to travel to the same conference.

I would like to thank my family and friends for their interest and encouragement during my studies.

Finally, a note of great appreciation to my long-suffering partner Ruth. Thanks for your continual support, patience and unconditional love.

Chapter 1

Introduction

1.1 Australian Pastures

Pastures in Australia form a substantial proportion of agricultural land and are one of the country's greatest resources. Over 460Mha (60% of the land surface) of natural grasslands and sown pastures are used for animal production and other purposes (Kemp and Michalk 1993), directly and indirectly generating $12 billion per annum for Australia through wool, meat and dairy production (Ockwell 1990). Apart from livestock production, pastures improve soil fertility necessary for crop production and help to maintain soil structure, halting land degradation (Reeves 1987).

In Australia, a large variety of legumes are utilized for temperate and tropical pastures. The majority of these temperate legumes originate in the Mediterranean and have shown excellent adaptation to Australian edaphic conditions (Crawford 1983; Crawford *et al.* 1989).

1.1.1 Annual Medic Pastures

Annual medics (*Medicago* spp.) are the dominant annual legumes of alkaline soils in southern Australian low rainfall (250-800mm AAR) environments (Puckridge and French 1983; Carter 1987). Annual medics have a high degree of hard-seededness, which has allowed the development of a farming system known as a "ley" where the rotation consists of alternating crops (often cereals) and legume pastures, each for a varying number of seasons (Reeves and Ewing 1993). The hard-seededness allows some medic seed to remain dormant in the soil through

the cropping phase until the following pasture phase, when a proportion of the seed population provides a regenerating pasture (Carter 1987). This system, in contrast to a continuous cropping system, provides opportunities for weed management, pest and disease control, build up of soil fertility and greater diversification of enterprises (Reeves 1987; Reeves and Ewing 1993).

1.1.2 The Need for Pasture Diversity

The range of annual legume pasture species commercially available for low rainfall alkaline soils in southern Australia has, until recently, been restricted to a few medic (*Medicago*) species (*M. littoralis, M. truncatula, M. polymorpha*; Crawford *et al.* 1989; Oram 1993). This limited range of legume genetic resources has restricted biological N_2 fixation, because the legumes grown have not always been well adapted to edaphic conditions (Cocks 1995; Howieson *et al.* 2000b). Concurrently, a decline in the productivity of annual medic pastures has been suggested and there is an interest in changing cropping rotations to favour longer phases of cereal crops, interspersed with phases of legumes (Denton and Bellotti 1996; Coventry *et al.* 1998; Howieson *et al.* 2000b). There is a recent emphasis to redress this limited legume diversity through Australian legume genetic resources and breeding programs. A huge array of annual legumes is currently being evaluated with the expectation that this diversity will provide alternative legumes to benefit Australian pastures.

Many clover (*Trifolium*) species have not been commercially grown in low rainfall environments of Australia, yet could provide additional plant genetic diversity for annual pastures. Many of these "*alternative*" clovers (eg. *T. resupinatum, T. michelianum, T. purpureum*) have been collected from low rainfall (<375mm), alkaline soil sites in the Mediterranean, and may fulfil niches which are traditionally designated for *Medicago* spp. In addition, there are a number of naturalised small-seeded clover species (eg. *T. glomeratum, T. tomentosum*) that are widespread in Australia and

show tremendous adaptation to southern Australian conditions (Fortune *et al.* 1995) although their productivity is just beginning to be understood (Bennett 1997). Many of these clovers are *aerial seeding*, producing seed heads that are raised up above the shoots, in contrast to subterranean clover, which buries its seeds below ground. Aerial seeding provides an opportunity for environmentally sustainable harvesting, in contrast to harvesting seeds produced close to the soil (eg. annual medics) which is often destructive to the soil structure. Some alternative clovers (eg. *T. purpureum, T. resupinatum*) have other characteristics which may make them suitable for Australian agriculture including: deep rooting morphology, small seeds (<1mg), efficient reproduction, early maturity, efficient water use, many seeds/pod, varying hardseededness and providing a range of shoot morphologies (Cocks 1995).

1.1.3 Rhizobial Requirements for Alternative Clovers

All legume species used in Australian agriculture have been introduced either purposefully or by accident, from overseas. Similarly, most economically important species of *Rhizobium* / *Sinorhizobium* / *Bradyrhizobium* are exotic to Australia. Pasture legumes often require specific strains of rhizobia to nodulate and fix nitrogen effectively (Brockwell and Hely 1966; Ballard 1996). Hence, the introduction of a new pasture species requires that a suitable microsymbiont also be introduced if no suitable strain exists within the target soil environment (Howieson *et al.* 2000a). Newly introduced pasture legumes may either 1) fail, 2) not reach production potential, or 3) be overlooked if suitable rhizobia are not provided (Bowman *et al.* 1998). The introduction of aerial seeding clovers into agriculture will therefore necessitate suitable *Rhizobium leguminosarum* bv. *trifolii* being available to fix sufficient nitrogen.

1.1.4 The Influence of Edaphic Factors and Resident Soil Rhizobia on the Introduction of Rhizobial Inoculants

Medics are adapted to alkaline soils (pH 7-9) and in these environments greater legume diversity is being sought. Soil environments have a profound effect upon rhizobial colonization (Bushby 1982) and while low soil pH is often a limiting factor for rhizobial colonization of soils (Howieson and Ewing 1986; Brockwell *et al.* 1991; Howieson *et al.* 1992b), there is currently little understanding of how high pH and alkaline soil reaction influences the colonization and persistence *of R. leguminosarum* bv. *trifolii*.

Additionally, the presence, size and effectiveness of *R. leguminosarum* bv. *trifolii* populations in low rainfall, alkaline soil environments is currently not known. Some indications are that *R. leguminosarum* bv. *trifolii* may be widespread in some areas (Ballard 1993) and it is likely that naturalised rhizobial populations may form a significant barrier to the introduction of highly effective rhizobial strains, as occurs in other systems (Streeter 1994; Howieson and Rome 1996).

1.2 Aims and Structure of this Thesis

1.2.1 Aims

The purpose of this thesis is to address the question:

What role do naturalised soil populations of *Rhizobium leguminosarum* bv. *trifolii* have on the successful introduction of rhizobial inoculants for alternative clovers on alkaline soils?

The hypothesis is that:

Naturalised *Rhizobium leguminosarum* bv. *trifolii* populations will form a barrier to the successful introduction of rhizobial inoculants on alkaline soils.

1.2.2 Structure of this Thesis

A flow chart, indicating the organization of the literature review and the order in which questions are addressed in this thesis, is provided in Fig. 1.1.

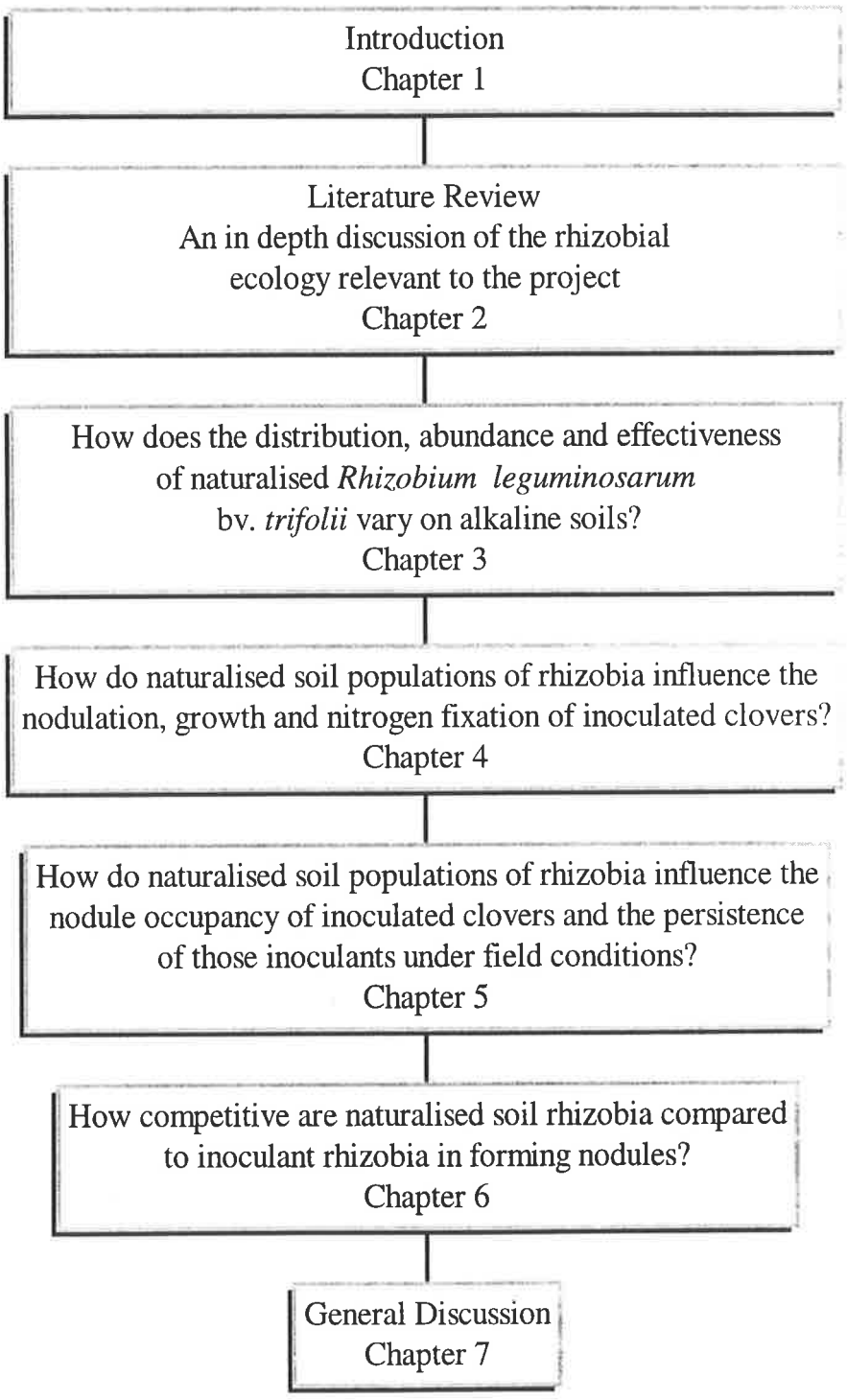

Fig. 1.1 Thesis Structure

Chapter 2

Literature Review

2.1 Introduction

The purpose of this review is to 1) provide background information to later chapters, 2) clarify our current understanding of rhizobial ecology and 3) determine the areas of rhizobial ecology in which our understanding is lacking. The biology of rhizobia, biochemistry and genetics of N_2 fixation and quantities of N_2 fixed in farming systems are background to chapters 3 to 6 and will be discussed briefly. Rhizobial ecology in field situations will be discussed in more detail and some techniques relevant to this research area will be described. At the end of this discussion, the aims of the project will be presented.

2.2 Establishment of Symbiotic Nitrogen Fixation in Legumes

2.2.1 Introduction

The establishment of biological N_2 fixation occurs through the symbiotic cooperation of legumes and rhizobia, which ultimately produces a unique structure, the nodule, following a complex series of signals. Bacteria within the nodule (bacteroids) reduce N_2 gas into ammonia, which is then exported to the plant for assimilation into proteins. The legume provides sugars to the bacteria, which are used as substrates. The process is regulated by the coordinated expression of more than 50 symbiotic genes.

2.2.2 Legumes

Fabaceae (Leguminosae), is the third largest family of angiosperms, and

includes annual herbs and large trees and is separated into three subfamilies: Mimosoideae, Papilionoideae and Caesalpinioideae with 674 genera (Allen and Allen 1980). The majority of legumes are able to fix N_2 when nodulated with suitable rhizobia: most species in the Mimosoideae (eg. *Acacia*), and Papilionoideae (eg. most agronomically important species). Only restricted species in the Caesalpinioideae (eg. *Cassia*) can fix N_2 (De Faria *et al.* 1989). Only one non-legume, *Parasponia*, can form symbiotic root nodules with a rhizobia (*Bradyrhizobium*) (Long 1989).

2.2.3 Rhizobia

Rhizobia are classified in the family Rhizobiaceae and are gram-negative flagellated bacteria 0.5-0.9μm wide and 1.2-3.0μm long. Rhizobia are able to form root and occasionally stem nodules (Loureiro *et al.* 1994) on legumes (Paul and Clark 1996). These diazotrophic bacteria are heterotrophic and microaerobic, only fixing nitrogen at less than half atmospheric oxygen when in symbiosis with legumes.

There are currently seven genera in the Rhizobiaceae family: *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Agrobacterium*, *Phyllobacterium*, *Mesorhizobium* and *Sinorhizobium*, the last two genera having only been recently separated from *Rhizobium* (van Berkum and Eardly 1998). *Agrobacterium* and *Phyllobacterium*, however, do not participate in symbiotic association with legumes. Based on extensive phenotypic and genetic characterization, *R. trifolii*, *R. phaseoli* and *R. leguminosarum* were reclassified as *R. leguminosarum*. Subspecific ranks (biovars *trifolii*, *phaseoli* and *viciae*) are used to distinguish plant affinities (clovers, beans, peas) (Jordan 1984). The most widely studied genera *Rhizobium* and *Bradyrhizobium* are pragmatically characterized as the fast-growing (acid producing, forming colonies of 2-4mm in 3-5 days) and slow-growing (alkali producing, colonies not exceeding 1mm after 5-7 days), respectively. All symbiotic N_2 fixing genera will collectively be referred to

as "rhizobia" through this review, unless specified.

2.2.4 Nodule Formation

Nodulation involves the attraction of rhizobia to roots through bi-directional signaling, the formation of an infection thread and the eventual formation of a nodule, in which N_2 fixation occurs. While the mechanisms of this interaction have been comprehensively studied (Brewin 1991; Hirsch 1992), only a fundamental description will be provided in this discussion.

Rhizobia are flagellated and move towards attractants (root exudates, amino acids, sugars and carbohydrates) (Bergman *et al.* 1988; Triplett 1990b) and away from irritants (bacteriocins) as demonstrated by testing motile and nonmotile mutant rhizobia (Ames and Bergman 1981). Legumes exude a range of flavonoids, such as luteolin and naringenin, which induce rhizobial nodulation (*nod*) genes (Phillips *et al.* 1994) although some flavonoids, such as umbelliferone, can inhibit *nod* genes on particular legume hosts (Djordjevic *et al.* 1987). Nodule initiation follows the bi-directional interaction of an appropriate flavonoid from the legume and nod genes. The nod factor is the product of the expression of *nodABC* genes, produced after *nodD* has interacted with flavones (Brewin 1991; Hirsch 1992) (Fig. 2.1), and is one component that determines host specificity.

Rhizobia then proliferate on the root surface and attach to root hairs by mechanisms involving lectins; another mode of host specificity (Bohlool and Schmidt 1974; Dazzo *et al.* 1993). Host attachment is aided by the presence of exopolysaccharides and lipopolysaccharides (Brewin 1991) and calcium (Caetano Anolles *et al.* 1989). After rhizobial attachment, the root hair begins to deform and curl, and an infection thread is then formed by enzymatic dissolution of the root-hair cell wall (Djordjevic and

Weinman 1991). The plant synthesizes new material to encase the invading rhizobia and the plant cell nucleus controls the ingrowth of the infection thread (Djordjevic and Weinman 1991; Hirsch 1992). Rhizobia are then released into cortical cells from the infection thread, penetrate intercellular spaces and proliferate (Brewin 1991). The rhizobial cells then become larger and lose their previous rod-shape, developing enzyme complexes required for N_2 fixation and become *bacteroids* (Werner 1992). Up to 2×10^4 bacteroids can be found per root cell (Bergersen and Goodchild 1973). The bacteroids in nodules are enclosed singly or in bundles by a bacteroid membrane, a peri-bacteroid membrane and the plant-derived plasmalemma.

2.2.5 Nitrogen Fixation

2.2.5.1 General Requirements

The plant membrane envelopes are biochemically important in controlling the diffusion of oxygen and substrates between plant cells and bacteroids. Bacteroids are completely reliant upon the plant for nutrients to maintain cellular function (Rosendahl *et al.* 1991).

The conversion of N_2 into ammonia requires the MoFe and Fe proteins of the nitrogenase enzyme, a source of electrons and a supply ofmgATP. C_4 carbon compounds from the legume cells (Dilworth and Glenn 1984) and possibly ferredoxin and flavodoxin (Atkins and Rainbird 1982) supply an electron supply for the nitrogenase. ATP is generated in the bacteroids in the presence of O_2, which poses a major problem for the N_2 fixation process, because O_2 irreversibly inactivates nitrogenase and suppresses its synthesis (Hill *et al.* 1981).

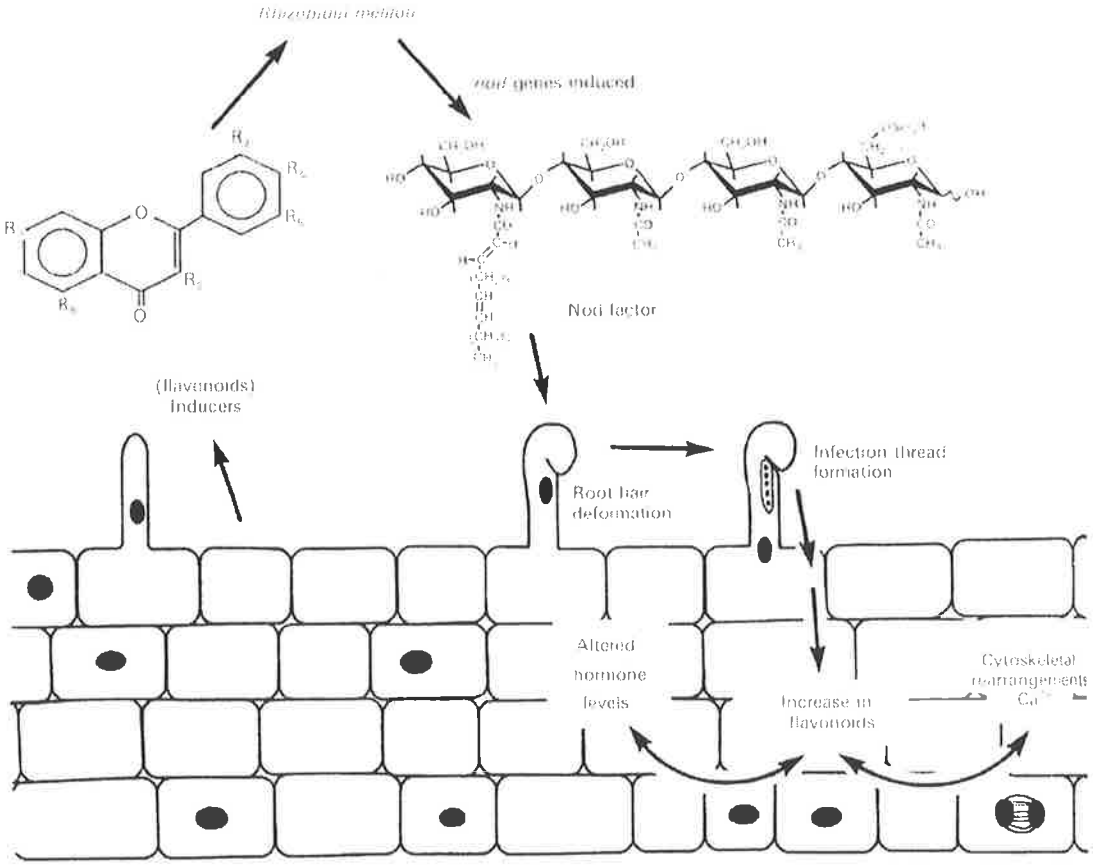

Fig. 2.1 Diagrammatic representation of nodule initiation indicating the signaling between plants and rhizobia as shown by the production of flavonoids by the legume and nod factors by the rhizobia (Hirsch 1992).

The problem is solved by leghaemoglobin (Lb) (Burris and Haas 1994), which binds with O_2 and facilitates O_2 diffusion through bacteroids at a low and buffered concentration. Lb delivers sufficient O_2 within bacteroids to allow for ATP production while maintaining O_2 concentration at the bacteroid surface sufficiently low to allow nitrogenase function (Bergersen and Goodchild 1973). Leghaemoglobin is derived from globin proteins, coded for by plant genes, and haem synthesis, which was traditionally thought to have been of bacterial origin (Dilworth and Glenn 1984). Recent evidence suggests that the haem group may be synthesized by the plant (O'Brian 1996). The precise location of leghaemoglobin in bacteroids is uncertain (Dilworth and Glenn 1984).

The N_2 fixation process produces hydrogen and utilizes 8 electrons:

$$N_2 + 16MgATP + 8H^+ + 8\ e^- \rightarrow 2NH_3 + 16MgADP + 16Pi + H_2$$

The production of H_2 is mediated by uptake hydrogenase and is thought to be of use in 1) reducing O_2 to protect nitrogenase, 2) for energy recovery (Atkins 1984) and 3) as an electron source (Robson and Postgate 1980).

2.2.5.2 Carbon Sources

Bacteroids utilize a wide range of carbon compounds for development: C_4 dicarboxyllic acids , C_6 and C_{12} sugars and amino acids, provided through the phloem (Vance 1997) (Fig. 2.2). These compounds, many of which are photosynthates, are contributed from plant cells and metabolised via the TCA cycle, glycolysis, the Entner Doudoroff pathway and gluconeogenesis (Rosendahl *et al.* 1991) (Fig. 2.2). Carbon compounds utilized by bacteroids are converted to sugars, cyclitols, organic acids and amino acids for use by the bacteroids (Dilworth and Glenn 1984). The theoretical amount of carbon used to fix N_2 has been estimated at 1.4–5.8 (g C expended/g N fixed) (Atkins 1984).

2.2.5.3 Nitrogen Assimilation

Fixation of N_2 by nitrogenase produces NH_3, which is assimilated into an organic form in the infected plant cells. Ammonia diffuses into plant cells and is metabolised by glutamine synthetase and glutamate synthase, which maintains the diffusion gradient (Vance 1997; Fig. 2.2). Amides (asparagine, glutamine) and ureides (allantoin, allantoic acid) are then transported in the xylem away from the nodule. Asparagine production is common for temperate legumes, whereas ureide production occurs in tropical species (Atkins 1987).

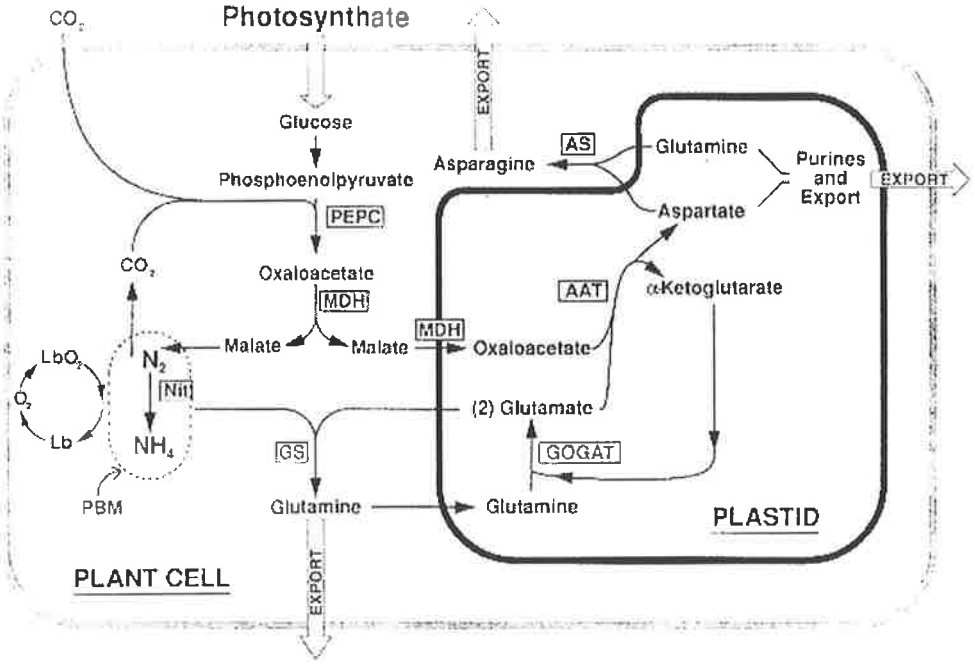

Fig. 2.2 A generalized scheme of carbon pathways and nitrogen assimilation in legume root nodules, including the enzymes involved. Glutamine, asparagine and ureides derived from purines are the primary nitrogenous compounds transported to other cells and plant organs. Photosynthate is used via glycolysis and the tricarboxylic acid (TCA) cycle to generate carbon skeletons for amino acid biosynthesis. Substantial carbon for amino acids can also be derived from non-photosynthetic CO_2 fixation via phosphoenolpyruvate. Enzymes involved are within blocks: AAT, aspartate aminotransferase; AS, asparagine synthetase; GOGAT, glutamate synthase; GS, glutamine synthetase; MDH, malate dehydrogenase, PEPC, phosphoenolpyruvate carboxylase; Nit, nitrogenase. Lb refers to leghaemoglobin (Vance 1997).

2.2.6 Genetics of Nitrogen Fixation

2.2.6.1 Genetic Organization of Nodulation and Nitrogen Fixation Genes

In addition to a chromosome, rhizobia possess large plasmids, which are circular DNA that replicate independently of the chromosome. The genes that enable *Rhizobium* and *Sinorhizobium* to recognize a plant host, to invade, to induce nodule formation and to fix atmospheric N_2, are located on the large Symbiotic plasmid (pSym) (Rolfe and Richardson 1987). In *Bradyrhizobium* the *nod*, *nif* and *fix* genes occur on the chromosome

(Long 1989). Most *Rhizobium* strains carry only one *Sym* plasmid and when this is removed they are unable to interact with their legume host. Genes required for metabolic pathways, energy production, electron transport, metabolite uptake systems, polysaccharide synthesis and some genes affecting the nitrogen-fixation phenotype are, however, located on the *Rhizobium/Sinorhizobium* chromosome (Rolfe and Richardson 1987).

2.2.6.2 Nodulation Genes

Genes involved in infection and nodule formation are the nodulation (*nod*) genes. The genes *nodA*, *nodB* and *nodC* are common for all rhizobia (including *Bradyrhizobium*) and produce the primary Nod factor structure. These genes are located adjacent to *nodD*, which regulates *nodABC* genes. Transfer of *nodDABC* genes between different *Rhizobium* stains does not inhibit nodulation, however, mutation in the genes stops root hair curling and halts nodulation (Long 1989). The *nodI* and *nodJ* genes are also common to all rhizobia and are transporters of Nod factors (Downie 1998).

In addition to *nodDABC*, over 50 nodulation genes have been identified, which are specific to particular species or genera of the Rhizobiaceae and these have been designated *nol* and *noe* (Downie 1998). The function of the gene products for many of these genes has been determined but for others it is unclear, possibly due to duplication of gene function. Some genes (*nodD*, *nodV*, *nodW*, *nolA*, *nolD*, *nolR* and *syrM*) appear to be involved in the regulation of other *nod* genes. Other genes are involved in: 1) biosynthesis and modification of lipo-chito-oligosaccharide Nod factors, 2) Nod factor secretion, 3) protein secretion and 4) genes of undefined function (Downie 1998). A number of these eg. *nodE*, *nodF*, *nodG*, *nodH*, *nodI*, *nodL*, *nodZ* are host specificity determinants.

2.2.6.3 Nodulation Factors

Nod factors, the products of *nod* genes, are made up of a backbone of β-1,4-linked *N*-acetyl glucosamine residues, which have a precursor of UDP-*N*-acetyl-D-glucosamine (Downie and Young 1996). Specific *nod* genes add further products to the Nod factor backbone, eg. glycosyl residues, sulfate, carbamyl, methyl or glycerol groups (Carlson *et al.* 1994) and confer specificity to the legume-*Rhizobium* interaction. Different species of Rhizobiaceae produce different Nod factors; up to 70 have been found for *Rhizobium* sp. NGR234 (Price *et al.* 1992). Nod factors are involved in root hair curling, infection thread development and cortical cell divisions (Long 1996; Pueppke 1996).

2.2.6.4 Genes Involved in Nitrogen Fixation

Genes for N_2 fixation in *Rhizobium* are divided into two groups: *nif* and *fix* genes. Genes homologous with the free-living N_2 fixing bacteria, *Klebsiella pneumoniae*, are *nif* genes; those that are restricted to symbiotic organisms are *fix* genes (Long 1989). The *nif* genes are located on the *Sym* plasmid 16–30 kilobases from the *nod* genes (Rolfe and Richardson 1987). The *nifH* gene encodes for the Fe-protein and the *nifD* and *nifK* encode the two subunits of the MoFe protein (Dean and Jacobson 1992). Ten *nif* genes have been found so far for *Rhizobium*. Full assembly of nitrogenase requires the product of other *nif* genes, in particular, *nifB*, *nifE* and *nifN*, which are required for the biosynthesis of the FeMo-cofactor (Kaminski *et al.* 1998). The *nifA* gene regulates all other *nif* genes and its function is itself regulated by oxygen concentration.

The *fixABCX* genes were originally discovered in *S. meliloti* and homologues have since been found in *B. japonicum*, *R. leguminosarum* and *Azorhizobium caulinodans*. The *fixABCX* genes are located close to

the *nif* cluster on the Sym plasmid (Kaminski *et al.* 1998). Both *nif* and *fix* mutants are able to cause nodule development, but the nodules do not fix nitrogen (Nod^+ Fix^-) (Long 1989).

2.3 Biological Nitrogen Fixation in Farming Systems

2.3.1 Introduction

Biological nitrogen fixation (BNF) by legumes makes a significant contribution to the productivity and sustainability of Australia's low input, extensive agriculture. Pasture and grain legumes are used extensively to provide for human and animal nutrition. In addition, the N supplied by BNF maintains cereal quantity and quality and provides N that would otherwise be considered unprofitable (Evans 1996). Biological N_2 fixation, in contrast to the use of N fertilizers, provides organic C and N inputs.

2.3.2 Quantities of Nitrogen Fixed

A number of techniques are used for determining the amount of nitrogen fixation of field or pot grown legumes (Peoples *et al.* 1993). Two widely used techniques for quantification of N_2 fixed are using ^{15}N isotopes either applied to crops and pastures (McNeill *et al.* 1998; Unkovich and Pate 1998) or the natural abundance ^{15}N technique (Pate *et al.* 1994; Unkovich *et al.* 1994). Most soils are enriched with ^{15}N, compared to atmospheric N_2, making it possible to estimate the amount of N_2 fixation by comparing the ^{15}N contents of the target legume and a non-legume accessing the same soil N pool (Unkovich *et al.* 1994). Soils in Australia are often sufficiently enriched with ^{15}N to provide an accurate estimate of N_2 fixation (Sanford *et al.* 1993; Unkovich *et al.* 1997).

The formula for the amount of N_2 fixation derived from the atmosphere ($\%N_{dfa}$) determined using the ^{15}N natural abundance technique, uses the equation:

$$\% N_{dfa} = (\delta^{15}N \text{ reference plant} - \delta^{15}N \text{ legume}) / (\delta^{15}N \text{ reference plant} - B)$$

where *B* refers to the $\delta^{15}N$ value of the effectively nodulated legume grown in media totally lacking combined nitrogen (Unkovich *et al.* 1997).

Quantities of biological nitrogen fixed (QBNF) measured by the above equation are usually determined by:

$$QBNF = LDM \times NC \times PF$$

where, LDM is legume dry matter, NC is the nitrogen content of the dry matter and PF ($=\%N_{dfa}$) is the proportion of the nitrogen derived from N_2 fixation (Evans 1996). Some estimates for amounts of nitrogen fixed are given in Table 2.1. Proportions of N_2 fixed ($\%N_{dfa}$) vary markedly depending upon soil nitrogen levels and the effectiveness of nodulation. The gross amount of N_2 fixation achieved is highly dependent upon the legume dry matter (Rifkin *et al.* 1999b) and the proportion of nitrogen fixed (Unkovich *et al.* 1997); nitrogen content remains relatively constant and has little influence in QBNF (Evans 1996).

2.3.3 Benefits of Nitrogen Fixation

Biological N_2 fixation is a renewable resource of N, compared to nitrogenous fertilizers, and if legume crops are managed well, BNF forms an economical means of increasing soil N fertility (Peoples *et al.* 1995a). The nitrogen contribution of BNF made to the soil pool of nitrogen is given by:

$$\text{N-balance} = (N_2 \text{ fixed}) - (\text{harvested N})$$

Table 2.1 Range of estimates of the proportions $\%N_{dfa}$ and amounts of plant nitrogen derived from fixation for Australian crops. This table refers to above ground biomass only.

Species	N yield (kg/ha)	$\%N_{dfa}$	Amount Fixed (kgN/ha)	Reference
Subterranean clover	-	50-96	2-238	(Peoples and Baldock 1999)
	3-261	0-100	0-188	(Unkovich *et al.* 1997)*
Balansa clover	-	61-72	66-111	(Rifkin *et al.* 1999a)
Persian clover	-	53-57	31-51	(Rifkin *et al.* 1999a)
Annual medics	-	51-99	2-109	(Peoples and Baldock 1999)
Lucerne	390-420	44-80	172-336	(Peoples *et al.* 1992)
Field Peas	143-191	20-92	28-177	(Evans *et al.* 1989)
	61-227	31-95	26-183	(Unkovich *et al.* 1997)*
Lupins	57-120	60-78	34-97	(Evans *et al.* 1989)
	53-322	29-97	30-283	(Unkovich *et al.* 1997)*
Chickpeas	83-146	37-86	43-124	(Unkovich *et al.* 1997)*
Soybeans	250-255	25-70	62-178	(Peoples *et al.* 1992)

* from a number of references cited in (Unkovich *et al.* 1997).

which is the amount of nitrogen derived from fixation after seed or biomass have been removed from the system. This measure is usually associated with crop legumes. Nitrogen derived from BNF is highest in seed pods compared with any other part of legumes (Ladd *et al.* 1986; Peoples *et al.* 1995b). This calculation does not consider losses of N from the system through volatilization, leaching or burning of stubbles (Peoples *et al.* 1994) but is still a useful tool in determining the fluxes of nitrogen from a legume in any particular year.

Nitrate in the soil is often enhanced during and after a legume crop due to reduced nitrate use or "nitrate sparing" that occurs when legumes fix nitrogen and produce more nitrogen than is lost in seed grain (Herridge *et al.* 1995). Soybeans grown after cropping fixed proportionally more nitrogen (PF: 67 cf. 34%) compared with those grown after fallow (Table 2.2). This resulted in an appreciable difference in N-balance (Bergersen *et al.* 1985).

Table 2.2 Effect of cropping sequence upon the potential residual value of fixed N after seed harvest (from Bergersen *et al.* 1985)

Previous Cropping Sequence	Crop N Total (kgN/ha)	N_2 Fixation PF	N_2 Fixation Amount (kgN/ha)	Seed N (kgN/ha)	Residual Benefit (kgN/ha)
fallowed	417	34	143	187	-44
cropped	363	67	244	205	+39

Only recently has the accumulated N invested below ground in roots, nodules and root exudates been assessed (McNeill *et al.* 1997). Crop legumes typically invest 40–50% of total plant nitrogen into below ground structures, with chickpeas investing up to 25% of total nitrogen in nodules (Unkovich and Pate 2000). Annual medic species typically invest only 25% of total nitrogen in below ground structures, the majority in roots (A. McNeill, unpublished data). The recovery of the total root system is difficult and below ground N from main roots comprises only 30-62% total N, the rest being lost via fine roots and exudates (McNeill *et al.* 1997). Typically, the below ground input of nitrogen from roots, nodules and root exudates has been ignored for calculations of BNF in field studies. Therefore the amount of inputs of N from legumes, particularly pulses has been substantially underestimated.

2.3.4 The Fate of Fixed Nitrogen

Nitrogen derived from fixation is made available to non-legumes upon breakdown of the legume residues, mediated by soil organisms, soil moisture and soil temperatures. Work from Ladd *et al.* (1986) has shown that decay of legume residues is a slow process: about 30% of the organic matter remained in the soil after 4 months declining to 11% after 8 years. Legume N showed an even slower release: after 0.3 years two thirds of the nitrogen remained and after 8 years one third of the nitrogen remained. Legume N is therefore a sustainable N source to provide for future crops.

Apart from the breakdown of residues, the major limitation to utilization of fixed N is limited uptake of N from legume residues. Plant (cereal) uptake of fixed N from soils is often only 6-28% of organic residues (cf. 41-50% using fertilizer N) because much of the N is immobilized in microbial biomass or semi stable organic materials (Ladd *et al.* 1986; Peoples *et al.* 1995a). While these rates appear to be small, losses of biologically fixed N from plant and soil pools (generally <15%) are significantly less than losses associated with N fertilizers (30 -50%; Peoples *et al.* 1995a).

2.3.5 Techniques to Increase Biological Nitrogen Fixation

There are a number of factors that contribute to the BNF of pasture or grain legumes that can be manipulated to influence the amount of fixation. These manipulations generally alter the proportion of N_2 fixed (N_{dfa}) or increase legume dry matter production.

Increasing legume dry matter production has been achieved through application of general agronomic principles including: provision of optimum plant nutrition, soil amelioration with respect to low pH or nutrient constraints, management of timing of sowing, correct cultivar or species choice and grazing management to alter pasture plant density

and composition (Herridge and Holland 1992; Herridge and Danso 1995; Peoples and Baldock 1999).

Soil nitrate plays a significant role in limiting the fixation of legumes (Streeter 1985; Becana *et al.* 1989) and there are a number of strategies in managing soil nitrate to maximize the proportion of nitrogen that is fixed. When non-legumes are grown for sufficiently long periods, nitrate levels are reduced. This assists in maximizing BNF in the legume phases of the rotation (Alston and Graham 1982; Bergersen *et al.* 1985). Excessive cultivation can oxidize organic matter leading to high levels of soil nitrate. In contrast, direct drilling limits oxidization and the consequent reduction in nitrate availability can increase fixed N by up to 50kgN/ha (Herridge and Holland 1992). The selection of suitable cultivars and species can have a significant effect upon BNF due to innate or bred tolerances to soil nitrate. A number of legumes have been bred for high N_2 fixation in the presence of combined nitrogen eg. lucerne (J.Lamb, pers. comm.) and soybeans (Herridge and Danso 1995). Management of rhizobial populations can certainly improve BNF, often through improving the proportion of N_2 fixed and is the subject of the next section.

2.4 Competition for Nodulation in the Field

2.4.1 Introduction

Rhizobial competition for nodulation of legume roots in a field situation is complex, involving the interaction of rhizobial genomes, a plant genome and environmental factors (Dowling and Broughton 1986; Vlassak and Vanderleyden 1997). Competitive ability has been defined as the ability to occupy nodules of a suitable legume when challenged with simple mixtures or naturalised soil populations of rhizobia (Sadowsky and Graham 1998). It should be considered, however, that non-symbiotic (non-nodulating) rhizobia can constitute a substantial portion of soil rhizobia,

but should not affect competition for nodulation (Soberon-Chavez and Najera 1988; Sivakumaran *et al.* 1997; Hartmann *et al.* 1998). Segovia *et al.* (1991) for example, found 40 non-infective rhizobia for each nodulating isolate recovered.

Various techniques have been utilized to measure the competitive ability of strains, mostly under artificial conditions. A number of methods have compared the proportions of nodules formed by two competing rhizobia compared to the proportions of these rhizobia present in the inoculum (Amarger 1981b; Amarger and Lobreau 1982). The recognition of dual occupation of nodules by rhizobia increased the accuracy of assessment (Beattie *et al.* 1989). Other studies have compared the proportions of inocula represented on the root surface with corresponding nodule occupancies (Marques-Pinto *et al.* 1974; Labandera and Vincent 1975).

2.4.2 Saprophytic Competence

Rhizobia are considered facultative symbionts, adapted to existence in a free-living state in soil when a suitable legume host is absent for some years (Sanginga *et al.* 1994). This is unlike mycorrhizal fungi, which are obligate symbionts (Smith and Read 1997). Existence of rhizobia outside the nodule, in soil or in the rhizosphere of a plant has been deemed to be an important characteristic of persistence of rhizobia in soils (Howieson 1995) and has been labeled *saprophytic competence* (Chatel *et al.* 1968). Rhizobia exist outside of the nodule in bulk soil, but migrate toward the rhizosphere of a compatible legume, a non-host legume (Robinson 1967) or non-legume (Rovira 1978). The ability to survive in soil without the aid of a homologous host is most important in acid soils (Thornton and Davey 1984; Howieson 1995; Ballard 1996). The current understanding of saprophytic existence of rhizobial populations is, however, limited (Bottomley *et al.* 1991).

2.4.3 Motility of Rhizobia

Rhizobia are flagellated organisms and mutant rhizobia lacking flagella sometimes show reduced motility in soil (Soby and Bergman 1983; Parco *et al.* 1994), although not always (Liu *et al.* 1989; Zdor and Pueppke 1991). However, the movement of rhizobia by the use of flagella is restricted to continuous water-filled pore spaces (Hamdi 1971; Issa *et al.* 1993a; 1993b) and movement is restricted even when soil moisture is sufficient for legume germination (Hamdi 1971). Movement of rhizobia is also restricted in clay soils, compared to coarser textured soils, possibly due to the adsorption of rhizobia to clay particles (Hamdi 1971; Issa *et al.* 1993a). The movement of rhizobia has been shown to be a major limitation in colonizing nodules because rhizobial motility is unable to match root growth (McDermott and Graham 1989).

2.4.4 Speed of Infection

The speed of infection of root hairs by rhizobia (nodule forming efficiency) also impacts on nodulation competitiveness. Bhuvaneswari and coworkers found that the pre-emergent root hair zone of soybeans immediately above the root tip susceptible to infection for only 4 to 6 hours (Bhuvaneswari *et al.* 1980; 1981). This developmental constraint means that to be competitive, strains need to initiate and complete the complex sequence of infection events within a few hours. A number of authors have shown that the ability to form nodules quickly is correlated with nodulation competitiveness (Dowling and Broughton 1986; Sargent *et al.* 1987; McDermott and Graham 1990; Onishchuk *et al.* 1994). Autoregulatory responses are also thought to be activated, so that once sufficient nodules have been formed, nodulation is suppressed by the legume (Pierce and Bauer 1983; Kosslak and Bohlool 1984; Heron and Pueppke 1987; Stephens and Cooper 1988), which provides another constraint to rhizobia competing for nodule occupancy.

2.4.5 Legume Interactions with Rhizobia

Legume selection of rhizobia plays a significant role in determining the outcome of competition for nodulation (Brockwell and Hely 1966; Date and Brockwell 1978; Hardarson and Jones 1979; Amarger 1981b; Djordjevic and Weinman 1991; Materon 1991). The plant genome will often exert a strong selection pressure upon a mixed population of rhizobia so that not all rhizobial genotypes have an equal chance to nodulate (Triplett and Sadowsky 1992; Toro 1996; Thies *et al.* 2000).

Host preference for rhizobial strains has been recognized in the *Medicago-Rhizobium* symbiosis. Recently, *Sinorhizobium meliloti* was found to form two distinct groups (Eardly *et al.* 1990; Gordon *et al.* 1995; Brunel *et al.* 1996). Type 1 nodulated well with 5 *Medicago* species (including *M. rigidula*) but produced only rudimentary nodules on *M. polymorpha*, while type 2 was effective with *M. polymorpha* but not *M. rigidula* (Brunel *et al.* 1996). This type of ineffective symbiosis may be important in both competition for nodulation and N_2 fixation in the field.

Host restriction of nodulation is an extreme example of selectivity, when a plant is unable to nodulate with a strain of rhizobia. *M. laciniata*, for example showed restricted nodulation and N_2 fixation with a number of isolates from the Darling River basin; *M. sativa* was promiscuous (Brockwell and Hely 1966). A much quoted example of host restriction of nodulation is Afghanistan pea (*Pisum sativum*), which is unable to nodulate with most European strains of *R. leguminosarum* bv. *viciae*, even though it nodulates with many strains of Middle Eastern origin (Sadowsky and Graham 1998). Similarly, *T. subterraneum* cultivar Woogenellup is unable to nodulate properly with *R. leguminosarum* bv. *trifolii* strain TA1 (Gibson 1968; Gibson and Brockwell 1968).

A number of studies have suggested that host legumes optimize N_2

fixation through selection of rhizobia that are effective, from a background of rhizobia with varying effectiveness (Robinson 1969; George and Robert 1991). Conversely, another study showed that effective and (mutant) ineffective strains were equally competitive in nodulating lucerne (Amarger 1981a). The discrepancy in these reports has not been resolved. One explanation may be that legume-*Rhizobium* selectivity differs between legumes that nodulate with *R. leguminosarum* (Robinson 1969; George and Robert 1991) compared to *S. meliloti* (Amarger 1981a).

2.4.6 The Effect of Combined Nitrogen on Rhizobial Competition and N_2 Fixation

Soil nitrogen has a profound effect upon the competition of rhizobia for nodulation (Vlassak and Vanderleyden 1997). The presence of soil nitrogen, usually nitrate or ammonium, restricts nodulation in a number of ways. Combined N inhibits attachment of rhizobia to root hairs, and alters *nod* gene induction, Nod factor production and Nod factor excretion and a range of enzymes necessary for N_2 fixation (Streeter 1988; Becana and Sprent 1989; Wang and Stacey 1990; McKay and Djordjevic 1993).

While N_2 fixation is reduced in legumes by combined nitrogen, legumes differ in their tolerance to soil nitrate (Harper and Gibson 1984). Rhizobia also show tolerance to soil nitrate: strain WU95 in the presence of soil nitrate still achieved N_2 fixation at a level of 80% of a treatment without applied nitrate, while most naturalised strains achieved <50% (Unkovich and Pate 1998). The presence of nitrogen in the soil has the potential to reduce, delay or halt nodulation in the field (McNeil 1982). In particular, soil N may reduce nodulation by inoculant rhizobia, due to delayed early nodulation, but this phenomena requires further investigations (Unkovich and Pate 1998).

2.4.7 Introducing Rhizobia into Fields with a Naturalised Rhizobial Population

2.4.7.1 Characterization of Naturalised Populations

Rhizobia usually occur in low numbers in land that has not previously carried legumes (Sadowsky and Graham 1998). Where legumes are grown, rhizobial populations of <10 to 10^7/g soil (0-10cm) are commonly encountered, regardless of inoculation history (Sadowsky and Graham 1998). In southern Australia, the native legume flora nodulates with fast and slow growing rhizobia (Lawrie 1983; Lafay and Burdon 1998), although these bacteria are limited in their associations with crop and pasture legumes of Mediterranean origin (Lange 1961). Agriculturally important rhizobia in soils of southern Australia have been purposefully or accidentally introduced, as have their legume hosts. The resulting rhizobia found in agricultural soils are probably a complex of introduced and endemic types with a degree of genetic exchange between various components and other soil bacteria. Populations of rhizobia are therefore *naturalised* but probably not *native* or *indigenous*.

It is particularly important to gain an understanding of the rhizobia resident in soils, so that we can understand how genotypically and phenotypically diverse they are, how adapted to soil constraints they are, and how they interact with legumes. It is also necessary to know how we can utilize existing populations of rhizobia or introduce new rhizobia, to maximize the benefits of N_2 fixation from legume crops and pastures.

2.4.7.2 Inoculation Methodology

Inoculation of legumes has, as its primary aim, to deliver rhizobial inoculants to a legume such that the possibility of forming nodules is maximized. The necessity for inoculation is not always straightforward and has been defined by different researchers as depending on: legume specificity, the history of growing legumes in the soil, the time elapsed since similar legumes were previously grown, the amount of soil nitrate and the size of the naturalised rhizobial population (Thies *et al.* 1991b; Brockwell *et al.* 1995). A number of inoculant carriers have been utilized for rhizobia, the most common being gamma sterilized peat. Alternatives to peat have been tested: polyacrylamide gel, polymers, cellulose gels, lyophilized cultures of rhizobia in vegetable oil, and vermiculite, although only vermiculite and peat have been accepted commercially (Brockwell *et al.* 1995). Inoculant carriers need to retain enough moisture and contain no inhibitory chemicals in order to achieve a rhizobial density of $\geq 1 \times 10^9$ cells/mL.

Peat inoculants are often applied directly to seeds with a cellulose sticking agent. A number of criteria should be met in order that seed inoculants remain viable. Hazards to rhizobial longevity include: drying, exposure to fertilizers and pesticides and detrimental soil factors. Lime pelleting inoculated seed often enhances survival of rhizobia and the addition of molybdenum can assist N_2 fixation in acid soils (Gault and Brockwell 1980; Coventry *et al.* 1985a; Evans *et al.* 1993). Application of rhizobia to legumes has also been achieved using solid inoculant or liquid inoculants (Ciafardini and Barbieri 1987); the success of the latter is often dependent on infiltration of the soil. Pre-inoculation of seeds is a further technique that has been utilized particularly well in New Zealand (Brockwell and Bottomley 1995) and has some commercial applications. Recently, the inoculation of tropical legumes has been successful with

freeze-dried inoculants, with excellent cell survival at 50°C, compared to peat inoculants (A. McInnes, pers. comm.). Current limitations in inoculant technology indicate a need for improved inoculant delivery to legume roots such that the best use of elite rhizobial strains is made.

2.4.7.3 The Importance of Inoculum Placement for Nodule Occupancy

Irrespective of the fact that rhizobia are motile, applied rhizobia are often insufficiently mobile to form nodules much below the crown region (0-5cm) of the root (Worrall and Roughley 1976; Ciafardini and Barbieri 1987; Beattie *et al.* 1989; McDermott and Graham 1989). Nodulation further down the root is generally restricted to naturalised rhizobia. A number of studies have indicated that rhizobia applied in a cover (water) inoculation (Ciafardini and Barbieri 1987; Ciafardini and Lombardo 1991) or post emergent water suspension (Danso and Bowen 1989) provided significantly greater nodule occupancy by inoculant strains further down the soil profile on the lower tap root and lateral roots.

Nodules that form on the lateral roots often contribute to a significant proportion of N_2 fixed by legumes, particularly late in the growing season (Hardarson *et al.* 1989; McDermott and Graham 1989). If inoculant rhizobia fail to nodulate on the lateral roots and lower sections of the taproot, then a loss of N_2 fixation potential may be experienced.

2.4.7.4 The Influence of Resident Soil Rhizobia on Inoculation Failure

Inoculation of legumes with rhizobia does not always increase shoot or grain yield (Thies *et al.* 1991a; Streeter 1994; Howieson and Rome 1996; Vlassak and Vanderleyden 1997). Often the inoculant fails to occupy a significant proportion of nodules (Thies *et al.* 1991a; 1991b), and there are a plethora of reasons for this (Dowling and Broughton 1986; Vlassak and Vanderleyden 1997; Sadowsky and Graham 1998).

In soils containing little or no established rhizobia, the induction of nodulation is usually feasible (Roughley *et al.* 1976; Brockwell *et al.* 1987; Howieson and Ewing 1989; Barran and Bromfield 1997; Hebb *et al.* 1998). Acid soils often contain low rhizobial populations (Brockwell *et al.* 1991; Carter *et al.* 1994) although *Bradyrhizobium* populations of 10^6 /g soil have been observed (Chatel *et al.* 1968). The problem of introducing rhizobia into these harsh soils (pH($CaCl_2$) <4.9) has been suitably overcome by the judicious use of acid-tolerant rhizobia from homologous soil environments (Howieson *et al.* 1991; Howieson and Rome 1996).

The problem of introducing rhizobia into more benign soils with established populations of rhizobia is entirely different. A typical soil population of 1 x 10^4 rhizobia will outnumber inoculant rhizobia per gram by 250:1 (Brockwell *et al.* 1995). Many soils provide aggressive competition for nodule occupancy which produce unacceptably low nodule occupancies of inoculant strains. In one large survey, the most significant environmental variable influencing competition for nodule occupancy was size of the indigenous rhizobial population (Thies *et al.* 1992). A number of studies have shown that large populations of rhizobia significantly reduce the chances of successful colonization of soils by inoculated rhizobia or displace the inoculant strains of rhizobia after some years (Singleton and Tavares 1986; Bottomley *et al.* 1990; Thies *et al.* 1991a; Streeter 1994; Evans *et al.* 1996).

Whether inoculation is at all necessary to meet the N demands of the legume will depend mostly upon the size and effectiveness of soil rhizobial populations, but also upon the soil nitrate reserves (Fig. 2.3). The N demand of the crop may be met by soil nitrate or nodulation by naturalised rhizobial populations, such that inoculation responses are not realized (Thies *et al.* 1991b). Symbiotic effectiveness of individual rhizobial populations is thought to be normally distributed, so that highly

effective or highly ineffective strains are not commonly encountered (Barran and Bromfield 1997). Assessment of naturalised strains shows a huge degree of diversity of N_2 fixation potential (Holding and King 1963; Rys and Bonish 1981; Mytton and Livesey 1983; Singleton and Tavares 1986) and a more holistic assessment of N_2 fixation potential from a whole soil inoculation is probably a more suitable approach (Brockwell *et al.* 1988b). The most critical problem occurs when naturalised rhizobia are less effective than commercial inoculant strains, so that N_2 fixation is limited by reduced nodulation of inoculant strains (Gibson *et al.* 1975; Bottomley and Jenkins 1992).

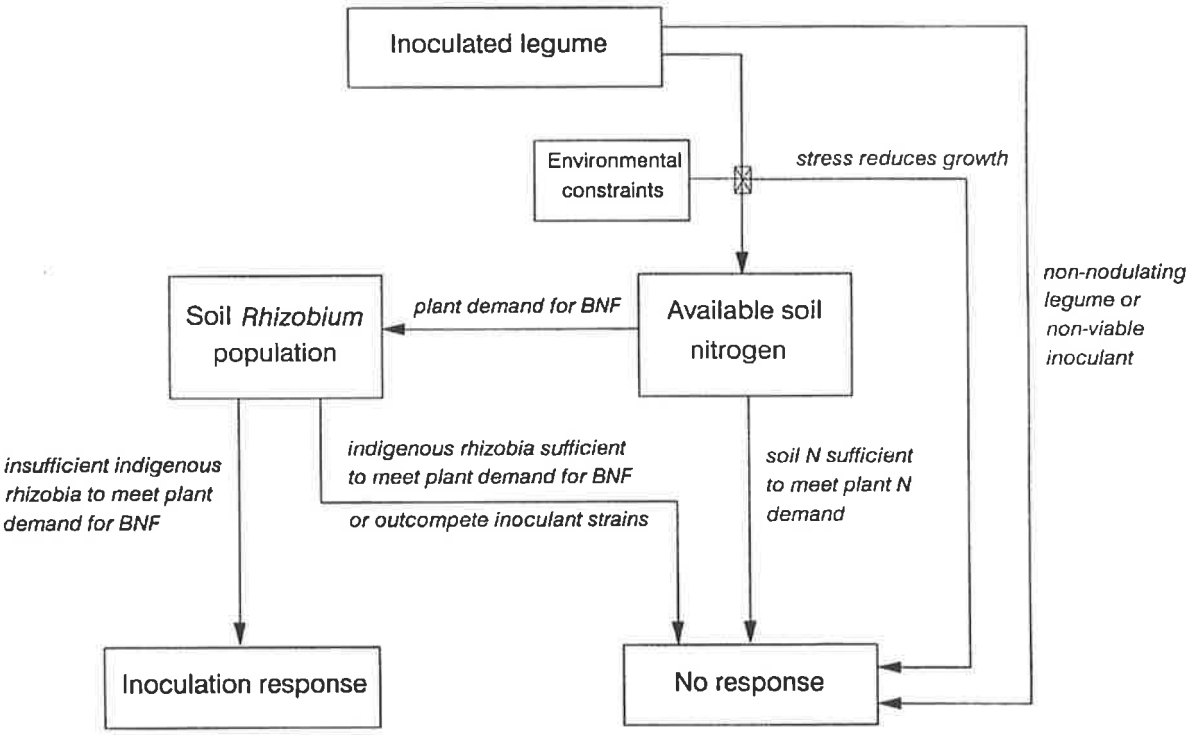

Fig. 2.3 The interaction of soil N and naturalised rhizobial populations in providing legume N requirements (Thies *et al.* 1991b).

A number of researchers have investigated rhizobial numbers in soils and seed inoculants to estimate whether an inoculation response will occur when sowing into a field. When faba beans and peas were inoculated and

sown into fields with background populations of <30, 537 and 1.7×10^5 *R. leguminosarum* bv. *viciae*/g soil, nodule occupation by inoculant rhizobia of 63-79%, <15% and <5%, respectively were achieved. From this data, it was estimated that inoculation responses would not occur with >1000 rhizobia/g soil (Evans *et al.* 1996). Weaver and Frederick estimated that legumes sown into soils with ≥1000 rhizobia/g soil were unlikely to be nodulated extensively by inoculant strains (Weaver and Frederick 1974a). They estimated that an inoculation rate of 1000 times the soil population would need to be used to achieve 50% of the nodules colonized by applied rhizobia (Weaver and Frederick 1974b). This is unrealistic for most soil populations (Brockwell *et al.* 1995). However, in other trials as few as 50 rhizobia/g soil eliminated an inoculation response; a minimum of 66% nodule occupancy and a doubling of nodule mass by inoculant rhizobia were required to significantly increase yield over uninoculated plots (Thies *et al.* 1991a).

Adding to the problem of establishing inoculant rhizobia into soils is that resident soil rhizobia are often better adapted to the physical and chemical environment and probably better in terms of saprophytic competence (Vlassak and Vanderleyden 1997; Sadowsky and Graham 1998). Some studies have shown that indigenous strains are competitive in forming nodules in field situations but are often inferior when competition studies are conducted in non-soil environments (McLoughlin and Dunican 1985; Leung *et al.* 1994b). This may indicate that these indigenous strains of rhizobia are better adapted to the specific soil conditions.

2.4.7.5 Genetic Diversity of Naturalised Rhizobia

A further complication in understanding the reaction of legumes to naturalised rhizobial populations is the amount of genetic diversity exhibited in these populations (Dowling and Broughton 1986; Triplett

1990b; Triplett and Sadowsky 1992; Vlassak and Vanderleyden 1997). Similarly, genetic instability of introduced strains and genetic exchange between soil microorganisms may complicate our understanding of naturalised populations and introduced rhizobia (Triplett and Sadowsky 1992; Sullivan *et al.* 1995; Thies *et al.* 2000).

A number of molecular techniques have been developed recently which assist in the understanding of genetic diversity of rhizobia (as reviewed in Thies *et al.*, 2000). Some of the difficulties in understanding rhizobial genetic diversity in soils relate to 1) the widespread distribution of rhizobia, 2) our poor understanding of evolutionary mechanisms for these organisms and 3) sampling strategies which have targeted a limited number of microsymbionts. Also, studies of "genetic diversity" using one or a few legume species are unlikely to represent the total population in soil and produce only a biased assessment (Hartmann *et al.* 1998). Newer techniques, such as the T-RFLP (Liu *et al.* 1997) allow sampling directly from soil and without the need for culturing, which reduces these problems.

Studies of the genetic diversity of fields inoculated or uninoculated attest to the fact that a large number of strain "types" are often present (Dowling and Broughton 1986; Harrison *et al.* 1987; Demezas *et al.* 1991; Bottomley *et al.* 1994) although this will obviously depend upon the technique used and its level of resolution (Thies *et al.* 2000). Many studies have indicated the dominance of particular strain types extracted, and while this may be selective for the legume cultivar used, it is nevertheless important. In soils in Oregon, a major rhizobial serotype was identified (Leung *et al.* 1994a) that occupied more than 50% of nodules (Leung *et al.* 1994c). In a field experiment in Tasmania, three common isolates were identified from uninoculated plots at levels of 7-12%, but were not observed from these plots in the following year (Hebb

et al. 1998). Similarly, a number of dominant isolates of *S. meliloti* were repeatedly observed in soils in the UK (Gandee *et al.* 1999) and Canada (Bromfield *et al.* 1986). Interestingly, (Shishido and Pepper 1990) found that 4 types of *S. meliloti* (based on plasmid profile analysis and intrinsic antibiotic resistance) were found from 5 widespread locations in Arizona.

While understanding genetic stability of rhizobial strains introduced into soil is crucial to monitoring inoculant rhizobia in soil, reports on instability are rare. Gibson *et al.* (1991) reported significant variability in the symbiotic potential within strains of *R. leguminosarum* bv. *trifolii* from laboratory cultures and field isolates, while *Bradyrhizobium japonicum* re-isolated from soils 9 years after inoculation had remarkably stable RFLP patterns.

The role of genetic exchange in increasing rhizobial diversity has received little attention from researchers, but may cause difficulty when tracking rhizobia inoculated into soils. Exchange of symbiotic plasmids has been demonstrated in *R. leguminosarum* bv. *trifolii* (Schofield *et al.* 1987) and *S. meliloti* (Dowling and Broughton 1986). A recently documented example of genetic instability in the field has been the transfer of a "symbiotic island" from *Mesorhizobium loti* (Sullivan *et al.* 1995). Symbiotic genes of *Mesorhizobium loti* are integrated into the bacterial chromosome, but were laterally transferred to other soil bacteria within 7 years of strain introduction. These few examples indicate the problems associated with understanding the introduction of rhizobial inoculants into field soils and highlight the need to develop molecular tools to understand how genetic exchange occurs.

2.4.7.6 Strategies to Overcome the Dominance of Soil Populations

Given that naturalised populations of rhizobia are so prevalent and that inoculant rhizobia are not sufficiently mobile to properly nodulate

legumes, Brockwell *et al.* (1995) has advocated utilizing the naturalised rhizobia to the benefit of legume N_2 fixation. A number of strategies have been suggested (Streeter 1994; Howieson and Rome 1996) to assist in overcoming the dominance of soil populations, such as:

- Using increased or repeated inoculation (even in high nitrate soils BNF has been improved with inoculation of excessively large numbers of rhizobia) (Meade *et al.* 1985; Martensson 1989; Herridge *et al.* 1990).
- Using a host legume with high specificity that is adapted to the environmental conditions but unable to nodulate with the naturalised population (Howieson *et al.* 1995).
- Selection of a "promiscuous" legume that is able to nodulate effectively with naturalised strains (Mytton *et al.* 1988).
- Manipulate the strain or host genetically to alter nodulation specificities (utilizing *nod* genes and Nod factors) (Montealegre *et al.* 1995).
- Modify the background rhizobial population through manipulation of strain population dynamics or strain DNA (Maier and Triplett 1996)
- Using bacteriocidal strains with resistance to those toxins (Triplett 1990a).
- A further alternative would be to improve the delivery of rhizobia to legumes sufficiently to overcome the constraints that currently exist (Brockwell and Bottomley 1995).

These strategies provide some hope for attempting to overcome the problem of nodule dominance by strains inferior in N_2 fixation. The role of genetics should not be underestimated in providing useful tools in the

manipulation of soil populations of rhizobia to help overcome these problems.

2.4.8 Response of Legumes and Rhizobia to Edaphic Constraints

Soil physical and chemical characteristics have a profound effect upon the ability of rhizobia to colonize a soil (Bushby 1982). Particularly important characteristics influencing rhizobial colonization are the effects of organic matter (C), soil particle size, soil nutrition, moisture and temperature (Bottomley 1992). Additionally, soil pH has been shown to have a major role in influencing the survival and growth of rhizobia, which may be important for the successful colonization of *R. leguminosarum* bv. *trifolii* in alkaline soils.

2.4.8.1 Soil pH

Soil pH is an important characteristic in determining the successful colonization of the soil through survival, growth, nodulation and N_2 fixing ability of rhizobia (Brockwell *et al.* 1991; Young and Brockwell 1992; Barclay *et al.* 1994). Acid soils and their consequential nutritional limitations provide the greatest problems to successful functioning of many symbioses in field soils (Howieson *et al.* 1988; Young and Brockwell 1992; Barclay *et al.* 1994; Slattery and Coventry 1995).

Rhizobial species display distinct adaptations to pH ranges: *R. meliloti* are more acid-sensitive than *R. leguminosarum* bv. *trifolii* which are not as tolerant as the slower growing *Bradyrhizobium* (Graham and Parker 1964). Apart from the direct effects of hydrogen ions, acid soils frequently have low levels of phosphorus, calcium and molybdenum and high concentrations of aluminium and manganese (Helyar 1987) and these elements often interact with pH in determining rhizobial response.

Richardson and co-workers suggested that the most acid sensitive stage for rhizobia was during the initial infection process, prior to nodule formation (Richardson *et al.* 1989). The expression of *nod* genes was restricted at low pH (Richardson *et al.* 1988c), being stimulated by additions of calcium and inhibited by the additions of aluminium ions. Acid tolerant annual medics have a greater ability to produce exudates inducing the expression of nod genes at low pH (Howieson *et al.* 1992a).

Growth of rhizobia in acid conditions is highly dependent upon calcium concentration in solution. Increasing calcium had no effect on growth at neutral pH but increased growth and survival at low pH for *Sinorhizobium meliloti* (Howieson *et al.* 1992b; Reeve *et al.* 1993) and *R. leguminosarum* bv. *trifolii* (Watkin *et al.* 1997). Similarly, root exudation compounds that induce *nod* genes were reduced by pH<4.0 and increased by calcium ions (Richardson *et al.* 1988a). Further to this, Caetano Anolles *et al.* (1989) established that the adsorption of rhizobia to alfalfa roots was dependent upon divalent cations (especially Ca, but alsomg) and the concentration of Ca required increased with decreasing pH. The actual role of calcium in assisting metabolism of bacteria at low pH may, however, be complex (Norris *et al.* 1991).

The influence of calcium carbonate (lime) in improving the survival, growth and nodulation of rhizobia has long been known in field situations. In low pH soils the addition of lime increased the populations of rhizobia (Coventry *et al.* 1985b; Richardson and Simpson 1988; Coventry and Hirth 1992) the number of nodules present (Coventry *et al.* 1985b; Richardson *et al.* 1988b) and, the N_2 fixation achieved by pastures (Coventry *et al.* 1985a; Evans *et al.* 1993).

Acid-tolerant strains of rhizobia have some mechanistic functions that enable them to tolerate acidic conditions. Acid tolerant strains were able

to maintain an alkaline intracellular pH at an external pH of 5.6 whereas acid-sensitive strains could not (O'Hara *et al.* 1989). Electron microscopy indicated that acid-tolerant *R. leguminosarum* bv. *trifolii* strain WSM409 was able to alter its morphology in response to low pH, whereas the acid sensitive strain TA1 could not (E.L.Watkin, pers. comm.).

2.4.8.2 Alkaline Soil Reaction of Legumes and Rhizobia

Soils with pH greater than 7 are associated with carbonates in soil and soil solution. Calcite is the most abundant form of calcium carbonate ($CaCO_3$) and the principle component of limestone; it profoundly effects soil properties through control of pH (Rowell 1994). Calcite has a low solubility that is dependent upon surface area, but in arid soils there is too little water to remove significant calcium carbonate. Dissolution of calcite forms carbonate (CO_3^{2-}) which hydrolyses to form bicarbonate ions (HCO_3^-). Bicarbonate is the predominant anion in soils of pH 6 to 9, and increases 10-fold for each increase in pH unit (Rowell 1988). Above pH 10 carbonate is predominant. Measurements of pH usually reflect the solubility of the carbonates, rather than the total amount of carbonate present (Troeh and Thompson 1993).

Alkaline (calcareous) soils often present physical problems to plant growth, the severity dependent upon how high the pH is. Physical limitations exist due to the dispersion of soil colloids, which block pores, can form a crust and reduce permeability (Troeh and Thompson 1993). Plant response to calcareous soils can be divided into 3 groups: those that grow well (calcicoles), those that do not grow well (calcifuges) and the third group has a wide range of tolerance of pH (Ehrman and Cocks 1990). Unlike acid soils and with the exception of boron, alkaline soils have few nutrient toxicities, which are usually overcome through foliar or soil additions of fertilizer (Rowell 1988).

It is not known whether the bicarbonate ion has a direct effect upon plant

growth because changes in bicarbonate cause changes in pH, Ca concentration and the form of phosphate ions. High bicarbonate ion concentrations generally cause iron chlorosis (Papastylianou 1989) reducing the solubility of iron and manganese, reducing adsorption and translocation of iron and inhibiting cytochrome oxidase (Rowell 1988). Yield decline in calcifuges is more closely related to soil pH and "active" $CaCO_3$, a measure of the extent and reactivity of the carbonate surfaces. Manganese, copper and zinc availability can also be reduced in highly calcareous soils (Rowell 1994).

Available phosphate is often found to be limiting in Mediterranean type soils and additions of phosphorus to these soil often fail to achieve the required growth response (Matar *et al.* 1992; Tyler 1992). In calcareous soils precipitation of insoluble Ca-P is considered to be a major factor in the loss of P availability (Afif *et al.* 1993) and a number of studies have reported that reduction in P availability is correlated to $CaCO_3$ content (Matar *et al.* 1992). Legume requirement for P is comparatively higher than that of cereals (Keay *et al.* 1970), so that P availability may limit the extent or success of introduction of legumes on these soils. Some legumes have the ability to solubilize P through acidifying the rhizosphere, which may assist their P nutrition in calcareous soil conditions (Hinsinger and Gilkes 1995).

A particular problem in many alkaline soils of southern Australia is the carbonate layers that commonly exist 0.3-0.6m below the surface (Wetherby and Oades 1975). In these carbonate layers the concentration of boron is often high, leading to the commonly occurring problem of boron toxicity of cereals (Cartwright *et al.* 1984; Cartwright *et al.* 1986; Holloway and Alston 1992). While some legumes (eg. *Medicago littoralis*) are thought to possess a degree of boron tolerance (W. Bellotti, pers. comm.) the introduction of new legumes could be affected by this toxicity.

Studies of legumes grown in alkaline and calcareous soils are generally limited to grain legumes (Coulombe *et al.* 1984; White and Robson 1990; Brand *et al.* 1999), with little information on pasture legumes. Evidence from studies on *Bradyrhizobium* in alkaline conditions suggested that pH above 6.0 was suitable for the growth of adapted lupin species, but decreased nodule initiation, nodule numbers and nodule mass (Tang and Robson 1993). The problems of nodulation in lupin appear to be related to poor root growth and impaired root physiology at high pH, compared to direct problems associated with initiation of nodulation by *Bradyrhizobium* (Tang *et al.* 1995; Tang *et al.* 1996). There are also large distinctions between *Bradyrhizobium* and *Rhizobium* in pH tolerances; *Bradyrhizobium* is more acid tolerant and is generally less adapted to alkaline soils compared to *R. leguminosarum bv. trifolii* (Graham and Parker 1964).

Ecological studies suggest that clover species respond differently to pH and lime in soils, based upon the centres of origin (Ehrman and Cocks 1990). *T. subterraneum*, for example is limited by lime content in soils, not alkalinity, whereas a number of clover species (*T. resupinatum*, *T. purpureum*, *T. cherleri*) have adaptations to lime and high pH and appear to act as calcicoles (Ehrman and Cocks 1990; Ehrman and Cocks 1996; Francis 1997). These species may prove useful in providing legume diversity for Australian farming systems. The response of clover rhizobia and N_2 fixation in alkaline calcareous soils, however, remains unknown.

2.4.8.3 Nutrition

To achieve optimal N_2 fixation, nutrition requirements need to be provided for the legume, the rhizobia and the symbiosis. Annual pasture legumes usually have a greater requirement for phosphorus compared with non-legumes (Robson *et al.* 1981; Bolland and Paynter 1994). Specific nutritional requirements for N_2 fixation including those

necessary for: rhizobial growth (Ca, Fe); nodule initiation (Co); nodule development (B); nodule function (Mo) and nitrogen fixation (Ca, Co, Cu, Ni and Fe) (O'Hara *et al.* 1988; Evans *et al.* 1985).

Apart from the specific needs of the fixation process, nutrition also plays a role in the competitive abilities and adaptation of rhizobia in nodulating with legumes. Attainment of P for functioning is important for rhizobia (Smart *et al.* 1984) and the additions of phosphate to soil have been shown to change the dominance of serogroups of *R. leguminosarum* bv. *trifolii* on a low P soil (Almendras and Bottomley 1987). Specific nutritional limitations may occur due to an interaction with low pH (low Ca, P, Mo; high Al, Mn) or high pH (high Ca; low Fe, P) (Tang *et al.* 1992) and may influence the dominance of particular strains due to soil adaptation of rhizobia or the nodulation process.

2.4.8.4 The Development of Pasture Legumes and their Microsymbionts for Difficult Soils

Annual legume pastures in the semi-arid Mediterranean type environments of southern Australia have typically consisted of annual *Medicago* species (Puckridge and French 1983; Carter 1987) and subclover (*Trifolium subterraneum*; Rossiter 1966). Prompted by problems associated with poor growth of legumes in difficult soils such as low pH and low fertility and the economic and environmental constraints of modern farming systems, a need for greater diversity of pastures was recognized, as reviewed by Howieson *et al.* (2000b). In these environments, typical of south western Australia, an additional constraint is that the survival of rhizobia is generally low and often thought of as limiting symbiotic N inputs (Robson 1969; Robson and Loneragan 1970; Parker *et al.* 1977).

A number of collections of legume genetic material has been made in the

Mediterranean basin (Francis 1997), and increasingly these have been directed toward locations that share similar edaphic conditions to the target environments in Australia. In addition, the combined collection of rhizobia from similar soil types has assisted in the development of legumes with suitable microsymbionts that are adapted to target soil conditions (Howieson and Loi 1994; Nutt *et al.* 1996).

The outcome of collection and evaluation of legume genetic material has been the introduction of a number of new legume species and genera. The last 2 decades have seen the introduction of new cultivars of *Medicago polymorpha*, *M. murex* and *M. sphaerocarpus* (Gillespie 1987; Ewing and Howieson 1989; Gillespie 1989; Howieson and Ewing 1989). Newly released species, such as *Biserrula pelecinus* and *Ornithopus sativus* have added to the diversity of legume pastures (Howieson *et al.* 1995; Ballard 1996; Loi *et al.* 1999).

The concurrent evaluation and introduction of rhizobia has assisted in the adaptation of the legume-rhizobia symbiosis to Australian conditions. Acid tolerant strains of rhizobia originating in the Mediterranean basin are now represented in the Australian commercial inoculants. Strain WSM409, which originates from Sardinia, is the commercial inoculant for a number of alternative clovers and a new inoculant for *T. subterraneum*. Strain WSM1274, the commercial inoculant for faba beans and lentils originates from Greece (Howieson *et al.* 2000a).

Collection of legumes and rhizobia from their centres of origin in the Mediterranean basin will no doubt continue to provide genetic material important for Australian farming systems. While the selection of legumes adapted to alkaline soils may not be as crucial compared to collection for highly acidic soils, continued selection of genetic material will strengthen the diversity of legume pastures to sustain these farming systems.

2.4.9 Other Factors Influencing Rhizobial Competition

Many other aspects can affect competition for nodulation in the field, both biotic and abiotic. Abiotic factors that influence competition include: soil moisture, soil nutrition, soil type, temperature and organic matter, herbicides and pesticides (Dowling and Broughton 1986; Barran and Bromfield 1997). Biotic factors include the production of rhizopines (Murphy *et al.* 1995), trifolitoxins (Triplett 1999), lipo-polysaccharides and exopolysaccharides (Sadowsky and Graham 1998), lysing by phages and predation by protozoa (Parker *et al.* 1977; Postma *et al.* 1990).

The genetic control of competition is far from being well understood. The mutation of many genes reduces competitive ability, but whether this has a direct role in competition is sometimes questionable (Sadowsky and Graham 1998). Mutations in a particular DNA region, called *nfe* (nodule formation efficiency) cause a delay in nodule formation and reduce competitiveness (Sanjuan and Olivares 1989).

2.5 Recently Developed Techniques Aiding the Study of Rhizobial Ecology

2.5.1 Introduction

The isolation, identification, quantification and classification of soil bacteria is a difficult exercise due to the nature of the organisms, their size and the complexity of the environments in which they are found. The development of knowledge about soil bacteria is therefore highly constrained by techniques available to isolate and identify the organisms. As the study of economically important soil microbes has progressed, techniques have evolved which have improved the accuracy and reduced the time taken to identify and quantify organisms. The advent of molecular techniques, in particular, has established powerful tools to

enhance our understanding of soil microorganisms (Thies *et al.* 2000). Many molecular techniques are now used as standard tools to aid in our understanding of the ecology of soil organisms such as *Rhizobium* and no doubt will be improved further to aid our understanding of soil ecology. In the next section, two techniques that are applicable to the current research: the polymerase chain reaction and GUS maker genes, will be discussed.

2.5.2 Identification of Rhizobia Using Polymerase Chain Reaction Techniques

Since its initial serendipitous discovery in 1983 (Mullis 1990), the polymerase chain reaction (PCR) has had a profound effect upon molecular biology. The technique, which allows the manufacture of nucleic acid molecules *in vitro*, has wide application in a multitude of areas. PCR technology can provide accurate strain-specific rhizobial fingerprints (Richardson *et al.* 1995; Laguerre *et al.* 1996) and can be used to understand the genetic variation in populations of rhizobia (Dooley *et al.* 1993; Laguerre *et al.* 1996).

PCR is an *in vitro* method for enzymatically synthesizing defined sequences of DNA. The reaction utilizes oligonucleotide primers that hybridize to the opposite strands of the target DNA sequence and determine the starting and ending points of replication. Elongation of the primers is catalyzed by *Taq* DNA polymerase, a heat-stable polymerase originally isolated from the thermophilic bacterium, *Thermus aquaticus* (or alternatively *Tth* from *Thermus thermophilus*). The three cycles of 1) DNA template denaturation, 2) primer annealing to the single strands of DNA and 3) extension of the annealed primers by *Taq* polymerase are repeated a number of times in order to replicate the target DNA. Cycles are mediated through temperature control for each part of the reaction: 92-95°C for DNA denaturation, 30-70°C for primer annealing and 72°C

for extension using Taq DNA polymerase, each step ranging from 1 second to 30 minutes. Because replication by Taq polymerase proceeds in the 5' to 3' direction, the products are defined by the 5' ends of the primers. The products formed in any cycle then become the template in the next cycle, and the number of target DNA copies doubles every cycle. Therefore, after 20 cycles of PCR, the yield of target DNA is about a million copies (2^{20}).

Other components of PCR reactions include dNTP's from which nucleotides are constructed,$mgCl_2$, bovine serum albumen, or dimethyl sulphoxide, which influence the stringency of the enzyme activity and template buffer to maintain optimal pH for the reaction. The PCR reaction is performed in a thermocycler that provides accurate and reproducible temperature control.

A multitude of different primer types has been developed to discriminate between rhizobial strains and amplification of one or a few specific fragments of DNA is one generalized group of identification methods. This group includes the analysis of portions of DNA from the rRNA or tRNA operon, or intergenic regions between the 16S and 23S RNA genes (Welsh and McClelland 1991; Jensen *et al.* 1993; Laguerre *et al.* 1994; Brunel *et al.* 1996).

Primers used to generate randomly amplified polymorphic DNA (RAPD) (Harrison *et al.* 1992; Richardson *et al.* 1995) bind arbitrarily with DNA and can produce bands that are unique to rhizobial strains. Depending upon the target genomes, a number of primers may need to be tested until a suitable primer is found, depending upon the primer/template interactions (Harrison *et al.* 1992; Richardson *et al.* 1995).

A second primer variation is those that bind with repetitive sequences such as the REP -primer (repetitive extragenic palindromic), which binds

to naturally occurring interspersed repetitive sequences, found in many (possibly all) bacteria (Schneider and de Bruijn 1996). In addition to the 35-40 bp REP sequences, 124-127 bp enterobacterial repetitive intergenic consensus sequences (ERIC) and 154 bp BOX elements have also been identified in diverse bacterial species (Lupiski and Weinstock 1992) and have been used as PCR priming sites (de Bruijn *et al.* 1992; Versalovic *et al.* 1994). In PCR of these repetitive primers, it is the intervening genomic DNA that is amplified to produce strain-specific fingerprints.

Another method of PCR is to utilize known DNA sequences to generate primers that bind to conserved regions of rhizobial genomes. The RPO1 primer, a 20bp primer binds to the *nifH* region of rhizobia (Schofield and Watson 1985) and can be used successfully to discriminate *R. leguminosarum* bv. *trifolii* (Richardson *et al.* 1995) and other rhizobial species at the strain level.

One of the useful characteristics of PCR is that whole bacterial cells can be used in the reaction and the initial annealing temperature is sufficiently high to lyse cells and render genomic DNA available for the reaction (Harrison *et al.* 1992; Versalovic *et al.* 1994). In addition, PCR has been applied to rhizobia within nodules, leading to implications for simpler analysis (Harrison *et al.* 1992; Richardson *et al.* 1995). Rhizobia from field isolates have also been used successfully for PCR (Hebb *et al.* 1998; Gandee *et al.* 1999) however, the direct use of field grown nodules in PCR is still uncertain because of contaminating soil organisms.

PCR is a powerful tool for *Rhizobium* ecology, enabling the accurate typing of rhizobial strains (laboratory or field) and a determination of their genetic relatedness (Schneider and de Bruijn 1996; van Berkum *et al.* 1996). However, there are a number of factors that constrain the techniques such as the susceptibility of the reaction to minor changes in component concentrations (primer,mgCl_2 concentrations etc.). In addition,

compared to marker genes, PCR is technically difficult, expensive and labour intensive (Wilson 1995). Even with these limitations, and the understanding that PCR is not sufficiently developed to provide quantitative information on strains, it is an excellent method for use in field studies to accurately identify *Rhizobium* strains.

2.5.3 The Use of GUS Marker Genes in Rhizobial Ecology

Molecular markers, including green fluorescent protein (GFP) (Gage *et al.* 1996) and the *gusA* gene expressing β–glucuronidase (GUS) (Wilson *et al.* 1995b) are powerful tools in the study of rhizobial ecology. Nodules formed by *Rhizobium* marked with the *gusA* gene turn blue upon treatment in buffer, while unmarked strains remain unstained. This process enables the visual detection of rhizobial occupants of nodules, because marked and unmarked strains can be easily distinguished. The simplicity of this system in identifying nodule occupants has many benefits to offer rhizobial ecology, because the assay is rapid and allows entire plant root systems to be tested (Wilson *et al.* 1999).

The *gusA* gene, which codes for β–glucuronidase, was originally isolated from *Escherichia coli* (Jefferson *et al.* 1986). β–glucuronidase is a hydrolase that catalyses the cleavage of a wide variety of β–glucuronide substrates, such as XGlcA (5-bromo-4-chloro-3-indolyl β-D-glucuronide) (Jefferson *et al.* 1986). When β–glucuronidase cleaves XGlcA, an indoxyl derivative is released, which, on dimerization, gives rise to an indigo precipitate (Wilson 1995), which allows the visual detection of a marked strain. Most importantly, there is no reported endogenous activity of GUS in any plant or rhizobial species so far examined (Wilson *et al.* 1995b), so untransformed rhizobia do not exhibit GUS activity.

The insertion of *gusA* into rhizobia is accomplished through the biparental mating of the target *Rhizobium* with *E. coli* that contains the

appropriate plasmids. A number of minitransposons containing *gusA* have been constructed by Wilson *et al.* (1995b) from the minitransposon Tn*5*s (mTn*5*s) developed by De Lorenzo *et al.* (1990) and Herrero *et al.* (1990). These provide a set of derivatives that restrict further transpositional events, so that *gusA* is permanently inserted into the chromosome of the rhizobia. The transposons constructed by Wilson *et al.* (1995b) carry streptomycin (Sm) and spectinomycin (Sp) resistance so that transconjugants are selected on media containing these antibiotics.

A number of useful *gusA* constructs have been developed which express GUS activity under the influence of different promoters (Wilson 1995). A constitutive promoter is useful for applications where free-living rhizobia need to be marked to assess early infection or colonization of the rhizosphere. The P*nifH-gusA* containing minitransposons (mTn*5*SS*gusA*30 and mTn*5*SS*gusA*31, developed for *Rhizobium* and *Bradyrhizobium*, respectively) express GUS in the oxygen deficient environment of nodules, but do not express in the free-living state. A number of promoterless constructs have also been developed (Wilson *et al.* 1995b) which have applications for GUS expression under defined gene regulation (Reeve *et al.* 1999).

One of the favourable characteristics of GUS is the ease of visual assessment of nodulation, compared to *luxAB* and *luc* markers, which require sophisticated amplification devices for detection (Wilson 1995). Other marker systems (eg. *lacZ(YA), phoA*) have endogenous activity which limits their use (Sessitsch *et al.* 1998). Unexplained "background" GUS activity can be observed after insertion of GUS plasmids into rhizobial genomes due to read-through transcription but this is usually minimal (Jefferson 1987).

One problem that arises from insertion of marker genes into bacteria, is the influence of gene insertion on competitive and symbiotic properties of

the organism. Studies of rhizobia and other bacteria indicate that the competitive ability of mutant bacteria is often unchanged, but sometimes reduced or even enhanced, compared to the wildtype.

Analysis of 1200 mutants of *Pseudomonas putida* containing a promoterless lacZ gene indicated that the majority of isolates did not differ from the wildtype in the ability to colonize roots (Lam *et al.* 1990). Tn*5* insertion did not affect the fixation or competitive ability of *Mesorhizobium* ciceri (Sharma *et al.* 1991) but did reduce competitive ability and actually increased symbiotic effectiveness in some *R. leguminosarum* biovars *viciae* and *phaseoli* (Brockman *et al.* 1991). Other experiments with GUS insertions caused little change to competitive ability or N_2 fixation (Wilson *et al.* 1995b; Sessitsch *et al.* 1997).

Problems of gene insertion may depend upon whether insertion is random or targeted, because most effects of gene insertion on fitness depend upon the position of insertion, rather than the inherent properties of the transposons themselves (Sessitsch *et al.* 1997). Inserted gusA mini-transposons may affect 1) the expression of the *gusA* gene, 2) the expression of the selectable marker in the mini-transposon, or 3) the phenotype of the cell (Sessitsch *et al.* 1997). Wilson *et al.* (1995a) have speculated that reporter genes such as *gusA* may revolutionize the ability to measure such traits as symbiotic N_2 fixation in field situations. In addition, the use of the *celB* gene in conjunction with GUS, has provided a system which allows several *Rhizobium* strains to be detected (including multiple-strain occupancy) on legumes (Sessitsch *et al.* 1996; Sessitsch *et al.* 1998). Therefore, it is likely that gene reporter systems will play a significant role in furthering our understanding of bacterial ecology, physiology and biochemistry.

2.6 Conclusion and Thesis Aims

This literature review has covered information on the process of establishing N_2 fixation in legume nodules and the interactions involved in rhizobial competition to form nodules. It has discussed the complex nature of rhizobial interactions in soils in a field, particularly the problem of overcoming the dominance of naturalised soil rhizobial populations in competition for nodulation. In addition, some techniques have been discussed which aid the study of rhizobial ecology relevant to the following experimental work.

A number of issues have been highlighted which indicate that our understanding in some areas of *Rhizobium* ecology is limited. In particular, there are gaps in our understanding of the genetic diversity of rhizobia and the roles of genetic instability and exchange of genetic information in contributing to this diversity. Fortunately, a number of genetic tools have recently been developed that provide the basis to further explore these issues (Thies *et al.* 2000).

There is a limitation in the technology of delivering inoculant rhizobia to legumes such that nodulation of lower sections of the taproot and lateral roots by inoculant rhizobia is maximized. This is a significant constraint to the commercial success of rhizobial inoculants.

Our understanding of how legumes, rhizobia, and in particular, the symbiosis between these organisms functions under alkaline calcareous soil conditions is incomplete. In addition, how naturalised populations of rhizobia influence the introduction of inoculant rhizobia under alkaline, calcareous conditions is not clear. These problems need to be addressed in order to maximize the potential of newly released legumes to perform well under the edaphic constraints of alkaline soils.

The purpose of this thesis is therefore to address the question:

What role do naturalised soil populations of *R. leguminosarum* bv. *trifolii* have on the successful introduction of rhizobial inoculants for alternative clovers on alkaline soils?

The hypothesis is that:

Naturalised *R. leguminosarum* bv. *trifolii* populations will form a barrier to the successful introduction of rhizobial inoculants on alkaline soils.

The following chapters specifically address the questions:

What is the size and effectiveness of soil rhizobial populations in the target geographical regions? (Chapter 3)

What is the influence of naturalised soil rhizobial populations on the ability of inoculant rhizobia to nodulate alternative clovers and to fix N_2? (Chapter 4)

What influence does the naturalised population of rhizobia have on the ability for inoculant rhizobia to occupy nodules of alternative clovers on alkaline soils? (Chapter 5)

How competitive are field dominant strains of rhizobia for nodulation of alternative clovers? (Chapter 6)

The final chapter (7) will discuss the implications of the findings.

Chapter 3

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

3.1 Introduction

There is a current need to seek alternative legumes to provide greater diversity of legume pastures in southern Australia. Alternative clovers may fulfil a niche in low rainfall, alkaline soil environments, typically sown to annual medic (*Medicago*) species (see Chapter 1). Commercial clover species have never been grown on many of the alkaline soils in the Mid North, Yorke and Eyre Peninsulas and, subsequently, information on the rhizobial status of these soils (numbers, effectiveness and the need for inoculation) is deficient.

If alternative clovers are to provide optimal N_2 fixation, it is essential that adequate numbers of effective clover rhizobia are present in soils. The need for inoculation of legumes with rhizobia can be influenced by a complex of factors including: the hostility of the soil environment, the frequency of the host legume in a rotation, specificity of the host's rhizobial requirements, soil nitrate dynamics and the nature of the resident rhizobial population (Thies *et al.* 1991a; Brockwell *et al.* 1995). The resident rhizobial population can have a significant effect on the establishment of N_2 fixing pastures, restricting particular species to little or no fixation (Bowman *et al.* 1998). For the low rainfall, alkaline environments in the present study, the influence of the resident rhizobial populations and plant-rhizobial interactions were considered to be of great importance.

This chapter outlines the results from a survey conducted in low rainfall,

alkaline soil environments in South Australia. The aim of the survey was 1) to determine the abundance, distribution and symbiotic nitrogen fixing potential of clover rhizobia from the target environment and 2) to gain an understanding of what environmental variables influence population sizes of clover rhizobia in these environments.

3.2 Methods

3.2.1 Survey Details

Survey work was conducted in South Australia. Collection areas (transects) were selected on eastern (n=19) and western (n=16) Eyre Peninsula, on upper Yorke Peninsula (NE of Moonta; n=10), on the northern Adelaide plains (NW of Roseworthy; n=10) and at Roseworthy Campus (n=6)(Fig. 3.1). Western Eyre Peninsula included landforms with calcareous soils (grey-brown calcareous loamy earths) and shallow soils over sheet calcrete. Eastern Eyre Peninsula covered areas with parallel siliceous sand ridges (grey-brown calcareous loamy earths) and deep sandy alkaline yellow duplex soils (Jeffery and Hughes 1995). Transects on upper Yorke Peninsula covered areas of sandy ridges and loamy mallee, grey mallee or calcareous loam flats. The northern Adelaide plains area was characterized by calcareous loams and grey calcareous sand (Northcote 1960). Sites surveyed at Roseworthy campus were solonized brown soils and clayey red-brown earths (Northcote 1960). In each of these regions, land use is predominantly a mixture of cropping and grazing enterprises.

The survey was conducted during October-November 1996 and pasture paddocks were sampled every 10 ± 1km along transects. At each sampling site, 20 random soil cores (10cm deep x 2.5cm diameter) were collected, bulked, thoroughly mixed and stored briefly before analysis. Where *Trifolium* species were present at a site, a second sample of soil cores was taken to include clover roots. An assessment of relative legume

abundance was made using the vegetation analysis package "Botanal" (Tothill *et al.* 1992), taking 20 rank abundance measures at each sampling site (3 species contributing most to biomass in a quadrat) weighted by standard multipliers.

To verify plant identification, additional soil samples were collected, the soil spread in trays, watered, and the seeds in the soil allowed to germinate and grow under glasshouse conditions until they were large enough to identify.

3.2.2 Enumeration of Rhizobial Populations

A serial dilution, plant infection technique (Brockwell 1980) was used to estimate the most probable number of rhizobia (MPN) in each soil sample. To determine whether estimated populations of rhizobia differed according to the legume used, *T. subterraneum* cv Clare and *T. resupinatum* cv. Nitro were used as test plants. These plants were grown in a glasshouse with 25°C/17°C day and night temperatures.

3.2.3 Symbiotic Effectiveness of Rhizobial Populations

Symbiotic effectiveness of soil rhizobia was tested with *T. resupinatum* cv. Nitro (Persian clover) and *T. purpureum* cv. Paratta (purple clover) using the whole-soil inoculation method (Brockwell *et al.* 1988b). This method uses rhizobial populations directly from soil to make an assessment of the N_2 fixation potential from a field, rather than a strain-specific measurement of N_2 fixation.

One of the limitations of the technique is that the effectiveness of soil populations of <300 rhizobia/g soil cannot be accurately assessed. This limitation was accepted in the present study and only 27 soils with rhizobial populations of >1000/g soil were used: 24 soils were tested using *T. resupinatum* cv. Nitro and 20 soils using *T. purpureum* cv. Paratta, due to poor survial in some treatments.

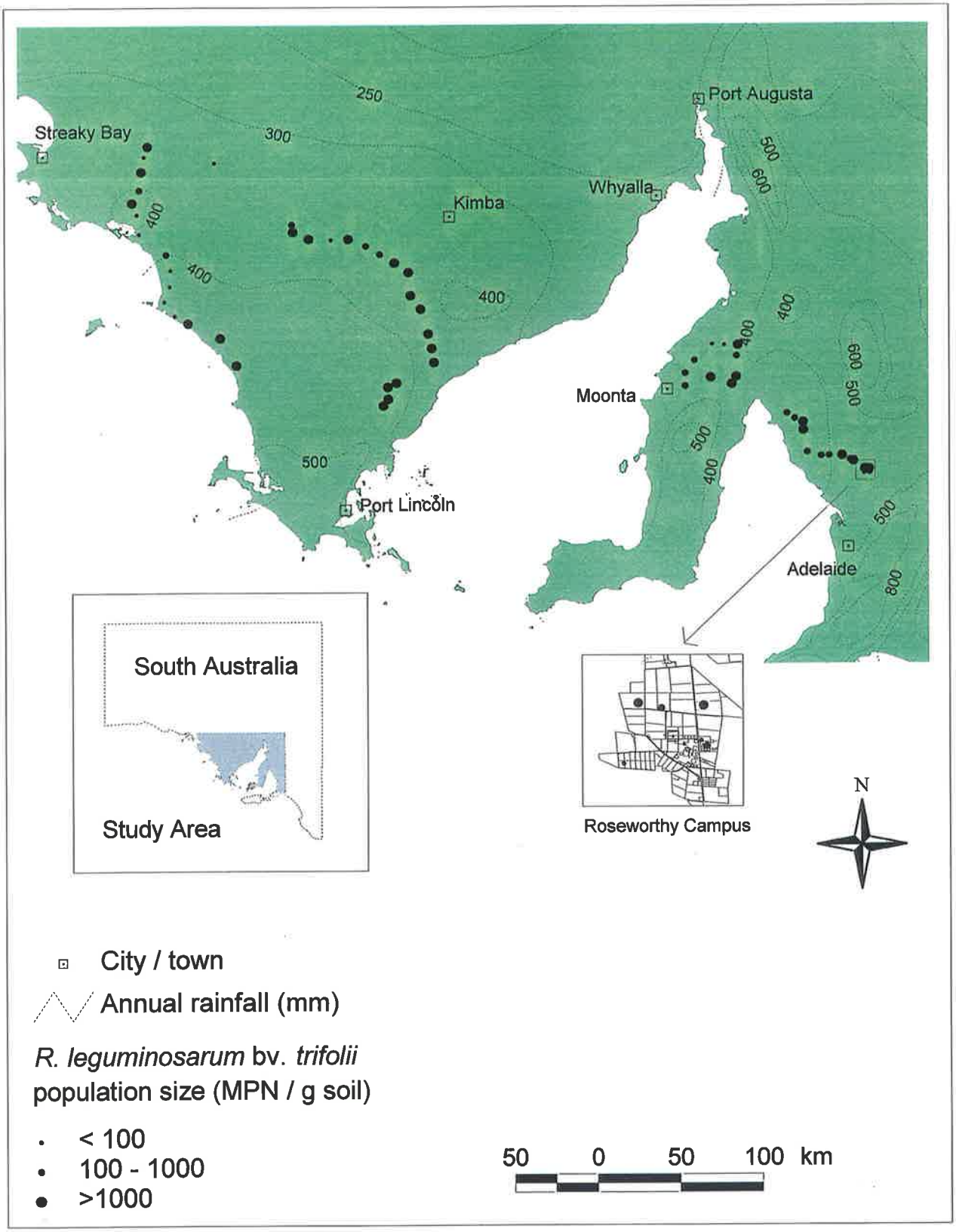

Fig. 3.1 Distribution of 61 survey sites across South Australia indicating rainfall isohyets and rhizobial population size groupings.

Clovers were grown under sterile conditions in vermiculite-filled, foil-capped test tubes (18 × 2.5cm diameter), watered with N-free Mc Knight's solution of pH 6.8 (Gibson 1980) and inoculated with a 1mL soil suspension (10g soil in 90mL Mc Knight's solution) when 5 days old. As positive controls, two reference (commercial) rhizobial strains were used for each species (TA1, CC2483g for *T. resupinatum*; TA1, WSM409 for *T. purpureum*). In addition, there were 2 uninoculated control treatments: minus nitrogen and plus nitrogen (14.7mg N/plant, as NH_4NO_3). Plants were grown in a controlled environment room with 21°C/17°C day and night temperatures and a light intensity of 300-400μmol/m^2/s. Ten replicate plants were used for each treatment in a randomized complete block design and plants were grown for 6 weeks. Shoot biomass was dried (80°C for 2d.), weighed and regarded as the index of N_2 fixation. Nodules were scored using the methods of (Corbin *et al.* 1977).

3.2.4 Soil Factors

To establish whether rhizobial populations varied with soil conditions, a number of soil factors were measured. Soils were sieved and the <2mm fractions analyzed for available phosphorus and potassium (Colwell method), organic carbon (Walkley and Black method), electrical conductivity, pH (H_2O and $CaCl_2$), cation exchange capacity, aluminium and total nitrogen; all these methods are given in Rayment and Higginson (1992). The amount of carbonates in the soil was determined using the pressure-calcimeter method (Nelson 1982). Particle-size analysis was performed using the Hydrometer method with a pre-treatment for removal of carbonates when these constituted >5% of the total dry soil mass (Gee and Bauder 1986).

3.2.5 Statistical Analysis

To determine whether sites formed defined groups based on environmental conditions, environmental variables were analyzed using

principal components analysis performed on the correlation matrix using the S-plus statistical package (Becker *et al.* 1988). The relationship between rhizobial populations and environmental variables was analyzed for all environmental variables with multiple linear regression and backward elimination using Genstat 5 (Payne 1993). T-tests were used to compare rhizobial populations between host species and shoot biomass between species when inoculated with soil suspensions of rhizobia. Comparisons between field and clover rhizosphere samples were analyzed with paired t-tests using Genstat 5. Data were transformed, where necessary, to satisfy assumptions of normality; values for MPN were transformed ($\log_{10}$) for analysis and then back-transformed and presented as geometric means.

3.3 Results

3.3.1 Rhizobial Populations

Over half of the sites surveyed had populations of less than 1000 clover rhizobia/g soil; 33 sites for *T. subterraneum* and 38 sites for *T. resupinatum*, (Fig. 3.2). Rhizobia were not detected at 5 to 7 sites (Fig. 3.2), and populations varied from 0 (undetectable) to 4.27×10^5 rhizobia/g soil (*T. resupinatum*) and from 0 to 4.24×10^4 rhizobia/g soil (*T. subterraneum*).

On a regional basis, eastern Eyre Peninsula had the highest rhizobial populations with 2300 rhizobia/g soil (median for both host legumes). Moderate populations of clover rhizobia were found on Yorke Peninsula (795/g soil), the northern Adelaide plains (463/g soil) and the Roseworthy campus (368/g soil). Western Eyre Peninsula had the smallest populations (315/g soil), although variation in rhizobial population size was large.

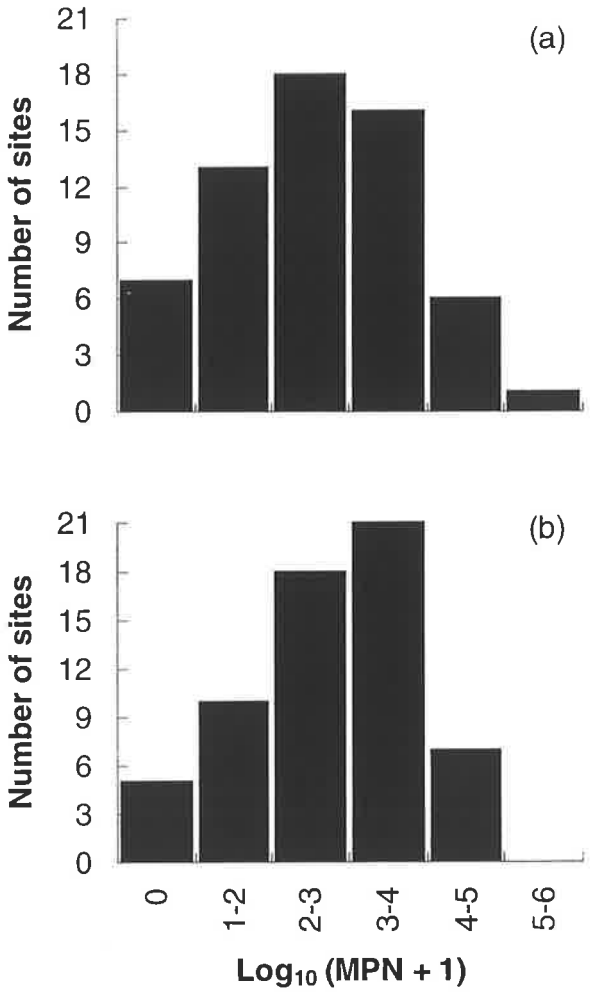

Fig. 3.2 Frequency of population sizes of *R. leguminosarum* bv. *trifolii* from 61 survey sites in South Australia estimated using (a) *T. resupinatum* cv. Nitro and (b) *T. subterraneum* cv. Clare as host plants.

3.3.2 Influence of Clover Presence Upon the Rhizobial Populations

At least one clover species (*T. tomentosum, T. glomeratum, T. campestre, T. arvense,* or *T. subterraneum*) was identified at 17 of the 61 survey sites. At these 17 sites, clover contributed from 0 (only 1 plant found in a field) to 21% of the total plant composition, contributing to only 5% of composition on average.

The presence or absence of clover within a field had a significant effect

upon clover rhizobia populations. Rhizobial populations were significantly larger when sampling included a clover rhizosphere (20000 rhizobia /g soil) compared to the mean field value for these 17 sites (3200 rhizobia /g soil, mean of both species; $P<0.05$; Fig. 3.3).

3.3.3 Symbiotic Effectiveness of Rhizobial Populations

At 60% of survey sites the mean level of N_2 fixation achieved by soil populations of rhizobia was less than half that of the reference rhizobia (WSM409/CC2483g) (Fig. 3.4). Shoot biomass of *T. purpureum* ranged from 5mg (N-free control, site 51) to over 100mg (site 34; Fig. 3.4). Strain TA1 performed only moderately with *T. purpureum*, as previously reported (Howieson *et al.* 2000a). Shoot biomass of *T. resupinatum* varied between 7mg (uninoculated N-free control, sites 27 and 32) and 75mg for the best treatments (CC2483g; Fig. 3.4).

Shoot biomass of *T. purpureum* inoculated with rhizobia from different sites was 11 to 89% of the reference strain WSM409; biomass of *T. resupinatum* similarly inoculated, varied from 10 to 85% of the reference strain, CC2483g. Rhizobia were generally ineffective (defined as <50% of shoot biomass of the reference strain) on one (eg. sites 34 and 41) or both plant species (eg. sites 27 and 51).

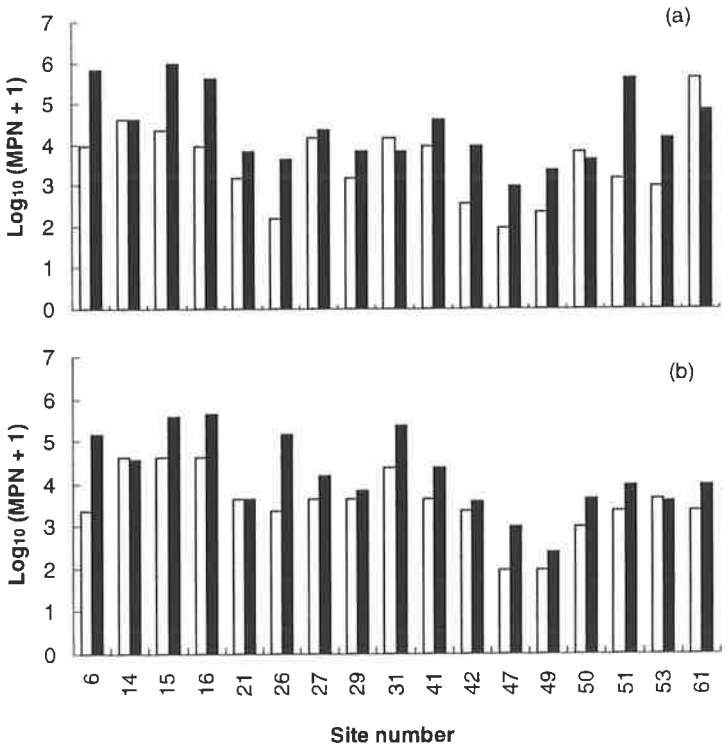

Fig. 3.3 Rhizobial populations from 17 survey sites where clover was present, estimated using paired samples from 1) field samples (□) and 2) field samples that included clover rhizospheres (_). Hosts used were (a) *T. resupinatum* and (b) *T. subterraneum*.

Only two sites (14 and 53) had effective rhizobia for both plant species. Effectiveness varied between the 5 different regions: only 3 out of 21 treatments inoculated with soil from eastern Eyre Peninsula produced plants with more than 50mg of shoot biomass, while the rhizobia from Roseworthy soils produced over 45mg shoot biomass on each of the 3 treatments tested. Soil rhizobia from western Eyre Peninsula, Yorke Peninsula and the northern Adelaide plains varied considerably in N_2 fixation.

Mean nodule score varied from 2 to 4.3 and was significantly correlated with shoot biomass for both *T. purpureum* (r^2=0.65, P<0.001) and *T. resupinatum* (r^2=0.61, P<0.001; Fig. 3.5). Nodule score was, however, independent of rhizobial population size (P>0.05). White (ineffective) nodules were commonly observed, but are not included in the nodule scoring system (Corbin *et al.* 1977), which applies only to pink nodules.

The relationship between shoot biomass and MPN indicates the symbiotic potential of the rhizobial populations from selected sites (Fig. 3.6).

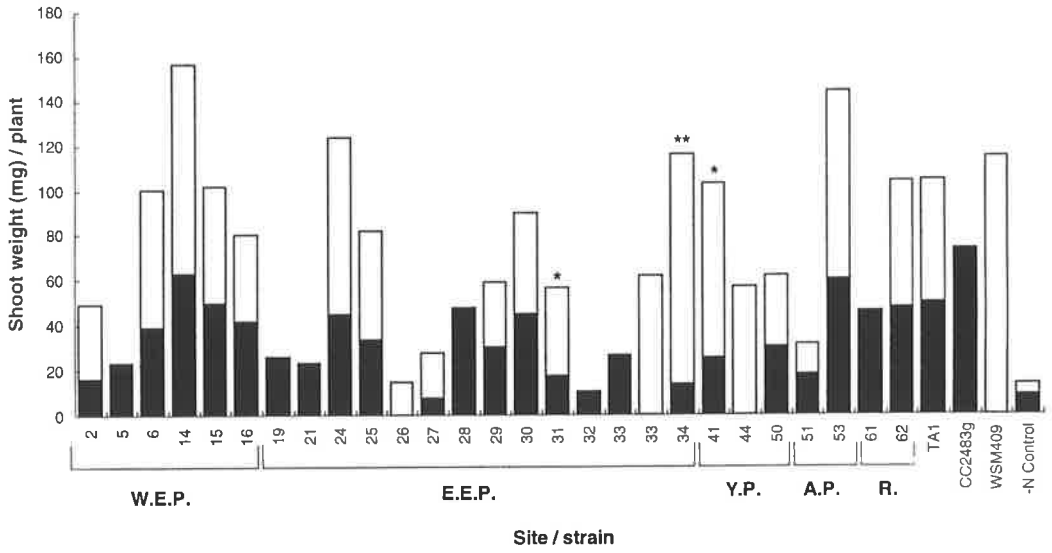

Fig. 3.4 Symbiotic effectiveness of soil populations of rhizobia from sampling sites inoculated on *T. purpureum* (□) and *T. resupinatum* (γ). Treatments include reference (commercial) strains (WSM409, CC2483g and TA1) and uninoculated controls (-N). Regions indicated are western Eyre Peninsula (W.E.P.), Eastern Eyre Peninsula (E.E.P.), Yorke Peninsula (Y.P.), the Adelaide Plains (A.P.) and Roseworthy (R.). Data are cumulative for a soil sample. Some treatments were only tested on one plant species, but in treatments where plant species were compared, these are indicated on the graph as * P<0.05 and ** P<0.01.

There was no significant correlation (P>0.05) between symbiotic potential and size of the rhizobial population for sites with >1 x 10^3 cells/g soil.

3.3.4 Environmental Factors

The survey covered regions with mean annual rainfall varying between 320 and 437mm, with a median of 376 mm (Table 3.1; Appendix A contains raw data for each site). Survey sites were high pH soils (median 8.4, H_2O) with generally low organic carbon (median 1.5%).

Soil P had a median value of 24 ppm and ranged between 3 and 57ppm. There were 2 unusual sites: less than 1ppm soil P (a non-arable site with no fertilizer inputs) and 204ppm (a site with a history of application of piggery effluent). Sites surveyed varied from highly calcareous (up to 78%

$CaCO_3$) to those with no reaction to acid. Clay content varied from 2% in sandy soils to 32%. Electrical conductivity was low for all sites (<0.21 dS/m).

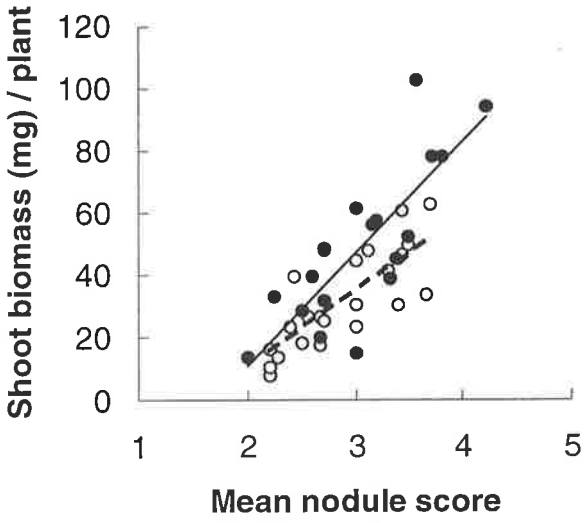

Fig. 3.5 Relationship between shoot biomass and nodule scores using host plants *T. purpureum* (●, r^2=0.65, P<0.001) and *T. resupinatum* (○, r^2=0.61, P<0.001).

Mean pasture legume composition across all sites was 35.3% and varied from legume-free sites (n=4) to one site consisting entirely of legumes (Table 3.1). Barrel medic (*Medicago truncatula*) was the most abundant legume, comprising 16.6% of the total biomass across all survey sites, followed by strand medic (*M. littoralis* 9.5%) and burr medic (*M. polymorpha* 6.4%). At sites where these species occurred, they contributed up to 75% of the foliage composition at a site. Minor legume species were woolly burr medic (*M. minima*) and woolly clover (*Trifolium tomentosum*) each contributing 1% on average of the total composition from all sites.

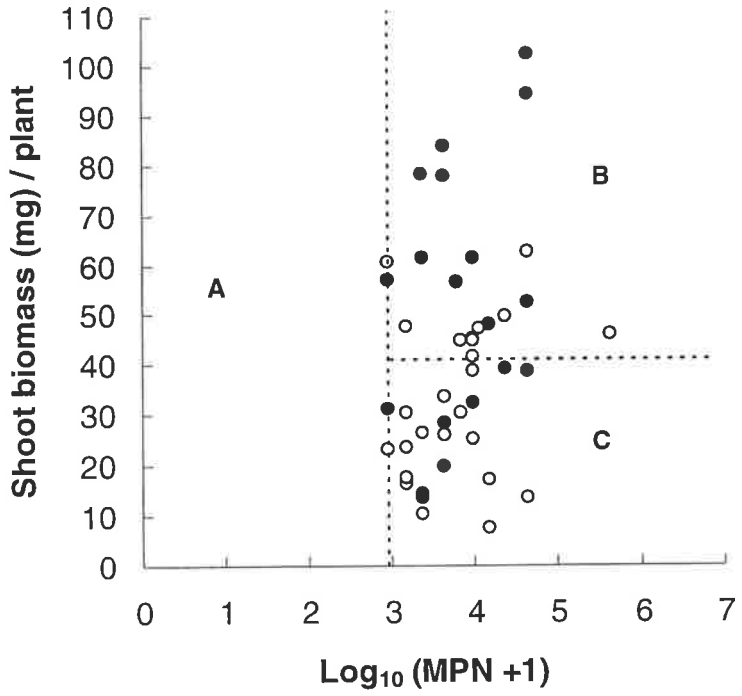

Fig. 3.6 Relationship between symbiotic effectiveness and rhizobial population size determined for selected sites using host plants *T. purpureum* (●) and *T. resupinatum* (○). Areas represent conditions A) where pastures may benefit from inoculation, B) where inoculation is unlikely to improve nitrogen fixation and C) where large populations of ineffective rhizobia are present (see text for discussion). Only sites with >1 × 10^3 rhizobia/g soil were used to estimate symbiotic effectiveness.

The first 4 components of the principal components analysis explained 75% of the variation in environmental characteristics (Table 3.2). Principal component 1 (PC1) described the major influencing factors: organic carbon, base nutrient ions, total nitrogen, pH (water) and mean annual rainfall. PC2 included the influence of clay content (%), mean annual rainfall, soil phosphorus and $CaCO_3$. Considering these first 2 components, which together accounted for 52% of environmental variation, the 5 regions of the survey formed four relatively distinct groups (Fig. 3.7).

Table 3.1. Environmental variables measured for the survey sites (n=61).

Environmental Variable	Mean (±s.e.)	Median	Range	
			Minimum	Maximum
Mean annual rainfall (mm)	379 ± 5	376	320	437
pH (H_2O)[A]	8.2 ± 0.1	8.4	6.2	8.9
pH ($CaCl_2$)	7.52 ± 0.06	7.7	5.2	8.0
Soil P (Colwell) (mg/kg)[B]	25.0 ± 3	24	0.5	204
Soil K (Colwell) (mg/kg)	501.2 ± 29.7	513	60	1279
Total soil N (%)	0.12 ± 0.008	0.11	0.01	0.3
Organic C (%)	1.51 ± 0.07	1.49	0.36	3.4
$CaCO_3$ (%)	11.5 ± 2.6	2.28	0	78.0
Clay content (%)	18.6 ± 1.0	19.0	2	32
Cation exchange capacity (cmol/kg)	21.20 ± 1.14	22.4	2.5	39.5
Electrical conductivity (dS/m)	0.12 ± 0.006	0.131	0.03	0.21
Legume composition (%)	35.3 ± 4.1	20.1	0	100
Clover composition (%)	1.4 ± 0.53	0	0	21.2

[A] soil variables were determined from a sampling depth of 0 to 10cm.

[B] two extreme outliers were not included in the calculation of the mean value (see text).

The sites from Roseworthy were characterized as high rainfall and high phosphorus and formed a group in the positive quarter of PC1 and PC2, excepting one low pH site. Sites from western Eyre Peninsula had positive PC1 values, negative PC2 values and were generally characterized as sites that were high in pH and $CaCO_3$ (Fig. 3.7). Sites from eastern Eyre Peninsula had predominantly negative values for PC1 and were characterized by low cations, organic carbon and total N. Sites from Yorke Peninsula and the Adelaide plains tended towards zero in both principal components.

3.3.5 Variables Correlated to Rhizobial Population Size

The size of populations of clover rhizobia estimated using *T. resupinatum* as the host plant was negatively influenced by pH and $CaCO_3$ and positively correlated with clay content and the presence of a homologous

host plant (Table 3.3). Rhizobial populations estimated using subterranean clover as host plant showed a positive correlation with total N, and a negative correlation with $CaCO_3$, soil P and pH (Table 3.3).

3.4 Discussion

3.4.1 Rhizobial Populations from Alkaline Soils of South Australia

Naturalised soil rhizobial population size is one important factor in determining a response to inoculation with a commercial rhizobial strain (Thies *et al.* 1991a). Populations of clover rhizobia in the low rainfall, alkaline soil environments of South Australia were generally small (median 230-920 rhizobia/g soil), although some sites had large populations.

When Persian and purple clovers were inoculated with large rhizobial populations from survey sites, more than half of the rhizobial populations were shown to be ineffective, compared to commercial strains. Similar findings have been reported using the whole-soil inoculation technique (Quigley *et al.* 1997; Unkovich and Pate 1998) and from inoculating with individual soil isolates (Gibson *et al.* 1975; Gaur and Lowther 1980).

In the present study, symbiotic effectiveness was unrelated to rhizobial density. No attempt was made to relate symbiotic effectiveness to other site variables. While population sizes of rhizobia are often correlated to environmental factors, due to their influence on bacterial survival, there is no *a priori* reason why rhizobial effectiveness should relate to environmental variables (Gibson *et al.* 1975; Quigley *et al.* 1997).

Table 3.2. Normalized principal component loadings from principal component analysis of 10 attributes of 61 survey sites.

Attribute	Components				
	1	2	3	4	5
$CaCO_3$	0.249	-0.309	0.345	0.158	0.580
Clay Content		-0.573	0.133	-0.321	0.257
Soil phosphorus	0.117	0.436	0.123	0.510	0.429
Organic carbon	0.502	-0.158		-0.157	
pH (H_2O)	0.317	-0.264	-0.134	0.443	-0.342
Base nutrient ions	0.489		-0.119		-0.318
Total nitrogen	0.475	0.170			
Legume composition		-0.241	-0.567	0.418	0.205
Clover composition			-0.700	-0.299	0.354
Mean annual rainfall	0.305	0.449		-0.356	0.163
Cumulative percentage of variance	33.5	52.8	65.6	75.2	84.1

Nodule score was positively correlated with symbiotic effectiveness (Fig. 3.5). White (ineffective) nodules were frequently observed on roots and perhaps contributed to a carbon drain, limiting shoot biomass in some treatments.

Table 3.3. Regression models describing *R. leguminosarum* bv. *trifolii* populations at 61 survey sites captured using 2 host legumes.

Host Plant	Intercept	Coefficients			
T. resupinatum	10.43	-1.06	-0.018	+ 0.048	+ 0.081
Factor		(pH)	($CaCO_3$)	(clay)	(%Clover)
Significance		***	*	*	*
T. subterraneum	8.115	-0.027	-0.017	-0.625	+ 4.92
Factor		($CaCO_3$)	(P)	(pH)	(Total N)
Significance		***	**	*	*

*P<0.05; **P<0.01, ***P<0.001.

3.4.2 Influence of Clover Presence upon Rhizobial Numbers

While the survey area comprised sites typically sown to annual *Medicago* spp., naturalised *Trifolium* species are often found as volunteers (Sweeney *et al.* 1993; Fortune *et al.* 1995). Interestingly, clover rhizobia occurred at most sites, despite the absence of clovers.

Clover rhizobia can multiply in the rhizospheres of *Trifolium* or *Medicago* species (Robinson 1967) which may explain their survival in the absence of clover roots. Rhizobial populations were higher in paddocks where clover occurred compared to those without clover. In addition, greater numbers of rhizobia occurred in the rhizospheres of clover compared to samples in the surrounding field. It has been demonstrated (Thies *et al.* 1995) that enrichment of *Bradyrhizobium* spp. populations occurs through the cropping of a homologous host, due mainly to an increase in soil rhizobia from senescing nodules, rather than a build up of rhizobia in the rhizospheres. The increase in rhizobia in the presence of the host will be limited by the constraints placed upon rhizobial multiplication by the soil environment. Our data indicate that multiplication of clover rhizobia in many of the soils sampled could be substantial under the influence of a suitable host plant, but is site specific.

3.4.3 Implications of Rhizobial Populations and Effectiveness

Positive response to inoculation (yield increase) is determined by a complex of factors including yield potential of the legume, availability of soil N and the ability of the indigenous rhizobial population to provide sufficient N_2 fixation (Thies *et al.* 1991a). Inoculation responses are not common under conditions where soil rhizobial populations exceed 1000/g soil (Thies *et al.* 1991a; Thies *et al.* 1994) but have been demonstrated for field pea under Australian conditions with <1000 rhizobia/g soil (Evans *et al.* 1993). In the current survey 60% of sites sampled contained <1000 rhizobia/g soil.

The need for inoculation of a particular legume also depends on the symbiotic effectiveness of the naturalised rhizobia. Where populations are small there is an opportunity to change the composition of the rhizobial microflora through inoculation, to favour strains selected for their effectiveness (Fig. 3.6, condition A).

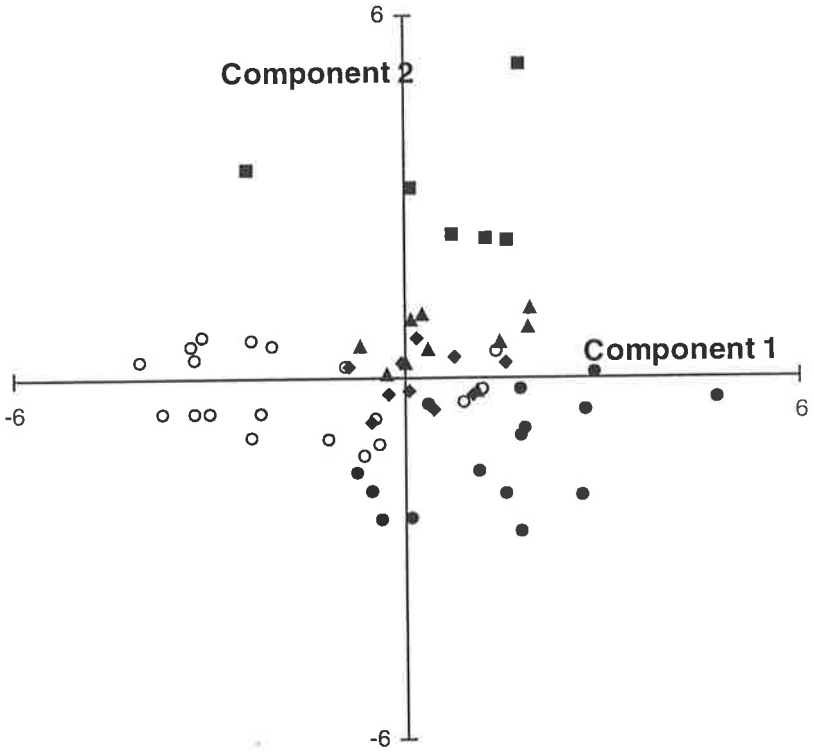

Fig. 3.7 Distribution of sites in relation to principal components 1 and 2. Sites represented are eastern Eyre Peninsula (O) and western Eyre Peninsula (●), Roseworthy Campus (■), Yorke Peninsula (◆) and the northern Adelaide Plains (π).

Where populations of rhizobia are large (eg. >1000 rhizobia/g soil) and effective for the particular host plant, inoculation provides little improvement of N_2 fixation (Fig. 3.6, condition B). If, however, populations are large and ineffective (eg. 25% of sites from the present survey; Fig. 3.6, condition C), as was found for the region of eastern Eyre

Peninsula, then replacing ineffective rhizobia with more effective strains is a substantial challenge (Brockwell *et al.* 1995). A number of methods have been suggested to alter the balance between poorly effective populations resident in the soil, to provide a population of rhizobia that will contribute to greater N_2 fixation (Streeter 1994; Howieson and Rome 1996). By using a legume which is adapted to the soil environment but highly specific in its nodulation requirements, symbioses can be restricted to preferred symbiotic partners eg. *Biserrula pelecinus* (Howieson *et al.* 1995). Secondly, breeding or selection of host legumes, non-specific in their rhizobial requirements, that nodulate effectively with background rhizobia, should improve N_2 fixation (Herridge and Danso 1995). Genetic manipulation of the host legume or rhizobia to encourage a specific symbiosis is a third approach (Triplett 1990a). A further method of improving N_2 fixation involves manipulating rhizobial population dynamics. The addition of lime or phosphate can alter nodule occupancies of plants and subsequently improve N_2 fixation (Almendras and Bottomley 1987). Whatever opportunities exist for improving the symbiosis of alternative clovers using the methods outlined above, they will depend upon our understanding of 1) the rhizobial specificities of alternative clovers, 2) dynamics and genetics of naturalised soil rhizobia, and 3) our ability to alter the abiotic soil conditions through agronomic management.

3.4.4 Influence of Environmental Variables Upon Rhizobial Populations

Several environmental factors influence the size of rhizobial populations. Rhizobia are positively correlated with organic matter, clay content, soil pH, base saturation, mean annual rainfall, irradiance and presence of legumes or legume growth (Bottomley 1992). A number of environmental variables (pH, O.C.%, annual rainfall, elevation, %legume cover) were used to predict native rhizobial populations in Hawaii (Woomer *et al.* 1988). The predictive model was, however, not easily translated to highly managed sites (Thies *et al.* 1995), nor was it applicable to the environments of southern Australia ($P<0.05$, data not shown). Models of this type may be region-specific or at least environment-specific.

Sites surveyed in this study fell into distinct regions, based upon abiotic characteristics. The different regions may pose different problems for the introduction and management of alternative clovers and their rhizobia. Naturalised rhizobial populations were negatively correlated with pH and $CaCO_3$, indicating that the more alkaline and calcareous sites (such as the regions of western Eyre Peninsula) supported fewer rhizobia, possibly the first report of high soil pH associated with a lowering of numbers of clover rhizobia. The influence of pH <7 in reducing rhizobial multiplication (Watkin *et al.* 1997) and reducing field rhizobial population size is well established (Richardson and Simpson 1988; Coventry and Hirth 1992). In addition, soil cultivation can substantially reduce populations of rhizobia, probably due to disruption of favourable microsites (Coventry and Hirth 1992). Conversely, little is known about the influence of alkaline soil reaction on clover rhizobia. Soils of pH >7 support large populations of *R. meliloti* (Brockwell *et al.* 1991), however, *R. meliloti* has greater tolerance to moderately high pH than *R. leguminosarum* bv. *trifolii* (Graham and Parker 1964). The effect of some high pH soils in limiting N_2 fixation is thought to be related to high soil

salinity, bicarbonate and borate (Bordeleau and Prevost 1994). The effect of pH >8.0 and high $CaCO_3$ upon the ability of commercial clover rhizobial strains to colonize soils, compete in the rhizosphere and contribute adequate N_2 fixation, remains to be investigated.

Positive correlations were found between rhizobial populations and percentage clay content in the soil, percentage clover at the site and total nitrogen. Increased clay content is thought to aid the survival of rhizobia, primarily through providing favourable microsites and providing associated nutrition and protection from predators (Bottomley 1992). The presence of clover can assist the multiplication of rhizobia by providing nodules and rhizospheres for rhizobial multiplication. Increased total nitrogen may be indicative of sites with higher organic matter and rainfall, higher fertility or previous legume growth, and conditions benign for rhizobial growth and survival. The reasons for the negative correlation between rhizobial populations and soil phosphorus are unclear.

Chapter 4

The Influence of Inoculation on the Nodulation, Growth and N_2 Fixation of Alternative Clovers in Alkaline Field Soils

4.1 Introduction

A number of "alternative" clover species (eg. *T. cherleri, T. echinatum, T. michelianum, T. purpureum, T. resupinatum*) have shown adaptation to alkaline calcareous soils (calcicoles), based on ecological studies conducted in their centres of origin (Ehrman and Cocks 1990) and in field studies in Australia (Gierke 1996). Some of these clovers have potential economic value in increasing the legume diversity on alkaline soils in Australia, where annual medics are typically grown. In addition, alternative clovers have other ecological characteristics that may be useful attributes for farming systems. Alternative clovers are generally small seeded and are able to be harvested by conventional harvesters, due to their morphology of producing seeds aerially, unlike subterranean clover (Cocks 1995; Ehrman and Cocks 1996).

Since alternative clovers have rarely been grown on alkaline soils in Australia, we do not understand how effectively they will fix N_2 with naturalised strains. The previous chapter identified that naturalised *R. leguminosarum* bv. *trifolii* is often low in number and poorly effective on alkaline soils. Equally, we do not understand how commercial strains of *R. leguminosarum* bv. *trifolii* will react in alkaline soils and whether the symbiosis will be affected by soil pH >8.0.

In order to test whether inoculation of *R. leguminosarum* bv. *trifolii* into

alkaline soils increased nodulation, growth or biological N_2 fixation, we measured these parameters in two field experiments. Three alternative clover species (*T. alexandrinum*, *T. purpureum*, *T. resupinatum*) were chosen, due to their adaptation to alkaline soils and a number of commercial rhizobia were used as inoculants.

4.2 Methods

4.2.1 Introduction

Sites were located in field North 4A at Roseworthy Campus, the University of Adelaide and on a local farm 5km north of Mallala (70km north of Adelaide). These fields had not been used to grow clover in the last 20 years and have not been inoculated with clover rhizobia.

4.2.2 Soil Descriptions

A random sample of 25 soil cores 2.5cm diameter × 10cm depth was taken from each site and mixed. Soil was sieved and the <2mm fractions were analyzed for available phosphorus and potassium (Colwell method), organic carbon (Walkley and Black method), electrical conductivity, pH (H_2O and $CaCl_2$), cation exchange capacity, aluminium and total nitrogen; all these methods are given in (Rayment and Higginson 1992). Micronutrients were also analyzed.

4.2.3 Soil Nitrogen

Soil nitrogen, in the form of nitrate and ammonium, was assessed at both field sites. Soil was sampled in 10cm sections to a maximum depth of 40cm and three replicate samples of soil were analyzed. Soil was air dried and mineral nitrogen was extracted using a 2M KCl extraction according to Rayment and Higginson (1992), excepting that a 1:5 ratio of soil to KCl was used. Samples were analysed for nitrate-N (+nitrite-N) and

ammonium-N using an Alpkem two channel autoanalyser. Soil N results were expressed in terms of oven dry soil (105°C).

4.2.4 Experimental Design

The experiment was designed to determine the influence of seed inoculation of commercial rhizobia on growth, nodulation and N_2 fixation of alternative clovers growing in alkaline soil. Three clover species were chosen for the experiment, based upon their adaptation to alkaline soils (Table 4.1). There were four treatments applied to each clover species. Clover seed was:

- inoculated with one of two strains of rhizobia (Table 4.1),
- sown uninoculated (surface sterilized), or
- left uninoculated and nitrogen (NH_4NO_3) was applied to the plots (200kgN/ha in 3 split applications) to determine what influence N addition had upon clover growth.

Treatments were arranged in a complete randomized block design with five replicates per treatment (plot size 1.8× 8). Strict attention was paid to hygiene and plots were separated by 1.2 m buffers to avoid interplot contamination. The two field sites were arranged and sown in a similar manner, excepting that clovers were sown at 15 kg/ha at the Roseworthy site and 6 kg/ha at the Mallala site and sites were sown on 19 June and 12 June, respectively.

4.2.5 Most Probable Number of Rhizobia

A serial dilution, plant infection technique (Brockwell 1980) was used to estimate the most probable number of rhizobia (MPN) before seasonal rains and at the time of sowing.

4.2.6 Rhizobia

Strains of *R. leguminosarum* bv. *trifolii* were obtained from E. Hartley, Australian Inoculant Research and Control Services, NSW Department of Agriculture (Table 4.2).

Table 4.1 Species of clover and inoculation treatments used in the experiments.

Species	Common name	Seed size (No./kg)	Treatments*	Replicates
T. alexandrinum cv. Elite II	Egyptian clover	330 000	TA1, WU95, Uninoculated, +Nitrogen	5
T. purpureum cv. Paratta	Purple clover	800 000	TA1, WSM409, Uninoculated, +Nitrogen	5
T. resupinatum cv. Nitro	Persian clover	1.5×10^6	TA1, CC2483g, Uninoculated, +Nitrogen	5
Total				60

*200 kg/ha N was added to the +Nitrogen treatments in 3 split applications.

Rhizobial strains were used to prepare peat inoculants. Strains were grown in 100mL sterile YMB (Appendix A) at 27°C for 2 days until cells achieved high concentration (approximately 10^9 cells/ml). Gamma sterile peat bags (final moist weight 250g, obtained from Inoculant Services®) were inoculated with 30mL of inoculant broth and 85mL SDDW. Peat bags were mixed and incubated at 28°C for 2 weeks after which rhizobia were counted using serial dilution plates, to ensure that peat inoculants contained $\geq 2 \times 10^9$ viable cells/g peat.

Table 4.2 Commercial strains of *R. leguminosarum* bv. *trifolii* used in the field experiments.

Strain	Attributes
CC2483g	Current commercial strain for *T. resupinatum*. Collected by J. Katznelson, on roadside in Macedonia, between Skopje and Belgrade, 1965 from *T. subterraneum* ssp. *yanninicum*, probably mildly acidic soil (Brockwell *et al.* 1998).
TA1	Current commercial strain for *T. alexandrinum* and previous commercial strain for *T. subterraneum* and *T. purpureum*. Collected by H.V. Rees, in Bridport, Tasmania, approx. 1953 from *T. subterraneum*, probably neutral to mildly acidic soil (Brockwell *et al.* 1998).
WSM409	Current commercial strain for *T. purpureum* and *T. subterraneum*. Collected by Gus Gintzburger / G. Sardara in Sardinia in April 1981 from *T. subterraneum* in soil of pH 6.0 (J. Howieson, pers. comm.).
WU95	Long-standing commercial strain for *T. subterraneum* (recently superceded). Collected in Western Australia from *T. subterraneum* approx. 1962, probably mildly acidic soil (Brockwell *et al.* 1998).

Seed was inoculated with rhizobial strains and coated with Plastaid™ lime pellet. Seed was sown at 15 and 6 kg/ha, at the Roseworthy and Mallala sites, respectively. The clover seeds were sown in plots (8m × 1.8m) and lightly raked in so that seed was covered by soil. A portion of seed was set aside and after sowing, measurements of the number of viable bacteria per seed were made. One hundred seeds from each clover × rhizobia treatment were washed in 10mL of 0.85% saline and shaken for 5 minutes. Ten-fold dilutions of this solution were then plated on YMA and the original number of rhizobia on a seed sample calculated.

4.2.7 Site Maintenance 1997

Preparation of the field sites involved application of 12kgP/ha as double super phosphate to the site followed by light harrowing. Weeds were controlled prior to sowing using 1L/ha glyphosate. Insect pests were controlled with 1L/ha Endosulfan®. A later application of 375mL/ha

Targa® plus 100mL/ha dimethoate and wetter was used to control rye grass, barley grass and lucerne flea. Weeds were also controlled by hand weeding.

4.2.8 Site Maintenance 1998

Plants were allowed to regenerate from seed produced the previous year and where this was insufficient (eg. *T. alexandrinum* at Roseworthy and Mallala, *T. purpureum* at Mallala), plots were resown with 40kg/ha surface sterilized seed (an amount of seed based on 1997 production). The Mallala site had a light cultivation early Autumn 1998 to control weeds. Weeds were also controlled by hand weeding.

4.2.9 Experimental Sampling

Rhizobial counts and plant samples for yield and nodulation were taken during the 1997 growing season only. In treatments with applied N, samples were taken for above ground parameters only. Establishment of clovers was determined using 3 quadrat counts/plot at 35 days after sowing (DAS). Quadrats (25 × 10cm) were thrown randomly into plots to determine the number of clovers established.

4.2.10 Rhizosphere Populations of Rhizobia

Populations of rhizobia were determined from the rhizospheres of plants from plots using the techniques of Brockwell *et al.* (1991). Three plants were sampled from each plot approximately 21 DAS, depending upon plant development at a site. All soil was removed from the roots, excepting that which was strongly attached to the root hairs, constituting the "rhizosphere". Rhizobia were then enumerated from the three seedlings using a MPN dilution series with plants grown in tubes with vermiculite (Brockwell 1980) and watered with sterile McKnights solution (Appendix A).

4.2.11 Nodulation

Plants were sampled from every plot approximately 50DAS and roots were washed in water and stored at -20°C until needed. Once thawed, 100 plants from each treatment (20/plot) were assessed for nodule number and were given a nodulation score based upon nodule position, size and number (Howieson and Ewing 1989). Below ground effects were not measured for the applied nitrogen (+N) treatments.

4.2.12 Shoot and Seed Biomass

Plant samples were harvested in late spring to determine maximum biomass using two 40 × 40cm quadrats/plot. Shoot biomass was cut at ground level from quadrats and weeds were removed. Shoots were then dried for 3 days at 80°C and weighed. Seed biomass was collected after senescence at the Roseworthy site, by collecting seed pods from two 40 × 40cm quadrats/plot. Pods were threshed and the seed was weighed.

4.2.13 Measurement of Biological Nitrogen Fixation

The amount of biological N_2 fixed by clovers in this trial was assessed using the ^{15}N natural abundance technique (Unkovich *et al.* 1994). The underlying assumption of this technique is that the proportion of ^{15}N present in soil N is higher than that in atmospheric N_2. Increased legume fixation therefore dilutes ^{15}N present in shoots, compared to non-legumes. Measurements of ^{15}N from legumes and non-legumes that are able to access the same pool of soil N are used to calculate the proportion of N_2 fixed.

Duplicate shoot samples of clover and available non-legume weeds, thought to possess similar rooting systems, were sampled late in the 1997 season where the two species were growing within 5cm of each other. Clover and capeweed (*Arctotheca calendula*) were sampled at the

Roseworthy site; clover and ryegrass (*Lolium rigidum*) were sampled from the Mallala site. In addition, samples of Indian hedge mustard (*Sisymbrium orientale*) were obtained from the Mallala site and used to calculate N_2 fixation.

B values (see below) were used to define the maximum limit of ^{15}N that could be determined from a clover completely reliant upon N_2 fixation for its N requirements (Unkovich *et al.* 1994). In order to generate B values, 3 replicate pots of the clover × strain combinations, as used in the field experiment, were grown in sterile mix of 1:1 vermiculite and sand supplied with McKnights N-free nutrient solution (Gibson 1980). These plants were grown in a glasshouse (17-25°C) and were harvested when they achieved a similar growth stage to when field plants were harvested.

All shoot samples were dried at 60°C for 3 days and ground finely using a grinding mill (Labtechnics Laboratory Pulverising Mill). Approximately 3mg of dried and finely ground shoot material was accurately measured and sealed into tin capsules. Analyses were carried out by Automated Nitrogen Carbon Analysis-Mass Spectrometry. Tin foil capsules were combusted and the reaction products were separated by GC to give pulses of pure nitrogen and carbon dioxide (removed by trapping in Carbosorb) for analysis of total N and ^{15}N by the mass spectrometer (20-20, Europa Scientific, Crewe, U.K.). Analysis of the $\delta^{15}N$ was performed at CSIRO Division of Land and Water, Adelaide.

The amount of N_2 fixation derived from the atmosphere ($\%N_{dfa}$) was determined using the equation:

$$\% N_{dfa} = (\delta^{15}N \text{ reference plant} - \delta^{15}N \text{ legume}) / (\delta^{15}N \text{ reference plant} - B)$$

where B refers to the $\delta^{15}N$ value of the effectively nodulated legume grown in media totally lacking combined nitrogen (Unkovich *et al.* 1994).

4.2.14 Statistical Analysis

Data for each experiment were analyzed separately with 2 factor Anovas, taking into account the blocking of experimental treatments into replicates. Data were analyzed using Genstat 5 (Payne 1993). All three clover species were inoculated with strain TA1 and one other strain of rhizobia, and these treatments were analyzed using the common strain (TA1) and grouping the other three strains into a single grouping ("Other"). In cases where data did not satisfy assumptions of normality, data were log (X+1) transformed.

4.3 Results

4.3.1 Site Description

The soil at both experimental sites was slightly alkaline loam soil with 23-28% clay, 1.5% organic carbon and ≥25ppm P in the 0-10cm layer (Table 4.3). The electrical conductivity was low for the sites, cation exchange capacity was high and the majority of micronutrients were in adequate levels, boron was moderate and chloride and aluminium were low (Table 4.3). Previous cropping rotation histories indicated a mixture of cereals, grain legumes and pasture legumes (annual *Medicago* spp.); clover had not been grown on these sites in the last 20 years. Consequently neither site had been inoculated with *R. leguminosarum* bv. *trifolii* during that time.

4.3.2 Precipitation

Annual rainfall during the trial period was above average, except at Roseworthy in 1998 (Table 4.4). More importantly, rainfall varied between years and in 1997 both experiments received sporadic rain during the season (Fig. 4.1). Both sites experienced a month of drought in the 1997 season, from late September to late October. Precipitation was

more uniform during the 1998 season (Fig. 4.1). The April-October rainfall, generally considered to be the growth period for annual pasture, was 265-305mm (Table 4.4).

Table 4.3 Soil characteristics from the Roseworthy and Mallala sites where field experiments were conducted, sampled from 0-10cm.

Attribute	Unit	Roseworthy	Mallala
Texture		loam	loam
pH water		8.0	8.5
pH $CaCl_2$		7.3	7.7
Percent clay	%	28	~23*
Extractable P	mg/kg	25	34
Extractable K	mg/kg	564	653
Extractable S	mg/kg	6.7	4.8
Org. C	%	1.64	1.37
E.C. (1:5)	dS/m	0.15	0.10
ECe (est.)	dS/m	1.40	1.00
Free lime		slight	moderate
Nitrate	mg/kg	14	10
Exchangeable Ca	mequiv/100g	21.00	17.40
Exchangeablemg	mequiv/100g	3.21	1.72
Exchangeable Na	mequiv/100g	0.49	0.16
Exchangeable K	mequiv/100g	1.65	1.63
Exchangeable Al	mequiv/100g	0.00	0.00
C.E.C.	mequiv/100g	26.35	20.91
Extractable Cu	mg/kg	2.3	1.9
Extractable Zn	mg/kg	3.1	3.4
Extractable Mn	mg/kg	150.0	40.3
Extractable Fe	mg/kg	39.0	11.0
Extractable Al	mg/kg	0.1	0.1
Extractable B	mg/kg	1.8	1.5
Extractable Cl	mg/kg	42.0	20.0

*determined from a soil nearby the Mallala site.

4.3.3 Soil Nitrogen

Samples taken from the field sites at the time of sowing indicated large variation in the amount of soil nitrate and ammonium across both sites (Table 4.5). Soil nitrate was highest, and most variable, 10-30cm down the soil profile at the Roseworthy site; at Mallala soil nitrate decreased with depth (Table 4.5). Ammonium in the soil was unevenly distributed in the soil and also variable.

Table 4.4 Rainfall at the experimental field sites.

Location	Year	Long-term annual rainfall (mm)	Annual rainfall (mm)	April-October rainfall (mm)
Roseworthy		430		
	1997		445	291
	1998		340	265
Mallala*		402		
	1997		508	305
	1998		462	299

* rainfall data from Mallala Post office, approximately 5km south of the field site.

4.3.4 Naturalised and Inoculant Rhizobia

Rhizobial counts indicated that naturalised rhizobia were present at both experimental sites and increased slightly after rainfall (Table 4.6). The number of rhizobia estimated, differed according to the clover species and the time of sampling, but in all cases MPN estimates were less than 500rhizobia/g soil (Table 4.6).

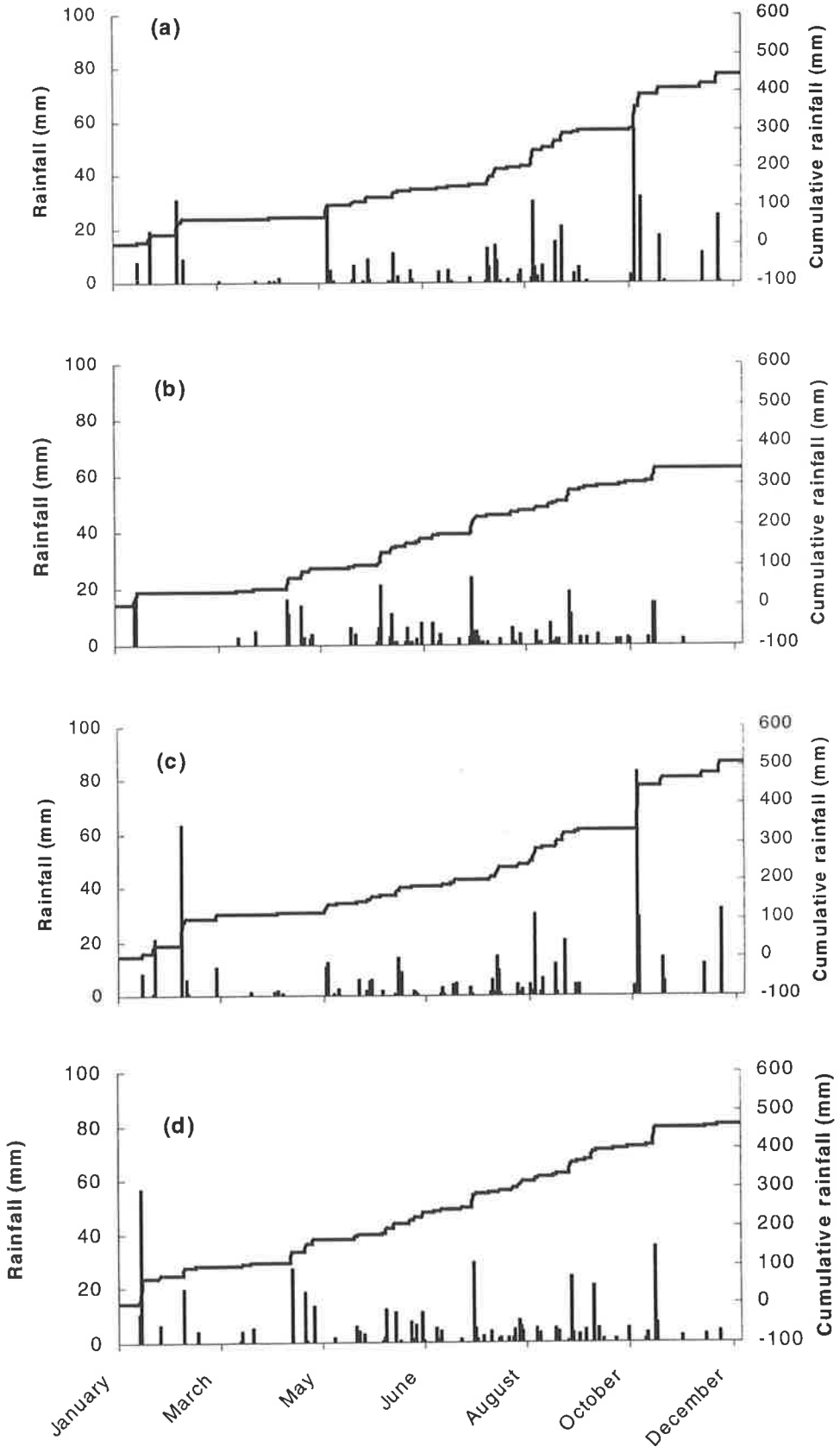

Figure 4.1 Rainfall events (bars) and cumulative rainfall (line graph) for the Roseworthy (a,b) and Mallala (c,d) field sites for 1997 and 1998, respectively.

Table 4.5. Soil nitrate and ammonium from the Roseworthy and Mallala sites, sampled at the time of sowing, 1997.

Site	Depth (cm)	Nitrate-N (mg/kg)	se	Ammonium-N (mg/kg)	se
Roseworthy	0-10	3.3	0.6	7.2	4.7
	10-20	13.3	8.0	0.8	0.4
	20-30	17.3	8.5	8.4	8.0
	30-40	4.6	3.4	1.6	0.8
Mallala	0-10	7.6	1.1	3.5	0.6
	10-20	5.0	1.9	3.0	0.2
	20-30	4.0	1.0	4.1	2.2
	30-40	2.1	1.4	9.9	5.3

The number of viable rhizobia present on seed at the time of sowing indicated that greater numbers of rhizobia were associated with the larger seeds of *T. alexandrinum*, compared to the smaller *seeded T. resupinatum* and *T. purpureum* (Table 4.7). The number of rhizobia per gram of inoculated seed was similar across sites, plant species and strains of rhizobia, except for the treatment *T. resupinatum* × CC2483g, which was poorly inoculated (Table 4.7).

4.3.5 Plant Establishment

Establishment of clovers varied according to species of clover sown and to the density sown. *T. resupinatum* had a high emergence rate at the Roseworthy site, while there was significantly lower emergence of *T. alexandrinum* and *T. purpureum* (Table 4.8). At the Mallala site *T. alexandrinum* established poorly; there were twice as many *T. purpureum* in plots at that site and twice as many plants again in plots containing *T. resupinatum*. Plant establishment did not vary according to

the rhizobial treatment applied at either site.

4.3.6 Rhizosphere Populations of Rhizobia

Populations of rhizobia from the rhizospheres of approximately 3 week old clovers were determined from the Roseworthy site (Fig. 4.2). Rhizosphere populations of *R. leguminosarum* bv. *trifolii* appeared to be highest when TA1 was the inoculant, compared to when other strains were inoculants or no inoculants were used. Populations varied from 1.6 to 2.9 on the log scale, but three replicates of each species × rhizobia treatment was insufficient to indicate significant effects of either plant or inoculants on rhizosphere populations ($P>0.05$).

Table 4.6 Rhizobial estimates (Most Probable Number) from the Roseworthy and Mallala field experiments. All estimates are site means measured in rhizobial numbers /g soil.

Site	Trap species	1996 Oct	1997 prior to breaking rain	1997 at sowing	1998 at sowing
Roseworthy	*T. alexandrinum*	-	30	230	420
	T. purpureum	-	0	360	325
	T. resupinatum	40	30	40	420
	T. subterraneum	420	-	-	-
Mallala	*T. alexandrinum*	-	0	40	0
	T. purpureum	-	0	40	325
	T. resupinatum	-	160	0	40

Table 4.7 Number of rhizobia per seed and per gram of inoculated seed, at sowing.

Site	Plant species	Strain*	Number of rhizobia/seed**	Number of rhizobia/g inoculated seed
Roseworthy	*T. alexandrinum*	TA1	7500	1.28×10^5
		WU95	n.d.	n.d.
		UC	0	0
	T. purpureum	TA1	4200	1.62×10^5
		WSM409	7100	2.23×10^6
		UC	0	0
	T.resupinatum	TA1	2300	2.22×10^5
		CC2483g	185	1.22×10^5
		UC	0	0
Mallala	*T. alexandrinum*	TA1	n.d.	n.d.
		WU95	n.d.	n.d.
		UC	n.d.	n.d.
	T. purpureum	TA1	4050	1.64×10^5
		WSM409	6000	2.21×10^5
		UC	0	0
	T.resupinatum	TA1	1200	1.11×10^5
		CC2483g	48	4×10^5
		UC	0	0

* UC indicates the uninoculated control (no addition of N).

**n.d.- not detected due to insufficient seed or contaminated plates.

4.3.7 Nodulation

The total number of nodules were counted and a nodule score was assigned, according to nodule size and position (on upper taproot or further down on laterals) (Howieson and Ewing 1989). At the Roseworthy site, applications of rhizobia did not influence the number of nodules (Fig. 4.3). The number of nodules (Fig. 4.3) and the nodule score (Table 4.9) both varied according to plant species: *T. resupinatum* had more nodules and larger nodule scores compared to *T. alexandrinum,* which had more nodules and a higher score than *T. purpureum* ($P<0.001$ for nodule number and nodule score). The taproot nodule score was defined by both the plant species ($P<0.001$) and the application of rhizobia ($P<0.05$). *T. resupinatum* had a higher taproot nodule score than either *T. purpureum* or *T. alexandrinum* (Table 4.9). Plants inoculated with TA1 or uninoculated had the lowest taproot nodule scores, treatments inoculated with other strains of rhizobia (WU95, WSM409, CC2483g) had significantly larger taproot nodule scores.

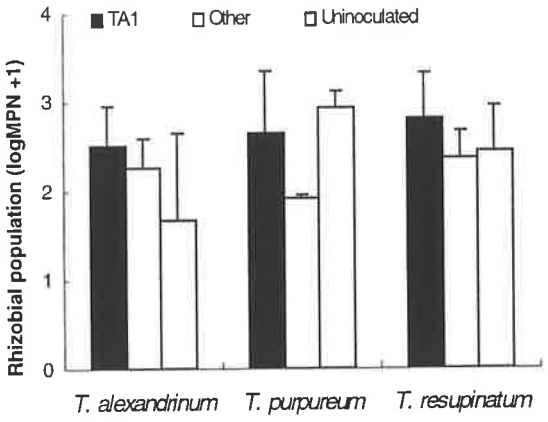

Fig. 4.2 Rhizosphere populations of rhizobia from Roseworthy site in 1997. Error bars indicate standard error.

Table 4.8 Establishment of clover species at the Roseworthy and Mallala field experiments in 1997.

Site	Species	Sowing Density kg/ha	Seed size No./ kg	Emergence (plants/m²)*
Roseworthy	*T. alexandrinum*	15	330 000	167[a]
	T. purpureum	15	800 000	154[a]
	T. resupinatum	15	1.5×10^6	579[b]
Mallala	*T. alexandrinum*	6	330 000	52[a]
	T. purpureum	6	800 000	117[b]
	T. resupinatum	6	1.5×10^6	223[c]

*Values followed by the same letter within a site do not differ significantly at the 0.05% level.

At the Mallala site, the number of nodules ($P<0.05$) and the nodule scores ($P<0.01$) were influenced by the interaction of clover species × inoculation (Fig. 4.3, Table 4.9). The number of nodules formed by *T. alexandrinum* and *T. purpureum* did not differ due to applied rhizobia, while a greater number of nodules were formed when *T. resupinatum* was inoculated (Fig. 4.3). For *T. purpureum,* nodule score did not increase due to inoculation, while nodule scores of *T. resupinatum* increased due to inoculation (Table 4.9). *T. alexandrinum* inoculated with TA1 had a higher nodule score than *T. alexandrinum* that was uninoculated; the treatment inoculated with WU95 was intermediate. The score of taproot nodules varied according to plant species: *T. alexandrinum* had a higher taproot nodule score than *T. resupinatum*, which was higher than *T. purpureum* ($P<0.001$; Table 4.9).

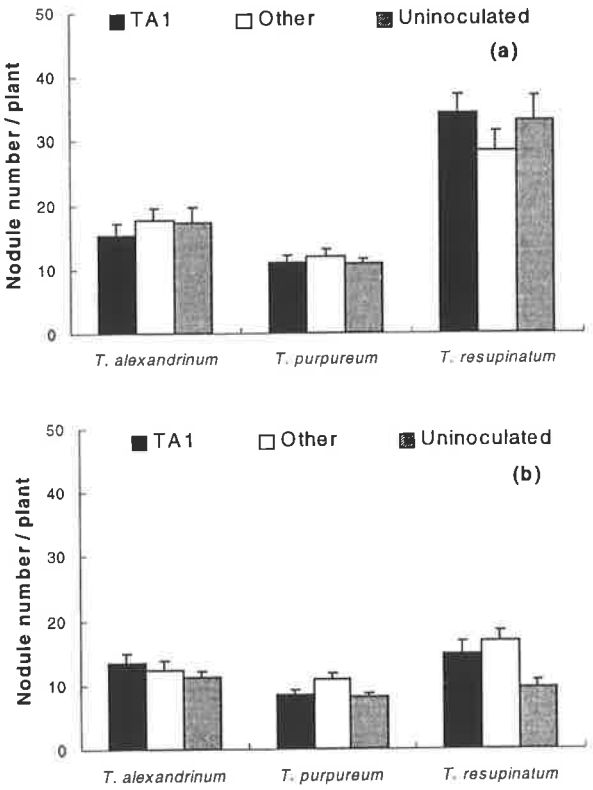

Fig. 4.3 Nodule numbers from the Roseworthy (a) and Mallala (b) field sites in 1997. Error bars indicate standard error.

4.3.8 Pasture Growth

Neither inoculation with rhizobia nor the addition of 200kg/ha of N increased pasture shoot growth, compared to uninoculated treatments at both field sites ($P>0.05$; Fig. 4.4). At the Roseworthy site, shoot biomass of purple (*T. purpureum*) and Persian (*T. resupinatum*) clovers did not differ significantly, but both were larger than the shoot biomass of *T. alexandrinum* (Fig. 4.4). Significantly less shoot biomass was produced at the Mallala site (2.6 t/ha) compared with the Roseworthy site (mean 4.2 t/ha). *T. purpureum* produced significantly more shoot biomass than *T. alexandrinum* or *T. resupinatum* at the Mallala site ($P<0.001$; Fig. 4.4).

4.3.9 Seed Production

Seed produced at the Roseworthy experiment was governed by the species of clover and did not differ according to the rhizobial treatment applied to the clovers (Fig. 4.5). The late flowering Egyptian clover, *T. alexandrinum* produced significantly less seed (211kg/ha) compared to *T. resupinatum* (673kg/ha) and *T. purpureum* (728kg/ha), which did not differ in seed production ($P<0.001$).

4.3.10 Nitrogen Accumulation

The N concentration (%) in shoots was dependent upon clover species: at Roseworthy %N was highest for *T. resupinatum* (2.52); *T. alexandrinum* (2.25) had a higher %N than did *T. purpureum* (2.06) ($P<0.001$). At the Mallala site *T. purpureum* had the lowest %N (2.24); %N did not differ between *T. resupinatum* (2.66) and *T. alexandrinum* (2.56) ($P<0.001$).

The concentration of N in shoots was combined with the total shoot biomass to determine the total N yield (kgN/ha) of shoots (Fig. 4.6). At the Roseworthy site, the N yield of shoots was affected by the species of clover and the inoculation of rhizobia or application of N. Persian clover (*T. resupinatum*) had a higher N yield than *T. alexandrinum* and *T. purpureum*, while the latter two did not differ in total N yield ($P<0.001$). Treatments with N applied to plots or strain TA1 applied to seed had a greater N yield than uninoculated plots; treatments that had other rhizobial strains applied were intermediate ($P<0.01$). At the Mallala site, the applied N treatment produced shoots with a higher N-yield than the uninoculated treatment; treatments with applied rhizobia were intermediate and plant species did not alter N yield ($P<0.05$).

Table 4.9 Whole root nodule scores and taproot nodule scores from the Roseworthy and Mallala field sites in 1997.

Site	Species	Applied rhizobia*	Nodule score	se	Tap root nodule score	se
Roseworthy	*T. alexandrinum*	UC	51.3	5.8	19.0	2.9
		TA1	45.4	4.9	19.8	2.6
		WU95	52.1	4.6	22.1	2.8
	T. purpureum	UC	34.7	2.5	19.0	2.2
		TA1	36.7	3.8	16.1	2.3
		WSM409	43.5	4.8	23.3	3.1
	T. resupinatum	UC	78.0	8.5	26.3	2.1
		TA1	80.5	6.2	32.5	2.3
		CC2483g	67.2	6.7	26.6	2.0
Mallala	*T. alexandrinum*	UC	36.0	2.4	17.8	1.9
		TA1	46.1	3.9	23.4	2.8
		WU95	38.5	4.0	16.1	2.0
	T. purpureum	UC	22.1	1.5	9.4	1.4
		TA1	23.2	1.7	10.3	1.1
		WSM409	28.3	2.1	10.2	1.4
	T. resupinatum	UC	24.6	2.3	12.0	1.1
		TA1	37.3	4.2	14.9	1.8
		CC2483g	41.0	3.9	16.3	2.1

*UC indicates uninoculated control.

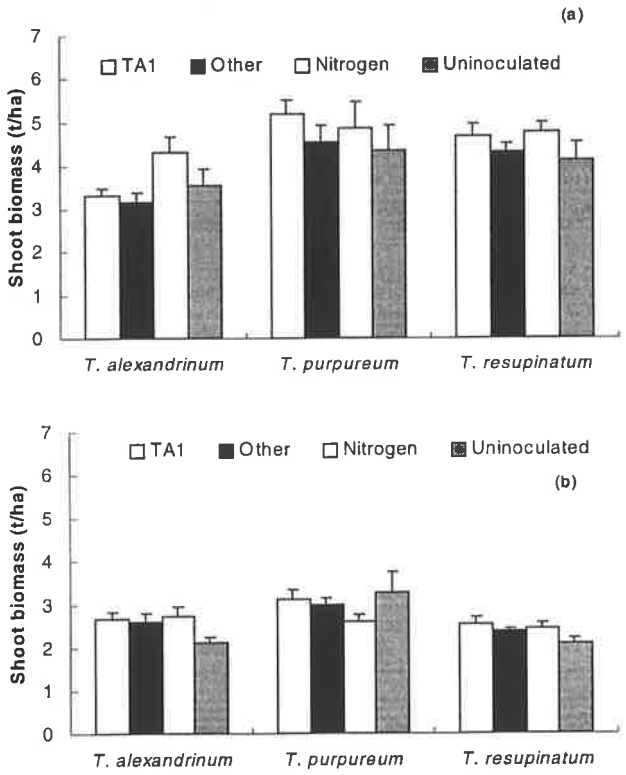

Fig. 4.4 Shoot biomass produced at the Roseworthy (a) and Mallala (b) field sites. Error bars indicate standard error.

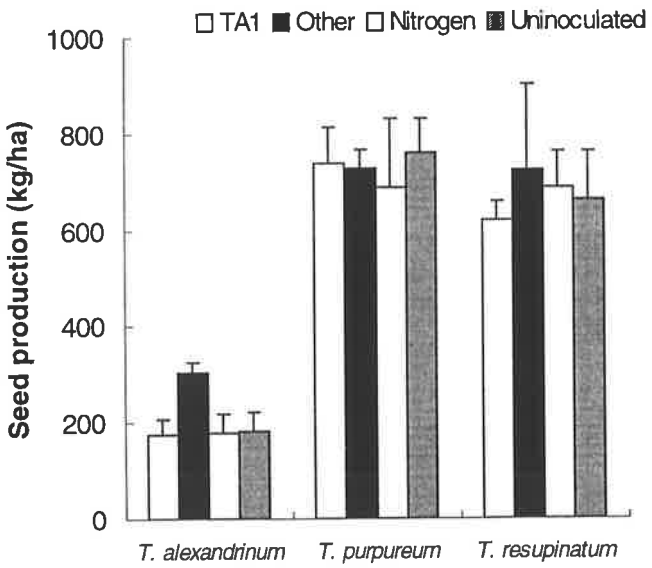

Fig. 4.5 Seed production at the Roseworthy field site. Error bars indicate standard error.

Most $\delta^{15}N(‰)$ values obtained from non-legumes at the experimental sites were considered acceptably high to define the upper limit of N_2 fixation (Table 4.10). Positive values were obtained for non-legumes *Arctotheca calendula* (capeweed) at Roseworthy and *Lolium rigidum* (ryegrass) and *Sisymbrium orientale* (Indian hedge mustard) at the Mallala site, and negative values were observed for clover shoots, indicating dilution of ^{15}N due to N_2 fixation (Table 4.10). However, $\delta^{15}N(‰)$ values for non-legumes sampled in the +N treatments were often very low and highly variable (eg. –8.13 to 3.48), particularly at the Roseworthy site (Table 4.10). These values were therefore considered unreliable for analysis. Plants from plots with applied N would be considered to have somewhat suppressed N_2 fixation compared to plants without added N.

Analysis of the data showed that non-legumes from either site did not differ in $\delta^{15}N(‰)$, while $\delta^{15}N(‰)$ for clovers differed according to clover species. At the Roseworthy site, $\delta^{15}N(‰)$ measured on clover were lowest for *T. alexandrinum,* but did not differ between *T. resupinatum* and *T. purpureum* ($P<0.001$). *T. alexandrinum* at the Mallala site had a significantly lower $\delta^{15}N(‰)$ compared to *T. purpureum*, while *T. resupinatum* was intermediate ($P<0.05$). Analysis of shoot material from the Mallala site indicated that *S. orientale* (Indian hedge mustard) provided a higher $\delta^{15}N(‰)$ compared to *L. rigidum* (ryegrass) (Table 4.10). B values of $\delta^{15}N(‰)$ obtained for all clover species were negative, with some values, eg. *T. alexandrinum*, exceptionally low (Table 4.10). *T. alexandrinum* and *T. purpureum* showed marked differences in $\delta^{15}N(‰)$ due to inoculation with different strains of rhizobia ($P<0.01$), but $\delta^{15}N(‰)$ measured for *T. resupinatum* did not vary between the two applied inocula (Table 4.11). Average B values (mean of 6 estimates) from each clover were used for uninoculated treatments (Table 4.11).

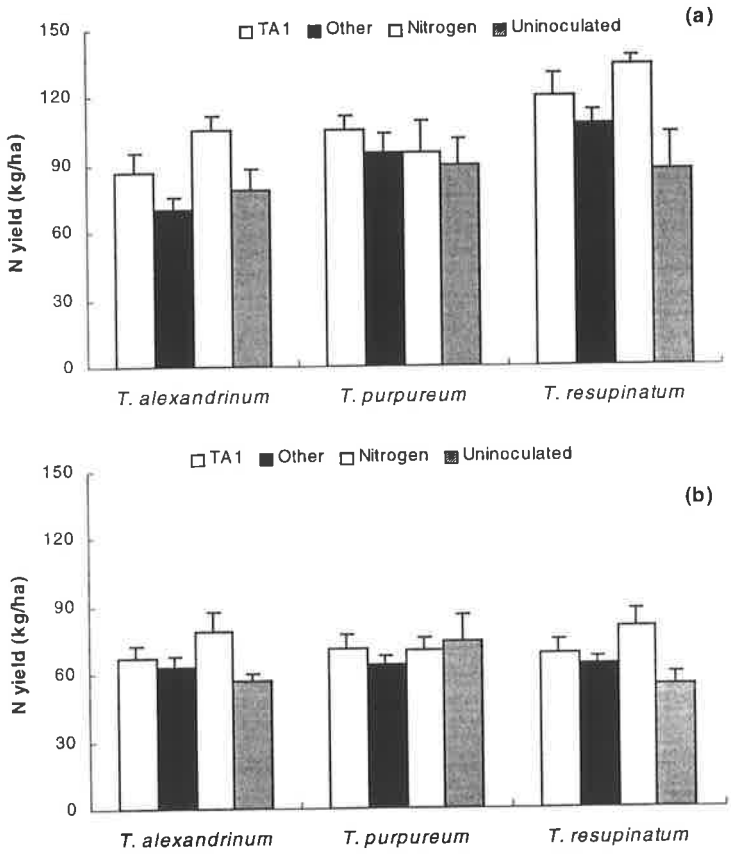

Fig. 4.6 N yield in shoots of clovers from field trials at (a) Roseworthy and (b) Mallala. Error bars indicate standard error.

The percentage of N derived from fixation ($\%N_{dfa}$) was calculated using the ^{15}N natural abundance technique (Table 4.12). $\%N_{dfa}$ at both field sites was influenced by the interaction of clover species × inoculation ($P<0.001$ all sites and reference plants). At Roseworthy, *T. alexandrinum* fixed nitrogen best with strain TA1, while $\%N_{dfa}$ of plants inoculated with WU95 was not different from the uninoculated treatment. *T. purpureum* inoculated with WSM409, and uninoculated, fixed a higher proportion of N than did *T. purpureum* with TA1 applied; *T. resupinatum* derived more than 70% of its N from fixation in all treatments, which did not differ significantly (Table 4.12).

Table 4.10 Delta ^{15}N values for shoots of non-legume reference plants and clovers from the Roseworthy and Mallala field sites, sampled in 1997.

Site	Plant species	Applied Treatment*	Non-legume $\delta^{15}N$(‰)**	se	Clover $\delta^{15}N$(‰)	se
Roseworthy	*T. alexandrinum*	UC	1.70	0.30	-1.14	0.18
		TA1	2.08	0.22	-1.14	0.06
		WU95	1.85	0.29	-1.43	0.08
		+Nitrogen	-5.66	0.94	-3.63	0.45
	T. purpureum	UC	2.13	0.34	-0.92	0.14
		TA1	1.80	0.30	-0.71	0.10
		WSM409	2.07	0.36	-0.74	0.17
		+Nitrogen	-3.18	0.85	-3.47	0.27
	T. resupinatum	UC	2.64	0.34	-0.88	0.15
		TA1	2.21	0.19	-0.99	0.13
		CC2483g	2.05	0.23	-1.05	0.10
		+Nitrogen	-3.71	1.08	-4.16	0.14
Mallala	*T. alexandrinum*	UC	0.78	0.04	-0.80	0.13
		TA1	0.57	0.19	-0.71	0.13
		WU95	1.20	0.16	-0.91	0.03
		+Nitrogen	0.31	0.33	-1.07	0.20
	T. purpureum	UC	0.95	0.22	-0.68	0.13
		TA1	0.41	0.31	-1.28	0.22
		WSM409	1.00	0.24	-0.65	0.20
		+Nitrogen	1.13	0.37	-0.89	0.65
	T. resupinatum	UC	0.77	0.12	-0.88	0.10
		TA1	0.86	0.21	-0.68	0.10
		CC2483g	1.17	0.15	-0.41	0.06
		+Nitrogen	0.76	0.37	-0.39	0.42
Mallala	*L. rigidum*	Mean	0.82	0.08		
	S. orientale	Mean	2.09	0.20		

* UC indicates the uninoculated control, +Nitrogen indicates the plots with 200kg/ha of applied nitrogen.

** Non-legumes sampled were *A. calendula* (capeweed) at the Roseworthy site and *L. rigidum* (ryegrass) at the Mallala site. *S. orientale* (Indian hedge mustard) was sampled from Mallala for preliminary analysis.

Table 4.11 B values used in the experiment.

Clover species	Strain	δ15N(‰)*	se
T. alexandrinum	TA1	-2.42	0.76
	WU95	-5.52	0.68
	Mean	-3.97	0.76
T. purpureum	TA1	-3.66	0.57
	WSM409	-1.93	0.50
	Mean	-2.80	0.50
T. resupinatum	TA1	-2.25	0.05
	CC2483g	-2.03	0.07
	Mean	-2.14	0.07

*Mean B values were used for control treatments in calculating $\%N_{dfa}$.

When mean values of Indian hedge mustard from the Mallala site were used as the non-legume reference, *T. alexandrinum* fixed the largest proportion of N_2 when inoculated with TA1; the proportion was smaller when uninoculated and even less when inoculated with WU95 (Table 4.12). *T. purpureum* fixed N_2 best with strain WSM409, while inoculation with strain TA1 and the uninoculated treatment did not differ. *T. resupinatum* fixed more than 60% of its N regardless of inoculation; a greater proportion of N_2 was fixed when this species was uninoculated.

When ryegrass was used as a reference plant for clovers at the Mallala site, *T. alexandrinum* again had the greatest proportion of N_2 fixed when in combination with strain TA1; other treatments were significantly lower (Table 4.12). *T. purpureum* also fixed N_2 best with WSM409; when strain TA1 was applied to *T. purpureum*, $\%N_{dfa}$ was lower than the uninoculated control. The percentage of fixed N_2 was again highest for *T. resupinatum*, but did not differ according to inoculation.

The absolute amount of shoot N fixed (kgNfixed/ha) at Roseworthy was determined by the interaction of clover species × applied rhizobia ($P<0.01$; Table 4.12). At Roseworthy, *T. alexandrinum* fixed 60 kg N/ha when inoculated with strain TA1, while the other treatments were significantly lower (Table 4.12). *T. purpureum* fixed the greatest amount of nitrogen (65kgN/ha) when inoculated with strain WSM409, slightly less when uninoculated, and significantly less when inoculated with strain TA1. *T. resupinatum* fixed N_2 well with both CC2483g and TA1 (>80kgN/ha), but still fixed more than 60kg of N when uninoculated.

When mean values for Indian hedge mustard were used as the reference for the Mallala site, the amount of N_2 fixed/ha was again determined by the interaction of clover species × rhizobial treatment ($P<0.05$). Amounts of N_2 fixed did not differ due to inoculation for *T. purpureum* or *T. resupinatum*. *T. alexandrinum* fixed the largest amount of N_2 when inoculated with strain TA1, while N_2 fixation was significantly lower when WU95 was the inoculant or there was no inoculation (Table 4.12).

When ryegrass was used as the non-legume reference plants at Mallala, the amount of N_2 fixed by clovers varied according to plant species * rhizobial treatments ($P<0.01$), although absolute amounts of N_{dfa} were less. *T. alexandrinum* responded in a similar trend to Roseworthy, although only fixed ~20kgN/ha. *T. purpureum* fixed a similar amount of N (>30kgN/ha) when inoculated with WSM409 or sown uninoculated, and less (~20kgN/ha) when inoculated with TA1. *T. resupinatum* did not respond to inoculation, but fixed >30kgN/ha, in all treatments.

Table 4.12 Percentage and amount of total shoot N derived from atmospheric N_2. Non-legume reference plants used to determine these values are capeweed (*A. calendula*) at the Roseworthy (RW) site and Indian hedge mustard (*S. orientale*) and ryegrass (*L. rigidum*) at the Mallala (Mal) site.

Site	Species *	Applied Rhizobia	%N_{dfa}	se	N_{dfa} (kg/ha)	se	%N_{dfa}	se	N_{dfa} (kg/ha)	se
			(Broadleaf)**				(*L. rigidum*)***			
RW	Alex	UC	50	2.0	39	4.2				
		TA1	71	2.2	61	5.0				
		WU95	44	1.9	31	1.5				
	Purp	UC	62	3.2	55	6.2				
		TA1	45	2.9	48	5.4				
		WSM409	70	4.9	65	5.2				
	Res	UC	73	3.0	63	10.8				
		TA1	72	2.2	86	7.1				
		CC2483g	75	3.4	81	5.2				
Mal	Alex	UC	49	2.2	28	1.9	33	2.4	20	1.7
		TA1	64	2.9	43	4.8	52	5.3	34	5.9
		WU95	40	0.4	25	1.8	31	1.6	21	1.0
	Purp	UC	55	2.7	40	6.0	44	2.5	29	3.7
		TA1	48	3.8	35	4.6	26	6.1	19	4.5
		WSM409	65	5.0	42	4.6	54	4.8	35	3.4
	Res	UC	70	2.3	39	5.1	58	3.9	34	5.2
		TA1	66	2.2	46	4.6	56	2.0	36	2.1
		CC2483g	61	1.4	39	1.7	49	3.4	30	1.5

*Alex= *T. alexandrinum*, Purp = *T. purpureum*, Res = *T. resupinatum*.

** %N_{dfa} and N_{dfa} determined using broadleaf plants: *A. calendula* as reference plant at Roseworthy and mean values of *S. orientale* from the Mallala site.

*** %N_{dfa} and N_{dfa} for Mallala determined using *L. rigidum* as reference plant.

4.4 Discussion

4.4.1 The Influence of Inoculation and Nitrogen Addition on Pasture Growth and Nodulation

Legume dry matter increases due to inoculation are often not achieved in field situations (Streeter 1994; Vlassak and Vanderleyden 1997; Sadowsky and Graham 1998). In the current experiments, pasture growth of alternative clovers did not increase due to inoculation. This suggests that the naturalised populations of rhizobia, estimated at <500/g soil at sowing, sufficiently nodulated clovers such that N was not limiting pasture growth, even though the sites had not received application of rhizobia in the previous 20 years and had not been used to grow clovers. Alternatively, the soil N pool was adequate so that the growth of clovers was not limited. Significantly greater pasture growth achieved at the Roseworthy site (mean 4.2t/ha), compared to the Mallala site (2.6t/ha), was probably due to the higher seeding rate (15kg/ha cf. 6 kg/ha) and higher plant establishment at the Roseworthy site.

N demands of a legume grown in a field can be met by supply from soil N, indigenous rhizobia or inoculant rhizobia (Thies *et al.* 1992). The addition of 200kgN/ha did not improve pasture dry matter, compared to uninoculated treatments. This result may indicate that the soil N pool was sufficient to provide N requirements for clovers, or so variable that treatment differences were obscured. Alternatively, naturalised *R. leguminosarum* bv. *trifolii* provided sufficient N_2 fixation so that uninoculated pasture, with no additional N, produced similar pasture growth to those supplemented with N.

Increases in nodule numbers are often observed due to inoculation onto soils with few rhizobia, or where resident soil populations do not interact with sown legumes (Hebb *et al.* 1998; Howieson *et al.* 2000a). In the

present experiments, nodules were formed in all treatments sampled, although nodule number varied mainly according to clover species. Nodule numbers on Persian clover (*T. resupinatum*) did increase due to inoculation at the Mallala site, where the number of naturalised rhizobia able to nodulate this plant was 0-40 rhizobia/g soil.

Seed biomass produced was similarly unaffected by applied inocula or N. The early flowering species, *T. purpureum* (140-150 days until first flower) and *T. resupinatum* (115 dff) produced acceptable levels of seed (728 and 673 kg/ha) compared to the late flowering *T. alexandrinum* (211 kg seed/ha; 191 dff) (Oram 1990; J. Howie, S. Hughes, pers. comm.).

4.4.2 The Influence of Inoculation and Nitrogen Addition on N Accumulation

While pasture growth did not respond to applied inocula or 200kgN/ha, inoculation consistently increased the N-yield, the proportion ($\%N_{dfa}$) and amounts of N_2 fixed by clovers. All clovers fixed N_2 most efficiently when they were inoculated with their specific commercial inoculant, other strains were less efficient. *T. purpureum*, for example fixed N_2 best with WSM409, but fixed N_2 poorly when inoculated with strain TA1, which has been shown to be an inferior combination (Howieson *et al.* 2000a). *T. resupinatum* was symbiotically competent with CC2483g and TA1 and with the naturalised soil rhizobia.

The proportions of N_2 derived from fixation and absolute amounts of N_2 fixed in the current experiments were similar to many other reports for *T. subterraneum, T. resupinatum* and *T. repens* (Sanford *et al.* 1993; Evans 1996; Unkovich *et al.* 1997; Peoples and Baldock 1999; Riffkin *et al.*1999a), except that amounts of N_2 were constrained by low legume content in dairy pastures (60-67 $\%N_{dfa}$; Rifkin *et al.* 1999b). The absolute

amounts of N derived from fixation (kgNfixed/ha) were higher at Roseworthy than Mallala, due to differences in legume DM, which has been previously observed (Bolger *et al.* 1995).

In this experiment, N_2 fixation was determined for shoot growth only and thus did not account for N accumulation in roots (both large and fine), nodules and N exuded from roots. While Unkovich and Pate have cautioned the direct use of extrapolating values of N_2 fixed to include root material (Unkovich and Pate 2000), recent glasshouse studies provide a guide to the proportion of N_2 fixation directed below ground (McNeill *et al.* 1997; Unkovich and Pate 2000). Grazed legume pastures (*M. truncatula*) also have a substantial proportion of root N (2.02-2.40%; Crawford *et al.* 1997). If it is assumed that clovers in the present study have a similar shoot:root N ratio to *T. subterraneum* (3:1), *Medicago littoralis* (2.7) or *M. truncatula* (3.4), then estimates for N_2 fixation will be ~25% higher (McNeill *et al.* 1997; Unkovich and Pate 2000). The most competent alternative clovers will be fixing 80-100kgN/ha, an appreciable input of organic N to the system.

Nitrogen yield (kgN/ha) was highest in shoots from plots with applied N (200kg/ha), and increased due to inoculation. While *T. purpureum* had the greatest dry matter production, *T. resupinatum* had a significantly larger shoot N concentration and N yield. This may indicate that *T. resupinatum* has greater pasture quality and possibly a lower C:N ratio, compared to *T. purpureum.* Other cultivars of *T. resupinatum* had significantly greater N, lower fibre and higher digestibility than other clover species, including *T. subterraneum* and *T. purpureum* (Kelleway *et al.* 1993). This indicates the potential of *T. resupinatum* as a high quality fodder species for low rainfall areas.

Rain-fed farming systems in southern Australia are characterized by Mediterranean type environments, with low and variable winter rainfall

(Coventry *et al.* 1998). The effects of variable rainfall through the 1997 growing season may have reduced nodulation by inoculant rhizobia, biomass production and N accumulation. Lack of moisture may have limited pasture potential such that it reduced the expression of clover × rhizobia interactions in the production of DM. Moisture stress can effect N_2 fixation through a reduction in nitrogenase activity, and by a reduced supply of photosynthates to nodules (Pankhurst and Sprent 1975). Davey and Simpson (1989) found that smaller, more numerous nodules on subterranean clover were more severely stressed by moisture deficit compared to the larger nodules produced in more acidic soils. Frequent moisture stress is therefore thought to be a significant factor in reducing N_2 fixation of pasture legumes in low rainfall alkaline environments.

The utilization of atmospheric N_2, as opposed to removing soil N for growth, may reduce the deficit of soil N (Bergersen *et al.* 1985; Herridge *et al.* 1995), allowing greater N accumulation for following cereals. The reliance on symbiotic N by pasture legumes is important in the context of low input Mediterranean type farming systems, as plants fixing appreciable amounts of N_2 provide pasture quality without mining soil N. Accumulation of biologically fixed N may therefore increase the yield and protein content of following non-legume crops, to sustain these low input farming systems.

4.4.3 Nitrogen Fixation by Naturalised Populations of Rhizobia

Field estimates indicate large variation in the proportion of N_2 fixed by pasture legumes, which can be influenced by a number of soil and environmental factors (Evans 1996). More specifically, Unkovich and Pate (1998) reported that rhizobial populations from different locations altered $\%N_{dfa}$. Rhizobial population size (MPN) was also a significant factor in the proportion of N_2 fixed in dairy pastures (Riffkin *et al.* 1999b). The size of naturalised populations of rhizobia at the two experimental

sites was considered low compared with populations generally encountered in alkaline soil regions of South Australia (Chapter 3). While most rhizobial populations sampled previously (Chapter 3) were thought to restrict N_2 fixation, naturalised rhizobia from the two field sites nodulated alternative clovers adequately. The proportions of N_2 fixed by alternative clovers nodulated with naturalised populations varied from 50-73% (Roseworthy) to 37-56% (Mallala), providing a maximum of 63kg fixed N/ha (Roseworthy) and 36kg fixed N/ha (Mallala). This result suggests that even sites that appear to have small populations of *R. leguminosarum* bv. *trifolii* are able to fix substantial amounts of N_2.

4.4.4 Measurements of N_2 Fixation by ^{15}N Natural Abundance

The ^{14}N isotope is usually assimilated more readily than ^{15}N in plants, but isotopic fractionation depends upon the relative proportions of isotopes in applied fertilizer the absolute amount of soil N and the ability of the plant to fractionate isotopes of N (Unkovich *et al.* 1994). Concentrations of ^{15}N also vary between whole plants and shoots (Unkovich and Pate 2000). The values of δN^{15} from the +N treatments were highly variable and often low, indicating that some type of fractionation of ^{15}N may have occurred.

Measurements of $\delta^{15}N(‰)$ were higher for Indian hedge mustard than ryegrass translating to higher $\%N_{dfa}$ and amounts of N_{dfa}. Ryegrass may not be an optimal reference plant because $\delta^{15}N(‰)$ values of ryegrass vary depending upon whether ryegrass is grown with or without subterranean clover (Pate *et al.* 1994). It was recognized, however, that the root system of ryegrass would mimic more closely the root architecture of clovers compared to Indian hedge mustard, as determined from root morphology. Consequently, both Indian hedge mustard and ryegrass were used for assessing N_2 fixation of clovers. Absolute values varied when the two reference plants are used, but relative differences were generally not

altered.

B values obtained differed according to clover species and strains of rhizobia inoculated, as has been previously reported (Ledgard 1989). The B values for these clovers (-2.0 to –5.5) were significantly lower than those from a number of legumes previously studied (-0.2 to –0.9) (Unkovich *et al.* 1994), but were similar to recently reported values for white clover (*T. repens*) (-1.48 to –3.93; Riffkin *et al.* 1999b). Low B values indicated a high concentration of ^{15}N in fully symbiotic plants, and these values influence the estimates obtained for $\%N_{dfa}$ and absolute amounts of N_{dfa} from field grown pastures. An alternative option to using B values obtained from single strains of rhizobia may be to use a soil dilution of rhizobia (containing negligible N), which may provide a more realistic B value for a mixed population of rhizobia from field soil (Unkovich and Pate 1998). There is some evidence to suggest that B values may differ according to the medium in which plants are grown. Growing plants to obtain B values in sand is thought preferable to growing plants in media containing vermiculite, as the latter has been shown to provide spurious (high $\delta^{15}N$ values) B values at times (Turner and Bergersen 1983). Exactly how B values may change due to different growing media is not well understood but could occur if vermiculite contained nitrogen of which a large percentage is ^{14}N.

4.5 Conclusion

These experiments highlight the value of inoculating alternative clovers. While increases in pasture DM may not have been realized, increased N_2 fixation did occur due to inoculation of 2 clovers with commercial strains of rhizobia.

Since an inoculation response was not observed in terms of pasture DM, it is possible that available soil N at the site was sufficiently large (or

variable) so that inoculation responses did not occur. Alternatively, rainfall was insufficient to allow the expression of N_2 fixation or naturalised rhizobia may have provided sufficient N_2 fixation compared to inoculated treatments. In addition, nodule occupancy by the commercial inoculant rhizobia may have been insufficient to cause an inoculation response. To answer these questions, more detail is required regarding the extent to which strains have formed nodules in the different clover × rhizobia combinations. For these reasons, the success of inoculation in these experiments, as determined by the proportion of nodules containing commercial inoculants, is the subject of the next chapter.

Chapter 5

Competition for Nodule Occupancy between Inoculant and Naturalised Rhizobia in a Field Environment

5.1 Introduction

While the influence of inoculation in the field can be measured by shoot or grain biomass or biological N_2 fixed, the contribution of particular inoculant strains to N_2 fixation can only be measured by understanding what proportion of nodules are occupied by those inoculant strains or naturalised soil rhizobia. Similarly, lack of an inoculation response cannot be fully understood unless some measure of nodule occupancy can be determined. Nodule occupancy is therefore used as an indication of the competitive ability of strains, judged by the relative occupancy of nodules by inoculant strains in competition with a naturalised population.

Over the last decade, a number of new molecular techniques have emerged that have advanced our ability to identify nodule isolates (Thies *et al.* 2000). PCR (polymerase chain reaction) primers have been developed that target random DNA sequences (Harrison *et al.* 1992), repeated sequences (de Bruijn *et al.* 1992), ribosomal DNA (Laguerre *et al.* 1996) or defined regions, such as the *nif* promoter region (Richardson *et al.* 1995). The value of PCR methods, over many previous techniques such as ELISA, is that PCR methods can provide information about the structure and diversity of a number of organisms in a community, rather than just confirmation of the presence of a particular strain. Molecular techniques allow identification of strains resident in a soil, providing an understanding of genetic diversity from unknown field samples (Dye *et al.* 1995).

This chapter examines the use of PCR amplification to identify strains from nodule isolates from the field experiments (Chapter 4), in order to determine nodule occupancy. PCR has been widely used to accurately type rhizobial strains in laboratories (de Bruijn *et al.* 1992; Laguerre *et al.* 1994; Laguerre *et al.* 1996; Schneider and de Bruijn 1996), and more recently PCR has been applied to ecological studies in the field (Hebb *et al.* 1998; Gandee *et al.* 1999; Loi *et al.* 1999).

5.2 Methods

5.2.1 General

The experimental design and procedures used for maintaining the field trials are detailed in Chapter 4. Importantly, it should be noted that clovers in the field trial were inoculated only in 1997, in order that the survival of rhizobia from a single inoculation event could be determined over the 2 years of the field trial. Inoculation records at the field sites indicate that inoculation with clover rhizobia had not occurred prior to this study, and there was a background of <500 *R. leguminosarum* bv. *trifolii* /g soil for the Roseworthy and Mallala sites (see Chapter 4).

5.2.2 Collection of Rhizobia from Nodules

Rhizobia were isolated from nodules sampled from field plots in 1997 and 1998. If possible, rhizobia were extracted from thirty nodules from each clover × strain treatment, using nodules from all 5 replicate plots. In 1997 limited success in rhizobial isolation and recovery of stored isolates restricted the number of rhizobia isolated to less than thirty isolates per clover × strain treatment. One nodule per plant, taken from the top 5cm of root that was on or close to the taproot was sampled. Nodules were sterilized in 95% ethanol for 30 sec; then 3-6% sodium hypochlorite for 1.5-3 min (depending upon nodule size) and washed 6-8 times in sterile water. Nodules were then crushed and streaked on YMA and incubated

for 4 days at 28°C. To obtain a pure colony, a single isolate from each nodule was re-streaked and used for subsequent work.

5.2.3 Authentication

In order to verify that field isolates were rhizobia, the majority of *R. leguminosarum* bv. *trifolii* field isolates were inoculated onto clovers (homologous to the collection plant) which were grown on agar slopes (Gibson 1980). Plants were grown until nodules appeared (which occurred for 99% of isolates tested). Uninoculated control plants did not develop nodules, indicating that contamination by rhizobia was not occurring.

5.2.4 DNA Extraction

Total genomic DNA was extracted from rhizobial isolates and purified using a Qiagen® QIAamp™ kit, but provided the same results as did DNA isolated using the freeze-thaw method (Richardson *et al.* 1995) and the latter was used in standard PCR reactions. Rhizobia from a 3-day old culture grown on YMA were used. A loopful of bacteria was resuspended in 50µL of SDDW in a 1.5mL Eppendorf® tube. Cell suspensions were subjected to 5 alternating cycles of freezing in liquid N_2 and heating in a 65°C water bath, each for 1minute. Cell debris was then pelleted by centrifugation (14000rpm × 2min.) and the supernatant was used as template DNA for amplification reactions. Template DNA was stored at -20°C and reused when necessary.

5.2.5 Polymerase Chain Reaction Methods

PCR amplification using the RP01 primer followed standard methods (Richardson *et al.* 1995), using 10µL reactions in capillary tubes and a Corbett Research™ Model FTS 4000 thermal sequencer. Reagents used are given in Appendix B. RP01 is a primer of 20 nucleotides (5'-AATTTTCAAGCGTCGTGCCA-3') that corresponds to the sequence of a conserved domain within the *R. leguminosarum* bv. *trifolii nifHDK*

promoter (Watson and Schofield 1985). The RP01 primer was supplied by A.E. Richardson, CSIRO, Plant Industry, Canberra. Temperature cycling for the RP01 primer was 5 cycles of 30s at 92°C, 2min at 50°C and 90s at 72°C; followed by 35 cycles of 5s at 92°C, 25s at 55°C, 90s at 72°C; and a final cycle of 10s at 92°C, 20s at 55°C and 5 min at 72°C (Richardson *et al.* 1995).

ERIC primers were used to verify results obtained with the RP01 primer, based upon previously published protocols (de Bruijn *et al.* 1992; Versalovic *et al.* 1994). ERIC primers target enterobacterial repetitive intergenic consensus sequences (intergenic repeat units) which contain highly conserved palindromic inverted repeat sequences (de Bruijn 1992). ERIC primers consist of two primers ERIC1R (3'-CACTTAGGGGTCCTCGAATGTA-5') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3'), each 22 nucleotides in length. The cycling of ERIC primers was performed in the same thermal cycler as for primer RP01 and the reagents are listed in Appendix B. Total reaction volume used for ERIC primers was 15µL. The temperature regime for ERIC primers consisted of 34 cycles of: 1min at 94°C, 1min at 40°C and 8min at 65°C; and a final extension for 16min at 65°C.

5.2.6 Optimization

PCR reactions were optimized by a number of methods. Initially, the published methods for amplification were used, which produced sufficient and reproducible amplification (Versalovic *et al.* 1994; Richardson *et al.* 1995). Different template DNA concentrations (2ng, 20ng and 200ng/reaction), tested with all other parameters held constant, did not change the amplification (Fig. 5.1). Primer concentrations of 10, 20, 40 and 80ng/µL were trialled with all other concentrations held constant and all except the lowest primer concentration provided optimal amplification (Fig. 5.1). PCR buffer (Appendix B) (Richardson *et al.* 1995) was used in

most PCR reactions, and produced similar profiles and reproducibility to commercially produced buffer. Standard annealing temperatures and concentrations of$mgCl_2$ (Richardson *et al.* 1995) produced optimal PCR products and only 3-4μL of PCR reaction products was analyzed in gels from the total 10μL reactions. Preheating the thermal cycler to 92°C to provide a "hot start", gave less repeatable results compared to the standard temperature cycles. Consequently, routine amplifications used 40ng/μL RP01 primer, 20ng DNA template, standard $mgCl_2$ and standard temperature regimes.

5.2.7 Controls

Controls for testing field isolates of *R. leguminosarum* bv. *trifolii* in PCR included the use of a commercial inoculant strain (positive control), for the comparison with field isolates, and a negative (no template) control to test for the presence of contaminating PCR products in the reaction.

Occasionally, bands were observed in the negative (nil DNA) controls, possibly due to contamination of PCR components (Boettger 1990). Contamination of no DNA controls was greatest using the RP01 primer and occurred to a lesser extent using ERIC primers. Primer dimerisation, the binding of primers to themselves, is another potential source of contamination that is often observed in no-DNA controls (Williams *et al.* 1990; Lanham and Fennel 1992; Li 1994; Richardson *et al.* 1995; Pan *et al.* 1997), though seldom reported (Pan *et al.* 1997).

The potential for amplification from small amounts of contaminating DNA, however, is generally negligible in the presence of large quantities of template DNA (Richardson *et al.* 1995). Occasional amplification of small products in nil DNA controls did not interfere with the interpretation of results, because fragments amplified in the negative controls were not consistently observed in the DNA templates used in any experiment. In cases where interpretation of results was difficult, PCR

reactions were repeated.

5.2.8 Determination of Multiple Occupancy in Nodules

A single rhizobial isolate was examined from each nodule to ensure that a pure sample was analyzed. However, a single nodule can contain more than one strain of rhizobia (Thies *et al.* 1992; Sessitsch *et al.* 1997; Heinrich *et al.* 1999). In order to estimate the frequency of multiple occupancy in this study, four isolates from a single nodule from the field were assessed, for selected plots. Seven to ten nodules per plot were used and the similarity between the four PCR fingerprints was assessed.

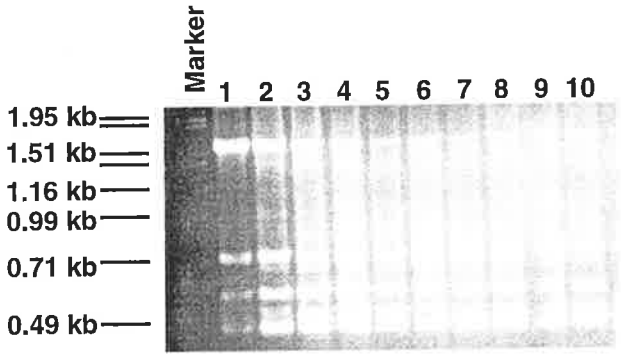

Fig. 5.1 PCR profiles using strain TA1 with varying concentrations of RP01 primer (10, 20, 40 and 80ng per reaction; lanes 1, 2, 3, 4, respectively) and template DNA concentrations (2, 20 and 200 ng per reaction, lanes 5, 6, 7). Lanes 8 to 10 are isolates from three different TA1 cultures.

5.2.9 Gel Products

PCR products were size fractionated using non-denaturing, vertical acrylamide gels (Richardson *et al.* 1995) or (routinely) using 1.5-2.0% agarose gels run in 1 × TBE buffer (Sambrook *et al.* 1989) at 75-90V. A Bio-rad® wide mini sub cell gel rig was used with a 15 × 10cm gel tray and a 20 slot comb. Standard DNA molecular weight marker, SPP1 bacteriophage digested with *Eco*R1 was used in all gels at 0.5 to 1.0µg per lane (Richardson *et al.* 1995). This marker has fragments of 81, 359, 492, 710, 992, 1164, 1412, 1515, 1882, 1953, 2799, 3639, 4899, 6106, 7427 and

8557 base pairs. Generally, 3 to 4μL of amplified product was run per lane of gel for the RP01 primer, while 15μL of product was run for the ERIC primers. Loading buffer (see Appendix B) was used to determine gel running time. Gels were stained in ethidium bromide, destained at 4°C in water for 30-60min and photographed using Polaroid type 665 film.

5.3 Results

5.3.1 Characterization of Inoculant Strains

Total genomic DNA from commercial strains TA1, WSM409, WU95 and CC2483g was subjected to PCR to determine whether characteristic banding patterns enabled discrimination of strains from one another.

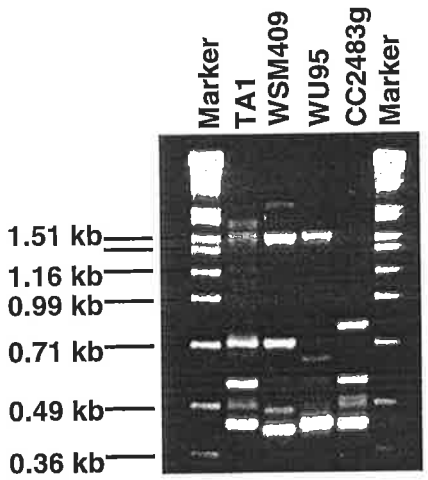

Fig. 5.2 PCR banding profiles of commercial strains TA1, WSM409, WU95 and CC2483g using *nif*-directed primer RP01.

Using the *nif*-directed primer RP01, different strains showed banding patterns that could be distinguished from one another (Fig. 5.2). Banding patterns from these strains consisted of 4 to 7 strong bands, and were consistently reproducible between different PCR reactions. In addition, banding patterns were similar to fingerprints produced in other laboratories (J. Thies, pers. comm.) and to those in published work (Hebb *et al.* 1998; Thies *et al.* 2000).

5.3.2 Determination of Multiple Occupancy of Nodules

Dual occupancy of field nodules occurred for a small proportion of nodules assessed (Fig. 5.3, Table 5.1). Of the nodules assessed, 0 to 14% were occupied by more than one strain. Where multiple occupancy occurred, usually only one isolate differed from the other three that were isolated from the nodule.

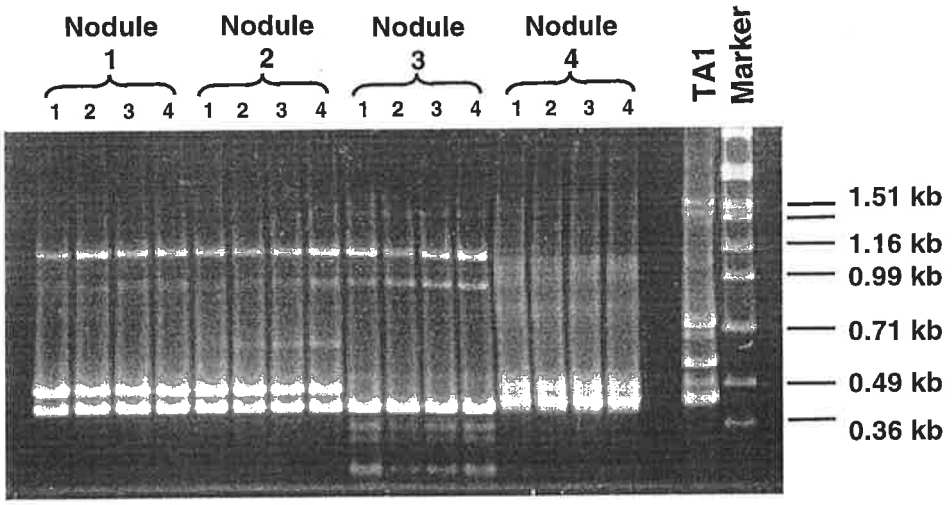

Fig. 5.3 Determination of multiple occupancy of nodules from plot 78 at the Mallala site, using the RP01 primer. Within each nodule, the four isolates show the same banding pattern, indicating that each nodule was occupied by a single strain type. TA1 is shown as a positive control.

Table 5.1 Occurrence of multiple occupancy in field nodules

Plot Details	Multiple occupancy of nodules (%)	Number of nodules occupied by >1 strain
Plot 76	0	0/8
Plot 78	14	1/7
Plot 91	11	1/9

5.3.3 Nodule Occupancy

Rhizobia isolated from field plots that had been inoculated with commercial strains were accurately identified by comparing amplification patterns of the field isolates with those of commercial strains (Fig. 5.4). Isolates from the Roseworthy field site confirmed as being commercial strain TA1 are indicated (lanes 4, 5, 12, 13, 14 and 15, Fig. 5.4 a) as are those positively identified as being commercial strain CC2483g (lanes 5, 10, 13, Fig. 5.4b). Most fingerprints from field isolates varied markedly from the control strains, which clarified identification of nodule isolates. Isolates were also positively identified from the Mallala site (lanes 7, 8 and 9 of Fig. 5.5).

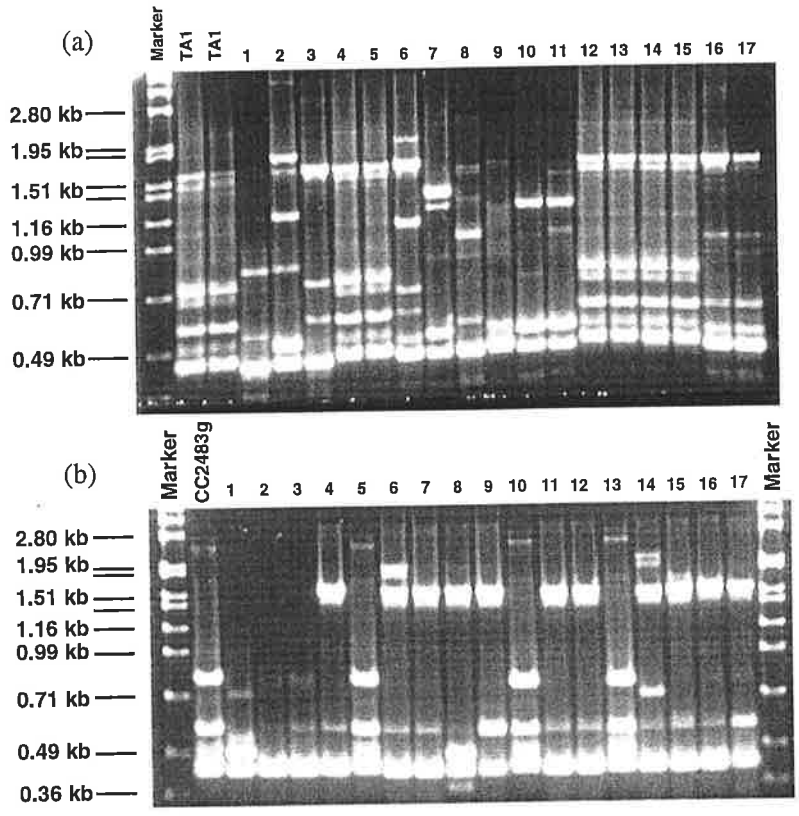

Fig. 5.4 PCR profiles of isolates from field plots amplified using primer RP01. These nodule isolates were collected from (a) *T. alexandrinum* inoculated with TA1 (lanes 1 to 17) and (b) *T. resupinatum* inoculated with CC2483g inoculant (lanes 1 to 17). Amplification profiles for commercial *R. leguminosarum* bv. *trifolii* strains TA1 and CC2483g are indicated in the lanes on the left of photographs. Matching amplification profiles for inoculant strain TA1 are shown in lanes 4, 5 and 12, 13, 14 and 15 (a) and matching amplifications for CC2483g are shown in lanes 5, 10 and 13 (b).

Isolates of all four commercial strains were positively identified from field nodules at the Roseworthy site (Table 5.2). Strain TA1 showed poor colonization of plots in the first year of the trial (Tables 5.2), except for *T. alexandrinum*, which had almost 40% of nodules occupied by TA1. In the second year, TA1 was seldom recovered from nodules, occurring in just 1 nodule of *T. alexandrinum* (Table 5.2). Strain WU95 occurred in a number of nodules in 1997, but was not recovered in 1998.

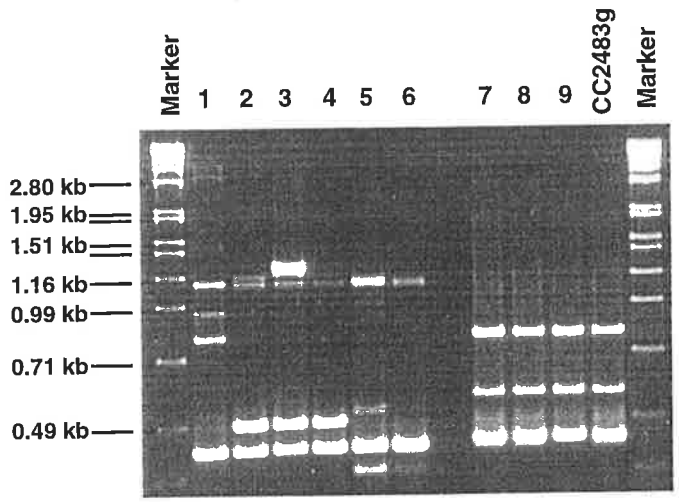

Fig. 5.5 PCR profiles indicating banding patterns generated using primer RP01. Lanes 1 to 6 indicate six dominant field isolates from the Mallala field site; "type1" to "type 6", as discussed in the text. Lanes 7 to 9 are field isolates positively identified as CC2483g.

Both WSM409 inoculated onto *T. purpureum*, and CC2483g inoculated onto *T. resupinatum*, showed initial colonization in the year of inoculation (31% and 21%, respectively) and persisted into the following season to occupy a similar proportion of nodules tested.

At the Mallala site, TA1 again performed poorly, and was not identified in a single nodule in 1997. Strain TA1 was, however, present in one *T. purpureum* nodule at the Mallala site in 1998 (Table 5.3). WU95 was not positively identified at the Mallala site at all (Table 5.3). Strain WSM409 occupied 13% of nodules in 1997 and persisted to nodulate a similar

number of nodules in 1998, while CC2483g showed initial colonization of nodules (19%) but was not recovered in the following year (Table 5.3).

Table 5.2 Frequency of nodules occupied by inoculant strains at the Roseworthy field site. Numbers in parentheses indicate number of positives found compared to the number of positives analysed.

Rhizobial strain	Host clover	Nodule Occupancy 1997	Nodule Occupancy 1998
TA1	*T. alexandrinum*	39% (7/18)	3% (1/30)
TA1	*T. purpureum*	0% (0/15)	0% (0/30)
TA1	*T. resupinatum*	0% (0/16)	0% (0/30)
WU95	*T. alexandrinum*	36% (5/14)	0% (0/30)
WSM409	*T. purpureum*	31% (4/13)	30% (9/30)
CC2483g	*T. resupinatum*	21% (3/14)	27% (8/30)

Table 5.3 Frequency of nodules occupied by inoculant strains at the Mallala field site.

Rhizobial strain	Host clover	Nodule Occupancy 1997	Nodule Occupancy 1998
TA1	*T. alexandrinum*	0% (0/17)	0% (0/30)
TA1	*T. purpureum*	0% (0/20)	3% (1/30)
TA1	*T. resupinatum*	0% (0/28)	0% (0/30)
WU95	*T. alexandrinum*	0% (0/30)	0% (0/30)
WSM409	*T. purpureum*	13% (4/30)	10% (3/30)
CC2483g	*T. resupinatum*	19% (5/27)	0% (0/30)

Cross over of strains from one plot to another was rarely observed, even in uninoculated plots. In the only recording of cross over of strains, an isolate of WSM409 was identified from a plot inoculated with strain TA1 at the Roseworthy site.

5.3.4 Confirmation of Nodule Isolates Using ERIC Primers

In addition to using RPOl, amplification of template DNA was performed using ERIC primers (Fig. 5.6). ERIC primers amplify regions of DNA between intergenic repetitive units and therefore produced a different banding pattern to RP01, which amplifies the conserved *nif* promoter region. Inoculant strains could be distinguished from one another, but banding patterns produced by ERIC primers were very similar for strains WU95 and for CC2483g, with only a few minor band differences (Fig. 5.6). Amplifications using ERIC primers produced 2 major bands for strains TA1 and WSM409. In addition to commercial strains, two field isolates previously confirmed as inoculant strains using RP01 (eg. lane 5 Fig. 5.4 (b) and lane 4 Fig. 5.4 (a)) were amplified using ERIC primers (lanes 1 and 2 Fig. 5.6). These isolates were also confirmed as CC2483g and TA1, respectively, using ERIC primers.

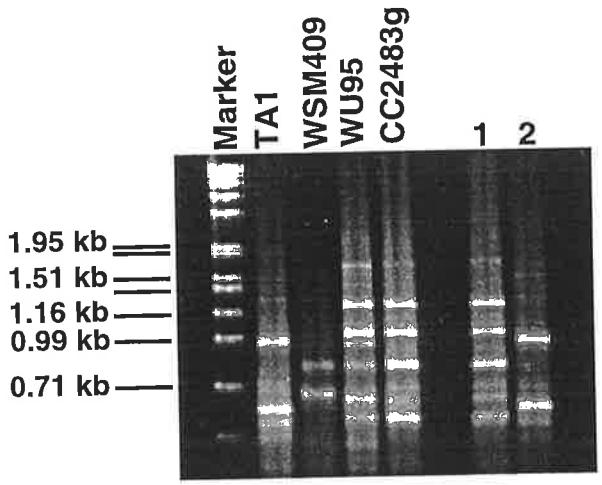

Fig. 5.6 PCR banding profiles of commercial strains TA1, WSM409, WU95 and CC2483g using ERIC primers. Also shown are isolates 1 and 2, previously confirmed as CC2483g and TA1, respectively, using RP01 primer.

5.3.5 Common Field Isolates

A number of isolates with similar banding patterns, which were distinct from commercial strains were consistently observed (Fig. 5.7, see also lanes 4, 7, 9, 11, 12 and 15, 16, 17 of Fig. 5.4 (b)). This isolate type, designated "isolate 18-5" was found in nodules sampled in 1997 and 1998 from the Roseworthy field plot (Table 5.4). In addition, it was found in nodules of all three clover species used in the experiment, and was deemed to be a dominant isolate at that site. Isolate 18-5 occupied 19% of nodules from the entire site and 24% of nodules from uninoculated plots at the Roseworthy site (Table 5.4). Although isolate 18-5 showed some similarities to WU95, when amplified using RP01, it lacked 2 bands, a 650 bp fragment and a 2300 bp fragment that are present in strain WU95 (Hebb *et al.* 1998; Thies *et al.* 2000) (Fig. 5.7).

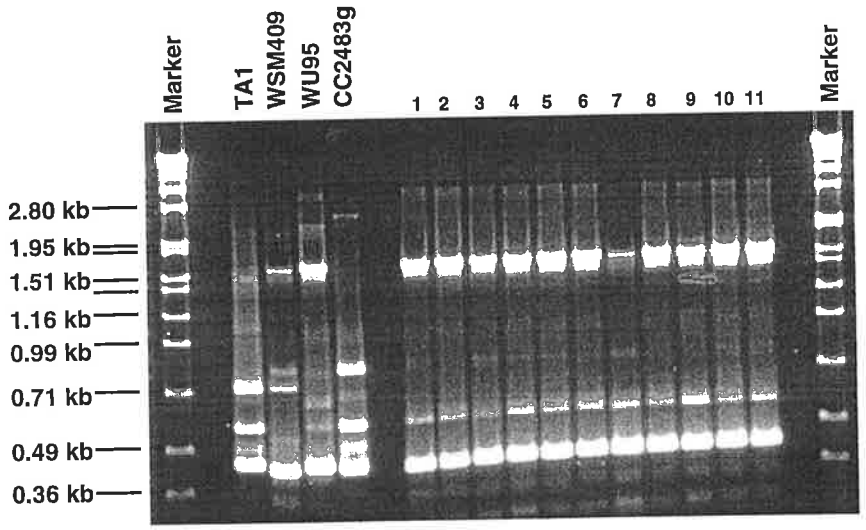

Fig. 5.7 A common field isolate amplified using the RP01 primer. The four commercial strains TA1, WSM409, WU95 and CC2483g are indicated and lanes 1 to 11 show banding patterns from field isolates sampled in 1997 (lanes 1 to 7) and 1998 (lanes 8 to 11). Clover species that these isolates were sourced from were *T. purpureum* (lanes 1,2,3,9,10,11), *T. resupinatum* (lanes 4 to 7) and *T. alexandrinum* (lane 8).

Another banding pattern commonly found, "isolate 855-16" was distinct from commercial strains and showed a similar banding pattern to isolate 18-5, with the addition of a ~450 bp band (Fig. 5.8, Table 5.4).

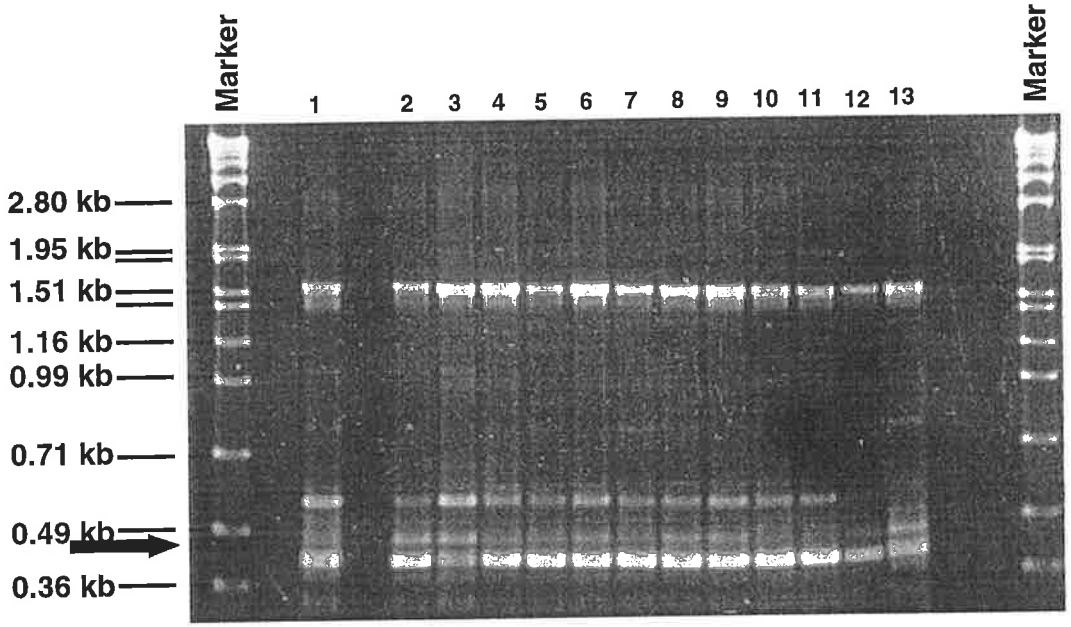

Fig. 5.8 A second common field isolate from the Roseworthy site, amplified using the RP01 primer. Lanes 10 and 11 show a banding pattern of the common field isolate 18-5. The banding patterns in lanes 1 to 9 exhibit an extra band at approximately 450 bp, indicated by the arrow on the left. Clover species that these strains were isolated from are *T. alexandrinum* (lanes 1, 4, 5, 8, 9); *T. purpureum* (2, 3, 6, 10, 11); and *T. resupinatum* (7, 12, 13).

Isolate 855-16 was found in 6% of all nodules across the Roseworthy site and in 18% of nodules from uninoculated plots. This isolate cross inoculated all 3 species of clover. When isolates 18-5 and 855-16 were amplified using ERIC primer, both strains showed the same banding pattern which was also similar to that from WSM409 (Fig. 5.9).

Six different isolate types were frequently encountered in nodules from the Mallala site in similar proportions in 1997 and 1998 (lanes 1 to 6, Fig. 5.5). Each of these isolates was able to nodulate all 3 species of clover used and ranged in frequency from 4 to 10% occupancy of nodules at the Mallala site (Table 5.4). These six isolate types together represented 44% (146) of nodules from the Mallala site (Table 5.4).

Table 5.4 Frequency of dominant field strains observed in nodule isolates from the Roseworthy and Mallala sites.

Site*	Isolate name	Mean occurrence in nodules of all species in 1997 (%)	Mean occurrence in nodules of all species in 1998 (%)	Mean occurrence in nodules of all species in both years (%)	Occurrence in uninoculated plots in 1998 (%)
RW	18-5	22	17	19	24
	855-16	6	6	6	18
Total		28	23	25	-
Mallala	type 1	12	7	9	-
	type 2	9	7	8	-
	type 3	7	9	8	-
	type 4	10	10	10	-
	type 5	3	7	5	-
	type 6	3	6	4	-
Total		44	43	44	-

* RW – Roseworthy field site

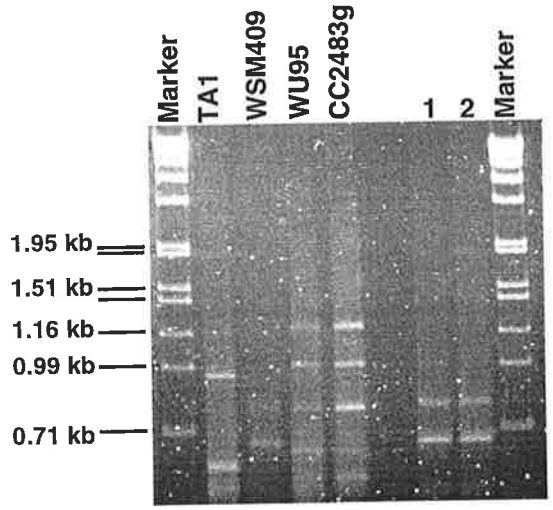

Fig. 5.9 Amplification patterns of commercial strains and isolates 18-5 and 855-16 (1 and 2 respectively), using ERIC primers.

5.4 Discussion

5.4.1 Nodule Occupancy

The use of the polymerase chain reaction in this study was shown to accurately detect commercial inoculant strains from unknown rhizobial isolates from field nodules. In particular, amplification of rhizobial isolates using the primer RP01 provided reproducible banding patterns that discriminated *R. leguminosarum* bv. *trifolii* at the strain level and enabled the identification of commonly-occurring field isolates. Confirmation of results was provided through the use of ERIC primers, which showed good agreement to RP01 in identification of commercial strains from field isolates.

Assessment of competition for nodulation was restricted to *R. leguminosarum* bv. *trifolii* that could form nodules. A number of other studies have identified free-living rhizobia that are unable to nodulate (Soberon-Chavez and Najera 1988; Sivakumaran *et al.* 1997; Hartmann *et al.* 1998) which would have been overlooked in the present study. However, only rhizobia that are able to nodulate can influence N_2 fixation, and identifying strains that influence N_2 fixation is the overall aim of this work. In addition, plant-bacterial interactions favour particular strains of rhizobia for nodulation by a particular legume host. Therefore our assessment of rhizobia at a field site is biased to those rhizobia with which the host clover species (*T. alexandrinum*, *T. purpureum*, *T. resupinatum*) preferentially nodulate (Hardarson and Jones 1979; Thies *et al.* 2000). In this study nodule occupancy was assessed using nodules from taproot nodules at 0-5cm depth, to maximize the chance of encountering inoculant rhizobia, which are generally found in nodules close to the crown in the year of inoculation (Danso and Bowen 1989; McDermott and Graham 1989).

Brockwell *et al.* (1982) defined first and second year success of inoculant strains in occupying nodules as indices of: 1) competitiveness and 2) persistence, respectively. On this basis, three types of responses were noted in the experiment: strains either 1) failed to nodulate in the first year, 2) nodulated in the first year but did not persist to nodulate in the second year, or 3) nodulated in the first year of the trial and persisted to nodulate in the following year. The following discussion will consider the reactions of rhizobia according to these three categories.

Strain TA1 failed to nodulate in the year of sowing in all treatments, except TA1 × *T. alexandrinum* at Roseworthy, and therefore did not persist into the second year. Interestingly, strain TA1 was only identified in 1 nodule from the Mallala site, in the year after inoculation, even though it was not identified in the year of inoculation. Strain WU95 at Mallala also failed to nodulate *T. alexandrinum* in the year of inoculation.

A possible reason for low nodule occupancy by a commercial inoculant in some treatments may be low survival of rhizobia on the seedcoat after sowing. Rhizobial numbers estimated from a subsample of seed, post sowing were all >300/seed, except for the CC2483g × *T. resupinatum* treatments (Chapter 4). Even with poor inoculation, strain CC2483g was still recovered from nodules, indicating that a sufficient number of TA1 inoculant should have been present on seed. While a protective coat of Plastaid™ lime pellet was applied to the inoculated seed to aid rhizobial survival, sporadic rainfall following sowing (Chapter 4) may have caused substantial loss of the inoculum post sowing and prior to seedling germination. In addition high or variable soil nitrogen may delay early nodulation, restricting the nodule occupancy by inoculant strains. High temperature can significantly decrease rhizobial survival on seed (Brockwell *et al.* 1988a) but was not experienced in the present study.

Plant preference for particular rhizobial strains (Triplett and Sadowsky

1992) may have influenced the lack of success of a number of inoculants. While strain TA1 is able to nodulate and fix N_2 with *T. purpureum* and *T. resupinatum*, commercial strains for these clovers (WSM409, CC2483g, respectively) showed a greater ability to form nodules (Howieson *et al.* 2000a). Even so, strain TA1, the commercial strain for *T. alexandrinum*, performed poorly on this clover species at the Mallala site, indicating general poor performance. In other field trials, TA1 has been recovered in 100% of nodules sampled from *T. purpureum* (Hebb *et al.* 1998). While preference for nodulation may mean that particular strains eg. strain TA1, are absent in nodules, this does not indicate their absence in soil (Sadowsky and Graham 1998). Also, there is a possibility that genetic exchange or genetic instability of rhizobia may occur (Gibson *et al.* 1991) which would limit the recovery of inoculant rhizobia.

Secondly, some strains established well initially, but did not persist into the second year, such as strains WU95 and TA1 inoculated on *T. alexandrinum* at the Roseworthy site and *T. resupinatum* × CC2483g at the Mallala site. This reduction in nodule occupancy in years following inoculation has been noted by a number of researchers as the inoculum fails to persist in soil and provide future nodulation (Parker *et al.* 1977; Brockwell *et al.* 1982; Bottomley *et al.* 1991). At one site with no background of rhizobia, inoculant strains decreased from 100% to 73-94% in the following year (Roughley *et al.* 1976). Similarly, Hebb and coworkers reported mean nodule occupancies of 77% in the year of inoculation which decreased to 45% in the following year, for a number of clover and strain combinations (Hebb *et al.* 1998). This reduction in the capacity to nodulate may indicate that resident soil rhizobia are better adapted to the soil constraints, compared to the commercial inoculants. The ability for strains to persist in soil in the absence of a legume host has been labeled *saprophytic competence* (Chatel *et al.* 1968; Bottomley *et al.* 1991) and is important in acid soils (Thornton and Davey 1984; Howieson 1995). It appears that limited saprophytic competence may be a

significant constraint to the introduction of commercial *R. leguminosarum* bv. *trifolii* into alkaline soils.

Thirdly, two treatments, *T. purpureum* × WSM409 and *T. resupinatum* × CC2483g at the Roseworthy site provided nodulation in the year of inoculation and persisted through the summer without a legume host to nodulate clovers in the following season. Similar occupancies of nodules (20-49%) were found for subclover inoculated with WU95 and NA3001 in acidic soils by the use of ELISA (Slattery and Coventry 1999). Other studies, reported comparatively higher nodule occupancies of inoculant rhizobia (frequently 100%) although occupancy decreased substantially as the number of indigenous rhizobia increased (Roughley *et al.* 1976; Brockwell *et al.* 1982; Hebb *et al.* 1998).

5.4.2 Implications of Nodule Occupancies

Even though some strains performed reasonably, most commercial strains of rhizobia were poorly competitive, despite the small naturalised populations of rhizobia, <500 rhizobia/g soil. While these naturalised population of *R. leguminosarum* bv. *trifolii* were considered small, they were aggressive for nodule occupancy. Populations as low as 50 rhizobia/g soil are able to limit the response to inoculation (Thies *et al.* 1991a). In addition, MPN measurements are estimates, and should be seen as a guide only to the number of rhizobia inhabiting a soil at a certain point in time. Whether MPN measurements in bulk soil provide an accurate assessment of the number of rhizobia in the rhizospheres of clover is questionable, because strains of rhizobia differ when measured in bulk soil compared to rhizospheres (Barclay *et al.* 1994; Chapter 3). In particular, the soils in these current experiments are alkaline and may provide a benign environment for rhizobial multiplication in clover rhizospheres, particularly for adapted strains, compared to the harsh conditions of low pH soils (Brockwell *et al.* 1991).

Another implication is that the commercial strains tested, in particular strain TA1, may have a poor alkaline soil reaction, which makes it uncompetitive on alkaline soils. Soil pH in the present study was 8.0 (H_2O, 0-10cm; Roseworthy) and 8.5 (Mallala). Many of these rhizobial strains have performed well on slightly acidic soils, typically comprising 100% occupancy of nodules. The pH values (H_2O, 0-10cm) for similar competition experiments using *R. leguminosarum* bv. *trifolii* were 5.4-6.2 (Roughley *et al.* 1976), 5.6-6.2 (Brockwell *et al.* 1982) and 5.85 (Hebb *et al.* 1998). The effects of soil pH may provide an explanation for why strains that have performed well in other environments, failed to nodulate significant numbers of plants and in many cases failed to persist into the second year of the trial in the present study.

Host-selectivity for *R. leguminosarum* bv. *trifolii* (Sadowsky and Graham 1998) appeared important in the success of nodule colonization by inoculant strains. Non-optimal clover × rhizobia combinations (*T. alexandrinum* × WU95, *T. purpureum* × TA1) were poorly competitive when in competition with an apparently small but aggressive naturalised population of rhizobia. This was not the case for previous studies, in which TA1 formed a significant proportion of nodules on *T. purpureum* (Hebb *et al.* 1998). Poor nodule occupancy with inferior clover × *Rhizobium* treatments led to inferior N_2 fixation for these combinations (Chapter 4). Conversely, inoculation of optimal strains of rhizobia (TA1 × *T. alexandrinum,* WSM409 × *T. alexandrinum,* CC2483g × *T. resupinatum*) produced the highest nodule occupancies by inoculant strains. Even though these nodule occupancies were not high, these plant/rhizobial combinations often provided the largest nodule scores and amounts of N_{dfa} (Chapter 4). This result suggests that optimal strains of *R. leguminosarum* bv. *trifolii* are necessary for alternative clovers in alkaline soil environments, even though optimal clover × *Rhizobium* combinations were not required for high nodule occupancy in studies conducted on more acidic soils (Roughley *et al.* 1976; Brockwell *et al.*

1982; Hebb *et al.* 1998).

The results of this study therefore question the practice of introducing many current commercial rhizobia into alkaline soils, because of poor recovery of strains, possibly due to inadequate adaptation to soil constraints. It is possible that inoculants with superior adaptation to alkaline soils could be found, and it is suggested that rhizobial inoculants be sought from these soil types to maximize the chance that the symbiosis functions well under alkaline conditions.

It is somewhat surprising that strain WSM409 had the greatest nodule occupancy in the alkaline soils of these trials. Strain WSM409 exhibits acid tolerance in laboratory culture (Watkin *et al.* 1997) and in field trials (Howieson *et al.* 2000a, Watkin *et al.* in press), compared to the acid-sensitive strain, TA1. For these reasons, it was expected that strain TA1 may perform better than WSM409 on the alkaline soils. While strain WSM409 shows better performance under acid soil conditions, it also appears that this strain has superior performance that is independent of soil pH. This finding is timely, because the Australian inoculant industry has recently selected this strain as the commercial inoculant for *T. subterraneum* and for a range of alternative clovers (J. Howieson, pers. Comm.).

A further implication is that the occupancy of nodules by commercial strains may be insufficient to produce an inoculation response (increase in biomass due to inoculation). Results in chapter 4 indicated that while inoculation of clovers with their commercial strain was sufficient to increase N_2 fixation, increases in biomass above that of seed sown uninoculated were not realized. Studies in Hawaii determined that a minimum of 66% nodule occupancy and a doubling of nodule mass by the inoculant rhizobia were required to achieve an inoculation response (Thies *et al.* 1991a), and that these increases usually occurred on soils with <10 rhizobia/g soil. Therefore the absence of an inoculation response

in DM production in the present study (see Chapter 4) could have been due to limited nodule occupancy by inoculant strains, even though N_{dfa} in shoots increased. An alternative possibility is that the naturalised rhizobia present in the soil provide adequate N_2 fixation and therefore do not limit N_2 fixation of clovers in these environments. In order to test this hypothesis, repeated inoculation could be attempted to improve occupancy of nodules by commercial strains and to determine whether an inoculation response is then achieved. The effectiveness of field isolates could also be tested to determine how they compare to the commercial strains; this will be discussed in more detail in Chapter 6.

5.4.3 Multiple Occupancies

Dually occupancy occurred to a limited extent for the samples tested. Occupancy of nodules by more than one strain indicates an error of approximately 10% in the final nodule occupancies determined. In one nodule tested in this study, WSM409 was found in only 1 out of the 4 isolates tested, indicating that occupancy by this strain could have easily been overlooked. It was however, considered more appropriate to isolate pure colonies of rhizobia as opposed to using nodule crushes, for routine PCR identifications, to simplify the identification of nodule isolates. Amplification of rhizobia directly from nodules has been shown for laboratory grown plants (Richardson *et al.* 1995) although it has been suggested recently that PCR amplification from dually occupied nodules generally favours 1 strain (Thies *et al.* 2000). Therefore, dual occupancy of nodules may not be detected even when using nodule crushes. Dual or multiple occupancy of nodules usually occurs at low levels (less than 15%) (Beattie *et al.* 1989; Sessitsch *et al.* 1997; Heinrich *et al.* 1999), but has been shown to occur in up to 46% of soybean nodules under high competition pressure (McDermott and Graham 1990). The mechanism for formation of dual occupancy of nodules is probably due to infection of root hairs in close proximity to one another by different rhizobia. In the

current study multiple occupancy was determined using fingerprints of a number of isolates from a single nodule, while laboratory studies usually rely upon the use of strains with differing antibiotic resistance. Regardless of the method of determination, no information is gained on the different proportions of strain (bacteroid) types represented within a nodule and, therefore, how these strains may contribute to N_2 fixation.

5.4.4 Field Dominant Strains

One of the advantages of PCR over strain specific techniques (eg. ELISA and strain-specific DNA probes; Matheson *et al.* 1997) is that the former provides some idea of the rhizobial community into which these inoculant rhizobia are placed. A number of fingerprint patterns were repeatedly encountered from both field sites, which had amplification patterns that differed from commercial strains using two primer types. Even though amplifications of isolates 18-5 and 855-16 appear similar to WSM409 when amplified with ERIC primers, amplification using primer RP01 confirmed that these strains were not WSM409.

The presence of these "dominant field isolates" in both years of the trial (Table 5.4) indicates that a number of rhizobial strains were present in the soil during both years of the trial which could nodulate at least three species of clover at a frequency of 3 to 19% of the total nodules at a site. The two sites differed in the proportions of dominant strains. At the Roseworthy site only two dominant strains were found, contributing to 25% of the nodule isolates (68 nodules) at that site. At the Mallala site, 6 dominant isolates were observed, which together occupied 44% of the nodules tested (146 nodules). The two sites (approximately 25 km apart) were exclusive in the rhizobial isolates they contained: the 2 dominant isolates from the Roseworthy site were not represented at Mallala site and the six isolates from Mallala (types 1 to 6) were not encountered in Roseworthy isolates. Each site also contained many other naturalised rhizobial isolates, each occurring in less than 2% of nodules, indicating

that both sites were diverse in the *R. leguminosarum* bv. *trifolii* that they contained.

A greater understanding of the genetic diversity of these two sites could be uncovered through further analysis of the banding patterns of all naturalised strains. To examine the genetic diversity of these isolates, employment of other PCR primers may clarify the level of genetic diversity. PCR using primers that target the conserved region of the intergenic spacer between genes coding for 16S and 23S rRNA (16S and 23S rDNA; Laguerre *et al.* 1996) may produce a better understanding of strain relatedness. Similarly, the analysis of genetic structure may be better understood using multi-locus enzyme electrophoresis (Selander *et al.* 1986; Strain *et al.* 1994). These different techniques may not identify *R. leguminosarum* bv. *trifolii* at the strain level very well, but provide a higher level of resolution (Thies *et al.* 2000), possibly indicative of the evolutionary conservation of different genetic sequences (Gogarten 1995). Even though all dominant isolates of *R. leguminosarum* bv. *trifolii* captured in the present study cross inoculated the 3 clover species, the host plant may influence the type of rhizobial isolates captured (Bottomley *et al.* 1994), even though varieties of a species may not (Harrison *et al.* 1987). Capturing rhizobia using a particular host plant will influence our understanding of the genetic diversity. Newer techniques identifying N_2 fixing genes without the need for isolation or culture could also be implemented (Noda *et al.* 1999).

Dominant field isolates such as those in the present study have previously been reported: thirteen dominant isolates came from clover pastures in Oregon (Leung *et al.* 1994a) with a major serotype occupying more than 50% of nodules (Leung *et al.* 1994c). In a field experiment in Tasmania, three common isolates were identified from uninoculated plots at levels of 7-12%, but were not observed from these plots in the following year (Hebb *et al.* 1998). Similarly, a number of dominant isolates of *S.*

meliloti were repeatedly observed in soils in the UK (Gandee *et al.* 1999) and Canada (Bromfield *et al.* 1986). Interestingly, (Shishido and Pepper 1990) found that 4 types of *S. meliloti* (based on plasmid profile analysis and intrinsic antibody resistance) were found from 5 widespread locations in Arizona.

There are numerous reasons to explain why isolates 18-5 and 855-16 formed from a large number of nodules at the Roseworthy site. Dominant field isolates may have an enhanced ability to cope with the physical constraints of the soil type (carbon requirements, temperature, alkaline soil reaction, low moisture status, saprophytic competence), or possess a greater ability to nodulate with the selected host clovers over the commercial *R. leguminosarum* bv. *trifolii* strains (plant-rhizobial gene interactions) (Vlassak and Vanderleyden 1997). Similarly, dominance of isolate types in nodules could reflect relative proportions in the field population, where the dominant field isolates are numerically favoured for nodulation, compared to inoculant rhizobia supplied on a small clover seed (Brockwell *et al.* 1995). Whether dominance of these field isolates in nodules from the field site is due to 1) large numbers of rhizobia in the soil or 2) an enhanced competitive ability over commercial inoculant strains in nodule formation, will be the subject of the next chapter.

Chapter 6

Competition between Commercial and Naturalised Soil Rhizobia for Nodule Occupancy.

6.1 Introduction

In the previous chapter it was concluded that commercial *R. leguminosarum* bv. *trifolii* inoculants (particularly strain TA1) compete poorly on alkaline soils. Naturalised strains of *R. leguminosarum* bv. *trifolii* appeared to outcompete commercial inoculants for nodule occupancy. For example, two naturalised isolates (strains 18-5 and 855-16) occupied 19% and 6% of the total number of nodules sampled at the Roseworthy site, respectively. These common field isolates are potentially adapted for survival in alkaline soils and may possess useful characteristics for an inoculant strain on alkaline soils, provided they are both symbiotically effective and highly competitive for nodule occupancy.

Strain competitiveness can be assessed by inoculating seedlings with varying proportions of two rhizobial strains and determining nodule occupancy using a suitable marker (Sessitsch *et al.* 1997). The *Escherichia coli gusA* gene encoding β-glucuronidase (GUS) (Jefferson *et al.* 1987; Wilson *et al.* 1994) is a very convenient and suitable marker for these purposes, since GUS activity has not been reported in either plants or rhizobia (Wilson *et al.* 1995b). This reporter has been used to visually assess nodule occupancy and has proven to be useful in rhizobial competition experiments (Streit *et al.* 1995; Wilson *et al.* 1995b; Sessitsch *et al.* 1997). Visual assessment avoids the otherwise time consuming requirement of isolating strains from nodules prior to performing antibiotic sensitivity or serology tests to distinguish one strain from

another.

The *gusA* reporter can be inserted into a strain to provide a convenient 'tag'. The derivative strain is then co-inoculated with an 'untagged' strain onto germinated seedlings. Upon harvest, nodules on the root system are stained with XGlcA substrate (5-bromo-4-chloro-3-indolyl β-D-glucuronide) and those containing a strain marked with *gusA* stain intense blue, those containing an unmarked strain remain unstained (Wilson *et al.* 1994).

It is important to determine that the insertion of the *gusA* gene into the rhizobial genome does not affect symbiotic functions, due to disruption of essential genes (Sessitsch *et al.* 1997). Preliminary experiments were therefore necessary to determine that the ability to form nodules, to fix N_2 and to compete to form nodules did not decrease in field dominant rhizobia due to the insertion of *gusA* genes.

The following chapter outlines experiments performed to address the issue of whether the potentially alkaline tolerant field isolates of *R. leguminosarum* bv *trifolii* can outcompete commercial inoculants. It describes (1) the construction of *gusA* marked strains from dominant field isolates using the technique of minitransposon mutagenesis, (2) experiments that verify that the *gusA* marked strains are able to nodulate *T. alexandrinum* and fix N_2 indistinguishably from their parent strain, (3) experiments to verify that *gusA* marked strains are equally competitive for nodulation as their parent strain and (4) experiments designed to test how competitive *gusA* marked naturalised strains are against the commercial inoculant TA1.

6.2 Methods

6.2.1 Strains, Plasmids and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table 6.1. *Escherichia coli* BW20767 was grown at 37°C in LB medium; *Rhizobium* strains were grown in TY or JMM media (Appendix A) and incubated at 28°C to obtain log phase cells for use in experiments. If required, media were supplemented with the (µg/mL): streptomycin (100) and spectinomycin (100) antibiotics.

6.2.2 Minitransposon Mutagenesis of R. leguminosarum bv. trifolii

Permission to use recombinant material was gained through the Genetic Manipulations Advisory Committee and all procedures were performed in PC2 laboratories or a PC2 containment glasshouse. Two replicate strains of clover rhizobia from the two isolate types, (18-5 / 851-7) and (855-16 / 837-7) were isolated from previous field experiments at Roseworthy Campus (Chapter 5). These strains were marked with the *Escherichia coli gusA* reporter (Jefferson *et al.* 1986) using minitransposon mutagenesis (Reeve *et al.* 1999). Strains of *R. leguminosarum* bv. *trifolii* were conjugated with *E. coli* BW20767 (pCAM130) overnight on TY plates at 28°C. A total of 16 biparental matings (one per TY plate) were performed; four for each of the Roseworthy isolates. Controls included 1 plate for each parent strain. After overnight incubation, cells were resuspended in saline and then plated on JMM minimal medium containing streptomycin and spectinomycin. No growth was observed on the JMM plates for any of the controls. Rhizobial transconjugants containing a minitransposon insertion were obtained at a frequency of approx. 1 in 10^6. One transconjugant was selected from each mating to provide 4 independent insertions in each isolate. Strains were stored at –80°C in 15% glycerol.

Table 6.1 Source of bacteria and plasmids used in this study.

Strain	Characteristics	Experiment	Origin
Escherichia coli			
BW20767	RP4-2-tet:Mu-1kan::Tn7 integrant leu-63::IS10 recA1 creC510 hsdR17 endA1 zbf-5 uidA(ΔMluI):pir$^+$ thi		Metcalf *et al.* (1996)
R. leguminosarum* bv. *trifolii			
WSM409	Commercial strain	1	
CC2483g	Commercial strain	1	
TA1	Commercial strain	1,3,4	
18-5	Isolate from Roseworthy Campus	1,2	This study
18-5A*	18-5::mTn*5*SS-*gusA*30; Sm^r, Sp^r	1	This study
18-5B	18-5::mTn*5*SS-*gusA*30; Sm^r, Sp^r	1	This study
18-5C	18-5::mTn*5*SS-*gusA*30; Sm^r, Sp^r	1,2	This study
18-5D	18-5::mTn*5*SS-*gusA*30; Sm^r, Sp^r	1,2,3,4	This study
851-7	Isolate from Roseworthy Campus	1	This study
851-7A	851-7::mTn*5*SS-*gusA*30; Sm^r, Sp^r	1	This study
851-7B	851-7::mTn*5*SS-*gusA*30; Sm^r, Sp^r	1	This study
851-7C	851-7::mTn*5*SS-*gusA*30; Sm^r, Sp^r	1	This study
851-7D	851-7::mTn*5*SS-*gusA*30; Sm^r, Sp^r	1	This study
855-16	Isolate from Roseworthy Campus	1,2	This study
855-16A	855-16::mTn*5*SS-*gusA*30; Sm^r, Sp^r	1	This study
855-16B	855-16::mTn*5*SS-*gusA*30; Sm^r, Sp^r	1	This study
855-16C	855-16::mTn*5*SS-*gusA*30; Sm^r, Sp^r	1,2,3,4	This study
855-16D	855-16::mTn*5*SS-*gusA*30; Sm^r, Sp^r	1,2	This study
837-7	Isolate from Roseworthy Campus	1	This study
837-7A	837-7::mTn*5*SS-*gusA*30; Sm^r, Sp^r	1	This study
837-7B	837-7::mTn*5*SS-*gusA*30; Sm^r, Sp^r	1	This study
837-7C	837-7::mTn*5*SS-*gusA*30; Sm^r, Sp^r	1	This study
837-7D	837-7::mTn*5*SS-*gusA*30; Sm^r, Sp^r	1	This study
Plasmids			
pCAM130	PUT derivative containing mTn*5*SS-*gusA*30		Wilson *et al.* (1995b)

*Four independent insertions of the mTn*5*SS-*gusA*30 element in each rhizobial strain isolated from the Roseworthy field site (18-5, 851-7, 855-16 and 837-7) were constructed by Dr. Wayne Reeve at the Centre for *Rhizobium* Studies, Murdoch University.

6.2.3 Plant Growth Conditions

All plant experiments were conducted in the PC2 containment glasshouse at the Waite Campus. The glasshouse temperatures were maintained between 17-25°C. The glasshouse was shaded with 70% white shade cloth and plants received no additional lighting other than natural light. At the start of experiments pots were watered to 70% of pore filled space with McKnights nutrient solution (N- free, pH 7.5; Gibson 1980) and autoclaved. For experiment 4 the pots were watered to 60% pore filled space with quarter strength McKnights solution. Plants were watered later with sterile water, as required.

6.2.4 Experiment 1: Effectiveness of Mutant Strains.

The objective of experiment 1 was to establish 1) that the mutants could nodulate and fix N_2; 2) that nodulation and fixation by a mutant was comparable to the wild-type strain; and 3) that the *nifH-gusA* fusion was being expressed effectively in mutant strains harbored in a nodule environment to aid visual identification of nodule occupancy. All field dominant strains and their mutant derivatives were tested (Table 6.1).

Plants were grown in polycarbonate pots (13 × 9cm diam.) containing 800g sand and vermiculite (1:1, v/v) which had lids with small holes through which to plant seedlings. Seedlings were germinated in sterile conditions on 1% water agar, sown into pots and allowed to establish for 5 days. Each plant was then inoculated with 0.8mL of either TY (control) or rhizobial suspension in TY at 1 x 10^9 cells/mL, 5 days after planting. Cell density was estimated using optical density, measured at 600nm using a spectrophotometer. Cell counts were based on the assumption that an OD_{600} of 1 corresponds to approximately 10^9 cells/mL. In addition, results were verified using serial dilution counts. A nitrogen supplied treatment (3mL/plant of a 4g NH_4NO_3/L solution at 12 and 15 days after inoculation

and 3mL/plant of a 12 g NH_4NO_3/L solution at 19 and 21 days after inoculation) was used as a control. Treatments were arranged in a split pot design with 2 seedlings of *T. alexandrinum*, *T. purpureum*, and *T. resupinatum* per pot (split treatments) and rhizobial (main) treatments, with 4 replicates per treatment. Plants were harvested at 30 days after sowing (DAS), after which nodulation was assessed and shoots were harvested and dried for 3d at 70°C. Assessment of N_2 fixation was based on shoot biomass produced in the absence of combined N.

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

The aim of experiment 2 was to assess the ability of the mutant strains to compete effectively against parent wild-types, to establish that the insertion of the minitransposon did not create a non-competitive strain. Four mutant strains (855-16C, 855-16D, 18-5C and 18-5D) were each co-inoculated with their wild-type strain (855-16 or 18-5) at ratios of 1:1, 1:2 and 2:1 (Table 6.2) onto germinated seedlings of *T. alexandrinum*. Cell numbers were estimated as determined for experiment 1.

Eight plants were grown in a pot of sand and vermiculite and each plant was inoculated with 1mL TY containing 10^4 cells (in log phase) of the appropriate strains. Treatments were arranged in a randomized complete block design.

The accuracy of the ratios of mutant (resistant) to wild type (sensitive) strains was assessed by spreading 100-150 colonies from the inoculum on TY and TY containing 100μg/mL of Sm/Sp. Plants were thinned to 3/pot after 1 week and were harvested at 30DAS, as for experiment 1.

Table 6.2 Experimental design for Experiment 2.

Strains Used	Ratios Tested*	Test	Replicate Pots
18-5	1	Test for absence of GUS activity	3
855-16	1	as above	3
18-5C	1	Test for GUS activity	3
18-5D	1	as above	3
855-16C	1	as above	3
855-16D	1	as above	3
18-5 and 18-5C	1:1, 1:2, 2:1	Visual assessment of stained versus unstained nodules	5 (× 3)
18-5 and 18-5D	1:1, 1:2, 2:1	as above	5 (× 3)
855-16 and 855-16C	1:1, 1:2, 2:1	as above	5 (× 3)
855-16 and 855-16D	1:1, 1:2, 2:1	as above	5 (× 3)
Total			78 pots

* seedlings were inoculated with 10^4 cells/plant in all treatments.

6.2.6 Experiment 3: Competitive Abilities of Commercial and Naturalised Rhizobia

The aim of experiment 3 was to determine the ability of strains 18-5D and 855-16C to compete with the commercial strain TA1, co-inoculated onto *T. alexandrinum* (Table 6.3). The pot arrangement and growing conditions were the same as reported for experiment 2. Proportions of inoculum were supplied at 10^4 cells/plant at ratios of 0:1, 1:0, 1:9, 1:3, 1:1, 3:1, 9:1 (TA1:mutant strain) arranged in a complete randomized block design. Viable cell counts were determined from the inoculum as described for experiment 2. Plants were thinned to 6/pot and harvested at

33 DAS, as for experiment 1.

Table 6.3 Experimental design for Experiment 3.

Strains Used	Ratios Tested*	Test	Replicate Pots
18-5C	1	Presence of GUS activity	3
855-16D	1	as above	3
TA1	1	Absence of GUS activity	3
TA1 and 18-5C	9:1, 3:1, 1:1, 1:3, 1:9	Visual assessment of stained versus unstained nodules	4 (× 5)
TA1 and 855-16D	9:1, 3:1, 1:1, 1:3, 1:9	as above	4 (× 5)
Total			**49 pots**

* seedlings were inoculated with 10^4 cells/plant in all treatments.

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

Experiment 4 determined the ability of commercial inoculant strain TA1 applied to the seed coat, to compete with a mutant strain applied to the soil. Roseworthy soil was collected (0-10cm) from the experimental site (see Chapter 4 for soil details), sieved and the <5mm fraction used. A 1:1 (v/v) mixture of Roseworthy soil and coarse river sand (double washed, 2 mm particle size) was mixed in a concrete mixer, steam sterilized and 800g was added to each sterile pot. *T. alexandrinum* grown in similarly treated soil prior to this experiment failed to nodulate.

An inoculation slurry was made using 100mL 0.5% methyl cellulose and 25g peat culture (which contained 2.8×10^9 cells, confirmed as TA1 using

PCR). Seeds were inoculated at three rates: 1× standard inoculation (0.4mL slurry/20g seed), 1/10× standard inoculation (substituting sterile peat) and uninoculated onto *T. alexandrinum* (Table 6.4). After inoculation, a count of viable rhizobia was made and the seeds were left overnight, prior to sowing. Uninoculated seed was surface sterilized in 99% ethanol.

Different concentrations of mutant rhizobia (18-5D, 855-16C), from approximately 10^2 to 10^6 rhizobia/g soil, were applied to the pots in quarter strength McKnights solution, which percolated through the soil (Table 6.4). Twenty uninoculated or inoculated seeds, depending upon treatment, were sown into the pots the same day (Table 6.4). Plants were allowed to germinate and were thinned to 6 /pot after one week.

Bacterial counts of soil applied rhizobia were made at the time of inoculation and one week later, using 1 g of soil, from 0-1cm depth using serial dilution plate counts on (low nutrient) Medium A + 50mg/L Sm/Sp (see Appendix A). Plants were harvested at 45 DAS, as for previous experiments, excepting that nodule counts were partitioned in the top 5cm of root and the lower (>5cm) sections.

6.2.8 Determination of Nodule Occupancy

Nodules were stained for GUS activity as previously described (Wilson *et al.* 1995b). Roots were placed in an aerated buffer (Appendix C) at 37°C, containing 100-200mg/L XGlcA for 1-3 days. Clearing of roots was performed by soaking in 2.5% sodium hypochlorite followed by 8 washes in water. Nodulation was assessed by comparing the number of stained and unstained, and partially stained (a nodule showing both stained and unstained regions) nodules. Nodules partially stained were visually assessed under a microscope following sectioning.

Table 6.4 Experimental design for Experiment 4.

Seed inoculant rate of TA1	Soil applied rhizobia*	Soil rhizobia density (No./g soil)	Replicate Pots
0	0	0	5
1/10	0	0	5
1	0	0	5
0	18-5D	10^2	5
0	18-5D	10^4	5
0	18-5D	10^6	5
1/10	18-5D	10^2	5
1/10	18-5D	10^4	5
1/10	18-5D	10^6	5
1	18-5D	10^2	5
1	18-5D	10^4	5
1	18-5D	10^6	5
0	855-16C	10^2	5
0	855-16C	10^4	5
0	855-16C	10^6	5
1/10	855-16C	10^2	5
1/10	855-16C	10^4	5
1/10	855-16C	10^6	5
1	855-16C	10^2	5
1	855-16C	10^4	5
1	855-16C	10^6	5
			105 pots

*indicates rhizobia inoculated into the pots prior to the experiment starting.

6.2.9 Assessment of Competitiveness for Nodulation

The competitive abilities of wild type and mutant rhizobia in forming nodules was assessed using the relationship:

$$\log[(P_X+P_{both})/(P_Y+P_{both})] = C_{X:Y} + k[\log(I_X/I_Y)]$$

(Beattie *et al.* 1989; Sessitsch *et al.* 1997) where strain X and Y are the two competing strains; P_X and P_Y are the proportion of nodules occupied by X and Y, respectively; P_{both} is the proportion of nodules occupied by both strains. I_X and I_Y represent the concentrations of strains X and Y in the inoculum (Fig. 6.1). The intercept of this equation, $C_{X:Y}$, is defined as the competitiveness index: a statistically significant positive value indicates that strain X is more competitive than strain Y, and a negative value indicates that it is less competitive (Fig. 6.1). The slope, k, gives the rate at which the nodule occupancy ratio chances as the inoculum ratio changes (Beattie *et al.* 1989; Sessitsch *et al.* 1997).

6.2.10 Statistical Analysis

Analysis was performed using Genstat 5 (Payne 1993). Anovas were performed taking into account the experimental design. Differences between treatments were tested using least significant differences at P=0.05. Assessment of nodulation competitiveness for experiment 2 was performed using linear regressions for the relationship between nodule occupancies and inoculum proportions, as described above. Experiment 4 was analyzed by comparing across all 21 treatments (1-factor Anova) with blocking. In addition, by limiting treatments to those with applied soil rhizobia or to those where seed inoculum was applied, the factors of seed inoculation, soil-applied strain type and soil rhizobial density were more powerfully analyzed (3-factor Anovas). Transformations of log (X+1) and arcsin$\sqrt{}$ were applied to nodule data, where necessary, to conform

data to the assumptions of these tests, and data were back-transformed.

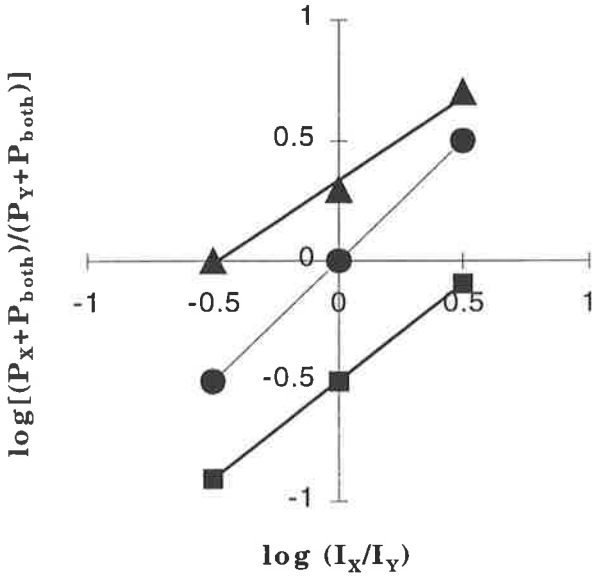

Fig. 6.1 Illustration of the response of rhizobial strain competition between 2 strains, "*X*" and "*Y*" using the methods of Beattie and coworkers (Beattie *et al.* 1989). The x-axis indicates the ratio of strain *X* and *Y* in the inoculum, and the y-axis indicates the proportion of nodules occupied by strain *X*, "P_X" or strain *Y* "P_Y"or those nodules dually occupied "P_{both}". The intercept of the line is $C_{X:Y}$ and the slope of the line (the rate of change of nodule occupancy) is *k*. Nodule competitiveness is determined by whether the line passes through the origin. The three lines represent conditions where strain *X* is more competitive than strain *Y* (triangles); strain *X* is less competitive than strain *Y* (squares) and where there is no difference between the competitiveness of strain *X* and strain *Y* (circles).

6.3 Results

6.3.1 Minitransposon Mutagenesis of R. leguminosarum *bv.* trifolii

The two strains 18-5 and 851-7 displayed the same PCR banding profile after amplification with the RPO1 primer; this banding profile was generated for 19% of the nodule isolates from the Roseworthy site (Fig. 6.2). In a similar fashion, the banding profile generated from strains 855-16 and 837-7 were the same and occurred for 6% of the nodule isolates at

the Roseworthy site (Fig. 6.2). Banding patterns were similar for the duplicate samples (eg. for 18-5, 851 and for 855-16, 837-7) using ERIC and BOXA1R primers (data not shown).

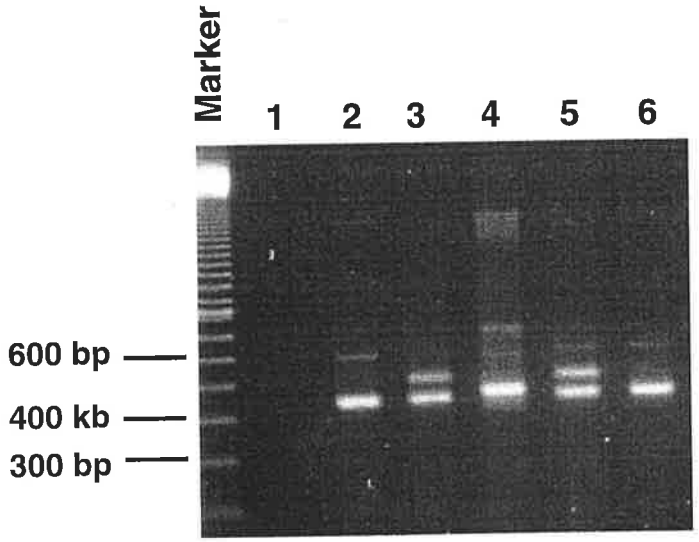

Fig. 6.2 PCR amplification of: 1) nil DNA (negative control), 2) strain 851-7, 3) 837-7, 4) WU95, 5) 855-16 and 6) 18-5, using the primer RP01 and protocols from Richardson *et al.* (1995). Products were run on a 2% agarose gel and the marker is a 100bp molecular weight marker from Amersham Pharmacia (gel photo courtesy of Dr. A.M. Vachot, University of Western Sydney).

To examine the issue of whether potentially alkaline tolerant field isolates of *R. leguminosarum* bv *trifolii* could outcompete commercial inoculants, the four wild-types were labeled with a *gusA* marker to enable the identification of nodule occupants.

These four wild-type strains were mutagenised with the minitransposon mTn*5*SS-*gusA*30 to construct 4 mutants of each parent. Each of the mutants should have the minitransposon located in a unique insertion site since the mutants were selected from independent conjugations. The mTn*5*SS-*gusA*30 minitransposon (containing the omega streptomycin /spectinomycin resistance cassette and a $P_{nifH\text{-}gusA}$ fusion; Fig. 6.3) was chosen on the basis that expression of the fusion in *Rhizobium* appears

consistent regardless of the age of the plants at harvest (Wilson *et al.* 1995b).

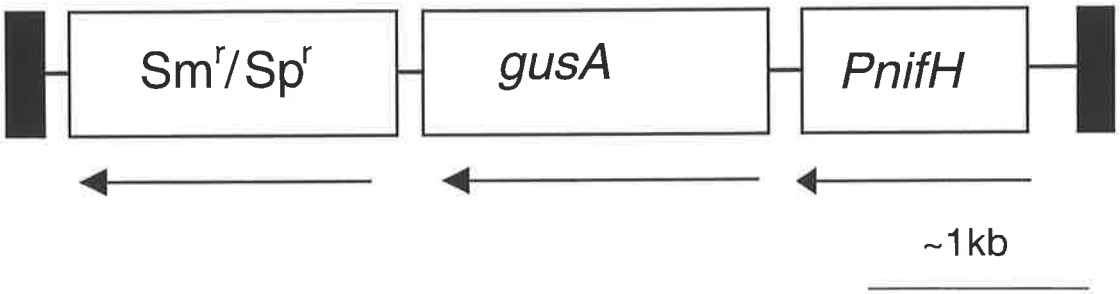

Fig. 6.3 Structure of the minitransposon mTn*5*SS-*gusA*30 present in the plasmid pCAM130 (Wilson *et al.* 1995b). The features of this minitransposon include: a symbiotically active promoter, *PnifH*; the *gusA* marker gene; the omega Sm/Sp cassette conferring resistance to the antibiotics streptomycin and spectinomycin; and the filled boxes represent the Tn*5* 19 bp inverted repeat sequences required for transposition.

However, the insertion of the *gusA* marker into the rhizobial genome could potentially affect nodulation, N_2 fixation, or competitiveness of the strain (Wilson *et al.* 1995). It was therefore necessary to determine that these functions have not been compromised in the 'tagged' strains constructed in this study.

6.3.2 Experiment 1: Effectiveness of Mutant Strains

The ability of the four field dominant strains, 16 *gusA*-labeled mutants and commercial rhizobial inoculants to produce nodules and fix N_2 was compared when inoculated onto *T. alexandrinum*, *T. purpureum* and *T. resupinatum*.

All strains of rhizobia and mutant derivatives were able to form nodules (Fig. 6.4). Nodulation was not observed on uninoculated plants. The number of nodules produced was influenced by the rhizobial inoculant and plant species (both $P<0.001$). *T. resupinatum* (18.4 nodules/plant) produced more nodules than *T. alexandrinum* (14.8 nodules/plant), which

had more nodules than *T. purpureum* (8.4 nodules/plant) (Fig. 6.4). In general, the number of nodules produced by a wild-type field dominant strain did not vary from the number produced by its mutant derivatives. However, there was a greater number of nodules induced by strain 851-7B compared to its wild-type or to mutants 851-7A, 851-7C and 851-7D. A significantly greater number of nodules was formed by strain 855-16 and its mutants in comparison to the 3 other groups of dominant field isolates/mutants. These nodules were notably smaller than those from other treatments. Nodule numbers did not differ between the three other field dominant strain groups and the commercial strains.

Nodules on the root systems of plants inoculated with mutant rhizobia all stained blue in the presence of the substrate XGlcA (Fig. 6.5b). Nodules containing mutants derived from 18-5, 837-7 and 851-7 all stained intensely dark blue. Interestingly, nodules formed by mutant derivatives of 855-16 (855-16A, 855-16B, 855-16C or 855-16D) stained a lighter blue and often contained sections of darker cobalt blue (Fig. 6.5b). There was a small degree of staining on roots, possibly due to high numbers of rhizobia in the rhizosphere, but this was minor and did not interfere with identification of nodule occupants. As expected, nodules on plants containing wild-type (or commercial) strains failed to stain in the presence of XGlcA (Fig. 6.5a).

Shoot weight produced in the absence of applied N was used as a measure of the amount of N_2 fixed. Analysis indicated a strong interaction effect of species × strain type ($P<0.01$; Fig. 6.6). The (-N) controls were significantly smaller than all other treatments ($P<0.001$). With one exception, multiple comparisons indicated no differences between wild type strains and their mutant derivatives for any strain-host combination. Inoculation of *T. purpureum* with 855-16 produced significantly less biomass than its mutant strains ($P<0.05$; Fig. 6.6).

Each dominant field strain was then grouped together with its corresponding mutant strains for analysis. *T. alexandrinum* produced significantly less shoot biomass when inoculated with strain 855-16 (and its derivatives 855-16A to 855-16D) than when inoculated with other dominant rhizobial groups ($P<0.05$). Strain 855-16 was ineffective and strain 837-7 effective when inoculated onto *T. alexandrinum*, even though these strains had similar PCR banding patterns, when amplified using RP01. Shoot biomass of *T. purpureum* and *T. resupinatum* did not differ when plants were inoculated with different groups of field dominant rhizobial strains.

Therefore, mutant strains were generally able to form nodules and fix N_2 equally to the parental strains. Strain 855-16 and all mutants were ineffective when inoculated on *T. alexandrinum*.

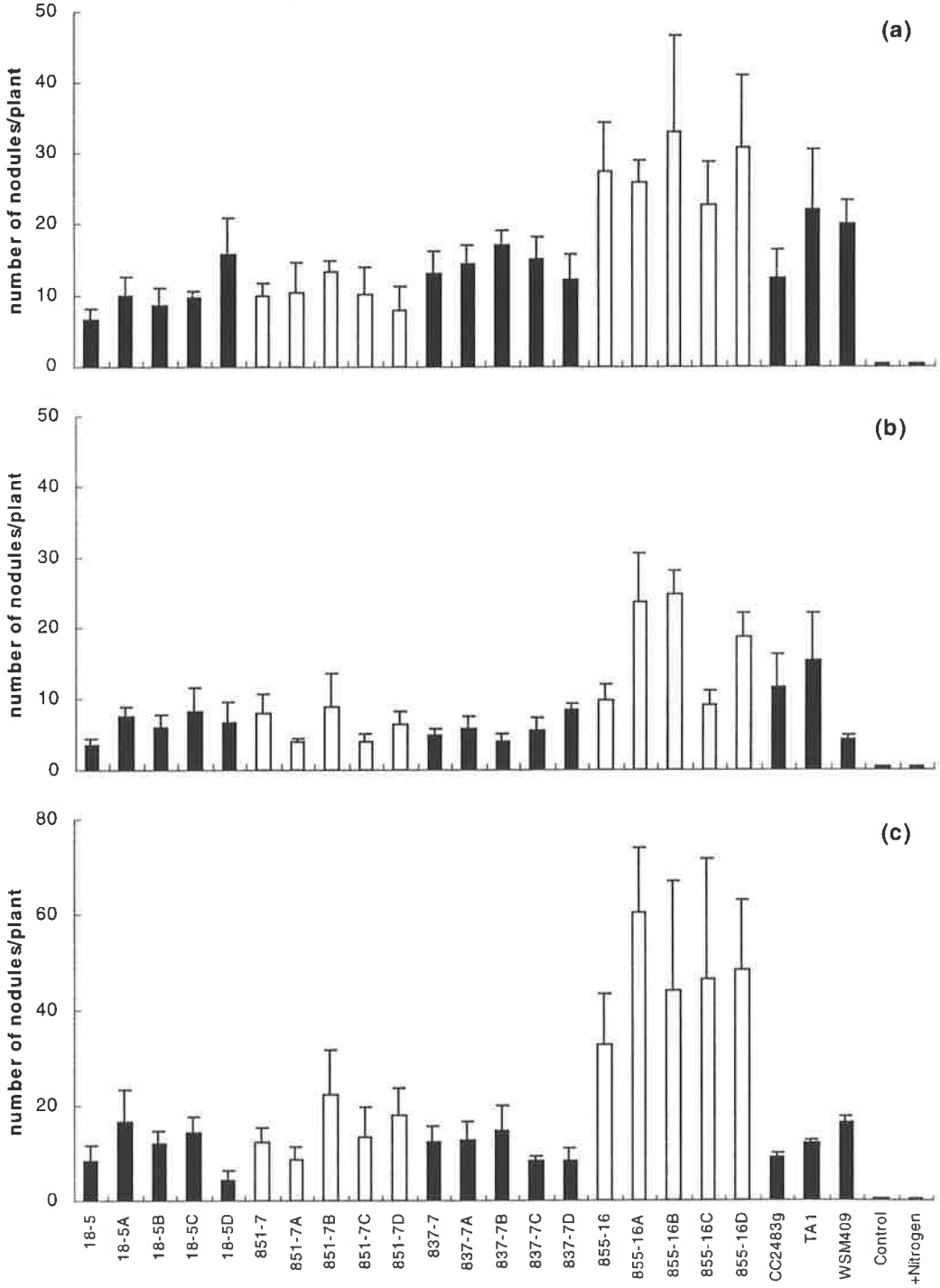

Fig. 6.4 The number of nodules per plant induced on (a) *T. alexandrinum*, (b) *T. purpureum* and (c) *T. resupinatum* in experiment 1. The sequential display of strains used as inoculants include a Roseworthy wild-type strain (either 18-5, 851-7, 837-7 or 855-16) followed by its 4 mutant derivatives containing *gusA* insertions (eg. 18-5A to 18-5D etc.), followed by the commercial strains (CC2483g, TA1, WSM409) and uninoculated controls (±N). Bars indicate standard error.

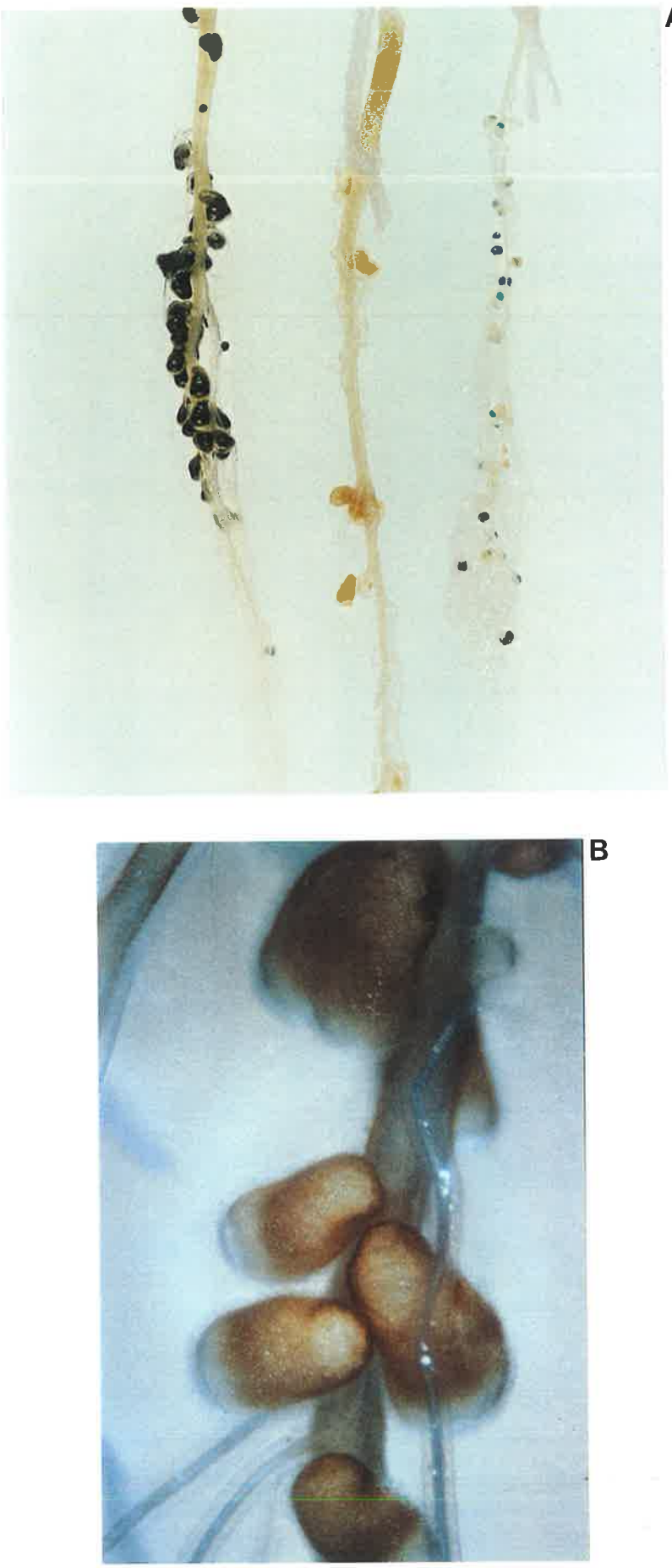

Fig. 6.5a Root systems of *T. alexandrinum* inoculated with 18-5D, 18-5 and 855-16C, left to right respectively (A) and nodules of 18-5 (B).

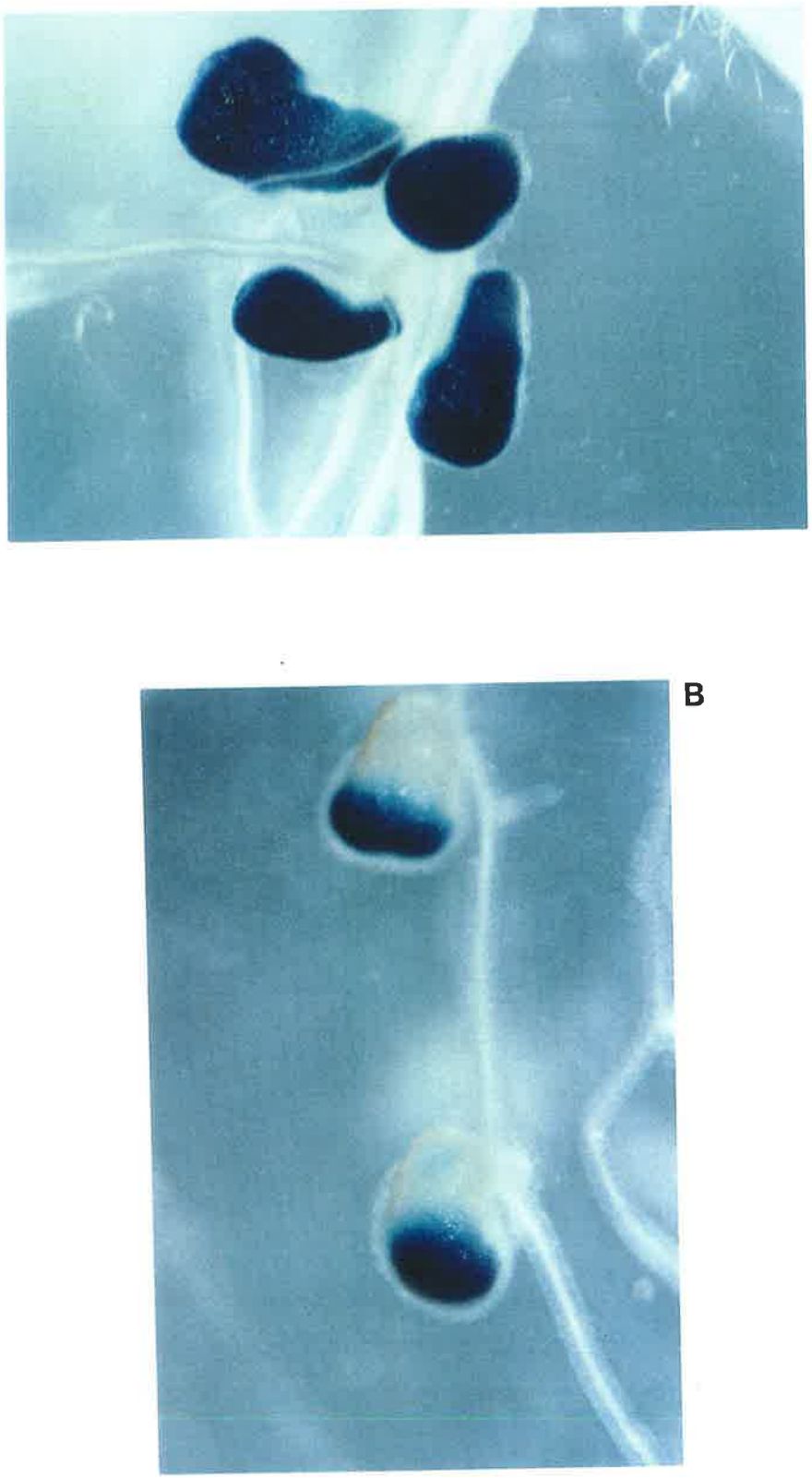

Fig. 6.5b Nodules formed on *T. alexandrinum* inoculated with 18-5D (A) and 855-16C (B).

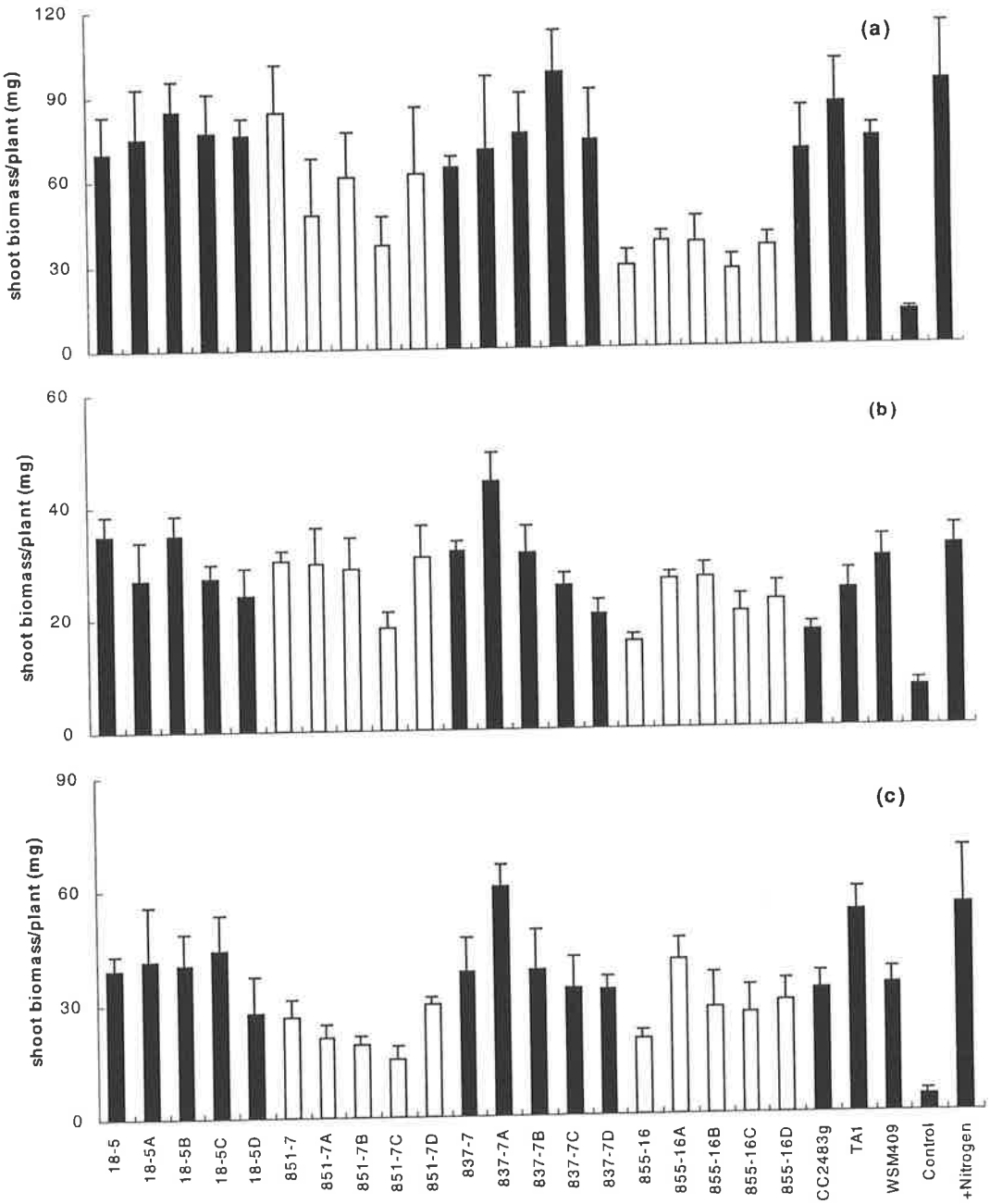

Fig. 6.6 A comparison of the symbiotic effectiveness of dominant strains of *R. leguminosarum* bv. *trifolii* isolated from the Roseworthy field site and their *gusA*-labeled mutants. The shoot biomass produced by a) *T. alexandrinum*, b) *T. purpureum* and c) *T. resupinatum* is presented. Treatments are listed in the same order as presented in the legend for Fig. 6.4. Error bars indicate standard error.

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

Although N_2 fixation generally did not differ within groups of field dominant rhizobia and their mutants, it was necessary to verify that insertion of the minitransposon did not create a non-competitive phenotype in the mutants. Strains 18-5C, 18-5D (effective on *T. alexandrinum*) and 855-16C, 855-16D (ineffective on *T. alexandrinum*) were co-inoculated along with the respective wild-type to examine competitiveness for nodule occupancy of *T. alexandrinum*.

Plants inoculated with either 18-5, either of the 2 minitransposon-induced mutants (18-5C, 18-5D) or combinations thereof (mutant co-inoculated with wild-type) did not show any difference in the number of nodules produced (Table 6.5). Although the numbers of nodules induced by either 855-16 and minitransposon induced derivatives (855-16C, 855-16D) were more variable and generally higher than those formed by 18-5 and its derivatives, nodule numbers did not differ significantly within these treatments (Table 6.5).

After XGlcA staining, nodules inoculated with a single strain of *Rhizobium* stained as expected: plants inoculated with mutant strains produced blue nodules, those containing a wild-type strain remained unstained (Table 6.5). Proportions of nodules that were stained and unstained following treatment in XGlcA, were generally proportional to the number of mutant and wildtype strains present in the inoculum (Table 6.5, Figure 6.7). In addition, inoculation with two strains of *R. leguminosarum* bv *trifolii* produced a small number of nodules (0-7% of nodules on each plant, 0-4% on average per treatment) that appeared to be dual occupied by visual assessment of the staining pattern (Table 6.5).

Sections of these nodules clearly revealed regions that were stained and unstained (Fig. 6.7).

Table 6.5 Nodule number and strain occupancy of nodules induced on plants co-inoculated with different ratios of wild-type strains (18-5, 855-16) and their minitransposon-marked derivatives.

Bacterial Strains	Measured Strain Ratio*	Mean total nodules**	Percent unmarked (pink) nodules	Percent GUS-marked nodules	Percent mixed nodules
18-5	1	29[a]	100	0	0
18-5C	1	32[a]	0	100	0
18-5:18-5C	57:43	37[a]	67	30	3
18-5:18-5C	40:60	37[a]	66	34	0
18-5:18-5C	73:27	27[a]	92	6	2
18-5D	1	26[a]	0	100	0
18-5:18-5D	70:30	35[a]	64	33	3
18-5:18-5D	54:46	40[a]	49	51	0
18-5:18-5D	82:18	39[a]	80	20	0
855-16	1	47[a]	100	0	0
855-16C	1	74[a]	0	100	0
855-16:855-16C	47:53	45[a]	50	47	3
855-16:855-16C	31:69	78[a]	39	61	0
855-16:855-16C	64:36	42[a]	71	27	2
855-16D	1	59[a]	0	100	0
855-16:855-16D	47:53	31[a]	37	59	4
855-16:855-16D	31:69	60[a]	25	73	2
855-16:855-16D	64:36	45[a]	74	25	1

*each plant was inoculated with 10^4 cells, regardless of treatment.

**values within each column for a strain followed by the same letter do not differ, P=0.05.

The competitive abilities of mutant strains against wild types was assessed using established methods (Beattie *et al.* 1989; Sessitsch *et al.* 1997; see methods). Increasing proportions of a mutant rhizobia (eg. 18-5C, 18-5D, 855-16C or 855-16D) in the inoculum caused a greater proportion of the nodules to be occupied by that mutant strain (Fig. 6.8).

When the raw data were submitted for regression analysis, coefficients of determination (R^2) explained 0.46 to 0.76 of the variation (Table 6.6). The y-intercepts of 3 mutants were not statistically different from zero (Probability that $C_{X:Y}$ =0; >0.05; Table 6.6). One exception to this was observed in co-inoculation experiments containing strain 18-5C (Fig 6.8). The probability that the line traversed the origin was <0.05 (Probability that $C_{X:Y}$ =0 <0.05; Table 6.6) and the intercept of the line was –0.367. Strains 18-5D, 855-16C and 855-16D were therefore equally competitive for nodulation as their parental strains; strain 18-5C was less competitive than 18-5 (Table 6.6).

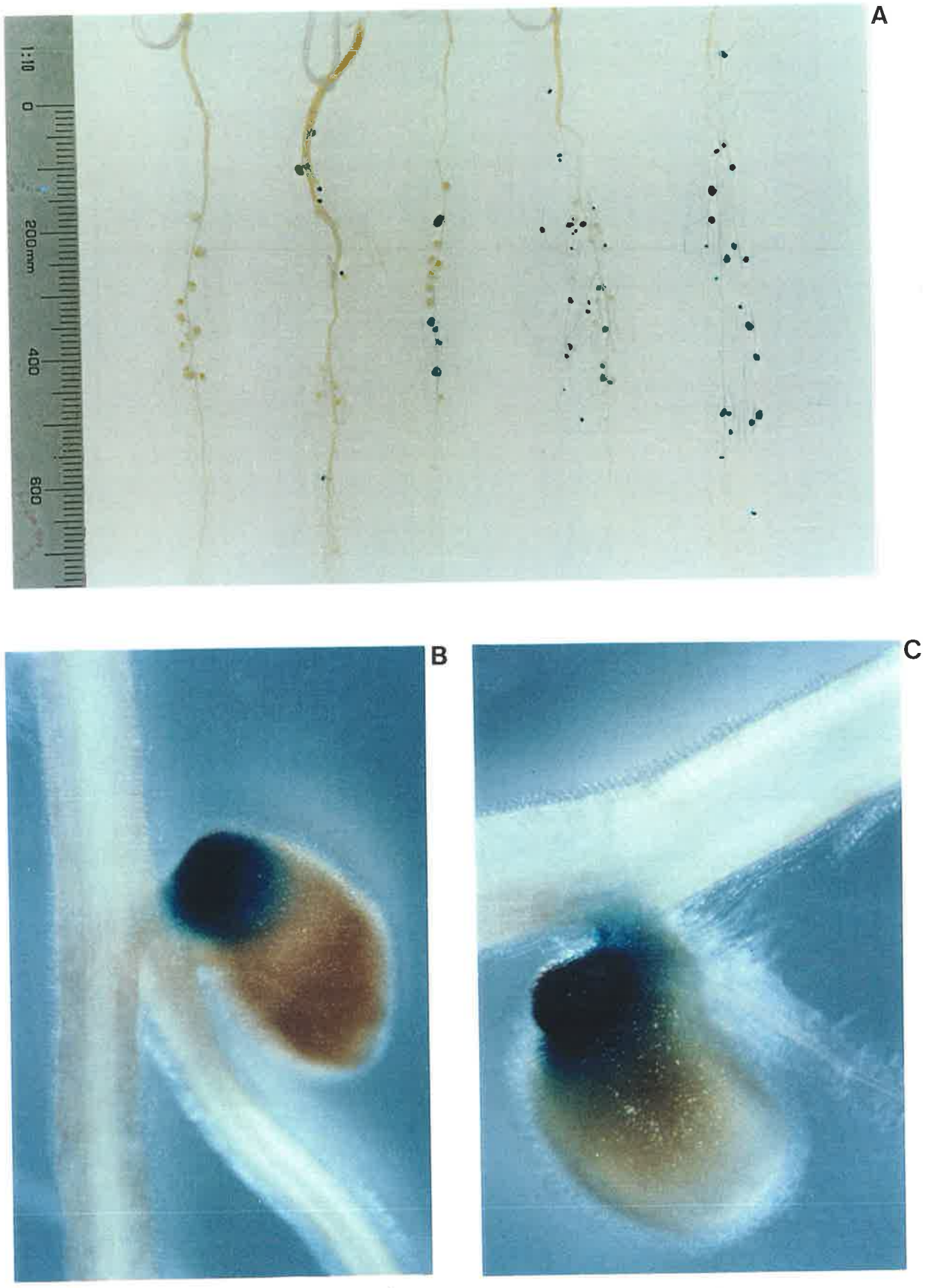

Fig. 6.7 *T. alexandrinum* inoculated with 18-5:18-5D at ratios of 1:0, 2:1, 1:1, 1:2 and 0:1 (A) and nodules dually occupied by 18-5 and 18-5D as evidenced by nodules containing blue and pink sections (B and C).

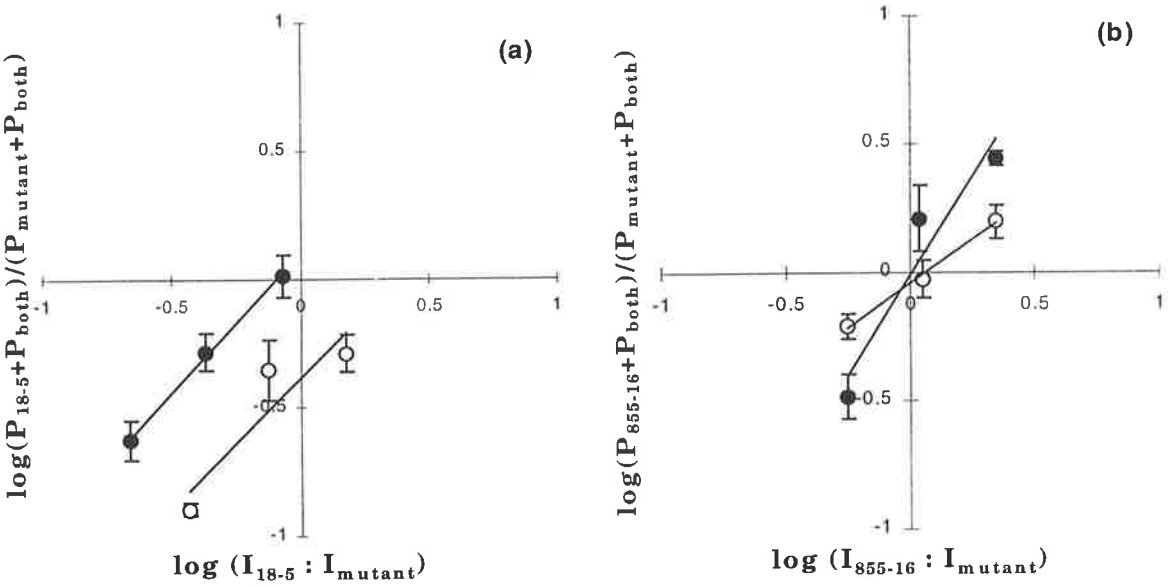

Fig. 6.8 The proportion of nodules produced by mutant and wild-type strains ($\log(P_X+P_{both})/(P_Y+P_{both})$) in comparison to the proportion of mutant and wild-type cells in the inoculum ($\log(I_X/I_Y)$). The intercept of the line is $C_{X:Y}$ and the slope is k. Indicated on the graph are (a) mutants 18-5C ○ and 18-5D ● and (b) mutants 855-16C ○ and 855-16D ●, in competition with their wild type parents 18-5 and 855-16, respectively. All y-intercepts are not significantly different from zero, except for strain 18-5C, which has a y-intercept significantly less than zero (Table 6.6). This indicates that strain 18-5C is less competitive than its parental strain 18-5.

Table 6.6 Competitive index (y-intercept, $C_{X:Y}$) and slope (k) for mutant strains relative to the parental strains, calculated using raw data for percentage nodulation.

Strain	Coefficient (R^2)	Probability that k=0	k	$C_{X:Y}$	Probability that $C_{X:Y}$ = 0	Competitive ability*
18-5C	0.46	0.02	0.879	-0.367	0.001	R
18-5D	0.76	<0.001	1.094	0.105	0.25	S
855-16C	0.66	<0.01	0.693	-0.047	0.28	S
855-16D	0.74	<0.001	1.551	-0.003	0.962	S

* competitive ability represents the statistically significant position relative to the parent strain 18-5 or 855-16: S = competitive ability unaffected by the *gusA* insertion; R = reduced competitive ability compared to parental strain.

6.3.4 Experiment 3: Competitive Abilities of Commercial and Naturalised Rhizobia

Strains 18-5D and 855-16C did not differ from their parental strains in nodulation competitiveness or effectiveness. These mutant strains were therefore used in place of the parental strains to test their competitiveness against commercial strain TA1.

Shoot biomass of *T. alexandrinum* did not differ whether it was inoculated with TA1, strain 18-5D (effective) or any combination of these 2 strains (Fig. 6.9 and Fig. 6.10). However, poor shoot biomass (26mg/plant) for *T. alexandrinum* was observed when the ineffective strain 855-16C was inoculated by itself (Fig. 6.9 and 6.10). Plants inoculated with different ratios of 855-16C and TA1 produced biomass similar to plants inoculated with TA1, except for the 3:1 ratio which had significantly greater shoot biomass (74mg/plant; Fig. 6.9).

Nodule numbers varied between 27 to 46 per plant for both the treatments (Table 6.7). When nodules were stained for GUS activity using XGlcA, the majority of nodules (>80%) were shown to be occupied by strain TA1. Strains 18-5D and 855-16C inoculated by themselves produced nodules that all stained blue (Table 6.7). Even in conditions that strongly favored the mutant 18-5D (ie. comprising 94% of the inoculum) the commercial inoculant TA1 was still able to occupy 83% of the nodules. Increasing the proportion of TA1 in the inoculum decreased the proportion of nodules occupied by 18-5D.

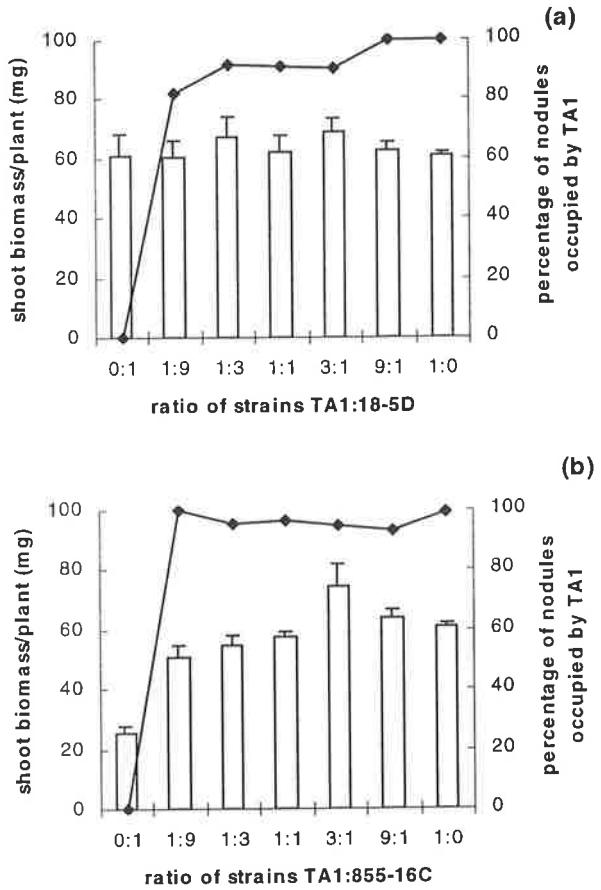

Fig. 6.9 Biomass accumulated by *T. alexandrinum* co-inoculated with different proportions of commercial inoculant TA1 and a *gusA*-labeled isolate of *R. leguminosarum* bv. *trifolii* (bar graph). All plants were inoculated with 10^4 cells per plant. TA1 and either 18-5D (effective) (a) or 855-16C (ineffective) (b) were inoculated in ratios of 0;1, 1:9, 1:3, 1:1, 3:1, 9:1 and 1:0. The figure also indicates the percentage of nodules occupied by TA1 (filled diamond, line graph) for each treatment (generally ≥90% for treatments containing strain TA1).

In mixed inocula with varying proportions of 855-16C and TA1, 855-16C only managed to colonize 0-5% of the nodules, despite comprising up to 88% of the inoculum applied to *T. alexandrinum*. The dominant field strains were therefore uncompetitive when co-inoculated with TA1 onto *T. alexandrinum*. The proportion of dual occupied nodules per plant varied between 0-10%, and from 0-3% of nodules per treatment.

Fig. 6.10a Shoot growth of *T. alexandrinum* inoculated with rates of TA1:18-5D (effective) of 0:1, 1:9, 1:1, 9:1 and 1:0 (A) and nodulation patterns of *T. alexandrinum* inoculated with TA1:18-5D at rates of 0:1, 1:9,, 1:3, 1:1, 3:1, 9:1 and 1:0 (B), indicating the dominance of TA1 in competing for nodulation.

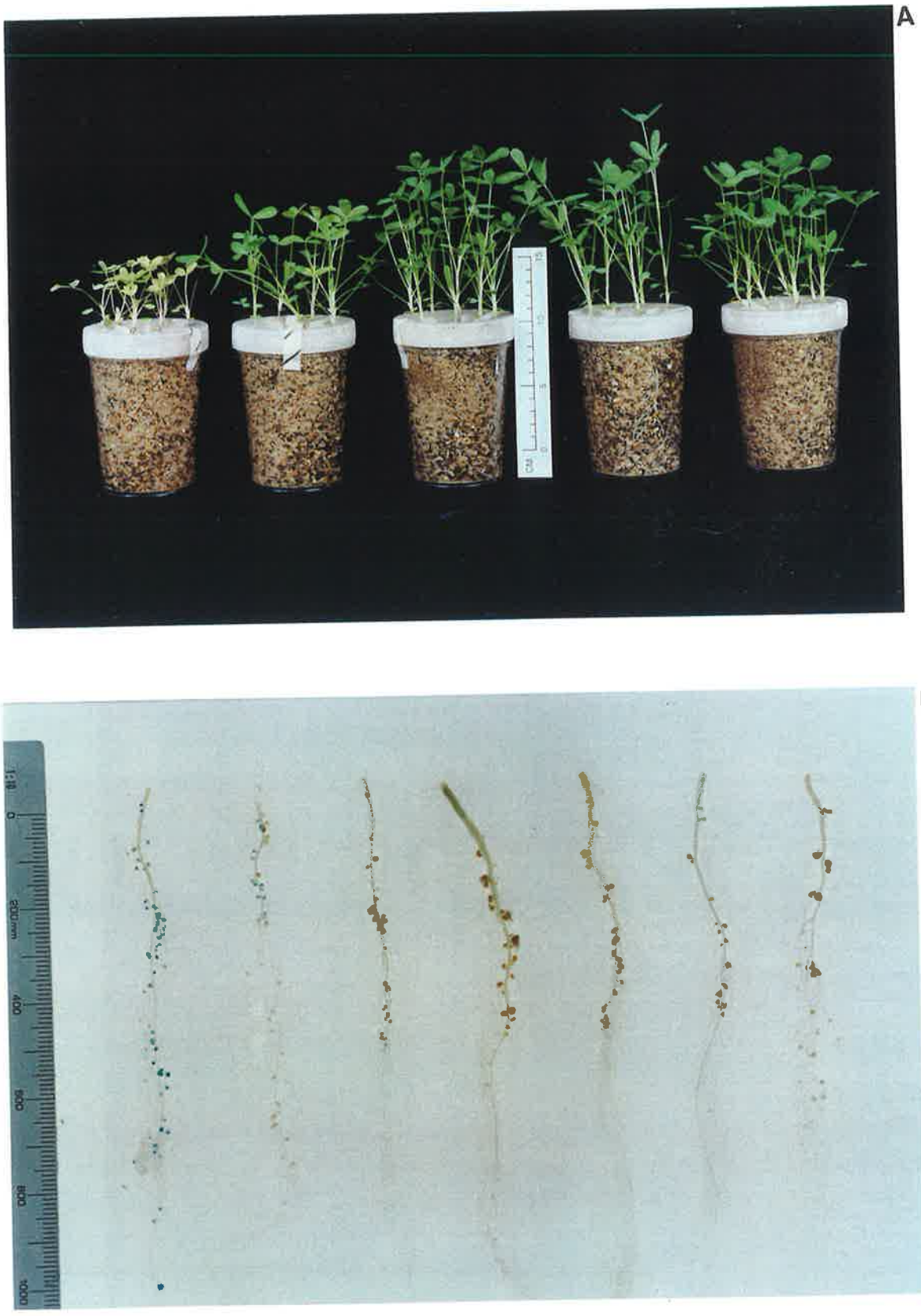

Fig. 6.10b Shoot growth of *T. alexandrinum* inoculated with rates of TA1:855-16C (ineffective) of 0:1, 1:9, 1:1, 9:1 and 1:0 (A) and nodulation patterns of *T. alexandrinum* inoculated with TA1:855-16C at rates of 0:1, 1:9, 1:3, 1:1, 3:1, 9:1 and 1:0 (B) indicating the dominance of strain TA1 in competing for nodulation.

Table 6.7 Nodule occupation of *T. alexandrinum* by *R. leguminosarum* bv. *trifolii* TA1 in comparison to a *gusA*-labeled Roseworthy isolate to provide a measure of competitiveness.

Strain	Strain ratio	Measured strain ratio*	Total nodules**	% TA1 nodules	% GUS-marked nodules	% Both
TA1	1	1	32^{ab}	100^{c}	0^{a}	0^{a}
18-5D	1	1	28^{ab}	0^{a}	100^{c}	0^{a}
TA1:18-5D	1:9	6:94	24^{a}	82^{b}	17^{b}	1^{a}
	1:3	23:77	29^{ab}	92^{bc}	8^{ab}	0^{a}
	1:1	46:54	46^{b}	91^{bc}	7^{ab}	3^{a}
	3:1	75:25	38^{b}	90^{bc}	8^{ab}	2^{a}
	9:1	92:8	27^{a}	99^{c}	1^{a}	0^{a}
TA1	1	1	32^{a}	100^{b}	0^{a}	0^{a}
855-16C	1	1	43^{a}	0^{a}	100^{b}	0^{a}
TA1:855-16C	1:9	12:88	42^{a}	95^{b}	2^{a}	3^{a}
	1:3	28:72	39^{a}	96^{b}	2^{a}	2^{a}
	1:1	51:49	29^{a}	95^{b}	3^{a}	1^{a}
	3:1	68:32	35^{a}	94^{b}	5^{a}	1^{a}
	9:1	88:12	27^{a}	99^{b}	0^{a}	1^{a}

*all treatments were inoculated with 10^4 cells/plant.

**values within each column followed by the same letter do not differ at the 0.05 level.

6.3.5 Experiment 4: Competitiveness of a Soil-Applied Mutant Strain Against a Seed-Applied Commercial Strain in Forming Nodules

In the final experiment, the ability for TA1 applied as a seed inoculant to compete for nodulation against mutant rhizobia (18-5D or 855-16C), applied separately to soil in pots, was assessed.

Seed was inoculated with 2 rates of strain TA1 and left overnight in the laboratory to sow the following day, in which time, viable cell density on

the seed coat halved (Table 6.8). The number of viable rhizobia/seed counted at sowing was ~9000 for the 1× inoculation and 900 for the 1/10× inoculation (Table 6.8). Uninoculated seed (surface sterilized) contained no viable rhizobia.

Table 6.8 Number of rhizobial cells/seed in Experiment 4.

Testing period	Treatment (TA1)	Rhizobial cells per seed
30 minutes after inoculation	1× inoculation	22300 ± 2960
	1/10× inoculation	1800 ± 115
	uninoculated	0
Immediately following sowing (24 hours after inoculation)	1× inoculation	8900 ± 800
	1/10× inoculation	900 ± 170
	uninoculated	0

Mutant rhizobia were added to the soil in estimated (log) rates of 2, 4 and 6 cells/g soil, which were assessed using serial plate counts (Table 6.9). When 1 g of soil from the top 1cm of pots was measured 1 week later, soil in all treatments contained greater than 6 (log scale) rhizobia/g soil (Table 6.10).

Table 6.9 Rhizobial numbers inoculated into pots, cells/g soil.

Strain	Estimated Density	Measured Density	se	Measured Density (log)*	se
18-5D	10^6	3.41×10^6	3.91×10^5	6.53[a]	5.59
18-5D	10^4	3.41×10^4	7.24×10^3	4.53[b]	3.86
18-5D	10^2	279	48	2.45[c]	1.68
855-16C	10^6	2.25×10^6	4.22×10^5	6.35[a]	5.62
855-16C	10^4	2.12×10^4	3.86×10^3	4.33[b]	3.59
855-16C	10^2	231	22	2.36[c]	1.35

* cell densities followed by the same letter do not differ significantly at P=0.05.

Table 6.10 Rhizobial densities in pots 7 days after inoculation, cells/g soil.

Strain	Estimated Density	Measured Density	se	Measured Density (log)*	se
18-5D	10^6	6.22×10^7	3.88×10^7	7.79[a]	7.59
18-5D	10^4	7.14×10^7	4.39×10^7	7.85[a]	7.64
18-5D	10^2	6.74×10^6	1.61×10^6	6.83[b]	6.21
855-16C	10^6	5.34×10^7	1.76×10^7	7.73[a]	7.25
855-16C	10^4	3.21×10^7	6.49×10^7	7.51[a]	6.81
855-16C	10^2	6.02×10^6	9.26×10^5	6.78[b]	5.97

* cell densities followed by the same letter do not differ significantly at P=0.05.

Nodules did not form in the uninoculated treatment (Table 6.11). The total number of nodules varied depending upon whether 18-5D or 855-16C was applied to soil. When strain 855-16C was applied to the soil, the

total number of nodules was influenced by the interaction of seed inoculation × density of 855-16C in soil ($P<0.05$). At low (1/10) seed inoculation rates, total nodules increased as the density of strain 855-16C in the soil decreased; at high (1×) seed inoculation rates, total nodules did not differ with the density of 855-16C in soil. Increasing density of strain 18-5D applied to the soil decreased total nodule number ($P<0.01$). Partially stained nodules made up a small proportion of nodules: 0-13% of individual plants and 0-6% of treatments (Table 6.11). Shoot biomass varied from 100-145mg/plant (Table 6.11).

When TA1 was applied to seed at the low (1/10) rate, in the absence of soil rhizobia, only 50% of the nodules formed were in the top 5cm of root. In other treatments the majority (>80%) of nodules formed by seed applied rhizobia (TA1) were restricted to the top 5cm of the root system (Table 6.12).

Analysis was restricted to plants that were seed inoculated (1 or 1/10 strength), to determine the effects of rates of seed and soil applied rhizobia on the production of pink (TA1-containing) nodules. The number of nodules formed by strain TA1 in the top 5cm of roots decreased as seed inoculation decreased ($P<0.01$) and as the density of soil-applied rhizobia increased ($P<0.001$) (Table 6.12). The number of nodules formed by TA1 on roots below 5cm decreased with increasing soil applied density of rhizobia ($P<0.01$). Nodulation by strain TA1 did not differ due to the different strains of soil-applied rhizobia, 18-5D and 855-16C.

Table 6.11 Nodule numbers and shoot biomass for Experiment 4.

Seed inoculant rate of TA1	Soil applied rhizobia	Soil rhizobia density (No./g soil)	Mean nodule number / plant	Partially stained nodules	Shoot biomass (mg/plant)
0	0	0	0^{a}	0	120^{b}
1/10	0	0	59^{d}	0	128^{bc}
1	0	0	40^{cd}	0	136^{bc}
0	18-5D	10^{2}	35^{c}	0	141^{bc}
0	18-5D	10^{4}	45^{cd}	0	116^{ab}
0	18-5D	10^{6}	22^{bc}	0	141^{bc}
1/10	18-5D	10^{2}	29^{c}	5.6	143^{bc}
1/10	18-5D	10^{4}	29^{c}	4.5	137^{bc}
1/10	18-5D	10^{6}	26^{bc}	2.6	116^{ab}
1	18-5D	10^{2}	45^{cd}	0.8	140^{bc}
1	18-5D	10^{4}	28^{c}	1.5	142^{bc}
1	18-5D	10^{6}	20^{bc}	4.8	134^{bc}
0	855-16C	10^{2}	90^{e}	0	106^{a}
0	855-16C	10^{4}	48^{cd}	0	100^{a}
0	855-16C	10^{6}	48^{cd}	0	124^{b}
1/10	855-16C	10^{2}	22^{bc}	0	124^{b}
1/10	855-16C	10^{4}	21^{bc}	0	130^{bc}
1/10	855-16C	10^{6}	9^{b}	0	111^{ab}
1	855-16C	10^{2}	39^{cd}	1.6	127^{bc}
1	855-16C	10^{4}	26^{c}	0.0	143^{bc}
1	855-16C	10^{6}	37^{cd}	0.0	145^{c}

*indicates rhizobia inoculated into the pots prior to the experiment starting.

Inoculation of seed with TA1 decreased the number of stained (*gusA*-labeled) nodules in the top 5cm of the root compared to uninoculated seed ($P<0.001$). The two different seed inoculation rates did not alter nodulation by *gusA*-labeled rhizobia (Table 6.12). The proportion of stained nodules formed by *gusA*-labeled rhizobia in the top 5cm of root were usually low, as a proportion of the total number of stained nodules on a root (22-77%; Table 6.12). Strain 18-5 produced a greater proportion of nodules in the top 5cm of root compared to strain 855-16 ($P<0.01$), and the proportions of blue nodules in this section were higher for plants inoculated with 1× inoculation compared to 1/10× inoculation ($P<0.01$).

Nodulation of the root sections below 5cm by *gusA*-labeled strains was influenced by the interactions of seed inoculation*density of rhizobia (soil applied) ($P<0.05$) and seed inoculation*strain type (soil applied) ($P<0.01$). Generally, seed inoculation decreased the number of stained nodules at >5cm depth, but the number of blue nodules was sometimes high when the highest seed inoculation treatment was applied, particularly nodules formed by strain 855-16C.

The ratio of stained (*gusA*-labeled rhizobia) to unstained (TA1-containing) nodules formed by plants varied so markedly that no significant differences in proportions were due to either seed inoculant strength or density of soil applied rhizobia (Table 6.13). The ratio (stained:unstained nodules) in the top 5cm of root varied was 1 or less when soil rhizobial populations were applied at low levels (10^2 rhizobia /g soil). The ratio was usually highest when soil rhizobia were applied in high density (10^6 rhizobia /g soil). Higher ratios of blue nodules:TA1 nodules were formed by the effective strain 18-5D; strain 855-16C appeared less competitive (Table 6.13). At the extremes: nodules formed by mutant rhizobia outnumbered those formed by TA1 by 5:1 and nodules formed by strain TA1 outnumbered blue nodules 15:1 (Table 6.13).

Table 6.12 Numbers of pink (TA1-containing) and blue (*gusA*-labeled) nodules in the top 5cm of roots and the proportion these nodules represent compared to the total number of pink or blue nodules on the entire root system.

Seed inoculant rate of TA1	Soil applied rhizobia	Soil rhizobia density (No./g soil)	No. TA1 (pink) nodules in the top 5cm of root ±se	Percent of pink nodules in top 5cm *	No. *gusA*-marked (blue) nodules in the top 5cm of root ±se	Percent of *gusA*-labeled nodules in top 5cm **
0	0	0	0	-	0	-
1/10	0	0	29.2±3.08	53	0	-
1	0	0	30.6±2.77	81	0	-
0	18-5D	10^2	0	-	13.2±3.8	52
0	18-5D	10^4	0	-	23.5±6.5	53
0	18-5D	10^6	0	-	16.0±2.9	77
1/10	18-5D	10^2	16.4±2.3	87	2.8±0.6	43
1/10	18-5D	10^4	16.3±1.6	88	3.8±2.7	35
1/10	18-5D	10^6	5.4±1.7	98	9.4±5.1	44
1	18-5D	10^2	20.6±4.6	84	10.1±9.0	44
1	18-5D	10^4	16.8±2.8	86	6.2±3.5	75
1	18-5D	10^6	8.8±4.4	99	6.1±2.8	67
0	855-16C	10^2	0	-	69.9±15.3	55
0	855-16C	10^4	0	-	31.0±9.5	49
0	855-16C	10^6	0	-	17.6±.7	38
1/10	855-16C	10^2	15.1±0.95	89	1.0±0.7	22
1/10	855-16C	10^4	12.2±3.1	97	5.4±3.1	38
1/10	855-16C	10^6	4.3±0.32	100	1.5±1.0	33
1	855-16C	10^2	17.5±3.0	86	8.0±4.8	31
1	855-16C	10^4	20.9±6.3	86	1.5±1.2	70
1	855-16C	10^6	15.5±1.7	92	16.1±8.9	34

* the proportion of pink nodules in the top 5cm of root compared to the total number of pink nodules on a root system.

** the proportion of *gusA*-labeled (blue) nodules in the top 5cm of root compared to the total number of *gusA*-labeled nodules on a root system.

Table 6.13. The proportion of blue (*gusA*-containing) to pink (TA1-containing) nodules in the top 5cm portion of roots.

Seed inoculant rate of TA1	Soil applied rhizobia	Soil rhizobia density (No./g soil)	Ratio of blue:pink nodules in the top 5cm of root ± se
0	0	0	-
1/10	0	0	-
1	0	0	-
0	18-5D	10^2	1.00
0	18-5D	10^4	1.00
0	18-5D	10^6	1.00
1/10	18-5D	10^2	0.19±0.06
1/10	18-5D	10^4	0.25±0.17
1/10	18-5D	10^6	4.59±3.70
1	18-5D	10^2	1.01±0.96
1	18-5D	10^4	0.54±0.37
1	18-5D	10^6	1.55±0.80
0	855-16C	10^2	1.00
0	855-16C	10^4	1.00
0	855-16C	10^6	1.00
1/10	855-16C	10^2	0.06±0.04
1/10	855-16C	10^4	0.89±0.53
1/10	855-16C	10^6	0.41±0.30
1	855-16C	10^2	0.68±0.53
1	855-16C	10^4	0.13±0.12
1	855-16C	10^6	1.32±0.78

6.4 Discussion

6.4.1 Competition Using gusA-Marked Strains

gusA-marked strains provide a convenient and rapid means to establish nodule occupancy (Wilson *et al.* 1995b; 1999). In this chapter the competitiveness of the commercial TA1 was assessed against field dominant *R. leguminosarum* bv *trifolii* isolates from Roseworthy, which were marked with *gusA*. The minitransposon mTn*5*SS-*gusA*30 was chosen as the tool to deliver *gusA* because: 1) it contains *gusA* transcribed by the *nifH* promoter which is active in the nodule environment; 2) expression of GUS from this fusion is reliable, consistent and not subject to variations in nodule age; and 3) the insertion of the minitransposon can be considered permanent since the minitransposon does not contain the *tnpA* gene (encoding the Tn*5* transposase). Strains of minitransposon mutants of Roseworthy isolates were not different from the parental strain in that: 1) the mutants could nodulate clovers; 2) the derivatives could fix N_2; and 3) could compete for nodulation equally against their wild-type. This behaviour meant that mutant strains were suitable substitute strains for the common field strains in testing competitive abilities with commercial strain TA1. Berseem clover (*T. alexandrinum*) was used in the experiments because: (1) it was used in the field trials (Chapters 4 and 5), (2) it has strain TA1 as its commercial inoculant and (3) it allowed the comparison of an ineffective and effective strain, as determined here in experiment 1.

6.4.2 Nodule Occupancies

Strain 855-16 was ineffective when inoculated onto *T. alexandrinum*, producing small nodules typical of an ineffective strain (Amarger 1981a). Interestingly, strain 837-7 had the same RP01 PCR banding pattern as strain 855-16 (Fig. 6.2), but was effective on *T. alexandrinum*. Phenotypic

differences between rhizobia with similar genotypes has been previously reported (Thies *et al.* 2000). Strain 18-5 produced comparatively larger nodules than 855-16 and was equally effective as the commercial strain TA1. Both field strains (18-5 and 855-16) were non-competitive when co-inoculated with strain TA1 on *T. alexandrinum*, grown in vermiculite and sand (Table 6.7). Even when strains were applied in ratios that should favor 855-16 or 18-5, TA1 clearly formed the majority of nodules and provided similar shoot biomass to TA1 applied alone.

Partially occupied nodules occurred only in treatments that contained wild type and *gusA*-labelled inoculum. This, and previous reports of the occurrence of dually occupied nodules in studies using *gusA*-labelled rhizobia (Wilson *et al.* 1995b; Sessitsch *et al.* 1997) indicates that the partially occupied nodules in the present study were likely to have been dually occupied.

The ability of commercial inoculant strain TA1 applied to the seed coat to compete with a field dominant strain (*gusA*-marked) applied to the soil was also tested. Soil from the Roseworthy field site was used; this was thought to provide conditions that more closely resembled the field soil physical and chemical characteristics. In particular, the influences of pH (Frey and Blum 1994) and the percent of clay and organic colloids (Hamdi 1971; Issa *et al.* 1993a) can influence rhizobial movement and competitive abilities.

This study focussed on the top 5cm of the root because this region is where the majority of nodules form due to inoculant strains (Worrall and Roughley 1976; Ciafardini and Barbieri 1987; Hardarson *et al.* 1989; McDermott and Graham 1989). This is also the region where nodules were sampled for the main field trial (Chapters 4 and 5).

After one week the numbers of soil applied rhizobia in the top 1cm of soil

were all $>10^6$ /g soil (Table 6.10). Even though rhizobia in different treatments appeared to multiply to similar levels in the first week of the experiment, nodulation data indicated that rhizobial density of the soil significantly affected the outcome of nodulation.

6.4.3 Implications of these Nodule Occupancy Studies

The previous chapter identified a number of field dominant strains that occupied many nodules in the field experiment. Strains 18-5 and 855-16 occupied 19% and 6% of all the nodules collected at the Roseworthy site, respectively. These strains were thought to be adapted to alkaline soils, and if they proved to be effective strains on alternative clovers, they may have some characteristics eg. saprophytic competence, that make them suitable for further consideration as commercial strains for alkaline soils.

These common field isolates performed poorly when co-inoculated with strain TA1 on *T. alexandrinum* in slightly alkaline media. Strain TA1 formed the majority of nodules, even when strains 18-5D and 855-16C represented 88% or more of rhizobia in the inoculum. The reasons that common field strains failed to perform in glasshouse competition trials could be numerous. The dominant field strains may be adapted to the specific conditions of alkaline soils and may be poorly adapted to competition in the non-soil environment, whereas TA1 performs well in vermiculite media (Brockwell and Gibson 1968; Robinson 1969; Brockwell *et al.* 1988b). A number of studies have reported poor performance of dominant field strains when competition studies are conducted in non-soil glasshouse conditions (McLoughlin and Dunican 1985; Leung *et al.* 1994b). Reasons for poor competitiveness of field dominant strains in non-soil media may relate to inherent differences in the media used in the experiment compared to the environmental conditions present at the field site. Soil temperature and soil pH, for example, have been implicated in modifying the competitive success of rhizobia in soil (Dughri and

Bottomley 1983; Dowling and Broughton 1986; Graham 1992; Streit *et al.* 1992). Competition in a sterilized soil may alter the physiology of rhizobia compared to competition in field soil with a diverse microflora. Strain TA1 was originally isolated from an Australian soil: a mildly acidic loam (exact pH unknown) as a naturalised isolate in 1953 from Bridport, Tasmania (Brockwell *et al.* 1998), and this strain may not be well adapted to competing in the specific alkaline conditions at the field site.

Some studies have suggested that the host legume is able to exert a selection pressure to "select" effective rhizobia to nodulate with, from a population of mixed effectiveness (Robinson 1969). The current experiments show that strain TA1 is superior in nodulating *T. alexandrinum* under non-soil conditions in preference to an equally effective field strain (18-5) and an ineffective strain (855-16). *T. alexandrinum* is likely to be highly compatible in forming nodules with strain TA1, which may have given TA1 a selective advantage in non-soil conditions. The current study suggests that the reason for optimal nodulation between TA1 and *T. alexandrinum* is due to host selectivity (possibly mediated by lectins or *nod* gene interactions etc.). Strain TA1 is an Australian commercial inoculant for white clover (*T. repens*) and berseem clover (*T. alexandrinum*) and was the commercial strain for *T. subterraneum* until it was discovered to be incompatible with the subterranean clover cultivar Woogenellup (Gibson 1968; Gibson and Brockwell 1968). TA1 was applied to seed 24 hours prior to sowing into soil in the pot experiment; seed for the field trial was inoculated the same day as sowing. It could be hypothesized that nodulation by strain TA1 was enhanced in the pot experiment due to pre-exposure of the TA1 causing early initiation of nod gene induction in this strain.

An alternative explanation for why field dominant strains formed few nodules in co-inoculation experiments in the glasshouse, is that field

dominant strains may multiply to form large proportions at the field site, so that they had a numerical advantage over inoculant rhizobia in forming nodules. When field dominant strains were tested in glasshouse experiments they were poorly competitive for nodulation, even when they made up 90% of the inoculum. Rhizobia present in field soils may not be equally present within the rhizosphere, so that particular strains are favoured (Barclay *et al.* 1994; Hatzinger and Alexander 1994).

A further experiment conducted in Roseworthy soil was thought to more closely mimic inoculation into a field situation. When different populations of rhizobia were inoculated into soil, and measured 7 days later, they had multiplied to $>10^6$ rhizobia/g soil, a microbiological effect of inoculation into sterile soil (Erceg *et al.* 1994; Hatzinger and Alexander 1994) that was not entirely unexpected. However, soil applied densities of rhizobia did significantly affect the nodulation of *T. alexandrinum,* indicating that nodule forming efficiency (the speed in which nodulation needs to occur) may have advantaged initially high soil rhizobial populations (Bhuvaneswari *et al.* 1980, 1981; McDermott and Graham 1990). When *T. alexandrinum* was inoculated with TA1, the soil-applied field dominant strains often formed a significant proportion of nodules in the top 5cm of the root, similar to the situation of the field trial (Chapters 4 and 5). These experiments were probably more representative of the field situation, in that commercial inoculum was applied only to the seed. The experiments therefore suggest that the reason strains 18-5 and 855-16 were commonly found as nodule isolates in the field trial (Chapter 5) may be due to their numerical abundance in the soil (Brockwell *et al.* 1995). This result was not entirely conclusive and an alternative explanation is that the presence of common field strains in a significant proportion of nodules in 0-5cm region could be due to adaptation of these strains to alkaline calcareous soils, as outlined above.

In order to test the hypothesis that the dominance of strains 18-5 and 855-16 is due to adaptation to alkaline soils, it is suggested that strain TA1 and these common field strains be tested by co-inoculation experiments in sterile alkaline field soil. Alternatively, TA1 could be labeled with the *gusA* gene and competed against the natural populations in soil. Improvements to such an experiment could be made by using soil cores from the field, so that soil structure is not compromised.

If strain 18-5 is able to compete well with commercial strains in alkaline soil conditions, it may have characteristics that warrant further investigation as a commercial strain for alkaline soils. Strain 18-5 was shown to be equally effective in producing shoot growth to TA1 in glasshouse conditions on *T. alexandrinum,* and produced a high proportion of nodules in field trials. This strain is likely to be saprophytically competent, surviving in the rhizospheres of *Medicago* spp. and non-legumes.

The selection of strains from alkaline calcareous soils may prove worthwhile in order to isolate strains that have characteristics that enable them to perform well in these soils. The majority of strains selected for clover rhizobia previously have been influenced by the need to select for acid tolerance. If alternative clovers are to be grown on alkaline soils, then similar selection criteria could be considered to maximize the potential of these legumes.

In the more likely scenario, that high nodule occupation by strains 18-5 and 855-16 in the field experiment (Chapter 4 and 5) was due to large numbers of these strains in the field soil, then high N_2 fixation may be limited if the population of rhizobia is poorly effective with clovers. While clovers were able to nodulate and fix N_2 with naturalised rhizobia in the field trial (Chapter 4), the occupancy of nodules by inoculant strains (Chapter 5) was insufficient to achieve differences in pasture DM

(Chapter 4). Management of naturalised rhizobial populations needs to be developed. A limited number of studies have indicated that changing the growth conditions (lime input, soil P) can alter the major strain types inhabiting nodules, increasing the amount of N_2 fixed by pastures (Almendras and Bottomley 1987), and these ideas should be further explored. The selection of alternative clovers that fix N_2 suitably with a number of soil rhizobial populations could also be considered.

In this study, >80% of nodules formed by the seed inoculant (TA1) were formed in the top 5cm of the root. Similar findings have previously been reported (Worrall and Roughley 1976; Ciafardini and Barbieri 1987; Beattie *et al.* 1989; Hardarson *et al.* 1989; McDermott and Graham 1989). Nodules formed below 5cm on the lateral roots have been implicated in late season N_2 fixation in field grown plants (Hardarson *et al.* 1989; McDermott and Graham 1989), by which time early formed nodules on the crown may be senescing. Therefore, if elite strains are restricted to the top few centimetres of root, N_2 fixation may be significantly reduced if the nodules formed in lower sections of root form from poorly fixing naturalised strains.

Reducing the seed inoculum rate to 1/10 reduced nodulation by the seed-inoculant strain (TA1) in the top 5cm of roots. However, the inoculum applied in the 1/10 treatment was still quite high at 900 cells/seed and similar to inoculation in the field experiment. In addition, the seed was sown into soil moisture and temperatures that provided optimal conditions for rhizobial survival and plant germination. Seedlings in this experiment germinated quickly so that in less than 24 hours roots were actively growing and shoot growth was visible at 48 hours. While efforts are made to sow legumes in optimal soil conditions on farms, it is likely that sowing often occurs when soil temperature is too low or high, which may reduce early root growth, reduce survival of rhizobia and therefore

reduce nodulation by inoculant strains (Dowling and Broughton 1986; Brockwell *et al.* 1988a; Bordeleau and Prevost 1994). Furthermore, soil moisture may rarely be optimal and dry periods or excessive rain following sowing may reduce survival of rhizobia (Parker *et al.* 1977; Dowling and Broughton 1986; Vincent 1988) and sporadic rainfall was experienced in the field experiments. In addition, available soil N early in the season (Unkovich and Pate 1998) may delay nodulation, which may limit or severely halt nodulation by inoculant rhizobia. The factors of temperature, soil moisture and soil N may interact, so that inoculant rhizobia have a much poorer chance of nodulating clovers grown in fields compared to the current pot experiments.

6.4.4 Assessment of Nodulation Competitiveness

One limitation in the use of mutant bacteria in ecological experiments is the possibility that the site of mutation could affect the competitive ability of the organism. Insertions of marker genes (eg. *gusA*, *lacZ*) into bacteria does not normally compromise root colonization (Lam *et al.* 1990; Brockman *et al.* 1991; Sharma *et al.* 1991). Problems may, or may not arise, depending upon whether the insertion is random or targeted. The impact on symbiotic effectiveness will depend on position of the insertion and whether an essential gene is disrupted (Sessitsch *et al.* 1997). In this study, *gusA* was inserted randomly using minitransposon mediated delivery. Using this approach, 3 out of 4 mutants were equally competitive as the parental strain, demonstrating the importance of determining whether the insertion has made a deleterious impact on symbiotic properties of the rhizobia.

The assessment of nodulation competitiveness in the present work followed the methods of Beattie *et al.* (1989). Both McDermott and Graham (1990) and Amarger and Lobreau (1982) assessed nodulation based upon the number of nodules formed by competing rhizobia and the

proportion of those strains in the inoculum. Other studies have compared the proportions of inocula occupying nodules with those represented on the root surface (Marques-Pinto *et al.* 1974; Labandera and Vincent 1975). Beattie *et al.* (1989) determined that the assessment of competitive abilities of rhizobia could be improved by including dually occupied nodules into the equation. Beattie and coworkers therefore developed the relationship:

$\log[(P_X+P_{both})/(P_Y+P_{both})] = C_{X:Y} + k[\log(I_X/I_Y)]$ (see methods for details). This equation is essentially a $y = mx + c$ relationship that assesses the relationship between the inoculum applied to legume roots and the resultant nodule occupancies, by incorporating dual occupancy. Nodulation is then analyzed by applying various co-inoculation ratios to linear regression and assessing whether the intercept traverses the origin. Beattie *et al.* (1989) applied raw data of nodule occupancies to this relationship, whereas Sessitsch *et al.* (1997) used mean data to predict the competitiveness of strains. This distinction is important: using means, as opposed to raw data, in a regression analysis decreases the variation in the regression, increasing the coefficient of determination (R^2) and may decrease the likelihood that a line will go through the origin. This will affect the final determination of whether strains are competitive. Although Sessitsch and coworkers reported that a number of strains were less competitive, or more competitive than the wild type CIAT899 (Sessitsch *et al.* 1997), using raw data may have shown that these strains had competitive abilities indistinguishable from wild-type strains. Whether mean data should be used in a regression equation is questionable in a statistical sense, because averaging hides the variation inherent in the raw data. Using means is therefore not suggested on the grounds that it obscures variation evident in the actual data, particularly when a small number of levels of inoculation are assessed. The model described by Beattie *et al.* (1989) is therefore recommended as the best

estimate of competitive ability. In the present study, using means (n=3) increased the R^2 value, reduced the likelihood that lines passed through the origin and suggested that only 2 out of the 4 strains were as competitive as their parental strains. Using means, strains 18-5D and 855-16C were not different from the wild type strain in competitiveness.

6.4.5 Future Use of GUS in Rhizobial Ecology

The use of GUS as a marker could provide a means to answer more comprehensive aspects of *Rhizobium* ecology under controlled conditions. In particular, our understanding of how various rates of inoculum placed on seeds and indeed how different seed sizes and legume types may influence the ability for rhizobial inoculum to be delivered to the infectible region of the root may be enhanced. *T. alexandrinum*, for example, has a seed weight approximately four times that of *T. resupinatum* (Chapter 4) and a much larger surface area in which to carry inoculant. This may impact on the ability of inoculant strains to form nodules on these two clovers.

The GUS system allows rapid observation of nodules, compared to other molecular techniques (Wilson *et al.* 1999) which means that nodulation patterns can readily be assessed. The delivery of inoculants to legume seeds is an area where improvements need to be made (Brockwell and Bottomley 1995) and may be assisted by the use of GUS marker strains. Marker technology may also be useful in modeling nodulation within the rhizosphere, and understanding spatial localization of nodules.

Chapter 7

General Discussion

7.1 Discussion of Results

Inoculation of legumes does not always result in the desired effect of increasing shoot or grain yield (Thies *et al.* 1991a; Streeter 1994; Howieson and Rome 1996; Vlassak and Vanderleyden 1997). The work in this thesis examined the role of naturalised soil populations of *R. leguminosarum* bv. *trifolii* in influencing the nodulation of alternative clovers. The question addressed was:

What role do naturalised soil populations of *R. leguminosarum* bv. *trifolii* have on the successful introduction of rhizobial inoculants for alternative clovers on alkaline soils?

To answer this question, four specific questions were addressed:

What is the size and effectiveness of soil rhizobial populations in the target geographical regions? (Chapter 3)

What is the influence of naturalised soil rhizobial populations on the ability of inoculant rhizobia to nodulate alternative clovers and to fix N_2? (Chapter 4)

What influence does the naturalised population of rhizobia have on the ability for inoculant rhizobia to occupy nodules of alternative clovers on alkaline soils? (Chapter 5)

How competitive are field dominant strains of rhizobia for nodulation of alternative clovers? (Chapter 6)

The specific answers to these questions are summarised below:

1) A survey determined that the majority of populations of *R. leguminosarum* bv. *trifolii* in the target region of alkaline soils were small in number and poor in their capacity to fix N_2 with Persian and purple clovers. *R. leguminosarum* bv. *trifolii* populations were found even where clover was not present. Rhizobial population size was negatively correlated to pH and to the percent of $CaCO_3$ in the soil, and was significantly increased in the rhizospheres of naturalised clover.

2) When alternative clovers were grown on two soils with small populations of *R. leguminosarum* bv. *trifolii*, inoculation with commercial rhizobia or the application of 200kgN/ha failed to increase pasture DM above uninoculated treatments. While *T. purpureum* produced the greatest biomass, it had a low N concentration in shoots. *T. resupinatum* formed a greater number of nodules, regardless of inoculation, had a higher N concentration, and fixed a greater amount of N_2 than *T. purpureum*. However, all clovers fixed N_2 best when inoculated with their specific commercial strain, indicating that productivity due to nodulation with naturalised rhizobia was not optimal.

3) The ability for commercial strains to compete with naturalised rhizobia to occupy nodules was determined for plants from the field trials. All commercial inoculant strains achieved <40% nodule occupancy. Strain TA1 was poorly competitive for nodulation with the exception that it nodulated *T. alexandrinum* up to 39% at the Roseworthy site. Commercial strains CC2483g (*T. resupinatum*) and WSM409 (*T. purpureum*) produced reasonable colonization of nodules in the first year of inoculation and showed persistence into the second year at both field sites. A number of isolates with similar banding patterns, distinct from commercial strains, were consistently observed from field isolates. These common field isolates cross-inoculated all 3 clover species and occurred in

both years of the field trials, each isolate occupying 4 to 19% of the total nodules at a field site. The two field sites sampled were unique in the dominant strains of *R. leguminosarum* bv. *trifolii* they contained.

4) Two dominant field isolates (18-5, effective and 855-16, ineffective) were assessed for their ability to compete against commercial strain TA1. Strains marked with the *gusA* gene did not differ from their parental strains in effectiveness (plant biomass) or in competitiveness for nodulation. Competition studies using TA1 as a seed inoculant, with *gusA*-marked strains applied to soil, indicated that increasing both density of rhizobia in the soil and reducing TA1 seed inoculant by one tenth reduced the number of nodules formed by strain TA1 in the top 5cm of root. The proportion of *gusA*-marked:TA1 strains varied enormously. The results suggested that the poor nodule occupancy by inoculant strains in the field study was most likely due to a numerical advantage of the naturalised strains of rhizobia. Variable soil N and soil moisture in the field experiment, which was not present in the pot experiment, may have contributed to reduced survival of inoculant rhizobia.

7.2 The Influence of Naturalised Populations of *R. leguminosarum* bv. *trifolii* in Alkaline Soils of Southern Australia

7.2.1 Introduction

Southern Australian farming systems rely substantially upon the use of legumes to provide N which sustains cropping and livestock industries, particularly in Mediterranean type environments (Reeves and Ewing 1993). In the more marginal regions there is an even greater need for organic N that is inexpensive (Coventry *et al.* 1998).

The current economic and environmental pressures on farming systems have demanded new roles for legumes in southern Australia (Howieson *et al.* 2000b). A large array of legumes has the potential to meet some of these needs, due to the huge diversity of legumes within locations such as the Mediterranean basin (Cocks and Bennett 1999). An essential part of the development and release of these legumes is that rhizobia well-matched to both soil and plant characteristics are available. Identifying suitable rhizobia is crucial to the success of alternative clovers on alkaline soils.

7.2.2 Naturalised Populations of R. leguminosarum *bv.* trifolii

Naturalised populations of *R. leguminosarum* bv. *trifolii* in the target regions were generally small but were aggressive in competing against inoculant strains for nodule occupancy. These naturalised strains also had variable effectiveness in fixing N_2 with alternative clovers. It was assumed that many of these naturalised populations were saprophytically competent (Chatel *et al.* 1968; Bottomley *et al.* 1991), existing in the alkaline soil in the rhizospheres of annual *Medicago* and non-legumes (Robinson 1967), since clovers were rarely encountered in these environments.

The naturalised *R. leguminosarum* bv. *trifolii* populations under study (selected using the particular clover hosts) were shown to contain a similar genetic structure to many other studies (Bromfield *et al.* 1986; Shishido and Pepper 1990; Leung *et al.* 1994a). While a number of strains were dominant in the sampled populations, there was also a high degree of relative diversity (probably genotypic and phenotypic).

Naturalised populations of rhizobia often inhibit nodulation by inoculant strains of rhizobia in field situations (Streeter 1994, Howieson and Rome 1996). It has been estimated that 1000 rhizobia/g soil is a sufficiently

large population of naturalised rhizobia to limit nodulation by inoculant strains of rhizobia (Weaver and Frederick 1974b; Evans *et al.* 1993; Brockwell *et al.* 1995), although much lower populations of rhizobia can limit nodulation by inoculant strains (Thies *et al.* 1991). In the present study, when commercial inoculants were applied to clovers growing in glasshouse conditions, the commercial inoculant (strain TA1) formed the majority of nodules in the upper root sections, even when inoculated into a high background of naturalised rhizobia (up to 10^6 rhizobia/g soil). This result is therefore contrary to many studies which suggest that inoculant strains fail to form a significant number of nodules when seeds are sown into high background populations of rhizobia (references cited above).

The majority of nodules formed by the inoculant strain (>80%) were, however, in the top 5cm of roots, indicating the limitations of seed inoculants to colonize lower sections of roots (Hardarson *et al.* 1989; McDermott and Graham 1989; Brockwell and Bottomley 1995). Nodulation by applied strains is usually restricted to the top portions of roots due to limited motility of rhizobia (Soby and Bergman 1983; Parco *et al.* 1994). Nodulation of lower tap roots and lateral roots also provides substantial late season N_2 fixation (Hardarson *et al.* 1989; McDermott and Graham 1989). Therefore the colonisation of lower roots is still a pragmatic problem which needs to be addressed for optimal commercial seed inoculation.

7.2.3 *Inoculation of Alternative Clovers with Commercial* R. leguminosarum *bv.* trifolii *in the Field*

In the more realistic and complex system of the field environment, nodulation of the upper sections of the clover root by inoculant strains was less successful. Inoculation of clovers with commercial rhizobia achieved up to 40% nodule occupancy by the inoculant strains. Most nodules were therefore occupied by naturalised field strains. Inoculation

using the specific commercial strain of rhizobia achieved the best nodule occupancy; inoculation of clovers with other strains of rhizobia produced limited nodule occupancy by those inoculant strains. In other environments, optimal nodulation has been achieved (100% occupancy of nodules; Roughley *et al.* 1976; Brockwell *et al.* 1982; Hebb *et al.* 1998) even though clover-*Rhizobium* matches were not optimal (Howieson *et al.* 2000a). This requirement for host-rhizobial compatibility is an important finding, although the reason that host selectivity is necessary in alkaline soil environments is not clear.

The discrepancy in low nodule occupancies of inoculants in the field experiments and high nodule occupancies under controlled (glasshouse) studies is difficult to explain. Differences in the ratios of seed inoculant to indigenous populations of rhizobia did not explain the results. Poor nodule occupancy in the field by inoculant rhizobia may relate to poorer early growth of clovers and the possible loss of inoculant due to environmental conditions. Under controlled condtions in the glasshouse, seeds were sown into optimal temperature and moisture conditions which enabled excellent early growth. In comparison, the field experiment experienced variable rainfall, particularly early in the season, which may have contributed substantially to poor survival of inoculants. Similarly, variable soil nitrogen may have delayed early nodulation, leading to a low proportion of nodules formed by inoculants. Alternatively, the rhizobial strains may be poorly adapted to alkaline soil conditions, compared to the naturalised populations and this may have limited inoculant survival.

7.2.4 Recommendations for the Production of Alternative Clovers

Alternative clovers are currently being evaluated as pastures for low rainfall alkaline environments, typical of the geographical areas covered in this study. Information from this study should assist in providing some information which is of benefit to the production of these pasture species

and the interactions expected with naturalised rhizobial populations.

The clovers in the current study showed substantial dry matter accumulation in the experiments, considering the rainfall received. Lack of response to applied inoculants and N suggests that other factors were limiting growth (nutrients, disease, moisture). The most likely constraints to dry matter accumulation by clovers was variable rainfall during the growth periods and variable soil nitrogen. While inoculation with particular strains did not achieve greater biomass in the year of the trial, in years with more consistent rainfall, inoculation may cause DM increases.

Alternative clovers (eg. *T. purpureum*) can be highly specific in their rhizobial requirements compared to subterranean clover (Howieson 2000a). In addition to superior nodule occupancies, clover species inoculated with their specific commercial rhizobia often produced the highest nodule scores on taproots and whole roots and consistently fixed the highest proportions of N_2. Conversely, N_2 fixation by clovers nodulated with other strains of rhizobia or naturalised strains produced less N_2 fixation. In addition, this study reported that populations of *R. leguminosarum* bv. *trifolii* are generally small and poorly effective and may therefore provide suboptimal symbioses. Small populations of rhizobia also make the introduction of inoculant rhizobia more feasible. On these grounds, the inoculation of clovers in alkaline soils is justified, particularly if there is high compatibility between clover species and rhizobia.

The development of new clover cultivars for these regions should therefore involve the concurrent selection of rhizobia for these species. The current constraints in the inoculant industry require that a limited number of commercial inoculants are available for all clover species (J. Howieson, pers. comm.). An additional constraint is that new rhizobial

inoculants should not compromise the growth of existing important agricultural species such as *T. subterraneum* (Howieson *et al.* 2000a). To this end, strain WSM409 has shown excellent performance on a number of alternative clover species, and particularly in this study, on alkaline soils. Strain WSM409 is an efficient strain for *T. purpureum* and *T. glanduliferum* but does not compromise N_2 fixation by *T. subterraneum* (Howieson *et al.* 2000a).

Selecting strains of *R. leguminosarum* bv. *trifolii* that are well adapted to the alkaline soil environments from Australia or overseas may therefore be worthwhile in producing optimal inoculants. One hypothesis is that if rhizobia are well adapted to soil conditions, they will perform well, regardless of the size of the naturalised populations of rhizobia (Howieson 2000c), although this hypothesis is yet to be tested in alkaline soils. While it was assumed that rhizobial strains commonly found in alkaline soils had an ability to survive without a legume host, saprophytic competence of potential inoculant strains needs to be established (Chatel *et al.* 1968; Bottomley *et al.* 1991) as one criteria in the selection of commercial inoculants (Brockwell and Bottomley 1995).

A second approach to legume evaluation is selecting rhizobially promiscuous legumes, which nodulate and fix N_2 well with naturalised rhizobia from a number of sites (Herridge and Danso 1995; Ballard *et al.* 2000). Responses of alternative clovers to inoculation on alkaline soils was dependent on the host response to naturalised populations of rhizobia. Some clovers (eg. *T. resupinatum*) demonstrated an ability to nodulate and fix N_2 successfully with naturalised rhizobia. Other species (*T. alexandrinum* and *T. purpureum*) fixed lower proportions and amounts of N_2 with naturalised rhizobial populations. Evaluation of the performance of alternative clovers assessed with and without applied rhizobial inoculants is therefore likely to favour different species. Clover

species with an ability to efficiently nodulate and fix N_2 with the naturalised populations of rhizobia are more likely to be favoured over species with more specific rhizobial requirements. The success of other widely used pasture legumes eg. subterranean clover and lucerne, may be partly attributable to the fact that they can nodulate and fix N_2 broadly with naturalised rhizobia.

Selecting promiscuous clovers, able to nodulate with naturalised rhizobia, reduces the constraints of the need to match particular legumes with a limited number of inoculants, but puts greater reliance upon naturalised rhizobia to provide nodulation and N_2 fixation. Given that populations of *R. leguminosarum* bv. *trifolii* in the target regions are generally small and ineffective, this strategy of clover selection is likely to limit N_2 fixation, compared to evaluation of clover species well matched to rhizobial inoculants. The approach of concurrent selection of alternative clovers and suitable microsymbionts is therefore favoured.

References

Afif, E., Matar, A. and Torrent, J. (1993). Availability of phosphate applied to calcareous soils of West Asia and North Africa. *Soil Science Society of America Journal*, **37**: 756-760.

Allen, O.N. and Allen, E.K. (1980). *The Leguminosae, a source book of characteristics, uses and nodulation*. Madison, WI: The University of Wisconsin Press.

Almendras, A.S. and Bottomley, P.J. (1987). Influence of lime and phosphate on nodulation of soil-grown *Trifolium subterraneum* L. by indigenous *Rhizobium trifolii*. *Applied and Environmental Microbiology*, **53**: 2090-2097.

Alston, A.M. and Graham, R.D. (1982). The influence of soil nitrogen status and previous crop on nitrogen fixation (Acetylene reduction) in barrel medic, *Medicago truncatula* Gaertn. *Australian Journal of Soil Research*, **20**: 51-59.

Amarger, N. (1981a). Competition for nodule formation between effective and ineffective strains of *Rhizobium* meliloti. *Soil Biology & Biochemistry*, **13**: 475-480.

Amarger, N. (1981b). Selection of *Rhizobium* strains on their competitive ability for nodulation. *Soil Biology & Biochemistry*, **13**: 481-486.

Amarger, N. and Lobreau, J.P. (1982). Quantitative study of nodulation competitiveness in *Rhizobium* strains. *Applied and Environmental Microbiology*, **44**: 583-588.

Ames, P. and Bergman, K. (1981). Competitive advantage provided by bacterial motility in the formation of nodules by *Rhizobium meliloti*. *Journal of Bacteriology*, **148**: 728-729.

Atkins, C.A. (1984). Efficiencies and inefficiencies in the legume / *Rhizobium* symbiosis - A review. *Plant and Soil*, **82**: 273-284.

Atkins, C.A. (1987). Metabolism and translocation of fixed nitrogen in the nodulated legume. *Plant and Soil*, **100**: 157-169.

Atkins, C.A. and Rainbird, R.M. (1982). Physiology and biochemistry of biological nitrogen fixation in legumes. In: *Advances in Agricultural Microbiology*. (Subba Rao, N.S., Ed.). London, Butterworth Scientific: 25-51.

Ballard, R.A. (1993). *Development of rhizobia for alternative temperate pasture legumes*. Proceedings of the second national alternative pasture legumes workshop, South Australia, Primary Industries.

Ballard, R.A. (1996). Assessment of strains of *Bradyrhizobium* sp. (*Lupinus*) for serradellas (*Ornithopus* spp.). *Australian Journal of Experimental Agriculture*, **36**: 63-70.

Ballard, R.A., Charman, N., Craig, A.D. and Hughes, S.J. (2000). Symbiotic performance of pasture legumes with naturalised soil rhizobia. In: CahiersOptions Mediteraneennes. (L. Sulas, Editor). **45**: Legumes for Mediterranean forage crops, pastures and alternative uses'. pp 315-319. (CIHEAM, Zaragosa.)

Barclay, R.M., Hebb, D.M. and Brockwell, J. (1994). Differential rhizobial colonisation of the roots of sown and volunteer annual species of *Medicago* in an acid soil. *Australian Journal of Experimental Agriculture*, **34**: 745-752.

Barran, L.R. and Bromfield, E.S.P. (1997). Competition among rhizobia for nodulation of legumes. In: *Biotechnology and the improvement of forage legumes.* (McKersie, B.D. and Brown, D.C.W., Eds.). Cambridge, University Press: 343-374.

Beattie, G.A., Clayton, M.K. and Handelsman, J. (1989). Quantitative comparison of the laboratory and field competitiveness of *Rhizobium leguminosarum* biovar. *phaseoli. Applied and Environmental Microbiology*, **55**: 2755-2761.

Becana, M., Minchin, F.R. and Sprent, J.I. (1989). Short-term inhibition of legume nitrogen fixation by nitrate: I. Nitrate effects on nitrate reductase activities of bacteroids and nodule cytosol. *Planta*, **180**: 40-45.

Becana, M. and Sprent, J.I. (1989). Effect of nitrate on components of nodule leghemoglobins. *Journal of Experimental Botany*, **40**: 725-732.

Becker, R.A., Chambers, J.M. and Wilks, A.R. (1988). *The new S language.* New York: Chapman and Hall.

Bennett, S.J. (1997). Genetic variation between and within 2 populations of *Trifolium glomeratum* (cluster clover) in Western Australia. *Australian Journal of Agricultural Research*, **48**: 969-976.

Bergersen, F.J. and Goodchild, D.J. (1973). cellular location and concentration of leghaemoglobin in soybean root nodules. *Australian Journal of Biological Sciences*, **26**: 741-756.

Bergersen, F.J., Turner, G.L., Gault, R.R., Chase, D.L. and Brockwell, J. (1985). The natural abundance of ^{15}N in an irrigated soybean crop and its use for the calculation of nitrogen fixation. *Australian Journal of Agricultural Research*, **36**: 411-423.

Bergman, K., Gulash-Hoffee, M., Hovestadt, R.E., Larosiliere, R.C., Ronco, P.G. and Su, L. (1988). Physiology of behavioural mutants of *Rhizobium meliloti*: evidence for a dual chemotaxis pathway. *Journal of Bacteriology*, **170**: 3249-3254.

Beringer, J.E. (1974). R factor transfer in *Rhizobium leguminosarum. Journal Of General Microbiology*, **84**: 188-198.

Bhuvaneswari, T.V., Bhagwat, A.A. and Bauer, W.D. (1981). Transient susceptibility of root cells in four common legumes to nodulation by rhizobia. *Plant Physiology*, **68**: 1144-1149.

Bhuvaneswari, T.V., Turgeon, B.G. and Bauer, W.D. (1980). Early events in the infection of soybean (*Glycine max* L. Merr). *Plant Physiology*, **66**: 1027-1031.

Boettger, E.C. (1990). Frequent contamination of Taq polymerase with DNA. *Clinical Chemistry*, **36**: 1258-1259.

Bohlool, B.B. and Schmidt, E.L. (1974). Lectins: a possible basis for specificity in the *Rhizobium*-legume root nodule symbiosis. *Science*, **185**: 269-271.

Bolger, T.P., Pate, J.S., Unkovich, M.J. and Turner, N.C. (1995). Estimates of seasonal nitrogen fixation of annual subterranean clover-based pastures using the ^{15}N natural abundance technique. *Plant and Soil*, **175**: 57-66.

Bolland, M.D.A. and Paynter, B.H. (1994). Critical phosphorus concentrations for burr medic, yellow serradella, subterranean clover, and wheat. *Communications in Soil Science and Plant Analysis*, **25**: 3-4.

Bordeleau, L.M. and Prevost, D. (1994). Nodulation and nitrogen fixation in extreme environments. *Plant and Soil*, **161**: 115-125.

Bottomley, P.J. (1992). The ecology of *Bradyrhizobium* and *Rhizobium*. In: *Biological Nitrogen Fixation*. (Stacey, G., Burris, R.H. and Evans, H.J., Eds.). New York, Chapman and Hall: 293-348.

Bottomley, P.J., Cheng, H.H. and Strain, S.R. (1994). Genetic structure and symbiotic characteristics of a *Bradyrhizobium* population recovered from a pasture soil. *Applied and Environmental Microbiology*, **60**: 1754-1761.

Bottomley, P.J. and Jenkins, M.B. (1992). Some characteristics of *Rhizobium meliloti* isolates from alfalfa fields in Oregon. *Soil Science Society of America Journal*, **47**: 1153-1157.

Bottomley, P.J., Leung, K., Strain, S.R., Yak, K., Dashti, N. and Claycomb, P. (1990). The ecology of indigenous populations of *Rhizobium leguminosarum* bvs. *trifolii* and *vicieae*. In: *Nitrogen Fixation: achievements and objectives*. (Gresshoff, P. Roth, S. Stacey, G. and Newton, W. Eds.). New York, Chapman and Hall: 371-378.

Bottomley, P.J., Maggard, S.P., Leung, K. and Busse, M.D. (1991). Importance of saprophytic competence for introduced rhizobia. In: *The rhizosphere and plant growth*. (Keister, D.L. and Cregan, P.B., Eds.). Netherlands, Kluwer Academic Publishers: 135-140.

Bowman, A., .M., Hebb, D.M., Munnich, D.J. and Brockwell, J. (1998). *Rhizobium* as a factor in the re-establishment of legume-based pastures on clay soils of the wheat belt of north-western New South Wales. *Australian Journal of Experimental Agriculture*, **38**: 555-566.

Brand, J.D., Tang, C. and Rathjen, A.J. (1999). Adaptation of *Lupinus angustifolius* L. and *L. pilosus* Murr. to calcareous soils. *Australian Journal of Agricultural Research*, **50**: 1027-1034.

Brewin, N.J. (1991). Development of the legume root nodule. *Annual Review of Cell Biology*, **7**: 191-226.

Brockman, F.J., Forse, L.B., Bezdicek, D.F. and Frederickson, J.K. (1991). Impairment of transposon-induced mutants of *Rhizobium leguminosarum*. *Soil Biology & Biochemistry*, **23**: 861-867.

Brockwell, J. (1980). Experiments with Crop and Pasture Legumes-Principles and Practice. In: *Methods for evaluating biological nitrogen fixation*. (Bergerson, F.J., Ed.). Brisbane, Australia, John Wiley and Sons: 488.

Brockwell, J. and Bottomley, P.J. (1995). Recent advances in inoculant technology and prospects for the future. *Soil Biology & Biochemistry*, **27**: 683-697.

Brockwell, J., Bottomley, P.J. and Thies, J.E. (1995). Manipulation of rhizobia microflora for improving legume productivity and soil fertility: a critical assessment. *Plant and Soil*, **174**: 143-180.

Brockwell, J., Gault, R.R., Zorin, M. and Roberts, M.J. (1982). Effects of environmental variables on the competition between inoculum strains and naturalised populations of *Rhizobium trifolii* for nodulation of *Trifolium subterraneum* L. and on rhizobia persistence in the soil. *Australian Journal of Agricultural Research*, **33**: 803-815.

Brockwell, J. and Gibson, A.H. (1968). Root-nodule bacteria for some cultivated species of *Trifolium*. *The Journal of the Australian Institute of Agricultural Science*, **34** : 224-227.

Brockwell, J. and Hely, F.W. (1966). Symbiotic characteristics of *Rhizobium meliloti*: An appraisal of the systematic treatment of nodulation and nitrogen fixation interactions between hosts and rhizobia of diverse origins. *Australian Journal of Agricultural Research*, **17**: 885-899.

Brockwell, J., Herridge, D.F., Morthorpe, L.J. and Roughley, R.J. (1988a). Numerical effects of *Rhizobium* population on legume symbiosis. In: *Nitrogen fixation by legumes in Mediterranean agriculture*. (Beck, D.P. and Materon, L.A., Eds.). Netherlands, International Centre for Agricultural Research in Dry Areas: 179-193.

Brockwell, J., Holliday, R.A. and Pilka, A. (1988b). Evaluation of the symbiotic nitrogen-fixing potential of soils by direct microbiological means. *Plant and Soil*, **108**: 163-170.

Brockwell, J., McIlroy, R.A. and Hebb, D.M. (1998). The Australian collection of *Rhizobium* strains for temperate legumes. Canberra, CSIRO, Plant Industry.

Brockwell, J., Pilka, A. and Holliday, R.A. (1991). Soil pH is a major determinant of the numbers of naturally occurring *Rhizobium meliloti* in non-cultivated soils in central New South Wales. *Australian Journal of Experimental Agriculture*, **31**: 211-219.

Brockwell, J., Roughley, R.J. and Herridge, D.F. (1987). Population dynamics of *Rhizobium japonicum* strains used to inoculate three successive crops of soybean. *Australian Journal of Agricultural Research*, **38**: 61-74.

Bromfield, E.S.P., Sinha, I.B. and Wolynetz, M.S. (1986). Influence of location, host cultivar, and inoculation on the composition of naturalised populations of *Rhizobium meliloti* in *Medicago sativa* nodules. *Applied and Environmental Microbiology*, **51**: 1077-1084.

Bromfield, E.S.P., Wheatcroft, R. and Barran, L.R. (1994). Medium for direct isolation of *Rhizobium meliloti* from soils. *Soil Biology & Biochemistry*, **26**: 423-428.

Brunel, B., Rome, S., Ziani, R. and Cleyet-Marel, J.C. (1996). Comparison of nucleotide diversity and symbiotic properties of *Rhizobium meliloti* populations from annual *Medicago* species. *FEMS Microbiology Ecology*, **19**: 71-82.

Burris, R.H. and Haas, E. (1994). The red pigment of leguminous root nodules. *Journal of Biological Chemistry*, **155**: 227-229.

Bushby, H.V.A. (1982). Ecology. In: *Nitrogen Fixation*. (Broughton, W.J., Ed.). Oxford, England, Clarendon Press. **2:** 35-75.

Caetano Anolles, G., Lagares, A. and Favelukes, G. (1989). Adsorption of *Rhizobium meliloti* to alfalfa roots: Dependence on divalent cations and pH. *Plant and Soil*, **117**: 67-74.

Carlson, R.W., Price, N.P. and Stacey, G. (1994). The biosynthesis of rhizobial lipo-oligosaccharide nodulation signal molecules. *Molecular Plant-Microbe Interactions*, **7**: 684-695.

Carter, E.D. (1987). Establishment and natural regeneration of annual pastures. In: *Temperate pastures: their production, use and management.* (Wheeler, J.L., Pearson, C.J. and Robarbs, G.E., Eds.). Australia, CSIRO: 35-51.

Carter, J.M., Gardner, W.K. and Gibson, A.H. (1994). Improved growth and yield of faba beans (*Vicia faba* cv. Fiord) by inoculation with strains of *Rhizobium leguminosarum* biovar. *viciae* in acid soils in south-west Victoria. *Australian Journal of Agricultural Research*, **45**: 613-623.

Cartwright, B., Zarcinas, B.A. and Mayfield, A.H. (1984). Toxic concentrations of boron in a red-brown earth at Gladstone, South Australia. *Australian Journal of Soil Research*, **22**: 261-272.

Cartwright, B., Zarcinas, B.A. and Mayfield, A.H. (1986). Boron toxicity in South Australian barley crops. *Australian Journal of Agricultural Research*, **37**: 351-359.

Chatel, D.L., Greenwood, R.M. and Parker, C.A. (1968). Saprophytic competence as an important character in the selection of *Rhizobium* for inoculation. *Proceedings of the 9th International Congress on Soil Science*, **2**: 65-73.

Ciafardini, G. and Barbieri, C. (1987). Effects of cover inoculation of soybean on nodulation, nitrogen fixation, and yield. *Agronomy Journal*, **79**: 645-648.

Ciafardini, G. and Lombardo, G.M. (1991). Nodulation, dinitrogen fixation, and yield improvement in second-crop soybean cover-inoculated with *Bradyrhizobium japonicum*. *Agronomy Journal*, **83**: 622-625.

Cocks, P.S. (1995). *New approaches to the development of pasture legumes for southern Australia.* Pasture decisions for the cereal-livestock zone, Turretfield, South Australia, Australian Society of Animal Production.

Cocks, P.S. and Bennett, S.J. (1999). Introduction: role of pasture and forage legumes in Mediterranean farming systems. In: *Genetic resources of Mediterranean pasture and forage legumes*. (Bennett, S.J. and Cocks, P.S., Eds.). Netherlands, Kluwer Academic Publishers.

Corbin, E.J., Brockwell, J. and Gault, R.R. (1977). Nodulaton studies on chickpea (*Cicer arietinum*). *Australian Journal of Experimental Agriculture and Animal Husbandry*, **17**: 126-134.

Coulombe, B.A., Chaney, R.L. and Wiebold, W.J. (1984). Use of bicarbonate in screening soybeans for resistance to iron chlorosis. *Journal of Plant Nutrition*, **7**: 411-425.

Coventry, D.R. and Hirth, J.R. (1992). Effects of tillage and lime on *Rhizobium trifolii* populations and survival in wheat-subterranean clover rotation in southeastern Australia. *Soil and Tillage Research*, **25**: 67-74.

Coventry, D.R., Hirth, J.R., Reeves, T.G. and Burnett, V.F. (1985a). Growth and nitrogen fixation by subterranean clover in response to inoculation, molybdenum application and soil amendment with lime. *Soil Biology & Biochemistry*, **17**: 791-796.

Coventry, D.R., Hirth, J.R., Reeves, T.G. and Jones, H.R. (1985b). Development of populations of *Rhizobium trifolii* and nodulation of subterranean clover following the cropping phase in crop-pasture rotations in southeastern Australia. *Soil Biology & Biochemistry*, **17**: 17-22.

Coventry, D.R., Holloway, R.E. and Cummins, J.A. (1998). *Farming fragile environments: low rainfall and difficult soils in South Australia.* Proceedings of the 9th Australian Agronomy Conference, Wagga Wagga, Australian Society of Agronomy.

Crawford, E.J. (1983). Selecting cultivars from naturally occurring genotypes: evaluating annual *Medicago* species. In: *Genetic resources of forage plants.* (Melvor, J.G. and Bray, R.A., Eds.)., CSIRO: 203-215.

Crawford, E.J., Lake, A.W.H. and Boyce, K.G. (1989). Breeding annual *Medicago* species for semiarid conditions in Southern Australia. *Advances in Agronomy*, **42**: 399-437.

Crawford, M.C., Grace, P.R., Bellotti, W.D. and Oades, M.J. (1997). Root production of a barrel medic (*Medicago truncatula*) pasture, a barley grass (*Hordeum leporinum*) pasture, and a faba bean (*Vicia faba*) crop in southern Australia. *Australian Journal of Agricultural Research*. **48**: 1139-1150.

Danso, S.K.A. and Bowen, G.D. (1989). Methods of inoculation and how they influence nodulation pattern and nitrogen fixation using two contrasting strains of *Bradyrhizobium japonicum*. *Soil Biology & Biochemistry*, **8**: 1053-1058.

Date, R.A. and Brockwell, J. (1978). *Rhizobium* strain competition and host interaction for nodulation. In: *Plant relations in Pastures.* (Wilson, J.R., Ed.). East Melbourne, Australia, CSIRO: 202-216.

Davey, A.G. and Simpson R.G. (1989). Nitrogen fixation by subterranean clover (*Trifolium subterraneaum* L.) in an acid soil in response to moisture deficits. *Soil Biology & Biochemistry*, **21**: 9-14.

Dazzo, F.B., Truchet, G.L., Sherwood, J.E., Hrabak, E.M., Abe, M. and Pankratz, S.H. (1993). Specific phases of root hair attachment in the *Rhizobium trifolii*-clover symbiosis. *Applied and Environmental Microbiology*, **48**: 1140-1150.

de Bruijn, F.J. (1992). Use of Repetitive (Repetitive Extragenic Palindromic and Enterobacterial Repetitive Intergeneric Consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Applied and Environmental Microbiology*, **58**: 2180-2187.

de Bruijn, F.J., Sadowsky, M. and Schneider, M. (1992). *Repetitive (REP and ERIC) sequences and PCR to fingerprint the genomes of Rhizobium and Bradyrhizobium strains*. New Horizons in Nitrogen Fixation, Proceedings of the 9th International Congress on Nitrogen Fixation, Mexico, (Palacios, R., Mora, J., Newton, W.E.). Dordrecht: Kluwer.

De Faria, S.M., Lewis, G.P., Sprent, J.I. and Sutherland, J.M. (1989). Occurrence of nodulation in the Leguminosae. *New Phytologist*, **111**: 607-620.

De Lorenzo, V., Herrero, M., Jakubzik, U. and Timmis, K.N. (1990). Mini-Tn*5* Transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *Journal of Bacteriology*, **172**: 6568-6572.

Dean, D.R. and Jacobson, M.R. (1992). Biochemical genetics of nitrogenase. In: *Biological Nitrogen Fixation.* (Stacey, G., Burris, R.H. and Evans, H.J., Eds.). New York, Chapman and Hall.

Demezas, D.H., Reardon, T.B., Watson, J.M. and Gibson, A.H. (1991). Genetic diversity among *Rhizobium leguminosarum* bv. *trifolii* strains revealed by allozyme and restriction fragment length polymorphism analyses. *Applied and Environmental Microbiology*, **57**: 3489-3495.

Denton, M.D. and Bellotti, W.D. (1996). *Factors involved in medic decline syndrome in the Murray Mallee, South Australia*. Proceedings of the 8th Australian Agronomy Conference, (Asghar, M.). Carlton, Australian Society of Agronomy.

Dilworth, M.J. and Glenn, A.R. (1984). How does a legume nodule work? *Trends in Biochemical Science*, **9**: 519-522.

Djordjevic, M.A., Redmond, J.W., Batley, M. and Rolfe, B.G. (1987). Clovers secrete specific phenolic compounds which either stimulate or repress *nod* gene expression in *Rhizobium trifolii*. *The EMBO Journal*, **6**: 1173-1179.

Djordjevic, M.A. and Weinman, J.J. (1991). Factors determining host recognition in the clover-*Rhizobium* symbiosis. *Australian Journal of Plant Physiology*, **18**: 543-558.

Dooley, J.J., Harrison, S.P., Mytton, L.R., Dye, M., Cresswell, A., Skot, L. and Beeching, J.R. (1993). Phylogenetic grouping and identification of *Rhizobium* isolates on the basis of random amplified polymorphic DNA profiles. *Canadian Journal of Microbiology*, **39**: 665-673.

Dowling, D.N. and Broughton, W.J. (1986). Competition for nodulation of legumes. *Annual Review of Microbiology*, **40**: 131-157.

Downie, J.A. (1998). Functions of rhizobial nodulation genes. In: *The Rhizobiaceae*. (Spaink, H.P., Kondorosi, A. and Hooykaas, P.J.J., Eds.). Dordrecht, Kluwer Academic Publishers: 387-402.

Downie, J.A. and Young, J.P.W. (1996). *Evolution of specificity in legume nodulating rhizobia*. 11th Australian Nitrogen Fixation Conference, Perth, Co-operative Research Centre for Legumes in Mediterranean Agriculture.

Dughri, M.H. and Bottomley, P.J. (1983). Effect of acidity on the composition of an indigenous soil population of *Rhizobium trifolii* found in nodules of *Trifolium subterraneum* cultivar Mt. Barker. *Applied and Environmental Microbiology*, **46**: 1207-1213.

Dye, M., Skot, L., Mytton, L.R., Harrison, S.P., Dooley, J.J. and Cresswell, A. (1995). A study of *Rhizobium leguminosarum* biovar *trifolii* populations from soil extracts using randomly amplified polymorphic DNA profiles. *Canadian Journal of Microbiology*, **41**: 336-344.

Eardly, B.D., Materon, L.A., Smith, N.H., Johnson, D.A., Rumbaugh, M.D. and Selander, R.K. (1990). Genetic structure of natural populations of the nitrogen-fixing bacterium *Rhizobium meliloti*. *Applied and Environmental Microbiology*, : 187-194.

Ehrman, T. and Cocks, P.S. (1990). Ecogeography of annual legumes in Syria: distribution patterns. *Journal of Applied Ecology*, **27**: 578-591.

Ehrman, T. and Cocks, P.S. (1996). Reproductive patterns in annual legume species on an aridity gradient. *Vegetatio*, **122**: 47-59.

Erceg, D., O'Brien, P., Bulman, T.L. and Glenn, A.R. (1994). Survival and movement of a recombinant strain of *Rhizobium leguminosarum* biovar *trifolii* in intact soil core microcosms. *Soil Biology & Biochemistry*, **26**: 1097-1098.

Evans, H.J., Hanus, F.J., Haugland, R.A., Cantrell, M.A., Xu, L.S., Russell, S.A., Lambert, G.R. and Harker, A.R. (1985). Hydrogen recycling in nodules affects nitrogen fixation and growth of soybeans. *In:* World Soybean Research Conference III, Proceeding. (Ed. R. Shibles.). pp.935-942. (Westcourt Press: Boulder, CO).

Evans, J. (1996). *N_2 fixation and the environment*. 11th Australian Nitrogen Fixation Conference, The University of Western Australia, Co-operative Research Centre for Legumes in Mediterranean Agriculture.

Evans, J., Gregory, A., Dobrowolski, N., Morris, S.G., O'Connor, G.E. and Wallace, C. (1996). Nodulation of field-grown *Pisum sativum* and *Vicia faba* - competitiveness of inoculant strains of *Rhizobium leguminosarum* bv *viciae* determined by an indirect, competitive elisa method. *Soil Biology & Biochemistry*, **28**: 247-255.

Evans, J., O'Connor, G.E., Turner, G.L., Coventry, D.R., Fettell, N., Mahoney, J., Armstrong, E.L. and Walsgott, D.N. (1989). N_2 fixation and its value to soil N increase in lupin, field pea and other legumes in South-Eastern Australia. *Australian Journal of Agricultural Research*, **40**: 791-805.

Evans, J., Wallace, C., Dobrowolski, N., Pritchard, I. and Sullivan, B. (1993). Requirement of field pea for inoculation with *Rhizobium* and lime pelleting in soils of Western Australia. *Australian Journal of Experimental Agriculture*, **33**: 767-773.

Ewing, M.A. and Howieson, J.G. (1989). *The development of Medicago polymorpha L. as an important pasture species for southern Australia.* Proceedings of the XVI International Grassland Congress, Nice, University of California, Berkeley and Davis.

Fortune, J.A., Cocks, P.S., Macfarlane, C.K. and Smith, F.P. (1995). Distribution and abundance of annual legume seeds in the wheatbelt of Western Australia. *Australian Journal of Experimental Agriculture*, **35**: 189-197.

Francis, C.M. (1997). *The need to collect new pasture and forage species.* Proceedings International Conference on genetic resources of Mediterranean pastures, York, Western Australia.

Franco, A.A. and Vincent, J.M. (1976). Competition amongst rhizobial strains for the colonization and nodulation of two tropical legumes. *Plant and Soil*, **45**: 27-48.

Frey, S.D. and Blum, L.K. (1994). Effect of pH on competition for nodule occupancy by type I and type II strains of *Rhizobium leguminosarum* bv. *phaseoli. Plant and Soil*, **163**: 157-164.

Gage, D., Bobo, T. and Long, S.R. (1996). Use of green fluorescent protein to visualize the early events of symbiosis between *Rhizobium meliloti* and alfalfa (*Medicago sativa*). *Journal of Bacteriology*, **178**: 7159-7166.

Gandee, C.M., Harrison, S.P. and Davies, W.P. (1999). Genetic characterisation of naturally occurring *Rhizobium meliloti* populations and their potential to form effective symbioses with lucerne. *Letters in Applied Microbiology*, **28**: 169-174.

Gault, R.R. and Brockwell, J. (1980). Effects of incorporation of molybdenum compounds in the seed pellet on inoculant survival, seedling nodulation and plant growth of lucerne and subterranean clover. *Australian Journal of Experimental Agriculture and Animal Husbandry*, **20**: 63-71.

Gaur, Y.D. and Lowther, W.L. (1980). Distribution, symbiotic effectiveness and fluorescent antibody reaction of naturalised populations of *Rhizobium trifolii* in Otago, New Zealand, soils. *New Zealand Journal of Agricultural Research*, **23**: 529-532.

Gee, G.W. and Bauder, J.W., Eds. (1986). *Particle-size analysis.* Methods of soil analysis. Madison, American Society of Agronomy, Inc. Soil Science Society of America, Inc.

George, M.L.C. and Robert, F. (1991). Autoregulation response of *Phaseolus vulgaris* L. to symbiotic mutants of *Rhizobium leguminosarum* biovar *phaseoli. Applied and Environmental Microbiology*, **57**: 2687-2692.

Gibson, A.H. (1968). Nodulation failure of *Trifolium subterraneum* L. cv. Woogenellup (syn. Marrar). *Australian Journal of Agricultural Research*, **19**: 907-918.

Gibson, A.H. (1980). Methods for Legumes in glasshouses and controlled environment cabinets. In: *Methods for evaluating biological nitrogen fixation.* (Bergerson, F.J., Ed.). Brisbane, Australia, John Wiley and Sons: 139-184.

Gibson, A.H. and Brockwell, J. (1968). Symbiotic characteristics of subspecies of *Trifolium subterraneum* L. *Australian Journal of Agricultural Research*, **19**: 891-905.

Gibson, A.H., Curnow, B.C., Bergersen, F.J., Brockwell, J. and Robinson, A.C. (1975). Studies of field strains of *Rhizobium trifolii* associated with *Trifolium subterraneum* L. pastures in south-eastern Australia. *Soil Biology & Biochemistry*, **7**: 95-102.

Gibson, A.H., Demezas, D.H., Gault, R.R., Bhuvaneswari, T.V. and Brockwell, J. (1991). Genetic stability in rhizobia in the field. In: *The rhizosphere and plant growth.* (Keister, D.L. and Cregan, P.B., Eds.). Kluwer Academic Publishers: 141-148.

Gierke, S. (1996). Alternative pasture legumes for the low rainfall wheat belt of South Australia. *Agronomy and Farming Systems.* Roseworthy, The University of Adelaide.

Gillespie, D.J. (1987). Murex, a new medic for acid soils. In: *Temperate pastures: their production, use and management.* (Wheeler, J.L., Pearson, C.J. and Robarbs, G.E., Eds.). Australia, CSIRO: 172-174.

Gillespie, D.J. (1989). The development of Murex medic as a new pasture species for acid soils in Australia. *Proceedings of the XVI International Grassland Congress*, Nice: France. 1989, 215-216.

Gogarten, J.P. (1995). The early evolution of cellular life (review). *Trends in Ecology and Evolution*, **10**.

Gordon, D.M., Wexler, M., Reardon, T.B. and Murphy, P.J. (1995). The genetic structure of *Rhizobium* populations. *Soil Biology & Biochemistry*, **27**: 491-499.

Graham, P.H. (1992). Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditons. *Canadian Journal of Microbiology*, **38**: 475-484.

Graham, P.H. and Parker, C.A. (1964). Diagnostic features in the characterisation of the root-nodule bacteria of legumes. *Plant and Soil*, **20**: 383-396.

Hamdi, Y.A. (1971). Soil-water tension and the movement of rhizobia. *Soil Biology & Biochemistry*, **3**: 121-126.

Hardarson, G., Golbs, M. and Danso, S.K.A. (1989). Nitrogen fixation in soybeans (*Glycine max* L. Merrill) as affected by nodulation patterns. *Soil Biology & Biochemistry*, **21**: 783-787.

Hardarson, G. and Jones, D.G. (1979). The inheritance of preference for strains of *Rhizobium trifolii* by white clover (*Trifolium repens*). *Annals of Applied Biology*, **92**: 329-333.

Harper and Gibson, A.H. (1984). Differential nodulation tolerance to nitrate among legume species. *Crop Science*, **24**: 797-801.

Harrison, S.P., Jones, D.G. and Young, J.P.W. (1989). *Rhizobium* population genetics: Genetic variation within and between populations from diverse location. *Journal of General Microbiology*, **135**: 1061-1070.

Harrison, S.P., Mytton, L.R., Skot, L., Dye, M. and Cresswell, A. (1992). Characterisation of *Rhizobium* isolates by amplification of DNA polymorphisms using random primers. *Canadian Journal of Microbiology*, **38**: 1009-1015.

Harrison, S.P., Young, J.P.W. and Jones, D.G. (1987). *Rhizobium* population genetics: Effect of clover variety and inoculum dilution on the genetic diversity sampled from natural populations. *Plant and Soil*, **103**: 147-150.

Hartmann, A., Giraud, J.J. and Catroux, G. (1998). Genotypic diversity of *Sinorhizobium* (formerly *Rhizobium*) *meliloti* strains isolated directly from a soil and from nodules of alfalfa (*Medicago sativa*) grown in the same soil. *FEMS Microbiology Ecology*, **25**: 107-116.

Hatzinger, P.B. and Alexander, M. (1994). Relationship between the number of bacteria added to soil or seeds and their abundance and distribution in the rhizosphere of alfalfa. *Plant and Soil*, **158**: 211-222.

Hebb, D.M., Richardson, A.E., Reid, R. and Brockwell, J. (1998). PCR as an ecological tool to determine the establishment and persistence of *Rhizobium* strains introduced into the field as seed inoculant. *Australian Journal of Agricultural Research*, **49**: 923-934.

Heinrich, K., Gordon, D.M., Ryder, M.H. and Murphy, P.J. (1999). A rhizopine strain of *Sinorhizobium meliloti* remains at a competitive nodulation advantage after an extended period in the soil. *Soil Biology & Biochemistry*, **31**: 1063-1065.

Helyar, K.R. (1987). Nutrition of plants on acid soils. In: *Temperate pastures: their production, use and management.* (Wheeler, J.L., Pearson, C.J. and Robarbs, G.E., Eds.). Australia, CSIRO: 501-515.

Heron, D.S. and Pueppke, S.G. (1987).Regulation of nodulation in the soybean-*Rhizobium* symbiosis. *Plant Physiology*, **84**: 1391-1396.

Herrero, M., De Lorenzo, V. and Timmis, K.N. (1990). Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *Journal of Bacteriology*, **172**: 6557-6567.

Herridge, D.F., Bergersen, F.J. and Peoples, M.B. (1990). Measurement of nitrogen fixation by soybean in the field using the ureide and natural abundance methods. *Plant Physiology*, **93**: 708-716.

Herridge, D.F. and Danso, S.K.A. (1995). Enhancing crop legume N-2 fixation through selection and breeding. *Plant and Soil*, **174**: 51-82.

Herridge, D.F. and Holland, J.F. (1992). Production of summer crops in northern New South Wales. I. Effects of tillage and double cropping on growth, grain and N yields of six crops. *Australian Journal of Agricultural Research*, **43**: 105-122.

Herridge, D.F., Marcellos, H., Felton, W.L., Turner, G.L. and Peoples, M.B. (1995). Chickpea increases soil N fertility in cereal systems through nitrate sparing and N_2 fixation. *Soil Biology & Biochemistry*, **27**: 545-551.

Hill, S., Kennedy, C., Kavanagh, E., Goldberg, R.B. and Hanau, R. (1981).Nitrogen fixation gene (*nifL*) involved in oxygen regulation of nitrogenase synthesis in *Klebsiella pneumoniae*. *Nature*, **290**.

Hinsinger, P. and Gilkes, R.J. (1995). Root-induced dissolution of phosphate rock in the rhizosphere of lupins grown in alkaline soil. *Australian Journal of Soil Research*, **33**: 477-489.

Hirsch, A.M. (1992). Developmental biology of legume nodulation. *New Phytologist*, **122**: 211-237.

Holding, A.J. and King, J. (1963). The effectiveness of indigenous populations of *Rhizobium trifolii* in relation to soil factors. *Plant and Soil*, **18**: 191-197.

Holloway, R.E. and Alston, A.M. (1992). The effects of salt and boron on growth of wheat. *Australian Journal of Agricultural Research*, **43**: 987-1001.

Howieson, J.G. (1995). Rhizobial persistence and its role in the development of sustainable agricultural systems in Mediterranean environments. *Soil Biology & Biochemistry*, **27**: 603-610.

Howieson, J.G. and Ewing, M.A. (1986). Acid tolerance in the *Rhizobium meliloti-Medicago* symbiosis. *Australian Journal of Agricultural Research*, **37**: 55-64.

Howieson, J.G. and Ewing, M.A. (1989). Annual species of *Medicago* differ in their ability to nodulate on acid soils. *Australian Journal of Agricultural Research*, **40**: 843-50.

Howieson, J.G., Ewing, M.A. and D'Antuono, M.F. (1988). Selection for acid tolerance in *Rhizobium meliloti*. *Plant and Soil*, **105**: 179-188.

Howieson, J.G., Ewing, M.A., Thorn, C.W. and Revell, C.K. (1991). Increased yield in annual species of *Medicago* grown in acidic soil in response to inoculation with acid tolerant *Rhizobium meliloti*. In: *Plant-soil interacrions at low pH*. (Wright, R.J. Ed.). Netherlands, Kluwer Academic Press: 589-595.

Howieson, J.G. and Loi, A. (1994). The distribution and preliminary evaluation of alternative pasture legumes and their associated root-nodule bacteria collected from acid soils of Greece (Serifos), Morocco, Sardinia and Corsica. *Agricola Mediterranea*, **124**: 1-17.

Howieson, J.G., Loi, A. and Carr, S.J. (1995). *Biserrula pelecinus* L.- a legume pasture species with potential for acid, duplex soils which is nodulated by unique root-nodule bacteria. *Australian Journal of Agricultural Research*, **46**: 997-1009.

Howieson, J.G., Malden, J., Yates, R.J. and O'Hara, G.W. (2000a). Techniques for the selection and development of elite inoculant strains of *Rhizobium leguminosarum* in southern Australia. *Symbiosis*, **28**: 33-48.

Howieson, J.G., O'Hara, G.W. and Carr, S.J. (2000b). Changing roles for legumes in Mediterranean agriculture: developments from an Australian perspective. *Field Crops Research*, **65**: 107-122.

Howieson J.G, O'Hara G.W and Loi A. (2000c). Legume-Rhizobia relationship.In *Legumes for Mediterranean Forage Crops, Pastures and Alternative Uses*.(L. Sulas, Editor). Cahiers Options Mediterraneennes **45**: 305-314.

Howieson, J.G., Robson, A.D. and Abbott, L.K. (1992a). Acid-tolerant species of *Medicago* produce root exudates at low pH which induce the expression of nodulation genes in *Rhizobium meliloti*. *Australian Journal of Plant Physiology*, **19**: 287-296.

Howieson, J.G., Robson, A.D. and Abbott, L.K. (1992b). Calcium modifies pH effects on the growth of acid-tolerant and acid-sensitive *Rhizobium meliloti*. *Australian Journal of Agricultural Research*, **43**: 765-772.

Howieson, J.G. and Rome, S. (1996). *Rhizobial ecology- challenges and opportunities emerging from a marriage of molecular biology and applied science.* 11th Australian Nitrogen Fixation Conference, The University of Western Australia, Co-operative Research Centre for Legumes in Mediterranean Agriculture.

Issa, S., Wood, M. and Simmonds, L.P. (1993a). Active movement of chickpea and bean rhizobia in dry soil. *Soil Biology & Biochemistry*, **7**: 951-958.

Issa, S., Wood, M. and Simmonds, L.P. (1993b). Passive movement of chickpea and bean rhizobia through soils. *Soil Biology & Biochemistry*, **7**: 959-965.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Molecular Biology Reporter*, **5**: 387-405.

Jefferson, R.A., Burgess, S.M. and Hirsh, D. (1986). β-glucuronidase from *Esherichia coli* as a gene-fusion marker. *Proceedings of the National Academy of Science, USA*, **83**: 8447-8451.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987). GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal*, **6**: 3901-3907.

Jeffery, P. and Hughes, B. (1995). Land zones of Eyre Peninsula. Adelaide, Primary Industries (South Australia).

Jensen, M.A., Webster, J.A. and Straus, N. (1993). Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Applied and Environmental Microbiology*, **59**: 945-952.

Jordan, D.C. (1984). Family III Rhizobiaceae. In: *Bergey's Manual of Systematic Bacteriology*. (Krieg, N.R. and Holt, J.G., Eds.). Baltimore, Williams and Wilkins. **1:** 234-256.

Kaminski, P.A., Batut, J. and Boistard, P. (1998). A survey of symbiotic nitrogen fixation by rhizobia. In: *The Rhizobiaceae*. (Spaink, H.P., Kondorosi, A. and Hooykaas, P.J.J., Eds.). Dordrecht, Kluwer Academic Publishers**:** 431-460.

Keay, J., Biddiscombe, E.F. and Ozanne, P.G. (1970). The comparative rates of phosphate absorption by eight annual pasture species. *Australian Journal of Agricultural Research*, **21**: 33-44.

Kellaway, R.C., Ison, R.L., Li, X. and Annison, G. (1993). *Nutritive value of alternative pasture legumes.* Proceedings of the second national alternative pasture legumes workshop, South Australia, Primary Industries.

Kemp, D.R. and Michalk, D.L. (1993). Introduction. In: *Pasture management technology for the 21st century*. (Kemp, D.R. and Michalk, D.L., Eds.). East Melbourne, Victoria, CSIRO Australia**:** ix-xi.

Kosslak, R.M. and Bohlool, B.B. (1984). Suppression of nodule development of one side of a split-root system of soybeans caused by prior inoculation of the other side. *Plant Physiology*, **75**: 125-130.

Labandera, C.A. and Vincent, J.M. (1975). Competition between an introduced strain and native Uruguayan strains of *Rhizobium trifolii*. *Plant and Soil*, **42**: 327-347.

Ladd, J.N., Butler, J.H.A. and Amato, M. (1986). Nitrogen fixation by legumes and their role as sources of nitrogen for soil and crop. *Biological Agriculture and Horticulture*, **3**: 296-286.

Lafay, B. and Burdon, J.J. (1998). Molecular diversity of rhizobia occurring on native shrubby legumes in southeastern Australia. *Applied and Environmental Microbiology*, **64**: 3989-3997.

Laguerre, G., Allard, M.R., Revoy, F. and Amarger, N. (1994). Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. *Applied and Environmental Microbiology*, **60**: 56-63.

Laguerre, G., Mavingui, P., Allard, M.R., Charnay, M.P., Louvrier, P., Mazurier, S.I., Rigottier Gois, L. and Amarger, N. (1996). Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: Application to *Rhizobium leguminosarum* and its different biovars. *Applied and Environmental Microbiology*, **62**: 2029-2036.

Lam, S.T., Ellis, D.M. and Ligon, J.M. (1990). Genetic approaches for the studying rhizosphere colonization. *Plant and Soil*, **52**: 11-18.

Lange, R.T. (1961). Nodule bacteria associated with the indigenous Leguminosae of south-western Australia. *Journal of General Microbiology*, **61**: 351-359.

Lanham, P.G. and Fennel, S. (1992). Detection of polymorphic loci in *Arachis* germplasm using random amplified polymorphic DNAs. *Genome*, **35**: 885-889.

Lawrie, A.C. (1983). Relationships among rhizobia from native Australian legumes. *Applied and Environmental Microbiology*, **45**: 1822-1828.

Ledgard, S.F. (1989). Nutrition, moisture and rhizobial strain influence isotopic fractionation during N_2 fixation in pasture legumes. *Soil Biology & Biochemistry*, **21**: 65-68.

Lee, C.K. and Reed, K.F.M. (1993). *Persian clover (Trifolium resupinatum) improvement.* Proceedings of the second national alternative pasture legumes workshop, South Australia, Primary Industries.

Leung, K., Strain, S.R., De Bruijn, F.J. and Bottomley, P.J. (1994a). Genotypic and phenotypic comparisons of chromosomal types within an indigenous soil population of *Rhizobium leguminosarum* bv. *trifolii*. *Applied and Environmental Microbiology*, **60**: 416-426.

Leung, K., Wanjage, F.N. and Bottomley, P.J. (1994b). Symbiotic characteristics of *Rhizobium leguminosarum* bv. *trifolii* isolates which represent major and minor nodule-occupying chromosomal types of field-grown subclover (*Trifolium subterraneum* L.). *Applied and Environmental Microbiology*, **60**: 427-433.

Leung, K., Yap, K., Dashti, N. and Bottomley, P.J. (1994c). Serological and ecological characteristics of a nodule-dominant serotype from an indigenous soil population of *Rhizobium leguminosarum* bv. *trifolii*. *Applied and Environmental Microbiology*, **60**: 408-415.

Li, W.L. (1994). Standardization of RAPD (randomly amplified polymorphic DNA) using Opti-Taq system. *Biosolutions News*. **1:** 1-3.

Liu, R., Tran, V.M. and Schmidt, E.L. (1989). Nodulating competitiveness of a nonmotile Tn*7* mutant of *Bradyrhizobium japonicum* in nonsterile soil. *Applied and Environmental Microbiology*, **55**: 1895-1900.

Liu, W.T., Marsh, T.L., Cheng, H. and Forney, L. (1997). Characterisation of microbial diversity by determining Terminal Restriction Fragment Lenth Polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology*, **63**: 4516-4522.

Loi, A., Howieson, J.G., Cocks, P.S. and Carr, S.J. (1999). Genetic variation in populations of two Mediterranean annual pasture legumes (*Biserrula pelecinus* L. and *Ornithopus compressus* L.) and associated rhizobia. *Australian Journal of Agricultural Research*, **50**: 303-313.

Long, S.R. (1989). *Rhizobium*-legume nodulation: life together in the underground. *Cell*, **56**: 203-214.

Long, S.R. (1996). *Rhizobium* symbiosis: Nod factors in perspective. *The Plant Cell*, **8**: 1885-1898.

Loureiro, M.F., de Faria, S.M., James, E.K. and Pott, A. (1994). Nitrogen-fixing stem nodules of the legume, *Discolobium pulchellum* Benth. *New Phytologist*, **128**: 283-295.

Lupiski, J.R. and Weinstock, G.M. (1992). Short, interspersed repetitive DNA sequences in prokaryotic genomes. *Journal Of Bacteriology*, **174**: 4525-4529.

Maier, R.J. and Triplett, E.W. (1996). Toward more productive, efficient, and competitive nitrogen-fixing symbiotic bacteria. *Critical Reviews in Plant Sciences*, **15**: 191-234.

Marques-Pinto, C.M., Yao, P.Y. and Vincent, J.M. (1974). Nodulating competitiveness amongst strains of *Rhizobium meliloti* and *R. trifolii*. *Australian Journal of Agricultural Research*, **25**: 317-329.

Martensson, A.M. (1989). Competitiveness of inoculant strains of *Rhizobium leguminosarum* bv. *trifolii* in red clover using repeated inoculation and increased inoculum levels. *Canadian Journal of Microbiology*, **36**: 136-139.

Matar, A., Torrent, J. and Ryan, J. (1992). Soil and fertilizer phosphorus and crop responses in the dryland Mediterranean zone. *Advances in Soil Science*, **18**: 81-146.

Materon, L.A. (1991). Symbiotic characteristics of *Rhizobium meliloti* in west Asian soils. *Soil Biology & Biochemistry*, **23**: 429-434.

Matheson, V.G., Munakata-Marr, J., Hopkins, G.D., McCarty, P.L., Tiedje, J.M. and Forney, L.J. (1997). A novel means to develop strain-specific DNA probes for detecting bacteria in the environment. *Applied and Environmental Microbiology*, **63**: 2863-2869.

McDermott, T.R. and Graham, P.H. (1989). *Bradyrhizobium japonicum* inoculant mobility, nodule occupancy, and acetylene reduction in the soybean root system. *Applied and Environmental Microbiology*, **55**: 2493-2498.

McDermott, T.R. and Graham, P.H. (1990). Competitive ability and efficiency in nodule formation of strains of *Bradyrhizobium japonicum*. *Applied and Environmental Microbiology*, **56**: 3035-3039.

McKay, I.A. and Djordjevic, M.A. (1993). Production and excretion of Nod metabolites by *Rhizobium leguminosarum* bv. *trifolii* are disrupted by the same environmental factors that reduce nodulation in the field. *Applied and Environmental Microbiology*, **59**: 3385-3392.

McLoughlin, T.J. and Dunican, L.K. (1985). Competition studies with *Rhizobium trifolii* in laboratory experiments. *Plant and Soil*, **88**: 139-143.

McNeil, D.L. (1982). Variations in ability of *Rhizobium japonicum* strains to nodulate soybeans and maintain fixation in the presence of nitrate. *Applied and Environmental Microbiology*, **44**: 647-652.

McNeill, A.M., Zhu, C. and Fillery, I.R.P. (1997). Use of *in situ* ^{15}N-labelling to estimate the total below-ground nitrogen of pasture legumes in intact soil-plant systems. *Australian Journal of*

Agricultural Research, **48**: 295-304.

McNeill, A.M., Zhu, C. and Fillery, I.R.P. (1998). A new approach to quantifying the N benefit from pasture legumes to succeeding wheat. *Australian Journal of Agricultural Research*, **49**: 427-436.

Meade, J., Higgins, P. and O'Gara, F. (1985). Studies on the inoculation and competitiveness of *Rhizobium leguminosarum* strains in soils containing indigenous rhizobia. *Applied and Environmental Microbiology*, **49**: 899-903.

Metcalf, W.W., Jiang, W., Daniels, L.L., Kim, S., Haldimann, A. and Wanner, B.L. (1996). Conditionally replicative and conjugative plasmids carrying lacZα for cloning, mutagenesis and allele replacement in bacteria. *Plasmid*, **35**: 1-13.

Miller, J.H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Montealegre, C., Graham, P.H. and Kipe-Nolt, J.A. (1995). Preference in the nodulation of *Phaseolus vulgaris* cultivar RAB39. *Canadian Journal of Microbiology*, **41**: 992-998.

Mullis, K.B. (1990). The unusual origin of the polymerase chain reaction. *Scientific American*, **262**: 36-43.

Murphy, P.J., Wexler, M., Grzemski, W., Rao, J.P. and Gordon, D. (1995). Rhizopines- their role in symbiosis and competition. *Soil Biology & Biochemistry*, **27**: 525-529.

Mytton, L.R. and Livesey, C.J. (1983). Specific and general effectiveness of *Rhizobium trifolii* populations from different agricultural locations. *Plant and Soil*, **73**: 299-306.

Mytton, R.L., Hughes, D.M. and Kahurananga, J. (1988). Host-*Rhizobium* relationships and their implications for legume breeding. In: *Nitrogen fixation by legumes in Mediterranean agriculture*. (Beck, D.P. and Materon, L.A., Eds.) Martinus Nijhoff, Dordrecht: 179-193.

Nelson, R.E., Ed. (1982). *Carbonate and Gypsum*. Methods of soil analysis. Madison, American Society of Agronomy, Inc. and Soil Science Society of America, Inc.

Noda, S., Ohkuma, M., Usami, R., Horikoshi, K. and Kudo, T. (1999). Culture-independent characterisation of a gene responsible for nitrogen fixation in the symbiotic microbial community in the gut of the termite. *Applied and Environmental Microbiology*, **65**: 4935-4942.

Norris, V., Chen, M., Goldberg, M., Voskull, J., McGurk, G. and Holland, I.B. (1991). Calcium in bacteria: a solution to which problem? *Molecular Microbiology*, **5**: 775-778.

Northcote, K.H. (1960). Atlas of Australian Soils. Explanatory data for sheet 1, Port Augusta-Adelaide-Hamilton. Adelaide, CSIRO.

Nutt, B.J., Carr, S.J. and Samaras, S. (1996). Collection of forage, pasture and grain legumes and their associated rhizobia from selected Greek Islands, CLIMA Occasional Publication.

O'Brian, M.R. (1996). Heme synthesis in the *Rhizobium*-legume symbiosis: a palette for bacterial and eukaryotic pigments. *Journal Of Bacteriology*, **178**: 2471-2478.

Ockwell, A. (1990). In: *Australia and the Australian Economy*. (Williams, D.B., Ed.). Sydney, Sydney University Press: 27-49.

O'Hara, G.W., Boonkerd, N. and Dilworth, M.J. (1988). Mineral constraints to nitrogen fixation. *Plant*

and Soil, **108**: 93-110.

O'Hara, G.W., Goss, T.J., Dilworth, M.J. and Glenn, A.R. (1989). Maintenance of intracellular pH and acid tolerance in *Rhizobium meliloti*. *Applied and Environmental Microbiology*, **55**: 1870-1876.

Onishchuk, O.P., Sharypova, L.A. and Simarov, B.V. (1994). Isolation and characterisation of the *Rhizobium meliloti* Tn*5*-mutants with impaired nodulation competitiveness. *Plant and Soil*, **167**: 267-274.

Oram, R.N. (1993). *The case for increasing biodiversity in the legume component of southern Australian pastures*. Proceedings of the second national alternative pasture legumes workshop, South Australia, Primary Industries.

Pan, Y.B., Burner, D.M., Ehrlich, K.C., Grisham, M.P. and Wei, Q. (1997). Analysis of primer-derived, nonspecific amplification products in RAPD-PCR. *BioTechniques*, **22**: 1071-1077.

Pankhurst, C.E. and Sprent, J.I. (1975). Effects of water stress on the respiratory and nitrogen-fixing activity of soybean root nodules. *Journal of Experimental Botany*. **26**: 287-304.

Papastylianou, I. (1989). Effect of selected soil factors on chlorosis of peanut plants grown in calcareous soils in Cyprus. *Plant and Soil*, **117**: 291-294.

Parco, S.Z., Dilworth, M.J. and Glenn, A.R. (1994). Motility and the distribution of introduced root nodule bacteria on the root system of legumes. *Soil Biology & Biochemistry*, **26**: 297-300.

Parker, C.A., Trinick, M.J. and Chatel, D.L. (1977). Rhizobia as soil and rhizosphere inhabitants. In: *A treatise on Dinitrogen Fixation IV Agronomy and Ecology*. (eds R.W.F. Hardy and A.H. Gibson, Wiley and Sons, New York).

Pate, J.S., Unkovich, M.J., Armstrong, E.L. and Sanford, P. (1994). Selection of reference plants for ^{15}N natural abundance assessment of N-2 fixation by crop and pasture legumes in south-west Australia. *Australian Journal of Agricultural Research*, **45**: 133-147.

Paul, E.A. and Clark, F.E. (1996). *Soil microbiology and biochemistry*. San Diego: Academic Press.

Payne, R.W. (1993). *Genstat 5 release 3 reference manual*. Oxford: New York: Clarendon Press; Oxford University Press.

Peoples, M.B. and Baldock, J.A. (1999). *A review of inputs of fixed nitrogen by pasture legumes*. The 12th Australian Nitrogen Fixation Conference, Wagga Wagga.

Peoples, M.B., Bergersen, F.J., Brockwell, J., Fillery, I.R.P. and Herridge, D.F. (1994). Management of nitrogen for sustainable agricultural systems. In: *Nuclear Related Methods in Soil/Plant Aspects of Sustainable Agriculture*. . Vienna, FAO/IAEA.

Peoples, M.B., Brockwell, J. and Bergersen, F.J. (1992). Factors affecting nitrogen fixation: scope for improvement? In: *Transfer of biologically fixed nitrogen to wheat.* (Angus, J.F., Ed.). Grains Research and Development Corporation: 21-32.

Peoples, M.B., Faizah, A.W., Rerkasem, B. and Herridge, D.F. (1993). *Methods for evaluating biological nitrogen fixation by nodulated legumes in the field*. Canberra: Australian Centre for International Agricultural Research.

Peoples, M.B., Herridge, D.F. and Ladha, J.K. (1995a). Biological nitrogen fixation: An efficient source of nitrogen for sustainable agricultural production? *Plant and Soil*, **174**: 3-28.

Peoples, M.B., Ladha, J.K. and Herridge, D.F. (1995b). Enhancing legume N-2 fixation through plant and soil management. *Plant and Soil*, **174**: 83-101.

Phillips, D.A., Dakora, F.D., Sande, E., Joseph, C.M. and Zon, J. (1994). Synthesis, release and transmission of alfalfa signals to rhizobial symbionts. *Plant and Soil*, **161**: 69-80.

Pierce, M. and Bauer, W.D. (1983). A rapid regulatory response governing nodulation in soybean. *Plant Physiology*, **73**: 286-290.

Postma, J., Hok, A.H.C.H. and Van Veen, J.A. (1990). Role of microniches in protecting introduced *Rhizobium leguminosarum* biovar *trifolii* against competition and predation in soil. *Applied and Environmental Microbiology*, **56**: 495-502.

Price, N.P.J., Relic, B., Talmont, F., Lewin, A., Prome, D., Pueppke, S.G., Maillet, F., Denarie, J., Prome, J.-C. and Broughton, W.J. (1992). Broad-host range *Rhizobium* species strain NGR234 secretes a family of carbomoylated, and fucosylated, nodulation signals that are O-acetylated or sulphated. *Molecular Microbiology*, **6**: 3575-3584.

Puckridge, D.W. and French, R.J. (1983). The annual legume pasture in cereal-ley farming systems of southern Australia: A Review. *Agriculture, Ecosystems and Environment*, **9**: 229-267.

Pueppke, S.G. (1996). The genetic and biochemical basis for nodulation of legumes by rhizobia. *Critical Reviews in Biotechnology*, **16**: 1-51.

Quigley, P.E., Cunningham, P.J., Hannah, M., Ward, G.N. and Morgan, T. (1997). Symbiotic effectiveness of *Rhizobium leguminosarum* bv. *trifolii* collected from pastures in south-western Victoria. *Australian Journal of Experimental Agriculture*, **37**: 623-630.

Rayment, G.E. and Higginson, F.R. (1992). *Australian laboratory handbook of soil and water chemical methods.* Melbourne, Australia: Inkata Press.

Reeve, W.G., Tiwari, R.P., Dilworth, M.J. and Glenn, A.R. (1993). Calcium affects the growth and survival of *Rhizobium meliloti*. *Soil Biology & Biochemistry*, **25**: 581 - 586.

Reeve, W.G., Tiwari, R.P., Worsley, P.S., Dilworth, M.J., Glenn, A.R. and Howieson, J.G. (1999). Constructs for insertional mutagenesis, transcriptional signal localisation and gene regulation studies in root nodule and other bacteria. *Microbiology*, **145**: 1307-1316.

Reeves, T.G. (1987). Pastures in cropping systems. In: *Temperate pastures: their production, use and management.* (Wheeler, J.L., Pearson, C.J. and Robarbs, G.E., Eds.). Australia, CSIRO: 501-515.

Reeves, T.G. and Ewing, M.A. (1993). *Is ley farming in Mediterranean zones just a passing phase?* Proceedings of the XVII International Grassland Congress, Wellington, SIR Publishing.

Richardson, A.E., Djordjevic, M.A., Rolfe, B.G. and Simpson, R.J. (1988a). Effects of pH, Ca and Al on the exudation from clover seedlings of compounds that induce the expression of nodulation genes in *Rhizobium trifolii*. *Plant and Soil*, **109**: 37-47.

Richardson, A.E., Djordjevic, M.A., Rolfe, B.G. and Simpson, R.J. (1989). Expression of nodulation genes in *Rhizobium* and acid-sensitivity of nodule formation. *Australian Journal of Plant Physiology*, **16**: 117-129.

Richardson, A.E., Henderson, A.P., James, G.S. and Simpson, R.J. (1988b). Consequences of soil acidity and the effect of lime on the nodulation of *Trifolium subterraneum* L. growing in acid soil. *Soil Biology & Biochemistry*, **20**: 439-445.

Richardson, A.E. and Simpson, R.J. (1988). Enumeration and distribution of *Rhizobium trifolii* under a subterranean clover-based pasture growing in an acid soil. *Soil Biology & Biochemistry*, **4**: 431-438.

Richardson, A.E., Viccars, L.A., Watson, J.M. and Gibson, A.H. (1995). Differentiation of *Rhizobium* strains using the polymerase chain reaction with random and directed primers. *Soil Biology & Biochemistry*, **27**: 515-524.

Richardson, A.R., Simpson, R.J., Djordjevic, M.A. and Rolfe, B.G. (1988c). Expression of nodulation genes in *Rhizobium leguminosarum* biovar *trifolii* is effected by low pH and by Ca and Al ions. *Applied and Environmental Microbiology*, **54**: 1-8.

Rifkin, P., Evans, P., Chin, J., Wright, T. and Cameron, N. (1999a). *Alternative legumes for pasture-crop rotations*. The 12th Australian Nitrogen Fixation Conference, Wagga Wagga.

Rifkin, P., Quigley, P., Cameron, F., Peoples, M. and Thies, J. (1999b). Annual nitrogen fixation in grazed dairy pastures in south-western Victoria. *Australian Journal of Agricultural Research*, **50**: 273-281.

Robinson, A.C. (1967). The influence of host on soil and rhizosphere populations of clover and lucerne root nodule bacteria. *Journal of the Australian Institute of Agricultural Science*, **33**: 207-209.

Robinson, A.C. (1969). Competition between effective and ineffective strains of *Rhizobium trifolii* in the nodulation of *Trifolium subterraneum*. *Australian Journal of Agricultural Research*, **20**: 827-841.

Robson, A.D. (1969). Soil factors affecting the distribution of annual *Medicago* species. *The Journal of the Australian Institute of Agricultural Science*, **35**: 154-167.

Robson, A.D. and Loneragan, J.F. (1970). Nodulation and growth of *Medicago truncatula* on acid soils. II-Colonization of acid soils by *Rhizobium meliloti*. *Australian Journal of Agricultural Research*, **21**: 435-445.

Robson, A.D., O'Hara, G.W. and Abbott, L.K. (1981). Involvement of phosphorus in nitrogen fixation by subterranean clover (*Trifolium subterraneum* L.). *Australian Journal of Plant Physiology*, **8**: 427-436.

Robson, R.O.D. and Postgate, J.R. (1980). Oxygen and hydrogen in biological nitrogen fixation. *Annual Review of Microbiology*, **34**: 183-207.

Rolfe, B.G. and Richardson, A.E. (1987). *The genetic organization of nitrogen-fixing Rhizobium species: past and future applications to agriculture*. Proceedings of the 4th Australian Agronomy Conference, La Trobe University.

Rosendahl, L., Glenn, A.R. and Dilworth, M.J. (1991). Organic and inorganic inputs into legume root nodule nitrogen fixation. In: *Biology and Biochemistry of Nitrogen Fixation*. (Dilworth, M.J. and Glenn, A.R., Eds.). Amsterdam, Elsevier: 259-292.

Rossiter, R.C. (1966). Ecology of the Mediterranean annual-type pasture. *Advances in Agronomy*, **18**: 1-56.

Roughley, R.J., Blowes, W.M. and Herridge, D.F. (1976). Nodulation of *Trifolium subterraneum* by introduced rhizobia in competition with naturalised strains. *Soil Biology & Biochemistry*, **8**: 403-407.

Rovira, A.D. (1978). Microbiology of pasture soils and some effects of microorganisms on pasture plants. In: *Plant relations in Pastures.* (Wilson, J.R., Ed.). East Melbourne, Australia, CSIRO: 95-110.

Rowell, D.L. (1988). Soil acidity and alkalinity. In: *Russell's soil conditions and plant growth.* (Wild, A., Ed.). Avon, Bath Press: 844-898.

Rowell, D.L. (1994). *Soil science: methods and applications.* Essex: Longman scientific and technical.

Rys, G.J. and Bonish, P.M. (1981). Effectiveness of *Rhizobium trifolii* populations associated with *Trifolium* species in Taranaki, New Zealand. *New Zealand Journal of Experimental Agriculture*, **9**: 3-4.

Sadowsky, M.J. and Graham, P.H. (1998). Soil Biology of the Rhizobiaceae. In: *The Rhizobiaceae.* (Spaink, H.P., Kondorosi, A. and Hooykaas, P.J.J., Eds.). Dordrecht, Kluwer Academic Publishers: 155-172.

Sambrook, J., Fritsch, E.J. and Maniatis, T. (1989). *Molecular cloning, a laboratory manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanford, P., Pate, J.S. and Unkovich, M.J. (1993). A survey of proportional dependence of subterranean clover and other pasture legumes on N_2 fixation in south-west Australia utilizing ^{15}N natural abundance. *Australian Journal of Agricultural Research*, **45**: 165-181.

Sanginga, N., Danso, S.K.A., Mulongoy, K. and Ojeifo, A.A. (1994). Persistence and recovery of introduced *Rhizobium* ten years after inoculation on *Leucaena leucocephala* grown on an Alfisol in southwestern Nigeria. *Plant and Soil*, **159**: 199-204.

Sanjuan, J. and Olivares, J. (1989). Implication of *nifA* in regulation of genes located on a *Rhizobium meliloti* cryptic plasmid that affect ndoulation efficiency. *Journal of Bacteriology*, **171**: 4154-4161.

Sargent, L., Huang, S.Z., Rolfe, B.G. and Djordjevic, M.A. (1987). Split-root assays using *Trifolium subterraneum* show that *Rhizobium* infection induces a systemic response that can inhibit nodulation of another invasive *Rhizobium* strain. *Applied and Environmental Microbiology*, **53**: 1611-1619.

Schneider, M. and de Bruijn, F.J. (1996). Rep-Pcr Mediated Genomic Fingerprinting Of Rhizobia and Computer-Assisted Phylogenetic Pattern Analysis. *World Journal of Microbiology and Biotechnology*, **12**: 163-174.

Schofield, P.R., Gibson, A.H., Dudman, W.F. and Watson, J.M. (1987). Evidence for genetic exchange and recombination of *Rhizobium* symbiotic plasmids in a soil population. *Applied and Environmental Microbiology*, **53**: 2942-2947.

Schofield, P.R. and Watson, J.M. (1985). Conservation of *nif-* and species-specific domains within repeated promoter sequences from fast- growing *Rhizobium* species. *Nucleic Acids Research*, **13**: 3407-3418.

Segovia, L., Pinero, D., Palacios, R. and Martinez-Romero, E. (1991). Genetic structure of a soil population of nonsymbiotic *Rhizobium leguminosarum. Applied and Environmental Microbiology*, **57**, 426-433.

Selander, R.K., Caugant, D.A., Ochman, H., Mussler, J.M., Gilmour, M.N. and Whittam, T.S. (1986). Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Applied and Environmental Microbiology*, **51**: 873-884.

Sessitsch, A., Hardarson, G., de Vos, W.M. and Wilson, K.J. (1998). Use of marker genes in competition studies of *Rhizobium*. *Plant and Soil*, **204**: 35-45.

Sessitsch, A., Jjemba, P.K., Hardarson, G., Akkermans, A.D.L. and Wilson, K.J. (1997). Measurement of the competitiveness index of *Rhizobium tropici* strain CIAT899 derivatives marked with the *gusA* gene. *Soil Biology & Biochemistry*, **29**: 1099-1110.

Sessitsch, A., Wilson, K.J., Akkermans, A.D.L. and De Vos, W.M. (1996). Simultaneous detection of different *Rhizobium* strains marked with either the *Eshcherichia coli gusA* gene or the *Pyrococcus furiosus celB* gene. *Applied and Environmental Microbiology*, **62**: 4191-4194.

Sharma, P.K., Anand, R.C. and Lakshminarayana, K. (1991). Construction of Tn5 tagged mutants of *Rhizobium* spp. (Cicer) for ecological studies. *Soil Biology & Biochemistry*, **23**: 881-885.

Shishido, M. and Pepper, I.L. (1990). Identification of dominant indigenous *Rhizobium meliloti* by plasmid profiles and intrinsic antibiotic resistance. *Soil Biology & Biochemistry*, **22**: 11-16.

Singleton, P.W. and Tavares, J.W. (1986). Inoculation response of legumes in relation to the number and effectiveness of indigenous *Rhizobium* populations. *Applied and Environmental Microbiology*, **51**: 1013-1018.

Sivakumaran, S., Lockhart, P.J. and Jarvis, B.D.W. (1997). Identification of soil bacteria expressing a symbiotic plasmid from *Rhizobium leguminosarum* bv. *trifolii*. *Canadian Journal of Microbiology*, **43**: 164-177.

Slattery, J.F. and Coventry, D.R. (1995). Acid-tolerance and symbiotic effectiveness of *Rhizobium leguminosarum* bv. *trifolii* isolated from subterranean clover growing in permanent pastures. *Soil Biology & Biochemistry*, **27**: 111-115.

Slattery, J.F. and Coventry, D.R. (1999). Persistence of introduced *Rhizobium leguminosarum* bv. *trifolii* in acidic soil of north-eastern Victoria. *Australian Journal of Experimental Agriculture*, **39**: 829-837.

Smart, J.B., Robson, A.D. and Dilworth, M.J. (1984). A continuous culture study of the phosphorus nutrition of *Rhizobium trifolii* WU95, *Rhizobium* NGR234 and *Bradyrhizobium* CB756. *Archives Of Microbiology*, **140**: 276-280.

Smith, S.E. and Read, D.J. (1997). *Mycorrhizal symbiosis*. London: Academic Press.

Soberon-Chavez, G. and Najera, R. (1988). Isolation from soil of *Rhizobium leguminosarum* lacking symbiotic information. *Canadian Journal of Microbiology*, **35**: 464-468.

Soby, S. and Bergman, K. (1983). Motility and chemotaxis of *Rhizobium meliloti* in soil. *Applied and Environmental Microbiology*, **46**: 995-998.

Stephens, P.M. and Cooper, J.E. (1988). Variation in speed of infection of "no root hair zone" of white clover and nodulating competitiveness among strains of *Rhizobium trifolii*. *Soil Biology & Biochemistry*, **20**: 465-470.

Strain, S.R., Leung, K., Whittam, T.S., de Bruijn, F.J. and Bottomley, P.J. (1994). Genetic structure of *Rhizobium leguminosarum* biovar *trifolii* and *viciae* populations found in two Oregon soils under different plant communities. *Applied and Environmental Microbiology*, **60**: 2772-2778.

Streeter, J.G. (1985). Nitrate inhibition of legume nodule growth and activity: 1. Long term studies with a continuous supply of nitrate. *Plant Physiology*, **77**: 321-324.

Streeter, J.G. (1988). Inhibition of legume nodule formation and N_2 fixation by nitrate. *CRC Critical Reviews in Plant Sciences*, **7**: 1-23.

Streeter, J.G. (1994). Failure of inoculant rhizobia to overcome the dominance of indigenous strains for nodule formation. *Canadian Journal of Microbiology*, **40**: 513-522.

Streit, W., Botero, L., Werner, D. and Beck, D. (1995). Competition for nodule occupancy on *Phaseolus vulgaris* by *Rhizobium etli* and *Rhizobium tropici* strains can be efficiently monitored in an ultisol during the early stages of growth using a constitutive GUS gene fusion. *Soil Biology & Biochemistry*, **27**: 1075-1081.

Streit, W., Kosch, K. and Werner, D. (1992). Nodulation competitiveness of *Rhizobium leguminosarum* bv. *phaseoli* and *Rhizobium tropici* strains measured by glucuronidase (*gus*) gene fusion. *Biology and Fertility Of Soils*, **14**: 140-144.

Sullivan, J.F., Patrick, H.N., Lowther, W.L., Scott, D.P. and Ronson, C.W. (1995). Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. *Proceedings of the National Academy of Science, USA*, **92**: 8985-8989.

Sweeney, G.C., Spencer, M.T. and Bellotti, W. (1993). *Annual pasture legumes for low rainfall, neutral-acid, red-brown earths of South Australia.* Proceedings of the second national alternative pasture legumes workshop, South Australia, Primary Industries.

Tang, C., Longnecker, N.E., Greenway, H. and Robson, A.D. (1996). Reduced root elongation of *Lupinus angustifolius* L. by high pH is not due to decreased membrane integrity of cortical cells or low proton production by the roots. *Annals in Botany*, **78**: 409-414.

Tang, C. and Robson, A.D. (1993). pH above 6.0 reduces nodulation in *Lupinus* species. *Plant and Soil*, **152**: 269-276.

Tang, C., Robson, A.D. and Adams, H. (1995). High Ca is not the primary factor in poor growth of *Lupinus angustifolius* L. in high pH soil. *Australian Journal of Agricultural Research*, **46**: 1051-1062.

Tang, C., Robson, A.D. and Dilworth, M.J. (1992). The role of iron in the (*Brady*)*rhizobium* legume symbiosis. *Journal of Plant Nutrition*, **15**: 2235-2252.

Thies, J.E., Bohlool, B.B. and Singleton, P.W. (1992). Environmental effects on competition for nodule occupancy between introduced and indigenous rhizobia and among introduced strains. *Canadian Journal of Microbiology*, **38**: 493-500.

Thies, J.E., Cook, S.E. and Corner, R.J. (1994). *Use of Bayesian inference in a geographical information system to determine regional legume inoculaton requirements.* Resource Technology '94, Melbourne, University of Melbourne.

Thies, J.E., Holmes, E.M. and Vachot, A.M. (2000). Application of molecular techniques to studies in *Rhizobium* ecology. *Australian Journal of Experimental Agriculture*, in press.

Thies, J.E., Singleton, P.W. and Bohlool, B.B. (1991a). Influence of the size of indigenous rhizobial population on establishment and symbiotic performance of introduced rhizobia on field-grown legumes. *Applied and Environmental Microbiology*, **57**: 19-28.

Thies, J.E., Singleton, P.W. and Bohlool, B.B. (1991b). Modeling symbiotic performance of introduced rhizobia in the field by use of indices of indigenous population size and nitrogen status of the soil. *Applied and Environmental Microbiology*, **57**: 29-37.

Thies, J.E., Woomer, P.L. and Singleton, P.W. (1995). Enrichment of *Bradyrhizobium* spp. populations in soil due to cropping of the homologous host legume. *Soil Biology & Biochemistry*, **27**: 633-636.

Thornton, F.C. and Davey, C.B. (1984). Saprophytic competence of acid tolerant strains of *Rhizobium trifolii* in acid soil. *Plant and Soil*, **80**: 337-344.

Toro, N. (1996). Nodulation competitiveness In the *Rhizobium* Legume Symbiosis. *World Journal of Microbiology and Biotechnology*, **12**: 157-162.

Tothill, J.C., Hargreaves, J.N.G., Jones, R.M. and McDonald, C.K. (1992). Botanal - A comprehensive sampling and computing procedure for estimating pasture yield and composition. St. Lucia, Brisbane, Queensland, Australia, CSIRO Division of Tropical Crops and Pastures: 1-24.

Triplett, E.W. (1990a). Construction of a symbiotically effective strain of *Rhizobium leguminosarum* biovar *trifolii* with increased nodulation competitiveness. *Applied and Environmental Microbiology*, **56**: 98-103.

Triplett, E.W. (1990b). The molecular genetics of nodulation competitiveness in *Rhizobium* and *Bradyrhizobium*. *Molecular Plant-Microbe Interactions*, **3**: 199-206.

Triplett, E.W. (1999). Antibiosis as a means to enhance nodulation competitiveness by *Rhizobium* inoculum strains under agricultural conditions. In: *Highlights of Nitrogen Fixation Research*. (Martinez and Hernandez, Eds.). New York, Kluwer Academic Publishers.

Triplett, E.W. and Sadowsky, M.J. (1992). Genetics of competition for nodulation of legumes. *Annual Review of Microbiology*, **46**: 399-428.

Troeh, F.R. and Thompson, L.M. (1993). *Soils and Soil Fertility*. New York: Oxford University Press.

Turner, G.L. and Bergersen, F.J. (1983). Natural abundance of ^{15}N in root nodules of soybean, lupin, subterranean clover and lucerne. *Soil Biology & Biochemistry*, **15**: 525-530.

Tyler, G. (1992). Inability to solubilize phosphate in limestone soils - Key factor controlling calcifuge habit of plants. *Plant and Soil*, **145**: 65-70.

Unkovich, M.J. and Pate, J.S. (1998). Symbiotic effectiveness and tolerance to early season nitrate in indigenous populations of subterranean clover rhizobia from S.W. Australian pastures. *Soil Biology & Biochemistry*, **30**: 1435-1443.

Unkovich, M.J. and Pate, J.S. (2000). An appraisal of recent field measurements of symbiotic N_2 fixation by annual legumes. *Field Crops Research*, **65**: 211-228.

Unkovich, M.J., Pate, J.S. and Sanford, P. (1997). Nitrogen fixation by annual legumes in Australian Mediterranean agriculture. *Australian Journal of Agricultural Research*, **48**: 267-293.

Unkovich, M.J., Pate, J.S., Sanford, P. and Armstrong, E.L. (1994). Potential precision of the $\delta^{15}N$ natural abundance method in field estimates of nitrogen fixation by crop and pasture legumes in south-west Australia. *Australian Journal of Agricultural Research*, **45**: 119-132.

van Berkum, P., Beyene, D. and Eardly, B.D. (1996). Phylogenetic relationships among *Rhizobium* species nodulating the common bean (*Phaseolus vulgaris* L). *International Journal of Systematic Bacteriology*, **46**: 240-244.

van Berkum, P. and Eardly, B.D. (1998). Molecular evolutionary systematics of the Rhizobiaceae. In: *The Rhizobiaceae*. (Spaink, H.P., Kondorosi, A. and Hooykaas, P.J.J., Eds.). Dordrecht, Kluwer Academic Publishers: 1-24.

Vance, C.P. (1997). Nitrogen Fixation Capacity. In: *Biotechnology and the improvement of forage legumes*. (McKersie, B.D. and Brown, D.C.W., Eds.). Cambridge, University Press: 357-407.

Versalovic, J., Schneider, M., DeBruijn, F. and Lupiski, J.R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cellular biology*, **5**: 25-40.

Vincent, J.M. (1988). Ecological aspects of the root-nodule bacteria: competition and survival. In: *Nitrogen fixation by legumes in Mediterranean agriculture*. (Beck, D.P. and Materon, L.A., Eds.). Netherlands, International Centre for Agricultural Research in Dry Areas: 165-171.

Vlassak, K.M. and Vanderleyden, J. (1997). Factors influencing nodule occupancy by inoculant rhizobia. *Critical Reviews in Plant Sciences*, **16**: 163-229.

Wang, S.P. and Stacey, G. (1990). Ammonia regulation of nod genes in *Bradyrhizobium japonicum*. *Molecular and General Genetics*, **223**: 329-331.

Watkin, E.L.J., O'Hara, G.W. and Glenn, A.R. (1997). Calcium and acid stress interact to affect the growth of *Rhizobium leguminosarum* bv. *trifolii*. *Soil Biology & Biochemistry*, **29**: 1427-1432.

Watkin, E.L.J., O'Hara, G.W., Howieson, J.G. and Glenn, A.R. (in press). Identification of tolerance to soil acidity in inoculant strains of *Rhizobium leguminosarum* bv. *trifolii*. *Soil Biology & Biochemistry*.

Watson, J.M. and Schofield, P.R. (1985). Species-specific, symbiotic plasmid-located repeated DNA sequences in *Rhizobium trifolii*. *Molecular and General Genetics*, **199**: 279-289.

Weaver, R.W. and Frederick, L.R. (1974a). Effect of inoculum rate on competitive nodulation of *Glycine max* L. Merrill. I. Glasshouse studies. *Agronomy Journal*, **66**: 229-232.

Weaver, R.W. and Frederick, L.R. (1974b). Effect of inoculum rate on competitive nodulation of *Glycine max* L. Merrill. II. Field studies. *Agronomy Journal*, **66**: 233-236.

Welsh, J. and McClelland, M. (1991). Genomic fingerprints produced by PCR with consensus tRNA gene primers. *Nucleic Acids Research*, **19**: 861-866.

Werner, D. (1992). *Symbiosis of plants and microbes*. London: Chapman and Hall.

Wetherby, K.G. and Oades, J.M. (1975). Classification of carbonate layers in highland soils of the northern Murray Mallee, South Australia, and their use in stratigraphic and land-use studies. *Australian Journal of Soil Research*, **13**: 119-132.

White, P.F. and Robson, A.D. (1990). Response of lupins (*Lupinus angustifolius*) and peas (*Pisum sativum*) to Fe deficiency induced by low concentrations of Fe in solution or by addition of HCO_3^-. *Plant and Soil*, **125**: 39-47.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, **18**: 6531-6535.

Wilson, K.J. (1995). Molecular techniques for the study of rhizobial ecology in the field. *Soil Biology*

& Biochemistry, **27**: 501-514.

Wilson, K.J., Parra, A. and Botero, L. (1999). Application of the GUS marker gene technique to high-throughput screening of rhizobial competition. *Canadian Journal of Microbiology*, **45**: 678-685.

Wilson, K.J., Peoples, M.B. and Jefferson, R.A. (1995a). New techniques for studying competition by rhizobia and for assessing nitrogen fixation in the field. *Plant and Soil*, **174**: 241-253.

Wilson, K.J., Sessitsch, A. and Akkermans, A. (1994). Molecular markers as tools to study the ecology of microorganisms. In: *Beyond the Biomass*. (Ritz, K., Dighton, J. and Giller, K.E., Eds.). London, Wiley-Sayce Publications: 149-156.

Wilson, K.J., Sessitsch, A., Corbo, J.C., Giller, K.E., Akkermans, A.D. and Jefferson, R.A. (1995b). β-glucuronidase (GUS) transposons for ecological and genetic studies of rhizobia and other Gram-negative bacteria. *Microbiology*, **141**: 1691-1705.

Woomer, P., Singleton, P.W. and Bohlool, B.B. (1988). Ecological indicators of native rhizobia in tropical soils. *Applied and Environmental Microbiology*, **54**: 1112-1116.

Worrall, V.S. and Roughley, R.J. (1976). The effect of moisture stress on infection of *Trifolium subterraneum* L. by *Rhizobium trifolii* Dang. *Journal of Experimental Botany*, **27**: 1233-1241.

Young, R.R. and Brockwell, J. (1992). Influence of soil pH on the development of symbiosis in field-grown acid-sensitive and acid-tolerant annual medics. *Australian Journal of Experimental Agriculture*, **32**: 167-173.

Zdor, R.E. and Pueppke, S.G. (1991). Nodulation competitiveness of Tn5-induced mutants of *Rhizobium fredii* USDA208 that are altered in motility and extracellular polysaccharide production. *Canadian Journal of Microbiology*, **37**: 52-58.

Appendix A

Raw Data from the Survey (Chapter 3).

Site	Location	Log (MPN+1)	Log (MPN+1	CaCO3	Clay	P	K	Org. C	EC	pH	pH	Cation EC	Total Soil N	Rainfall
		Persian	Sub clover	%	%	mg/kg	mg/kg	%	dS/m	(H_2O)	($CaCl_2$)		%	(mm)
1	WEP	1.61	2.18	5.47	32	37	695	1.24	0.113	8.6	7.6	n.d.	0.1	329
2	WEP	3.17	3.96	49.02	13	20	386	1.19	0.109	8.6	7.8	18.11	0.08	324
3	WEP	2.03	1.96	24.28	26	8	613	1.71	0.126	8.5	7.8	23.88	0.12	330
4	WEP	3.22	2.62	19.95	32	16	478	1.35	0.131	8.6	7.8	21.33	0.09	340
5	WEP	2.96	2.16	1.82	32	11	417	1.65	0.122	8.3	7.7	19.81	0.09	350
6	WEP	3.96	3.36	0.00	29	17	684	1.72	0.195	7.9	7.3	26.94	0.09	431
7	WEP	2.17	1.61	11.86	30	24	679	2.26	0.174	8.4	7.7	30.19	0.11	350
8	WEP	1.63	1.61	59.62	31	27	430	2.49	0.141	8.5	7.8	25.51	0.12	350
9	WEP	2.77	2.83	62.93	29	30	400	1.84	0.144	8.5	7.7	22.75	0.17	355
10	WEP	1.83	1.63	62.70	31	26	464	2.51	0.148	8.5	7.7	25.43	0.2	370
11	WEP	0.00	0.00	62.93	24	44	734	2.28	0.171	8.4	7.6	26.63	0.19	404
12	WEP	0.00	0.00	77.52	27	48	278	1.87	0.176	8.4	7.7	19.92	0.13	415
13	WEP	1.61	0.00	77.98	26	31	162	1.82	0.15	8.5	7.7	18.47	0.13	430
14	WEP	4.63	4.63	22.12	27	12	635	3.4	0.196	8.3	7.7	37	0.3	430
15	WEP	4.36	4.63	1.60	23	3	806	2.5	0.16	8.3	7.6	39.46	0.19	430
16	WEP	3.96	4.63	7.75	28	8	351	2.09	0.137	8.4	7.7	23.15	0.18	434
17	WEP	1.61	2.38	0.00	17	13	138	0.56	0.046	7.6	7	4.93	0.02	320
18	EEP	1.83	3.36	0.00	24	14	176	0.67	0.044	7.6	7	4.59	0.04	325
19	EEP	3.63	2.57	0.00	21	19	595	1.64	0.117	8.5	7.8	22.02	0.1	327
20	EEP	0.00	1.83	4.79	21	18	441	1.44	0.127	8.6	7.9	21.42	0.09	327
21	EEP	3.17	3.63	0.00	19	14	337	0.79	0.055	7.5	6.8	8.5	0.06	330
22	EEP	2.36	1.61	2.28	19	6	220	1.37	0.094	8.6	7.8	15.72	0.06	335
23	EEP	2.18	2.62	1.60	19	15	553	1.56	0.108	8.5	7.9	20.93	0.09	340
23.5	EEP	0.00	0.00	0.00	22	0.5	164	0.73	0.086	8.5	7.8	11.66	0.01	340
24	EEP	3.83	3.36	0.00	22	13	60	0.36	0.029	6.9	6.4	2.49	0.01	350
25	EEP	3.63	4.17	0.00	25	55	885	1.51	0.136	8.3	7.5	38.32	0.14	387
26	EEP	2.18	3.36	0.00	12	8	95	0.97	0.035	7	6.4	4.36	0.05	350
27	EEP	4.17	3.63	0.00	19	34	235	0.72	0.073	8.5	7.7	10.11	0.05	343
28	EEP	4.04	4.36	0.00	23	24	696	2.19	0.144	8	7.4	27.22	0.13	414

Continued over page.

Base Data from the Survey (Chapter 3) continued.

Sit	Location	Log (MPN+1)	Log (MPN+1	CaCO3	Clay	P	K	Org. C	EC	pH	pH	Cation EC	Total Soil N	Rainfall
		Persian	Sub clover	%	%	mg/kg	mg/kg	%	dS/m	(H_2O)	($CaCl_2$)		%	(mm)
29	EEP	3.17	3.63	8.30	22	20	543	2.01	0.14	8.3	7.7	31.97	0.1	405
30	EEP	3.96	3.96	0.00	19	15	268	1.04	0.068	7.6	7	9.87	0.04	395
31	EEP	4.17	4.36	0.00	20	23	525	1.19	0.153	8.3	7.5	16.27	0.06	385
32	EEP	3.36	3.17	0.00	19	17	209	0.62	0.044	7.3	6.7	5.18	0.04	350
33	EEP	3.36	3.96	0.00	19	40	349	0.89	0.055	7.5	6.8	9.84	0.05	360
34	EEP	4.63	4.63	0.00	18	21	62	0.76	0.039	7.2	6.7	4.87	0.03	370
41	YP	3.96	3.63	3.29	19	28	465	2.13	0.131	8.3	7.7	27.22	0.15	385
42	YP	2.57	3.36	0.00	19	13	673	1.59	0.181	8.2	7.6	25.07	0.07	387
43	YP	1.96	3.03	1.10	13	14	642	1.34	0.173	8.9	8	23.34	0.07	391
44	YP	2.96	2.96	2.93	20	21	479	1.49	0.116	8.4	7.8	28.74	0.07	376
45	YP	2.96	2.62	10.61	16	17	549	1.93	0.145	8.3	7.7	33.59	0.16	391
46	YP	2.01	1.97	18.06	18	38	513	1.66	0.138	8.4	7.8	26.68	0.11	375
47	YP	1.96	1.96	14.52	19	26	493	1.29	0.118	8.6	7.9	22.2	0.05	380
48	YP	0.00	3.63	0.00	14	32	533	1.5	0.129	8.5	7.8	25.97	0.08	396
49	YP	2.36	1.96	5.61	14	34	567	1.62	0.141	8.5	7.8	24.29	0.16	396
50	YP	3.83	2.96	0.24	13	28	319	1.21	0.108	8.3	7.7	15.97	0.12	374
51	AP	3.17	3.36	0.00	16	26	655	1.44	0.124	8	7.4	22.57	0.12	420
52	AP	2.16	2.18	0.00	14	27	674	1.91	0.206	8.1	7.6	32.88	0.22	420
53	AP	2.96	3.63	0.00	14	20	561	1.24	0.142	8.3	7.7	24.23	0.13	420
54	AP	0.00	2.56	16.10	12	31	724	1.89	0.178	8.5	7.8	29.54	0.2	400
55	AP	2.56	2.62	12.93	14	25	714	1.73	0.154	8.5	7.6	27.52	0.19	380
56	AP	2.03	2.56	10.49	13	36	732	1.89	0.138	8.5	7.8	26.26	0.2	370
57	AP	1.83	3.63	8.66	9	30	493	1.3	0.117	8.6	7.9	20.87	0.13	360
58	AP	2.18	3.36	1.71	11	37	512	1.13	0.103	8.7	7.9	15.66	0.11	360
59	AP	2.18	2.96	3.90	10	24	1279	1.41	0.122	8.7	7.9	21.94	0.17	355
60	AP	1.61	2.36	8.54	9	32	824	1.36	0.136	8.7	8	20.83	0.15	345
61	RW	5.63	3.36	0.00	6	57	529	0.84	0.056	6.2	5.2	6.79	0.11	437
62	RW	3.17	3.77	0.00	4	48	824	1.32	0.135	7.5	6.8	18.36	0.18	437
63	RW	1.61	2.62	0.00	5	25	672	1.5	0.147	8.2	7.6	27.89	0.19	437
64	RW	0.00	0.00	4.64	5	204	757	1.37	0.167	8.3	7.7	23.31	0.19	437
65	RW	1.96	2.96	3.42	4	38	331	1.42	0.112	8.4	7.8	21.96	0.15	437
66	RW	1.61	1.61	9.15	2	35	294	1.9	0.18	8.3	7.8	29.35	0.15	437

Appendix B

Media and Reagents

JMM Media (O'Hara *et al.* 1989)

	Amount/L
D-(+)-galactose	1.8 g
L-(+)-arabinose	1.5 g
L-glutamate (monosodium salt)	0.507 g
K_2HPO_4	26.1mg
KH_2PO_4	20.4mg
$FeSO_4.7H_2O$	5.5mg
$CaCl_2.2H_2O$	147mg
Thiamine hydrochloride	1.0mg
Pantothenic acid	1.0mg
Biotin	20μg
$Na_2MoO_4.2H_2O$	0.967mg
Na_2SO_4	100mg
$MgSO_4.7H_2O$	246.4mg
$MnSO_4.4H_2O$	1.114mg
$ZnSO_4.7H_2O$	1.08mg
$CuSO_4.5H_2O$	0.5mg

LB media (Miller 1972)

Tryptone	10 g/L
Yeast Extract	5 g/L
NaCl	5 g/L

pH is adjusted to 7.2, and autoclaved.

McKnights nutrient solution (Gibson 1980)

Nutrient	mg/L
$FeCl_3$	140
KH_2PO_4	200
$CaSO_4.2H_2O$	1500
$MgSO_4.7H_2O$	200
KCl	300
Trace Elements	1mL

Trace Elements: 2.86g H_3BO_3, 2.08 g $MnSO_4.7H_2O$, 0.22 g $ZnSO_4.7H_2O$, .079 g $CuSO_4.5H_2O$, 0.09 g $H_2MoO_4.H_2O$ / L.

Medium A (Bromfield *et al.* 1994)

Yeast Extract	100mg/L
Tryptone	400mg/L
$CaCl_2.H_2O$	100mg/L
cycloheximide	150mg/L
Congo-red	25mg/L
agar	10 g/L

Cycloheximide (filter sterilized) was added after autoclaving when solution had cooled to 50°C.

TY media (Beringer 1974)

Tryptone	5 g/L
Yeast Extract	3 g/L
$CaCl_2.2H_2O$	0.87 g/L

The pH is adjusted to 6.8 and autoclaved for 20 min at 121°C. TY agar: 15 g/L agar is added to TY broth prior to autoclaving.

YMA (Gibson 1980)

Nutrient	amount (g/L)
Yeast	0.5
Mannitol	5.0
L-glutamic acid Na salt	0.5
$Na_2HPO_4.12H_2O$	0.36
$MgSO_4.7H_2O$	0.1
$CaCl_2$	0.04
$FeCl_3$	0.004
Agar	16

pH is set to 6.8 and the solution is autoclaved for 20 min. at 121°C.

YMB (broth): no addition of agar.

Appendix C

PCR Reagents

PCR Reaction Cocktail for primer RP01 (per reaction)

Solution	Volume (μL)
SDDW	4.3
$MgCl_2$ (25 mM)	0.6
Primer (RPO1, 40ng/μL)	2.0
5 x buffer (see recipe below)	2.0
Taq polymerase (Boehringer Mannheim®)	0.1
Template	1.0

5 × RAPD buffer

Component	Final concentration	Volume
1.0 M Tris-HCl pH 8.8	335 mM	2.01mL
1.0 M $(NH_4)_2SO_4$	83 mM	498 μL
20 mM ATP*	1 mM	300 μL
20 mM TTP		300 μL
20 mM CTP		300 μL
20 mM GTP		300 μL
Triton X-100	2.25%	135 μL
SDDW		2.16mL
Total		6.003mL

*dNTPs sourced from Perkin Elmer®

Loading Buffer

glycerol	30mL
bromophenol blue	0.25g
Make up to 100mL with TE buffer	

Protocol for ERIC primer (for 8 reactions)

Solution	Volume (μL)
Primer ERIC1R	2.5
Primer ERIC2	2.5
10 mM dNTP mix (1.25 mM)	6
10 × REP buffer	12.5
Dimethyl Sulphoxide	12.5
SDDW	62.5
Taq polymerase	2
DNA template added to each reaction	2.5

10 × REP Buffer

Solution	Volume
1.66 M $(NH_4)_2SO_4$	1mL
1.34 M Tris-HCl pH 8.8	5mL
1 M$MgCl_2$	0.6mL
0.5 M EDTA pH 8.0	1.3 μL
BSA (bovine serum albumen)	17mg
SDDW	3.3mL

store at room temperature and use within 2 weeks. Just prior to use, add 7 μL mercaptoethanol/mL.

Appendix D

Staining root nodules for GUS activity (Wilson *et al.* 1995b)

GUS staining buffer

50mM $NaPO_4$ (250mL of 0.1M Na_2HPO_4 + 250mL of 0.1M NaH_2PO_4 /L, pH = 7)

0.1% Sarkosyl (10mL/L of 10% stock)

0.1% Triton X-100 (10mL/L of 10% stock)

1mM EDTA (2mL/L of a 0.5M stock at pH 8.0)

300 µg/mL XGlcA (300mg/L) can be reduced to 100µg/mL if longer staining time allowed)

100µg/mL chloramphenicol (dissolve 100mg in 1mL of methanol and add to 1 L)

Stain roots overnight (up to 72 hours) at 37°C (using 300µg/mL XGlcA). Oxygenate using an aerator set at very low aeration rate otherwise loss of buffer occurs due to frothing of detergent.

Root stain was cleared using 50% (w/v) household bleach (2.5% final concentration hypochlorite) for approximately 30 min, followed by 10 washes in water.

Appendix E

Publications arising from this work

REFEREED PAPERS

Denton, M.D. Coventry, D.R., Bellotti, W. D. and Howieson, J. G. (2000). Distribution, abundance and symbiotic effectiveness of *Rhizobium leguminosarum* bv. *trifolii* from alkaline pasture soils in South Australia. *Australian Journal of Experimental Agriculture*, 40: 25-35.

CONFERENCE ABSTRACTS / POSTERS

M.D. Denton, D.R. Coventry, J.G. Howieson P.J. Murphy and W.D. Bellotti. (1999). Establishing effective symbioses for alternative clovers in southern Australia. 12th International Congress on Nitrogen Fixation, Iguassu Falls, Brazil.

M.D. Denton, D.R. Coventry, J.G. Howieson, and W.D. Bellotti. (1999). The influence of naturalised soil *Rhizobium* populations on the nodulation and N_2 fixation of alternative clovers in southern Australia. 12th Australian Nitrogen Fixation Conference, Wagga Wagga. (prize for best poster).