

**Symbiotic Interactions of Geographically Diverse
Annual and Perennial *Trifolium* spp. with
Rhizobium leguminosarum bv. *trifolii***

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ABSTRACT

Perennial clovers are being evaluated for their potential to reduce groundwater levels in Australian cropping zones where many soils are considered too acidic for reliable lucerne nodulation. However, the release of effective inocula for perennial clovers into such areas where sub clover is the predominant legume, could potentially compromise nitrogen fixation from this valuable annual clover if the symbiosis between the new inoculants and sub clover is not optimal. Studies were therefore designed to increase our understanding of these symbiotic interactions to optimise the management of legume-rhizobia interactions to extend (rather than restrict) the use of legumes in new environments.

To assist the understanding of interactions between clovers and their micro-symbionts, a glasshouse-based study of the cross-inoculation characteristics of 38 strains of *Rhizobium leguminosarum* biovar *trifolii* (*R. l. trifolii*) associated with 38 genotypes of annual and perennial *Trifolium* spp. from world centres of diversity was undertaken. Rhizobial isolates and clovers were assembled from South and equatorial Africa, North and South America and the Euro-Mediterranean regions. There was substantial specificity amongst the African clovers for effective nodulation. No strain of rhizobia from the South American perennial *T. polymorphum*, or from the Ethiopian clovers, was able to nodulate sub clover effectively, whilst less than 33% of the 18 strains from these regions could form nodules with the less promiscuous Mediterranean annual *T. glanduliferum*. Seventy of 476 cross-inoculation treatments examined did not nodulate, whilst 81 treatments clearly demonstrated effective nodulation. The remainder of the cross-inoculation pairings revealed only partially effective or ineffective nodulation. Two barriers to effective nodulation were identified from the cross-inoculation study: a geographic barrier representing the broad centres of clover diversity, across which

few host-strain combinations were effective; and within each region, a significant barrier to effective nodulation between an isolate from an annual host on a perennial host, or *vice versa*. Clovers and their rhizobia from within the Euro-Mediterranean region of diversity were more able to overlap the annual/perennial barrier than genotypes from the other regions. The data indicate that it will be a substantial challenge to develop inocula for perennial clovers that do not adversely affect nitrogen fixation by sub clover and other annual clovers in commerce, especially if the perennial clovers originate from Africa or America.

To investigate the management of legume-rhizobia interactions when introducing legumes into new environments, a study was initiated in Uruguay (Mediterranean annual clovers were introduced into a predominantly perennial clover setting) that could be considered opposite to the situation emerging within southern Australia (perennial clovers evaluated in a predominantly annual clover setting). The Uruguayan grasslands contain populations of indigenous *R. I. trifolii* that nodulate endemic *T. polymorphum* but form ineffective nodules on clovers originating from the Mediterranean region. Importantly in the Uruguayan setting, Government policy has facilitated the introduction of numerous varieties of annual Mediterranean clovers with the aim of improving overall winter production in their naturally managed grasslands. In an attempt to understand the rhizobial ecology of this scenario, a cross-row experiment was set-up in 1999 in a basaltic, acid soil in Glencoe, Uruguay, to follow the survival and symbiotic performance of nine exotic strains of *R. I. trifolii*. In this thesis I report on the ability of the introduced strains to compete for nodule occupancy of Mediterranean clover hosts and show the impacts of the introduced strains on the productivity of the indigenous Uruguayan clover, *T. polymorphum*. Of the introduced strains, WSM1325 was a superior inoculant and remained highly persistent and competitive in forming effective symbioses with the Mediterranean hosts, *T. purpureum* and *T. repens*, in the

Uruguayan environment over a 3 year period. *T. purpureum* and *T. repens*, when inoculated with the introduced strains, did not nodulate with any indigenous *R. I. trifolii* as typed from nodules of *T. polymorphum*. Conversely, there were no nodules on the Uruguayan host *T. polymorphum* that contained the introduced *R. I. trifolii*. These results revealed that there were effective symbioses between strains of *R. I. trifolii* and clovers, even though the soil contained ineffective *R. I. trifolii* for all hosts. This represents the first reported example of selective nodulation for an effective symbiosis *in situ* with annual and perennial clovers in acid soils. This phenomenon raised the question of whether this was restricted to the particular edaphic scenario in Glencoe, Uruguay.

Glasshouse-based experiments in Australia were conducted to further understand the selection phenomenon. Two strains were selected for comparisons; strain WSM1325 isolated from an annual clover in the Mediterranean and WSM2304 isolated from the perennial clover *T. polymorphum* in Uruguay, South America. Variables that may have been specific to Glencoe were investigated. Thus, the effect of cell density and strain ratio at the time of inoculation, as well as soil pH, were examined on the two hosts (*T. purpureum* and *T. polymorphum*). Each was exposed to the same effective and ineffective micro-symbionts. In co-inoculation experiments at a cell density of 10^4 cells mL⁻¹, each host nodulated solely with its effective strain, even when this strain was out-numbered 100:1 by the ineffective strain. However, the selection process ceased when the effective strain was out-numbered 1000:1. At higher basal cell concentrations of 10^5 - 10^8 cells mL⁻¹, selection for WSM1325 to form effective nodules on *T. purpureum* was evident, but was significantly reduced as the ratio of ineffective cells in the inoculum increased above 4-fold. These results indicate that the selection mechanism is highly dependent upon the basal rhizobial cell density. Soil pH did not significantly

alter the process, which could not be simply explained by the rate of strain growth, or extent of nodulation.

Greater precision was sought in the terminology applied to nodulation outcomes where legumes have a choice of micro-symbiotic partners from within the same species of root-nodule bacteria. The nominated preferred terms are “non-selective”, “exclusive”, and “selective” nodulation.

In view of the difference in host range between WSM1325 and WSM2304 and the selective nodulation process, a preliminary investigation into the genetic backgrounds of WSM1325 and WSM2304 was conducted. A selected range of gene regions were amplified by PCR from each strain and sequenced. Comparative analysis of the nucleotide sequences revealed that although the 16S rRNA sequences were identical, the *atpD*, *GSII* and *nodD* sequences contained distinct differences revealing disparity between the pSym replicons and between the chromosomal replicons of these strains. Of the genes sequenced, the highest degree of divergence was noted for the symbiotic NodD protein products, which are known to be critical determinants in the nodulation of specific hosts. An examination of the *nodD* gene region of WSM1325 and WSM2304 revealed a further contrasting feature; the regulatory gene *nodR* was present in the *nodD* gene region of WSM1325 but absent in WSM2304. Since NodR is known to be required for adding highly unsaturated fatty acyl groups onto the Nod-factor backbone, I could now hypothesise that the nodulation incompatibility observed between *Trifolium* hosts and micro-symbionts obtained from different geographical locations may result from differences in Nod-factor decoration. With the full genome sequence of the two strains WSM1325 and WSM2304 soon to be available, the role of *nodR* and any link to the selection phenomenon described in this thesis can be addressed.

DECLARATION

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

Ronald John Yates

TABLE OF CONTENTS

	Page
PUBLICATIONS ARISING FROM THE THESIS	I
ACKNOWLEDGEMENTS	II
ABSTRACT	IV
CHAPTER 1: LITERATURE REVIEW	1
1.1 Symbiotic nitrogen fixation in legumes	2
1.1.1 Legumes and their importance	2
1.1.2 Rhizobia and <i>Rhizobium leguminosarum</i> biovar <i>trifolii</i>	5
1.1.3 Survival of rhizobia in soil	8
1.1.3.1 Diversity of soil rhizobia	9
1.1.3.2 Techniques to aid in identifying rhizobia	11
1.2 Legume nodulation – a complex relationship	12
1.2.1 Symbiotic communication, colonisation and effective nodule formation	13
1.2.1.1 Molecular signal recognition and interaction	14
1.2.1.2 Nodulation genes and host specificity	17
1.2.2 The physiology and epidemiology of nodule formation	19
1.2.2.1 Intracellular infection and nodule development	19
1.3 Challenges to legume symbioses in southern Australian agriculture ..	20
1.3.1 Salinity	20
1.3.2 A solution through the use of alternative perennial legumes	21
1.3.2.1 <i>Trifolium</i> L. (clovers)	22
1.3.3 Soil acidity	24
1.3.3.1 Effect of pH on the legume-rhizobia symbiosis	25
1.3.3.2 pH effects on free-living rhizobia in the soil	27
1.3.3.3 pH effects on symbiotic communication, attachment and infection thread formation	28
1.4 Nodulation outcomes in agricultural soils	29
1.4.1 Competition between rhizobia for nodulation	29
1.4.1.1 Competition of <i>R. l. trifolii</i> strains in agricultural soils	32
1.4.2 Soil and biological factors influencing rhizobial competition	35
1.4.2.1 Can legumes and rhizobia influence the establishment of their symbiosis?	37
1.5 Research Aims	42
1.5.1 Background	42
1.5.2 Aims for this research thesis	45

TABLE OF CONTENTS

	Page
CHAPTER 2: THE SYMBIOTIC INTERACTIONS OF <i>RHIZOBIUM LEGUMINOSARUM</i> BIOVAR <i>TRIFOLII</i> WITH ANNUAL AND PERENNIAL <i>TRIFOLIUM</i> SPP. FROM DIVERSE CENTRES OF ORIGIN.	46
2.1 Introduction	47
2.2 Materials and methods	49
2.2.1 Effectiveness experiments	49
2.2.1.1 Experimental preparation, inoculation and sowing	49
2.2.1.2 Growing conditions, harvesting and statistical analysis	49
2.3 Results	55
2.3.1 Experiment A	55
2.3.2 Experiment B	57
2.3.3 Experiment C	58
2.4 Discussion	61
CHAPTER 3: COMPETITIVE ABILITY AND PERSISTENCE OF EURO-MEDITERRANEAN CLOVERS AND THEIR MICRO-SYMBIONTS WHEN INTRODUCED INTO THE NATURAL GRASSLANDS OF URUGUAY.	67
3.1 Introduction	68
3.2 Materials and methods	71
3.2.1 Most probable number of rhizobia in Uruguayan soil	71
3.2.2 Experimental design (year 1)	71
3.2.3 Site and preparation	73
3.2.4 Inoculation and Sowing	73
3.2.5 Sampling (year 1)	75
3.2.6 Experimental design and sampling (year 2)	75
3.2.7 Isolation and authentication of rhizobia	75
3.2.8 Molecular fingerprinting with primer RPO1	76
3.3 Results	78
3.3.1 Soil analysis	78
3.3.2 Most probable number of rhizobia in Uruguayan soil	79
3.3.3 Strain performance (year 1)	79
3.3.4 Strain persistence (year 2)	81
3.3.5 Molecular fingerprinting with primer RPO1	83
3.4 Discussion	85

TABLE OF CONTENTS

	Page
CHAPTER 4: EVIDENCE OF SELECTION FOR EFFECTIVE NODULATION IN THE <i>TRIFOLIUM</i> SPP. SYMBIOSIS WITH <i>RHIZOBIUM LEGUMINOSARUM</i> BIOVAR <i>TRIFOLII</i>	87
4.1 Introduction	88
4.2 Materials and methods	90
4.2.1 Cross-row experiment (year 3)	90
4.2.2 Isolation and authentication of rhizobia	90
4.2.3 PCR amplification and analysis	91
4.2.4 Effectiveness studies	92
4.3 Results	94
4.3.1 PCR fingerprint analysis	94
4.3.2 Identification of nodule occupants	95
4.3.3 Effectiveness experiments	100
4.4 Discussion	102
CHAPTER 5: HOST-STRAIN MEDIATED SELECTION FOR AN EFFECTIVE N ₂ FIXING SYMBIOSIS BETWEEN <i>TRIFOLIUM</i> SPP. AND <i>RHIZOBIUM LEGUMINOSARUM</i> BIOVAR <i>TRIFOLII</i>	106
5.1 Introduction	107
5.2 Materials and methods	109
5.2.1 Hosts and bacterial strains	109
5.2.2 Growth rate determination	109
5.2.3 Design for GH experiments	109
5.2.3.1 Soil types and planting	110
5.2.3.2 Preparation of inoculant cultures for GH experiments I, II and III	111
5.2.3.3 GH experiment I - Nodule occupancy of <i>T. purpureum</i> and <i>T. polymorphum</i> after co-inoculation with WSM1325 and /or WSM2304 at low cell densities	112
5.2.3.4 GH experiment II - Nodule occupancy on <i>T. purpureum</i> grown in acid, neutral or alkaline soil after co-inoculation with WSM1325 and /or WSM2304 at low cell densities	113
5.2.3.5 GH experiment III - Nodule occupancy on <i>T. purpureum</i> following co-inoculation with WSM1325 and/or WSM2304 at high cell densities	114
5.2.3.6 GH experiment IV - Rate of nodule initiation	114
5.2.4 Assessment of nodule occupancy	115
5.2.4.1. ERIC DNA fingerprint construction and analysis	115

TABLE OF CONTENTS

	Page
5.2.5 DNA amplification for nodD sequencing	115
5.2.5.1 DNA sequencing and analysis	117
5.2.5.2 Sequence accessions	118
5.2.6 Construction of genetic maps of the nodD regions	118
5.2.7 Statistical analyses	118
5.3 Results	120
5.3.1 Glasshouse experiment I – Nodule occupancy of <i>T. purpureum</i> and <i>T. polymorphum</i> after co-inoculation at low cell densities	120
5.3.2 Glasshouse experiment II - Nodule occupancy of <i>T. purpureum</i> co-inoculated at low cell densities in an acid, neutral or alkaline soil	123
5.3.3 Glasshouse experiment III - Nodule occupancy of <i>T. purpureum</i> following co-inoculation at high cell densities	125
5.3.4 Validation of nodule occupancy	126
5.3.5 Experiment IV - Rate of nodule initiation	127
5.3.6 Phylogeny of WSM1325 and WSM2304 and gene similarity	127
5.3.7 Comparison of the nodD regions of <i>R. I. trifolii</i> strains WSM1325 and WSM2304	132
5.4 Discussion	134
CHAPTER 6: GENERAL DISCUSSION	141
6.1 Symbiotic interactions of annual and perennial <i>Trifolium</i> spp.with <i>R. I. trifolii</i>	142
6.2 Future work: Exploring the basis of incompatibility between annual and perennial clovers and their micro-symbionts	152
References	156

LIST OF FIGURES

		Page
Figure 1.1.	<i>T. polymorphum</i> at reproduction stage growing in a natural grassland located at Tacuarembó, Uruguay.	44
Figure 1.2.	The Euro-Mediterranean annual clover <i>T. purpureum</i> in flower.	44
Figure 2.1.	Dry weight of shoots (% of +N control) produced on the Euro-Mediterranean annual clovers <i>T. subterraneum</i> and <i>T. glanduliferum</i> and the South American perennial clover <i>T. polymorphum</i> by strains of <i>R. I. trifolii</i> from different centres of diversity.	56
Figure 2.2.	Dry weight of shoots (% of +N control) produced on African annual clovers (A) <i>T. rueppellianum</i> , <i>T. mattirolianum</i> and <i>T. steudneri</i> and perennial clovers (P) <i>T. cheranganiense</i> , <i>T. burchellianum</i> and <i>T. usamburense</i> , by strains of <i>R. I. trifolii</i>	58
Figure 3.1.	Experimental block design of treatments including 9 inoculant strains and an uninoculated treatment.	72
Figure 3.2.	Diagrammatic representation of the plot management over 2 years (1999-2000) at the Glencoe field trial site, Uruguay. In the first year, inoculated <i>T. vesiculosum</i> seed was introduced into the soil at 1 g m ⁻¹ . In the second year, uninoculated seed (surface sterilised) of <i>T. vesiculosum</i> , <i>T. purpureum</i> and <i>T. spumosum</i> was sown across the original row at 1 g m ⁻¹	74
Figure 3.3.	Shoot dry weight of <i>T. vesiculosum</i> (g plant ⁻¹) sown on the 20 th April 1999 and harvested on the 17 th September 1999 (18 weeks) when inoculated with 9 strains of <i>R. I. trifolii</i> and the uninoculated control (UNINOC) from the Glencoe field site, Uruguay (1999) in the first year of the cross-row experiment.	80
Figure 3.4.	Mean nodule number present per ten <i>T. vesiculosum</i> plants and percentage of plants not nodulated (% above columns) when harvested on the 17 th September 1999 after being inoculated with 9 strains of <i>R. I. trifolii</i> from the Glencoe field site, Uruguay (1999) in the first year of the cross-row experiment.	81
Figure 3.5.	Visual biomass ratings from the Glencoe field site on the 15 th September 2000, Uruguay, of inner (0-25 cm) and outer (26-50 cm) second year cross-rows of <i>T. purpureum</i> sown as uninoculated seed on the 15 th June 2000 into plots previously inoculated with nine strains of <i>R. I. trifolii</i> , and uninoculated controls.	82
Figure 3.6.	Second year cross-rows growth response at the Glencoe field site, Uruguay, when uninoculated <i>T. purpureum</i> seed was sown on the 15 th June into plots previously inoculated with 9 strains of <i>R. I. trifolii</i> , and uninoculated controls.	83

LIST OF FIGURES

		Page
Figure 4.1.	Diagrammatic representation of the individual trial plots that was managed over three years (1999-2001) at the Glencoe, Uruguay, field trial site.	91
Figure 4.2.	UPGMA cluster analysis dendrogram displaying similarity of fingerprint profiles generated from the ERIC primer pair for the 9 introduced strains of <i>R. I. trifolii</i>	96
Figure 4.3.	UPGMA cluster analysis dendrogram displaying similarity of fingerprint profiles generated from the ERIC primer pair for the 9 introduced strains of <i>R. I. trifolii</i> and 44 strains isolated from <i>T. polymorphum</i> sampled within plots inoculated with WSM1325 or WSM1328 two years previously.	97
Figure 5.1.	Mean total shoot dry weight (DW mg plant ⁻¹) expressed as a logarithm and the square root of the mean number of nodules [pink = effective or white = ineffective] produced by <i>T. purpureum</i> and <i>T. polymorphum</i> plants when inoculated separately with 1 mL [approximately 1 x 10 ⁴ total cells] of 9 different combinations of WSM1325 and WSM2304 in soil NG (pH (0.01M CaCl ₂) = 6.9), represented as logit % WSM2304 / total number of cells.	122
Figure 5.2.	Mean total shoot dry weight (DW mg plant ⁻¹) expressed as a logarithm and the square root of the mean number of nodules [pink = effective or white = ineffective] produced by <i>T. purpureum</i> plants when inoculated separately with 1 mL (approximately 2 x 10 ⁴ total cells) of 4 different combinations of WSM1325 and WSM2304 in soils HM (pH (0.01M CaCl ₂) = 7.9), NM (pH (0.01M CaCl ₂) = 6.9) and AM (pH (0.01M CaCl ₂) = 4.9), represented as logit % WSM2304 / total number of cells.	124
Figure 5.3.	Total shoot dry weight (DW mg plant ⁻¹) expressed as a logarithm and the square root of number of nodules [pink = effective or white = ineffective] produced by <i>T. purpureum</i> plants when inoculated separately with soil NG (pH (0.01M CaCl ₂) = 6.9) after co-inoculation with WSM1325 and WSM2304, represented as logit % WSM2304 / total number of cells.	126
Figure 5.4.	Nucleotide sequence identity % between strains WSM2304 and WSM1325, and a range of Type strains of rhizobia, for a 560 bp segment of the glutamine synthetase gene (<i>GSI</i>) and a 450 bp segment of the gene coding for the β subunit of the membrane ATP synthase (<i>atpD</i>).	129
Figure 5.5.	NodD phylogenetic tree of different <i>R. I. viciae</i> and <i>R. I. trifolii</i> strains.	130

LIST OF FIGURES

	Page
Figure 5.6. Diagrammatic representation of the single <i>nod</i> region in DNA of <i>R. l. trifolii</i> strains WSM2304 and WSM1325.	133
Figure 6.1. Conceptual diagram of nodule occupancy of a compatible host when inoculated with mixtures of strain A (effective) and strain B (ineffective) when displaying non-selective nodulation.	148
Figure 6.2. Representation of the combined results collated from this study displaying the nodule occupancy of <i>T. purpureum</i> when inoculated with mixtures of strain A (effective) and strain B (ineffective) with total number of cells at 10^4 and $>10^5$ cells mL ⁻¹	150

LIST OF TABLES

	Page
Table 1.1. Experimental reports that provide evidence of selection between effective (E) and ineffective (I) symbiotic partners, together with information on the experimental growth medium and pH, initial inoculation rate (cells mL ⁻¹), number of rhizobia with ability to nodulate the host in soil (if applicable), and method used to identify strains	40
Table 2.1. Strains of <i>R. I. trifolii</i> used in experiments A-C, their synonym and other details of their origin	50
Table 2.2. Trifolium accessions used in experiments A-C with notes on their growth cycle, origin and source.	51
Table 2.3. Host-strain interaction for 32 <i>Trifolium</i> spp. and 16 <i>R. I. trifolii</i> . Dark shading represents both geographical phenological homologies; light shading represents geographical homologies only. Host-strain reactions for N ₂ fixation are: E, effective; PE, partially effective; I, ineffective; X, no nodulation; dash, not tested. Origins of clover species are: NA, North America; SA, South America; Eur, Euro-Mediterranean; Afr, Africa. Growth cycles are: A, annual; P, perennial	60
Table 3.1. Origins of the R. I. trifolii inoculant strains	72
Table 3.2. Properties of vertisol soil in the basaltic region of Uruguay	78
Table 3.3. Predicted nodule occupancy of random <i>T. purpureum</i> plants excavated from the outer regions of the cross-rows at Glencoe Research Station, Uruguay, based on PCR analysis with primer RPO1. SBF – not distinguishable due to having similar banding fingerprints (WSM409, WSM1325 and WSM1328); Unidentified – a field isolate, not one of the 9 introduced strains	84
Table 4.1. Oligonucleotide primers used for PCR fingerprint profiling of rhizobial isolates in this study	92
Table 4.2. Summary of the results of fingerprint profiles generated by the use of rep-PCR to distinguish nine strains of <i>R. I. trifolii</i> introduced to the soil at the Glencoe field site, Uruguay	95
Table 4.3. Occupancy of nodules on <i>T. purpureum</i> , <i>T. repens</i> and <i>T. polymorphum</i> grown at the Glencoe field site, Uruguay, in the third year after inoculation.	99
Table 4.4. Total shoot dry weight (g plant ⁻¹) and nodulation effectiveness ratings expressed as a percentage of the +N control (KNO ₃), produced by <i>Trifolium purpureum</i> , <i>T. repens</i> and <i>T. polymorphum</i> when inoculated separately with three soil dilutions from native grasslands (Uruguay), under controlled conditions	100

LIST OF TABLES

	Page
Table 4.5. Total shoot dry weight (g plant ⁻¹) and nodulation effectiveness ratings expressed as a percentage of the +N control (KNO ₃), produced by <i>Trifolium purpureum</i> , <i>T. repens</i> and <i>T. polymorphum</i> when inoculated separately with 10 strains of rhizobia under controlled conditions.	101
Table 5.1. The collection site details and symbiotic phenotype of <i>R. I. trifolii</i> strains used in the study	110
Table 5.2. Physical and chemical propertiesA of the four soils used in glasshouse experiments I-IV	111
Table 5.3. Inoculant cell ratios and total cell number delivered to each clover seedling for glasshouse experiments I to III	113
Table 5.4. Oligonucleotides and PCR cycling conditions used for sequencing reactions.	116
Table 6.1 Terminology previously applied to nodulation events where legumes have a choice of micro-symbiotic partners within the same species of root-nodule bacteria. Column three provides a suggested preferred term with which to group these events	147