

***RHIZOBIUM* INOCULATION, CULTIVAR AND MANAGEMENT
EFFECTS ON THE GROWTH, DEVELOPMENT AND YIELD OF
COMMON BEAN (*Phaseolus vulgaris* L.)**

**A thesis
submitted in fulfilment of the
requirements for the degree of**

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New Zealand

By

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Lincoln University

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**Dedicated to my family
especially those lost during my quest for knowledge.**

**Frank Goodridge
Father
(1935-1990)**

**Lavinia Kellman
Grandmother
(1910-1991)**

**Donvid M. Kellman
Brother
(1970-2005)**

**I do not think there is any other quality
so essential to success of any kind
as the quality of perseverance.
It overcomes almost everything, even nature.**

John D. Rockefeller

Abstract

Abstract of a thesis submitted in the fulfilment of the requirements for the degree of Doctor of Philosophy at Lincoln University, Canterbury, New Zealand.

***Rhizobium* Inoculation, Cultivar and Management effects on the Growth, Development and Yield of Common Bean (*Phaseolus vulgaris* L.)**

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Genotypic differences in growth and yield of two common bean (*Phaseolus vulgaris* L) cultivars to *Rhizobium* inoculation and management were investigated. In 2003-04, the two bean cultivars (Scylla and T-49) were combined with three inoculant treatments (strains CC 511 and RCR 3644, and a control of no inoculation), two fertiliser levels (0 and 150 kg N ha⁻¹) and two irrigation treatments (irrigated and rainfed). There was no nodulation on either cultivar. To further investigate the symbiotic relationship, 16 rhizobial isolates, including the two used in the first field experiment, were combined with the cultivar Scylla and evaluated in a greenhouse. Subsequently, five *Rhizobium* isolates were chosen for further field evaluation, based on signs of early nodulation in the greenhouse trial. The second field experiment in 2004-05 combined the five inoculant strains (RCR 3644, UK 2, H 20, PRF 81, PhP 17 and a control) with two bean cultivars (Scylla and T-49).

In the greenhouse, nodule number varied from 7 (UK 2) to 347 (H 441) nodules plant⁻¹ at 51 DAS and from 13 (UK 1) to 335 (CIAT 899) nodules plant⁻¹ at 85 DAS. In 2004-05, in the field, nodulation was also variable, ranging between 1 and approximately 70 nodules plant⁻¹, with higher nodules numbers plant⁻¹ being found on cultivar T-49. Of the isolates used in the field, strains H 20, PRF 81 and PhP 17 produced 70, 25 and 12 nodules plant⁻¹ at 70, 40 and 54 DAS respectively. Nodules formed were of various sizes and more than 80 % were pink to dark red in colour denoting the presence of leghaemoglobin and active N fixation. The remaining nodules were either green or white.

The importance of selecting an appropriate cultivar for the growing conditions was highlighted in these experiments. Leaf area index, leaf area duration intercepted radiation and final utilisation efficiency were significantly affected by cultivar. In both seasons cv.

T-49 reached maturity (dry seed) before Scylla, while unirrigated plants reached green pod maturity seven days before irrigated plants. Plants of cv. Scylla gave a final TDM of 730 g m⁻²; compared to the 530 g m⁻² produced by T-49. The average growth rate was 7.0 and 5.2 g m⁻² day⁻¹ for Scylla and T-49 respectively (2003-04). Plants receiving 150 kg N ha⁻¹ produced 665 g m⁻² TDM which was 12 % more than was produced by unfertilised plants. The application of 150 kg N ha⁻¹ gave an average growth rate of 6.4 g m⁻² day⁻¹ compared to 5.7 g m⁻² day⁻¹ from plants with no N. Inoculation in the field had no significant effect on TDM in both seasons.

Temperature affected growth and DM accumulation. Accumulated DM was highly dependent on cumulative intercepted PAR. Air temperatures below the base temperature (10 °C) affected growth in 2004-05, resulting in plants accumulating just 0.24 g DM MJ⁻¹ PAR during early growth. This increased to 2.26 g DM MJ⁻¹ PAR when the temperature was increased above the base temperature. There was a strong relationship between LAI and intercepted PAR. A LAI of 4.0-4.5 was required to intercept 90-95 % of incident solar radiation. Cultivar significantly (p < 0.001) affected radiation use efficiency (RUE). Scylla had a RUE of 1.02 g DM MJ⁻¹ PAR compared to T-49 at 1.18 g DM MJ⁻¹ PAR.

Seed yield was significantly (p < 0.001) affected by cultivar and fertiliser application. Cultivar Scylla produced 467 g m⁻² which was 76 % more than T-49, while a 12 % increase in seed yield was observed in N fertilised plants over unfertilised plants. Only cultivar significantly affected HI, while the yield components that had the greatest effect on seed yield were hundred seed weight and pods plant⁻¹. Inoculation significantly (p < 0.05) affected 100 seed weight (2004-05). Plants inoculated with strain H 20 had the highest 100 seed weight at 25.2 g with cv. Scylla producing larger seeds than T-49.

The belief that local environmental conditions play a major role on field survival of bacteria, led to the use of PCR methods to identify field nodulating organisms. Amplification of genomic DNA from parent isolates using primers fC and rD generated a single band for each isolate. Isolates were identified to the species level as either *Rhizobium* or *Agrobacterium*, using the highly conserved internally transcribed spacer (ITS) region and are known to nodulate common bean. The DNA extracted from the isolates recovered from nodules of field grown beans gave multiple bands with primers fC and rD. Five distinct banding patterns were observed. All of these were different from those of parent isolates. Sequencing of the 16S rRNA demonstrated that nodules of field

grown beans in Canterbury were inhabited by *Pseudomonads* either alone or in association with other root nodulating organisms. The inability to identify the inoculant strains in nodules of field grown beans does not rule out their infection and nodulating function in the cultivars used. The results suggest the possibility of both *Rhizobium* and *Pseudomonads* cohabiting in the nodules of field grown beans. The aggressive nature of *Pseudomonads* on artificial media, possibly out competing the inoculant rhizobia is proposed, leading to the inability to identify the inoculant strain from the nodules of the field grown beans by PCR methods.

The need to identify the nodule forming or nodule inhabiting bacteria in the nodules is necessary to classify the importance of these organisms and their economic benefit to agricultural production. This study also underlines the importance of using PCR methods to gain valuable insights into the ecological behaviour of *Rhizobium* inoculants and nodule inhabiting organisms.

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Table of Contents

Abstract.....	iii
Acknowledgements	vi
Table of Contents	viii
List of Tables	xiii
List of Figures.....	xvi
List of Plates	xix
List of Abbreviations	xx
CHAPTER 1 General introduction.....	1
1.1 Objectives of the study	4
CHAPTER 2 Literature Review.....	6
2.1 Introduction	6
2.1.1 Origin and Distribution.....	6
2.1.2 Uses	7
2.1.3 Taxonomy and Botany	8
2.2 Environmental impact on crop phenology, growth and development.....	9
2.2.1 Phenology	9
2.2.2 Development.....	10
2.2.2.1 Effect of temperature.....	10
2.2.2.2 Effect of photoperiod.....	11
2.2.3 Crop Growth.....	12
2.2.3.1 Effect of water stress	13
2.2.3.2 Effect of temperature.....	14
2.2.3.3 Effect of nodulation.....	14
2.3 Biological Nitrogen Fixation.....	15
2.3.1 Rhizobium	17
2.3.2 Introduction of rhizobia into the soil	18
2.3.3 Inoculation	19
2.3.4 Soil colonisation	21
2.3.5 Host specificity and nodulation	22
2.3.6 Nodulation	23
2.4 Amount of nitrogen fixed	25
2.4.1 Methods of estimating nitrogen fixed.....	25
2.4.2 Amount of nitrogen fixed	27
2.5 Factors affecting nitrogen fixation	29
2.5.1 Soil acidity	29
2.5.2 Temperature.....	30
2.5.3 Soil moisture and water supply	31
2.5.4 Rhizobial competition	32
2.5.5 Chemical residues in the soil.....	32

2.5.6	Crop sequence and mechanisation.....	33
2.5.7	Fertiliser and soil nitrogen.....	33
2.6	Dry matter accumulation and yield.....	34
2.7	Seed yield.....	36
2.7.1	Yield components.....	37
2.7.2	Harvest index.....	38
2.8	Conclusions.....	39
CHAPTER 3 Plant growth and nodulation studies of common beans (<i>Phaseolus vulgaris</i> L.) inoculated with elite strains of <i>Rhizobium</i>.....		41
3.1	Introduction.....	42
3.2	Materials and Methods.....	44
3.2.1	Field experiments.....	44
3.2.1.1	Site description.....	44
3.2.1.2	Soil.....	44
3.2.1.3	History.....	44
3.2.1.4	Experimental design.....	45
3.2.1.5	Crop Husbandry.....	46
3.2.1.6	Sowing.....	47
3.2.1.7	Inoculation.....	47
3.2.1.8	Irrigation.....	47
3.2.1.9	Sampling.....	47
3.2.1.10	Measurements.....	48
3.2.2	Greenhouse experiment.....	49
3.2.2.1	Design.....	49
3.2.2.2	<i>Rhizobium</i> strains used.....	50
3.2.2.3	Potting Mix.....	50
3.2.2.4	Sowing.....	51
3.2.2.5	Nutrient solution.....	51
3.2.2.6	Media and growth conditions.....	51
3.2.2.7	Bacterial density.....	52
3.2.2.8	Harvest.....	53
3.2.2.9	Measurements.....	54
3.2.2.10	Statistical analysis.....	54
3.3	Results.....	55
3.3.1	Field experiment.....	55
3.3.1.1	Climate.....	55
3.3.1.2	Nodulation.....	57
3.3.1.3	Nodules plant ⁻¹	57
3.3.1.4	Root dry weight plant ⁻¹	59
3.3.1.5	Shoot dry weight plant ⁻¹	61
3.3.1.6	Plant Nitrogen Concentration.....	62
3.3.2	Greenhouse experiment.....	64
3.3.2.1	Nodulation pattern.....	64
3.3.2.2	Nodules plant ⁻¹	64
3.3.2.3	Shoot dry weight plant ⁻¹	65
3.3.2.4	Root dry weight plant ⁻¹	68
3.3.2.5	Shoot:root (S:R) ratio.....	69

3.3.2.6	Pods and pod dry weight plant ⁻¹ (Yield)	70
3.3.2.7	Plant Nitrogen Concentration	71
3.4	Discussion	75
3.4.1	Inoculation	75
3.4.2	Nodulation	77
3.4.3	Shoot and Root growth	81
3.4.4	Nitrogen	82
3.4.5	Plant Nitrogen Concentration	83
3.5	Conclusions	85
CHAPTER 4 Management, cultivar and <i>Rhizobium</i> inoculation effects on the growth and development of common beans (<i>Phaseolus vulgaris</i> L.): Phenology, growth and dry matter accumulation.....		86
4.1	Introduction	86
4.2	Materials and Methods	88
4.2.1	Measurements and Sampling	88
4.2.2	Growth analysis	89
4.3	Results	89
4.3.1	Climate	89
4.3.2	Phenology	89
4.3.3	Maximum total dry matter	93
4.3.4	Average growth rate	94
4.4	Discussion	96
4.4.1	Phenology	96
4.4.2	Maximum total dry matter production.....	98
4.4.3	Average growth rate	99
4.5	Conclusions	100
CHAPTER 5 The influence of inoculation, cultivar and fertiliser on leaf area index (LAI), radiation interception and radiation use efficiency (RUE)		101
5.1	Introduction	102
5.2	Materials and Methods	103
5.2.1	Measurements	104
5.3	Results	105
5.3.1	Climate	105
5.3.2	Leaf area index	105
5.3.3	Leaf area duration, dry matter accumulation and yield.....	105
5.3.4	Intercepted radiation and crop growth.....	108
5.3.5	Intercepted PAR, DM accumulation and yield.....	112
5.3.6	Radiation use efficiency (RUE).....	116
5.4	Discussion	122
5.4.1	Leaf area index and duration	122
5.4.2	Radiation interception.....	124
5.4.3	Intercepted PAR, dry matter and seed yield	125

5.5	Conclusions	129
CHAPTER 6 <i>Rhizobium</i> inoculation, cultivar and management effects on growth and development of common bean (<i>Phaseolus vulgaris</i> L.): Yield and yield components. .130		
6.1	Introduction	131
6.2	Materials and Methods	133
6.2.1	Site description	133
6.2.2	Statistical analysis	133
6.3	Results	134
6.3.1	Climate	134
6.3.2	Germination	134
6.4	Yield at the green pod stage	134
6.4.1	Total dry matter production	134
6.4.2	Green pod yield	134
6.5	Final harvest	137
6.5.1	Total dry matter production	137
6.5.2	Seed Yield	137
6.5.3	Harvest index	140
6.6	Yield components	141
6.6.1	Number of plants m ⁻²	141
6.6.2	Pods plant ⁻¹	142
6.6.3	Seeds pod ⁻¹	143
6.6.4	100 seed weight	145
6.6.5	Yield and yield components correlation	145
6.7	Discussion	146
6.7.1	Total dry matter and pod yield at the green pod stage	146
6.7.2	Total dry matter and seed yield	147
6.7.3	Harvest index (of TDM at final harvest)	149
6.7.4	Yield components	150
6.8	Conclusions	152
CHAPTER 7 Identification and characterisation of <i>Rhizobium</i> isolates nodulating different common bean (<i>Phaseolus vulgaris</i> L.) cultivars153		
7.1	Introduction	154
7.2	Materials and Methods	156
7.2.1	Part 1	156
7.2.1.1	Bacterial strains	156
7.2.1.2	Bacterial growth	156
7.2.1.3	Rapid DNA extraction for identification of parent species	157
7.2.1.4	Agarose gel preparation	157
7.2.1.5	Polymerase Chain Reaction (PCR)	158
7.2.1.6	Agarose gel electrophoresis	158
7.2.1.7	Sequencing	159
7.2.2	Part 2	159
7.2.2.1	Rhizobial bacteria isolation from nodules	159

7.2.2.2	DNA extraction from a single colony	159
7.2.2.3	Polymerase chain reaction	160
7.2.2.4	Sequencing of PCR products.....	161
7.2.3	Part 3.....	161
7.2.3.1	Authentication of isolates from nodules.....	161
7.3	Results	163
7.3.1	Part 1	163
7.3.1.1	Growth of isolates on media.....	163
7.3.1.2	Amplification of DNA extracted from original isolates.....	163
7.3.1.3	Sequence analysis of the rRNA.....	164
7.3.2	Part 2.....	166
7.3.2.1	Identification and confirmation of isolates and sequence analysis	171
7.3.3	Part 3.....	173
7.3.3.1	Authentication of nodule isolates	173
7.4	Discussion	174
7.4.1	Summary of results	174
7.4.2	Part 1	174
7.4.3	Part 2.....	177
7.4.4	Part 3.....	184
7.5	Conclusions	186
	CHAPTER 8 General Discussion	187
8.1.1	Inoculation and nodulation	187
8.1.2	Growth, TDM and Seed yield	190
8.1.3	Isolation and characterisation	195
8.2	Conclusions	198
8.3	Recommendations for future research.....	199
	References.....	200
	Appendices.....	247

List of Tables

Table 2.1. Legume inoculation techniques. (Deaker <i>et al.</i> , 2004).....	20
Table 2.2. Some <i>Rhizobium</i> -plant associations. (van Rhijn and Vanderleyden, 1995).	23
Table 2.3. Experimental estimates of the proportion (P_{fix}) and amount of N_2 fixed by important pulses and legume oilseeds. (Peoples <i>et al.</i> , 1995b) and (Wani <i>et al.</i> , 1995).....	29
Table 3.1. MAF soil quick test values for paddocks in the Horticultural Research area (0-20 cm depth), Lincoln University, Canterbury. 2003-04 and 2004-05.	45
Table 3.2. Treatments used in the 2003-04 growing season of common beans at Lincoln University.....	45
Table 3.3. Treatments used in the 2004-05 growing season of common beans at Lincoln University.....	46
Table 3.4. Bacterial strains used in the experiment and their source.....	50
Table 3.5. Nutrient solution for legumes in sand or perlite culture (Hansen, 1994)	52
Table 3.6. Bacterial concentrations of media from which 2 or 15 ml was used for inoculation of the seed in the greenhouse and field experiment (2004-05) expressed as colony forming units (cfu) ml^{-1} of broth	53
Table 3.7. The effect of inoculation treatment and cultivar on shoot dry weight $plant^{-1}$ (g) in field grown common bean in 2004-05.....	61
Table 3.8. The effect of inoculation with five different <i>Rhizobium</i> isolates on plant nitrogen concentration (%) of Scylla and T-49 field grown common bean in 2004-05.	63
Table 3.9. Effect of different <i>Rhizobium</i> strains on nodule $plant^{-1}$ and nodule dry weight $plant^{-1}$ on common beans cv. Scylla grown in a greenhouse.....	65
Table 3.10. The effect of inoculation of common bean cv. Scylla with different <i>Rhizobium</i> strains on shoot, leaf, stem, pod, and root nodule dry weight and nodule $plant^{-1}$ at 51 days after sowing grown in a greenhouse.....	67
Table 3.11. The effect of inoculation of common bean cv. Scylla with different <i>Rhizobium</i> strains on shoot, leaf, stem, and pod dry weight and the number of pods $plant^{-1}$ at 85 days after sowing when grown in a greenhouse.....	68
Table 3.12. The effect of inoculation of common bean cv. Scylla with different <i>Rhizobium</i> strains on root dry weight $plant^{-1}$ at 51 and 85 DAS grown in the greenhouse.....	69
Table 3.13. Shoot:root ratio at 51 and 85 days after sowing with common bean cv Scylla grown in a greenhouse with and without applied nitrogen.	72
Table 3.14. The effect of <i>Rhizobium</i> inoculation on plant dry weight (g) (PDW) and plant N concentration (%) and N uptake ($g N plant^{-1}$) at 51 and 85 days after sowing of common bean (<i>Phaseolus vulgaris</i>) cv. Scylla grown in a greenhouse with and without applied nitrogen.....	73
Table 4.1. Chronological time for four physiological growth stages [(sowing to emergence (S-E), emergence to flowering (E-F), flowering to podding (F-	

	P), Podding to physiological maturity (P-M)] of common bean cultivars under irrigated and non-irrigated conditions in Canterbury, New Zealand. ...	91
Table 4.2.	Thermal time for four physiological growth phases [(sowing to emergence (S-E), emergence to flowering (E-F), Flowering to podding (F-P), and podding to physiological maturity (P-M)] of common bean as affected by irrigation and cultivar in Canterbury, 2003-2005.....	92
Table 4.3.	The effects of inoculation, cultivar, fertiliser and irrigation on final maximum TDM (g m^{-2}) of common beans in Canterbury, 2003-04 and 2004-05.....	93
Table 4.4.	The effect of inoculation, cultivar, fertiliser and irrigation on the average growth rate ($\text{g m}^{-2} \text{d}^{-1}$) of common beans in Canterbury, 2003-04 and 2004-05.....	96
Table 5.1.	The effect of inoculation, cultivar, nitrogen fertiliser and irrigation on the leaf area duration (d) of common bean in Canterbury, 2003-04 and 2004-05.....	108
Table 5.2.	The effect of inoculation, cultivar, fertiliser and irrigation on total intercepted PAR (MJ m^{-2}) of common bean in Canterbury, 2003-04 and 2004-05.....	115
Table 5.3.	The cultivar by inoculation interaction on total intercepted PAR (MJ m^{-2}) of common bean in Canterbury, 2003-04.....	116
Table 5.4.	The effect of inoculation, cultivar, fertiliser and irrigation on final radiation use efficiency ($\text{g DM MJ}^{-1} \text{PAR}$) of common bean in Canterbury, 2003-04 and 2004-05.....	120
Table 5.5.	The cultivar by irrigation interaction on final radiation use efficiency (RUE) ($\text{g DM MJ}^{-1} \text{PAR}$) of common bean in Canterbury, 2003-04.....	120
Table 6.1.	The effect of inoculation, cultivar, fertiliser and irrigation on total dry matter at green pod harvest (g m^{-2}) of common bean in Canterbury, 2003-04 and 2004-05.....	135
Table 6.2.	The effect of inoculation, cultivar, fertiliser and irrigation on green pod yield (g m^{-2}) on a dry weight basis of common bean in Canterbury, 2003-04 and 2004-05.....	136
Table 6.3.	The effect of inoculation, cultivar, fertiliser and irrigation on total dry matter at final harvest (g m^{-2}) of common bean in Canterbury, 2003-04 and 2004-05.....	138
Table 6.4.	The effect of inoculation, cultivar, fertiliser and irrigation on seed yield at final harvest (g m^{-2}) of common bean in Canterbury, 2003-04 and 2004-05.....	139
Table 6.5.	The cultivar by fertiliser interaction for seed yield at final harvest (g m^{-2}) of common bean in Canterbury, 2003-04.....	139
Table 6.6.	The effect of inoculation, cultivar, fertiliser and irrigation on harvest index at final harvest (g m^{-2}) of common bean in Canterbury, 2003-04 and 2004-05.....	141
Table 6.7.	The cultivar by irrigation interaction on harvest index at final harvest (g m^{-2}) of common bean in Canterbury, 2003-04.....	142

Table 6.8. The irrigation by fertiliser interaction on harvest index at final harvest (g m ⁻²) of common bean in Canterbury, 2003-04.	142
Table 6.9. The interaction of irrigation by cultivar by fertiliser on harvest index at final harvest (g m ⁻²) of common bean in Canterbury, 2003-04.	142
Table 6.10. The effect of inoculation, cultivar, fertiliser and irrigation on yield components of common bean in Canterbury, 2003-04.	143
Table 6.11. The cultivar by fertiliser interaction on 100 seed weight (g) of common bean in Canterbury in 2003-04.	144
Table 6.12. The fertiliser by inoculation interaction on seeds pod ⁻¹ of common bean in Canterbury in 2003-04.	144
Table 6.13. The effect of inoculation, cultivar, fertiliser and irrigation on yield components of common bean in Canterbury, New Zealand, 2004-05.	144
Table 6.14. Correlation matrix between yield and yield components for common bean in Canterbury, New Zealand, 2003-04.	146
Table 6.15. Correlation matrix between yield and yield components for common bean in Canterbury, New Zealand, 2004-05.	146
Table 7.1 Bacterial strains used in the experiment and their source of origin.	157
Table 7.2. The treatments used in the authentication experiment	163
Table 7.3. Species identification of original parent isolates obtained from the GenBank comparison with the sequenced DNA	166
Table 7.4. The main banding patterns generated using primers fC and rD from nodules collected from the 2004-05 field experiment.	171
Table 7.5. Identification of isolates recovered from the nodules of field grown beans and the parents strain amplified with primers F27 and R1494 obtained from Genbank search used in this study.	173

List of Figures

Figure 1.1. Diagrammatic representation of the relationship of each chapter.....	5
Figure 3.1. Climate data for the 2003-04 (□) and 2004-05 (▨) growing seasons and the long term means (■) (1975-2002) for Lincoln University, Canterbury, New Zealand.	56
Figure 3.2. The number of nodules plant ⁻¹ at different harvests with five strain of <i>Rhizobium</i> on common beans cvs Scylla and T-49 in the 2004-05 field experiment.	58
Figure 3.3. The effect of (a) inoculation treatment and (b) cultivar on root dry weight production over time of field grown common beans in 2004-05 in Canterbury, New Zealand.	60
Figure 3.4. The relationship between percent N and the number of nodules plant ⁻¹ at 51 (●) and 85 (○) DAS in greenhouse grown common beans.	74
Figure 3.5. The relationship between pods plant ⁻¹ and shoot dry weight at final harvest (85 DAS) in the greenhouse.	75
Figure 4.1. The relationship between final maximum total dry matter (g m ⁻²) and days after sowing calculated over the linear portion of the growth phase for common beans in Canterbury, 2003-04. The relationship is represented by the equation $y = -0.0114 + 6.28x$ ($r^2 = 0.90$). The average growth rate over the season was 6.28 g m ⁻² d ⁻¹	94
Figure 4.2. The effects of fertiliser (a) and cultivar (b) on total dry matter (g m ⁻²) over time (days) for common beans in Canterbury, 2003-04. Average growth rates: Nitrogen = 6.7; No Nitrogen = 5.8; Scylla = 7.1; T-49 = 4.8 g m ⁻² d ⁻¹	95
Figure 5.1. The effect of a) irrigation, b) nitrogen fertiliser, c) cultivar and d) inoculation on leaf area index over the growing season of common bean in Canterbury in 2003-04.	106
Figure 5.2. The effect of a) cultivar and b) inoculation on leaf area index over the growing season of common bean in Canterbury in 2004-05.....	107
Figure 5.3. The proportion of intercepted radiation (F _i) up to maximum leaf area index by common beans in Canterbury in 2003-04 as affected by a) irrigation; b) nitrogen fertiliser; c) cultivar and d) inoculation.	110
Figure 5.4. The proportion of intercepted radiation (F _i) up to maximum leaf area index by common beans in Canterbury in 2004-05 as affected by a) cultivar and b) inoculation.....	111
Figure 5.5. The relationship between the proportion of intercepted radiation (F _i) and leaf area index in common bean in Canterbury, 2003-05.	112
Figure 5.6. The relationship between leaf area index (LAI) and ln (1-F _i) in common bean in Canterbury, in 2003-04 as affected by a) irrigation; b) nitrogen fertiliser; c) cultivar and d) inoculation.....	113
Figure 5.7. The relationship between leaf area index (LAI) and ln (1-F _i) in common bean in Canterbury, in 2004-05 as affected by a) cultivar and b) inoculation.....	114

Figure 5.8. Dry matter production during crop growth in relation to accumulation of photosynthetically active radiation (PAR) for common bean in Canterbury in 2003-04. a) irrigation; b) nitrogen fertiliser; c) cultivar and d) inoculation.....	117
Figure 5.9. Dry matter production during crop growth in relation to accumulation of photosynthetically active radiation (PAR) for common bean in Canterbury in 2004-05. a) cultivar and b) inoculation.....	118
Figure 5.10. The cultivar by fertiliser interaction on final radiation use efficiency (RUE) (g DM MJ ⁻¹ PAR) of common beans in Canterbury, 2003-04.	121
Figure 5.11. The relationship between predicted and observed seed yield of common bean in Canterbury in 2003-04.....	122
Figure 7.1. Primer sites for PCR. Primers fC and rD amplified the variable ITS 1 and 2 and the conserved 5.8S regions and primers F27, F968 and R1494 amplified the highly conserved 16S rRNA region	161
Figure 7.2. PCR products obtained with primers fC and rD of original <i>Rhizobium</i> isolates. From left to right: (1Kb Plus DNA ladder TM (Invitrogen), CARA 72 (1), H 20 (2), H 12 (3), PRF 81 (4), PhP 17 (5) H 441 (6), CESTA 221 (7), CIAT 899 (8), HT 2a2 (9), CARA 56 (10), RCR 3644 (11), CC 511 (12), UK 4 (13), UK 3 (14), UK 2 (15), UK 1 (16), Negative control (C).....	166
Figure 7.3. The PCR products obtained with primers fC and rD from nodules of plant inoculated with strain H 20. From left to right: 1Kb Plus DNA Ladder TM (Invitrogen), Parent DNA (P), lanes 1 to 7 represent individual nodules of cv. Scylla and lanes 8 to 15 cv. T-49, 16 negative control, 1Kb Plus DNA Ladder TM (Invitrogen). (Banding patterns present: Lane 9 = A, lane 7 = D, lane 5 = E, lane 8 = C). Asterisk represents fC and rD amplimers selected as a representative of the nodule inhabitants.....	167
Figure 7.4. The PCR products obtained with primers fC and rD from nodules of plant inoculated with strain PhP 17. From left to right: 1Kb plus DNA Ladder TM (Invitrogen), Parent DNA (P), lanes1 to 6 represent individual nodules of cv. Scylla and lanes 7 to 13 cv. T-49, C is a negative control, and 1Kb plus DNA Ladder TM (Invitrogen).(Banding patterns present: lane 10 = D, lane 5 = B, lane 4 = A, Lane 2 = C). Asterisk represents fC and rD amplimers selected as a representative of the nodule inhabitants.....	168
Figure 7.5. The PCR products obtained with primers fC and rD from nodules of plant inoculated with strain RCR 3644. From left to right: 1Kb Plus DNA Ladder TM (Invitrogen), Parent DNA (P), lanes 1 to 3 represent individual nodules of cv. Scylla, and lanes 4 to 7 represent individual nodules of cv. T-49, C is a negative control, and 1Kb Plus DNA Ladder TM (Invitrogen).(Banding patterns present: lane 3 = D, lane 7 = E). Asterisk represents fC and rD amplimers selected as a representative of the nodule inhabitants	168
Figure 7.6. The PCR products obtained with primers fC and rD from nodules of plant inoculated with strain PRF 81. From left to right: 1Kb plus DNA Ladder TM (Invitrogen), Parent DNA (P), lanes 1 to 6 represent individual nodules of cv. Scylla and lanes 7 to 12 cv. T-49, C is a negative control, and 1Kb plus DNA Ladder TM (Invitrogen).(Banding patterns present: lane	

1 = D, lanes 7 = C, lane 9 = Y(indistinguishable). **Asterisk** represents fC and rD amplimers selected as a representative of the nodule inhabitants..... 169

Figure 7.7. The PCR products obtained with primers fC and rD from nodules of plant inoculated with strain UK 2. From left to right: 1Kb Plus DNA Ladder™ (Invitrogen), (P) Parent DNA, lanes 1 to 6 represent individual nodules from cv. Scylla and lanes 7 to 14 cv. T-49, C is a negative control, and 1Kb Plus DNA Ladder™ (Invitrogen).(Banding patterns present: lane 1 = C, lane 2 = D , lane 11 = B and lanes 14 = A). **Asterisk** represents fC and rD amplimers selected as a representative of the nodule inhabitants..... 170

Figure 7.8. The PCR products obtained with primers fC and rD from nodules of the uninoculated control plants. From left to right: 1Kb Plus DNA Ladder™ (Invitrogen), lanes 1 to 6 represents individual nodules of cv. Scylla and lanes 7 to 12, cultivar T-49 and 1Kb Plus DNA Ladder™ (Invitrogen). (Banding patterns present: lanes 12 = E, lane 8 = B and lane 10 = likely to be C). **Asterisk** represents fC and rD amplimers selected as a representative of the nodule inhabitants..... 170

Figure 7.9. The PCR products obtained with primers F27 and R1494 in preparation for sequencing. From left to right 1Kb Plus DNA ladder™ (Invitrogen), 1 to 16 are representative nodules that had produced patterns A, B, C, D, and E with the fC and rD primers, C – control and another 1Kb Plus DNA Ladder™ (Invitrogen) patterns A (lane 11), B (lanes 1, 2, 7 and 8), D (lanes 3, 4, 5) E (lane 6 and 9) Y (lane 10). Lanes 12-16 was PCR product from the parent isolates UK 2, PRF 81, RCR 3644, H 20 and PhP 17 respectively..... 171

List of Plates

- Plate 3.1. The nodulation pattern of field grown common bean (*Phaseolus vulgaris*) cvs Scylla (left) and T-49 (right) inoculated with *Rhizobium* isolate H 20..... 59
- Plate 3.2. Nodulation pattern of greenhouse grown common bean cv. Scylla) at 85 days after sowing inoculated with *Rhizobium* isolate H 20. 66
- Plate 7.1. Original cultures of isolates growing on Rhizobia Defined Media (RDM). From left to right CARA 72 (1), H 20 (2), H 12 (3), PRF 81 (4), PhP 17 (5), H 441 (6), CESTA 221 (7), CIAT 899 (8), HT 2a2 (9), CARA 56 (10), RCR 3644 (11), CC 511 (12), UK 4 (13), UK 3 (14), UK 2 (15), UK 1 (16). 165

List of Abbreviations

Symbols	Descriptions
a.i.	Active ingredient
ANOVA	Analysis of variance
BNF	Biological nitrogen fixation
bv.	biovar
CIAT	International Center for Tropical Agriculture
CGR	Crop growth rate
C_m	maximum crop growth rate
cv.	cultivar
CV	Coefficient of variation
d	day
DAS	Days after sowing
DM	Dry matter
DNA	Deoxyribonucleic acid
DUR	Duration of crop growth
EPT	Penman evapo-transpiration
FAO	Food and Agricultural Organisation
F_i	Proportion of radiation intercepted
HI	Harvest index
h	hours
HRU	Horticultural Research Unit
ICRISAT	International Crop Research Institute for the Semi-Arid Tropics
IGS	Intergenic spacer
ITS	Internally transcribed spacer
-k	extinction coefficient
kPa	Kilopascals
LAD	Leaf area duration
LAI	Leaf area index
MAF	Ministry of Agriculture and Fisheries
MaxDM	Maximum total dry matter
MLP	Maximum Likelihood Programme

N	Nitrogen
N ₂	Atmospheric Nitrogen
Ndfa	Nitrogen derived from the atmosphere
nov.	novel
PAR	Photosynthetically active radiation
PCR	Polymerase chain reaction
r ²	Coefficient of determination
rDNA	ribosomal deoxyribonucleic acid
RDW	Root dry weight
RFLP	Restriction Fragment Length Polymorphism
RUE	Radiation use efficiency
rRNA	ribosomal ribonucleic acid
S _a	Intercepted solar radiation
SEM	Standard error of mean
SDW	Shoot dry weight
sp(p).	Specie(s)
Si	Total incident PAR
S:R	Shoot root ratio
T _b	Base temperature
TDR	Time Domain Reflectometry
TDM	Total dry matter
T _{max}	Average maximum daily temperature
T _{min}	Average minimum daily temperature
TN	Total nitrogen
TSY	Total seed yield
T _t	Accumulated temperature
TY	Tryptone-Yeast extract
WMAGR	Weighted mean absolute growth rate

CHAPTER 1

General introduction

Grain legumes are widely recognised as an important source of food and feed proteins, (Cristou, 1997; Duranti and Guis, 1997) and have become very important in human nutrition and as a feed for domestic animals (Egli, 1998; Cummings *et al.*, 2001). Additionally, the increasing interest in low input sustainable agricultural systems on cropping farms from an economic, managerial and environmental standpoint opens the door for continued interest in grain legumes (White, 1989). The importance of legumes to these systems is not only for their nitrogen (N) fixing capabilities but also their ability in breaking the cycles of diseases and pests affecting other crops (White, 1989; Cummings *et al.*, 2001). Amongst the legumes, the common bean (*Phaseolus vulgaris* L.) is second only to soybean (*Glycine max* (L.) Merr.) in importance and is the major cultivated representative of the genus (Cristou, 1997).

Common bean is an interesting crop from the point of view of the consumer, farmer and processor. For the consumer, bean is important for its nutritive composition and its variable uses in different culinary forms. For farmer, the crop contributes N to the soil which is often low. While dry seed and fresh pod of specific landraces attract a high market price. The fresh pod crop, mainly field grown can be produced during the coolest season in glasshouses. For the processor, common bean provides many possibilities such as canned or frozen seeds and pods (Escribano *et al.*, 1997).

Common beans are known by many common names in languages around the world and are classified by their agronomic, physical and consumption characteristics (Voysesst and Dessert, 1991). They are grown extensively in five major continental areas: Eastern Africa, North and Central America, South America, Eastern Asia, and Western and South-eastern Europe (Adams *et al.*, 1985). Green beans as vegetables are a particularly important class in developed countries such as those of Europe and North America (Voysesst and Dessert, 1991). Green beans are classified according to many criteria but the most important are those related to their marketing and agronomic characteristics. Group classifications of green beans are also on pod colour (green and yellow or wax-podded), pod texture (fleshy, slender, or blue-lake), and pod shape (flat, oval or round) and these

classifications are based on the needs of the food processing industry (Voyses and Dessert, 1991).

By far the most important classes of beans throughout the world are dry beans with the most important production areas being Mexico, Central and most of South America and the highlands of eastern Africa where they are a preferred food because of their high protein content and storability (Voyses and Dessert, 1991).

Historically, *Phaseolus vulgaris* was the first legume in which the *Rhizobium* symbiosis was identified (Taylor *et al.*, 1983), however, conflicting evidence of the value of the symbiosis still remains. In comparison to other legumes, the species symbiotic nitrogen-fixation (SNF) potential is considered to be very low (Graham, 1981; Bliss, 1993b; Hardarson, 1993). Because of the weak performance of the symbiotic system in *Phaseolus vulgaris*; *Rhizobium phaseoli* inoculants are not widely used (Taylor *et al.*, 1983). This makes N fertilization a common practice in most areas especially where growth conditions are sub-optimal in relation to *Rhizobium* inoculation (Muller *et al.*, 1993).

One of the reasons documented for the poor performance of the *Phaseolus-Rhizobium* association is that many of the indigenous strains of *Rhizobium leguminosarum* bv. *phaseoli* which nodulate common bean, have been characterized as either poor (Moxley *et al.*, 1986), moderately effective or completely ineffective in N fixation (Hardaker and Hardwick, 1978). Research has identified high N fixing strains of *Rhizobium etli*. However, these often do not provide agronomic benefits in the field because they are excluded from the nodules of host plants by indigenous *Rhizobium* strains in the soil. The latter are often more competitive for nodulation than strains applied as inoculants (Graham, 1981).

Host-endophyte interactions have been investigated in some major field crops but only recently has common bean received attention (Hohenberg *et al.*, 1982). This leaves considerable scope for selecting and modifying the genotypes of both *Phaseolus vulgaris* and *Rhizobium phaseoli* (Sprent, 1982). To aid this, efficient strains must be matched with compatible hosts and the resulting association selected for suitable environments (Rhy and Bonish, 1981). Large differences among locations, and seasons have been found for both % N derived from the atmosphere (Ndfa) and total Ndfa. These differences were

documented as a reflection of the sensitivity of the bean/*Rhizobium* symbiosis to environmental factors that can have an enhancing or reducing effect on N fixation (Hardarson *et al.*, 1993). Where environmental factors could not be readily altered, emphasis was placed on maximizing exploitation of ecological adaptation of different components of the agricultural system (Giller and Cadish, 1995). Excellent results were achieved in the selection of both pasture legumes and effective rhizobia for acid, aluminium, rich soils of the South American savannas, described as one of the most hostile soil environments (Giller and Wilson, 1991; Thomas, 1995).

Enhancement of the N-fixing capacity of common bean is a major agronomic goal. The ability to fix N₂ is variable among genotypes of common bean and ranges from 4-59 % of plant N being derived from the atmosphere (Hardarson *et al.*, 1993; Pena-Cabriaes and Castellanos, 1993; Pena-Cabriaes *et al.*, 1993). Crop response to inoculation with selected strains of *Rhizobium* is often low, frequently due to the high competitive ability of native strains (Vasquez-Arroyo *et al.*, 1998). In Mexico (one of the centres of diversity), a large number of diverse rhizobial strains nodulate common beans (Araujo *et al.*, 1986; Pinero *et al.*, 1988). Nine gene pools make up the genetic diversity of *Phaseolus vulgaris* and three different groups of bean rhizobia nodulate them (Martinez-Romero *et al.*, 1991; Singh *et al.*, 1991). The diverse climates and environments in which these gene pools are found (Gepts and Debouck, 1991) would lead one to believe that the possibility exists for specific interactions between cultivars of beans and strains of rhizobia in relation to different climates, environments and specific locations.

This study was undertaken to determine the extent of cultivar/*Rhizobium* interaction in *Phaseolus vulgaris* under the mild temperate conditions of Canterbury, New Zealand. Cultivar/*Rhizobium* interactions on the growth, development and yield of beans were explored, as well as the identification and molecular characterization of the inoculant strains used. The nodulation and competitive abilities of inoculant strains were also explored in the hope of selecting an effective, and efficient, strain of rhizobia for use on the Canterbury plains or the wider bean producing areas of New Zealand.

1.1 Objectives of the study

The work was based on the following objectives:

1. to identify responses of different cultivars of *Phaseolus* beans to various strains of rhizobia.
2. to quantify this response in relation to irrigation, growth, development and yield.
3. to measure the effect of the interaction on N fixation in the plant.
4. to quantify the effect of N on the cultivar/*Rhizobium* interaction.
5. to compare the effectiveness of nodulation/inoculation of the different Rhizobial strains.
6. to determine N₂ fixation and competitive ability of selected strains.
7. to determine the persistence of inoculated *Rhizobium* strains, and identify the nodulating strain from field produced nodules.

This thesis is presented in eight chapters describing three major experiments, two large field experiments and one greenhouse experiment (Figure 1.1). Chapter one is a short introduction of the topic being researched. A review of previously published literature on the topic is presented in Chapter 2. Nodulation studies in the greenhouse and both field experiments are covered in Chapter 3. The influence of *Rhizobium* inoculation, cultivar and management on growth, biomass accumulation and partitioning (Chapter 4); and leaf area development, radiation interception and radiation use efficiency (Chapter 5) is examined. Chapter 6 describes yield, both green pod and dry seed yield and the yield components of the dry seed as influenced by factors discussed in Chapters 3, 4 and 5. Chapter 7 covers the isolation and identification of the nodulating *Rhizobium* strains (Chapter 3) and Chapter 8 covers the results obtained in a general discussion and the conclusions that can be drawn from the research.

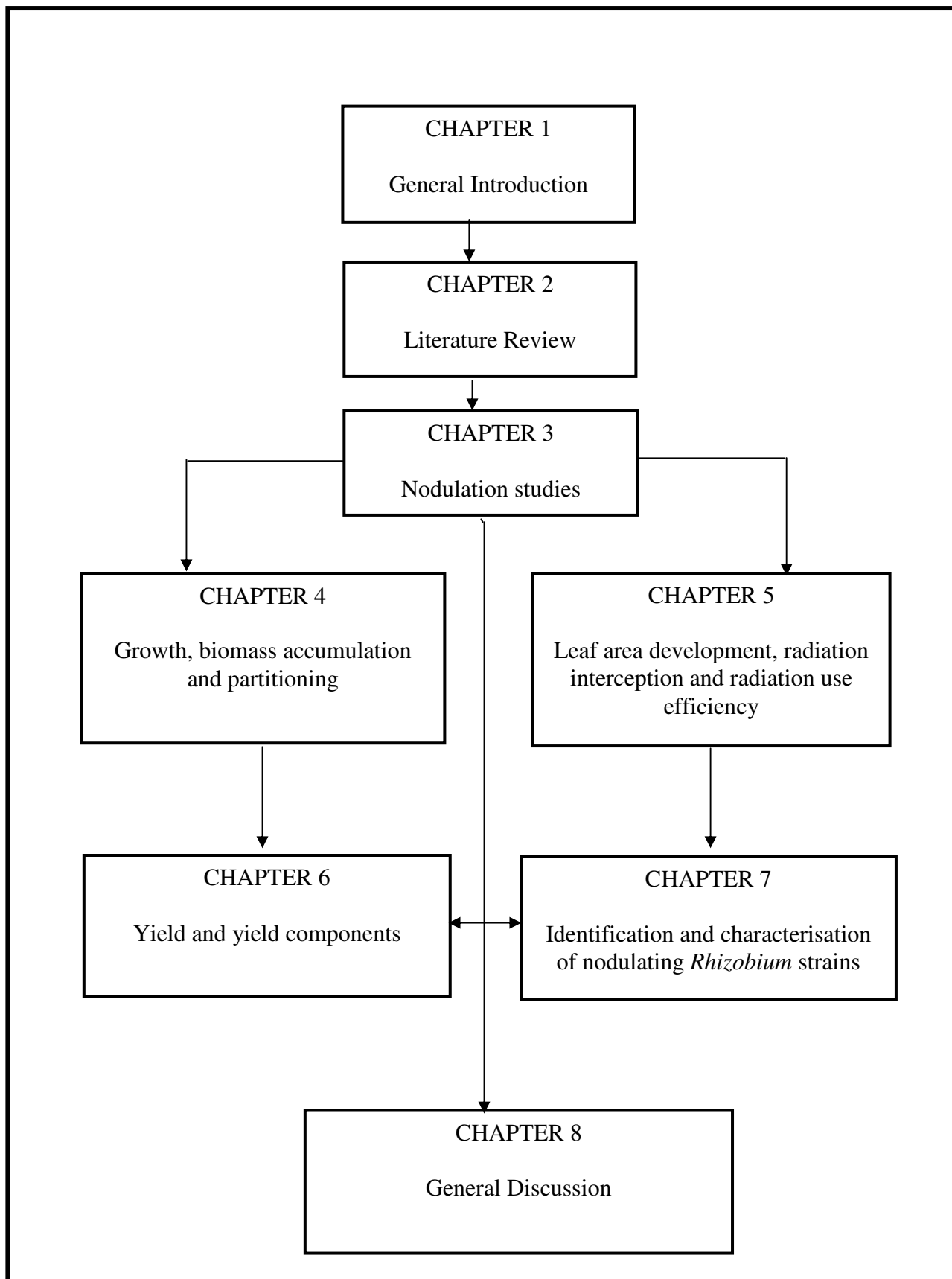


Figure 1.1. Diagrammatic representation of the relationship of each chapter.

CHAPTER 2

Literature Review

2.1 Introduction

The world population has increased approximately 1,000 fold since the beginning of agriculture (Cohen, 1995). Food supply has increased with population but concern has been expressed as to whether this parallel increase can continue with current agricultural practices (Evans, 1993). The world's food supply is dominated by 15 plant species from two families, the Poaceae (Gramineae) and Fabaceae (Leguminosae) (Egli, 1998). These species account for over 90 % of the total production of the 19 major seed crops (Egli, 1998). Three major crops, wheat (*Triticum aestivum* L.), maize (*Zea mays* L.) and rice (*Oryza sativa* L.) account for three quarters of this supply (Harlan, 1995).

The seed of wheat, maize and rice, provide carbohydrates for much of the world's population and are complemented by the pulses or grain legumes (poor man's meat) (Heiser, 1973; ICRISAT, 1991); which exhibit more variation in carbohydrate and oil concentration but have a high protein content (ICRISAT, 1991; Egli, 1998). Legumes are not only important as a source of food, feed and fuel but are also a renewable source of N, via N fixation for agriculture (Jensen and Hauggaard-Nielsen, 2003). In crop rotations they improve soil fertility and are vital for yield sustainability (ICRISAT, 1991). In addition, the legume family shows the most promise for producing the vastly increased supply of vegetable protein the world will need in the near future (National Academy of Sciences, 1979). Among the legumes, *Phaseolus* beans, of American origin, are among the most edible pulses in the world and are second only to the soybean (*Glycine max* (L.) Merr.) in the Americas (Debouck, 1991).

2.1.1 Origin and Distribution

Phaseolus vulgaris L. (the common bean) has been identified as having two centres of origin (Gepts and Debouck, 1991; Graham and Ranalli, 1997; Martinez-Romero, 2003); one in Mesoamerica yielding small-seeded cultivars and the other in the Andes, of South America, yielding large-seeded cultivars (Gepts *et al.*, 1986; Gepts, 1990; Gepts and Debouck, 1991). These centres have been separated by archaeological evidence (Kaplan

and Kaplan, 1988; Araujo *et al.*, 1998). Crop domestication originally occurred over 8,000 years ago in Mexico, Peru and Colombia (van der Maesen and Somaatmadja, 1989). This is supported by carbon-dated evidence of *Phaseolus* bean remains found in Mexican caves (Purseglove, 1974; Kaplan and Kaplan, 1988; ICRISAT, 1991). More recently, Kaplan and Lynch (1999) indicated that domestication began approximately 4,000 years ago by selection among wild *Phaseolus vulgaris* ecotypes for larger seeds and plants.

Dispersal from the Andean centre of origin of large-seeded bean types was via the Iberian Peninsula into Europe following the discovery of the Americas (Kaplan, 1965; Purseglove, 1974). Subsequent spread occurred into Africa during the slave trade and colonial period, and into the northeast of the United States of America via immigration (Graham and Ranalli, 1997). The small-seeded beans followed a route through Mexico and Central America, via the Caribbean and Northern South America to Brazil (Kaplan, 1965; Gepts, 1988b, a). Over the period of domestication and dispersal, the plant evolved from a wild vine into a major legume food crop, grown world wide, in a broad range of environments and cropping systems.

2.1.2 Uses

Common bean is used both as a pulse and as a green vegetable (van der Maesen and Somaatmadja, 1989). In both the developed and the developing world, *Phaseolus vulgaris* is consumed in many different forms (Rachie and Roberts, 1974; Silbernagel *et al.*, 1991). Developing countries are the major producers of dry beans, while developed countries produce more green beans (Rachie and Roberts, 1974). Consumption of dry beans is most frequent but other forms are also important. Seed can be consumed as immature green grain. Dehulled seed may be boiled, parched, roasted, germinated, fermented or cooked in different ways to suit specific tastes (ICRISAT, 1991). In some parts of the tropics, the young leaves are used like spinach. In temperate regions, common bean is grown mainly for green immature pods that are eaten as a vegetable, and are also canned and frozen (Purseglove, 1974; van der Maesen and Somaatmadja, 1989). Dried seeds are also cooked with tomato sauce and canned. The residual straw can be used as fodder and forage (Purseglove, 1974; van der Maesen and Somaatmadja, 1989; Silbernagel *et al.*, 1991).

2.1.3 Taxonomy and Botany

The genus *Phaseolus* belongs to the family Fabaceae which is enormous, and has worldwide distribution (Jones, 1967; Martin, 1984; Huda and Maiti, 1997). Current estimates are of 16,000-19,000 species in approximately 750 genera in this plant family. The Fabaceae is second only to the grasses (Poaceae) in economic importance and in size only the Orchidaceae and Asteraceae contain more species (National Academy of Sciences, 1979). Species of the family Fabaceae can be found in temperate zones, the humid tropics, arid zones, highlands, savannas and lowlands; there are even a few aquatic legumes.

The family Fabaceae is divided into three subfamilies the Caesalpinioideae, the Mimosoideae, and the Papilionoideae (Faboideae) (Isley and Polhill, 1980). The genus *Phaseolus* contains approximately 55 species, and belongs to the sub-tribe Phaseoline in the sub-family Papilionoideae (Isley and Polhill, 1980; Polhill, 1981). There are contrasting reports on the numbers of *Phaseolus* species and their characteristics (Kaplan, 1965; Purseglove, 1974; Graham and Halliday, 1977; Delago Salinas *et al.*, 1999). However, four agriculturally important species are normally recognised: *Phaseolus vulgaris* L. (common bean, haricot, navy, French or snap bean); *Phaseolus coccineus* L. (runner or scarlet bean); *Phaseolus lunatus* L. (Lima, sieva, butter or Madagascar bean); *Phaseolus acutifolius* Gray (tepariy bean) (Jones, 1967; Smartt, 1970; Graham and Halliday, 1977; Hancock, 2004).

Extensive botanical descriptions of the plant can be found in (Purseglove, 1974; Duke, 1983; van der Maesen and Somaatmadja, 1989; van Schoonhoven and Voysest, 1991; Graham and Ranalli, 1997).

The botanical description is:

The plant varies from being a climbing, viny or bushy, slightly pubescent annual herb ranging in height from 0.20 m to 3 m. The root system has a pronounced taproot with well-developed lateral and adventitious roots. Nodules are sometimes present and may be globular, irregular and knobbly. Stems are angular or nearly cylindrical. Leaves are alternate, trifoliate; with the petiole usually up to 15 cm long, grooved above with a marked pulvinus at the base. Basal leaflets are asymmetrical and the apical leaflet is symmetrical, usually ovate 7.5-14 cm × 5.5-10 cm. The inflorescence may be axillary or

terminal racemes with several to many white, pink, lilac or purple papilionaceous flowers. Flowers are zygomorphic with a bi-petalled keel, two lateral wing petals and a large outwardly displayed standard petal. Flower colour is genetically independent of seed colour, but an association between a particular flower and seed colour is common. The flower has ten stamens, nine fused and one free and a single multi-ovuled ovary. Flowers are predominately self-fertilised and develop into a straight or slightly curved pod. Seed may be round, elliptical, somewhat flattened or rounded elongate in shape and a rich assortment of coat colours and patterns exists. Seed size ranges from 50 mg seed⁻¹ in wild accessions collected in Mexico to more than 200 mg seed⁻¹ in some large seeded Colombian varieties. Hungria and Bohrer (2000) gave a more detailed seed description. Plants are propagated by seeds and have epigeal germination.

2.2 Environmental impact on crop phenology, growth and development

Plant growth and development are connected to the environment via a combination of linear and non-linear responses (Campbell and Norman, 1989). In some cases, as much as 80 % of the variability of agricultural production is due to variations in the weather, especially under rainfed conditions (Petr, 1991; Fageria, 1992; Hoogenboom, 2000). Agro-meteorological variables such as rainfall, soil and air temperature, wind, relative humidity or dew point temperature and solar radiation have major impacts on plants, pests and diseases (Hoogenboom, 2000). In turn, these influence crop growth and development and are some of the primary determinants of yield (Dapaah, 1997). Individual legume species or cultivars often require specific ecological niches for maximum production (Masaya and White, 1991), which should not be ignored when considering site suitability whether local, national or international (Valentine and Matthew, 1999; Rahman, 2002). In crop production, one of the first requirements is a site which is compatible with the day length, temperature and radiation requirements of the species or cultivar to be grown. A sound knowledge of the developmental and environmental factors contributing to yield and quality variation is therefore required to maximise yield of agricultural crops.

2.2.1 Phenology

Phenology is the study of the timing of biological events, the cause of their timing with regards to biotic and abiotic forces, and the interrelation among phases of the same or

different species (Shaykewich, 1995). Phenological development is described as the rate of progress through growth stages. It is controlled by temperature and photoperiod, but is mostly independent of crop management (Angus *et al.*, 1981). The life cycle of a bean plant (grain legumes) can be identified by several phenological stages (LeBaron, 1974; Maiti and Martinez-Lozano, 1997). These are emergence, vegetative growth, flowering, pod set and pod fill, physiological maturity and harvest maturity (Hadley *et al.*, 1983; McKenzie and Hill, 1989; Petr, 1991; Dapaah, 1997).

A quantitative understanding of phenological development in response to environmental factors is required to adequately predict crop yield. Chronological and calendar days are normally used to predict phenological development as days to maturity recommendations. The value of these recommendations has been marginalized because of the variability in growing environments between where the crops (seeds) were developed and where they are usually grown commercially (Greven, 2000). Many authors have described the use of photoperiod and, more importantly, the accumulated temperature or thermal time, as a more accurate method of determining the growing season (growth and development) of a crop (Summerfield and Wein, 1980; Hadley *et al.*, 1983; Husain *et al.*, 1988a; McKenzie and Hill, 1989; Summerfield *et al.*, 1994).

2.2.2 Development

2.2.2.1 Effect of temperature

Growth and development are related to temperature and can be quantified using thermal time (growing degree days or physiological day-scale). Thermal time is the accumulated temperature (T_t) above a crop specific base temperature (T_b) required between two developmental stages (Arnold and Monteith, 1974; Spitters, 1990; Gomez-Paniagua and Wallace, 1992; Gomez *et al.*, 1992). Gomez-Paniagua and Wallace (1992) used a simple formula to calculate thermal time in beans. This formula has been adopted by several authors to various New Zealand crops; for maize (Wilson and Salinger, 1994); pinto beans (Dapaah, 1997) and green beans (*Phaseolus vulgaris*) (Greven, 2000). Thermal time is calculated as:

$$T_t = \sum_{\text{Stage A}}^{\text{Stage B}} [[(T_{\min} + T_{\max})/2] - T_b] \dots \dots \dots \text{Equation 2.1}$$

where T_{\min} and T_{\max} are the mean minimum and maximum daily temperatures.

Mauromicale *et al.* (1988) reported the use of growing degree-days was a more reliable method of predicting crop development than chronological days. This was subsequently demonstrated and supported in common beans (Gomez *et al.*, 1992; Dapaah, 1997; Greven, 2000).

Temperature affects development rate, growth rate and other physiological processes, and any biochemical process in a plant has a characteristic temperature response (Masaya and White, 1991). Various developmental and growth processes in beans are affected by temperature: germination (Ladror *et al.*, 1986; Powell *et al.*, 1986); partitioning and root function (Masaya and White, 1991); and reproduction (Kemp, 1973; Marsh and Davis, 1985). Masaya and White (1991) found a bias towards reproductive growth under low temperatures and towards vegetative growth under warm temperatures. They concluded that reproductive growth predominated in cool climates and vegetative growth predominated in warm climates.

Common bean yields are usually higher in temperate than in tropical zones, although in temperate areas, common beans are grown in the warm season. Beans are intolerant of frost and a short period of exposure to 0 °C or below will kill bean tissue (Wallace and Enriquez, 1980). Soil temperature also has a drastic effect on beans with poor germination in soil cooler than 15 °C (Dickson, 1971; Kooistra, 1971). After emergence, day temperatures below 20 °C give very slow growth (Rajan *et al.*, 1973). Since increased temperature accelerates crop maturity and senescence (Khanna-Chopa and Sinha, 1987), supra-optimal temperatures are likely to shorten crop duration to the extent that dry matter (DM) accumulation and seed yield are decreased.

2.2.2.2 Effect of photoperiod

Responses to the relative duration of day and night are widely found in plants (Masaya and White, 1991). The life of a plant is critically dependent on the timing of events such as germination, flowering, seed filling, and maturity; unfortunately, many studies have included events or genetic variants where the differential response to photoperiod was chosen to be very dramatic (Masaya and White, 1991). This generated

the idea that the regulation of photoperiod events such as flowering is of an absolute nature. However, photoperiod affects the plant in more subtle and quantitative ways (Masaya and White, 1991). Adaptation and yield under specific agricultural management can also be changed by the photoperiod.

Seasonal variation in the length of day and night is important in determining the adaptation of plants to photoperiod. Night length is crucial for internal control of the differentiation of tissues and organs that constitute plant reproductive structures (Masaya and White, 1991). The rate of flower bud development strongly affects the time of anthesis and maturity. By changing the rate of flower bud development, and presumably of pod growth, temperature affects the duration of flowering and seed filling, and thus the time of maturity (Wallace, 1985). Wallace (1985) further showed that in the tropics at mean temperatures of between 19 °C and 29 °C, time of flowering across environments showed a U-shaped response curve.

2.2.3 Crop Growth

The literature often uses the term *growth and development* when describing a crop because to reach a marketable stage a crop needs to both develop morphologically and increase in weight (Wurr *et al.*, 2002). Growth and development are distinct, but related processes (Wilhelm and McMaster, 1995). However, these processes are often confused or conceived as being synonymous. Crop growth can be defined in several ways, but the most simple definition is the irreversible change in dry mass (weight) or change in size (area) or number of cells of a plant or its organs (Monteith, 1981; Gallagher *et al.*, 1983). Subsequently, crop development (phenology) can be defined as, the progress of a plant from germination to maturity through a series of stages, often measured as the numbers of nodes present (Monteith, 1981; Huda and Maiti, 1997). Photosynthesis, respiration and translocation contribute to the increase in dry mass while growth in area results from the division and expansion of cells followed by their shrinkage or death (Gallagher *et al.*, 1983; Atkinson and Porter, 1996).

Of the environmental factors that affect growth, DM accumulation and its partitioning in a common bean crop, soil and the atmospheric environment are the most important. In a production system, stresses such as drought, water logging, high and low temperatures affect crop growth and production. These are further influenced by biotic

factors and management (Huda and Maiti, 1997). It is therefore necessary to understand the influence of environmental parameters on crop growth and productivity.

2.2.3.1 Effect of water stress

In most regions where temperature is appropriate for plant growth, water is among the most limiting factors for plant production and growth rates are proportional to water availability (Pugnaire *et al.*, 1994). Because of its essential role in plant metabolism at both the cellular and whole-plant levels, decrease in water availability has an immediate effect on plant growth through processes ranging from photosynthesis to solute transport and accumulation (Hsiao *et al.*, 1976). The magnitude of the stress is determined by the extent and duration of the deprivation (Hsiao *et al.*, 1976). Plant growth is the result of cell division and enlargement and water stress directly reduces growth by decreasing CO₂ assimilation and reducing cell division and expansion (Pugnaire *et al.*, 1994). Although plant growth rates are generally reduced when soil water supply is limited, shoot growth is often inhibited more than root growth and in some cases the absolute root biomass of plants in a drying soil may increase relative to that of well-watered controls, as found in maize (Sharp and Davies, 1979).

Water stress during the vegetative phase in common beans which was severe enough to cause stunted growth did not reduce seed yield so long as there was a plentiful water during flowering (Stoker, 1974). Singh (1995) showed that in dry common beans, water stress during flowering and pod filling reduced yield and seed weight and accelerated crop maturity. Seed yield reduction in common bean was between 22 and 71 % as a result of water stress (Ramirez-Vallejo and Kelly, 1998). Yield reduction in dry common beans subjected to water stress was suggested to be caused mainly by abscission of flowers and young pods (Stoker, 1974). Adams *et al.* (1985) documented common bean response to water stress as leaf flagging, stomatal closure, and shedding of leaves, flowers and young fruits. Reductions in water use efficiency and harvest index (HI) were observed as a result of moderate moisture stress in common beans (Foster *et al.*, 1995). In the same crop, water stress reduced the number of trifoliolate leaves, stem height, the total number of main branches and the number of main stem nodes (Boutraa and Sanders, 2001a).

2.2.3.2 Effect of temperature

Growth responses are enhanced by certain diurnal temperature regimes compared with other regimes with the same mean temperature (Masaya and White, 1991). Masaya and White (1991) suggested that cool night temperatures have a greater effect on common beans based on daily mean temperatures. The temperature of maximum flowering rate (minimum days to first flower) increased with 3 °C versus 6 °C fluctuations, in 32 combinations of photoperiod and day/night temperatures in common beans (Wallace and Enriquez, 1980). This delaying of flowering effect of greater temperature differences was confirmed in other grain legumes (Wallace, 1985).

Bean seed germination is particularly sensitive to low temperatures although there are considerable cultivar differences (Masaya and White, 1991). This sensitivity has been cited as a problem in snap bean production, particularly in cool temperature regions, but not in the tropics (Masaya and White, 1991). Attempts to select for improved bean seed germination at temperatures below 15 °C have shown promise (Kooistra, 1971; Scully and Waines, 1987). Better germination was observed in large seeded beans compared to small-seeded beans at 12.5 °C (Austin and Maclean, 1972b). On the other hand, an estimated heritability of 33-41 % for cold tolerance in germination was observed for seed colour but not for seed size (Dickson, 1971).

Temperature effects on assimilate partitioning to leaves and roots may reflect effects on the rate of water uptake by roots, which cause shifts in the balance between root and shoot growth (Masaya and White, 1991). Cool temperatures markedly reduce root growth and restricted the rate of water uptake (Masaya and White, 1991). A root temperature of 5 °C reduced water uptake (McWilliam *et al.*, 1982). A temperature of 7 °C damaged floral primordia (Kemp, 1973), and 14 °C reduced seed set (Farlow *et al.*, 1979). Farlow (1981) subsequently found that these effects were due to ovule fertility and not pollen germinability. High temperature regimes of 35 °C/20 °C or 35 °C consistently reduced pollen viability compared with a 25 °C control (Halterlein *et al.*, 1980).

2.2.3.3 Effect of nodulation

Among factors contributing to plant growth, nutrient availability plays a vital role. Factors may simultaneously interact synergistically or antagonistically in the soil, in the plant or at absorption sites (Alam, 1994). In legumes, the development of effective root

nodules with the capacity for symbiotic N fixation involves a complex series of interactions between the host plant and the invading bacteria (Baird *et al.*, 1983). Many strains of bacteria have been identified, some more efficient than others at fixing atmospheric N (Whigham, 1983). Genetic variation for N₂ fixation has been identified in *Phaseolus vulgaris* (Graham, 1981; Westermann *et al.*, 1981; Rennie and Kemp, 1983b). Both growth habit and maturity responded to variation in N₂ fixation (Graham, 1981; Duque *et al.*, 1985). However, other factors such as nodule efficiency and earliness and duration of nodulation may be involved (Pacovsky *et al.*, 1984; Piha and Munns, 1987a; Chaverra and Graham, 1992).

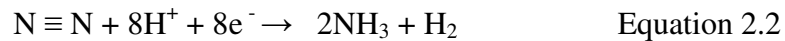
Studies with *Phaseolus vulgaris* cv. Light Red Kidney showed that nodules formed (following inoculation with a compatible strain) and fixed N until the start of reproductive development (Baird and Webster, 1982). However, with an ineffective strain, 14c, transformation to the bacteroid stage was delayed. Plant shoots were chlorotic and under N stress at flowering. Ineffective nodulation was expressed as poorly nodulated plants four weeks after inoculation (Baird and Webster, 1982). It is thought that low applications of N may stimulate N₂ fixation by improving early plant growth until N₂ fixation provides adequate N for plant growth and development (Muller *et al.*, 1993). On the other hand, fertilizer rates exceeding those exerting a “starter-N” effect, generally reduce nodulation and N₂ fixation (Kucey, 1989).

Park and Buttery, (1989) found that in common bean, nodulation is largely a function of plant size. Bigger plants produce more nodules, which in turn promoted further growth. Plant weight was positively correlated with nodule score (Park and Buttery, 1989). Rapid plant growth is directly linked to rapid N fixation in the absence of combined N (Gibson, 1987), which is directly related to rapid nodulation by effective rhizobia. Promotion of nodulation and N fixation may be of economic importance, particularly in common beans (Burdman *et al.*, 1997). Under field conditions, a greater number of active nodules can be expected to contribute fixed N and give higher yields (Burdman *et al.*, 1997).

2.3 Biological Nitrogen Fixation

Biological N fixation (BNF) is a fascinating biological phenomenon, which involves some legumes, whether grown as pulses for seed, as pasture, in agro-forestry or in natural ecosystems (Hardarson and Atkins, 2003). The process involves the reduction of

atmospheric dinitrogen to ammonia (NH₃) (Giller and Wilson, 1991). The reaction is carried out by the enzyme nitrogenase, which is found only in prokaryotes. Many N₂ fixing prokaryotes are diazotrophic, that is, they can grow using dinitrogen gas as their sole source of N while other organisms can fix N₂ only in symbiosis with another eukaryotic organism. The equation for the reaction is



At least two protons are reduced by hydrogen for each molecule of dinitrogen fixed, and because dinitrogen is very stable, the reaction has a high energy requirement. The energy required is greater than for nitrate assimilation (Mellor and Werner, 1990). However, in practice the energy cost for N₂ fixation can be borne by most field grown legumes with little loss in production (Herridge and Bergerson, 1988).

Nitrogen fixation involves a specialized and intricately evolved interaction between soil micro organisms (bacteria) and higher plants, which results in the formation of special organs, called nodules (Sessitsch *et al.*, 2002). The nodules are formed on the roots or in some cases the stems, where they harness and convert atmospheric N₂ (Bliss *et al.*, 1989; Tamimi and Timko, 2003) into a form (ammonium, nitrates and ammonia) that can be easily assimilated by the plant (Hardarson and Atkins, 2003; Prevost and Bromfield, 2003).

Globally, terrestrial BNF is estimated at between 100 and 290 million t of N year⁻¹ (Cleveland *et al.*, 1999; Jensen and Hauggaard-Nielsen, 2003). Of this, 40-48 million t is fixed by agricultural field crops (Galloway *et al.*, 1995; Jenkinson, 2001). There are extensive reviews of BNF, in various agro-ecosystems, in the literature (Giller and Wilson, 1991; Ledgard and Giller, 1995; Peoples *et al.*, 1995b; Peoples *et al.*, 1995c; Boddey *et al.*, 1997; Ladha, 1998).

Since biological nitrogen fixation makes atmospheric N₂ is a renewable resource, BNF in agricultural systems is a sustainable source of N in cropping systems (Bohloul *et al.*, 1992; Peoples *et al.*, 1995b; Peoples *et al.*, 1995c). Unlike the large amounts of fossil energy required for fertiliser N production in the Haber-Bosch process, the energy that drives BNF is virtually 'free' and is from photosynthesis. This makes BNF one of the most 'environmentally friendly' approaches to obtaining N in agro-ecosystems (Jensen and Hauggaard-Nielsen, 2003). The current international emphasis on environmentally

sustainable development with the use of renewable resources is refocusing attention on the potential of BNF in supplying N for agriculture. This combined with the contribution of BNF to the N-cycle and the fact that BNF can be controlled by manipulating physical, environmental, nutritional or biological factors (Hansen, 1994) make it an attractive management option, especially for organic growers.

2.3.1 Rhizobium

The family *Rhizobiaceae* is a group of genetically diverse and physiologically heterogeneous soil organisms collectively called rhizobia (Somasegaran and Hoben, 1994). Rhizobia is a term used to describe a range of soil bacterial genera including *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium*, and *Azorhizobium* that are able to enter into symbiosis and nodulate members of the plant family Leguminosae (Sprent, 2001; Sessitsch *et al.*, 2002; O' Hara *et al.*, 2003; Howieson and Ballard, 2004). Great variation in the specificity of interaction with rhizobia is observed among legume species. Some *Rhizobium*–legume associations are very specific and the legume will only form nodules when infected with a specific *Rhizobium*, while other legumes will form nodules with a range of rhizobia (Broughton *et al.*, 2000; Graham and Vance, 2000). Specificity involves the recognition of the bacterium by the host and of the host by the bacterium through the exchange of signals compounds, which induce differential gene expression in both partners (Broughton *et al.*, 2000).

The bacteria, which are able to form root nodules on common bean (described earlier) are classified into five species of the genus *Rhizobium*, *R. leguminosarum* biovar (bv.) *phaseoli* (Jordan, 1984) , *R. tropici* (Martinez-Romero *et al.*, 1991), *R. etli* bv. *phaseoli* (Segovia *et al.*, 1993), *R. gallicum* bvs *gallicum* and *phaseoli* (Amarger *et al.*, 1997), and *R. giardinii* bvs *giardinii* and *phaseoli* (Amarger *et al.*, 1997). Other, still unclassified but already genetically well characterised rhizobia such as *Rhizobium* sp. BR816 (Hungria and Phillips, 1993), *Rhizobium* sp. NGR234 (van Rhijn *et al.*, 1994) and *Rhizobium* sp. GRH2 (Herrera *et al.*, 1985) can also symbiotically infect *Phaseolus vulgaris*.

The distribution of rhizobia species that nodulate common bean varies among geographical locations (Mhamdi *et al.*, 2002) (for a detailed review see Amarger (2001)). *Rhizobium etli* and *R. tropici*, originally isolated in Meso - and South America respectively

are now distributed worldwide (Martinez-Romero *et al.*, 1991; Segovia *et al.*, 1993). The prevalence of *R. etli* as a common entity in bean populations has been widely reported in Europe (Sessitsch *et al.*, 1997; Herrera-Cervera *et al.*, 1999), Central and West Africa (Anyango *et al.*, 1995; Diouf *et al.*, 2000) and Central and South America (Souza *et al.*, 1994; Aguilar *et al.*, 1998). The predominance of *R. etli* strains in common and primitive beans in northwest Argentina suggest that this species may have coevolved with *Phaseolus vulgaris* in this region (Aguilar *et al.*, 1998; Aguilar *et al.*, 2004).

Strains of collections first described as *Rhizobium tropici* (Martinez-Romero *et al.*, 1991) were isolated in Brazil and Colombia. *Rhizobium tropici* is now predominant in the acid soils of France (Amarger *et al.*, 1994), and Kenya (Anyango *et al.*, 1995), but is sparsely represented in populations found in the neutral soils of Africa (Anyango *et al.*, 1995), and in Mexican and Argentinean populations (Aguilar *et al.*, 1998; Vasquez-Arroyo *et al.*, 1998). *Rhizobium leguminosarum* was most prevalent in European soils (Young, 1985; Geniaux *et al.*, 1993; Laguerre *et al.*, 1993b; Amarger *et al.*, 1994). This species was also a natural component of South American bean populations (Aguilar *et al.*, 1998). *Rhizobium gallicum* and *R. giardinii* first described in France (Geniaux *et al.*, 1993; Laguerre *et al.*, 1993b; Amarger *et al.*, 1994) are also found in Spanish bean populations (Herrera-Cervera *et al.*, 1999). *Rhizobium gallicum* was commonly found with *R. etli* in nodules of bean plants in Austrian soils (Sessitsch *et al.*, 1997). It appears from the different studies that *Phaseolus* rhizobia are very diverse at the species, intraspecies and population levels and the population of rhizobia present in the nodules of *Phaseolus* spp. are highly differentiated among sites (Amarger, 2001). Current evidence suggests difficulty in identifying factors involved in the distribution of the different rhizobial species among sites, although there is increasing evidence in the literature of parallel evolution between bacteria and the common bean plant (Aguilar *et al.*, 2004).

2.3.2 Introduction of rhizobia into the soil

Rhizobia are very widespread as a result of natural distribution and legume cultivation. In spite of this, there are still soils where strains of rhizobia specific for a legume crop are absent or are only present in low numbers (Amarger, 2001). Wherever a particular legume has long been established in an agricultural system or is considered a traditional crop, it is likely that there will be an adequate number of indigenous rhizobia for nodulation (Peoples *et al.*, 1995c). Beyond this generalisation, inoculation is the general

practice. Introduction of rhizobial strains may be desirable where soils harbour populations of rhizobia composed of a majority of strains which are symbiotically inactive with a particular legume (Hagedorn, 1978; Bottomley and Jenkins, 1983; Moawad *et al.*, 1998; Deaker *et al.*, 2004), or when better performing strains become available (Bosworth *et al.*, 1994).

2.3.3 Inoculation

Legume inoculation is an established agricultural practice that has been used for more than a century to introduce rhizobia into the soil; (for reviews on inoculation, inoculant production and use, see; Smith (1992); Somasegaran and Hoben (1994); Brockwell and Bottomley (1995); Stephens and Rask (2000) and Deaker *et al.* (2004). Inoculants are produced commercially in many countries. Their quality depends on both the number of rhizobia they contain and their effectiveness in fixing N with the target host (Amarger, 2001). Symbiotic effectiveness is one of the most important factors when selecting an inoculant strain (Date, 2000; Stephens and Rask, 2000). Other characteristics to be considered are genetic stability, ability to survive in inoculant, to persist in the soil, and to compete in nodule formation with soil rhizobia (Graham and Vance, 2000). In Brazil, Thies *et al.* (1991) found that re-inoculation of soybean guaranteed increased nodulation and nodule occupancy by the inoculated strains and increased N₂ fixation and crop yield.

Inoculants are produced in powdered, granular or liquid forms (Date, 2000; Deaker *et al.*, 2004). They can be applied directly onto seed, which is the traditional and most commonly used method of inoculation, on mineral granules (Wadoux, 1991) or into the seed bed (Hynes *et al.*, 1995). The quality of an inoculant is evaluated by the number of viable rhizobia it contains (Hiltbold *et al.*, 1980; Brockwell and Bottomley, 1995; Stephens and Rask, 2000). Quality is highly variable and a number of inoculants presently on the market, in countries where quality controls are not systematically practiced, are of poor quality (Catroux *et al.*, 2001). However, high quality inoculants are produced and are available in powdered or liquid form in North America, Europe, Australasia and some other countries. For soybean, storage at room temperature for up to a year would provide as many as 10⁶ viable rhizobia seed⁻¹. High quality inoculants can provide approximately 2 x 10¹¹ to 4 x 10¹¹ rhizobia ha⁻¹. This is about 1 % of the indigenous population of rhizobia present in the top 20 cm of soil (Catroux and Amarger, 1992).

Legume seeds may be inoculated by farmers immediately prior to sowing or custom inoculated by local seed merchants with coating facilities to be sown within a week (Deaker *et al.*, 2004). Inoculation techniques used in legumes are highly variable. For fundamental inoculation practices see Table 2.1.

Table 2.1. Legume inoculation techniques. (Deaker *et al.*, 2004)

Technique	Description
<u>Seed inoculation</u>	
Dusting	Peat inoculant is mixed with the seed without rewetting
Slurry	Seed is mixed with a water solution of peat often with the addition of an adhesive
Lime or phosphate pelleting	Seed is treated with a slurry peat inoculant followed by a coating of calcium carbonate (superfine limestone) or rock phosphate
Vacuum impregnation	Rhizobia is introduced into or beneath the seed coat under vacuum
<u>Soil inoculation</u>	
Liquid inoculation	Peat culture mixed with water or liquid inoculant applied to the seedbed at the time of sowing (liquid inoculants may also be applied to the seed)
Granular inoculation	Granules containing inoculum sown with seed in the seedbed.

Seed inoculation immediately prior to sowing is by far the most popular method used (Graham, 1981; FAO, 1993; Hungria *et al.*, 2003), but there is a growing demand for pre-inoculated seeds (seeds commercially inoculated and stored prior to sale) since its inception in 1971 in Australia (Deaker *et al.*, 2004). This seed coating technology was questioned after subsequent tests in 1972-1974 and 1999-2002 which revealed poor survival of the rhizobial inoculum (Deaker *et al.*, 2004). Other methods of inoculum

application, which involves the dusting of seed with peat culture results in the removal of most of the inoculum as the moist peat dries out (Gault, 1978; Deaker *et al.*, 2004). However, the addition of adhesives to the peat inoculant results in the retention of more peat on the seed coat and prevents the sloughing-off of the coating material and reduces damage to the cotyledons (Brockwell, 1977).

Inoculation of annual crop legumes has produced variable results when different inoculation methods were used. Granular seed inoculation gave increased seed yield compared with peat-based seed inoculants in dry peas (*Pisum sativum* L.) (McKenzie *et al.*, 2001; Clayton *et al.*, 2004a, b) and in chickpea (*Cicer arietinum* L.) (Kyei-Boahen *et al.*, 2002; Gan *et al.*, 2005). In common bean, the most common method of inoculation is to apply the culture of *Rhizobium* spp. to seed prior to sowing (Graham, 1981; Hungria and Vargas, 2000; Hungria *et al.*, 2003). An alternative method is application of the inoculant to the soil at the time of sowing. Soil application of inoculum has been successful in the annual legumes; soybean (Danso *et al.*, 1990) and field peas (*Pisum sativum* L.) (McKenzie *et al.*, 2001). In respect of the method used, the goal of inoculation is to supply a minimum of 10,000 rhizobia seed⁻¹ for small legumes such as clovers (*Trifolium* spp.) and increasing to 1 million rhizobia seed⁻¹ for large-seeded legumes such as soybean (Lupwayi *et al.*, 2000).

2.3.4 Soil colonisation

The ability of a legume crop to benefit from atmospheric N fixation will depend on the presence in the soil of rhizobia which are able to nodulate the legume and to form an effective N fixing symbiosis with the host plant under field conditions (Amarger, 2001). The formation of nodules on host plants by *Rhizobium* provides the location and the physiological environment for symbiotic N fixation. Inoculation provides the rhizobia necessary for soil colonisation and subsequent nodulation if rhizobia are not present in the soil.

Once applied to soil, *Rhizobium* from inoculants remain relatively immobile and, as a consequence, application on or with the seed, at sowing, results predominantly in crown and tap root nodulation (Hardarson and Atkins, 2003). The colonisation and survival of the inoculated rhizobia in the soil depends on how the strain responds to or resists the prevailing soil conditions. Multiple biotic and abiotic factors can affect the persistence of

rhizobia in soil (van Veen *et al.*, 1997; Sadowsky and Graham, 1998). Indigenous rhizobia vigorously compete with inoculant strains and frequently are more successful in nodulation (Ham, 1980; Carter *et al.*, 1995). Although in these cases inoculation has failed, it does not mean that the introduction of the rhizobia into the field has also failed (Amarger, 2001).

When soils are devoid of rhizobia, the inoculant strain nodulates the host legume and multiplies in the nodules (Amarger, 2001), the nodule represents an environment akin to a pure culture and great multiplication of rhizobia occurs. Subsequent nodule senescence and breakdown, results in the release of high numbers of viable cells into the soil (Kuykendall *et al.*, 1982; Moawad *et al.*, 1984; Thies *et al.*, 1995; van Rhijn and Vanderleyden, 1995). The released bacteria are a potent source of infection for subsequent crops and usually become persistent in the soil micro flora.

2.3.5 Host specificity and nodulation

The symbiosis between legumes and rhizobia leads to the formation of N₂ fixing root nodules. This symbiosis is specific and each strain has a characteristic host range as seen in (Table 2.2) (van Rhijn and Vanderleyden, 1995). Some strains have a very narrow host range as with *Rhizobium leguminosarum* bv. *trifolii*, while others like *Rhizobium* sp. strain NGR234, have a very broad host range. It is becoming increasingly clear the symbiotic relationship between legume species and rhizobia is quite complex as in the case of *Rhizobium* sp. strain NGR234 which has nodulated at least 35 different legume genera as well as the non-legume *Parasponia* (van Rhijn and Vanderleyden, 1995). *Phaseolus vulgaris* has been shown to nodulate when inoculated with *Azorhizobium caulinodans* (van Rhijn and Vanderleyden, 1995). *Phaseolus vulgaris* is a promiscuous host, and is nodulated by a range of rhizobia, including isolates taken from *Leucaena* sp. and other tropical legumes (Mhamdi *et al.*, 1999).

Table 2.2. Some *Rhizobium*-plant associations. (van Rhijn and Vanderleyden, 1995).

<i>Rhizobium</i>	Species	Biovar	Host plant(s)
<i>Rhizobium</i>	<i>leguminosarum</i>	<i>trifolii</i>	<i>Trifolium</i> (clover)
		<i>viciae</i>	Field pea/faba bean
	<i>meliloti</i>		<i>Medicago</i> (alfalfa)
	<i>loti</i>		<i>Lotus</i>
	<i>ciceri</i>		<i>Cicer</i> (chickpea)
	<i>fredii</i>		<i>Glycine</i> (soybean)
	sp. NGR234		Tropical legumes, <i>Parasponia</i> (non legume)
	<i>tropici</i>		Common bean <i>Leucaena</i>
	<i>etli</i>		Common bean
	<i>Bradyrhizobium</i>	<i>japonicum</i>	
<i>elkanii</i>			<i>Glycine</i> (soybean)

The host specificity of strains belonging to bv. *phaseoli* of *Rhizobium leguminosarum*, *R. etli*, *R. gallicum* and *R. giardinii* is restricted to plants of the genus *Phaseolus* (Amarger, 2001). *Rhizobium tropici*, *R. gallicum* bv. *gallicum*, and *R. giardinii* bv. *giardinii* have wider host ranges that include *Leucaena* sp. in addition to common bean (Amarger, 2001). Under laboratory conditions common bean is highly promiscuous and nodulates with many rhizobia both classified and unclassified (Michiels *et al.*, 1998) which, in most cases, form ineffective nodules. Field nodulation of common bean is often sparse and nodules may be absent from the roots of field-grown beans (Graham, 1981). However, soil populations of common bean rhizobia are commonly in the range 10^2 - 10^6 g⁻¹ of soil (Anyango *et al.*, 1995; Aguilar *et al.*, 1998).

2.3.6 Nodulation

Root infection is a precursor to nodulation which is a multi-step process that involves specific plant and bacterial gene expression (Hungria and Stacey, 1997). Before nodulation begins, the two partners; *Rhizobium*, the micro symbiont, and a host plant, the macro symbiont each exist as individual organism (Laeremans and Vanderleyden, 1998). The process starts with the multiplication of the bacteria in the rhizosphere (Becker *et al.*,

1991). Before infection can proceed, the plant and compatible rhizobia must recognise each other, and the rhizobia must colonise the root surface and attach themselves to the root hairs (Sprent, 2001).

Infection and nodule organogenesis occur simultaneously during root nodule formation. During the course of this interaction, the invading rhizobia attached to the emerging root hairs release Nod factors that induce a pronounced curling of the root hair cells (Laeremans and Vanderleyden, 1998). The cells most susceptible to infection are those located just above the region of root elongation (Bhuvaneshwari *et al.*, 1980). The rhizobia become enclosed in a small compartment formed by the curling of the root hair and stimulate non-dividing root cortical cells to divide and form a distinct area in the cortex, called a nodule primordium from which the nodule develops. The infection and differentiation of the cortical cells causes the induction of a series of plant genes to provide functions for the developing nodules (Hungria and Stacey, 1997).

The infection thread with proliferating rhizobia elongates through the root hair towards specialised cells in the developing nodule where it fuses with the plasma membrane, divides and begins to branch. Branching of the infection thread enables the bacteria to infect many cells (Mylona *et al.*, 1995). The bacteria then stop dividing and begin to enlarge and differentiate into N fixing endosymbiotic organelles called bacteroids. The infection process requires and is regulated by a range of molecular signals between the bacterium and the host plant (Mylona *et al.*, 1995).

The nodule as a whole develops such features as a vascular system (which facilitates the exchange of fixed N produced by the bacteroids for nutrients contributed by the plant) and a layer of cells to exclude O₂ from the root nodule interior. The nodule meristem contributes to the shape of the nodule, with some temperate legumes like peas having nodules which are cylindrical and elongated while the nodules of tropical legumes such as soybean, common bean and peanut (*Arachis hypogaea* L.) lack a persistent meristem and are spherical (Rolfe and Gresshoft, 1988). Rhizobia generally enter the plant through root hairs except in genera like *Arachis* and *Stylosanthes* where they enter through the sites of lateral root emergence (Kijne, 1992).

2.4 Amount of nitrogen fixed

2.4.1 Methods of estimating nitrogen fixed

Accurate measurement of symbiotic biological N fixation in legumes is important to improve the fixation efficiency and determine its contribution to an agricultural system (Rennie and Kemp, 1983a; Wani *et al.*, 1995). Many methods to estimate N fixation under field conditions have been developed. These methods include the DM yield method, the N-difference method, the N-fertiliser equivalence method, the ureide method, the acetylene reduction assay (ARA) and the ^{15}N isotope method. Full descriptions including the advantages and disadvantages of the various methods are reviewed by Bergerson (1980); LaRue and Patterson (1981); Hardarson and Danso (1993); Hansen (1994); Chalk and Ladha (1999) and Unkovich and Pate (2000).

Considerable effort towards the establishment of a better understanding of the advantages and limitation of the various techniques has been ongoing (Chalk, 1985; Boddey *et al.*, 1995; Danso, 1995; Chalk, 1996). All of the methods have limitations. To obtain accurate measurements of N fixation in the field, the limitations of each method must be recognised and procedures used to reduce their effect on the calculation of symbiotic activity. Thus, method choice will depend on the environmental conditions, the purpose of the experiment and the available resources.

Total N accumulated is the simplest estimate of the amount of N fixed. However, it overestimates N fixation because it assumes that the crop derives all its N from BNF (LaRue and Patterson, 1981). Under conditions where yield is limited by soil N, the DM produced by a legume is positively correlated with the amount of N fixed (Hardarson and Atkins, 2003). However, where soil N levels are significant, analysis of N in an accompanying non- N_2 fixing crop (for example a cereal or fix⁻ (ineffective symbiont) mutant) is needed to provide an estimate of the actual amount of fixed N in the legume “by subtraction” (Hardarson and Atkins, 2003). The number and mass of legume nodules may provide a rough guide to the level of N fixed (Hardarson and Atkins, 2003). Visual appraisal of the leghaemoglobin (degree of red pigmentation) in nodules provides a relative indication of their effectiveness (Unkovich and Pate, 2000; Hardarson and Atkins, 2003).

The ^{15}N isotope dilution technique provides a direct and precise measure of N fixation (St. Clair *et al.*, 1988; Danso, 1995; Unkovich and Pate, 2000). This method is expensive partly because the fertilisers used are enriched above normal atmospheric levels of ^{15}N (St. Clair *et al.*, 1988). Plant material is analysed for ^{15}N content and the dilution of the ^{15}N enriched fertiliser by ^{14}N derived from atmospheric N relative to that of the non-N-fixing plant is used to calculate the proportion of legume N coming from N fixed from the atmosphere (Unkovich and Pate, 2000). The ^{15}N method gives an integrated measure of biological N fixation, can be applied directly in the field and can distinguish fixed N from soil-derived N.

The acetylene reduction assay (ARA) measures nitrogenase activity. In recent years it has been widely criticised as being unreliable for quantitative field studies (Unkovich and Pate, 2000). It involves the incubation of detached nodules or nodulated root pieces with 10 % acetylene in a closed container of known volume (Peoples and Herridge, 1990; Hansen, 1994). The concentration of accumulated ethylene (reduction of acetylene to ethylene by nitrogenase) is then measured by gas chromatography (Hardy *et al.*, 1968; Hardy *et al.*, 1973; LaRue and Patterson, 1981). There is general agreement that this technique is not valid for quantifying N fixation but has other qualitative uses (Danso, 1995) such as establishing the time of the onset of N fixation or for screening rhizobia for ineffective strains to screening large collections of plants in plant breeding programmes for increasing crop BNF. It can be used as a standard method for screening potential non-N fixing crops for use as reference plants to estimate BNF by the ^{15}N soil labelling method.

The ureide or xylem exudate method is simple, inexpensive and very useful for comparing the N fixing abilities of different plant genotypes. This method only applies to tropical legumes such as soybean and field peas. The main drawback of the method is that each field measurement reflects N fixation by the crop at, or shortly before, the time of the assay (Unkovich and Pate, 2000). It is clearly unwise to extrapolate from a single measurements to predict % Ndfa (N derived from the atmosphere) for the whole growth period (Hansen *et al.*, 1993; Aveline *et al.*, 1995). In two samples taken for ureide analysis, one at early flowering (R2-R3) and one at seed fill (R6); the total N fixation was calculated by adding the amounts of N fixed for the periods sowing to flowering, and flowering to seed fill. This approach did not produce the most accurate estimate of time-integrated N fixation because of limited sampling frequency. However, it did offer a

useful means of cost effectively generating reliable data (Peoples *et al.*, 1995a). The method is limited to N fixing plants that produce ureide. This includes more than 25 tropical legumes in which the ureide:amide balance in the xylem sap offers promise in the estimation of symbiotic dependency (Peoples and Herridge, 1990). Common grain legumes such as soybean, cowpeas and common beans produce ureides which transports nitrogen in plants (Unkovich and Pate, 2000).

The N fertiliser equivalence method is simple and tends to overestimate N fixation (LaRue and Patterson, 1981). Nitrogen fertiliser in the soil is susceptible to volatilisation and leaching (LaRue and Patterson, 1981) and microbial immobilisation (Ledgard, 1989). The plants ability to recover fertiliser is reduced by these processes. In addition, fertiliser recovery is not linear with increased rates of fertiliser N. This gives erroneous estimations of biological N fixation. Nevertheless, the technique can provide a site-related estimate of the potential economic value of a legume in a rotation in terms of increased production and/or savings of fertiliser N (Peoples and Herridge, 1990).

The ^{15}N natural abundance (NA) technique relies on the slight natural enrichment of ^{15}N that is observed in many agricultural soils, relative to atmospheric N (Unkovich and Pate, 2000). Its principle advantage is that there is no requirement to add N, making it applicable at a farm, or even a landscape level, rather than on a plot basis which constrains the isotope dilution technique. The technique has recently been used in grain legumes such as narrow-leafed lupin (*Lupinus angustifolius* L.) (Unkovich *et al.*, 1994), field pea (Armstrong *et al.*, 1994) and chickpea (Doughton *et al.*, 1995; Herridge *et al.*, 1995). However, problems may arise when N fixation by the legumes is relatively low (Hansen, 1994).

In *Phaseolus vulgaris* quantification of N_2 fixation has been limited by the presence of indigenous, sometimes ineffective, *Rhizobium leguminosarum* biovar *phaseoli* in most soils used for bean production, the lack of non-nodulating isolines of test cultivars, and problems caused by differences in N uptake rates and rooting patterns between beans and reference crops (Henson, 1993).

2.4.2 Amount of nitrogen fixed

The amount of N fixed by most legumes is regulated by environmental and management constraints to plant growth associated with soil nutrients, water supply,

diseases and pests (Peoples and Herridge, 2000). Legumes commonly fix around 20-25 kg of shoot N for every tonne of shoot DM accumulated over a range of environments unless their capacity to fix N is restricted by local practices which either limit the presence of effective rhizobia (no inoculation, poor inoculant quality), lack of rhizobia or directly affect soil N fertility (excessive tillage, extended fallows, fertiliser N), and rotations (Peoples *et al.*, 2002). Legume roots may contribute up to 0.7 kg of fixed N accumulated in the DM of the shoots (Peoples and Herridge, 2000).

Nitrogen fixation rates similar to those of other legumes can be obtained from nodulated common bean under controlled conditions (Giller, 1990). However, field studies suggest that it has poor symbiotic fixation potential and biological N fixation is often unable to provide sufficient N for maximum yield (Huntington *et al.*, 1986; Buttery *et al.*, 1987; Piha and Munns, 1987b). Another factor limiting bean yields and N fixation rates is the poor availability of phosphorus (P) in soils in which beans are grown (Graham, 1981; Hungria *et al.*, 2003); P is an important element in nodule metabolism (Bethlenfalvay and Yoder, 1981).

The amount of N₂ fixed by grain legumes is controlled by two major factors (a) the proportion of plant N derived from symbiotically fixed N (P_{fix}), and (b) the amount of N accumulated during growth (Ladha, 1995; Peoples *et al.*, 1995c). Therefore, strategies which influence either the N derived from the atmosphere or legume growth will affect the total input of fixed N. There is a range of experimentally derived values for N₂ fixation, by legumes in the literature (Table 2.3). These values reflect the inherent capacity of legumes to fix and accumulate N, although the values are a function of experimental treatments and environmental and nutritional variables (Peoples *et al.*, 1995b; Peoples *et al.*, 1995c). Notwithstanding these variables, the crops can generate a large range of P_{fix} (0-98 %) and inputs of fixed N. The values in (Table 2.3) show the potential biological N fixation of legume crops to be 200 to 300 kg N ha⁻¹ per crop. The contribution of fixed N to the agricultural production system will be further reduced if legume crop residues are removed from the field for use as animal fodder, organic mulch or fuel, or if the trash and stubble remaining after seed harvest are burnt (Wani *et al.*, 1995).

Although values approaching the theoretical limits may be achieved under optimal conditions (high legume yield and low soil nitrate), in practice, levels of N fixation in farmers' fields are often only a fraction of potential fixation (Peoples *et al.*, 1995b; Peoples

et al., 1995c). Several ecological constraints can limit N₂ fixation by legumes. Identifying these constraints and the processes responsible for limiting N₂ fixation is important for both improved agronomic practices and crop breeding strategies.

Table 2.3. Experimental estimates of the proportion (P_{fix}) and amount of N₂ fixed by important pulses and legume oilseeds. (Peoples *et al.*, 1995b) and (Wani *et al.*, 1995)

Species	P _{fix} (%)	Amount of N ₂ fixed (kg ha ⁻¹)
Cool season legumes		
Chickpea (<i>Cicer arietinum</i>)	8-82	3-141
Faba bean (<i>Vicia faba</i>)	64-92	53-330
Lentils (<i>Lens culinaris</i>)	39-87	10-192
Lupin (<i>Lupinus angustifolius</i>)	29-97	32-288
Pea (<i>Pisum sativum</i>)	23-73	17-244
Warm season legumes		
Black gram (<i>Vigna mungo</i>)	37-98	21-140
Common bean (<i>Phaseolus vulgaris</i>)	0-73	0-125
Cowpea (<i>Vigna unguiculata</i>)	32-89	9-201
Green gram (<i>Vigna radiata</i>)	15-63	9-112
Groundnut (<i>Arachis hypogaea</i>)	22-92	37-206
Pigeon pea (<i>Cajanus cajan</i>)	10-81	7-235
Soybean (<i>Glycine max</i>)	0-95	0-450

2.5 Factors affecting nitrogen fixation

Rhizobium sp. survival in soil is influenced by a combination of factors including acidity (aluminium (Al) toxicity), salinity, alkalinity (high concentrations of calcium (Ca) and boron (B)), soil temperature, moisture, fertility - nutrient deficiencies and soil structure (Slattery *et al.*, 2001). In addition, rhizobia must compete with other soil organisms, including other bacteria, fungi and protozoa.

2.5.1 Soil acidity

Soil acidity is a major chemical imbalance affecting millions of hectares of agricultural land (Edwards *et al.*, 1991; von Uexkull and Mutert, 1995; Date, 2000). Much of this area is naturally acidic, although agricultural practices are further acidifying the soil. Acidity related factors (high Al, low Ca and low P) have a direct impact on rhizobia growth and persistence, on nodule initiation and N fixation effectiveness (Coventry and Evans, 1989). Limitations on *Rhizobium* survival, persistence in soil and subsequent root

colonization, infection and nodule activity, due to soil acidity, have been documented (Munns, 1968; Graham *et al.*, 1982; Brockwell *et al.*, 1991).

Soil pH is a crucial factor in *Rhizobium* spp. survival. In some situations, rhizobia strains differ in their ability to infect the host plant (Brockwell *et al.*, 1995). Recent developments in new pulse and pasture legumes, particularly in Australia, have emphasized the need for the selection of compatible *Rhizobium* spp. to optimize legume production (Slattery *et al.*, 2001). For example the chickpea-*Rhizobium* symbiosis is highly specific. As a consequence, rhizobial survival in soil and growth in the rhizosphere is most important. Working with strains of *Mesorhizobium cicer*, adapted to acid conditions, and chickpea, Rai (Rai, 1991), demonstrated that only 5 % of strains tested were suitable for nodulation and growth in strongly acidic environments. Poor *Rhizobium leguminosarum* bv. *viciae* persistence in acid soils has been shown and is reflected in low nodulation scores and poor plant growth. Some strains were more tolerant of acidic soils than others (Carter *et al.*, 1995).

2.5.2 Temperature

As well as having rhizobial strains which are suited to survival under different environmental conditions, rhizobial growth rate under such conditions must be considered (Howieson *et al.*, 1995). *Phaseolus vulgaris* has relatively specific temperature tolerances (17-40 °C) (Hungria and Vargas, 2000). The survival and multiplication of the bacteria in the soil is influenced by temperature. Excessively high and low temperatures depress bacterial growth and N fixation (Dart *et al.*, 1976; Graham, 1981). Reduction in the root zone temperature below optimal temperatures (17-25 °C) reduced N fixed and delayed the onset of N fixation in soybean (Boddey *et al.*, 1995; Montanez *et al.*, 1995). *Rhizobium leguminosarum* bv. *trifolii* showed a seasonal decline in numbers as soil dried and air temperatures increased (Chatel and Parker, 1973b; Evans *et al.*, 1988).

In tropical soils, the upper limits for rhizobial growth are between 32 and 47 °C, although tolerance varies among species and strains (Day *et al.*, 1978; Karanja and wood, 1988; Da Silva *et al.*, 1993; Hungria and Vargas, 2000). Hungria and Stacey (1997) showed that a difference of 6 °C at 5 cm soil depth reduced the soybean *Bradyrhizobium* population by more the 10,000 cells g⁻¹ of soil. Because high temperatures decrease

rhizobial survival and establishment in tropical soils, repeated inoculation of grain legumes and higher rates of inoculation may be required (Hungria and Vargas, 2000).

The exchange of molecular signals between the host plant and rhizobia is also affected by high temperature. At 39 °C, the release of nod-gene inducers from soybean and common bean was decreased (Hungria and Stacey, 1997; Hungria and Vargas, 2000). Temperature also affects nodule function (Piha and Munns, 1987a), nodule initiation, rhizobia release from the infection thread and bacteroid development (Roughley, 1970) and accelerates nodule senescence (Hungria and Franco, 1993).

2.5.3 Soil moisture and water supply

Soil moisture status has a major impact on nodulation and N fixation in legumes. Nodulation is affected by moisture stress through reduced rhizobial motility in the soil, insufficient rhizobia multiplication in the rhizosphere, reduced formation of infection threads and retarded nodule development (Sprent and Zahran, 1988; Worrall and Roughley, 1991; Griffith and Roughley, 1992). Water shortage can compromise both plant and rhizobial growth, and is a major cause of nodulation failure and low N₂ fixation (Hungria and Phillips, 1993). The loss of more than 20 % of a nodule's fresh weight can permanently terminate nodule activity (Pena-Cabriales and Castellanos, 1993). However, symbiosis sensitivity depends, to a large extent, on the bacterial strain (Griffith and Roughley, 1992).

Optimum soil moisture for symbiotic N₂ fixation occurs at near field capacity (Sprent, 1972). Increasing, or decreasing, soil water can greatly affect the rate of N₂ fixation because of inadequate aeration in saturated soil and reduced water availability in dry soils at critical times during development and growth (Sall and Sinclair, 1991; Pena-Cabriales and Castellanos, 1993; Peoples *et al.*, 1995b). Growth rates of rhizobia may be slow in situations of higher early soil moisture. In a drying, acid soil, soil moisture limitations can reduce nodulation and plant growth by reducing root growth and *Rhizobium* survival in the upper soil profile (Davey *et al.*, 1989; White and Molano, 1994). The availability of nutrients such as P is restricted in an acid, drying soil, which severely restricts root growth (Davey *et al.*, 1989). Restricted root growth limits infective sites for *Rhizobium* on the roots thus affecting nodulation and plant growth (Davey *et al.*, 1989). Novel irrigation management strategies have been developed (saturated soil culture) for

common beans (White and Molano, 1994), which have the potential to improve plant growth and N fixation. However, most grain legumes are grown under natural rainfall without irrigation (Peoples *et al.*, 1995b).

2.5.4 Rhizobial competition

Naturally occurring *Rhizobium* often exist at populations of 1.0×10^4 to 1.0×10^7 cells g^{-1} of soil (Brockwell *et al.*, 1995). Where naturalized rhizobia are few or absent the introduction of new strains by seed or soil inoculation is normally successful, provided due care is exercised (Brockwell *et al.*, 1995). On the other hand, where large populations of naturally occurring rhizobia exist, inoculation is invariably futile. At intermediate levels of naturalized rhizobia (10-1,000 g^{-1} of soil) competition between naturalised and introduced rhizobia for nodule formation is of practical concern (Brockwell *et al.*, 1995). To alleviate this, Brockwell *et al.* (1995) and Howieson (1999) proposed certain desirable characteristics when selecting rhizobial strains suitable for use as inoculants:

1. Ability to colonise the soil and tolerate environmental stresses.
2. Ability to compete for nodule formation with background population of rhizobia.
3. Ability to form effective nodules that fix N and to have no deleterious effects on non-target hosts.

Strains of rhizobia differ widely in their ability to survive, nodulate and fix N in the soil environment, thus strain selection plays a major part in its survival and infective ability (Brockwell *et al.*, 1995; Howieson, 1999). Additionally, population density, effectiveness and competitive ability are primary characteristics of indigenous rhizobial populations that affect inoculation responses (Thies *et al.*, 1991).

2.5.5 Chemical residues in the soil

Intensified cropping is often accompanied by increased reliance on chemicals for weed, pest and disease control. These chemicals whether used as a seed dressing or in pre and post emergent crop situations can decrease rhizobial growth, nodulation, N fixation and yield in legumes (Kooistra, 1971; Durgesha, 1994; Martinez *et al.*, 1996) . In glasshouse studies, Eberach and Douglas (1989) demonstrated that the herbicides Paraquat and Amitrole had a toxic effect on the growth, nodulation and nitrogenase activity of

subterranean clover (*Trifolium subterraneum* L.). Their studies illustrated that understanding the mode of action and management of herbicides can have a great influence on the survival and persistence of inoculated *Rhizobium*.

2.5.6 Crop sequence and mechanisation

Rhizobia survival and infectivity is also affected by crop and pasture rotation, as well as the length of the cropping phase, together with soil chemical properties (Slattery *et al.*, 2001). Mechanical cultivation causes soil disruption, which prevents the rapid colonisation of soil by *Rhizobium*. Cultivation also causes rapid oxidation and decomposition of soil organic matter, exposing *Rhizobium* spp. to a less active carbon pool for their energy needs leaving them more vulnerable to adverse conditions (Coventry *et al.*, 1985). Rosenberg *et al.* (1967) showed that in *Phaseolus vulgaris* nodulation was poor in compacted fields which gave poor plant growth and low yields.

In *Lupinus angustifolius*, the number of rhizobia declined after a cereal crop but was sufficient to enable adequate nodulation for subsequent lupin crops up to 6 years after initial lupin inoculation (Slattery and Coventry, 1989). Decomposing canola (*Brassica napus* L.) residues release biocidal compounds from their roots, which influence the survival of rhizosphere micro-organisms in particular, soil borne pathogens (Augus *et al.*, 1994). These compounds can also affect beneficial soil micro-organisms. Muehlchen *et al.* (1990) showed that nodulation of field pea was reduced following a mustard (*Brassica juncea* (L.) Czern) crop, which may relate to a reduced ability of the *Rhizobium* to survive in the soil.

2.5.7 Fertiliser and soil nitrogen

Mineral N can influence N₂ fixation and nodulation in various ways. The quantity of accessible mineral N determines whether it is stimulating or detrimental to N₂ fixation (Hansen, 1994). Large quantities of inorganic N in the rhizosphere generally inhibit N fixation (Vance *et al.*, 1988), by limiting the development of the *Rhizobium*-legume symbiosis (Dazzo and Truchet, 1884; Kijne, 1992) and reducing N fixation in already formed nodules (Beringer *et al.*, 1988).

If plant available soil N is restricted, legumes tend to undergo a 'N hunger period' (Pate and Layzell, 1990), which can retard development and may reduce yield. This may

be the reason why between 70 and 100 % of pulse and oilseed legume crops are routinely fertilised with “starter N” (Food and Agriculture Organisation, 1992). Small amounts of starter N tend to increase BNF (Becker *et al.*, 1991) but further increases in soil N decrease BNF, which may approach zero at high levels of soil N (George and Singleton, 1992; Sanginga *et al.*, 1996). The level of available soil N influences infection, nodule development, the rate of N₂ fixation and nodule senescence (Hardarson and Atkins, 2003).

Common bean has a high physiological demand for N, and the question has arisen as to how well they can use plant available soil N or symbiotic N₂ fixation to meet their N demand (Gan *et al.*, 2003). Available soil N is efficiently accumulated by common bean, and the nitrogenase activity of the *Rhizobium* bacteria is easily inhibited in the presence of mineral N (Kato *et al.*, 1997). Therefore, plant available soil N reduces the demand for symbiotically fixed N₂. This influence of plant available soil N is greatest during early vegetative growth, where N₂ fixation by common bean is very low (George and Singleton, 1992; Arima, 1993). Nitrogen fertilization has been shown to have a positive effect on the growth and yield of common beans (Rennie and Kemp, 1983a; Kucey, 1989; Muller and Pereira, 1995). It stimulated increased growth and the amount of N₂ fixed.

2.6 Dry matter accumulation and yield

Growth, accumulation of DM and its partitioning in grain legumes is strongly influenced by a number of key factors including genotype (Saxena and Hawtin, 1981), soil edaphic factors (Adams *et al.*, 1985), the amount of solar radiation intercepted (Monteith, 1977, 1981), water stress (Saxena and Hawtin, 1981) and the photo-thermal regime (Monteith, 1981; Gallagher *et al.*, 1983; Masaya and White, 1991; Dapaah, 1997). Dry matter accumulation in common bean plants is a direct result of the balance among photosynthesis, respiration, and losses caused by senescence and abscission. Partitioning establishes an equilibrium between vegetative and reproductive growth, which is integrated during common bean development, resulting in the end product of yield (Gallagher *et al.*, 1983; White and Izquierdo, 1991).

The rate of DM accumulation in many species is represented by a sigmoid growth curve (Herdina and Salisbury, 1990). This curve is characterized by 3 stages:

1. a period of slow growth up to the start of flowering.

2. a period of near constant growth over several development stages including stem elongation, pod formation, and early seed filling.
3. a cessation of growth during the later part of seed filling (Herdina and Salisbury, 1990).

Growth rates in chickpea averaging between 9.0-13.1 g DM m⁻² d⁻¹ were obtained in Canterbury, New Zealand (McKenzie and Hill, 1995). In Syria, in the Tel Hadya, region (Saxena *et al.*, 1983a), obtained rates of 2.0 g DM, m⁻² d⁻¹ during the initial slow growth phase in lentil. This rose to between 8 and 14 g DM m⁻² d⁻¹ during linear growth. Crop growth rates in beans are quite variable. In bush beans it ranges from 14-18 g DM m⁻² d⁻¹ (White and Izquierdo, 1991). In climbing beans, on a trellis, in Chapingo, Mexico, it reached 21 g DM m⁻² d⁻¹ (Fanjul *et al.*, 1982). The rate of DM accumulation is usually maximized during full canopy cover by management factors that optimise soil water, fertility and genetic factors that enhance photosynthesis (Boote and Toolenaar, 1994).

In most legumes, DM accumulation reaches a maximum around the start of seed fill. In common bean, root, petiole, stem and pod weight then remain constant. However, while seed weight increased, leaf weight decreased by about 45 % (Herdina and Salisbury, 1990). Total DM accumulation in common beans is quite variable but (Sinha, 1977) reported that common beans accumulated two-thirds of their overall DM before the end of flowering. In other legumes, soybean accumulates 30-67 % before flowering and lentils 16-30 % before flower initiation, with the majority of the DM being produced after anthesis (Pandey and Torrie, 1973). In most grain legumes, at harvest, total DM production is often underestimated due to the loss of leaves and petioles after physiological maturity (Khanna-Chopa and Sinha, 1987), flower loss, and pod abortion (Nleya *et al.*, 2001). Dapaah (1997) with pinto beans, in Canterbury, New Zealand, found seed yield was highly correlated with total DM, with a relatively stable harvest index. He considered that a high DM yield was probably a prerequisite for high pinto bean seed yield as found in chickpea by Saxena *et al.* (1990). By comparison, with DM accumulation in *Glycine max*, indeterminate plants produced more vegetative growth during flowering and pod set than determinate plants (Egli and Leggett, 1973). If this occurs in *Phaseolus* spp., competition between vegetative and reproductive sinks for photosynthate could be greater in indeterminate than in determinate plants.

2.7 Seed yield

Seed yield is the result of many plant growth processes, largely influenced by the environment, which are ultimately expressed in the plant yield components of pods plant⁻¹, seed pod⁻¹ and g seed⁻¹. In grain legumes, the total seed yield (TSY) is generally expressed by the following yield component equation:

$$\text{TSY} = \text{Plants unit}^{-1} \text{ area} \times \text{pods plant}^{-1} \times \text{seeds pod}^{-1} \times \text{weight seed}^{-1} \quad \text{Equation 2.2}$$

The yield component approach has been used extensively to calculate the yield of grain legumes (Fallon and White, 1978; Dapaah, 1997; Egli, 1998; Greven, 2000). The relationship between yield components has been described as compensatory or mutually interdependent (Wilson, 1987). Subsequently, even a large change in one yield component may not affect overall seed yield (Taweekul, 1999).

The functional approach analyses yield using simple models based on physical and physiological traits (Monteith, 1977). In the functional approach, seed yield per unit area (Y) can be expressed as the integral of the crop growth rate with time multiplied by the harvest index (Monteith, 1977):

$$Y = \int \text{CGR} \, dt * \text{HI} \quad \text{Equation 2.3}$$

where HI is the harvest index, CGR is the daily rate of aboveground DM production and dt is the time duration of growth. Crop growth is related to the amount of photosynthetically active radiation (PAR) intercepted by the crop assuming that the crop has adequate water and nutrients and is free of weeds, pests and diseases. The daily rate of above ground DM production (CGR) can be estimated using the following equation:

$$\text{CGR} = \text{RUE} \times S_a \quad \text{Equation 2.4}$$

where RUE is the efficiency with which a crop uses PAR to produce DM and S_a is the amount of PAR intercepted or absorbed by the crop canopy. Therefore growth and yield variation can be interpreted in terms of change in four parameters: HI, RUE, S_a and the growth duration. Highest seed yields are obtained when all of these components are maximized (Westermann and Crothers, 1977). Seed yield is a direct representation of the amount of DM accumulated during the growing season and its partitioning into seeds

(Muchow and Charles-Edwards, 1982). Monteith (1977) and Gallagher and Biscoe (1978) proposed the need for higher biomass accumulation and HI to obtain higher seed yields.

Positive correlations between seed yield and biomass production were reported for faba bean by Siddique *et al.* (1990) and high DM production was reported as a prerequisite for high seed yield in grain legumes by Saxena *et al.* (1990) and Ayaz *et al.* (1999). Due to the large influence of the environment on the processes of seed yield, common bean seed yield varies from site to site, location to location and from one season to the next (El Nadi, 1970). Yield variability in grain legumes is still a global problem (Ambrose and Hedley, 1984; Hedley and Ambrose, 1985).

The maximum seed yield potential of bush type *Phaseolus vulgaris* has been estimated as 4 to 6 t ha⁻¹ (White and Izquierdo, 1991). However, average yields of this crop are often less than 1.0 t ha⁻¹ in most developing countries. Farm yields average 1.4 to 1.8 t ha⁻¹ in North America and 3 t ha⁻¹ in the United Kingdom (Laing *et al.*, 1984; Hardwick, 1988). Where beans are cultivated as high value monocrops, namely in North America, Europe and a few areas of Brazil, yields of 3.0 t ha⁻¹ have been achieved (Adams *et al.*, 1985; Hardwick, 1988). Under experimental conditions in Canterbury, New Zealand (Goulden, 1974; Love *et al.*, 1988) navy bean yields of 3.0 to 3.3 t ha⁻¹ were reported and Dapaah *et al.* (2000) reported a seed yield equivalent to 3.8 t ha⁻¹ in pinto beans.

2.7.1 Yield components

One useful method of examining yield performance is to partition yield into its components. Management, genotype and environment can affect seed yield, expressed as the yield components of pods plant⁻¹, seeds pod⁻¹ and the mean seed weight. Yield components may help to explain why a reduction in yield has occurred (Gardner *et al.*, 1985; Ayaz *et al.*, 2004a). Efforts to improve seed yield through management have been geared towards improving each yield component (Ayaz *et al.*, 2004a). However results have been limited due to interdependent compensatory mechanisms among the components, as mentioned earlier (Wilson, 1987).

The number of pods per unit area is usually a contributing factor to seed yield variation, while in legumes the most stable yield component is mean seed weight (Littleton *et al.*, 1979; Saxena *et al.*, 1983a). Westermann and Crothers (1977) found a significant relationship between area plant⁻¹ and seed yield plant⁻¹ and suggested that some yield

components are influenced by the area plant⁻¹ in common bean. The number of pods plant⁻¹ had a dominant effect on seed yield in common bean (Duarte and Adams, 1972; Leakey, 1972); and soybean (Leakey, 1972; Pandey and Torrie, 1973). Generally, variation in the number of pods plant⁻¹ depends on crop species (Ayaz *et al.*, 2004a).

Variability in crop seed yield can sometimes be related to variability in yield components, as yield depends on the development of these components (Slinkard and Sindhu, 1988). Dapaah *et al.* (1999) showed an irrigation effect on yield and yield components of pinto beans in Canterbury, New Zealand. Pods plant⁻¹ increased by 21 and 41 % respectively, over two growing seasons, when compared to an unirrigated crop. There was an increase of 10-15 % in the number of seeds pod⁻¹ in the irrigated crop (Dapaah *et al.*, 1999). Pods plant⁻¹ was consistently and strongly correlated with seed yield, and irrigation accounted for an increased numbers of pods plant⁻¹ because it increased the number of branches plant⁻¹ (Dapaah *et al.*, 1999).

Dapaah *et al.* (1999) found that a low plant population gave a greater number of pods plant⁻¹ in a November sowing in Canterbury. This inverse relationship of increased pods plant⁻¹ with decreased plant population was also reported in field beans (Ishag, 1973) in the United Kingdom; chickpea (Hernandez and Hill, 1985), lentil (McKenzie, 1987) and in lentil, chickpea, pea and narrow-leafed lupin (Ayaz *et al.*, 1999) in Canterbury. Seeds pod⁻¹ also increased by 10 and 15 % respectively over two seasons. Increase in seeds pod⁻¹ was attributed to increased seed set due to adequate water supply and this was correlated with HI (Dapaah *et al.*, 1999). Siddique and Sedgeley (1986) reported similar results with chickpea in south Western Australia. The hundred seed weight in navy beans (Love *et al.*, 1988) was significantly correlated with seed yield.

2.7.2 Harvest index

In common beans, HI can be defined as the ratio of seed yield (economic yield) to total above ground biomass, or biological yield (TDM) at maturity (Muchow and Charles-Edwards, 1982). Crop HI (CHI) is a measure of the partitioning of photosynthate (Donald, 1958). In cereals and grain legumes, crops grown for their seeds, the proportion of the total DM accumulated during the growing season that is partitioned into seed is of vital economic importance (Muchow and Charles-Edwards, 1982). Harvest Index is very variable in grain legumes (Husain *et al.*, 1988a; McKenzie and Hill, 1990; Ayaz, 2001).

In common beans, considerable variation in HI has been reported (range 0.39-0.67) (Wallace and Munger, 1966; Wallace *et al.*, 1972); 0.53-0.69 in pinto beans (Dapaah, 1997) in New Zealand and 0.72 (White *et al.*, 1992b) for 62 common bean genotypes in Colombia. In comparison, Hay (Hay, 1995), in a review of the HI of some modern, high yielding varieties of selected crops, reported values in the range 0.40-0.60. Variability in HI was attributed to variability in yield components (Ayaz, 2001).

Increasing biological yield ha^{-1} increases seed yield where the HI is constant (Singh, 1991). Therefore, increasing biological yield (total DM) and/or HI can lead to increased seed yield. For some cereals (such as barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.), HI is relatively stable unless plants are exposed to extremes of temperature, moisture and density (Hay, 1995). Variation in HI and growth duration are major factors responsible for yield variability (Wilson, 1987). Harvest index usually declines as the maturity period increases. The partitioning efficiency of mung bean (*Vigna radiata* (L.) R. Wilczek) was 0.52 at maturity (65 days after sowing) which was higher than in soybean at 0.34 at (72 DAS) or peanut at 0.27 (103 DAS) (Angus *et al.*, 1983). In many indeterminate legumes, flower and pod abortion is as high as 95 % which reduces yield and severely affects HI (Summerfield and Wein, 1980).

2.8 Conclusions

Common bean has been an important crop for many centuries, but with increased pressure on feeding the world's population a cheap, energy efficient and environmentally friendly system of agricultural production is needed to produce protein to feed the world's population. The legume/*Rhizobium* symbiosis offers a natural system for supplying food and improving soil fertility.

1. Bean yield can be as high as 4-6 t ha^{-1} but crop yield is variable due to physical, agronomic, and environmental conditions and poor microbial associations.
2. The relationship between bean cultivar grown and *Rhizobium* used has been, and continues to be, a source of contention.
3. Dry matter production in common bean responds to N application but the effect of N on the *Rhizobium*/cultivar symbiosis is variable depending on the time, amount, and type of fertilizer N applied.
4. Environmental conditions (water availability, temperature, solar radiation) play a vital role in crop growth and development. They also affect the micro symbiont

and its survival in the soil and hence the total N fixing ability of the symbiosis as well as the overall growth and production of the crop.

5. The ability to identify strains of *Rhizobium*, which are suited to particular common bean cultivars under specific growing conditions, could go a long way towards increasing the productivity of the crop, opening new production areas and reducing the use of fertilizer N in crop systems.

CHAPTER 3

Plant growth and nodulation studies of common beans (*Phaseolus vulgaris* L.) inoculated with elite strains of *Rhizobium*

Summary

The effectiveness of sixteen rhizobial isolates was tested for nodulation and N-fixation (plant growth) with common bean cv. Scylla. The strains used were from the United Kingdom (UK 1, UK 2, UK 3, and UK 4), Brazil (PRF 81, H 12, and H 20) France (CARA 56, CARA 72, H 441, PhP 17, CESTA 221, CIAT 899 and HT 2a2) and Australia (RCR 3644 and CC 511). They were evaluated in three experiments (one in the greenhouse and two in the field) over two growing seasons (2003-04 and 2004-05) in Canterbury, New Zealand.

In 2003-04, two bean cultivars (Scylla and T-49) were combined with three inoculation treatments (rhizobia strains CC 511, RCR 3644 and a control) and two irrigation treatments. This gave no nodulation on either cultivar. Subsequently, five *Rhizobium* isolates were chosen, based on signs of early nodulation in the greenhouse trial, for further field evaluation. In the 2004-05 field experiment cvs Scylla and T-49 were combined with six inoculation treatments.

In the greenhouse, all inoculated strains induced nodules. The major region of nodule concentration was the top 10-20 cm of the root. Most of the isolates in the greenhouse had a positive effect on the number of nodules plant⁻¹, root and shoot dry weight plant⁻¹ and pod yield. In the second field experiment, nodulation was obtained in all treatments, including controls. Nodules were concentrated at the junction of the main and the secondary roots, and varied in size, colour and position. In both bean cultivars, strain H 20 gave the highest number of nodules plant⁻¹, while strain PRF 81 gave the highest shoot dry weight in Scylla. The highest shoot dry weight in T-49 was produced with strain H 20. Over all the experiment, the most effective *Rhizobium* was H 20 which helped plants produce 10.4 g TDM plant⁻¹, 5.5 g pods plant⁻¹ at 85 DAS and 3.31 % N in

the plant at 51 DAS. The results indicate that it should be possible to increase nodulation and yield of common beans, in Canterbury, by combining suitable bean cultivars with appropriate rhizobial strains.

3.1 Introduction

Legume plants can establish symbiotic associations that result in the formation of N fixing root nodules in the presence of effective soil rhizobia or through inoculation (Santalla *et al.*, 2001a; Santalla *et al.*, 2001b; Sessitsch *et al.*, 2002; Tamimi and Timko, 2003). *Phaseolus vulgaris* is a promiscuous legume that forms nodules with a diversity of rhizobial genotypes (Aguilar *et al.*, 2004). Despite this ability to enter into associations with N-fixing symbionts, under agronomic conditions, productivity of common beans is often limited by N deficiency (Graham, 1981; Rosas *et al.*, 1998). Production is dominated by the use of commercial N fertilisers (Muller *et al.*, 1993; Peoples *et al.*, 1995b; Peoples *et al.*, 1995c; Graham and Vance, 2000).

This symbiotic association is further affected by inadequate *Rhizobium*/Fabaceae technology, the presence of nodulating but ineffective native rhizobia, the suppression of biological nitrogen fixation (BNF) by high soil mineral N levels, inadequate quality control of inoculants and difficulties of inoculating under various agricultural conditions (Bantilan and Johansen, 1995). Inoculation with a suitable rhizobia is required in three situations: in the absence of compatible rhizobia; if the population of compatible rhizobia is too low and if indigenous rhizobia are less effective than alternative strains (Meade *et al.*, 1985; Hansen, 1994; Brockwell and Bottomley, 1995).

If commercially available rhizobial strains are not accessible or recommended strains do not produce the desired effect, selection of new strains will be required. Selection procedures are very labour intensive and should include strains from diverse geographic regions (Hansen, 1994; Rodriguez-Navarro *et al.*, 1999). These investigations usually commence with evaluation of strain infectivity under aseptic conditions followed by subsequent glasshouse tests to evaluate N fixation (Gibson, 1987). Initial investigations are followed by further strain eliminations in soil and field testing to obtain a complete range of relevant information (e.g. nodulation, nodule occupancy, N content, N fixation and yield) (Gibson, 1987; Hansen, 1994).

In the *Phaseolus/Rhizobium* symbiosis, both plant cultivar and *Rhizobium* strain can affect nodulation (Laeremans and Vanderleyden, 1998; Hungria and Bohrer, 2000; Mutch *et al.*, 2003). Additionally, accumulation of N in vegetative and reproductive tissues, partitioning of fixed N in the mature shoot and the remobilisation of stored N in vegetative organs to seeds (Hungria and Neves, 1987; Kapranov *et al.*, 1999) are also affected. In spite of over 100 years of published research related to this symbiosis (Zahran, 1999; Stephens and Rask, 2000); erratic nodulation and low rates of N fixation under field conditions are among the main causes of low N fixation (Graham, 1981; Piha and Munns, 1987a).

The wide genetic diversity of both the micro and macro symbionts (Martinez-Romero *et al.*, 1991; Singh *et al.*, 1991) strongly suggests the possibility of specific host-strain interactions (Chaverra and Graham, 1992). *Rhizobium* strains which perform well in symbiosis with a large number of host plants and cultivars, under widely different conditions have been utilised in inoculant production (Amarger, 1981; Mhamdi *et al.*, 1999; Rodriguez-Navarro *et al.*, 1999). However, there are some bacterial strains, which are exceptional under certain conditions on specific cultivars, but which may not perform well with similar or other cultivars under other conditions (Gibson *et al.*, 1975; Hardarson, 1993; Rosas *et al.*, 1998). Therefore it is necessary to continually evaluate new bacterial strains and cultivars to make better predictions of the possible field performance of the symbionts (Lie, 1971; Meade *et al.*, 1985).

In New Zealand, commercial sowings of *Phaseolus* beans are generally not inoculated (George Hill, personal communication). Responses to inoculation in experimental sowings have varied, from no response to substantial responses (Dapaah *et al.*, 1995; Dapaah, 1997). Similar results have been obtained in Brazil (Ramos and Boddey, 1987; Vargas *et al.*, 2000) and in other parts of the world (Graham and Temple, 1984). There is a growing realisation of co-evolution between *Rhizobium* strains and bean cultivars in their centres of diversification (Aguilar *et al.*, 2004). This highlights the need for continuous evaluation and selection to make maximum use of BNF to reduce, or eliminate, reliance on fertiliser N (White, 1989) and to better match micro and macro symbionts with the conditions to maximize yield potential.

The data presented in this chapter were derived from three experiments; two carried out in the field in the summers of 2003-04 and 2004-05 and one in a greenhouse during the

autumn/winter (March-June) of 2005. The greenhouse experiment was carried out in the Forrester greenhouse, Lincoln University, Canterbury, New Zealand to study common bean nodulation with elite strains of *Rhizobium* in order to:

1. Assess the effectiveness of introduced *Rhizobium* strains under the sub-humid conditions of Canterbury.
2. Select an efficient nodulating rhizobial strain under greenhouse conditions, to be field-tested as an inoculant for common beans grown in Canterbury.
3. Determine the effect of the selected strain(s) on nodulation, BNF, growth and yield of *Phaseolus vulgaris* plants.

3.2 Materials and Methods

3.2.1 Field experiments

3.2.1.1 Site description

The two field experiments were conducted in paddocks H2 (2003-04) and H14 (2004-05) of the Horticultural Research Area, Lincoln University, New Zealand (Lat. 43° 38' S, Long. 172° 30' E, 11 m above sea level). The Canterbury climate is considered to be sub-humid temperate. It is characterised by warm summers, cool winters and an annual rainfall of 600-750 mm with a slight summer maximum (White, 1999). Both paddocks had similar topography (relatively flat) and soil type.

3.2.1.2 Soil

The soil was a Wakanui silt loam (*Udic Ustochrept*, USDA Soil Taxonomy) with 1.8-3.5 m of fine textured material overlaying gravels (Cox, 1978). Wakanui silt loams have 0.3 m of uniform topsoil with a weakly developed granular structure underlain by layers of varying depth ranging from fine silt to loamy sand or sand in texture. Wakanui soils are imperfectly drained and display strong mottling below 0.7 m indicating periods of water logging (Watt and Burgham, 1992).

3.2.1.3 History

The paddocks had previously been planted in apples (*Malus* spp.). Following removal of the apples, the paddocks were drilled in barley cv. Liberty, followed by oats

(*Avena sativa* L.) cv. Hokanui. A Ministry of Agriculture and Fisheries (MAF) quick soil test for a depth of 0-20 cm gave the values shown in Table 3.1.

Table 3.1. MAF soil quick test values for paddocks in the Horticultural Research area (0-20 cm depth), Lincoln University, Canterbury. 2003-04 and 2004-05.

Season	pH	P	Ca	Mg	K	Na	Total N
2003/04	6.4	15	10	20	9	9	---
2004/05	6.3	28	9	21	14	8	0.20

Ca, P, K, Mg and Na are expressed in $\mu\text{g/g}$ soil and total N as a percentage

3.2.1.4 Experimental design

The 2003-04 experiment was a split plot randomised complete block design with three replicates, with irrigation as main plots. The treatments were two irrigation levels, three *Rhizobium* inoculations, two fertiliser N levels, and two common bean cultivars. Each replicate contained 24 plots and plots were 10 m by 2.25 m. There were 72 plots in a total. The treatments were designed to give a variety of *Rhizobium*-bean cultivar combinations for observation of plant response to different management conditions.

Table 3.2. Treatments used in the 2003-04 growing season of common beans at Lincoln University.

Treatment	
Irrigation (Main plots)	1. Full Irrigation 2. No Irrigation (rain-fed)
<i>Rhizobium</i> strain	1. Nil (no rhizobia) 2. CC 511 3. RCR 3644
Bean cultivar	1. Scylla 2. T-49
Fertiliser	1. Nil (no fertiliser) 2. Full nitrogen (150 kg N ha^{-1})

Scylla: a white seeded green bean cultivar; T-49: a white seeded navy bean cultivar

Peat-based inoculants containing *Rhizobium tropici* CC 511 (1.70×10^9 colony forming units (cfu) g^{-1} of moist peat) or *Rhizobium tropici* RCR 3644 (2.41×10^9 cfu g^{-1} of moist peat) (NODULAID™, Victoria, Australia), (*Rhizobium* counts (cfu g^{-1} of moist peat) supplied by the manufacturer)) obtained through Coated Seeds Ltd., Christchurch were used to inoculate the seed. Inoculant at 240 g of peat 100 kg seed^{-1} was made into a slurry of 20 g peat inoculant to 40 ml water and gently mixed in a plastic bag to coat the seeds, which were then air-dried before sowing. Calcium ammonium nitrate was broadcast onto

the +N plots on 7 January 2004 (30 DAS) at 150 kg N ha⁻¹ [Calcium ammonium nitrate (CAN) (27 %N) 13.5 % nitrate N, 13.5 % ammonium N and 8 % Calcium (CaCO₃)]. Seed of navy bean cv. T-49 (100 seed weight 24 g, germination 45 %) was purchased from Canterbury Seed Company Limited, Christchurch, New Zealand and green dwarf bean seed of cv. Scylla (100 seed weight 35.2 g, germination 85 %) was donated by Heinz Wattie's Australasia, Christchurch, New Zealand.

The 2004-2005 experiment was a randomised complete block design with four replicates. Treatments were the same two cultivars of *Phaseolus vulgaris* used in Experiment 1 (navy bean cv. T-49 and green dwarf bean cv. Scylla) and six rhizobia treatments (*Rhizobium* strains RCR 3644, UK 2, H 20, PRF 81, PhP 17 and an uninoculated control). Each replicate consisted of 12 plots and plots were 2 m by 0.75 m with 0.3 m between plots and 0.5 m between replicates.

Table 3.3. Treatments used in the 2004-05 growing season of common beans at Lincoln University

Treatment	
Bean cultivar	1. Scylla
	2. T-49
<i>Rhizobium</i> strain:	1. Control
	2. RCR 3644
	3. UK 2
	4. H 20
	5. PRF 81
	6. PhP 17

3.2.1.5 Crop Husbandry

In both 2003-2004 and 2004-2005, paddocks were sprayed with Glyphosate at 3 litre ha⁻¹ for control of couch grass (*Elytrigia repens* (L.) Desv). Cultivation included ploughing, harrowing and rolling to form a firm seedbed. Trifluralin was applied pre-emergence at 2 litre (800 g a.i.) ha⁻¹ for weed control before sowing. Hand weeding was

periodically carried out during the life of the crop to control spot infestation with black nightshade (*Solanum nigrum* (L.) and chickweed (*Stellaria media* (L.) Vill.)

3.2.1.6 Sowing

In 2003-04, plots were sown on 8 December 2003 with an Öyjord cone seeder. The sowing rate of T-49 was doubled due to its low germination. Each plot consisted of 12 rows, 15 cm apart. Seed was sown at 2-3 cm apart to obtain a target plant population of 60 plants m⁻². In 2004-05, plots were hand-sown on 11 January 2005. Planting holes were prepared with a dibble board. Holes were 5 cm deep and seed was sown into the holes. Each plot consisted of 5 rows 15 cm apart with 10 cm between plants. This gave 100 plants plot⁻¹ (67 plants m⁻²). After sowing, 2 ml of inoculant was applied to each seed hole onto the seed, which was then covered with soil and gently compacted.

3.2.1.7 Inoculation

Inoculants were prepared as described in the Material and Method section. Densities (concentration) of bacteria applied to the seed in the greenhouse and in the second field experiment are given in (Table 3.6). The rhizobial strains used in the second field experiment were selected based on early nodulation observed at thinning (14 DAS) in the preliminary greenhouse experiment.

3.2.1.8 Irrigation

In 2003-2004, the crop was irrigated with overhead sprinklers depending on the soil moisture status. When the soil moisture deficit in the 0-20 cm layer reached 50 % of plant available water, as determined by Time domain reflectometry (TDR, Model 6050X1, Soil Moisture Equipment Corp., Ca., USA), water was applied. A total 200 mm of water was applied over the growth of the crop. In the second season, irrigation was applied to eliminate any water stress during plant growth. Six applications, each lasting 2.5 h were applied by over head sprinkler. A total of 120 mm of water was applied.

3.2.1.9 Sampling

Samples were taken from the three centre rows of each plot, starting from a randomly selected end. The first two rows of plants were omitted to eliminate border effects and the sample size was approximately six plants (two along the row and three across rows). A buffer of three plants was used between samplings. Crop biomass was determined from

sequential 0.09 m⁻² samples taken to measure DM accumulation. Root and nodule samples were collected from the same 0.09 m⁻² sample areas to a depth of 0.20 m. Soil was washed off the roots in gently running water to ensure that roots and nodules remained intact.

3.2.1.10 Measurements

Climate data were recorded at Broadfields Meteorological Station situated 2 km from Lincoln University. In 2003-2004, leaf area index (LAI) was measured every 10-14 d from 25 DAS using a LI-COR LAI Plant Canopy Analyser (LI-COR Inc., Lincoln, Nebraska, USA). For each plot, one reading was taken above and five readings below the canopy of the growing plants (repeated five times). In 2004-05, readings were taken from 19 DAS, every 5-6 d, and one reading was taken per plot (one reading above and five readings below with no repetitions). The five below canopy readings were taken diagonally across the rows. A 45 ° lens cap was used on the leaf area meter probe to account for inter row gap effects and readings were taken under predominantly diffuse conditions on completely cloudy days.

In 2003-04 (Experiment 1) crop dry matter (DM) accumulation was determined from 0.2 m⁻² quadrat samples taken every 10-14 d from 23 DAS. Final plant population, DM and seed yield were determined from a 1.0 m⁻² quadrat sample taken from the 6 central rows of each plot at crop physiological maturity (when 95 % of plants were brown). Plants were cut at ground level, bagged, air-dried and machine threshed (Kurtpelz stationary thresher). Yield components were determined from six randomly selected plants from the 1.0 m⁻² quadrats. Samples were oven-dried, in a forced draught oven at 70 °C for 48 h to constant weight.

Green bean yield was taken from a 1.0 m⁻² quadrat at green bean maturity (Heinz Wattie's Australasia criteria) (Appendix 1A). Pods were sorted into total yield and marketable yield (Heinz Wattie's Australasia criteria) (Appendix 1B).

In 2004-05 (Experiment 2), five DM samples were taken as follows:

1. At 21 DAS,
2. When 50 % of plants had at least 1 open flower,
3. When > 50 % of plants had at least 1 green pod of 5 mm long,
4. At green bean harvest (Appendix 2) and
5. At dry bean harvest (when 95 % of plants were brown).

At each sampling, plants were collected to determine shoot and root dry weight, and nodule number. Plant tops were dried, immediately after harvest, in a forced draft oven at 70 °C for 48 h. Roots were washed in tap water; nodules were counted and then dried. Dried plant samples were ground to pass through a 1 mm screen (Cyclotec Mill, USA) and plant N concentration was determined by the Kjeldahl method. The following plant characteristics were analysed: nodules plant⁻¹, root dry weight, shoot dry weight (SDW) and whole plant N content. Yield was evaluated at green bean and at dry bean harvest stages from the harvest area of 0.09 m⁻² (six plants) taken from the three central rows of each plot.

Final DM and seed yield were estimated from six plants (0.09 m⁻²). Plants were cut at ground level, bagged, air-dried and hand threshed. There were five harvests, as described above. Green bean harvest was when the average seed length was approximately 11 mm. In 2004-05 the crop was harvested before 95 % of plants were brown due to late sowing and the risk of frost damage. Around 75 % of the plants were brown.

3.2.2 Greenhouse experiment

3.2.2.1 Design

The experiment was established in the Forrester greenhouse, Lincoln University on 7 March 2005, to explore the effect of different ‘elite’ *Rhizobium* strains on the nodulation, shoot and root growth of common bean cv. Scylla.

A randomised complete block design with three replicates was used. There were 19 treatments consisting of: 16 *Rhizobium* strains (Table 3.4), uninoculated plants with and without N, and plants growing in a recommended potting mix. The bean cultivar used was Scylla (a white seeded green bean, 100 seed weight 35.2 g and a germination minimum of 85 %). This gave a total of 114 experimental units.

Plants were grown for approximately 85 d in a glasshouse with average day and night temperatures of 26 °C, and 14 °C respectively. Two samplings were taken during the experiment at 51 and 85 DAS.

3.2.2.2 *Rhizobium* strains used

The bacterial strains used in this study were provided by D. Humphry (Department of Biology, University of York, Heslington, United Kingdom); J.J. Drevon (UMR Rhizosphère and Symbiose, I.N.R.A.-ENSA Centre de Montpellier, France); M. Hungria (EMBRAPA Londrina, PR, Brazil) and G. Laguerre (Laboratoire de Microbiologie des Sols, Centre du Microbiologie du Sol de l'Environnement, INRA, Dijon, Cedex, France). Two additional strains were isolated from peat-based inoculant purchased from NODULAITM (Victoria, Australia) through Coated Seeds Ltd., Christchurch.

Table 3.4. Bacterial strains used in the experiment and their source.

Strain	Country of origin	Strain	Country of origin
H 12	Brazil	CARA 72	France
H 20	Brazil	H 441	France
PRF 81	Brazil	PhP 17	France
UK 1	England	CESTA 221	France
UK 2	England	CIAT 899	France
UK 3	England	HT 2a2	France
UK 4	England	CARA 56	France
RCR 3644	Australia	CC 511	Australia

Strains from Brazil have all been shown to be symbiotically effective in *Phaseolus vulgaris* and have been recommended and adapted as commercial inoculants (Mostasso *et al.*, 2002; Hungria *et al.*, 2003). Strains from France were collected from the nodules of field grown *Phaseolus vulgaris* from various international locations and are stored in the collection of the Centre de Microbiologie du Sol et de l'Environnement, INRA Dijon France (Amarger *et al.*, 1997). CIAT 899 is universally recognised as an efficient nodulator of common beans and RCR 3644 and CC 511 are used in commercial inoculants in Australia and New Zealand.

3.2.2.3 Potting Mix

A 1:5 mix of sieved, washed river sand and perlite Grade C 400 (INPRO Minerals, Industrial Processors Ltd, Auckland, New Zealand) was used as the potting media. The Lincoln University 3-4 month potting mix consisted of a 4:1 mixture of composted horticultural bark (Grade 2) and pumice (3-6 mm) (Southern Horticultural Products Ltd. Main South Road, Weedons, Christchurch), 1,000 g of controlled release fertiliser Osmocote® Plus [(15-4.8-10.8) – 15 % N, 4.8% P and 1.8 % Potassium (K)] (Scotts

Australia Pty Ltd, NSW, Australia), 500 g of agricultural lime and 500 g of Hydrflo™ (wetting agent) (Scotts Australia Pty Ltd, NSW, Australia) in 500 litres. Pots were made of polyvinyl chloride (PVC) tubes (15 cm in diameter and 80 cm long) with a 0.82 mm quarantine mesh (Taylor Built Ltd., Auckland, New Zealand) taped to the bottom. This facilitated drainage and supported the media in the pot and enhanced maximum root growth. Pots were sterilised for 5 minutes in a 9:1 water to super strength bleach (Wilson Chemicals Ltd., Christchurch, New Zealand) solution, then rinsed three times with clean running water.

3.2.2.4 Sowing

Pots were watered and allowed to free drain to field capacity for over two days. Seed was surface sterilised in 70 % ethanol for 1 minute and then washed in clean running deionised water. On 7 March 2005, three healthy seeds were sown per pot at 4-5 cm deep and inoculated with the required inocula (bacterial strain). Uninoculated pots were sown first to avoid cross-contamination. Each pot was inoculated with 15 ml of inoculant (Table 3.6) on the seeds, to establish a vigorous population of the rhizobial applied strain for early and effective nodulation. Plants were thinned to one plant per pot at 14 d after sowing (DAS).

3.2.2.5 Nutrient solution

Hansen's (1994) nutrient solution (Table 3.5) was used. Stock solutions A, B, C, D, E and F (Table 3.5) were prepared separately and stored at 3-4 °C. Stock solutions were mixed at the required application rate when required for fertilisation. Nutrient solution application rates were calculated based on pot volume. It was applied every 4 d. A full strength solution was used throughout the experiment until final harvest.

Calcium ammonium nitrate (CAN) 27 % N) at the equivalent of 150 kg N ha⁻¹ was applied by volume (7.85 g pot⁻¹), and was incorporated into the pot before sowing in the N treatments. All pots, excluding the treatments with Lincoln University potting mix, were flushed with water weekly to avoid accumulation of unused salts.

3.2.2.6 Media and growth conditions

Rhizobial strains were grown on Tryptone-Yeast (TY) extract agar plates (Beringer, 1974; Somasegaran and Hoben, 1994) (Appendix 2B). The medium was made up to 1000 ml with 5 g Tryptone (DIFCO, Fort Richard, Auckland, New Zealand); 3 g Yeast Extract

(DIFCO, Fort Richard, Auckland); 15 g Technical agar (Davis Bacteriological, Christchurch, New Zealand); 0.87 g CaCl₂.H₂O and deionised water in a clean 1000 ml bottle. Boiling for 5-10 minutes dissolved the agar. The pH was adjusted within the range of 6.8-7.2 and the mixture autoclaved at 121 °C for 15-20 minutes. Fifteen ml of the cooled liquid was poured into individual Petri dishes (LabServe, Biolab, New Zealand).

Rhizobial strains were streaked onto the agar and incubated at 28 °C for 18 h. One hundred µl of sterile water was pipetted onto the agar plate and gently mixed with a sterile spreader. The mixed suspension was pipetted into a 250 ml sterile conical flask containing Tryptone-Yeast broth. The mixture of Tryptone-Yeast broth and bacterial suspension were incubated at 28 °C for 18 h and rotated at 150 rpm. One ml of the incubated mixture (inoculant) was specto-photometrically measured at Å600.

Table 3.5. Nutrient solution for legumes in sand or perlite culture (Hansen, 1994)

Solution	Constituent	Stock (g l ⁻¹)	Full strength (ml l ⁻¹)	¼ strength (ml l ⁻¹)
A	MgSO ₄ .7H ₂ O	246.47	2	0.50
B	KH ₂ PO ₄	68.00	1	0.25
	K ₂ HPO ₄	87.00		
C	CaCl ₂	110.99	2	0.50
D	K ₂ SO ₄	87.14	2	0.50
E	Sequestrene	18.70	4	1.00
	138Fe Iron Chelate			
F	Boric acid	5.72	1	0.25
	MnCl ₂ .4H ₂ O	0.72		
	ZnCl ₂	0.11		
	CuCl ₂ .2H ₂ O	0.05		
	NaMoO ₄ .2H ₂ O	0.03		
	CoCl ₂ .6H ₂ O	0.05		

CaSO₄ at a concentration of 17.2 g l⁻¹ in stock solution and 2 ml l⁻¹ in final solution was substituted for CaCl₂ and Ferrous sulphate (FeSO₄.7H₂O) at a concentration of 5.5 g l⁻¹ in the stock solution and 2 ml l⁻¹ in final solution was substituted for Sequestrene 138 Fe Iron Chelate.

3.2.2.7 Bacterial density

Fifty µl of the incubated Tryptone-Yeast broth/bacterial suspension mixture was placed in a sterile 500 µl tube with 450 µl of sterile water. The original broth culture was designated 10⁰ and the first dilution 10⁻¹. Fifty µl of well mixed broth was taken from 10⁻¹ dilution and added to 450 µl of sterile distilled water and thoroughly mixed to give 10⁻². This was repeated until the required dilution was obtained. An aliquot of 100 µl of the weak solution was pipetted onto a Tryptone-Yeast agar plate and lightly spread as evenly

as possible over the whole plate with a sterile spreader. Two replicates were prepared of each dilution. Plates were sealed with plastic cling film and incubated in the dark at 28 °C, until the number of colony forming units (cfu) were determined. Bacterial numbers expressed as colony forming units' ml⁻¹ broth are shown in (Table 3.6) for both the greenhouse and field experiments.

Table 3.6. Bacterial concentrations of media from which 2 or 15 ml was used for inoculation of the seed in the greenhouse and field experiment (2004-05) expressed as colony forming units (cfu) ml⁻¹ of broth

Greenhouse		Field	
<i>Rhizobium</i> strain	Bacteria concentration (cfu ml ⁻¹ broth)	<i>Rhizobium</i> strain	Bacteria concentration (cfu ml ⁻¹ broth)
CARA 72	2.7 x 10 ⁹	RCR 3644	3.8 x 10 ⁹
H 20	3.2 x 10 ⁹	UK 2	4.1 x 10 ⁹
H 12	2.9 x 10 ⁹	H 20	1.7 x 10 ⁹
PRF 81	3.5 x 10 ⁹	PRF 81	3.9 x 10 ⁹
PhP 17	2.9 x 10 ⁹	PhP 17	3.3 x 10 ⁹
H 441	1.3 x 10 ⁹		
CESTA 221	5.8 x 10 ⁹		
CIAT 899	4.4 x 10 ⁹		
HT 2a2	1.9 x 10 ⁹		
CARA 56	7.0 x 10 ⁸		
RCR 3644	2.4 x 10 ⁹		
CC 511	1.4 x 10 ⁹		
UK 4	9.7 x 10 ⁹		
UK 3	1.1 x 10 ⁹		
UK 2	7.8 x 10 ⁹		
UK 1	4.9 x 10 ⁹		

2 ml applied in the field and 15 ml applied in the greenhouse.

cfu = colony forming units.

Selection of rhizobial strains for the field experiment was based on signs of early nodulation in the preliminary greenhouse experiment at 15 d after inoculation.

3.2.2.8 Harvest

Destructive harvests were taken at two stages of plant development. The first harvest was taken at full bloom (51 DAS) and the second at 85 DAS (green pod harvest). At each harvest, half the plants in a replicate were randomly selected and removed from the pots. The shoots were cut from the roots, and partitioned into leaves (including petioles), stems and pods. Roots were carefully washed to remove perlite and sand particles. Nodules were then removed and counted. The individual components were weighed and all plant components were dried at 70 °C for 48 h to constant weight. Oven-dry weights were

recorded and plant samples were ground to pass through a 1 mm screen (Cyclotec Mill, USA) and analysed for total N by the Kjeldahl method.

3.2.2.9 Measurements

Growth parameters measured were leaf weight (including petiole), and the weight of stems, pods and nodules. Nodule number, root and whole plant weight and total N content plant⁻¹ were also recorded at each harvest.

3.2.2.10 Statistical analysis

All variates were analysed using analysis of variance (ANOVA). The statistical package used was GenStat (GenStat Release 6.1 Lawes Agricultural Trust, Rothamsted Experimental Station, Hertfordshire, UK). Standard errors of the mean (SEM), coefficient of variation (CV as a %) and percentage variation accounted for (r^2) were also calculated. ANOVA was done both with and without the potting mix data. All data was analysed twice. Once with potting mix included, and again with potting mix excluded. Generally the statistics presented are based on the analyses without potting mix as including potting mix nullifies the assumption of a normal distribution of mean values. However, for an indicator of the effects of the potting mix the analyses with the potting mix is used.

3.3 Results

3.3.1 Field experiment

3.3.1.1 Climate

The average monthly mean air temperatures, total rainfall, Penman evapotranspiration (EPT) and solar radiation for the period December to May are shown in Figure 3.1. Rainfall during December 03-April 04 was 163 mm. This was 31 % less than the long term average of 238 mm. Maximum and minimum mean air temperatures were approximately 20 °C and 10 °C respectively. These are similar to long-term averages. A mean monthly temperature of approximately 15 °C was also similar to the long term average. Solar radiation was also similar to the long term average of 2,668 MJ m⁻². Penman evapotranspiration (EPT) was 5 % higher than the long-term average at the Broadfields site.

In the second season, rainfall from January 05-May 05 was 200 mm. This was 16 % less than the long term average. The mean air temperature over the corresponding period was approximately 13.5 °C. Accumulated solar radiation over the five months from January-May 2005 was 2,156 MJ m⁻², about the same as the long term average. Penman evapo-transpiration over the same period was again similar to the long term average.

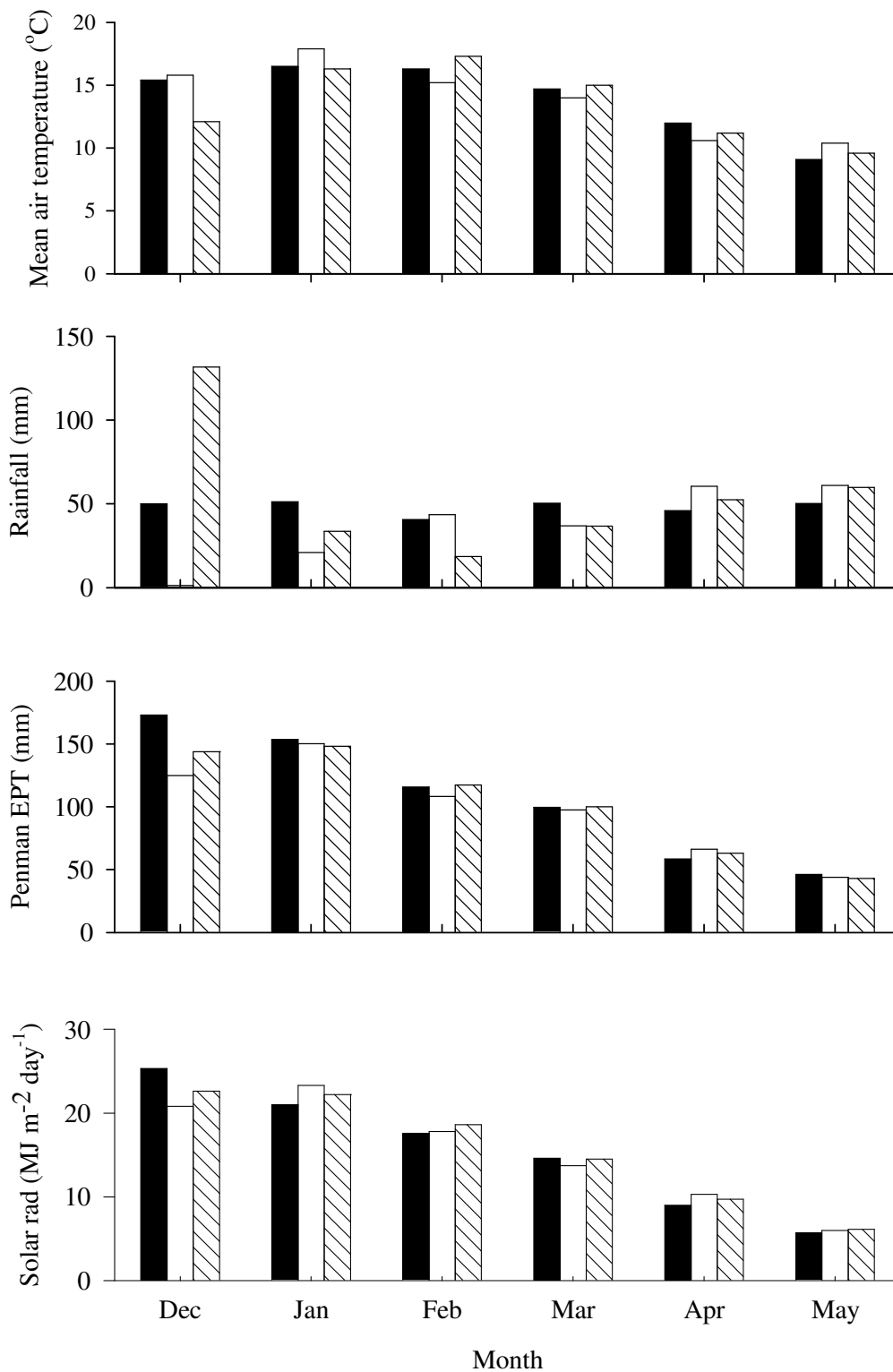


Figure 3.1. Climate data for the 2003-04 (□) and 2004-05 (▨) growing seasons and the long term means (■) (1975-2002) for Lincoln University, Canterbury, New Zealand.

3.3.1.2 Nodulation

In 2003-04, root samples were checked from 23 DAS every 10-14 d for the presence of nodulation. Signs of nodulation (development of nodules or nodule-like structures) were not observed on either bean cultivar. The absence of nodules could be attributed to a number of factors which will be discussed later. In 2004-05, there was considerable variation in the number of nodules produced (Figure 3.2) in response to *Rhizobium* strain used and the bean cultivar sown. Plate 3.1 shows well nodulated plants of cultivars Scylla and T-49 inoculated with *Rhizobium* isolate H 20. Nodules, when present, were concentrated at the root crown at the junction of the tap and secondary roots. Nodules produced were of various sizes and ranged in colour from light pink to deep red to brown (Plate 3.2) denoting the presence of leghaemoglobin.

Nodules formed in response to *Rhizobium* strains were globular on both Scylla and T-49. *Rhizobium* strains H 20, PhP 17 and PRF 81 produced large globular clusters of nodules on both bean cultivars. Nodules produced in response to strain PhP 17 were located along the top third of the secondary roots, while in response to H 20 and PRF 81 nodules were generally concentrated at the junction of the primary root and the secondary roots and along secondary roots. In both cultivars, some uninoculated plants produced nodules. However, they were not as well nodulated as inoculated plants. When present, nodules on uninoculated plants were small, round and white to pale pink.

3.3.1.3 Nodules plant⁻¹

In 2004-05, inoculation significantly ($p < 0.001$) increased the number of nodules plant⁻¹ at all harvests (Figure 3.2). Generally, no nodules were formed on uninoculated plants. However, as indicated above, in some cases a few nodules were present. In the well nodulated plants, the number of nodules plant⁻¹ increased as plants developed. Each inoculation treatment with good nodulation gave its maximum number of nodules plant⁻¹ at a different developmental stage. Plants inoculated with strain H 20 had a rapid increase in nodules plant⁻¹ from the first harvest and this continued until 70 DAS (Figure 3.2). Strain PRF 81 was the second most prolific at inducing nodule formation. Plants inoculated with strain PRF 81 showed increased numbers of nodules plant⁻¹ until the onset of flowering at 40 DAS: nodules plant⁻¹ declined rapidly thereafter (Figure 3.2). Plants inoculated with strains UK 2 and PhP 17 produced 3.1 and 3.3 nodules plant⁻¹ respectively. Control plants

and RCR 3644 averaged only 0 to 0.7 nodules plant⁻¹. No nodule counts were taken at final harvest (112 DAS) due the advanced decomposition of the nodules (Figure 3.2).

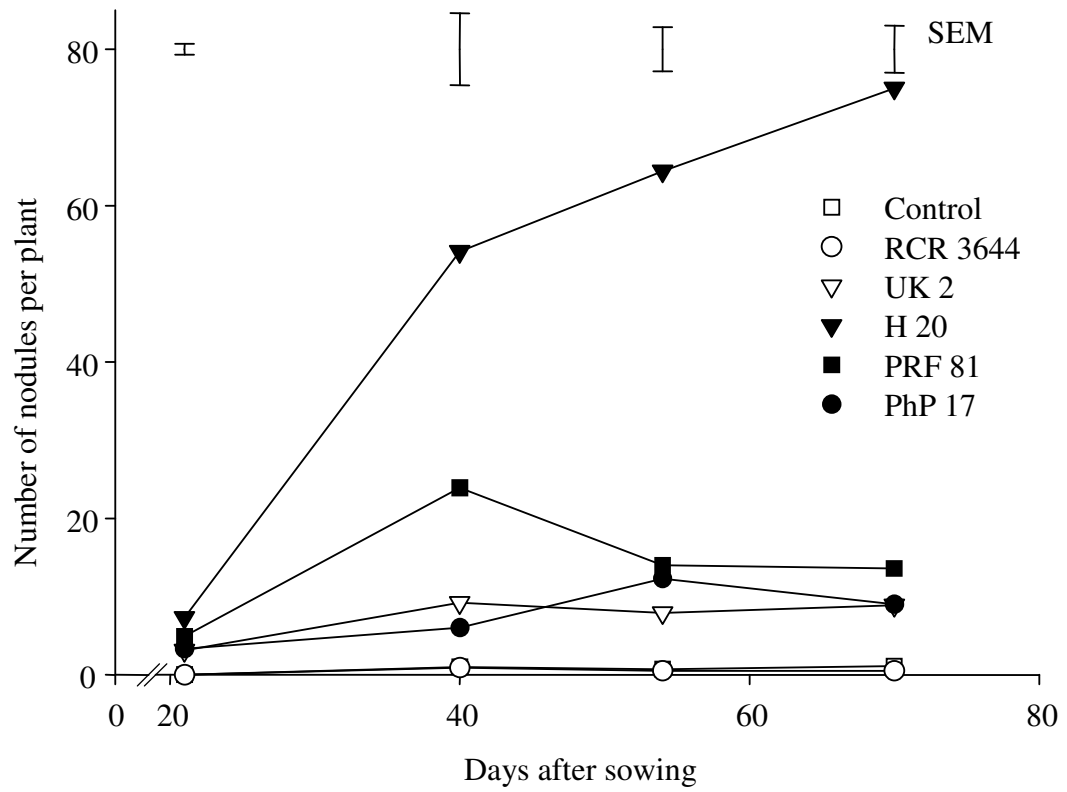


Figure 3.2. The number of nodules plant⁻¹ at different harvests with five strain of *Rhizobium* on common beans cvs Scylla and T-49 in the 2004-05 field experiment.



Plate 3.1. The nodulation pattern of field grown common bean (*Phaseolus vulgaris*) cvs Scylla (left) and T-49 (right) inoculated with *Rhizobium* isolate H 20.

3.3.1.4 Root dry weight plant⁻¹

In the 2004-05 field experiment, *Rhizobium* isolate effect was variable among the different harvests (Figure 3.3a). There were significant cultivar effects ($p < 0.001$) at 21, 40 and ($p < 0.05$) at 54 DAS (Figure 3.3b), but by 70 and 112 DAS cultivar did not affect RDW plant⁻¹. Root dry weight plant⁻¹ was greater for T-49 than Scylla at the first three harvests.

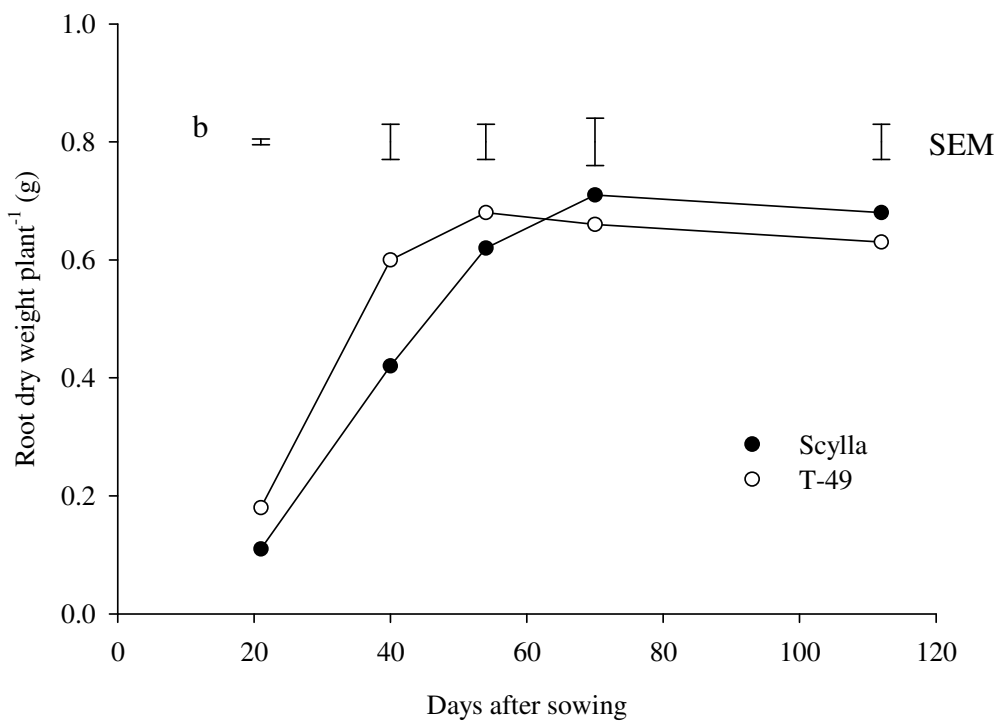
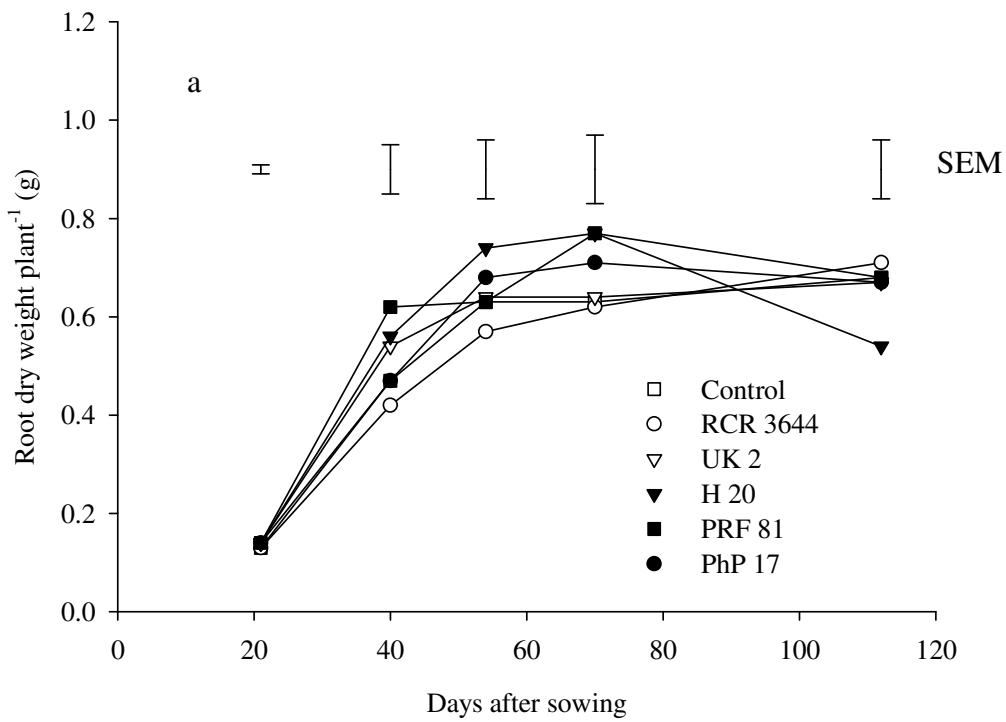


Figure 3.3. The effect of (a) inoculation treatment and (b) cultivar on root dry weight production over time of field grown common beans in 2004-05 in Canterbury, New Zealand.

3.3.1.5 Shoot dry weight plant⁻¹

In 2004-05, as with RDW plant⁻¹, SDW plant⁻¹ increased over time. Over time, the means over all inoculant treatments were 0.64, 5.05, 5.83, 5.81 and 15.01 g plant⁻¹ at 21, 40, 54, 70 and 112 DAS respectively. Similarly, SDW plant⁻¹ of the cultivars increased over time. The SDW plant⁻¹ increase after 54 DAS corresponded with green pod harvest. Cultivar effect on SDW plant⁻¹ was significant ($p < 0.001$) at 21 DAS and ($p < 0.05$) at 40 DAS but was not significant at any other harvest. Cultivar T-49 produced the greater SDW plant⁻¹ at 21 DAS, which was approximately twice as much as produced by Scylla. At 40 DAS (Table 3.7), T-49 produced 5.76 g SDW plant⁻¹ which was 32 % more than Scylla with 4.35 g SDW plant⁻¹.

Table 3.7. The effect of inoculation treatment and cultivar on shoot dry weight plant⁻¹ (g) in field grown common bean in 2004-05.

		Shoot dry weight plant ⁻¹ (g)				
Cultivar	21 DAS	40 DAS	54 DAS	70 DAS	112 DAS	
Scylla	0.49	4.35	5.5	5.91	15.32	
T-49	0.80	5.76	6.15	5.70	14.69	
SEM	0.04	0.41	0.49	0.60	0.98	
Significant	$p < 0.001$	$p < 0.05$	ns	ns	ns	
Treatments						
Control	0.70	5.28	6.28	5.38	13.55	
RCR 3644	0.64	4.16	4.90	4.84	17.70	
UK 2	0.60	4.74	5.91	4.74	14.34	
H 20	0.62	5.16	6.12	5.79	12.96	
PRF 81	0.68	6.24	5.43	7.65	16.08	
PhP 17	0.62	4.75	6.32	6.52	15.41	
SEM	0.06	0.72	0.85	1.0	1.7	
Significance	ns	ns	ns	ns	ns	
CV (%)	8.2	12.1	23.9	33.3	6.3	

3.3.1.6 Plant Nitrogen Concentration

Plant nitrogen concentration (Table 3.8) was significantly ($p < 0.01$) affected by *Rhizobium* isolate at 21, 54, 70 and 112 DAS. Strain PhP 17 gave the highest plant nitrogen concentration (2.09 %) at 21 DAS, followed by H 20 (1.08 %) at 54 DAS and H 20 with 1 % at 70 and 112 DAS respectively. There was a significant cultivar effect on plant nitrogen concentration at 21 and 40 DAS. Scylla had a significantly higher N concentration (2.11 %) at 21 DAS than T-49 (1.87 %) (Table 3.8). Nitrogen concentration generally decreased as plants developed and this trend was observed in relation to both *Rhizobium* inoculation and cultivar.

Inoculation with strain H 20 and PRF 81 usually gave plants with higher plant nitrogen concentrations than the control (uninoculated) plants (Table 3.8) at all harvest. Plants inoculated with strains UK 2 and RCR 3644 produced plants with plant nitrogen concentration below that of the other inoculated plants as well as the uninoculated control plants.

Table 3.8. The effect of inoculation with five different *Rhizobium* isolates on plant nitrogen concentration (%) of Scylla and T-49 field grown common bean in 2004-05.

Treatments	Nitrogen concentration (%)				
	21 DAS	40 DAS	54 DAS	70 DAS	112 DAS
Control	2.01	1.23	0.79	0.75	0.85
RCR 3644	1.94	1.05	0.75	0.69	0.86
UK 2	1.84	1.10	0.78	0.76	0.86
H 20	2.03	1.26	1.08	1.00	1.00
PRF 81	2.03	1.36	0.98	0.84	0.90
PhP 17	2.09	1.21	0.86	0.74	0.81
SEM	0.10	0.07	0.06	0.05	0.03
Significance	p < 0.01	ns	p < 0.01	p < 0.01	p < 0.01
Cultivar					
Scylla	2.11	1.27	0.85	0.78	0.88
T-49	1.87	1.13	0.89	0.81	0.89
SEM	0.06	0.04	0.04	0.03	0.02
Significance	p < 0.01	p < 0.05	ns	ns	ns
CV (%)	14.6	17.6	21.1	18.1	10.1
Significant					
Interactions	nil	nil	nil	nil	nil

3.3.2 Greenhouse experiment

3.3.2.1 Nodulation pattern

At the first harvest at 51 DAS, most plants were in full bloom, except the control (potting mix) treatment and Scylla plants treated with strains H 441 and UK 4, which already had immature green pods. All of the *Rhizobium* isolates used nodulated the cultivar Scylla. Irrespective of *Rhizobium* isolate used, nodules were scattered along the length of the root system. The region of highest nodule concentration was the top 5-15 cm (Plate 3.2) of the root system. Nodules produced were of various sizes, from small individual nodules to large globular clusters. Nodule colour ranged from light pink to rich red to brown. Both primary and secondary roots were evenly distributed with nodules. Root length varied with treatment used and they were 30 to 90 cm long (data not shown).

3.3.2.2 Nodules plant⁻¹

The number of nodules plant⁻¹ was variable on inoculated plants. Isolate H 441, apart from stimulating Scylla to a more advanced stage in its phenology gave the highest number of nodules plant⁻¹ (347). It was followed by H 12 (326), CIAT 899 (323), HT 2a2 (294) and PhP 17 (280) (Table 3.9). Six strains (CESTA 221, PRF 81, CARA 72, H 20, CARA 56 and UK 4) produced 100-200 nodules. Strains UK 2 and CC 511 produced the fewest nodules at 7 and 15 respectively (Table 3.9).

By 85 DAS, plants were at various stages of green pod development. Strain CIAT 899 gave the highest number of nodules (335), followed closely by strains HT 2a2 and PhP 17 with 333 and 328 nodules respectively. Strains UK 1 and UK 2 produced the fewest nodules at 13 and 22 respectively. The remaining strains produced 30 to 227 nodules plant⁻¹ (Table 3.9). Uninoculated plants (+N, 0 N and the potting mix treatments) did not form any nodules at either harvest. The large differences in nodule dry weight were significant ($p < 0.001$) at both harvests. The number of nodules greatly influenced nodule dry weight g plant⁻¹ (Table 3.9).

Table 3.9. Effect of different *Rhizobium* strains on nodule plant⁻¹ and nodule dry weight plant⁻¹ on common beans cv. Scylla grown in a greenhouse.

Strain	51 DAS		85 DAS	
	Nodules plant ⁻¹	NDW (g plant ⁻¹)	Nodules plant ⁻¹	NDW (g plant ⁻¹)
H 441	347	0.36	221	0.23
H 12	326	0.43	227	0.23
CIAT 899	323	0.33	335	0.26
HT 2a2	294	0.32	333	0.32
PhP 17	280	0.27	328	0.38
UK 4	196	0.23	82	0.19
CARA 56	187	0.25	144	0.25
H 20	132	0.24	146	0.25
CARA 72	127	0.17	74	0.16
PRF 81	99	0.17	100	0.18
CESTA 221	93	0.16	127	0.19
UK 1	72	0.12	13	0.07
UK 3	63	0.16	30	0.07
RCR 3644	60	0.11	100	0.15
CC 511	15	0.03	72	0.18
UK 2	7	0.03	22	0.03
Potting mix	0.0	0.0	0.0	0.0
Applied N (+N)	0.0	0.0	0.0	0.0
No applied N (-N)	0.0	0.0	0.0	0.0
SEM	42	0.06	37	0.05
Significance	p < 0.001	p < 0.001	p < 0.001	p < 0.001
CV (%)	6	8.7	11	34

Potting mix data as described earlier in the material and methods section was eliminated from the analyses when comparing isolates

DAS = Days after sowing

NDW = Nodule dry weight

3.3.2.3 Shoot dry weight plant⁻¹

At 51 DAS, total shoot dry (SDW) plant⁻¹ of N treated plants was 2.53 g plant⁻¹ (Table 3.10). This was 13 % more than that of the heaviest inoculated plant (2.24 g plant⁻¹). Application of N increased SDW by 100 % over 0 N treatments. The lowest SDW was 0.85 g plant⁻¹ produced with strain CC 511. Sixty two percent of isolates were effective at helping plants produce more SDW than the control (Table 3.10) and gave an over all average of 1.46 g plant⁻¹.

There were large differences among isolates at the two harvests. Strain H 12, which gave the highest total SDW at 51 DAS (2.24 g), was replaced by H 20 (7.95 g) by 85 DAS. The two isolates gave 0.25 and 0.23 g of nodule dry weight plant⁻¹ respectively (Table 3.9). Nitrogen increased SDW by 100 % over the 0 N treatments. At the final harvest, inoculation increased total SDW over the 0 N controls. By 85 DAS, 56 % of isolates were associated with plants which produced more total SDW than the overall mean.



Plate 3.2. Nodulation pattern of greenhouse grown common bean cv. Scylla) at 85 days after sowing inoculated with *Rhizobium* isolate H 20.

Table 3.10. The effect of inoculation of common bean cv. Scylla with different *Rhizobium* strains on shoot, leaf, stem, pod, and root nodule dry weight and nodule plant⁻¹ at 51 days after sowing grown in a greenhouse.

Treatment	Shoot (g plant ⁻¹)	Leaf (g plant ⁻¹)	Stem (g plant ⁻¹)	Pods (g plant ⁻¹)
Potting mix	23.31	14.8	5.23	3.27
Applied Nitrogen (+N)	2.53	1.80	0.68	
H 12	2.24	1.60	0.62	
CIAT 899	1.87	1.30	0.55	
H 441	1.83	1.30	0.50	0.08
UK 4	1.67	1.20	0.49	0.10
H 20	1.64	1.20	0.46	
PRF 81	1.57	1.10	0.49	
CARA 56	1.54	1.10	0.42	
HT 2a2	1.44	1.00	0.43	
CESTA 221	1.37	0.99	0.39	
CARA 72	1.30	0.92	0.38	
UK 1	1.27	0.86	0.41	
UK 3	1.26	0.89	0.37	
No applied N (-N)	1.26	0.87	0.39	
PhP 17	1.23	0.86	0.37	
RCR 3644	1.24	0.85	0.38	
UK 2	1.00	0.69	0.30	
CC 511	0.85	0.59	0.26	
SEM	0.27	0.20	0.07	0.03
Significance	p < 0.001	p < 0.001	p < 0.001	ns
CV (%)	5	5	3.3	173

Potting mix data as described earlier in the material and methods section was eliminated from the analyses when comparing isolates.

At 51 DAS, leaves were 70 % of the total SDW averaged over all treatments (Table 3.10). Plants inoculated with H 12, CIAT 899, H 20 and UK 4 gave 1.60, 1.30, 1.20 and 1.20 g plant⁻¹ of leaves respectively. By 85 DAS leaf, as a proportion of total SDW, had fallen to 9 % over all treatments. Strains H 20 and H 12 were among the top performing strains behind CARA 56, H 441 and CIAT 899. Nitrogen significantly (p < 0.001) increased leaf dry weight and gave a 107 % increase in SDW over the control. Nitrogen also gave a higher total leaf dry weight than inoculation.

Differences in stem weight (g plant⁻¹) were significant (p < 0.001) at 51 and at 85 DAS. Stems were 14 and 5 % of total SDW over all treatments at 51 and 85 DAS respectively. At 51 DAS plants receiving N had almost double the stem weight (0.68 g) of

the no N plants at 0.39 g. Plants which received nitrogen also produced more stem DM than all of the inoculation treatments (Table 3.10). At 85 DAS, average stem weight plant⁻¹ over the experiment was 0.49 g. Plants receiving N produced 1.34 g of stem dry weight plant⁻¹ which was 1.04 g more than the control and 0.71 g more than that produced by plants inoculated with the best isolate (CIAT 899) (Table 3.11).

Table 3.11. The effect of inoculation of common bean cv. Scylla with different *Rhizobium* strains on shoot, leaf, stem, and pod dry weight and the number of pods plant⁻¹ at 85 days after sowing when grown in a greenhouse.

Treatment	Shoot (g plant ⁻¹)	Leaf (g plant ⁻¹)	Stem (g plant ⁻¹)	Pods (g plant ⁻¹)	Pods plant ⁻¹
Potting mix	71.7	20.18	8.5	42.9	61.00
Applied Nitrogen (+N)	11.18	3.35	1.34	6.5	13.00
H 20	7.95	1.88	0.57	5.5	10.60
CARA 56	7.94	2.04	0.61	5.3	8.30
CIAT 899	7.76	2.14	0.63	5.0	10.30
H 12	6.68	1.88	0.51	4.3	8.30
HT 2a2	6.39	1.67	0.44	4.3	9.30
RCR 3644	6.36	1.76	0.56	4.1	10.70
UK 4	6.30	1.64	0.52	4.1	8.70
H 441	6.09	1.90	0.58	3.6	10.70
PhP 17	5.40	1.47	0.52	3.4	7.70
PRF 81	4.62	1.22	0.40	3.0	6.30
CARA 72	4.28	1.15	0.41	2.7	5.30
CESTA 221	3.96	1.05	0.38	2.5	5.70
CC 511	3.50	1.07	0.37	2.1	6.70
UK 3	1.85	0.74	0.34	0.8	5.70
UK 1	1.73	0.55	0.25	0.9	4.30
UK 2	1.50	0.48	0.24	0.8	4.30
No applied N (-N)	1.47	0.53	0.30	0.6	3.30
SEM	1.39	0.30	0.11	1.05	1.26
Significance	p < 0.001	p < 0.001	p < 0.001	p < 0.001	p < 0.001
CV (%)	10.5	5	3.4	14	4.8

Potting mix data as described earlier in the material and methods section was eliminated from the analyses when comparing isolates.

3.3.2.4 Root dry weight plant⁻¹

Average root dry weight (RDW) plant⁻¹ increased significantly (p < 0.001) during crop growth in the greenhouse. At 51 DAS, applied N (+N) increased the RDW by 2.35 g over the -N from 2.17 g plant⁻¹ to 4.52 g plant⁻¹ (Table 3.12). Most of the *Rhizobium*

isolates used did not perform better than the -N treatment at 51 DAS (Table 3.12). Only strain H 12 performed better than the -N treatment with 2.40 g root dry weight plant⁻¹. At 85 DAS, an increased RDW (3.56 g) was also observed in the +N over -N plants. Sixty-five percent of the isolates used performed better than the -N treatments at 85 DAS (Table 3.12). In +N treated plants the increase in root dry weight was 0.21 g plant⁻¹ compared to a 0.89 g plant decrease in 0 N plants between 51 and 85 DAS (34 days) (Table 3.12).

Table 3.12. The effect of inoculation of common bean cv. Scylla with different *Rhizobium* strains on root dry weight plant⁻¹ at 51 and 85 DAS grown in the greenhouse.

Treatment	RDW (g plant ⁻¹)	
	51 DAS	85 DAS
Potting mix	7.30	10.83
Applied Nitrogen (+N)	4.52	4.73
H 12	2.40	1.45
CIAT 899	2.17	1.64
No applied N (-N)	2.17	1.28
UK 4	1.94	1.47
H 441	1.92	1.45
UK 1	1.91	1.15
H 20	1.78	1.79
UK 3	1.78	1.14
PRF 81	1.74	1.23
CESTA 221	1.72	1.06
RCR 3644	1.65	1.38
CARA 56	1.60	1.65
PhP 17	1.58	1.81
HT 2a2	1.55	1.40
UK 2	1.53	0.87
CARA 72	1.52	1.26
CC 511	1.46	1.29
SEM	0.30	0.34
Significance	p < 0.001	p < 0.001
CV (%)	19	6.9

DAS = Days after sowing; RDW = Root dry weight.

Potting mix data as described earlier in the material and methods section was eliminated from the analyses when comparing isolates.

3.3.2.5 Shoot:root (S:R) ratio

Shoot:root ratios (S:R) were variable and were significantly ($p < 0.05$ and $p < 0.001$) affected by *Rhizobium* isolate at 51 and 85 days after sowing. At 51 DAS, +N treated

plants had the lowest S:R ratio at 0.57. This was similar to the 0.61 in 0 N plants. Plants inoculated with strain CC 511 had a ratio of 0.61, similar to the 0 N treatment. All the other *Rhizobium* strains had higher ratios (Table 3.13). Plants inoculated with strain CARA 52 produced the highest ratio (0.88). This was followed by PRF 81 (0.84), H 12 and H 441 (0.82) and H 20 (0.81). There was a significant ($p < 0.05$) difference among strains, in strains CARA 72, CESTA 221, CIAT 899, and HT 2a2 ratios ranged from 0.75 to 0.79, while in the other strains they were between 0.61 and 0.67 (Table 3.13).

By 85 DAS, + N plants had increased their S:R ratios nearly 5 times than at 51 DAS. In 0 N control plants, the increase in S:R ratio from 51 to 85 DAS was only 85 %. Ratios were variable among inoculated plants and ranged from 1.47 to 4.79. Inoculation significantly ($p < 0.001$) affected shoot to root ratios (Table 3.13). All inoculated plants had shoot:root ratios which were higher than that of the 0 N control plants. Strains H 20, H 12, H 441, CIAT 899, HT 2a2, CARA 56, RCR 3644, and UK 4 all had a shoot:root ratio > 4 which was 75 % higher than the plants with 0 N application. Plants inoculated with isolates CC 511, PRF 81, CESTA 221, CARA 72 and PhP 17 (Table 3.13) had shoot:root ratios in the range 2-4. Among isolate treated plants, 81 % had a shoot:root ratio which was higher than the 2.50 of +N plants. The remaining 19 % of isolates had shoot:root ratios between 1 and 2. Overall, *Rhizobium* isolates and N increased the shoot to root ratio of the cultivar Scylla when grown in a greenhouse.

3.3.2.6 Pods and pod dry weight plant⁻¹ (Yield)

The pod dry weight plant⁻¹ (yield) varied among inoculated plants (Table 3.11). It increased considerably by 85 DAS in +N plants over 0 N plants. Nitrogen treated plants had about 11 times more pod yield than 0 N control plants (0.6 g to 6.5 g plant⁻¹). Average pod yield over all the treatments (excluding potting mix) was 3.3 g plant⁻¹ at 85 DAS (Table 3.11).

At 51 DAS, only plants treated with strain H 441 and UK 4 had produced any pods at 0.08 and 0.10 g pod plant⁻¹ respectively. By 85 DAS plants inoculated with strains H 20, CIAT 899 and CARA 56 had produced 5 to 5.5 g pods plant⁻¹. Plants inoculated with strains PhP 17, H 441, RCR 3644, UK 4, HT 2a2 and H12 produced 3.4 to 4.3 g plant⁻¹. Inoculation with the other strains yielded less than the overall treatment mean of 3.3 g plant⁻¹ (Table 3.11). Pod yield of plants inoculated with strains UK 1, UK 2 and UK 3 was

0.9, 0.8, and 0.8 g plant⁻¹ respectively. The 0 N plants yielded 0.6 g plant⁻¹. Plants treated with strain CC 511 gave 2.1 g pods plant⁻¹.

3.3.2.7 Plant Nitrogen Concentration

Inoculation significantly ($p < 0.001$) affected plant N concentration at 51 DAS. Plants from the 0 N treatments had the lowest total plant N concentration at the two harvests at 0.96 and 1.24 % at 51 and 85 DAS respectively. Nitrogen increased total shoot N concentration by 2.3 % from 0.96 to 2.40 % over 0 N treated plants. At 51 DAS, all inoculated plants had a higher total plant N % concentration than 0 N plants. Fifty-six percent of the inoculant strains gave plants with a higher plant % N than the plants with applied N (Table 3.14). The highest plant N concentrations were in plants inoculated with isolates H 12 (3.50 %); CIAT 899 (3.41 %); CARA 56 (3.39 %) and H 20 (3.31 %). Strains H 441, UK 2, CARA 72, PRF 81 and HT 2a2 followed with N concentrations of 3.06, 2.74, 2.70, 2.69 and 2.55 %, respectively. These values were higher than the 2.40 % in +N treated plants.

Total plant N concentration had decreased in 63 % of the inoculated plants by 85 DAS. Plants inoculated with strains H 20 and H 12 had the largest decline at 0.51 and 0.50 % respectively. In contrast the largest increase was in plants inoculated with strain CC 511, which increased N from 1.43 % at 51 DAS to 2.59 % at 85 DAS. Plants inoculated with strain PhP 17 increased from 2.18 to 2.43 % (Table 3.14). At 85 DAS, 72 % of the inoculants used gave more plant N than the plants with applied N, while 18 % of isolates used produced less plant N than the plants with -N but not as much as the plants with + N (Table 3.14).

Table 3.13. Shoot:root ratio at 51 and 85 days after sowing with common bean cv Scylla grown in a greenhouse with and without applied nitrogen.

Strains	Shoot: root ratio	
	51 DAS	85 DAS
Potting mix	3.29	6.91
CARA 56	0.88	4.62
PRF 81	0.83	3.53
H 441	0.82	4.19
H 12	0.82	4.60
H 20	0.81	4.56
CESTA 221	0.79	3.53
UK 4	0.78	4.13
CARA 72	0.78	3.50
HT 2a2	0.77	4.52
CIAT 899	0.77	4.67
PhP 17	0.67	2.79
RCR 3644	0.67	4.79
UK 3	0.66	1.73
UK 1	0.65	1.47
UK 2	0.64	1.73
No applied N (0 N)	0.61	1.13
CC 511	0.61	2.84
Applied Nitrogen (+N)	0.57	2.50
SEM	0.07	0.68
Significance	p < 0.05	p < 0.001
CV (%)	17.4	1.7

Potting mix data as described earlier in the material and methods section was eliminated from the analyses when comparing isolates.

Table 3.14. The effect of *Rhizobium* inoculation on plant dry weight (g) (PDW) and plant N concentration (%) and N uptake (g N plant⁻¹) at 51 and 85 days after sowing of common bean (*Phaseolus vulgaris*) cv. Scylla grown in a greenhouse with and without applied nitrogen.

Treatment (strains)	51 DAS			85 DAS		
	PDW (g)	Plant N (%)	N uptake (g N plant ⁻¹)	PDW (g)	Plant N (%)	N uptake (g N plant ⁻¹)
Potting mix	30.57	3.32	101.66	82.51	1.96	160.4
Applied Nitrogen (+N)	7.05	2.40	16.87	15.91	2.21	34.3
H 12	5.03	3.50	17.28	8.14	3.00	23.7
CIAT 899	4.37	3.41	14.74	9.40	3.02	27.8
H 441	4.11	3.06	12.57	7.55	2.93	22.1
UK 4	3.84	2.74	10.68	7.77	2.50	21.1
H 20	3.66	3.31	11.91	9.74	2.80	27.1
PRF 81	3.49	2.69	9.69	5.85	2.41	15.8
No Applied N (-N)	3.43	0.96	3.20	2.75	1.24	3.4
CARA 56	3.40	3.39	11.32	9.60	2.72	26.6
HT 2a2	3.32	2.55	8.40	7.79	2.79	21.5
UK 1	3.29	2.05	6.50	2.88	1.79	5.6
CESTA 221	3.26	2.11	7.33	5.02	2.70	14.3
UK 3	3.19	2.21	7.17	2.99	2.05	6.4
PhP 17	3.07	2.18	6.98	7.21	2.34	18.1
CARA 72	2.99	2.70	8.30	5.54	2.54	14.2
RCR 3644	2.99	2.03	7.06	7.75	2.93	22.6
UK 2	2.55	1.18	2.78	2.37	1.73	4.1
CC 511	2.34	1.43	3.23	4.79	2.59	12.8
SEM	0.68	0.37	2.2	2.2	0.26	4.7
Significance	p < 0.001	p < 0.001	p < 0.001	p < 0.001	p < 0.001	p < 0.001
CV (%)	6.0	11.8	10.4	11.7	6.7	14.8

PDW: Plant dry weight

All plants grown in a perlite sieved and washed river sand mix

Potting mix data as described earlier in the material and methods section was eliminated from the analyses when comparing isolates.

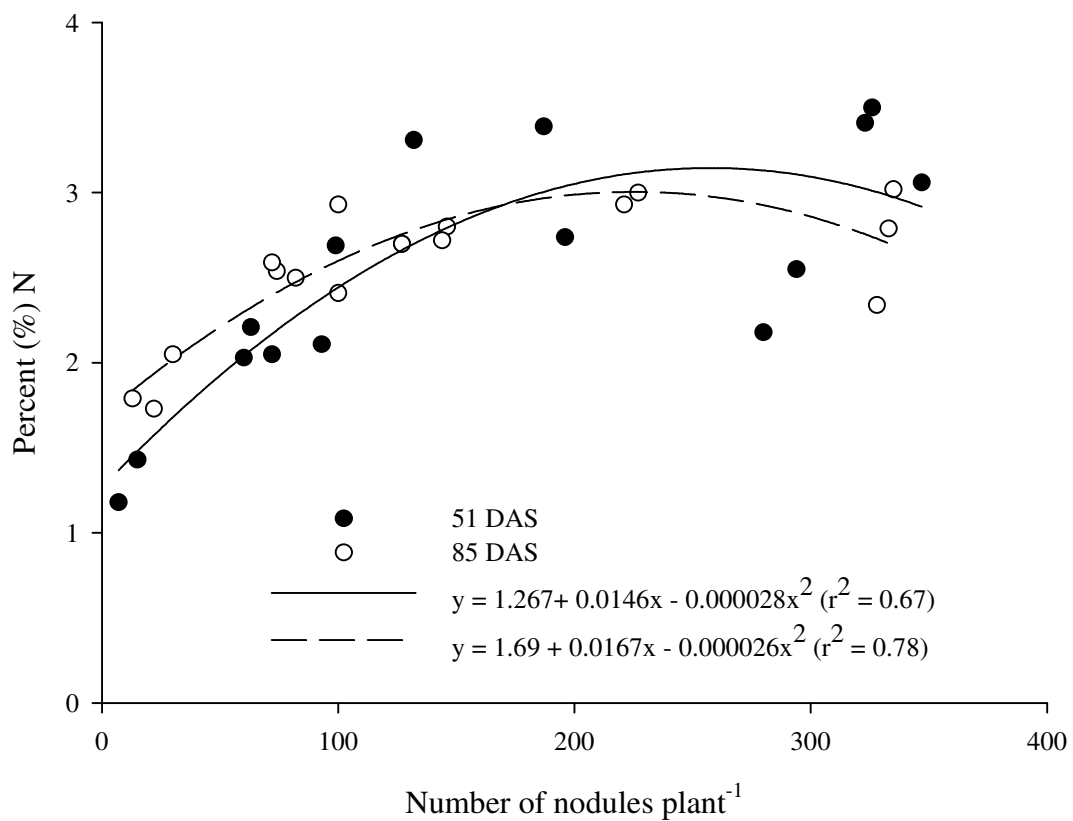


Figure 3.4. The relationship between percent N and the number of nodules plant⁻¹ at 51 (●) and 85 (○) DAS in greenhouse grown common beans.

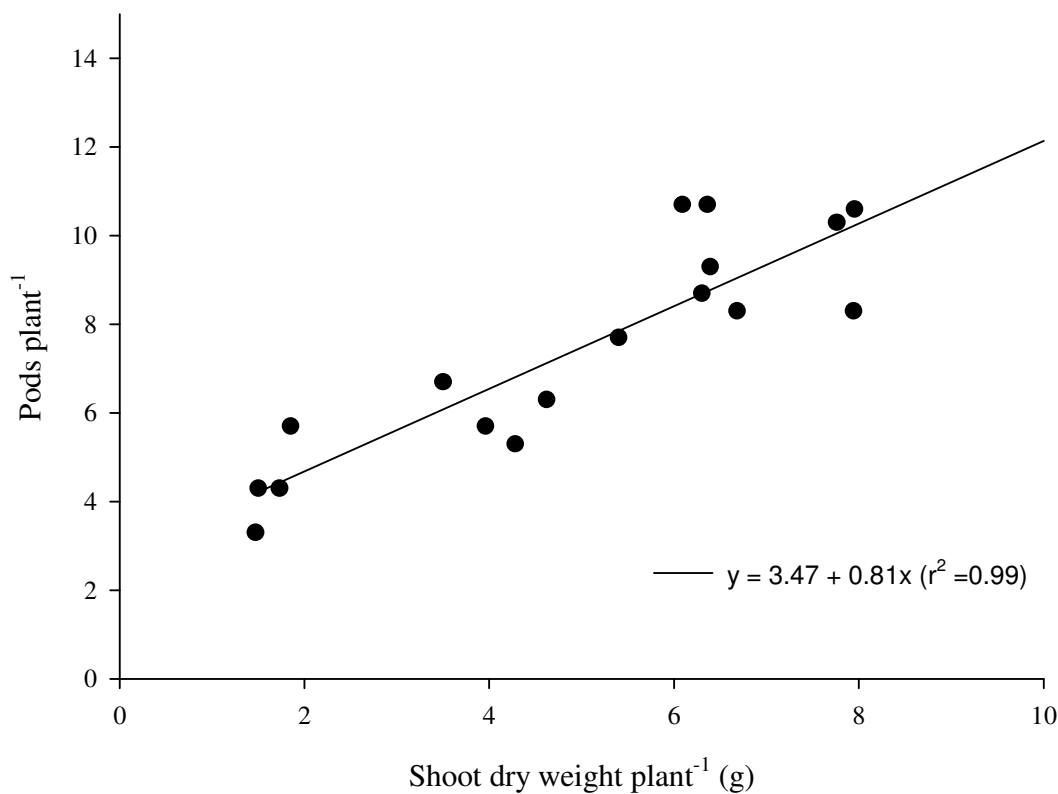


Figure 3.5. The relationship between pods plant⁻¹ and shoot dry weight at final harvest (85 DAS) in the greenhouse.

3.4 Discussion

3.4.1 Inoculation

The response of *Phaseolus vulgaris* to *Rhizobium* inoculation in many parts of the world, New Zealand included, is very inconsistent as observed in this study. Application of inoculum to the seed surface prior to sowing is the traditional, most commonly used and most user-friendly means of inoculation (Brockwell *et al.*, 1995). The primary objective of inoculation is to provide efficient *Rhizobium*, in adequate quantities, to colonise the legume rhizosphere and effect N₂ fixing nodules (Stephens and Rask, 2000; Deaker *et al.*, 2004).

In these experiments, inoculation produced varying results. In the field in 2003-04, inoculation gave no nodulation. However, in the field in 2004-05 and in a greenhouse study there was prolific nodulation with all the isolates tested. When inoculation resulted

in nodulation, it did not significantly increase either green bean or dry bean yield in the field. Generally in the field in 2004-05, and in the greenhouse experiment, inoculation increased total shoot weight, root weight, nodules plant⁻¹, pods plant⁻¹ and plant N concentration. Similar results have been reported with field grown common bean in Brazil (Hungria *et al.*, 2000; Mostasso *et al.*, 2002; Hungria *et al.*, 2003). The fact that *Rhizobium* inoculation affected bean nodulation at one field site and not at another indicates that other edaphic or environmental factors beyond inoculation could have influenced the ability of the inoculant to affect nodulation. Site specificity in plant response to microbial inoculants is not uncommon (Hungria and Bohrer, 2000; Hungria *et al.*, 2003). This is one of the challenges that need to be overcome if microbial inputs are to be more commonly used in agriculture.

The use of inoculation as a management tool in common bean production has been proposed by many authors (Barron *et al.*, 1999; Graham and Vance, 2000; Daba and Haile, 2002; Hungria *et al.*, 2003). In these experiments, the commercially available, and recommended inoculant strains failed in the first field trial (2003-04), performed poorly in the second experiment (2004-05) and virtually failed in the greenhouse even when applied as a liquid inoculant. It is possible that the bacteria in this inoculant did not survive the storage process and thus, were in poor condition at the time of their use. The significant positive effects of high rates of inoculation (high numbers of rhizobia seed⁻¹) have been demonstrated (Roughley *et al.*, 1993; Date, 2001). High numbers of viable cells on the seed or applied to the soil are an important criterion for good nodulation which was not always evident in these experiments.

Peat is universally recognised and widely accepted as the best carrier in the inoculant industry (Daza *et al.*, 2000; Date, 2001), and the benefits of using sterilised over unsterilised peat has been highlighted (Somasegaran, 1985; Catroux *et al.*, 2001). The possibility exists that the peat used may not have been sterilised thus reducing the quality of the product when used. Commercially, in inoculant technology, peat is manufactured to support bacterial populations at, or exceeding, 10⁹ cfu ml⁻¹ (Stephens and Rask, 2000). In these experiments, the peat-based inoculant used contained 2.41x 10⁹ cfu g⁻¹ of moist peat (manufacturer instructions). Working on the growth and survival of *Rhizobium* spp. in peat culture Roughley and Vincent (1967) showed a weekly logarithmic rate of death of bacteria in peat media ranging from nil in sterilised peat at 5 °C with minimal water loss to

0.19 at 25 °C under conditions which permitted considerable water loss. The commercial inoculant used in these experiments was in storage for just under a year which could have reduced bacterial numbers and hence viability in the inoculant (Brockwell and Bottomley, 1995; Catroux *et al.*, 2001). In comparison, the liquid inoculant used in the greenhouse and in the 2004-05 field experiment was freshly made and applied onto the seed in the seedbed. This inoculant was applied when the bacteria were in the exponential growth phase (Table 3.6). Exponential phase cells adapt better to survival in liquid formulations and to survival in soil microcosms resembling drought conditions; two situations commonly faced by commercially produced products (Brockwell and Bottomley, 1995; Soria *et al.*, 2006). One of the factors that will determine the success of a product is its capacity to overcome stress (Soria *et al.*, 2006). Although isolates from commercially available inoculants were used in the greenhouse and the 2004-05 field experiment in a liquid formulation, they still failed to cause nodulation. This was probably due to poor ability to overcome stress or a reduced number of rhizobia remaining in the peat media after more than 11 months storage. This leaves questions with regards to the shelf life and quality control of the inoculant product. In reviewing the status of inoculant technology, Brockwell and Bottomley (1995) proposed that most inoculants produced today are of poor quality and have no practical effect on the productivity of legumes on which they are used. This point could aid in explaining the poor nodulation result obtained with commercial inoculants in this experiment and the world wide poor response of common bean to inoculation.

3.4.2 Nodulation

Effective nodulation is essential for a functioning legume/*Rhizobium* symbiosis. Plants most susceptible to infection and capable of producing effective nodules should have greater potential to fix more atmospheric N. However, this assumption often depends on other factors such as the environment, crop management, choice of micro and macro symbiont and the ability of the plant to support high levels of N fixation (Pereira *et al.*, 1993). The results of this experiment showed great variability in nodulation between the two common bean cultivars and among the *Rhizobium* strains used. This suggests apparent differences in compatibility between the common bean cultivars and the *Rhizobium* strains used.

In 2003-04, two peat-based inoculums recommended for common beans in New Zealand were used. The result was no nodulation. This implies that either the isolates in the inoculum were dead, ineffective or were killed in the soil. The results also indicate an absence of indigenous *Rhizobium* in the soil able to nodulate *Phaseolus vulgaris*. A similar lack of response to bean inoculation was reported in Brazil (Pereira *et al.*, 1984; Ramos and Boddey, 1987). Lack of nodulation has been attributed to numerous factors (Graham, 1981; van Jaarsveld *et al.*, 2002). High levels of soil N have a negative effect on nodulation (Graham, 1981). This could have contributed to a lack of nodulation in the first growing season. In 2003-04, soil N content was not measured but in the 2004-05 the field used for the experiment had a similar cropping history and total N was measured (Table 3.1). The measured total soil N 0.2 % (Table 3.1) is in the range (0.1-0.3 %) which is considered low for New Zealand top soils (Cox, 1978; Watt and Burgham, 1992). In preparation for sowing, the inoculum was mixed into a slurry and applied to the seed. After inoculum application, 3 h was allowed to dry the seed before sowing. The seed was dried under cool room conditions which should not have affected the number of rhizobia introduced into the field.

Dry windy conditions during sowing may have desiccated the bacteria resulting in death and a lack of nodulation. Similar conditions were reported to be a contributing factor in nodulation failure of chickpea (Kyei-Boahen *et al.*, 2005). Working with inoculation methods, Hansen (1994) reported increased strain survival in peat-based inoculants compared to liquid carriers. Hansen (1994) further stated that peat carriers may increase strain survival by reducing desiccation or cell heat stress, a major factor in soil *Rhizobium* establishment. This observation warrants further evaluations in the dry windy conditions of Canterbury, New Zealand to make recommendations on suitable inoculations methods.

All of the *Rhizobium* strains evaluated in the greenhouse and in the 2004-05 field experiment induced nodules on the two common bean cultivars used. These results are in accordance with the assumption that common bean is a non-selective host for nodulation (Michiels *et al.*, 1998). Application of liquid inoculum onto the seed in the 2004-05 experiment gave a concentration of nodules at the root crown. It is well established that movement of *Rhizobium* in soil is limited (Madsen and Alexander, 1982). A concentration of nodules at the root crown is consistent with the results of Hardarson *et al* (1989) and Danso *et al* (1990) who worked with seed applied and granular applied inoculum placed at

seed depth. A preponderance of nodules in the root zone immediately in the region of inoculum deposition could be due to limited *Rhizobium* migration to other infection sites on the root. In comparison, liquid applied inoculum, in the greenhouse experiment, resulted in the formation of nodules along the entire length of the root. This difference in nodulation pattern could be attributed to the river sand/perlite medium used for greenhouse grown plants. Secondly, weekly flushing of the medium could have contributed by moving bacteria down the length of the pots. Inoculation strategies aimed at positioning inoculum so it is intercepted by lateral roots in chickpea have been proposed as a means of improving nodulation of the lower root system (Kyei-Boahen *et al.*, 2002) and improving N₂ fixation.

With soybean, Madsen and Alexander (1982) reported that *Bradyrhizobium japonicum* movement in soil was restricted in the absence of infiltrating water. They further proposed that nodulation of the entire root system requires the root to encounter the inoculant *Rhizobium* in the soil. This was possible in the sieved sand:perlite medium used in the greenhouse and resulted in nodulation of the entire root system. However, it did not occur in the field. The field nodulation pattern in this experiment highlights the poor mobility of *Rhizobium* in soil when it is placed on seed. This is supported by work on chickpea (Kyei-Boahen *et al.*, 2002).

The Phaseoleae are reported to be nodulated by both fast and slow growing bacterial symbionts (Dakora, 2000) and this could be the reason for some of the variation in nodulation. The initial steps towards nodulation are initiated by a number of biochemical signals from both symbionts (Brevin, 1991). The acceptance of these signals is responsible for initiation, continued development and final nodulation. Inconsistent nodulation, as seen in this experiment, could be the result of the highly promiscuous behaviour of *Phaseolus vulgaris* (Graham, 1981; Dakora, 2000) or to inadequate genetic compatibility between the bean cultivars used and *Rhizobium* strain applied (Milev and Genchev, 1997).

Several authors have suggested a reduction in nodulation ability of an inoculant due to extended storage (Stephens and Rask, 2000; Catroux *et al.*, 2001; Deaker *et al.*, 2004). The lack of nodulation in 2003-04 could have been due to a failure of the *Rhizobium* to establish in the rhizosphere, due to the inoculation method used (Brockwell and Bottomley, 1995) and the age of the inoculum (Catroux *et al.*, 2001). The shelf life and storage conditions of *Rhizobium* are important inoculum characteristics which affect the quantity

and quality of viable bacteria colonising the legume rhizosphere (Catroux *et al.*, 2001). In these experiments the inoculum was stored for one month before used at 3 °C and these conditions should not have affected the inoculum strain viability.

In soybean, Catroux *et al.* (2001) found decreased fitness of stored inoculum bacteria over time. The bacteria were also more sensitive to desiccation when inoculated onto seed. These conditions could have contributed to the lack of nodulation in the 2003-04 field experiment. In addition, after inoculation and subsequent seed drying before sowing (2003-04), further removal of bacteria could have occurred as the seed passed through the seeder, contributing to the lack of nodulation. Similar findings were reported for peat-based inoculants by Deaker *et al* (2004).

In comparison direct inoculation of seed with liquid inoculum, as used in the greenhouse and the 2004-05 field experiment could have reduced the loss of viable bacteria through the drill and by desiccation. These two experiments were hand sown and liquid inoculum was applied directly onto the seed in the planting hole. The inoculum used was made from fresh (24 h old) bacterial cultures. Thus the nodulation response observed in the two experiments could have been the result of more active bacteria in the vicinity of the seed, leading to more rapid colonisation of the rhizosphere when liquid inoculum was used compared to the peat-based inoculum.

The ability of indigenous and naturalized bacterial populations to prevent nodulation and displace applied inoculants as the nodulating organisms has been proposed (Graham, 1981; Brockwell *et al.*, 1995). In the 2003-04 field experiment, there was no plant nodulation which suggests a lack of indigenous or naturalised bacteria or a high soil N level. However, given that *Rhizobium* bacteria are said to be widespread in most soils sown to common bean (Vargas *et al.*, 2000), no attempt was made to determine the number of bacteria g soil⁻¹ after inoculation. In the 2004-05 field sowing, control plants which were not inoculated formed nodules. Some of the nodules were pink, denoting the presence of leghaemoglobin. The presence of leghaemoglobin has been documented as a good indicator of active N fixation by the microsymbiont (Ceccatto *et al.*, 1988). These nodules might have been due to the presence of indigenous *Rhizobium* in the soil. This suggested that a naturalised nodulating isolate was present in the soil. This possibility of naturalised nodulating isolates in Canterbury soils merits further exploration.

3.4.3 Shoot and Root growth

Several studies have shown that inoculation of legumes with *Rhizobium* isolates, which result in nodulation, have a positive effect on a number of plant growth parameters (Wange *et al.*, 1996; Rodriguez-Navarro *et al.*, 1999; Kyei-Boahen *et al.*, 2005). These differences in vegetative growth have been attributed to changes in assimilate partitioning (Rodriguez-Navarro *et al.*, 1999). In the pot (greenhouse) experiment top growth was positively influenced by *Rhizobium* inoculation (N availability) in all measured parameters (Table 3.10 and Table 3.11). Inoculation with strain H 12, produced 55 % more nodules plant⁻¹ than H 20 at 85 DAS, but produced less total SDW (Table 3.11). This underlines the importance of the continual screening of *Rhizobium* isolates to improve N₂ fixation. In the greenhouse, strain response to the cultivar used was quite variable. However shoot dry weight response was small but significant.

Variation in plant growth among plants in a treatment was high. Plants grown in potting mix grew faster and produced more plant biomass and yield than all the other treatments. Plant growth in the sand/perlite mix was not as expected. The sand/perlite mix did not appear to be ideal for growth of common beans in these experiments. The highest plant dry weight obtained from plants grown in this mixture was 7.05 g. At both harvests some inoculated plants had lower or only slightly higher shoot DM production than the control 0 N plants. Strains UK 3, UK 2, UK 1 and the control (0 N) gave the least SDM at final harvest. A sand/perlite mix was used in these experiments to reduce the effect of contamination from *Rhizobium* which may have been present if soil had been used. The results suggest the need for further evaluation of the bean/*Rhizobium* interaction in different potting mixtures to ascertain the effect of the mixtures on plant growth and development.

In the greenhouse at 85 DAS, there was a strong relationship between shoot growth and yield (pod plant⁻¹) (Figure 3.5). Inoculation and N, applied individually increased SDM and pod yield plant⁻¹. The 2004-05 field experiment did not give similar results. In the field, inoculation did not affect the SDW at any harvest. However, SDW was significantly affected by cultivar at 21 DAS (Table 3.7). Similar findings confirming the positive effects of N on SDM and yield were reported from field studies with *Phaseolus vulgaris* cv Carioca in Brazil (Vargas *et al.*, 2000).

During the greenhouse experiment there were significant variations in root dry weight. At the first harvest (51 DAS) root weight was higher than shoot weight. This was reversed by 85 DAS. In the field experiment, shoot weight was higher than root weight at all harvests. It is well documented that plants generally invest more biomass in shoots than roots when grown at high levels of N supply but the opposite is observed at low N levels (van de Werf and Nagel, 1996). On the other hand, Beck (1996) considered that higher plants, especially when pre-reproductive partition photosynthetically produced biomass between roots and shoots to effect optimal utilisation of all available resources. At 51 DAS (in the greenhouse) RDW was higher than the SDW. A higher RDW over SDW could be due to a shortage of N as a result of not developing a fully functional N₂ fixation system. Under nutrient and water stress more biomass is allocated relatively to the root system, resulting in an increase in the absorbing plant organ (the shoot) (Beck, 1996; van de Werf and Nagel, 1996).

In the greenhouse shoot:root ratios (S:R) were significantly affected by treatment. This occurred at both harvests. More DM was partitioned to shoots at the expense of the root during the latter half of plant growth. The higher S:R ratios in the greenhouse experiment could be the result of a fully functional N fixation system as a result of effective nodulation. Plants that have an adequate N supply from effective, functioning nodules on the root, direct biomass predominately to the shoot (Beck, 1996), as seen in the latter half of the greenhouse experiment. This could also explain the variation in S:R ratios over the experiment. The S:R ratios of plants are not fixed and vary within species-specific limits in response to the environment (McMichael and Quisenberry, 1991) as seen in these results.

3.4.4 Nitrogen

In the field experiments in both seasons, there were no visible signs of N deficiency. The cultivar, Scylla, grown in the greenhouse, was a determinate bush cultivar of *Phaseolus vulgaris*. Unlike the results of Owens y de Novoa (1980) this growth habit was maintained throughout the experiment. In her work Owens y de Novoa observed a change from bush to climbing growth habit. Clear, visible signs of N deficiency were observed in control plants (0 N) in the greenhouse experiment. Throughout the experiment plants inoculated with isolates UK 3, UK, 2, UK 1, CARA 72 and CC 511 periodically showed signs of N deficiency.

The negative effect of N fertiliser on bean nodulation is well documented (Graham, 1981; Graham *et al.*, 2003) continuing from early studies on this topic (Fred and Graul, 1916). However, farmers have gradually adopted the use of N fertilisers with bean crops, to maximise yields, particularly when irrigation is used. Under these conditions, *Rhizobium* inoculation is complemented by N fertilisation to give high yields (Vargas *et al.*, 2000). The influence of combined *Rhizobium* inoculation and N fertiliser was not explored in this work due to the lack of nodulation in the 2003-04 experiment. The 2004-05 experiment aimed at exploring the nodulating abilities of the isolates tested. There was also no combined *Rhizobium* inoculation and N fertiliser treatment in the greenhouse experiment.

In 2003-04, there was no significant effect of N on TDM, pod yield or HI at green pod harvest. In contrast, by final harvest there was a significant N effect ($p < 0.001$) on both TDM and seed yield. The significant response of TDM production and seed yield to N could be the result of low available soil N (Hungria and Vargas, 2000) induced by previous oat and barley crops. It is possible that N was utilised early during crop growth giving no significant N effect on the measured parameters. A similar, strong fertiliser N response for shoot biomass and grain yield in navy and culinary beans was reported by Redden and Herridge (1999) in Australia. They stated that to satisfy crop N requirements, culinary and navy beans required N fertilisation when grown in soil low in nitrates, due to inadequate N_2 fixation capacity.

3.4.5 Plant Nitrogen Concentration

In the greenhouse, at both harvests, N fertilised plants yielded more TDM plant⁻¹ and total plant N than 0 N control plants (Table 3.14). In the greenhouse at 51 DAS, 37 % of the isolates helped plants to produce more total plant dry matter than the uninoculated plants, but less than plants with applied N. At 85 DAS, 93 % of the isolates performed better than the -N treatment but were not better than the +N treatment in plant dry matter, confirming the preference of commercial lines of common bean to fertiliser N application (Vargas *et al.*, 2000). Total plant N concentration was higher in all plants at 51 DAS than at 85 DAS. All treatments gave a higher plant N concentration than the 0 N treated plants. The N concentration of plant DM varies between 1 and 6 % depending on plant species, age, plant organ and environment (Haynes *et al.*, 1986; Mengel and Kirkby, 1987). The results obtained here are well within these limits.

Plant N concentration declines as the plant grows, even when ample supplies of N and other nutrients are available (Greenwood *et al.*, 1991; Gastal and Lemaire, 2002). This is supported by the results of both the field and greenhouse experiments. In the greenhouse experiment, the number of nodules plant⁻¹ was poorly correlated with plant % N (Figure 3.4), although treatments with the highest number of nodules plant⁻¹ had the highest plant % N concentration (Table 3.9 and Table 3.14). The pattern, seen in these experiments, of decreased plant % N with increased growth (DAS) was similar to that observed in both vegetable and herbage crops (Greenwood *et al.*, 1991) provided growth was not limited by some other environmental factor. The decline in plant % N was attributed to the amount of N in photosynthetic and metabolic/structural components of the plants (Greenwood *et al.*, 1991) which fall in relation to one another. As plants emerge and develop through the constant phase, the photosynthetic component of the plant remains relatively constant (Greenwood *et al.*, 1991). Subsequently, the % N in the plant decreases as the plant develops towards the structural phase (Greenwood *et al.*, 1990). An exception to this rule was found in white lupin (*Lupinus albus* L.), where plant % N only varied according to plant density and between N nutrition regimes in non-inoculated plants (Duthion and Pigeaire, 1993). Inoculating lupin plants did not affect the trend of decreased plant % N (Duthion and Pigeaire, 1993) as seen in these experiments.

In grain legumes, plant % N is highly sensitive to early growth conditions of the plant which affect nodule formation and efficiency (Ney *et al.*, 1997). In addition, it is also influenced by the ratio of seed to other plant components (Ney *et al.*, 1997). In this work, plant % N was measured on composite plant samples which could have influenced any differences in N₂ fixation due to different *Rhizobium* strains. The incorporation of the seed could have affected the final plant % N, as N is transferred from other plant parts to the seed at the start of seed filling (Greenwood *et al.*, 1991). The fact that the well nodulated plants also had the highest plant N % concentration implies a positive effect of nodulation on plant % N. Hence the ability of the isolates to establish a symbiosis had an impact on symbiotic nitrogen fixation, plant growth and consequently plant % N. These findings require further examination. Future work should involve the evaluation of individual plant components of plant N %, which might give a better indication of plant N accumulation. A recent report by Tejera *et al.* (2005) working with bean showed a positive significant correlation between nodule number and shoot dry weight and nodule number and N % confirming the importance of symbiosis in N accumulation in legumes.

3.5 Conclusions

1. Inoculation can enhance nodulation and growth in common bean in Canterbury, however additional information is required to address the issue of inconsistent responses among sites.
2. Field surveys are required to determine the presence or absence of nodulating bacteria and delineate areas where inoculation failure has occurred.
3. Greenhouse work on the evaluation of *Rhizobium* isolates should be closely tied to field evaluation before recommendations on the effectiveness of isolates are made.
4. Further field evaluation is needed as inoculation and N significantly increased root and shoot growth, DM, number of nodules and green pod yield in common bean in the greenhouse.
5. Rhizobial strains H 20 and PRF 81 gave promising results in terms of nodulation, plant growth and yield and requires further evaluation at different sites in Canterbury.
6. Suitable *Rhizobium* strains should be continually tested to improve nodulation and N fixation in suitable bean varieties.

CHAPTER 4

Management, cultivar and *Rhizobium* inoculation effects on the growth and development of common beans (*Phaseolus vulgaris* L.): Phenology, growth and dry matter accumulation.

Summary

Between 2003-2005 field experiments were conducted to investigate the effect of *Rhizobium* inoculation, cultivar and management on the phenology, growth and dry matter (DM) accumulation of common beans (*Phaseolus vulgaris* L.) in Canterbury. Progression through the different physiological stages varied with chronological and thermal time. In both seasons, cultivar T-49 reached maturity (dry seed) before Scylla. On average, unirrigated plants reached green pod maturity seven days before irrigated plants. Scylla plants gave a final total dry matter (TDM) of 730 g m⁻²; while T-49 produced only 530 g m⁻². Plants receiving 150 kg N ha⁻¹ produced 665 g m⁻² TDM which was 12 % more than the production of unfertilised plants. Average growth rates were 7.0 and 5.2 g m⁻² day⁻¹ for Scylla and T-49 respectively (2003-04). Application of 150 kg nitrogen (N) ha⁻¹ gave an average growth rate of 6.4 g m⁻² day⁻¹ compared to 5.7 g m⁻² day⁻¹ in plants with no application of N. Nodulation had no significant effect on TDM in the field. In 2004-05, there was no significant effect with any of the treatments used.

4.1 Introduction

Crop productivity is the rate at which a crop accumulates DM per unit area per unit time (Acquaah, 2005). This process primarily depends on the rate of photosynthesis and the conversion of light energy to chemical energy by green plants (Tsubo and Walker, 2004). Accumulation of dry weight in plants is a direct result of the balance among photosynthesis, respiration and losses caused by senescence and abscission. Partitioning on the other hand; establishes an equilibrium between vegetative and reproductive growth

integrated during development, resulting in an end product of economic yield (White and Izquierdo, 1991).

Crop growth or DM production is defined as changes in dry mass, size or number of cells of a plant or its organs (Monteith, 1981; Gallagher *et al.*, 1983). It is strongly related to radiation interception during the season. In seed crops, common bean included, DM is initially slowly accumulated, followed by a rapid near exponential growth phase, which continues until flowering and the start of pod filling (White and Izquierdo, 1991). Subsequently, DM decreases due to the fall of senescent leaves, and loss of DM in leaves, stems and pod valves caused by retranslocation of assimilates to the seed (Stutzel and Aufhammer, 1992). Shoot biomass accumulation is an important trait to attain high seed yield in grain legumes, as is a long duration of the vegetative and reproductive stages (Rosales-Serna *et al.*, 2004).

The productivity of grain crops depends not only on DM accumulation, but also on its effective partitioning to economically important plant parts (Richards, 2000; Kumar *et al.*, 2006). Wallace *et al.* (1993) stressed the importance of increased partitioning of DM to reproductive yield components in beans for improved yield performance. In grain crops, the harvest index (HI) - a measure of the proportion of total dry matter (TDM) of a crop that represents economic yield is often used as a measure of partitioning. This measurement neglects changes in partitioning between vegetative and reproductive plant parts during growth and development of the crop because it is measured at maturity (Egli *et al.*, 1985).

Growth, DM accumulation and its partitioning in grain legumes, are affected by a number of factors, the most important of which are the photo thermal regimes, the amount of solar radiation intercepted, temperature, water stress and soil edaphic factors which can equally affect the symbiotic process. In Canterbury, inoculation with *Rhizobium* has given variable responses with the currently recommended inoculant strains for common beans. Two experiments were designed to evaluate the response to currently recommended inoculants and a number of new inoculant bacteria for their effect on the growth, DM accumulation and partitioning of common bean.

4.2 Materials and Methods

Experimental and husbandry details for the experiments described in this chapter are fully described in the previous chapter (Chapter 3, Materials and Methods). Therefore, only a brief description will be given here. This chapter is based on the results of two field experiments. In 2003-04, a split plot completely randomised block design was used, with irrigation as the main plot (nil and full). Sub-plot combinations were common bean cultivars (Scylla and T-49); three inoculation treatments (*Rhizobium tropici* strains CC 511 and RCR 3644 and a control (nil *Rhizobium*)) and two fertiliser treatments (nil and 150 kg N ha⁻¹ (Calcium ammonium nitrate 27 % N)). In 2004-2005, a completely randomised block design with 4 replicates and factorial combinations of bean cultivar (T-49 and Scylla) and inoculation treatments (strains RCR 3644, UK 2, H 20, PRF 81, PhP 17, and a nil *Rhizobium* control) was used.

4.2.1 Measurements and Sampling

Climate data was recorded at the Broadfields Meteorological station situated 2 km from the experimental area. In 2003-04, plant biomass from each plot was obtained from randomly selected 0.2 m² quadrats taken every 10-14 d from 23 days after sowing (DAS) for DM accumulation. One metre square quadrat sample were taken from the six central rows of each plot at harvest maturity for final plant population, DM and seed yield. Plants were cut at ground level, bagged, air-dried and machine threshed (Kurtpelz stationary thresher), then dried in a forced-draught oven at 70 °C for 48 h.

In 2004-2005, five sets of DM samples were taken: at 21 DAS; when 50 % of plants had at least one open flower; when over 50 % of plants had at least one green pod 5 mm long; at green bean harvest (Appendix 1A) and at dry bean harvest. At each harvest, six plants were taken from the three centre rows of each plot (0.09 m²) in sequential order from a randomly selected side of the plot. The first two rows were omitted at the beginning and a buffer of one row (three plants) was used between harvests. Seed yield, and pods plant⁻¹ were taken from the six plants at final dry bean harvest. The 100 seed weight was determined from a randomly selected sample of 50 pods taken from the three centre rows of each plot. Plant material was dried in a forced-draught oven at 70 °C for 48 h. Pods were hand threshed and weighed.

4.2.2 Growth analysis

All statistical analyses were computed using GenStat (GenStat Release 6.1 Lawes Agricultural Trust, Rothamsted Experimental Station, Hertfordshire, UK). Standard errors of the mean (SEM), the coefficient of variation (CV as a %) and percentage variation accounted for (r^2) were also calculated. Average growth rates were produced from the linear portion of the growth curves defined as the time between 5 and 95 % of maximum final TDM for each plot. The slopes were then analysed by ANOVA. Regression analysis was done with Sigmaplot 2002 for Windows Version 8.2 Copyright © 1986-2001 SPSS Inc.

4.3 Results

4.3.1 Climate

The climate experienced during the experimental period was described in Section 3.3.1.1.

4.3.2 Phenology

Phenological development, in this study, was recorded in both days and in thermal time. Only cultivar and irrigation had any effect on development. In the first season (2003-04), both cultivars reached 50 % emergence at 8 DAS. Cultivar T-49 took 38 d from 50 % emergence to 50 % flowering (E-F), three days less than Scylla. Both cultivars reached green pod harvest at 76 DAS (Table 4.1). Scylla reached dry bean maturity at 116 DAS, five days after T-49 at 111 DAS. Non irrigated plants reached green pod maturity (Appendix 1A) before irrigated plants. In 2004-05, days to 50 % flowering and green pod maturity were 6 d less than in 2003-04, while days to dry bean was similar in cv. T-49 at 112 days (Table 4.1). Scylla plants were also harvested at 112 DAS (Table 4.1), but this was before the defined dry bean harvest date.

The thermal time (T_t) above a T_b of 10 °C from sowing to emergence was similar (43 °C days) for both irrigated and non-irrigated plants in 2003-04. In 2004-05, thermal time increased to 48 °C days (Table 4.2). Cultivar affected accumulated T_t from E-F irrespective of irrigation treatment. Cultivar T-49 accumulated 280 °C days between E-F compared to 298 °C days for Scylla (Table 4.2). In 2003-04, plants accumulated 658 °C days until maturity compared to the 595 °C days accumulated in 2004-05. In 2004-05,

plants took 267 °C days from 50 % emergence to 50 % flowering (E-F), 185 °C days from 50 % flowering to podding (F-P) and 95 °C days from podding to physiological maturity (P-M) (Table 4.2).

Table 4.1. Chronological time for four physiological growth stages [(sowing to emergence (S-E), emergence to flowering (E-F), flowering to podding (F-P), Podding to physiological maturity (P-M)] of common bean cultivars under irrigated and non-irrigated conditions in Canterbury, New Zealand.

Genotypes	2003-04						2004-05					
	Non-irrigated			Irrigated			Non-irrigated			Irrigated		
	S-E (d)	E-F (d)	F-P (d)	P-M (d)	S-E (d)	E-F (d)	F-P (d)	P-M (d)	S-E (d)	E-F (d)	F-P (d)	P-M (d)
T-49	8	38	30	35	8	38	33	32	7	32	30	42†
Scylla	8	41	27	40	8	41	30	37	7	32	30	42†

Inadequate variation in the data for a proper analysis of variance.

In 2004-05, Scylla was harvested before dry bean harvest (when 95 % of the plants turn brown)

† harvested early due to disease

Table 4.2. Thermal time for four physiological growth phases [(sowing to emergence (S-E), emergence to flowering (E-F), Flowering to podding (F-P), and podding to physiological maturity (P-M)] of common bean as affected by irrigation and cultivar in Canterbury, 2003-2005.

Thermal time for phase (°C days) ($T_b = 10\text{ }^\circ\text{C}$)												
2003-04												
Cultivar	Non-irrigated					Irrigated						
	S-E	E-F	F-P	P-M	S-E	E-F	F-P	P-M	S-E	E-F	F-P	P-M
T-49	43	280	177	150	43	280	190	138	48	267	185	95†
Seylla	43	298	159	167	43	298	171	155	48	267	185	95†

Inadequate variation in the data for a proper analysis of variance.

In 2004-05, Seylla was harvested before dry bean harvest (when 95 % of the plants turn brown)

† harvested early due to disease

4.3.3 Maximum total dry matter

In 2003-04, N fertiliser significantly ($p < 0.001$) affected final maximum TDM production (Table 4.3). Plants given 150 kg N ha^{-1} produced the second highest DM at 665 g TDM m^{-2} , compared to 595 g m^{-2} from unfertilised plants. Cultivar significantly affected ($p < 0.001$) final maximum TDM. Scylla plants produced 730 g m^{-2} , of TDM. This was 200 g m^{-2} more than cv. T-49. Inoculation and irrigation had no significant effect on TDM and the grand mean was 630 g m^{-2} (Table 4.3). In 2004-05, final maximum TDM (Table 4.3) was not significantly affected by any of the treatments used and the grand mean was $1,044 \text{ g m}^{-2}$, 40 % more than in 2003-04.

Table 4.3. The effects of inoculation, cultivar, fertiliser and irrigation on final maximum TDM (g m^{-2}) of common beans in Canterbury, 2003-04 and 2004-05.

2003-04		2004-05	
Inoculation (IN)		Inoculation (IN)	
Nil	625	Nil	948
CC 511	640	RCR 3644	1227
RCR 3644	625	UK 2	1000
SEM	12.6	H 20	900
Significance	Ns	PRF 81	1117
		PhP 17	1072
		SEM	116.0
Cultivar (CU)		Cultivar (CU)	
Scylla	730	Significance	ns
T-49	530		
SEM	10.3	Scylla	1066
Significance	$p < 0.001$	T-49	1022
		SEM	67.0
Irrigation (IR)		Significance	
Nil	604		ns
Full	656		
SEM	25.9		
Significance	Ns		
Fertiliser (FE)			
0 (kg N ha^{-1})	595		
150 (kg N ha^{-1})	665		
SEM	10.3		
Significance	$p < 0.001$		
CV (%)	5.7		6.5
Significant	nil		nil
Interactions			

4.3.4 Average growth rate

The average growth rate was significantly affected ($p < 0.001$) by both cultivar and fertiliser (2003-04) (Table 4.4 and Figure 4.2a, b). Scylla plants had an average growth of $7.0 \text{ g m}^{-2} \text{ d}^{-1}$ which was $1.8 \text{ g m}^{-2} \text{ d}^{-1}$ more than T-49 plants. Plants receiving 150 kg N ha^{-1} had an average growth rate of $6.4 \text{ g m}^{-2} \text{ d}^{-1}$ which was $0.7 \text{ g m}^{-2} \text{ d}^{-1}$ more than the $5.7 \text{ g m}^{-2} \text{ d}^{-1}$ attained by the plants which received no N. In 2004-05, average growth rate was not affected by any of the treatments used Table 4.4.

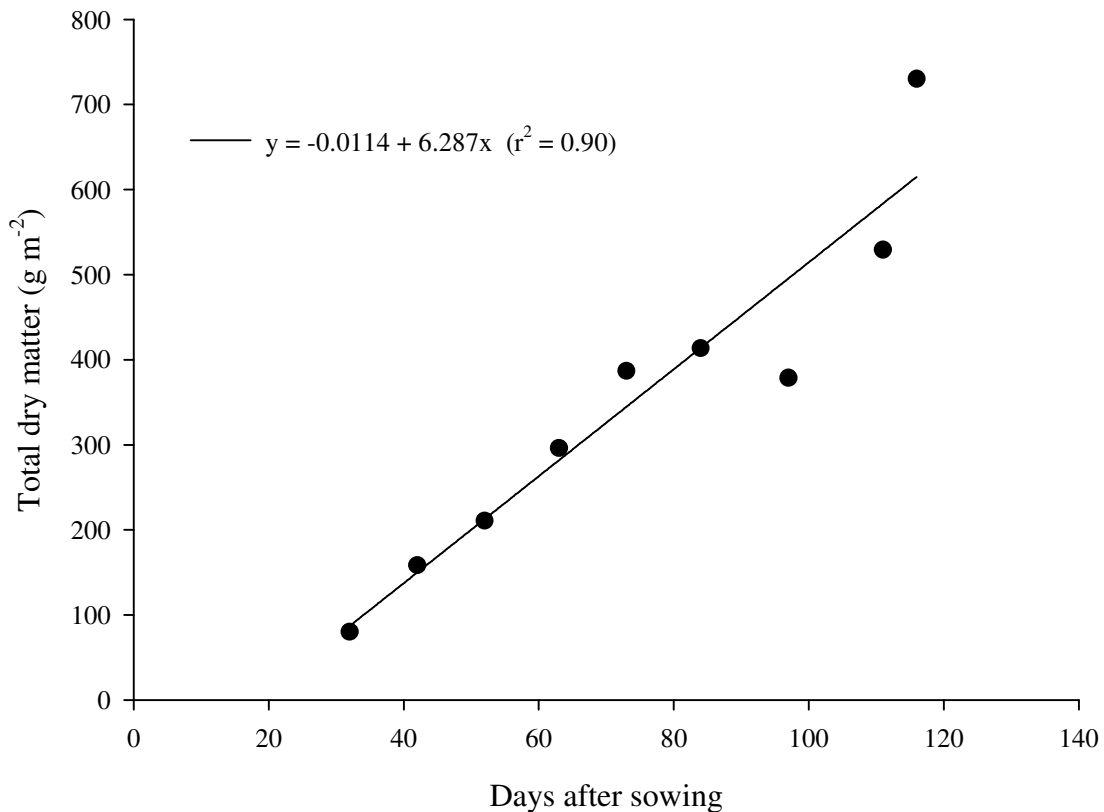


Figure 4.1. The relationship between final maximum total dry matter (g m^{-2}) and days after sowing calculated over the linear portion of the growth phase for common beans in Canterbury, 2003-04. The relationship is represented by the equation $y = -0.0114 + 6.28x$ ($r^2 = 0.90$). The average growth rate over the season was $6.28 \text{ g m}^{-2} \text{ d}^{-1}$.

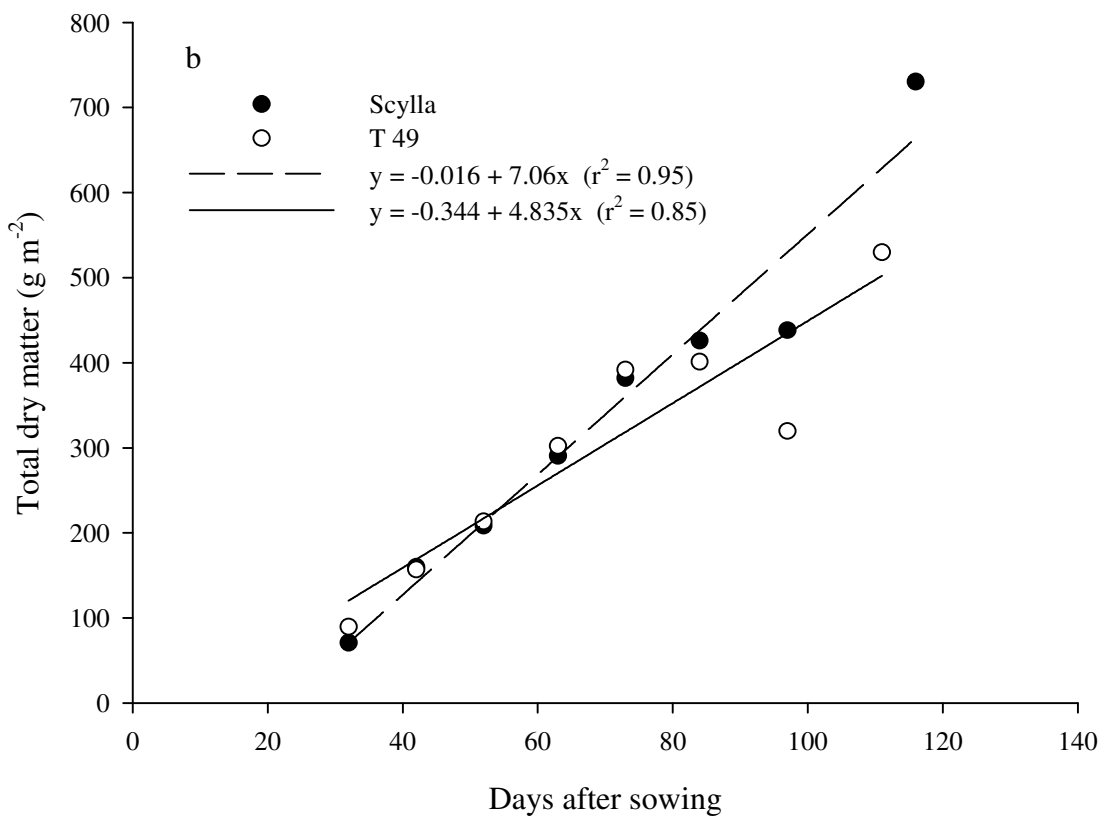
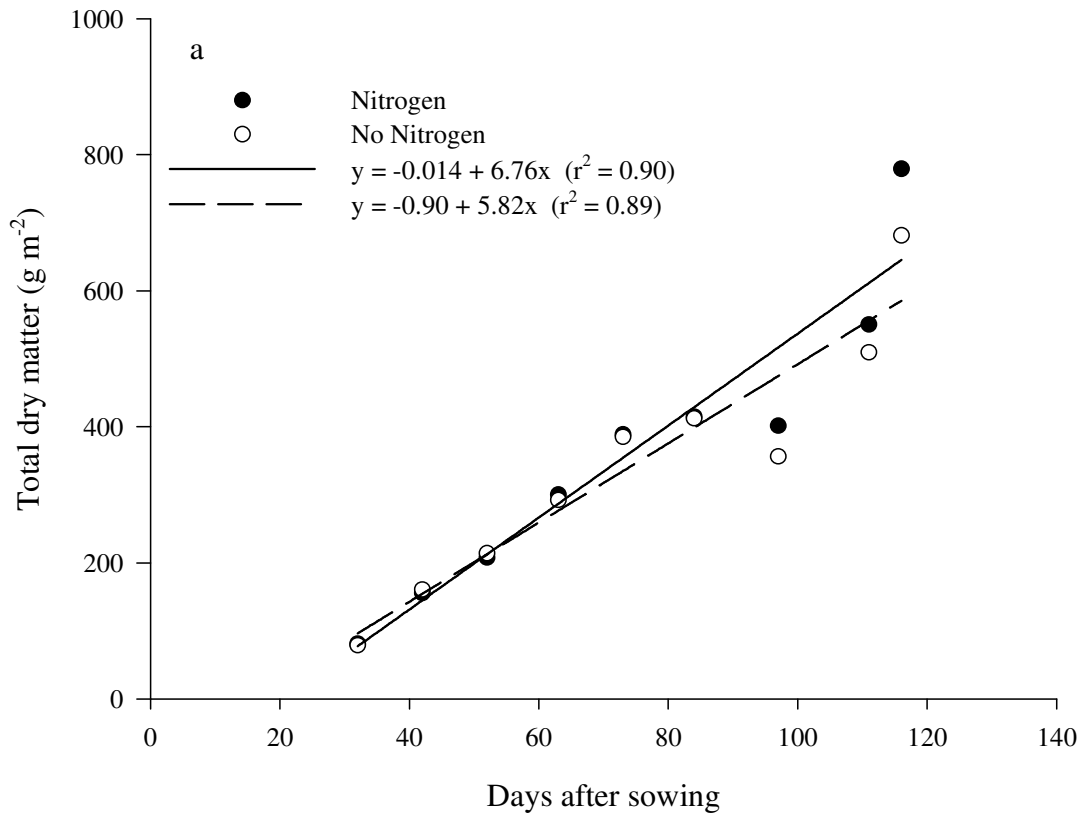


Figure 4.2. The effects of fertiliser (a) and cultivar (b) on total dry matter (g m^{-2}) over time (days) for common beans in Canterbury, 2003-04. Average growth rates: Nitrogen = 6.7; No Nitrogen = 5.8; Scylla = 7.1; T-49 = 4.8 $\text{g m}^{-2} \text{d}^{-1}$.

Table 4.4. The effect of inoculation, cultivar, fertiliser and irrigation on the average growth rate ($\text{g m}^{-2} \text{d}^{-1}$) of common beans in Canterbury, 2003-04 and 2004-05.

2003-04		2004-05	
Inoculation (IN)		Inoculation (IN)	
Nil	6.1	Nil	8.3
CC 511	6.1	RCR 3644	12.8
RCR 3644	6.0	UK 2	9.5
SEM	0.12	H 20	9.5
Significance	ns	PRF 81	11.3
		PhP 17	12.2
		SEM	1.12
Cultivar (CU)		Significance	ns
Scylla	7.0		
T-49	5.2		
SEM	0.096	Cultivar (CU)	
Significance	$p < 0.001$	Scylla	11.4
		T-49	9.8
		SEM	0.65
Irrigation (IR)		Significance	ns
Nil	5.7		
Full	6.4		
SEM	0.34		
Significance	ns		
Fertiliser (FE)			
0 (kg N ha^{-1})	5.7		
150 (kg N ha^{-1})	6.4		
SEM	0.096		
Significance	$P < 0.001$		
CV (%)	6.4		7.8
Significant	nil		nil
Interactions			

4.4 Discussion

4.4.1 Phenology

While water stress had no effect on TDM production, it accelerated the rate of plant development to maturity in unirrigated crops. Similar responses to water stress have been reported for other grain legumes, Kabuli chickpea (Anwar *et al.*, 2003), pinto bean (Dapaah *et al.*, 1999) and faba bean (De Costa *et al.*, 1997). There was a difference of three days from sowing to podding between irrigated and unirrigated plants (2003-04) and seven days from sowing to podding between irrigated plants (69 d) (2004-05) and

unirrigated plants (76 d) (2003-04) (Table 4.1). Sowing beans later than the recommended mid November sowing date (McKenzie, 1989) may explain why plants flowered and podded earlier in the second season. Later sowing (2004-05), resulted in seed being sown and germinating at higher average temperatures 16.3 °C (2004-05) compared to 15.8 °C (2003-04). Higher average temperatures contributed to faster development of the different phenological stages. For peas, Al-Karaki (1999) found that the rate of phenological development was increased with increased temperature. Crop development was generally faster in 2004-05 than in 2003-04. Water stress, resulted in higher leaf and canopy temperatures (Jackson, 1981; Siddique *et al.*, 2000) and results in a more rapid thermal time accumulation. According to Gardner *et al.* (1981) working with various crops and de Almeida Lobo *et al.* (2004) working with common bean the standard deviation values of plant canopy temperature ranged from 0.3 °C to 4.2 °C for well irrigated and stressed plants, respectively. With limited water, transpiration is reduced and leaf temperature increases due to absorbed radiation and warmer air temperatures around the leaves.

The days to reach different phenological crop growth stages varied between cultivars (Table 4.1). Cultivar T-49 reached physiological maturity in 111 d compared to 116 d in cv. Scylla (2003-04). In 2004-05, the crop was harvested before physiological maturity (111 d). In 2004-05, due to late sowing, the crop was maturing under decreasing temperatures (Figure 3.1). Low temperatures and high humidity during the period from podding to physiological maturity (2004-05) predisposed the crop to disease resulting in its early harvest. Genetic characteristics could explain why Scylla took longer to reach harvest maturity. Cultivar Scylla is a green bean cultivar (70-75 d to maturity) and would not normally remain in farmers fields until dry pod harvest. In this experiment, the climatic conditions (low temperature) during growth could have slowed growth causing the plants to take longer to reach maturity. Similar results were reported for soybean (Mayers *et al.*, 1991). The prevailing weather contributed to cv. Scylla being harvested before 95 % of the plants had turned brown.

Irrespective of irrigation treatment (2003-04), thermal time (T_t) accumulation from emergence to flowering (E-F) varied between cultivars (Table 4.2). Cultivar T-49 accumulated 280 °C d from E-F, 18 °C days less than Scylla at 298 °C d. Average accumulated thermal time over the season above a T_b of 10 °C was 656°C d and 575 °C d in 2003-04 and 2004-05 respectively. These values are much lower than the 800 to 960 °C

d range recorded by Greven (2000). Greven (2000) worked with dwarf French beans and the crop was sown in late October/early November, the recommended window for sowing *Phaseolus* beans in Canterbury (McKenzie, 1989). Results close to those obtained in this study were reported by Dapaah (1997) for pinto beans. In other grain legumes, accumulated T_t units of 1,824-2,628 °C d for field beans (Husain *et al.*, 1988a) and 1,480-2,080 °C d for lentil (McKenzie and Hill, 1989) were reported from sowing to harvest in Canterbury. Both field beans and lentils are long duration cool season crops with a T_b near 0 °C (Husain *et al.*, 1988a; McKenzie and Hill, 1989). This is the reason for the accumulation of their higher °C days compared to a short duration, warm season crop such as common bean. Ensuring crop phenology matches the environmental conditions (especially temperature and photoperiod) has been suggested as one of the most important steps towards maximising crop yield (Summerfield *et al.*, 1994; Al-Karaki, 1999; Anwar *et al.*, 2003). The narrow window for production of common beans in Canterbury makes cultivar selection an important management concern to reduce harvest losses. This was highlighted in this study in 2004-05, when sowing after the recommended mid November sowing date (McKenzie, 1989), resulted in the crop having to be harvested before 95 % of the plants had turned brown. Around 75% of the plants were brown at harvest.

4.4.2 Maximum total dry matter production

Previous studies in Canterbury have shown variable responses in maximum DM production to inoculation and additional N. No response to either inoculation or additional N was found in chickpea by Kosgey (1994). This was attributed to poor nodulation and mineralisation of organic N with rising spring temperatures. In contrast, increased maximum TDM in response to inoculation and additional N was reported in lentil (Turay, 1993) and chickpea (Hernandez, 1986) in a greenhouse. McKenzie (1987) found no response to either inoculation or additional N in field grown lentil. In this study, a limited increase in maximum TDM was observed with additional N (2003-04). This could be due to nodulation failure or an indication that the soil N level was relatively high.

Total dry matter showed a decline at around 90 DAS (Figure 4.1 and Figure 4.2). This decline corresponded to a period of cool temperatures and a frost which occurred on 25 February 2004. Leading up to 25 February 2004, minimum temperatures ranged from 9.1 to -2.7 °C with an average of 5.5 °C. In common bean, the negative effect of low temperature on growth and development has long been proposed (Peet *et al.*, 1977; Masaya

and White, 1991; Knell *et al.*, 2006). Frost caused premature leaf drop (2003-04) between 80 and 90 DAS which could have affected the TDM. Zero degrees Celcius is considered the lethal minimum temperature for which common bean is affected by frost (Knell *et al.*, 2006), although frost can occur at temperatures above 0 °C (Hawthorne, 2006; Knell *et al.*, 2006). In this period of the experiment, the temperature was well below the 10 °C base temperature for growth in common bean suggested by Greven (2000), and below the 15 °C shown by Austin and Maclean (1972a) to severely limit growth of dwarf beans in Britain. The reduction in TDM around 90 DAS could be the result of the plants having to recover from the damage or destruction of the terminal primordia due to exposure to low temperatures (Kemp, 1973). In addition to temperature, additional factors that can influence frost risk are crop variety, canopy management and row spacing, stubble presence and atmospheric and soil moisture levels (Knell *et al.*, 2006).

In 2004-05, there was good nodulation with some inoculant strains. Over all treatments, final maximum TDM was higher in 2004-05 than in 2003-04 (Table 4.3). The increased final maximum TDM in the second season was probably a result of better growing conditions. The growth promoting effect of the identified organisms (Chapter 7) could also partially account for the increased final maximum TDM.

4.4.3 Average growth rate

The average growth rate over the 2003-04 season was $6.28 \text{ g m}^{-2} \text{ d}^{-1}$ which is much lower than the weighted mean absolute growth rate (WMAGR) of $9.06 \text{ g m}^{-2} \text{ d}^{-1}$ for pinto beans reported by Dapaah (1997) and the $17.1 \text{ g m}^{-2} \text{ d}^{-1}$ for dwarf French beans reported by Greven (2000). The significant effect of cultivar and N on average growth rate as seen in these experiments supports reports of common bean cultivars being bred and selected for in areas of high soil fertility (Bliss, 1993b, a). Vargas *et al.* (2000) also reported significant growth rate responses in common bean in response to N application in Brazil, and suggested that N fertilisation may be necessary to reach maximum yields. Bean yields (TDM) frequently differ with cultivar differences in phenology, growth habit, seed size and other traits (White and Izquierdo, 1991; White *et al.*, 1992a). Increased crop yield arises out of the ability of the plant to intercept more solar radiation (Dawo *et al.*, 2007). More intercepted solar radiation should result in high biomass values as a result of increased growth rates in the absence of growth limiting factors (Lopez-Bellido *et al.*, 2005). This probably accounted for the higher biomass values in 2004-05 than in 2003-04.

4.5 Conclusions

1. Time of sowing and irrigation affected the rate of development toward different phenological stages in common bean grown in Canterbury.
2. Fertiliser and cultivar increased final TDM production by increasing the crop average growth rate.
3. The cv. Scylla had the higher average growth rate (2003-04) with no observed nodulation response.

CHAPTER 5

The influence of inoculation, cultivar and fertiliser on leaf area index (LAI), radiation interception and radiation use efficiency (RUE)

Summary

Studies on the effect of inoculation, cultivar and management on the leaf area index (LAI), radiation interception and radiation use efficiency (RUE) of common bean (*Phaseolus vulgaris* L.) cv. Scylla and T-49 were undertaken in Canterbury, New Zealand. In 2003-04, two irrigation treatments combined with two cultivars, two fertiliser and three inoculation treatments were used. In 2004-05, six inoculation treatments and two cultivars were studied. Leaf area index, LAD, intercepted radiation and final utilisation coefficient were significantly affected by cultivar. A maximum LAI of 6.5 was achieved by T-49 around 65 DAS in 2003-04 and 5.4 around 40 DAS in 2004-05. While Scylla attained a maximum LAI of 5.7 (2003-04) and 4.4 (2004-05) at 65 and 55 DAS respectively. An LAI of 4.0-4.5 was required to intercept a maximum of 90-95 % of incident solar radiation.

Over the experimental period, only cultivar (2004-05) affected LAD. There was a strong relationship between LAI and intercepted photosynthetically active radiation (PAR). Cumulative TDM and intercepted PAR had a strong positive relationship, with RUE values within the range of 0.80 and 1.49 g DM MJ⁻¹ PAR and r² values of 0.86 to 0.94 over both seasons. Cultivar significantly (p < 0.001) affected final RUE, with cv. Scylla producing an RUE of 1.02 g DM MJ⁻¹ PAR compared to 1.18 g DM MJ⁻¹ PAR produced by T-49. A significant cultivar by irrigation (p < 0.001) interaction affected final RUE. There was a significant (p < 0.05) cultivar by fertiliser interaction which also affected final RUE. Increasing rates of N fertiliser cause a reduction in the final RUE associated with plants of cultivar Scylla. The results indicate that cultivar, N fertiliser and irrigation are important variables in common bean in order to achieve early canopy expansion and ground cover, maximising, the amount of intercepted PAR, and eventually leading to a high TDM and seed yield.

5.1 Introduction

Incident radiation intercepted by leaves and other green organs of higher plants is absorbed and utilised as radiant energy in photosynthesis. The photosynthetic products are then partitioned and accumulated as plant biomass (Tsubo and Walker, 2004). Analysis of this process is meaningful under non-stress environmental conditions because radiation is the key driving force in an ideal growth environment (Tsubo and Walker, 2004). The dry matter (DM) production and yield of a crop may be defined in terms of the amount of solar radiation intercepted by the crop canopy and its utilisation and conversion into DM (Monteith, 1977). According to Gallagher and Biscoe (1978), crop growth or seed yield (Y) can be analysed using the following equation:

$$Y = S_a \times RUE \times HI \dots \dots \dots \text{Equation 5.1}$$

where S_a is intercepted solar radiation, RUE is radiation use efficiency and HI is harvest index.

Leaves are the primary photosynthetic organs of plants; therefore the amount or percentage of solar radiation intercepted by a crop depends on the LAI. The LAI of the crop and canopy architecture determine the amount of light intercepted, which is directly related to TDM production. This in turn can influence seed yield (Muchow *et al.*, 1993). Sinclair (1984) also identified LAI and LAD as having substantial influence on the growth of a crop. Total dry matter and grain yield were also strongly correlated with LAD in growth analysis studies of legumes (Hebblewithe, 1982; Husain *et al.*, 1988a).

Radiation use efficiency (g DM MJ^{-1} intercepted PAR) under non-stressed conditions is regarded as a fairly stable quantity (Gallagher and Biscoe, 1978; Muchow *et al.*, 1990; Sinclair, 1994; Sinclair and Muchow, 1999). However, RUE in a crop species does vary under different environments. Factors which are known to vary the RUE of a species are cultivar (Blum, 1990; Stutzel *et al.*, 1994), season, location and management practices (Hamblin *et al.*, 1990; Gregory *et al.*, 1992; Sinclair and Muchow, 1999). Under non limiting water and nutrient supply, Pengelly *et al.* (1999) reported that RUE was a key determinant of yield, while Monteith (1977); Gallagher and Biscoe (1978); Siddique *et al.* (1989) and Thomson and Siddique (1997) concluded that RUE is a conservative value and

considered crop growth or DM production was strongly related to radiation intercepted during the growing season.

In Canterbury, work on grain legumes indicated that TDM production and grain yield were directly related to intercepted PAR and RUE. Radiation use efficiency values in Canterbury ranged from 1.2, 1.6-1.8, and 2.0-2.5 g DM MJ⁻¹ PAR for field bean (Husain *et al.*, 1988a), lentil (Wilson *et al.*, 1983; McKenzie and Hill, 1991) and pea (Zain *et al.*, 1983), respectively. Dapaah (1997) obtained similar results with RUE values of 0.84-0.92 g DM MJ⁻¹ PAR for unirrigated and 0.94-1.15 g DM MJ⁻¹ PAR for irrigated plants of pinto bean. In comparison, values ranging between 0.62 and 1.16 g DM MJ⁻¹ were recorded in soybean, cowpeas (*Vigna unguiculata*) and mung bean (*Vigna radiata*), in Australia (Muchow *et al.*, 1993). Variability in the range of RUE values is also attributed to differences in growth patterns, photosynthetic functions and leaf size of the different crop species (Hipps *et al.*, 1983) and the method and instrument used to measure solar radiation interception (Sinclair and Muchow, 1999).

This chapter examines changes in leaf area development, radiation interception and its utilisation in common beans as influenced by *Rhizobium* inoculation, cultivar and management in Canterbury, New Zealand. It also aims to further clarify processes involved in plant growth (Chapter 4) and yield and yield components responses discussed in Chapter 3.

5.2 Materials and Methods

The experimental and husbandry methods are fully described in Chapter 3; however specific details regarding leaf area measurement, calculation and sampling will be discussed here. This chapter is based on the results of two field experiments. In 2003-04, the experiment was a split plot randomised complete block design, with irrigation as main plot (nil and full). Sub-plot combinations were bean cultivars Scylla and T-49; three inoculation treatments (*Rhizobium tropici* strains CC 511 and RCR 3644 and a control of uninoculated plants) and two N fertiliser treatments (nil and 150 kg N ha⁻¹). In 2004-2005, a completely randomised block design with 4 replicates and factorial combinations of cultivar (Scylla and T-49) and six inoculation treatments (strains RCR 3644, UK 2, H 20, PRF 81, PhP 17 and a control of no inoculation) was used.

5.2.1 Measurements

Canopy development was assessed as LAI and LAD. Leaf area index and the amount of radiation transmitted through the canopy were measured using a LICOR LAI 2000 Plant Canopy Analyser (LI-COR Inc., Lincoln, Nebraska, USA). Leaf area duration was calculated as the time integral of GAI (Hunt, 1982). In year one (2003-04), LAI and T_i were measured every 10-14 d from 21 DAS until near to harvest maturity, while in year 2 (2004-05), measurements were carried out every 4-7 d from 19 DAS. The proportion of radiation intercepted (F_i) by the canopy was calculated as (Gallagher and Biscoe, 1978):

$$F_i = 1.0 - T_i \dots \dots \dots \text{Equation 5.2}$$

The amount of PAR intercepted S_a was calculated as from Sceicz (1974):

$$S_a = F_i \times S_i \dots \dots \dots \text{Equation 5.3}$$

where S_i is the total incident PAR.

The extinction coefficient ($-k$) of the canopy was calculated from Equation 5.4:

$$-k = -\ln(1 - F_i) / \text{LAI} \dots \dots \dots \text{Equation 5.4}$$

Analysis of variance results were calculated for all data using GenStat Release 6.1 (Lawes Agricultural Trust, Rothamsted Experimental Station). Standard error of means (SEM), the coefficient of variation (CV as a %) and percentage variation accounted for in correlation coefficients (r^2) were also calculated. Regression analysis was done using Sigmaplot 2002 for Windows Version 8.2 Copyright © 1986-2001 SPSS Inc.

5.3 Results

5.3.1 Climate

The climatic information during the experimental period was described in Section 3.3.1.1

5.3.2 Leaf area index

In both seasons, significant cultivar ($p < 0.05$ 2003-04; $p < 0.001$ 2004-05) differences were observed in LAI at 24 DAS or later (Figure 5.1c and Figure 5.2a). In both seasons, cv. T-49 had the higher LAI. T-49 attained a maximum LAI of 6.5 at around 65 DAS in 2003-04 and 5.4 around 40 DAS in 2004-05. Scylla maximum LAI was 5.8 (2003-04) and 4.5 (2004-05) which was attained around 65 and 55 DAS respectively. Leaf area index was significantly ($p < 0.001$) affected by N fertiliser in 2003-04. *Rhizobium* inoculations in both seasons, did not significantly affect LAI. The LAI over the experimental period (grand means) ranged from 1.21 at 25 DAS to a maximum of 6.19 at 65 DAS, it then began a steady decline in 2003-04. In 2004-05, LAI ranged from 0.99 at 19 DAS and reached a maximum of 4.73 at 50 DAS; this was followed by a gradual decline. In 2004-05, between 52 and 60 DAS (Figure 5.2), there was a large drop in LAI which then increased from 60 DAS to around 65 DAS.

5.3.3 Leaf area duration, dry matter accumulation and yield

In 2003-04, all treatments had no effect on LAD (Table 5.1) with a grand mean of 298 d. There was a highly significant ($p < 0.001$) cultivar effect on LAD in 2004-05 (Table 5.1). Cultivar T-49 had a much longer LAD than Scylla, at 212 and 161 d respectively. Inoculation had no effect on LAD in 2004-05. In both seasons, TDM production and seed yield were poorly correlated (data not shown) with LAD.

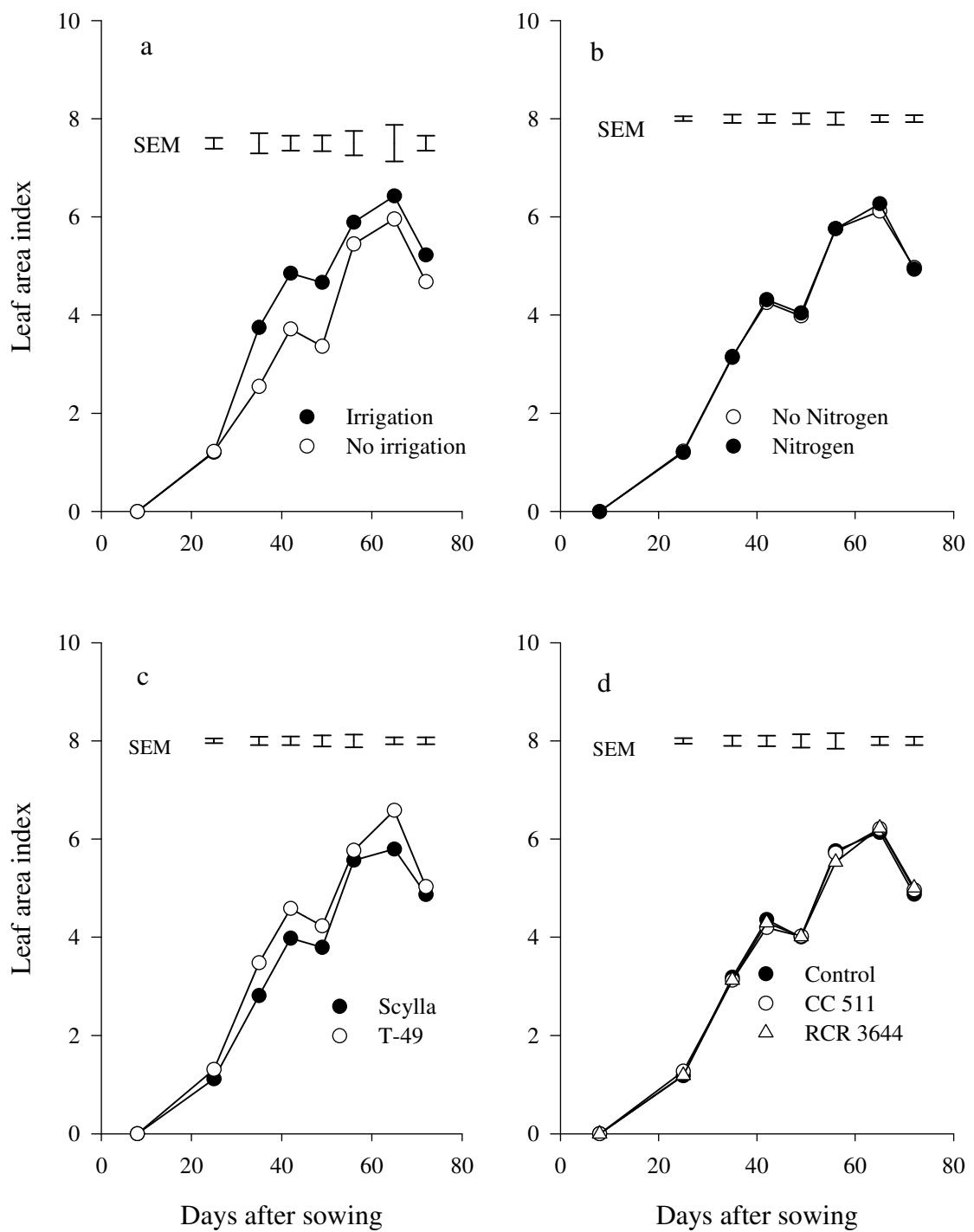


Figure 5.1. The effect of a) irrigation, b) nitrogen fertiliser, c) cultivar and d) inoculation on leaf area index over the growing season of common bean in Canterbury in 2003-04.

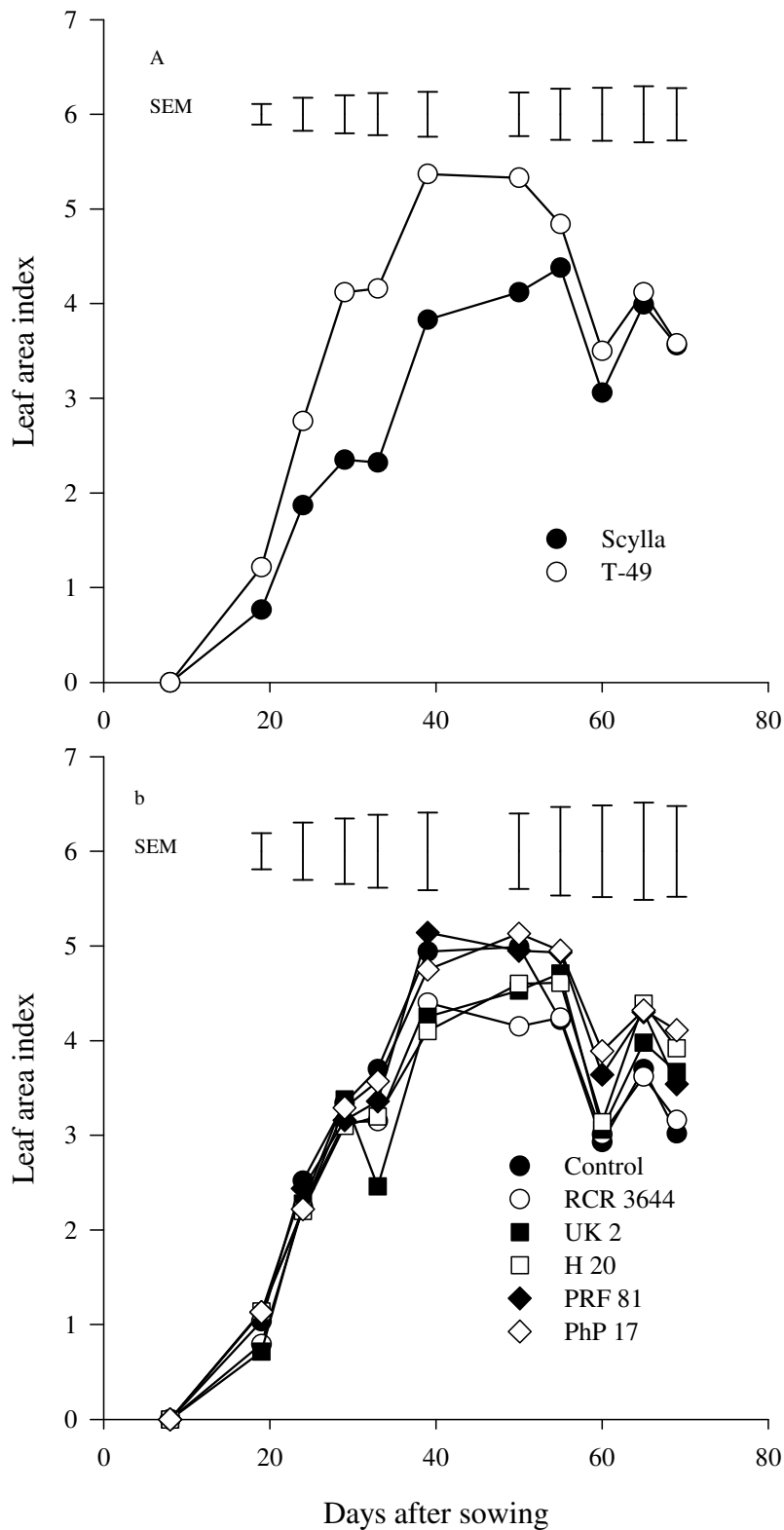


Figure 5.2. The effect of a) cultivar and b) inoculation on leaf area index over the growing season of common bean in Canterbury in 2004-05.

Table 5.1. The effect of inoculation, cultivar, nitrogen fertiliser and irrigation on the leaf area duration (d) of common bean in Canterbury, 2003-04 and 2004-05.

2003-04		2004-05	
Inoculation (IN)		Inoculation (IN)	
Nil	298	Nil	189
CC 511	297	RCR 3644	173
RCR 3644	298	UK 2	177
SEM	3.4	H 20	182
Significance	ns	PRF 81	199
		PhP 17	200
Cultivar (CU)		SEM	16.5
Scylla	296	Significance	ns
T-49	300		
SEM	ns	Cultivar (CU)	
Significance	2.8	Scylla	161
		T-49	212
Irrigation (IR)		SEM	9.5
Nil	274	Significance	p < 0.001
Full	322		
SEM	11.0		
Significance	ns		
Fertiliser (FE)			
0 (kg N ha ⁻¹)	295		
150 (kg N ha ⁻¹)	301		
SEM	2.8		
Significance	ns		
CV (%)	2.3		14.5
Significant	nil		nil
Interactions			

5.3.4 Intercepted radiation and crop growth

The proportion of intercepted PAR (F_i) showed a similar pattern of increase to LAI. In 2003-04, the amount of PAR intercepted by the canopy was significantly affected by cultivar at 35, 42 and 49 DAS. A similar significant result was observed in 2004-05 from 19 DAS ($p < 0.05$) until 50 DAS ($p < 0.01$). Irrigated plants had a higher F_i than unirrigated plants (Figure 5.3) and similar results was obtained for cultivar T-49 over Scylla in both seasons (Figure 5.3a, and Figure 5.4a). Canopies of fertilised and inoculated plants did not vary in the amount of F_i intercepted in 2003-04, both intercepting a maximum of 0.96 of the incident radiation over the season (Figure 5.3b, d). In 2004-05, inoculation had no effect on F_i with grand means over the season ranging from 0.51 at 19 DAS to 0.96 at 39 DAS and then remained constant (Figure 5.4b).

The LAI strongly influenced the amount of radiation intercepted. The relationship between LAI and the proportion of intercepted radiation showed a typical exponential function (Figure 5.5). An LAI of between 4.0-4.5 was required to intercept between 90 and 95 % of incident solar radiation. This is the definition of the critical LAI (LAI_{crit}).

The mean extinction coefficient (-k) for common bean in Canterbury over the two seasons (2003-2005) was 0.76 (Figure 5.5). Variation in k among all treatments over the two seasons was relatively stable and not significant. The average canopy -k values were derived from the relationship between LAI and $\ln(1-F_i)$. The canopy -k values for 2003-04 and 2004-05 are shown in Figure 5.6a, b, c, d and Figure 5.7a, b respectively.

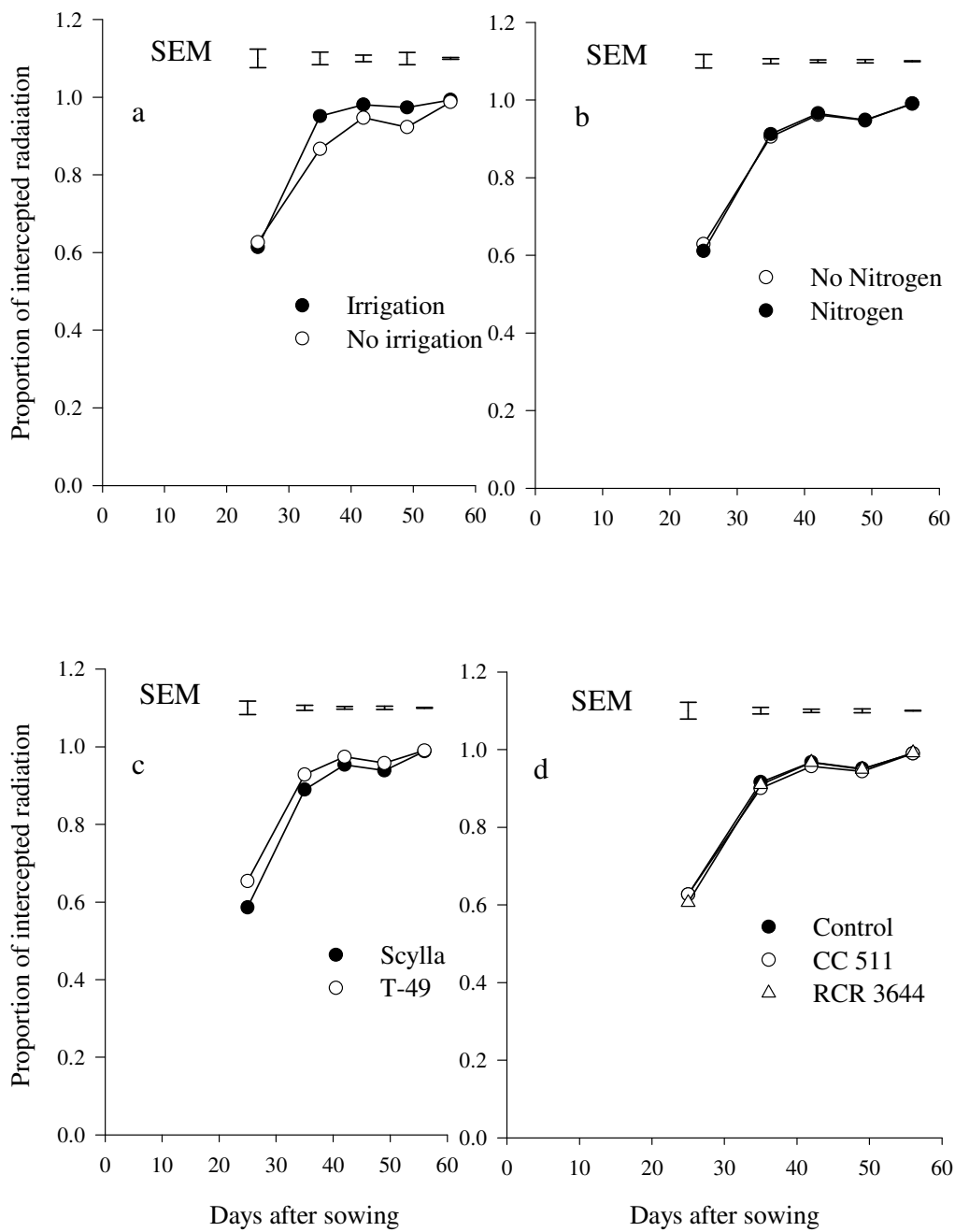


Figure 5.3. The proportion of intercepted radiation (F_i) up to maximum leaf area index by common beans in Canterbury in 2003-04 as affected by a) irrigation; b) nitrogen fertiliser; c) cultivar and d) inoculation.

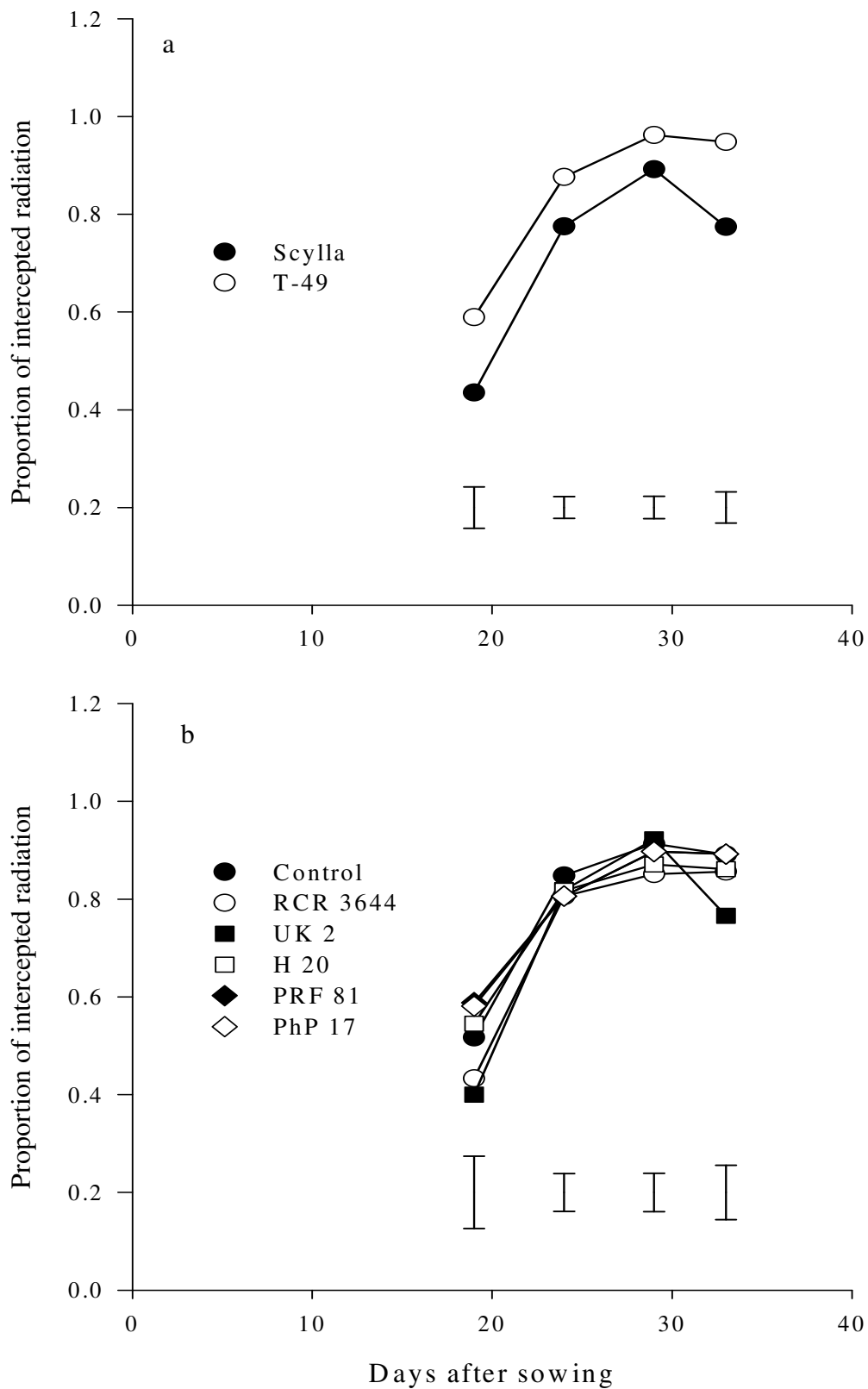


Figure 5.4. The proportion of intercepted radiation (F_i) up to maximum leaf area index by common beans in Canterbury in 2004-05 as affected by a) cultivar and b) inoculation.

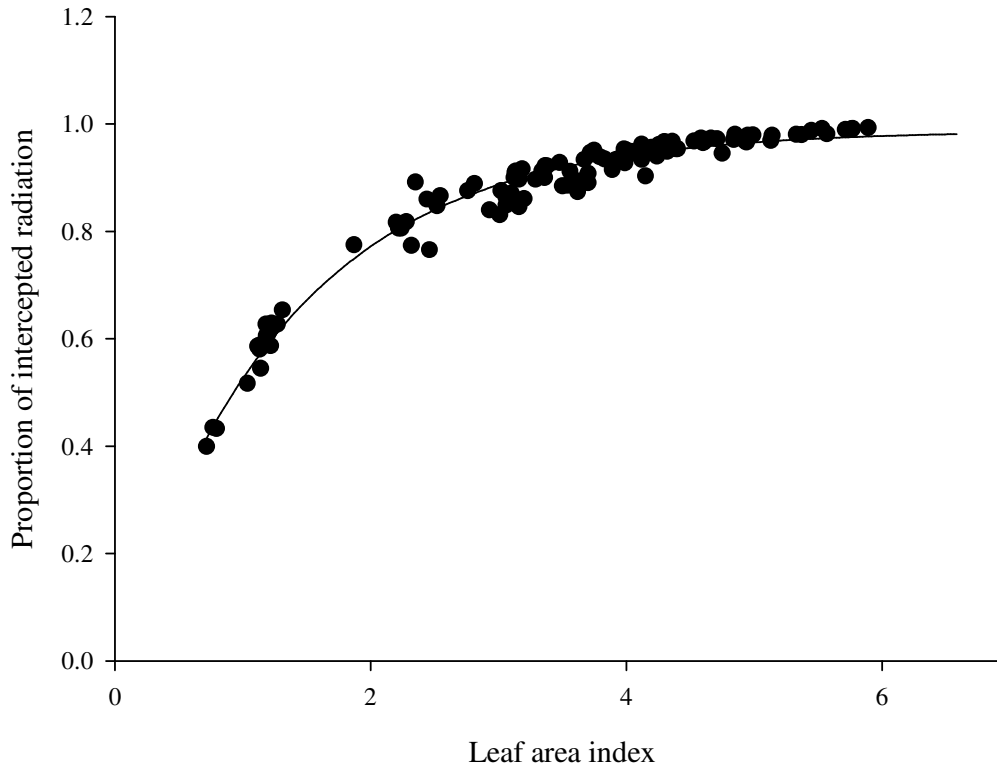


Figure 5.5. The relationship between the proportion of intercepted radiation (F_i) and leaf area index in common bean in Canterbury, 2003-05.

$$Y = 0.998 (e^{-0.7593LAI})$$

5.3.5 Intercepted PAR, DM accumulation and yield

Total intercepted PAR for the season was significantly ($p < 0.001$) affected by cultivar (Table 5.2). Scylla intercepted 744 MJ m^{-2} of PAR compared to 714 MJ m^{-2} of PAR intercepted by cv. T-49. Over the experiments, grand means were 729 MJ m^{-2} (2003-04) and 423 MJ m^{-2} (2004-05). A significant cultivar by inoculation interaction (2003-04) indicated that the intercepted PAR response to cultivar depended on inoculation treatment (Table 5.3). Scylla had the most radiation interception of 750 MJ m^{-2} when inoculated with strain RCR 3644 (Table 5.3). On the other hand, T-49 had the least radiation interception of 702 MJ m^{-2} when inoculated with RCR 3644.

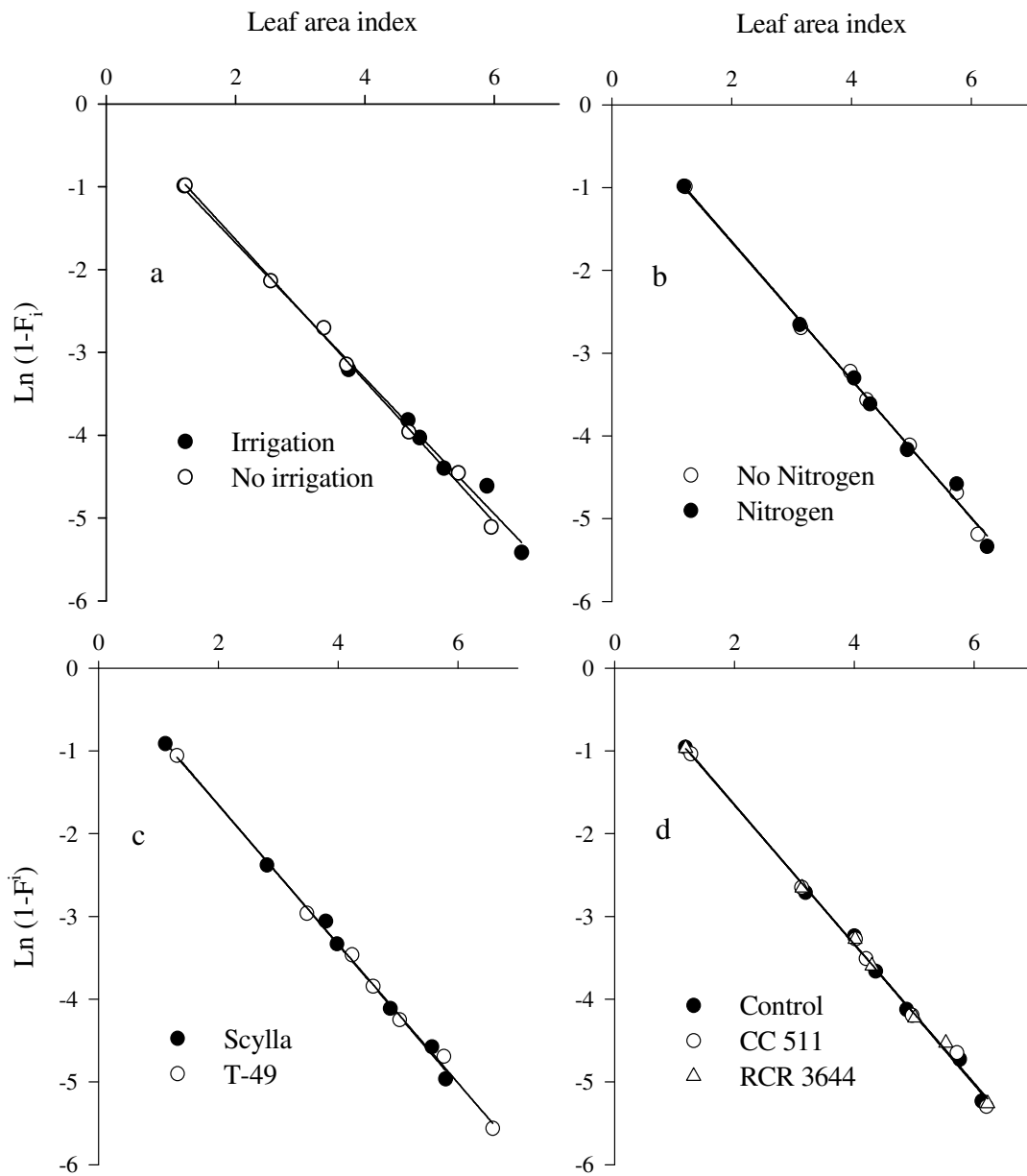


Figure 5.6. The relationship between leaf area index (LAI) and $\ln(1-F_1)$ in common bean in Canterbury, in 2003-04 as affected by a) irrigation; b) nitrogen fertiliser; c) cultivar and d) inoculation.

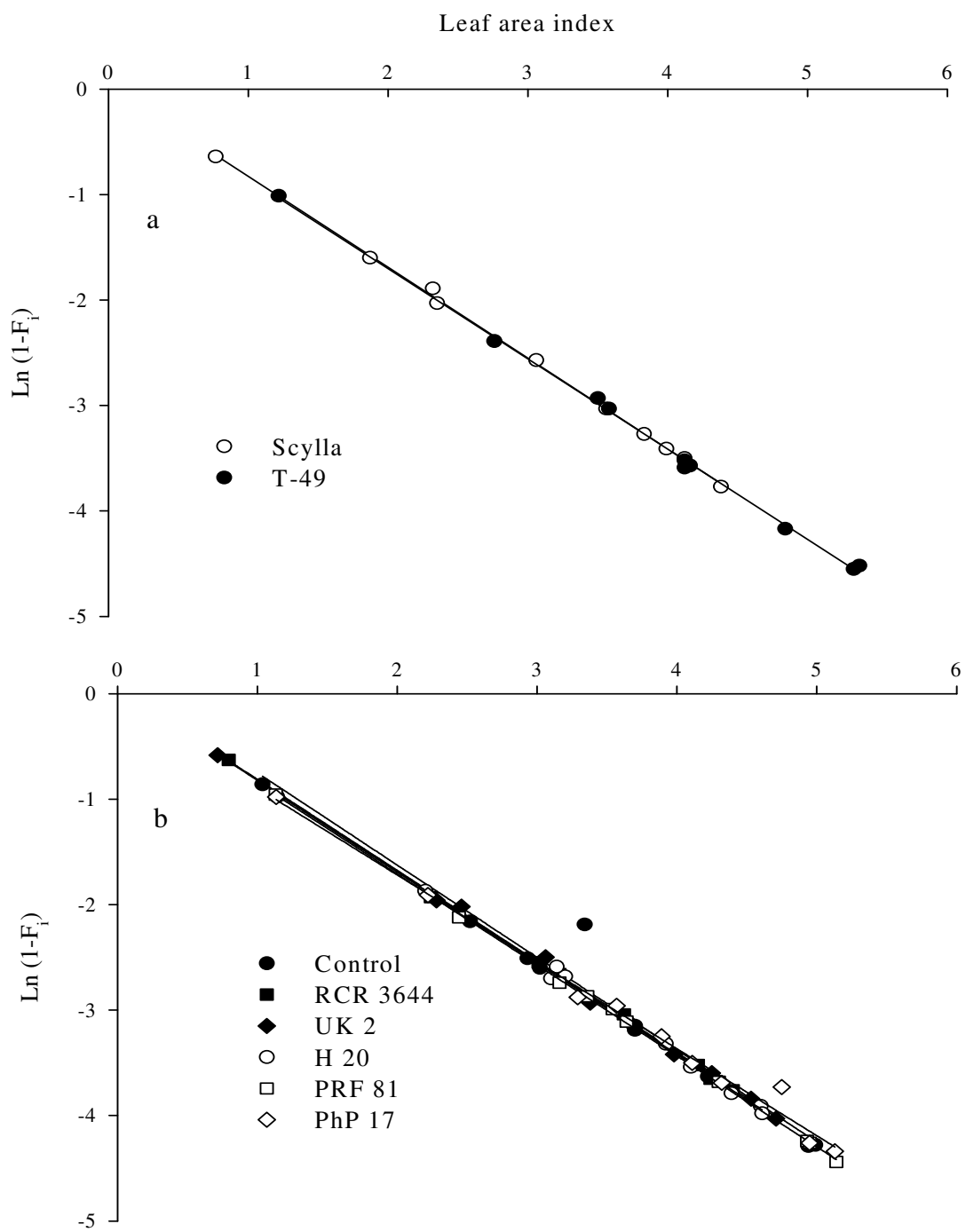


Figure 5.7. The relationship between leaf area index (LAI) and $\ln(1-F_1)$ in common bean in Canterbury, in 2004-05 as affected by a) cultivar and b) inoculation.

Table 5.2. The effect of inoculation, cultivar, fertiliser and irrigation on total intercepted PAR (MJ m^{-2}) of common bean in Canterbury, 2003-04 and 2004-05.

2003-04		2004-05	
Inoculation (IN)		Inoculation (IN)	
Nil	732	Nil	422
CC 511	729	RCR 3644	405
RCR 3644	726	UK 2	421
SEM	3.24	H 20	426
Significance	ns	PRF 81	432
		PhP 17	433
		SEM	19.5
Cultivar (CU)		Significance	
Scylla	744		ns
T-49	714		
SEM	2.65	Cultivar (CU)	
Significance	$p < 0.001$	Scylla	410
		T-49	436
		SEM	11.2
Irrigation (IR)		Significance	
Nil	721		ns
Full	737		
SEM	8.20		
Significance	ns		
Fertiliser (FE)			
0 (kg N ha^{-1})	726		
150 (kg N ha^{-1})	732		
SEM	2.65		
Significance	ns		
CV (%)	1.4		41.1
Significant Interactions	CV X IN		
	$p < 0.001$		nil

Table 5.3. The cultivar by inoculation interaction on total intercepted PAR (MJ m^{-2}) of common bean in Canterbury, 2003-04.

Cultivar	Inoculation		
	Control	CC 511	RCR 3644
Scylla	745	735	750
T-49	718	723	702
SEM		4.6	
CV (%)		1.4	

5.3.6 Radiation use efficiency (RUE)

In (2003-04), there was a highly significant linear relationship between accumulated TDM and accumulated intercepted PAR for all treatments (Figure 5.8). The regression accounted for 93 % of the variation over irrigated treatments, 94 % over the fertilised treatments, 92 % over cultivars and 94 % over inoculation treatments in 2003-04 (Figure 5.8). The slope of the lines indicated RUE of $0.80 \text{ g DM MJ}^{-1} \text{ PAR}$ for the irrigated, fertilised and inoculated plants and $0.84 \text{ g DM MJ}^{-1} \text{ PAR}$ for cultivar. There was a quadratic relationship in 2004-05 for accumulated TDM and accumulated intercepted PAR over cultivars and inoculation treatments (Figure 5.9a, b). The relationship accounted for 94 % of the variation in both treatments.

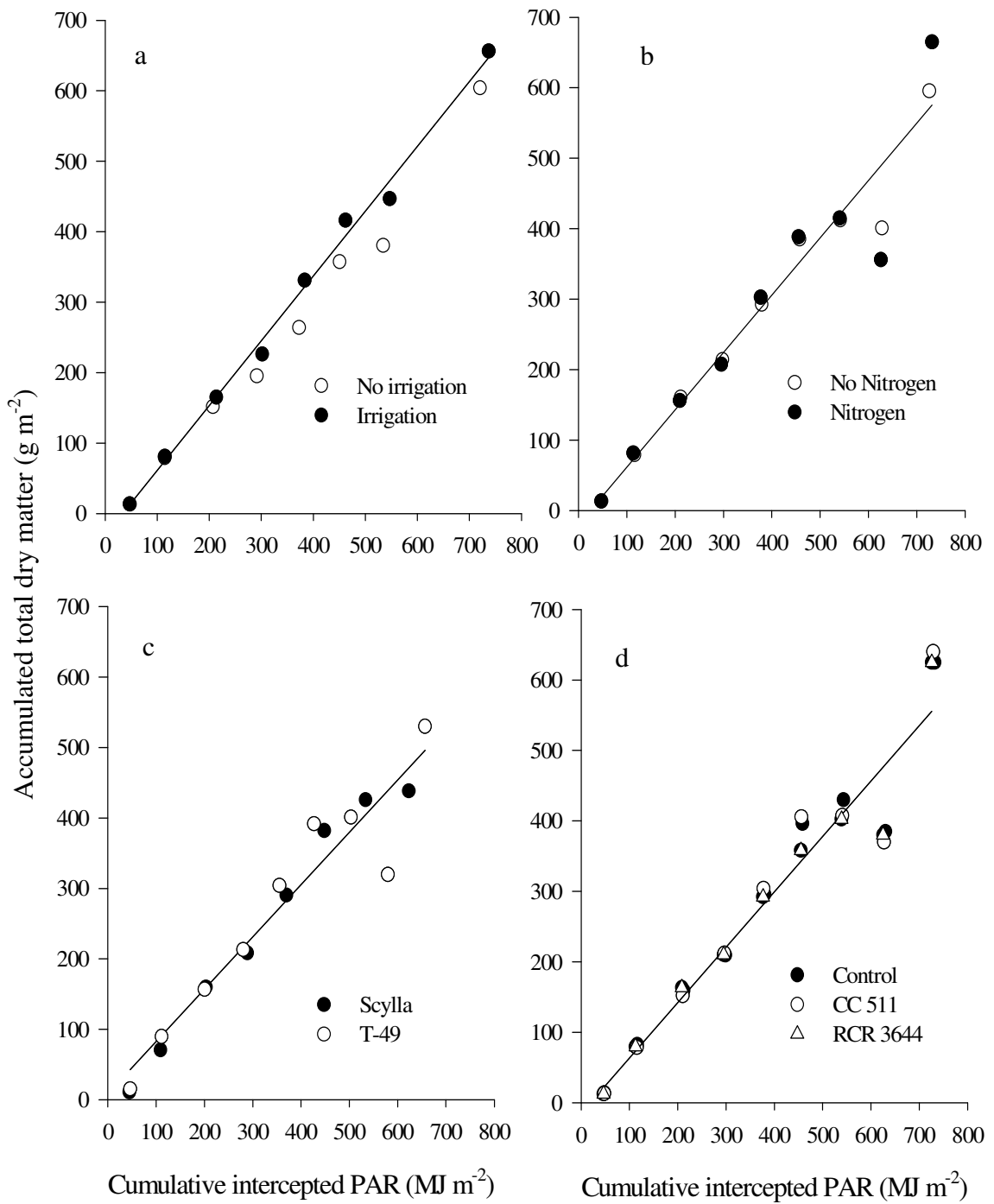


Figure 5.8. Dry matter production during crop growth in relation to accumulation of photosynthetically active radiation (PAR) for common bean in Canterbury in 2003-04. a) irrigation; b) nitrogen fertiliser; c) cultivar and d) inoculation.

(a) Irrigation: $y = -16.9 + 0.80x$ ($r^2 = 0.93$), (b); Fertiliser: $y = -16.3 + 0.80x$ ($r^2 = 0.94$)

(c) Cultivar: $y = -19.3 + 0.84x$ ($r^2 = 0.92$), (d); Inoculation: $y = -16.2 + 0.80x$ ($r^2 = 0.94$)

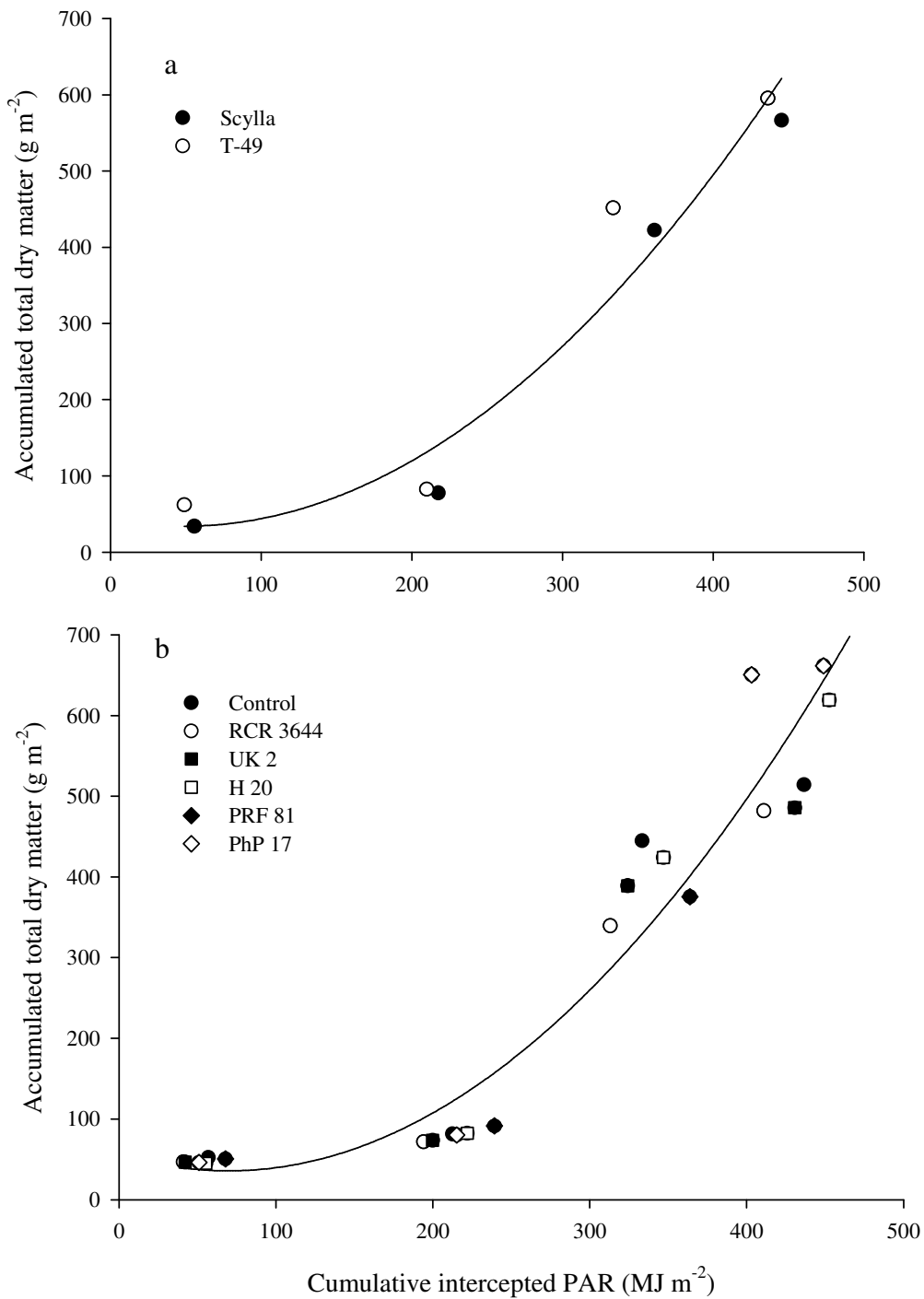


Figure 5.9. Dry matter production during crop growth in relation to accumulation of photosynthetically active radiation (PAR) for common bean in Canterbury in 2004-05. a) cultivar and b) inoculation.

(a) Cultivar: $y = 42.58 - 0.359x + 0.0037x^2$ ($r^2 = 0.94$)

(b) Inoculation: $y = 56.38 - 0.590x + 0.004x^2$ ($r^2 = 0.94$)

The final RUE (TDM divided by total PAR) was variable among treatments and seasons. In 2003-04 final RUE was significantly ($p < 0.001$) affected by cultivar (Table 5.4). Cultivar T-49 produced $1.18 \text{ g DM MJ}^{-1} \text{ PAR}$ compared to $1.02 \text{ g DM MJ}^{-1} \text{ PAR}$ for Scylla. Neither cultivar nor inoculation treatment affected final RUE in 2004-05.

A significant cultivar by irrigation interaction in 2003-04 indicated that the final RUE response to irrigation depended upon cultivar (Table 5.5). Whilst the RUE of both cultivars responded to irrigation, irrigated Scylla plants had a 6 % higher RUE than the non irrigated plants. In T-49, irrigated plants had a RUE which was 22 % higher than in non irrigated plants.

A significant ($p < 0.05$) cultivar by fertiliser interaction was also observed (Figure 5.10). The RUE of Scylla plants decreased from $1.08 \text{ g DM MJ}^{-1} \text{ PAR}$ to $0.96 \text{ g DM MJ}^{-1} \text{ PAR}$ as the fertiliser rate increased from 0 to 150 kg N ha^{-1} ; in T-49 plants, the RUE remained constant at $1.18 \text{ g DM MJ}^{-1} \text{ PAR}$ as fertiliser rate increased (Figure 5.10).

Table 5.4. The effect of inoculation, cultivar, fertiliser and irrigation on final radiation use efficiency (g DM MJ⁻¹ PAR) of common bean in Canterbury, 2003-04 and 2004-05.

2003-04		2004-05	
Inoculation (IN)		Inoculation (IN)	
Nil	1.20	Nil	1.21
CC 511	1.07	RCR 3644	1.16
RCR 3644	1.13	UK 2	1.11
SEM	0.026	H 20	1.34
Significance	ns	PRF 81	1.52
		PhP 17	1.47
Cultivar (CU)		SEM	0.200
Scylla	1.02	Significance	ns
T-49	1.18		
SEM	0.021	Cultivar (CU)	
Significance	p < 0.001	Scylla	1.29
		T-49	1.33
Irrigation (IR)		SEM	0.115
Nil	1.02	Significance	ns
Full	1.20		
SEM	0.041		
Significance	ns		
Fertiliser (FE)			
0 (kg N ha ⁻¹)	1.13		
150 (kg N ha ⁻¹)	1.07		
SEM	0.021		
Significance	ns		
CV (%)	9.5		29.5
Significant Interactions	CU X IR p < 0.01 CU X FE p < 0.05		nil

Table 5.5. The cultivar by irrigation interaction on final radiation use efficiency (RUE) (g DM MJ⁻¹ PAR) of common bean in Canterbury, 2003-04.

	Irrigation	
	Nil	Full
Scylla	0.99	1.05
T-49	1.06	1.29
SEM	0.048	
CV (%)	9.5	

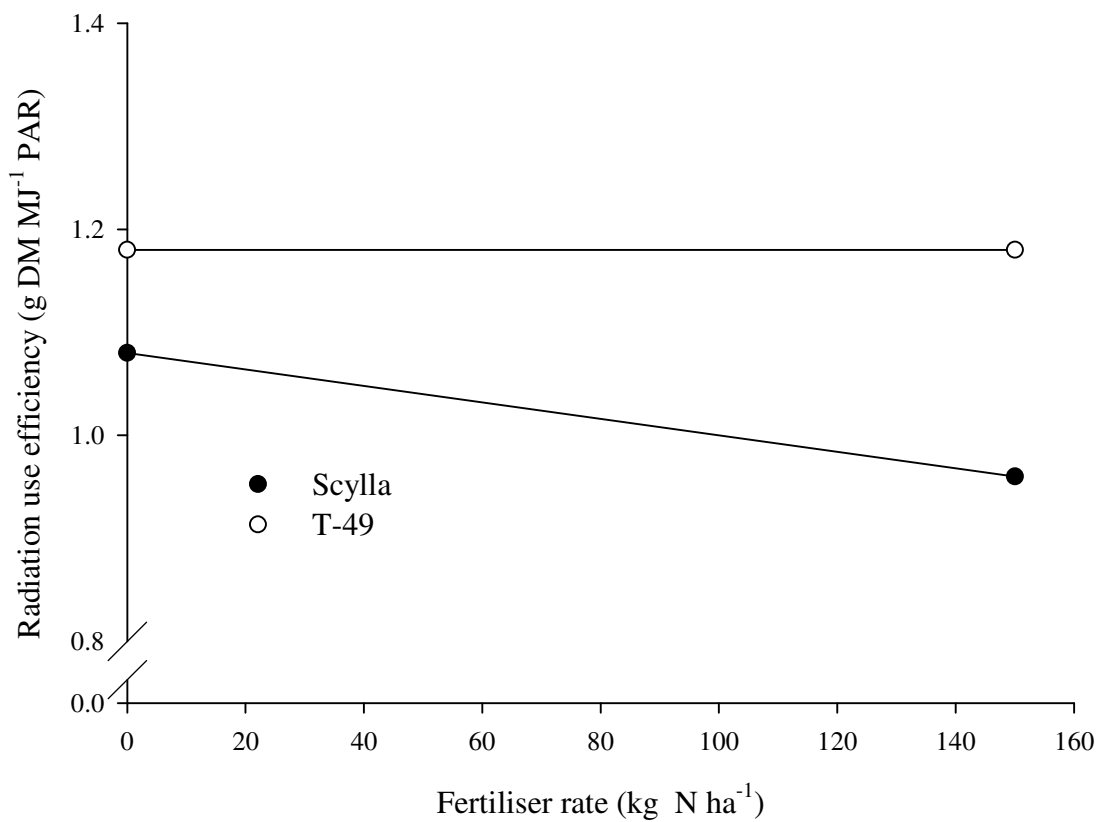


Figure 5.10. The cultivar by fertiliser interaction on final radiation use efficiency (RUE) (g DM MJ⁻¹ PAR) of common beans in Canterbury, 2003-04.

The equation $Y = S_a \times RUE \times HI$ was used to verify the predicted seed yield for common bean. The relationship between predicted and actual seed yield for all treatments over the 2003-04 season (Figure 5.11) was highly significant ($p < 0.05$) with an r^2 of 0.99. This indicates that intercepted PAR (S_a), the RUE and HI are useful indicators for the prediction of seed yield in common bean.

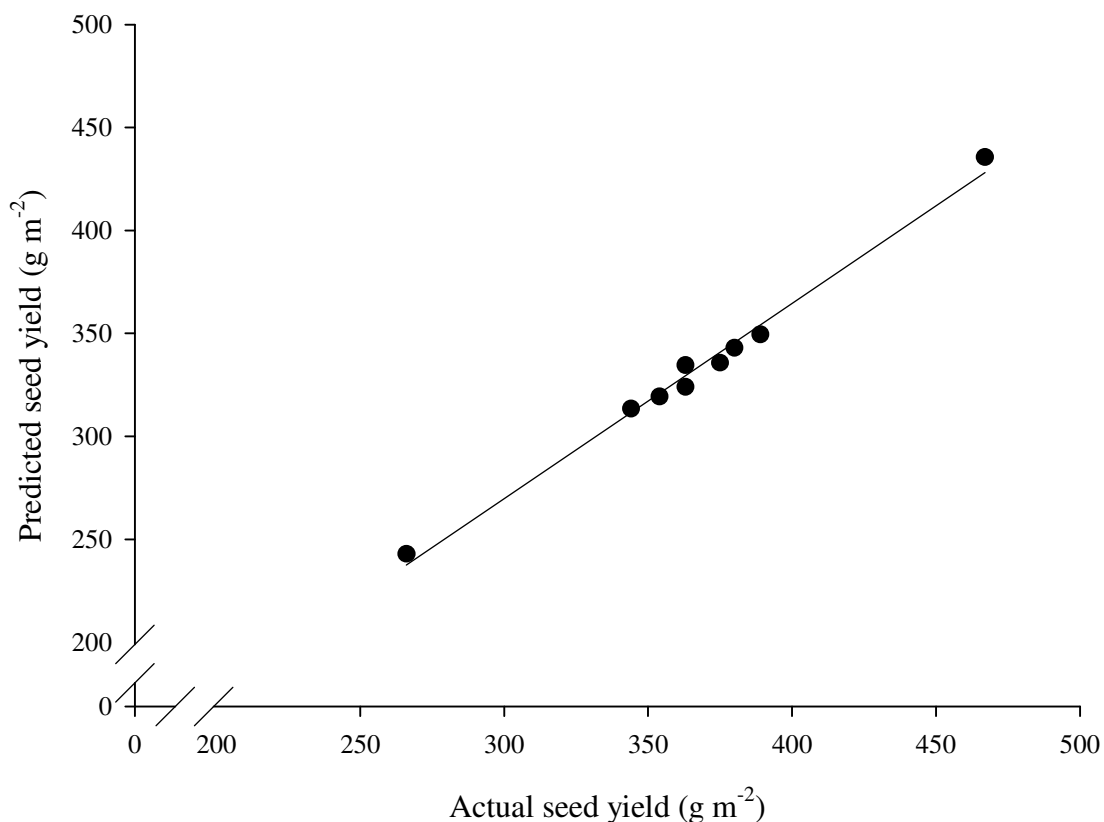


Figure 5.11. The relationship between predicted and observed seed yield of common bean in Canterbury in 2003-04.

$$Y = 14.7 + 0.95X, (r^2 = 0.99)$$

5.4 Discussion

5.4.1 Leaf area index and duration

Leaf area index (2003-04) increased from 24 DAS to 42 DAS when a decrease in LAI was observed (Figure 5.1). This decrease in LAI corresponded to a period of cool temperatures with a mean 3 day temperature of 5.6 °C and a minimum of 3 °C. Low temperatures (as low as -1.6 °C) had a similar effect on LAI in 2004-05 between 50 and 60 DAS (Figure 5.2). This temperature is well below the base temperature of 10 °C required for common bean growth in Canterbury (Greven, 2000). Temperatures below 7 °C are known to cause leaf damage in beans (Wilson, 1976) and other tropical and sub tropical crops (Lewis, 1956). A subsequent increase in LAI was observed from 49 DAS to 65 DAS when maximum LAI was attained followed by a decline coinciding with leaf senescence.

A maximum LAI of 6.4 was attained at around pod fill. The subsequent reduction in LAI can be attributed primarily to leaf senescence and/or translocation of assimilates from leaves to reproductive sinks (White and Izquierdo, 1991; Dapaah, 1997).

The main consequence of low temperature is a delay in plant development and DM production (Robin *et al.*, 2005). Temperature directly affects the rate of leaf production and the rate and duration of leaf expansion (Hay and Walker, 1989), and hence LAI. The decline in LAI seen between 40 and 50 DAS in 2003-04 (Figure 5.1a, b, c, d) and between 50 and 60 DAS in 2004-05 (Figure 5.2a, b) coincided with periods of low temperatures just above zero or frost. In 2004-05, the harvest at 60 DAS was after a period of low temperatures, with the five days immediately before harvest averaging 4.3 °C. Temperature effects on LAI as seen in these experiments, have also been documented by Regan *et al.* (2006) working with chickpea in the Mediterranean-type environment of south-western Australia and in a review on the implications low temperature stress on chickpea improvement (Croser *et al.*, 2003).

Cultivar significantly affected LAI (Section 5.3.2). Cultivar T-49, senesced earlier than Scylla and matured earlier. In both seasons, T-49 had the higher LAI 6.5 (2003-04) and 5.4 (2004-05). However, this did not convert into the higher DM production or seed yield. Working with different soybean cultivars, Rahman (2002) found that plants with a higher LAI had higher PAR interception, which contributed to greater DM accumulation and hence, a higher seed yield. Rahman (2002) attributed these results to slow leaf senescence and a prolonged “stay green” period during the seed filling. His results agreed with those of Kumudini *et al* (2001) who worked with soybean. However, Scylla intercepted more radiation due to a longer growing season.

In 2003-04, irrigation positively affected LAI compared to rainfed plants throughout crop growth. Anwar (2001) found irrigation was necessary to maximise LAI, especially during the vegetative and flowering stages in chickpea. Irrigation has a positive influence on LAI because adequate water is needed for leaf emergence and expansion (Lawn, 1982b, a; Squire, 1990). The negative effect of water stress on LAI was confirmed in these experiments (Figure 5.1a). Irrigated plants had a higher LAI at all sampling dates. The sensitivity of LAI to water deficit is well documented (Passioura *et al.*, 1995; Anwar, 2001), with several authors reporting maximum LAI with irrigation (Saxena *et al.*, 1990; Thomas *et al.*, 2004). In Canterbury, the effects of irrigation increasing the LAI in grain

legumes has also been reported for peas (Zain *et al.*, 1983), pinto bean (Dapaah *et al.*, 2000) and chickpea (Anwar, 2001).

Leaf area duration was shorter in 2004-05 than in 2003-04 and differed among treatments. A maximum LAD of 322 d was recorded in irrigated plants compared to 277 d for rainfed plants. In both seasons, LAD was only weakly correlated with TDM and seed yield. These results differed from those of Laing *et al.* (1984) working with eight grain legume species, who reported that LAD explained a large proportion of the variation in seed yield ($r^2 = 0.99$). Hebblewaithe (1982) and Zain *et al.* (1983) noted that a high correlation does not necessarily specify a simple cause and effect relationship as the number of pods plant⁻¹ was highly correlated with seed yield. In addition, in some cases when a high LAI (exceeding that required for 95 % interception) is produced (as in this study), LAD may not be closely related to yield because at LAIs above LAI_{crit.}, radiation interception will not be increased, while LAD will be (McKenzie, 1987; Husain *et al.*, 1988a). Leaf area duration does not take into account variations in leaf shading, canopy architecture or photosynthesis rate as leaves age (Monteith and Elston, 1983). Therefore, LAD is only an estimate of light utilisation over time. Measurements of irradiance and the interception of irradiance integrated over time should correlate better with yield than LAD (Sinclair and Muchow, 1999).

5.4.2 Radiation interception

In both years, cultivar had a significant effect on the proportion of intercepted radiation (Figure 5.3c and Figure 5.4a). However, in 2003-04 the sharp decline in LAI was not seen in F_i, probably due to absorption of radiation by senesced leaves, pods and stems particularly close to crop maturity. In Canterbury, a similar F_i trend, as seen in this study, in response to irrigation (Figure 5.3 and Figure 5.4) was reported by Anwar (2001) for chickpea and Dapaah *et al.* (2000) for pinto beans. The higher F_i with irrigation (Figure 5.3c) was attributed to a higher LAI and the time of canopy closure. The time of canopy closure and higher LAIs are major factors for radiation interception as plants with larger vegetative structures increase the capture of PAR (Gardner and Auma, 1989). Inoculation, in both seasons, and N fertiliser had no significant effect on F_i.

The relationship between LAI and the fraction of radiation intercepted by the canopy was exponential (Figure 5.5). Similar relationships have been found in other grain

legumes grown in Canterbury: field beans (Husain *et al.*, 1988a), pinto beans (Dapaah *et al.*, 2000), chickpea (Anwar, 2001) and soybean (Rahman, 2002) and in other crops such as maize (Yamoah, 1999), rape (*Brassica napus*) and rye corn (*Secale cereale*) (Isaac, 2001) and lucerne (*Medicago sativa*) (Teixeira, 2005). Pooled over both seasons, an estimated LAI of 4.0-4.5 was required to intercept 90-95 % of incident radiation. The relationship showed that when LAI was below this level, a small increment in LAI gave a large increase in intercepted radiation. In comparison, when the LAI was above this level, there was only a marginal increase in light interception. These results are similar to those of Dapaah *et al.* (2000) for pinto beans, but are low compared to 6.5 for field beans (Husain *et al.*, 1988a; Husain *et al.*, 1988b) and 7.0-11.0 for lentils (McKenzie and Hill, 1991). An LAI with a 95 % F_i has been adopted as the critical LAI by most crop physiologists (Gardner and Auma, 1989).

The extinction coefficient (-k) (the slope of the linear regression line between LAI and $\ln(1-F_i)$) gives a numerical indicator of the light reduction down through the canopy due to leaf architecture (Figure 5.5, Figure 5.8, Figure 5.9) (Szeicz, 1974). The amount of solar radiation intercepted depends on the LAI and the extinction coefficient. Over all treatments, in both seasons, a mean extinction coefficient (-k) of 0.84 was obtained indicating a crop with near-horizontal leaves on the top of the canopy. A similar -k value was obtained for chickpea (Anwar, 2001) and soybean (Rahman, 2002) in Canterbury. However this -k value is much higher than that reported by other authors in Canterbury for field beans (0.36-0.48; (Husain *et al.*, 1988a; Husain *et al.*, 1988b) or lentils (0.26; (McKenzie and Hill, 1991). In Australia, a -k value as high as 1.1 was reported for chickpea (Thomas and Fukai, 1995). Differences in -k values reflect differences in cultivar or species (Thomson and Siddique, 1997), environment (Muchow *et al.*, 1993), sun angle or elevation and leaf properties (Szeicz, 1974; Hay and Walker, 1989). Generally species with flat leaves, such as beans, will have a high -k while species with erect leaves such as lentils will have a low -k.

5.4.3 Intercepted PAR, dry matter and seed yield

More success was obtained in relating crop growth to solar radiation intercepted by the leaf during the growing season than with growth analysis based on LAD (Monteith and Elston, 1983). The amount of DM accumulated was found to be strongly correlated with seasonal radiation interception (Gallagher and Biscoe, 1978). This study showed a highly

significant linear relationship between accumulated TDM and cumulative intercepted PAR in 2003-04 (Figure 5.8a, b, c, d) and 2004-05 (Figure 5.9a, b). The slopes of these relationships gave RUE values of 0.81 and 1.37 g DM MJ⁻¹ of intercepted PAR respectively. Cultivar significantly affected total intercepted PAR (Table 5.2). The dominant influence of cultivar on total intercepted PAR was highlighted by the significant cultivar by inoculation interaction (Table 5.3). This interaction showed that Scylla intercepted the most radiation when inoculated with RCR 3644, while T-49 intercepted the least solar radiation with the same inoculant. Scylla had the highest intercepted PAR, even though T-49 had a marginally greater LAI. Scylla's increased radiation interception was probably due to a canopy which developed earlier and senesced later, giving greater PAR interception. Similar interception patterns were reported for pinto beans (Dapaah *et al.*, 2000) and for Kabuli chickpea (Anwar, 2001).

In 2003-04, a reduction in accumulated TDM was observed (Figure 5.8) when plants had accumulated approximately 610 MJ m⁻² PAR. This reduction was preceded by a frost which occurred on 25 February 2004. Zero degrees Celcius is considered a lethal temperature for common beans (Knell *et al.*, 2006), although frost can occur at air temperatures above 0 °C (Hawthorne, 2006; Knell *et al.*, 2006). In addition to temperature, additional factors which can influence frost risk are crop variety, canopy management and row spacing, stubble presence and atmospheric and soil moisture levels (Knell *et al.*, 2006). Leading up to harvest eight on the 11 March 2004, a number of cold temperatures were recorded with a minimum of -2.7 °C on the 25 February 2004. The frost and subsequent low temperatures would have affected plant growth rate. Leaf loss due to the frost is another factor that could have affected total accumulated DM as seen in Figure 5.8.

In this work, the relationship between intercepted PAR and accumulated DM for 2003-04 (Figure 5.8) is similar to the classic work of Monteith (1977). Crop DM accumulation is dependent on solar radiation interception, which is a function of leaf area development and incident radiation, and RUE which is a function of photosynthesis and respiration. Therefore rapid canopy closure is required to optimise PAR interception for high DM accumulation (Shibles and Weber, 1966; Taylor *et al.*, 1982). Similar conclusions were reported for soybean (Rahman, 2002), chickpea (Anwar, 2001) and other grain legumes (Ayaz, 2001) in Canterbury. In 2004-05, a similar relationship between PAR interception and accumulated DM (Figure 5.9) was not observed. Figure 5.9 shows

increased intercepted PAR with limited increase in accumulated DM between 50 and 225 MJ m⁻² of intercepted PAR. This corresponds to the period of growth between the first and second DM harvest 21 and 40 DAS, respectively. The minimum temperature during this period (20 d) was quite variable and ranged from 17.4 to 1.9 °C. Ten days after the first harvest at 21 DAS, the average minimum temperature was 10.9 °C, in the following 10 d, before the second harvest (40 DAS), the average minimum temperature was 7.3 °C. The base temperature for growth of common bean as reported and used in these experiments was 10 °C (Greven, 2000).

Average temperatures recorded during the period in question were 10.9 and 7.3 °C which were respectively just above and below the 10 °C base temperature required for growth. Although plants were receiving radiation, the temperature was below that required for growth and therefore there was no increase in DM (Figure 5.9). This gave very low RUE values early in the season giving the exponential curves shown in Figure 5.9. Data from Figure 5.9 was divided into two period of growth (see Appendix 5). Averaged over all treatments shown in Appendix 5, the plants accumulated 0.24 g DM MJ⁻¹ in the early growth period and this increased to 2.26 g DM MJ⁻¹ in the second growth period when temperatures were above base temperature (Appendix 5). The retarding effect of low temperature on the emergence, growth and flowering of legumes has been reported for chickpea (Croser *et al.*, 2003; Regan *et al.*, 2006) and in common bean (Dickson and Boettger, 1984 ; Melo *et al.*, 1997).

The final RUE, which is the TDM divided by total PAR, was significantly affected by cultivar (Table 5.3). Irrigated plants accumulated higher amounts of DM than unirrigated plants, which was associated with greater PAR interception and higher RUE. Similar results were reported in well watered crops with chickpea in Australia (Thomas and Fukai, 1995) and field beans (Husain *et al.*, 1988a), lentils (McKenzie and Hill, 1991) and chickpea (Anwar, 2001) in Canterbury. The RUE was much lower in 2003-04 than in 2004-05 (Table 5.5). Differences in solar radiation levels (Figure 3.1) over the two seasons could have contributed to differences in RUE. Decreasing solar radiation, particularly at daily radiation levels less than half that of a bright day, increase RUE (Sinclair and Muchow, 1999). Variations in RUE have also been attributed not only to radiation levels but also to the amount of diffuse radiation (Sinclair and Muchow, 1999).

The lower RUE observed in non irrigated T-49 plants might have been caused by water stress and possibly increased respiration with increased temperature (Muchow *et al.*, 1993). However, water stress affects leaf expansion well before it affects photosynthesis. The RUE is significantly decreased by water stress, indicating that water deficit affects DM accumulation through reducing RUE (Sinclair and Muchow, 1999). Low leaf water potential causes stomatal closure, reduced leaf conductance and CO₂ decline (Turner, 1997), which decreases the leaf photosynthesis rate. Increased leaf photosynthesis rate is directly linked to increased RUE (Sinclair and Muchow, 1999). While water stress can reduce RUE, leaf expansion is more sensitive to water stress than photosynthesis (Monteith and Scott, 1982; Alfredo *et al.*, 2004) therefore, water stress is likely to have a larger effect on radiation interception than on RUE. Many authors have reported a decline in RUE around the time of maximum pod biomass or near maturity in many other crops (Gallagher and Biscoe, 1978; Muchow *et al.*, 1993; Dapaah, 1997). This is usually the result of remobilisation of leaf carbon (C) and N to the grain and biomass loss due to leaf senescence (Muchow *et al.*, 1993). Legumes have lower RUEs than other crops (Sinclair and Muchow, 1999), the RUE values obtained here are lower than the 1.65 for soybean (Rahman, 2002), and 1.68 for field pea (Ayaz, 2004), but similar to the 1.40 for narrow-leaf lupin (Ayaz, 2004) and 1.06 to 1.34 for chickpea (Anwar, 2001).

Many authors have suggested that RUE is relatively stable over a range of environmental conditions and management variables (Gallagher and Biscoe, 1978; Sinclair, 1994; Sinclair and Muchow, 1999). However, variability in RUE clearly exists. The product of RUE and the amount of intercepted solar radiation (S_a) through the season provides an estimate of total biomass accumulated by the crop. Since RUE is a conservative value (Sinclair, 1994), DM accumulation as well as crop yield is mostly related to intercepted solar radiation (Speath *et al.*, 1987).

Solar radiation intercepted is a function of canopy development. Canopy development is related to the temperatures and photoperiod under which the crop is grown. The importance of canopy development and the duration of crop growth are reflected in the amount of solar radiation that can be intercepted and used to accumulate DM (Muchow *et al.*, 1990). The photosynthetic limit to DM production may ultimately limit yield in unstressed crops. The overall yield response was the net effect of radiation intercepted by a crop, DM accumulated, the RUE and HI. These variables can be used to accurately

predict seed yield in common bean (Figure 5.11). Similar results were reported in other grain legumes chickpea (Anwar, 2001), soybean (Rahman, 2002) and pinto bean (Dapaah, 1997).

5.5 Conclusions

- 1) A LAI of 4.0-4.5 was required to intercept 90-95 % of incident radiation.
- 2) Total DM produced was strongly correlated with intercepted PAR.
- 3) Low temperature retarded growth (accumulation of TDM) and RUE in common bean.
- 4) Choice of cultivar and irrigation are important variables in achieving early canopy closure to maximise the amount of PAR intercepted, leading to high DM accumulation and seed yield.
- 5) The seed yield of common beans can be predicted reasonably well from the proportion of intercepted radiation, RUE and HI.

CHAPTER 6

***Rhizobium* inoculation, cultivar and management effects on growth and development of common bean**

(*Phaseolus vulgaris* L.):

Yield and yield components.

Summary

The potential of common beans cvs Scylla and T-49 was examined in combination with different rhizobial strains over two growing seasons at Lincoln University, Canterbury New Zealand. In 2003-04, the two bean cultivars were combined with two irrigation treatments, three inoculation levels and two fertiliser levels. In 2004-05, six inoculation treatments were combined with the two cultivars. Growing conditions were similar during the two growing seasons with maximum and minimum temperature of 20 and 10 °C respectively. In 2004-05, the crop was sown on 11 January 2005 which was one month later than in 2003-04 and received 128 mm of rainfall. This was 26 mm more than in the first season (2003-04). These figures are 31 % (2003-04) and 16 % (2004-05) less than the long term average rainfall.

Cultivar had no effect on green pod yield or TDM at green pod harvest in either season. However, by final harvest in 2003-04 Scylla produced 76 % and 38 % more seed and TDM respectively. Plants inoculated with isolates H 20, PRF 81 and PhP 17 had increased green pod yield of 69, 49 and 49 g m⁻² respectively over uninoculated plants. Inoculation had no effect on seed yield. However, seed yield was affected by a cultivar by fertiliser interaction in 2003-04, which showed Scylla responded better to nitrogen than cv. T-49. Treatment effects on yield components were variable over the two seasons. In 2004-05, cultivar significantly affected the pods plant⁻¹ (p < 0.05) and 100 seed weight (p < 0.001). The 100 seed weight was significantly affected by cultivar and fertiliser and seeds pod⁻¹ by cultivar in 2003-04. The results suggest that improvements in yield of green bean and dry seed in common bean in Canterbury depend on cultivar and inoculation.

6.1 Introduction

The characteristics and functions of a crop plant depend on a series of interrelated events which are sequential in time (Adams, 1967). Additionally, these events are gene-regulated at critical times, and subject to modifying influences of non-genetic forces. Adams (1967) further stated that these events do not occur haphazardly, but follow an integrated pattern. Final seed yield is an obvious example of this integration in which the components of seed yield, are to some extent, interdependent in their development (Adams, 1967).

Plant population, number of pods plant^{-1} , the number of seeds pod^{-1} and the mean seed weight are the yield components in grain legumes (Westermann and Crothers, 1977; Summerfield *et al.*, 1980). Highest seed yields are obtained when all of these components are maximized. However, because of negative correlations between yield components from intra plant competition for nutrients, large seed yield changes may be prevented (Adams, 1967). The number of pods unit area^{-1} is usually the major cause of variation in seed yield, while the most stable yield component is mean seed weight (Littleton *et al.*, 1979; Saxena *et al.*, 1983b). Generally, environmental conditions under which a crop is grown have a major influence on yield component development (Egli, 1998).

The common bean has a poor reputation for yield but potential appears high. Although research programmes have reported experimental yields of over 5 t ha^{-1} in bush beans and have demonstrated genetic variability in yield potential, these findings have not led to dramatic increases in bean productivity like those which have occurred in other crops such as the cereals. These studies have however generated useful information for guiding agronomic management and suggesting strategies for varietal improvement (van Schoonhoven and Voysset, 1991). Agronomic management involves making critical decisions such as sowing date, choice of bean crop, inoculant use and irrigation needs, all of which can affect crop yield.

Dry *Phaseolus* beans are not an established crop in Canterbury because of the length of the growing season needed by the crop and the limited time window for sowing and harvest (Greven, 2000). Cultivar selection could play an important role in combating the variable yield and growing conditions. Stutzel and Aufhammer (1992) showed in faba bean that cultivar influences on yield were higher than those of plant density, but were

comparable to environmental effects. Comparisons between determinate and indeterminate bean cultivars showed that yield appeared to be related to the potential for extended root growth. This was evident the growth of cultivar of bean with an indeterminate growth habit inoculated with a single rhizobial strain (Morrison and Baird, 1987).

Internationally, field responses to inoculation in beans have been variable. Significant yield increases due to inoculation have been obtained in dry beans (Taylor *et al.*, 1983; Mostasso *et al.*, 2002; Hungria *et al.*, 2003), and in kidney beans (Bengtsson, 1991). However, other reports showed slight, or no effect, of inoculation despite increased nodulation (Buttery *et al.*, 1987; Ramos and Boddey, 1987; Hardarson *et al.*, 1993). In New Zealand, *Phaseolus* beans are not generally inoculated, but there is a realization of the importance of selecting efficient and effective strains of *Rhizobium* and cultivars of beans for local conditions as a way of reducing reliance on fertiliser N (White, 1989).

This chapter examines the effect of management, cultivar and *Rhizobium* inoculation on the growth, development and yield of two common bean cultivars. In 2003-04, two *Rhizobium* strains were evaluated with the two cultivars, with and without applied N fertiliser, under two irrigation regimes. In 2004-05, a range of *Rhizobium* strains was used with the same two cultivars under irrigation to verify the nodulation potential of the cultivars. The work had the following objectives:

1. To identify the response of different *Phaseolus* bean cultivars to varying rhizobial strains.
2. To quantify the accumulation of plant N in response to irrigation, cultivar and *Rhizobium* inoculation.
3. To investigate the effect of *Rhizobium* inoculation and bean cultivar on common bean green pod and dry bean yield and yield components.

6.2 Materials and Methods

6.2.1 Site description

The two experiments were conducted in paddocks H2 (2003-04) and H14 (2004-05) of the Horticultural Research Area, Lincoln University, New Zealand. Sections 3.2.1.1 to 3.2.1.10 describe the site, soil type, history, experimental design, crop husbandry, management and measurements employed during the experiments.

6.2.2 Statistical analysis

All variates were analysed using analysis of variance (ANOVA). The statistical package used was GenStat (GenStat Release 6.1 Lawes Agricultural Trust, Rothamsted Experimental Station, Hertfordshire, UK). Standard errors of the mean (SEM), coefficient of variation (CV as a %) and percentage variation accounted for (r^2) were also calculated. All data were analysed twice. Once with potting mix included, and again with potting mix excluded. Generally the statistics presented are based on the analyses without potting mix as including potting mix nullifies the assumption of a normal distribution of mean values. However, for an indication of the effect of the potting mix the analyses with the potting mix are used.

6.3 Results

6.3.1 Climate

Climatic information during the experimental period has been described in Section 3.3.1.1.

6.3.2 Germination

In both seasons germination was uniformly high, with emergence (cotyledons above the soil and displayed to the sun) of more than 80 % by 5 DAS irrespective of experimental treatment. An initial, before sowing germination test in 2003-04 gave T-49 a germination of 45 % and Scylla 85 %. As a result the sowing rate of T-49 was doubled to obtain a population density close to the required number of the recommended planting density of 60 plants m⁻² for *Phaseolus vulgaris* in Canterbury (McKenzie, 1989).

6.4 Yield at the green pod stage

6.4.1 Total dry matter production

In both growing seasons, TDM production at green pod harvest was not affected by any treatment. The grand mean in 2003-04 was 868 g m⁻² and in 2004-05 it was 581 g m⁻² (Table 6.1).

6.4.2 Green pod yield

In 2003-04, no treatments had any effect on green pod yield (on a dry weight basis) and the overall mean was 441 g m⁻² (Table 6.2).

In 2004-05, inoculation significantly ($p < 0.05$) affected green pod yield. The overall mean was 194 g m⁻² (Table 6.2). Plants inoculated with strains H.20, PRF 81 and PhP 17 produced 234, 214 and 214 g m⁻² respectively, all more than the control plants which yielded only 165 g pods m⁻². RCR 3644, the strain recommended for inoculation in New Zealand, produced 164 g m⁻²; this was also below the grand mean. Cultivar did not significantly affect green pod yield and the average was 198 g m⁻² (Table 6.2).

Table 6.1. The effect of inoculation, cultivar, fertiliser and irrigation on total dry matter at green pod harvest (g m^{-2}) of common bean in Canterbury, 2003-04 and 2004-05.

2003-04		2004-05	
Inoculation (IN)		Inoculation (IN)	
Nil	893	Nil	514
CC 511	819	RCR 3644	482
RCR 3644	890	UK 2	486
SEM	28.6	H 20	619
Significance	ns	PRF 81	723
		PhP 17	662
Cultivar (CU)		SEM	82.8
Scylla	872	Significance	ns
T-49	863		
SEM	23.4	Cultivar (CU)	
Significance	ns	Scylla	567
		T-49	595
Irrigation (IR)		SEM	47.8
Nil	841	Significance	ns
Full	894		
SEM	38.5		
Significance	ns		
Fertiliser (FE)			
0 (kg N ha^{-1})	855		
150 (kg N ha^{-1})	880		
SEM	23.4		
Significance	ns		
CV (%)	6.1		26.6
Significant Interactions	nil		nil

Table 6.2. The effect of inoculation, cultivar, fertiliser and irrigation on green pod yield (g m⁻²) on a dry weight basis of common bean in Canterbury, 2003-04 and 2004-05.

	2003-04		2004-05
Inoculation (IN)		Inoculation (IN)	
Nil	463	Nil	165
CC 511	414	RCR 3644	164
RCR 3644	446	UK 2	175
SEM	16.4	H 20	234
Significance	ns	PRF 81	214
		PhP 17	214
		SEM	22.2
Cultivar (CU)		Significance	p < 0.05
Scylla	448		
T-49	433	Cultivar (CU)	
SEM	13.4	Scylla	183
Significance	ns	T-49	214
		SEM	12.8
Irrigation (IR)		Significance	ns
Nil	411		
Full	471		
SEM	21		
Significance	ns		
Fertiliser (FE)			
0 (kg N ha. ⁻¹)	431		
150 (kg N ha. ⁻¹)	451		
SEM	13.4		
Significance	ns		
CV (%)	6.5		17.7
Significant	nil		nil
Interactions			

6.5 Final harvest

6.5.1 Total dry matter production

In 2003-04, TDM at final harvest was not affected by inoculation or irrigation (Table 6.3). However, both cultivar and N did have a significant effect. Scylla produced 27 % more TDM than T-49 (730 and 530 g m⁻²) respectively (Table 6.3). Plants which received 150 kg N ha⁻¹ produced 12 % more TDM than control plants with no N fertiliser application. In 2004-05, TDM was not affected by either factor and the grand mean was 1,044 g m⁻² (Table 6.3). In 2004-05, the crop produced 47 % more TDM than in 2003-04.

6.5.2 Seed Yield

Seed yield in 2003-04 ranged from 266 to 467 g m⁻² (Table 6.4). Seed yield was not affected by inoculation or irrigation. However, cultivar and N did produce a significant effect ($p < 0.001$). Scylla's seed yield (467 g m⁻²) was 76 % higher than that of T-49 (266 g m⁻²). There was also a yield increase of 12 % in N treated plants compared with unfertilised plants. In 2003-04, at final harvest, a strong cultivar by fertiliser interaction influenced seed yield (Table 6.4). Scylla's response to extra N was almost double (16 %) that of T-49 (9 %). In 2004-05, there was no significant main treatment effect on seed yield at final harvest (Table 6.4) and the grand mean was 535 g m⁻².

Table 6.3. The effect of inoculation, cultivar, fertiliser and irrigation on total dry matter at final harvest (g m^{-2}) of common bean in Canterbury, 2003-04 and 2004-05.

	2003-04		2004-05
Inoculation (IN)		Inoculation (IN)	
Nil	625	Nil	948
CC 511	640	RCR 3644	1227
RCR 3644	625	UK 2	1000
SEM	12.6	H 20	900
Significance	ns	PRF 81	1117
		PhP 17	1072
Cultivar (CU)		SEM	116.0
Scylla	730	Significance	ns
T-49	530		
SEM	10.3	Cultivar (CU)	
Significance	$p < 0.001$	Scylla	1066
		T-49	1022
Irrigation (IR)		SEM	67.0
Nil	604	Significance	ns
Full	656		
SEM	25.9		
Significance	ns		
Fertiliser (FE)			
0 (kg N ha^{-1})	595		
150 (kg N ha^{-1})	665		
SEM	10.3		
Significance	$p < 0.001$		
CV (%)	5.7		6.5
Significant	nil		nil
Interactions			

Table 6.4. The effect of inoculation, cultivar, fertiliser and irrigation on seed yield at final harvest (g m^{-2}) of common bean in Canterbury, 2003-04 and 2004-05.

2003-04		2004-05	
Inoculation (IN)		Inoculation (IN)	
Nil	363	Nil	442
CC 511	375	RCR 3644	635
RCR 3644	363	UK 2	522
SEM	8.9	H 20	505
Significance	ns	PRF 81	557
		PhP 17	545
		SEM	66.4
Cultivar (CU)		Cultivar (CU)	
Scylla	467	Scylla	572
T-49	266	T-49	497
SEM	7.2	SEM	38.3
Significance	$p < 0.001$	Significance	ns
Irrigation (IR)			
Nil	354		
Full	380		
SEM	14.9		
Significance	ns		
Fertiliser (FE)			
0 (kg N ha^{-1})	344		
150 (kg N ha^{-1})	389		
SEM	7.2		
Significance	$p < 0.001$		
CV (%)	6.9		6.1
Significant Interactions	CU x FE		nil
	$p < 0.05$		

Table 6.5. The cultivar by fertiliser interaction for seed yield at final harvest (g m^{-2}) of common bean in Canterbury, 2003-04.

Cultivar	Fertiliser	
	0 (kg N ha^{-1})	150 (kg N ha^{-1})
Scylla	433	501
T-49	254	278
SEM	10.2	
CV (%)	6.9	

6.5.3 Harvest index

In 2003-04, the HI was quite stable. Only cultivar significantly ($p < 0.001$) affected HI. Scylla had a HI of 0.64, while in T-49 it was only 0.50. In 2004-05, inoculation had no effect on HI, but again Scylla (0.56) had a higher HI than T-49 (0.51).

In 2003-04, there were significant ($p < 0.05$) irrigation by cultivar (Table 6.7), irrigation by fertiliser (Table 6.8) and irrigation by cultivar by fertiliser (Table 6.9) interactions on HI. In most of the interactions, Scylla out performed T-49 (Table 6.9). Although the cultivar by irrigation interaction was significant, its effect on HI was small. With Scylla, HI declined minimally from 0.64 without irrigation to 0.63 with irrigation, while with T-49, HI increased slightly from 0.50 with no irrigation to 0.51 with irrigation (Table 6.7)

In 2003-04, HI was significantly ($p < 0.05$) affected by an irrigation by fertiliser interaction (Table 6.8). The HI declined from 0.58 with N and no irrigation to 0.56 when no irrigation and no N were applied to the plants. Harvest index remained constant (0.57), in plants which received N and irrigation. This was also evident in plants with no N and irrigation. A significant cultivar by irrigation by fertiliser interaction (Table 6.9) only had a small effect on HI. In Scylla, HI increased from 0.62 without N and irrigation to 0.65 with irrigation and no N. In comparison, N and no irrigation led to an increase of 0.65, over the 0.64 for N fertilised, irrigated plants. The HI response of T-49 in the irrigation by cultivar by fertiliser interaction was more consistent at 0.50 at final harvest.

6.6 Yield components

6.6.1 Number of plants m⁻²

Generally, the plant population was slightly higher than the target population. In 2003-04, at harvest maturity, plant population was unaffected by any treatment and the grand mean was 87 plants m⁻² (Table 6.10). In 2004-05 plants were sown at 67 plant m⁻².

Table 6.6. The effect of inoculation, cultivar, fertiliser and irrigation on harvest index at final harvest (g m⁻²) of common bean in Canterbury, 2003-04 and 2004-05.

	2003-04		2004-05
Inoculation (IN)		Inoculation (IN)	
Nil	0.57	Nil	0.49
CC 511	0.57	RCR 3644	0.54
RCR 3644	0.57	UK 2	0.54
SEM	0.004	H 20	0.59
Significance	ns	PRF 81	0.52
		PhP 17	0.52
Cultivar (CU)		SEM	0.015
Scylla	0.64	Significance	ns
T-49	0.50		
SEM	0.003	Cultivar (CU)	
Significance	p < 0.001	Scylla	0.56
		T-49	0.51
Irrigation (IR)		SEM	0.009
Nil	0.57	Significance	p < 0.001
Full	0.57		
SEM	0.002		
Significance	ns		
Fertiliser (FE)			
0 (kg N ha ⁻¹)	0.57		
150 (kg N ha ⁻¹)	0.57		
SEM	0.003		
Significance	ns		
CV (%)	1.1		7.3
Significant Interactions	IR x CU*		nil
	IR x FE*		
	IR X CU x FE*		

Table 6.7. The cultivar by irrigation interaction on harvest index at final harvest (g m^{-2}) of common bean in Canterbury, 2003-04.

Cultivar	Irrigation	
	Nil	Full
Scylla	0.64	0.63
T-49	0.50	0.51
SEM	0.003	
CV (%)	1.1	

Table 6.8. The irrigation by fertiliser interaction on harvest index at final harvest (g m^{-2}) of common bean in Canterbury, 2003-04.

Fertiliser	Irrigation	
	Nil	Full
0 (kg N ha^{-1})	0.56	0.57
150 (kg N ha^{-1})	0.58	0.57
SEM	0.003	
CV (%)	1.1	

Table 6.9. The interaction of irrigation by cultivar by fertiliser on harvest index at final harvest (g m^{-2}) of common bean in Canterbury, 2003-04.

Irrigation	Cultivar		T-49		
	Fertiliser	Scylla	Scylla	T-49	
		0 (kg N ha^{-1})	150 (kg N ha^{-1})	0 (kg N ha^{-1})	150 (kg N ha^{-1})
Nil		0.62	0.65	0.50	0.51
Full		0.65	0.64	0.50	0.50
SEM		0.007			
CV (%)		1.1			

6.6.2 Pods plant⁻¹

In 2003-04, the number of pods plant⁻¹ at final harvest was not affected by inoculation, irrigation or fertiliser. However, Scylla had 7.6 pods plant⁻¹ which was 17 % more than T-49 at 6.3 (Table 6.10). There were no significant interactions. In 2004-05, the average number of pods plant⁻¹ was 12. This was an increase of 42 % from the 7 in 2003-04 (Table 6.13). Cultivar effect was significant and T-49 produced more pods plant⁻² (12.5) than Scylla (10.7).

6.6.3 Seeds pod⁻¹

Scylla produced 10 % more seeds pod⁻¹ (3.2) than T-49 (2.9) in 2003-04 but no other treatment had an effect (Table 6.10). In 2004-05, there was no treatment response on the number of seeds pod⁻¹ and the grand mean was 3.45 (Table 6.13).

Table 6.10. The effect of inoculation, cultivar, fertiliser and irrigation on yield components of common bean in Canterbury, 2003-04.

	Plant m ⁻²	Pods plant ⁻¹	Seeds pod ⁻¹	100 seed wt. (g)
Inoculation (IN)				
Nil	88	7.1	3.0	25.7
CC 511	88	6.6	3.1	26.1
RCR 3644	84	7.2	3.0	26.0
SEM	3.0	0.33	0.06	0.23
Significance	ns	ns	ns	ns
Cultivar (CU)				
Scylla	86	7.6	3.2	31.1
T-49	87	6.3	2.9	20.7
SEM	2.4	0.27	0.05	0.19
Significance	ns	p < 0.05	p < 0.001	p < 0.001
Irrigation (IR)				
Nil	83	6.81	2.9	26.1
Full	90	7.21	3.2	25.7
SEM	1.2	0.31	0.16	0.5
Significance	ns	ns	ns	ns
Fertiliser (FE)				
0(kg N ha ⁻¹)	86	6.74	3.0	25.1
150(kg N ha ⁻¹)	87	7.19	3.1	26.8
SEM	2.4	0.27	0.05	0.19
Significance	ns	ns	ns	p < 0.001
CV (%)	3	5.4	0.5	1.8
Significant interactions	nil	nil	FE x IN*	CU x FE**

Table 6.11. The cultivar by fertiliser interaction on 100 seed weight (g) of common bean in Canterbury in 2003-04.

Cultivar	Fertiliser (kg N ha ⁻¹)	
	0	150
Scylla	29.7	32.4
T-49	20.4	21.1
SEM	1.8	
CV (%)	0.27	

Table 6.12. The fertiliser by inoculation interaction on seeds pod⁻¹ of common bean in Canterbury in 2003-04.

Fertiliser	Inoculation	Nil	CC 511	RCR 3644
0 (kg N ha ⁻¹)		2.9	3.2	2.9
150 (kg N ha ⁻¹)		3.0	3.0	3.2
SEM		0.09		
CV (%)		0.5		

Table 6.13. The effect of inoculation, cultivar, fertiliser and irrigation on yield components of common bean in Canterbury, New Zealand, 2004-05.

	Pod plant ⁻¹	Seeds pod ⁻¹	100 seed wt.(g)
Inoculation (IN)			
Nil	10.9	3.3	22.2
RCR 3644	13.6	3.6	21.8
UK 2	11.5	3.4	22.6
H 20	10.0	3.5	25.2
PRF 81	11.6	3.5	22.7
PhP 17	12.1	3.5	22.7
SEM	1.1	0.15	0.66
Significance	ns	ns	p < 0.05
Cultivar (CU)			
Scylla	10.7	3.5	26.8
T-49	12.5	3.4	18.9
SEM	0.6	0.13	0.38
Significance	p < 0.05	ns	p < 0.001
CV (%)	8.1	3.9	3.9
Significant interactions	nil	nil	nil

6.6.4 100 seed weight

In 2003-04, fertiliser and cultivar significantly ($p < 0.001$) affected the 100 seed weight (Table 6.10). The 100 seed weight in Scylla (31.1 g) was 50 % higher than in T-49 (20.7 g). There was a significant 7 % increase in the 100 seed weight when 150 kg N ha⁻¹ was applied compared to the 0 kg N ha⁻¹ control. Irrigation and inoculation had no effect on 100 seed weight. In 2004-05, (Table 6.13) plants inoculated with strain H 20 had the highest 100 seed weight at 25.2 g and plants inoculated with strain RCR 3644 the lowest at 21.8 g. As in the previous season, Scylla had larger seeds than T-49.

6.6.5 Yield and yield components correlation

Simple correlation analysis between seed yield and yield components showed a significant correlation ($p < 0.01$), $r^2 = 0.94$ (2003-04) and $r^2 = 0.85$ (2004-05) between total DM and seed yield (Table 6.14). The 100 seed weight in 2003-04 was positively correlated ($p < 0.01$) with TDM, seed yield and HI, $r^2 = 0.62, 0.77$ and 0.88 respectively. In 2004-05, there was a positive correlation, $r^2 = 0.32$, between the 100 seed weight and HI (Table 6.15).

In both seasons, seeds pod⁻¹ were positively correlated ($p < 0.01$) with all the other yield components tested, except in 2004-05 when there was no significant relationship with HI and there was a negative relationship with plant m⁻² in 2003-04 (Table 6.14). In both seasons, the relationship between seed yield and HI was positive ($r^2 = 0.83$, $p < 0.01$) in 2003-04 and ($r^2 = 0.14$, $p < 0.05$) in 2004-05 (Table 6.14 and Table 6.15). Pods plant⁻¹ was negatively correlated with plants m⁻² ($r^2 = 0.27$, $p < 0.01$) in 2003-04 but in 2004-05, there was no relationship. All other yield components were positively correlated ($p < 0.01$) in both seasons.

Table 6.14. Correlation matrix between yield and yield components for common bean in Canterbury, New Zealand, 2003-04.

	Total DM	Seed yield	Harvest index	Plant m ⁻²	Pod plant ⁻¹	Seed pod ⁻¹
2003-2004						
Seed yield	0.97**					
Harvest index	0.80**	0.91**				
Plant m ⁻²	-0.10	-0.09	-0.05			
Pod plant ⁻¹	0.40**	0.39**	0.34**	-0.52**		
Seed pod ⁻¹	0.52**	0.49**	0.38**	-0.34**	0.36**	
100 seed weight.	0.79**	0.88**	0.94**	0.01	0.30*	0.34**

* = p < 0.05, ** = p < 0.01

Table 6.15. Correlation matrix between yield and yield components for common bean in Canterbury, New Zealand, 2004-05.

	Total DM	Seed yield	Harvest index	Pod plant ⁻¹	Seed pod ⁻¹
2004-2005					
Seed yield	0.92**				
Harvest index	-0.004	0.37*			
Pod plant ⁻¹	0.86**	0.74**	-0.16		
Seed pod ⁻¹	0.68**	0.71**	0.17	0.39**	
100 seed weight.	-0.05	0.16	0.57**	-0.37*	0.01

* = p < 0.05, ** = p < 0.01

6.7 Discussion

6.7.1 Total dry matter and pod yield at the green pod stage

Total DM at green pod harvest, averaged 868 g m⁻² in 2003-04 and 581 g m⁻² in 2004-05. In 2004-05, plants were sown on 11 January 2005, one month later than in 2003-04. The lower TDM production in 2004-05 sown plants could have been due to more rapid crop development. More rapid development allows less time for canopy development and PAR interception during vegetative growth, pod set and development stages. In this study at green pod harvest, there was no significant effect of irrigation on TDM, green pod yield or HI. These findings confirm the results of Martin (1982) who found that the response of *Phaseolus vulgaris* to irrigation varied between seasons and probably depended on soil

type and its water retention ability. Similar responses were reported for peas grown at Templeton, near Christchurch (Stoker, 1977).

Green bean yields in New Zealand have ranged from 11.6 to 20.9 t ha⁻¹ of fresh weight over four seasons (Martin, 1982), 1.3 t ha⁻¹ (Nichols, 1974) and 6.7 t ha⁻¹ (FAO, 2004). The yield of Scylla, the green bean cultivar used in this study confirms this variability of green bean production and highlights the unpredictability of producing this crop in Canterbury.

6.7.2 Total dry matter and seed yield

This study showed that both TDM and seed yield at final harvest were influenced by cultivar and N fertiliser. The cultivar effect was positive in these experiments with cultivar Scylla performing better than T-49 in most of the measured parameters over the experimental period. Common bean cultivar response to N fertiliser application was reported by Bliss *et al* (1989) and Bliss (1993b), (1993a). Commercial cultivars of common beans are usually bred and selected under conditions of high soil fertility leading to auto-selection of genotypes that prefer the use of combined, not biologically fixed N₂ (Greven, 2000; Provorov and Tikhonovich, 2003). Scylla, a more recent upright green bean, performed better than T-49 which is a dry navy bean in TDM at final harvest and green pod yield, seed yield, pods plant⁻¹ and seeds pod⁻¹ (2003-04). In both seasons there was a highly significant correlation between seed yield and TDM (Table 6.14) and (Table 6.15). High TDM production in grain legumes is a prerequisite for high seed yield, for example; chickpea (Saxena *et al.*, 1990), pinto beans (Dapaah *et al.*, 2000), and field peas, lentil, chickpea and narrow-leaved lupin (Ayaz *et al.*, 2004b).

Over the two years, the average TDM yield at final harvest was 804 g m⁻². In 2004-05 it was 1,000 g m⁻² which was 37 % higher than in 2003-04. This was 12 % more than the yield reported by Greven (2000) and 40 to 55 % more than the 450 and 600 g m⁻² reported by Owens y de Novoa (1980) and Dapaah *et al.* (1995) in common beans grown in Canterbury, New Zealand. One of the reasons for these differences among seasons could be the effect of nodulation and N fixation in 2005-04 compared with 2003-04. In 2003-04 inoculation did not result in nodulation while there was successful nodulation in 2004-05 (Chapter 3).

In 2003-04, applied N gave a positive response in TDM and seed yield which was probably due to inadequate available soil N (Table 3.1) (Edje *et al.*, 1975; Westermann *et al.*, 1981; McKenzie *et al.*, 1994). Unlike 2004-05, soil total N was not measured in 2003-04, so a comparison of soil N content could not be undertaken. The significant positive N fertiliser response in TDM at final harvest could also be a function of the combined effect of applied N and residual soil N from the previous cropping as described in Section 3.2.1.3. This increase in TDM due to applied N (Table 6.3) gave increased seed yield (Table 6.4). Judicious N application can increase branching, LAI and leaf area duration, which gives increased interception of solar radiation, higher TDM production and consequently higher seed yield (Hay and Walker, 1989).

Over the two seasons the maximum seed yield was 635 g m⁻² and the lowest was 266 g m⁻². In 2003-04, seed yield was very consistent over the experimental area. There was no significant interaction for inoculation (probably due to no nodulation) or irrigation but there was a significant (p < 0.001) interaction between cultivar and fertiliser. Table 6.5 shows that Scylla responded much better to applied N than T-49. Scylla is a much more vigorous cultivar than T-49 as shown in 2004-05.

In grain legumes, seed yields can be reduced when crops are sown at above optimum populations. High population results in reduced branching and pods plant⁻¹ (Hernandez and Hill, 1985; McKenzie *et al.*, 1994). In this study, an increase in plant population above the recommended 60 plants m⁻² (McKenzie, 1989) reduced seed yield (2003-04). The expected reduction in pods plant⁻¹ was also observed. The number of pods plant⁻¹ is a major factor affected by plant population (Ayaz *et al.*, 2004a). This was confirmed in this study. The number of pods plant⁻¹ depends on the number of reproductive sites produced plant⁻¹; it is possible that in the 2003-04, plants progressed towards the reproductive phase at a time when the temperature would have affected flower primordia and the pod development stage. Reduced pollen viability (Halterlein *et al.*, 1980) and pollen injury (Farlow, 1981; Monterroso and Wein, 1990) at low temperature have been implicated in reduced pod and seed set processes which could be the case in this study. In these experiments low temperatures of 3 °C in 2003-04 and -1.6 in 2004-05 were experienced during flowering and fruit set which could have affected the number of pods plant⁻¹ and seeds pod⁻¹.

6.7.3 Harvest index (of TDM at final harvest)

Harvest index (HI), ranged from 0.49 to 0.64 depending on cultivar and season. The difference in HI between the two seasons may have been due to the difference in the sowing date of the two experiments. In 2003-04, the experiment was sown on 8 December 2003; the second season's experiment was sown over a month later on 11 January 2005. In Canterbury, the recommended sowing time for common beans is mid to late November (McKenzie, 1989; Dapaah, 1997). The January 2005 sowing caused an early final harvest before 95 % of the plants had turned brown in Scylla to prevent crop loss from fungal infection of pods and seed. The early harvest affected the 100 seed weight and reduced the HI (Table 6.10) and (Table 6.13). These results are consistent with the results of Greven (2000).

High variability of HI in grain legumes has been previously reported in Canterbury (McKenzie, 1987; Moot and McNeil, 1995; Anwar *et al.*, 1999; Ayaz *et al.*, 2004b) and by White *et al.* (1992b), who worked with 62 common bean genotypes, in Colombia and in the USA. Ayaz *et al.* (2004b) working with lentil and narrow-leafed lupin found the highest HI in lentil and the lowest in narrow-leafed lupin. This was attributed to the shorter lentil stems. In this study, cultivar had a significant ($p < 0.001$) effect on HI. Scylla has shorter, more compact stems than T-49 which supports the results of Ayaz *et al.* (2004b) with lentil and narrow-leafed lupin in Canterbury. In most grain legumes, the HI is generally low when compared to cereals. The improved HI in cereal crops has generally been obtained by reduction in stem length and the diversion of more assimilates to grain production (Stanforth *et al.*, 1994). The variable HI in the common beans may also have been due to the concurrent accumulation of DM and N during the period of rapid seed growth (Saxena and Sheldrake, 1979a) reducing assimilate partitioning to developing seed, hence affecting the final HI.

Saxena and Sheldrake (1979b) stated that care should be taken when comparing the HIs of cereals with crops like grain legumes such as chickpea. The loss of leaves during senescence, leaving mainly stems and pods at harvest maturity usually gives an over estimate of the HI in the grain legume crops and this could be the case here. The harvest of the 2004-05 experiment before 95 % the plants had turned brown could have affected the HI. At harvest, the leaves were not senescent as in the first season's experiment, and this affected final TDM, seed yield and hence HI. Irrigation, a major factor in this study, did

not affect HI. The reported effect of irrigation reducing (McKenzie and Hill, 1990; Dapaah, 1997) or increasing (White *et al.*, 1982) HI was not observed in this study.

6.7.4 Yield components

The effect of yield components on final yield was variable over the two seasons. The TDM and seed yield were highly correlated ($r^2 = 0.94$) in 2003-04 and ($r^2 = 0.85$) in 2004-05. The single most important factor influencing seed yield in 2003-04 was the 100 seed weight ($r^2 = 0.77$), followed by seeds pod⁻¹ ($r^2 = 0.24$). However, in the second year, when the plant population was less variable, pod plant⁻¹ ($r^2 = 0.55$) and seed pod⁻¹ ($r^2 = 0.50$) were the major factors.

The variation in plant population from 83 to 90 plants m⁻² in 2003-04 to a consistent 67 plants m⁻² in 2004-05 was probably due to the different sowing methods used. In 2003-04 sowing was mechanical, using an Öyjord cone seeder. In 2004-05, sowing was by hand. Similar results were reported by Ayaz (2001) working with mechanically sown grain legumes in Canterbury. Second, the doubling of the sowing rate in 2003-04, to account for the poor seed germination, could have contributed to population variation in that season.

There was similar variation in pods plant⁻¹ over the two seasons. Pods plant⁻¹ was correlated ($r^2 = 0.49$) with seed yield in 2004-05. Similar correlations have been found in pinto bean, dwarf French bean and lentil (McKenzie, 1987; Dapaah, 1997; Greven, 2000). In 2003-04, yield was significantly ($p < 0.01$) affected by pods plant⁻¹ but the influence of this component was negligible ($r^2 = 0.15$). In 2004-05, pods plant⁻¹ was the single largest component affecting seed yield. This is consistent with work on chickpea (Siddique and Sedgley, 1986) and pea (Pandey and Grafton, 1975). Generally, species plays a large part in variation in pods plant⁻¹. However, the number of pods plant⁻¹ produced and maintained to final harvest in this study, can be attributed to the environmental conditions as discussed earlier. These results are consistent with previously published work (Knott, 1987).

In both years, the number of seeds pod⁻¹ was consistent over the experimental area and averaged between 3.0 and 3.5. The expected increase in seeds pod⁻¹ due to cultivar was observed in both seasons. In Canterbury, reductions in seeds pod⁻¹ due to variations in sowing dates were reported in chickpea (Verghis *et al.*, 1993; Verghis *et al.*, 1999). This reduction was attributed to unfavourable climatic conditions which were exceptionally cold

and wet when compared to the 50 year averages of air temperatures and rainfall in Canterbury. There were increases of 9 and 3 % in seeds pod⁻¹ in Scylla over T-49. The small increase in this component observed, especially in 2004-05, could be due to increased competition for assimilates from high vegetative growth in the January sowing. This may have reduced the number of seeds pod⁻¹ by increasing the number of seeds that failed to fill, a condition often reported as seed abortion (Dracup and Kirby, 1996). An increase in the number of seeds pod⁻¹ as observed here, with delayed sowing, was reported in chickpea in south-Western Australia by Siddique and Sedgley (1986).

Irrigation increased seeds pod⁻¹ by 9 % over unirrigated plants. This is consistent with the results of work on pinto beans (Dapaah, 1997), and navy beans (Love *et al.*, 1988). In contrast, irrigation increased TDM and the number of pods plant⁻¹ in these experiments but decreased the number of pods plant⁻¹ in chickpea in Canterbury (Verghis, 1996). This was attributed to competition between reproductive and vegetative structures for assimilate, resulting in seed abortion, as reported by Goldsworthy (1984).

In grain legumes, mean seed weight is considered to be the most stable yield component (Littleton *et al.*, 1979; Ayaz *et al.*, 2004a). However, both negative (Singh and Auckland, 1975) and positive (McKenzie, 1987; Dapaah, 1997) correlations with total seed yield have been reported. In this study, the 100 seed weight was significantly correlated with seed yield in 2003-04. However, in 2004-05, the relationship was not significant. This could be due to the environmental conditions experienced during seed set and development and the harvest of the crop before full harvest maturity (as discussed earlier). This early harvest could have reduced the amount of assimilate that partitioned from leaves to seeds in the 2004-05 growing season.

The 100 seed weight was significantly ($p < 0.001$) affected by cultivar and fertiliser in 2003-04, with a significant cultivar by fertiliser interaction (Table 6.10). This may be attributed to the low N status of the field (Table 3.1) and the crop response to applied N. The higher seed weight was probably a result of the lower seed pod⁻¹ in that season (3.0) and the provision of enough assimilate to fill the seeds produced. In both seasons, the 100 seed weight was positively and significantly correlated with HI. Cultivar influence (as mentioned earlier) on the 100 seed weight was strong. The cv. T-49 is small seeded and is never likely to produce a seed as large as cv. Scylla. The differences between Scylla and T-49 were 33 and 29 % in 2003-04 and 2004-05 respectively. The 100 seed weights in this

study were not similar to those obtained with pinto bean (Dapaah *et al.*, 1999) or dwarf French bean cultivars in Canterbury (Greven, 2000). Mean seed weight can vary with both genotype and environment (Hardwick *et al.*, 1978), as confirmed by comparing these results with previous reports of Dapaah *et al.* (1999) and Greven (2000) .

6.8 Conclusions

1. Late November and early December sowings can give high dry bean seed yields in Canterbury, provided the cultivars that are selected are well adapted to the production environment.
2. The cv. Scylla showed good adaptation to production conditions in Canterbury by producing the highest TDM at green pod and final harvest (2003-04) and the highest seed yield in both growing seasons.
3. Green bean yields, (immature pods), on a dry weight basis, can be as high as 470 g m⁻², while dry seed yields can reach 635 g m⁻².
4. Inoculation of compatible bean cultivars with an effective *Rhizobium* isolate can increase green pod yield as observed in cv. T-49 in 2004-05.
5. Selection of phenologically adapted bean cultivars with appropriate sowing times and bacterial associations may improve the growth and development of common bean in the South Island of New Zealand.
6. The 100 seed weight and the number of seeds pod⁻¹ were the yield components that had the greatest effect on seed yield.

CHAPTER 7

Identification and characterisation of *Rhizobium* isolates nodulating different common bean (*Phaseolus vulgaris* L.) cultivars

Summary

Phaseolus vulgaris is a promiscuous legume which is nodulated by rhizobia that are present in most fields. Eighteen *Rhizobium* isolates, recognised for their ability to nodulate common bean (Chapter 3), were examined in a greenhouse, five were selected, based on their ability to form nodules. Nodules were recovered from field grown plants that had been inoculated with these isolates and the nodulating organisms were identified. Polymerase Chain Reaction (PCR) amplification and DNA sequencing of the ribosomal DNA (rDNA) was used to identify these organisms after growing them on Tryptone-Yeast extract (TY) and Rhizobia Defined Media (RDM) agar and DNA extraction.

Genomic DNA extracted from the parent isolates was amplified using the primers fC and rD. Primers fC and rD amplified a single band from each parent isolate. These amplimers, encompassing the highly variable internally transcribed spacer (ITS) regions, were sequenced to identify the isolates to species level. Parent isolates were identified as either *Rhizobium* or *Agrobacterium* which are both known to nodulate common bean. In contrast, DNA extracted from isolates recovered from nodules of field grown plants produced multiple bands with primers fC and rD. Of 80 field grown nodules tested, five distinct banding patterns were observed with primers fC and rD. All patterns were different to those of the original parent isolates. The alternate, universal primers F27 and R1494 were then used and these produced a single amplimer from the recovered isolates that was suitable for DNA sequencing. This primer pair encompassed the conserved 16S region. While some contamination was possible, sequencing of these amplimers did not identify the isolates as *Rhizobium* but suggested that organisms, other than those used in the inoculum, were capable of inhabiting the nodules of common beans.

7.1 Introduction

Microbial diversity and its role in nature is poorly understood, mainly because traditional microbiological techniques, such as microscopy and culturing are only of limited use for microbial classification and identification (Muyzer, 1999). Muyzer (1999) further stated that classification of morphological traits is difficult because the organisms are small and look simple, lacking conspicuous external features for reliable and robust grouping. Further, classification of micro-organisms on their physiological and biochemical features is nearly impossible, because the majority (99 %) can not be isolated in pure cultures (Amann *et al.*, 1995).

Successful management of symbiotic associations between legumes and their bacterial endo-symbionts, *Rhizobium* (*Bradyrhizobium*) spp., requires that specific strains can be easily, rapidly and reliably identified (Richardson *et al.*, 1995; Selenska-Pobell *et al.*, 1995; Selenska-Pobell *et al.*, 1996). Traditional methods for identifying rhizobial strains are based on morphological, physiological and biochemical assays (Echeverrigaray *et al.*, 2000). These methods, however, frequently failed to identify *Rhizobium* strains within a species (Hameed *et al.*, 2004).

Recently, various methods based on PCR have been developed to characterise *Rhizobium* species and strains, and to examine genetic relationships in the *Rhizobium* group (Laguerre *et al.*, 1996). In addition to sequencing of ribosomal DNA, these methods include: restriction fragment length polymorphism (RFLP) (Demezas *et al.*, 1991; Amarger *et al.*, 1994; Laguerre *et al.*, 1994); the use of specific hybridisation probes (Watson and Schofield, 1985; Bjourson and Cooper, 1988; Amann *et al.*, 1995); and other methods that utilise the polymerase chain reaction (PCR) (Mullis and Faloona, 1987). The rRNA genetic locus (*rrn*) is found in both prokaryotic and eukaryotic organisms (Jensen *et al.*, 1993). The rRNA genetic locus in prokaryotes contains 16S, 23S and 5.8S genes which are highly conserved allowing RNA sequence information to be used in universal organisation of evolutionary relationships (Cedergren *et al.*, 1988).

The utility of the rRNA sequence as a taxonomic tool has been demonstrated in numerous bacterial species, where 16S rRNA sequence analyses have completely redefined phylogenetic relationships, previously dependent on characterisation of cellular metabolism (Woese, 1987). Sequencing of the 16S-23S rRNA IGS (intergenic spacer)

sequences has been used to differentiate within various bacterial species including chickpea rhizobia (Nour *et al.*, 1994), the new genus *Allorhizobium* (de Lajudie *et al.*, 1994) and the suggested combining of *Agrobacterium* and *Allorhizobium* into the genus *Rhizobium* (Young *et al.*, 2001). Direct gene sequencing of genes coding for 16S rRNA (16S rDNA) amplified by PCR (Young *et al.*, 1991; Laguerre *et al.*, 1993b) has been used to establish the genetic relationships of *Rhizobium* at the species and higher levels (Laguerre *et al.*, 1996). The method can also be used to trace the presence of target organisms in the environment (Tan *et al.*, 2001).

The regions between the 16S, 5.8S and 23S rDNAs are the internally transcribed spacer (ITS) regions (Bala *et al.*, 2001). The ITS regions are universally present in bacteria and show considerable sequence and length variations in species, even at the strain level (Jensen *et al.*, 1993; Gurtler and Stanisich, 1996). Use of PCR analysis of the ITS region has become a commonly used tool for intra species characterisation of rhizobia strains (Laguerre *et al.*, 1996; Paffetti *et al.*, 1996). The most direct and rapid method to determine the polymorphic character of the internal (*rrn*) spacer is amplification of ITS regions using primers from highly conserved flanking sequences. The length and sequence polymorphism present in the PCR product can then be used in recognition of genera or species (Jensen *et al.*, 1993).

One of the great advantages of the use of DNA amplification to study rhizobial ecology is that it requires no prior preparation for individual strains such as the preparation of antiserum (ELISA technique) or the introduction of marker genes (Wilson, 1995). The new technology offer significantly improved approaches to the study of rhizobial ecology by providing more accurate methods for the verification and identification of individual species in pure cultures and enabling rapid and reliable assessment of large numbers of rhizobial isolates (Wilson, 1995). The objectives of this chapter were to:

1. Identify the species of strains purchased in peat-based inoculum and obtained on slopes from the researchers described in the materials and methods section.
2. Identify the nodulating bacteria from inoculated plants in the field using PCR and DNA sequencing of ribosomal DNA.
3. Examine the nodulating effectiveness of the nodulated strains for nodulation.

7.2 Materials and Methods

7.2.1 Part 1

7.2.1.1 Bacterial strains

The bacterial strains used in this study were provided by David Humphry (Department of Biology, University of York, Heslington, United Kingdom); Jacques Jean Drevon (UMR Rhizosphere and Symbiose, INRA.-ENSA Centre de Montpellier, France); Mariangela Hungria (EMBRAPA Londrina, PR, Brazil) and Giselle Laguerre (Laboratoire de Microbiologie des Sols, Centre du Microbiologie du Sol de l'Environnement, INRA, Dijon, Cedex, France). Two further strains were isolated from peat-based inoculant purchased from NODULAID™ (Victoria, Australia) through Coated Seed Ltd., Christchurch (Table 7.1).

7.2.1.2 Bacterial growth

Rhizobial strains were grown on Tryptone-Yeast extract (TY) agar (Appendix 2B) plates (Beringer, 1974; Somasegaran and Hoben, 1994). The medium was made to 1 litre with 5 g Tryptone (DIFCO, Fort Richard, Auckland, New Zealand); 3 g Yeast Extract (DIFCO); 15 g Technical agar (Davis Bacteriological, Christchurch, New Zealand); 0.87 g CaCl₂.H₂O and deionised water in a clean 1000 ml bottle. Boiling for 5-10 minutes dissolved the agar. The pH was adjusted to 7.1 and the mixture autoclaved at 121 °C for 15-20 minutes. Fifteen ml of cooled liquid was poured into individual Petri dishes (LabServe, Biolab New Zealand). Strains were streaked onto the agar and incubated at 28 °C for 3-7 d. Cultures were stored in a refrigerator on TY agar plates for short term storage and on slopes of the same medium, replacing the technical agar from Davis Bacteriological with agar from Germantown Bacteriological, Auckland, New Zealand, for long-term storage.

Table 7.1 Bacterial strains used in the experiment and their source of origin.

Strain	Generic name	Origin
CARA 72 ^b	<i>Rhizobium tropici</i>	France
H 20 ^a	<i>Rhizobium tropici</i>	Brazil
H 12 ^a	<i>Rhizobium tropici</i>	Brazil
PRF 81 ^a	<i>Rhizobium tropici</i>	Brazil
PhP 17 ^b	<i>Rhizobium leguminosarum</i> bv. <i>phaseoli</i>	France
H 441 ^b	<i>Rhizobium leguminosarum</i> bv. <i>phaseoli</i>	France
CESTA 221 ^b	<i>Rhizobium tropici</i>	France
CIAT 899 ^c	<i>Rhizobium tropici</i>	France
HT 2a2 ^b	<i>Rhizobium tropici</i>	France
CARA 56 ^b	<i>Rhizobium tropici</i>	France
RCR 3644 ^d	<i>Rhizobium tropici</i>	Australia
CC 511 ^d	<i>Rhizobium tropici</i>	Australia
UK 4 ^e	Unknown	England
UK 3 ^e	Unknown	England
UK 2 ^e	Unknown	England
UK 1 ^e	Unknown	England

^a Source: EMBRAPA Londrina, PR, Brazil;

^b Laboratoire de Microbiologie des Sols, Centre du Microbiologie du Sol de l'Environnement, INRA, Dijon, Cedex, France

^c UMR Rhizosphere and Symbiose, I.N.R.A.-ENSA Centre de Montpellier, France

^d NODULAIDTM (Victoria, Australia)

^e Department of Biology, University of York, Heslington, United Kingdom

7.2.1.3 Rapid DNA extraction for identification of parent species

The DNA was extracted from pure cultures using the Chelex method, which is a rapid extraction method. A 5 % stock solution of Chelex resin (Chelex®100 Molecular Biology Grade Resin, 200-400 µm mesh Sodium Form, Bio-Rad Laboratories CA, USA) was prepared in distilled water. Fifty µl of Chelex resin, periodically swirled, was pipetted into sterile micro tubes placed on ice. A small amount of bacterial growth was scraped from the TY agar plate with a sterile 200 µl pipette tip and placed in the centrifuge tubes with the Chelex resin. Tube and contents were gently mixed and heated to 92 °C for 20 minutes in a thermal cycler (Bio-Rad iCYCLER, Bio-Rad Laboratories). Tubes were placed in the freezer for 20 minutes, thawed and gently mixed. The resin was pelleted by centrifuging at 20,000 x g for 2 minutes and the mixture stored at -80 °C until use.

7.2.1.4 Agarose gel preparation

The quantity and molecular weight of the PCR product was estimated using 1 % agarose gel electrophoresis. A 1 % agarose gel was prepared by combining 1 g agarose

(Roche Diagnostics, Mannheim, Germany) with 100 ml 1 X TAE buffer (40 mM Tris acetate, 2 mM Na₂EDTA, pH 8.5) in a clean 500 ml bottle. Boiling for 5-10 minutes dissolved the agarose, which was subsequently cooled to 60 °C. Molten agarose was poured into a horizontal E-C® Gel Electrophoresis Apparatus (E-C Apparatus Corporation, New York, USA) (10 cm × 21 cm) casting tray, the comb inserted and allowed to set for 30-45 minutes. The comb was removed, the gel placed in an electrophoresis chamber and covered with 1 X TAE buffer.

7.2.1.5 Polymerase Chain Reaction (PCR)

The bacterial genomic DNA, prepared using the Chelex resin method, was amplified using the following two primers fC (5'GGCTGGATCACCTCCTTTCT3') and rD (5'CCGGGTTTCCCATTCGG3') (Bala *et al.*, 2002) (Invitrogen Life Technologies Ltd California, USA.). This amplified the 5.8S and ITS regions. Each 25 µl amplification reaction contained 10 mM Tris-HCL, 1.5 mM MgCl₂, 50 mM KCl, 200 µM (each) dATP, dCTP, dGTP and dTTP (Roche Diagnostics), 5 pmoles of each primer (fC and rD) (Invitrogen Life Technologies Ltd) and 1.25 units of Hotmaster DNA Polymerase (Global Science and Technology Ltd, Auckland, New Zealand). A control containing nanopure water instead of genomic DNA was included to ensure the reaction mix was free of contamination. The contents of each tube were briefly vortexed and centrifuged at 10,000 x g for 10 seconds. Amplification was performed in an iCYCLER® Gradient PCR machine (Bio-Rad Laboratories) programmed for 2 minutes at 94 °C, then 35 cycles of 30 seconds at 94 °C, 30 seconds 58 °C and 45 seconds at 65 °C followed by 7 minutes at 65 °C and a 4 °C hold.

7.2.1.6 Agarose gel electrophoresis

The PCR samples were centrifuged at 10,000 x g for 10 seconds in preparation for electrophoresis. Subsequently, 5 µl PCR product and 2 µl 6 X loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF, 40 % (w/v) sucrose (Sigma Chemical Co., USA); in water) were combined for gel loading. A molecular marker was prepared in a similar manner with the 5 µl PCR product replaced by 2 µl High DNA Mass™ Ladder (Invitrogen Life Technologies). The samples were loaded into individual wells in the prepared 1 % agarose gel covered with 1 X TAE buffer. The samples were separated by electrophoresis at 10 volts cm⁻¹ for approximately 60 minutes. The gel was transferred into a plastic container and stained with ethidium bromide (0.5 µg/µl) for 30 minutes, destained

in water for 15 minutes and photographed using a Versadoc 3000 system (Bio-Rad Laboratories CA, USA). The resulting band intensity was compared with the High DNA Mass™ Ladder and the PCR product quantified by visual estimation.

7.2.1.7 Sequencing

The PCR product from the original parent samples was sequenced at The University of Auckland (DNA Sequencing Facility, Genomics Unit, Auckland, New Zealand). Primer rD (^{5'}CCGGGTTTCCCCATTCGG^{3'}) at a concentration of 5 pmoles/μl was used for sequencing of the almost complete 5.8S and ITS 1 and 2 regions. An analysis of the results was carried out via the BLASTn function in GenBank (<http://www.ncbi.nlm.nih.gov/BLAST>).

7.2.2 Part 2

7.2.2.1 Rhizobial bacteria isolation from nodules

Nodules were collected from the roots of field grown plants 70 d after inoculation to identify the nodulating species (Chapter 3). Roots were thoroughly washed and nodules collected which showed signs of activity by the presence of leghaemoglobin. One experimental block was selected as representative of the experiment. A maximum of 8 nodules were selected from each treatment for molecular characterisation. Each nodule was sterilised for 1-2 minutes in a 50/50 solution of 6 % hydrogen peroxide (H₂O₂) and 95 % ethyl alcohol (modified from Vincent (1970) and Somasegaran and Hoben (1985; Somasegaran and Hoben, 1994)) and washed five times in sterile deionised water. Individual nodules were crushed in a small volume of sterile deionised water (Vincent, 1970), streaked on Rhizobia Defined Media (Ronson *et al.*, 1984) (Appendix 2A) and incubated at 28 °C, for 48 h. Glucose was the carbon substrate used from Appendix 2A. Subsequent colony streaking and growth was undertaken to obtain a single colony from each nodule for DNA extraction and identification by sequencing of rDNA.

7.2.2.2 DNA extraction from a single colony

One colony was aseptically removed from the Rhizobia Defined Media (Ronson *et al.*, 1984) agar plate and inoculated into a universal tube containing 5.0 ml of TY broth. The universal tube was incubated at 28 °C overnight on an orbital rotary shaker (Chiltern Scientific, Auckland, New Zealand) at 150 rpm. High quality DNA of bacteria isolated

from nodules of field grown plants was extracted using a PureGene™ kit as described by the manufacturer (Gentra Systems, ProGenz Scientific Supplies, Auckland, New Zealand). The PureGene kit was used to extract high quality DNA of bacteria isolated from the nodules because it was possible that multiple PCR reactions would be necessary. Typically, 500 µl of cell suspension was placed in a 1.5 ml centrifuge tube on ice and centrifuged at 13,000-16,000 x g for 5 seconds to pellet the cells. The supernatant was carefully removed with a pipette and the cells were resuspended in 300 µl of cell lysis solution (Gentra Systems) and incubated at 80 °C for five minutes.

One and a half µl of RNase solution (20 µg/µl) (Gentra Systems) was added to the cell lysate. The sample was mixed by inverting the tube 25 times and incubated at 37 °C for 60 minutes. The solution was cooled to room temperature and 100 µl of protein precipitation solution (Gentra Systems) added to lyse the cells. The solution was mixed at high speed (vortex) for 20 seconds and centrifuged at 13,000-16,000 x g for 3 minutes to pellet the proteins. The supernatant containing the DNA was poured into a clean 1.5 ml centrifuge tube containing 300 µl of 100 % isopropanol (2-propanol) and mixed by gently inverting 50 times. The sample was centrifuged 13,000-16,000 x g for 1 minute to pellet the DNA. The supernatant was discarded and the DNA washed in 300 µl of 70 % ethanol. The DNA pellet was air dried for 10-15 minutes.

Fifty µl of DNA hydration solution (Gentra Systems) was added to the DNA pellet and the DNA sample was rehydrated by incubating for 60 minutes at 65 °C and/or overnight at 4 °C. For long term storage, samples were stored at -20 °C or -80 °C. The DNA was quantified using a Nanodrop® ND-1000 Spectrophotometer (Nanodrop® Technologies Inc., Montchanin, USA), and diluted to 10 nanograms (ng) for PCR.

7.2.2.3 Polymerase chain reaction

The polymerase chain reaction (PCR) amplification of the 5.8S and ITS 1 and 2 regions was achieved using primers fC (5'GGCTGGATCACCTCCTTTCT^{3'}) and rD (5'CCGGGTTTCCCCATTCGG^{3'}) (Bala *et al.*, 2002), (as described in section 7.2.1.5) Two other universal bacterial primers F27 (5'AGAGTTTGATCMTGGCTCAG^{3'}) (Kane *et al.*, 1993) and R1494 (5'CTACGGYTACCTTGTTACGAC^{3'}) (Weisburg *et al.*, 1991) were used to amplify the 16S rRNA region (Figure 7.1). The same reaction mix was used as in

Section 7.2.1.5. Amplification was performed in an iCYCLER® Gradient PCR machine (Bio-Rad Laboratories) programmed for 2 minutes at 94 °C, then 35 cycles of 30 seconds at 94 °C, 30 seconds at 58 °C and 1 minute at 68 °C followed by 7 minutes at 68 °C and a 4 °C hold. The PCR product obtained from the parent and the bacteria isolate of the selected plant nodules were separated by electrophoresis on a 1 % agarose gel.

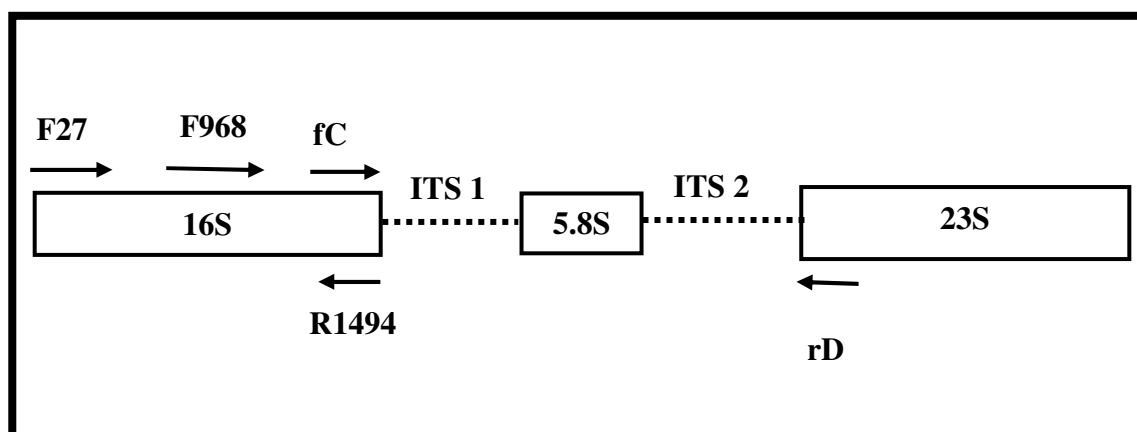


Figure 7.1. Primer sites for PCR. Primers fC and rD amplified the variable ITS 1 and 2 and the conserved 5.8S regions and primers F27, F968 and R1494 amplified the highly conserved 16S rRNA region

7.2.2.4 Sequencing of PCR products

To identify the bacterial species isolated from nodules of field grown beans used in these experiments, amplicons produced by primers F27 and R1494 were sequenced. The amplicons were sequenced at the Waikato DNA Sequencing Facility (Hamilton, New Zealand). An internal primer F968 ($5'$ ACCGCGAAGAACCTTAC $3'$) (Heuer *et al.*, 1997) at a concentration 5 pmoles/ μ l was used for sequencing the 16S rRNA gene. Results were analysed using the BLASTn function in GenBank (<http://www.ncbi.nlm.nih.gov/BLAST>).

7.2.3 Part 3

7.2.3.1 Authentication of isolates from nodules

Strains isolated from nodules of field grown plants and identified by sequencing were authenticated as nodulating strains using pure cultures to reinoculate bean seeds under *in vitro* conditions (Somasegaran and Hoben, 1985). Rhizobial strains were grown on Tryptone-Yeast extract (TY) agar plates (Beringer, 1974; Somasegaran and Hoben,

1994) (Appendix 2B). Strains were streaked onto the agar and incubated at 28 °C for 18 h. One hundred µl of sterile water was pipetted onto the agar plate and gently mixed with a sterile spreader. The mixed suspension was pipetted into a 250 ml sterile conical flask containing Tryptone-Yeast broth. The mixture of Tryptone-Yeast broth and bacterial suspension was incubated at 28 °C for 18 h at 150 rpm. One ml of broth culture was used as inoculum.

Seedling agar (Broughton and Dilworth, 1971) was prepared by mixing 0.5 ml each of solutions 1, 2, 3 and 4 (Appendix 2C) in 1 L of deionised water containing 25 g of bacteriological agar (Germantown Bacteriological). Solutions were poured into 640 ml glass specimen jars, sealed with cotton wool and autoclaved at 121 °C (138-140 kPa), for 15 minutes. The agar was allowed to set on an angle in 640 ml glass specimen jars to form slopes so the agar reached halfway up the bottle.

Seed was covered in 95 % ethanol for 10-30 seconds, and rinsed 10 times in sterile deionised water. Seed was soaked in 2 % commercial bleach for 5 minutes with stirring and rinsed three times in deionised water. Seed was then placed in a fridge overnight after imbibing water for 15-60 minutes (Somasegaran and Hoben, 1985). Seed was then sown on water agar (Brown, 1924) (Appendix 2D) plates (20 seeds plate⁻¹) and incubated inverted in the dark at 20 °C until the radicle emerged.

Germinated seeds were transferred under aseptic conditions with a sterile loop onto prepared seedling agar in growth bottles. Seeds were placed towards the top of the slope with the radicle facing down. One ml of inoculum was then placed on the germinating seed and radicle and seeds were grown at room temperature in the laboratory for one month.

The experimental design was a randomised complete block design with three replicates. There were 14 treatments (Table 7.2). In the treatments, seedlings of two bean cultivars (Scylla and T-49) were inoculated with one of the five distinct isolates retrieved from nodules, the parent strain RCR 3644, or inoculated broth. The treatments were designed to give a *Rhizobium*-cultivar combination for observing the nodulating ability of the isolates recovered from the field grown nodules. Isolates were assigned according to the following designation: 1² = treatment 1 nodule 2; 5⁸ = treatment 5 nodule 8; 9⁴ = treatment 9 nodule 4; 7² = treatment 7 nodule 2; 6⁷ = treatment 6 nodule 7. The

experiment was monitored daily for visual signs of nodule initiation and scored for nodulation as E effective nodules, I ineffective nodules, 0 no nodules.

Table 7.2. The treatments used in the authentication experiment

Cultivar	Isolate	Cultivar	Isolate
Scylla	1 ²	T-49	1 ²
	5 ⁸		5 ⁸
	9 ⁴		9 ⁴
	7 ²		7 ²
	6 ⁷		6 ⁷
	RCR 3644		RCR 3644
	control		control

RCR 3644: - reference strain is the recommended bean inoculant in New Zealand

7.3 Results

7.3.1 Part 1

7.3.1.1 Growth of isolates on media

The appearance of the original parent isolates grown on plates of Rhizobia Defined Media (RDM) was very similar. Most plates showed growth which was wet and gummy (Plate 7.1) and had a characteristic pungent smell. Isolates 3, 11 and 12 (Plate 7.1) were distinctly dry colonies without the gummy mucilaginous substance on the borders of the advancing colony. Isolates showed varying degrees of colour intensity on the RDM plates, ranging from pale to a deep rich yellow (Plate 7.1). All isolates grown on the RDM plates caused a colour change on the plates. The original colour of the agar plates was rich green. Bacterial growth on the plates changed colour from rich green to a translucent white. This was due to the acidifying ability of the multiplying bacteria.

7.3.1.2 Amplification of DNA extracted from original isolates

The PCR amplication of the internally transcribed spacer (ITS) and 5.8S region from the DNA of the original isolates using the primers fC (5' GGCTGGATCACCTCCTTTCT^{3'}) and rD (5' CCGGGTTTCCCCATTCGG^{3'}) generated amplimers of varying sizes (Figure 7.2). All isolates except H 12, and RCR 3644 generated a single band. Isolate H 12 generated three bands, the smallest was 400 base pairs (bp), through to 650 bp to the largest at 850 bp in length. Four bands were generated by RCR 3644, the smallest through to largest were approximately 400, 650, 850 and 1200 bp respectively. Only one fragment

was generated by CC 511 which was 650 bp long. Strains UK 1-4 (lanes 13-16) (Figure 7.2) each produced a single band, which was 1,100 bp in length. All the other isolates produced a single band with estimated size between 1,100 and 1,200 bp.

7.3.1.3 Sequence analysis of the rRNA

All PCR products that produced a single band of approximately 1,100-1,200 bp (Figure 7.2) were sequenced using primer rD. The sequence obtained spanned the ITS 1, 5.8S and ITS 2 regions of the rDNA. When aligned, these sequences showed varying percent identity with sequences of *Rhizobium* species deposited in GenBank (Table 7.3). Sequence comparison using the PCR products produced by amplification of parent strains H 12, RCR 3644 and CC 511 was not possible because of the multiple, or unexpected size, of the bands produced. Sixty two percent (8) of the submitted sequences matched the genus *Rhizobium*, and the remaining 38 % (5) were identified as from the genus *Agrobacterium*. The results compared favourably with the species identifications that were supplied with the original isolates. Of the original (parent) isolates 75 % were designated as from the genus *Rhizobium*, and the remaining 25 % were designated as from the genus *Agrobacterium* (Table 7.1). The original isolates were supplied with the species names *Rhizobium tropici* and *leguminosarum* bv. *phaseoli*, while sequencing of ITS regions produced the additional species names *hainanense*, *trifolii* and *rhizogenes* and *Agrobacterium tumefaciens* (Table 7.3). However, most of the GenBank matches produced by sequencing of the original parent isolates were not 100 %.

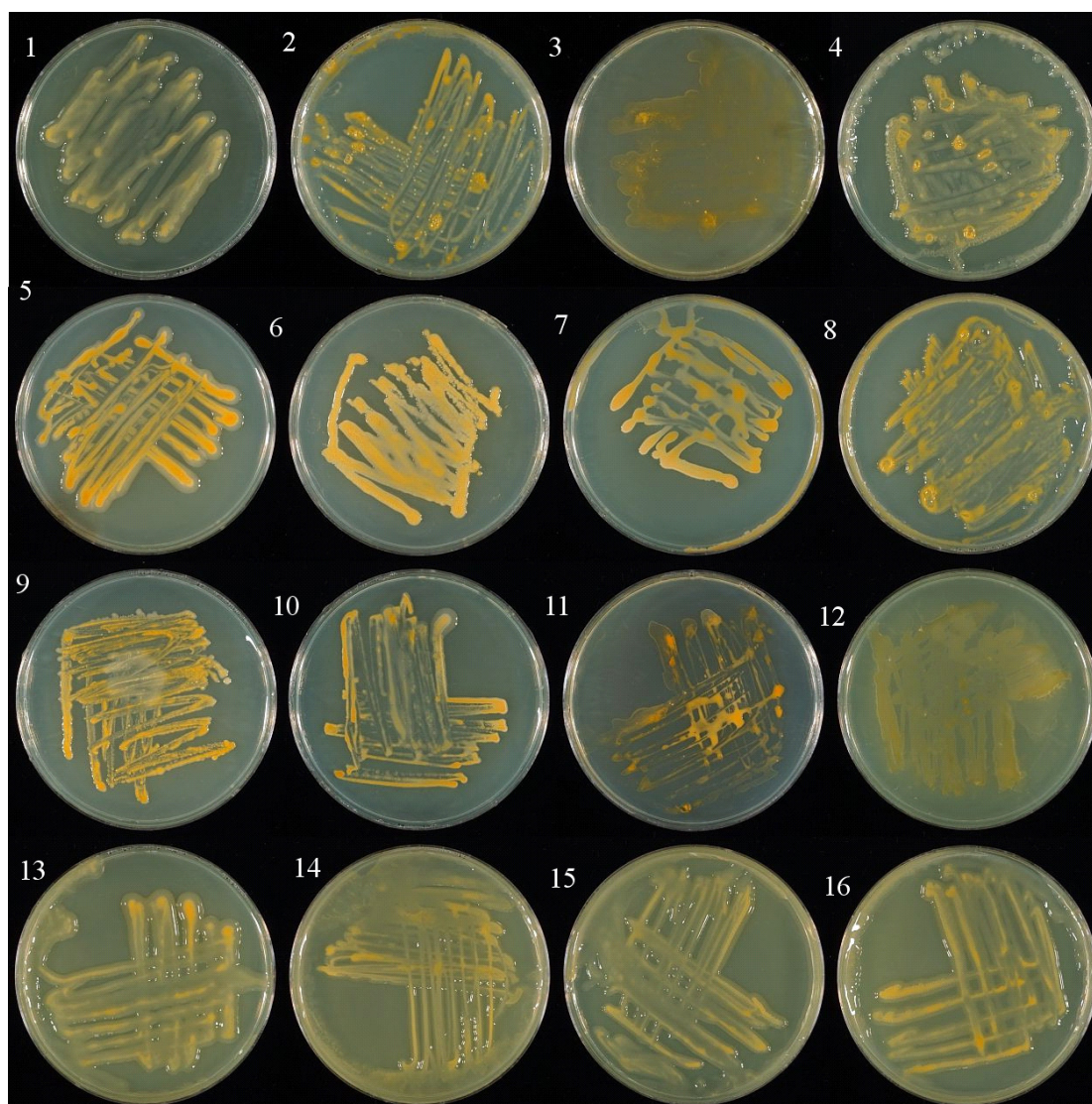


Plate 7.1. Original cultures of isolates growing on Rhizobia Defined Media (RDM). From left to right CARA 72 (1), H 20 (2), H 12 (3), PRF 81 (4), PhP 17 (5), H 441 (6), CESTA 221 (7), CIAT 899 (8), HT 2a2 (9), CARA 56 (10), RCR 3644 (11), CC 511 (12), UK 4 (13), UK 3 (14), UK 2 (15), UK 1 (16).

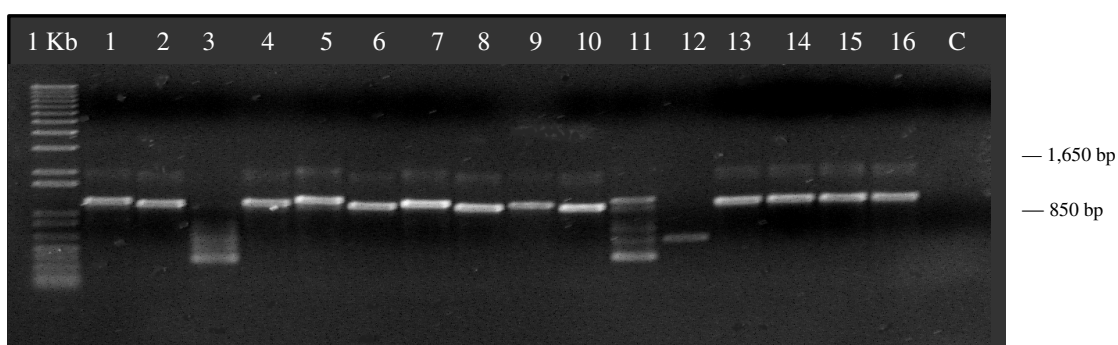


Figure 7.2. PCR products obtained with primers fC and rD of original *Rhizobium* isolates. From left to right: (1Kb Plus DNA ladder TM (Invitrogen), CARA 72 (1), H 20 (2), H 12 (3), PRF 81 (4), PhP 17 (5) H 441 (6), CESTA 221 (7), CIAT 899 (8), HT 2a2 (9), CARA 56 (10), RCR 3644 (11), CC 511 (12), UK 4 (13), UK 3 (14), UK 2 (15), UK 1 (16), Negative control (C).

Table 7.3. Species identification of original parent isolates obtained from the GenBank comparison with the sequenced DNA

Isolate name	Amplimer length in base pairs (bp)	Species	Identity (%)	E value
1:CARA 72	690	<i>Rhizobium rhizogenes</i> (AF345275*)	93	10 ⁻¹³⁹
2:H 20	540	<i>Rhizobium tropici</i> (AY491966*)	98	0.00
3:H 12	-	-	-	-
4:PRF 81	680	<i>Rhizobium hainanense</i> (AF345269*)	97	10 ⁻⁸³
5:PhP 17	540	<i>Rhizobium leguminosarum</i> bv. <i>trifolii</i> (DQ196423*)	99	0.00
6:H 441	590	<i>Rhizobium leguminosarum</i> bv. <i>phaseoli</i> (AY491963)	99	0.00
7:CESTA 221	775	<i>Rhizobium</i> sp. (AF510910*)	91	10 ⁻¹⁷⁰
		<i>Rhizobium</i> sp. (AF510906*)	91	10 ⁻¹⁷⁰
8:CIAT 899	590	<i>Rhizobium tropici</i> (AY491966*)	99	0.00
9:HT 2a2	590	<i>Rhizobium</i> sp. (AF510917*)	92	10 ⁻⁵⁰
10:CARA 56	640	<i>Agrobacterium tumefaciens</i> (AB102735*)	99	0.00
11:RCR 3644	-	-	-	-
12:CC 511	-	-	-	-
13:UK 4	590	<i>Agrobacterium tumefaciens</i> (AB102735*)	98	0.00
14:UK 3	773	<i>Agrobacterium tumefaciens</i> (AB102735*)	99	0.00
15:UK 2	550	<i>Agrobacterium tumefaciens</i> (AB102735*)	98	0.00
16:UK 1	550	<i>Agrobacterium tumefaciens</i> (AB102735*)	98	0.00

* Accession number in parenthesis

7.3.2 Part 2

Amplification of the ITS region of isolates recovered from nodules of field grown plants was also carried out with primers fC and rD. In contrast to the parent strains, where

the majority only produced a single amplicon of approximately 1,100 bp, the size and number of bands generated from the bacteria colonising the nodules varied among strains and between cultivars. The H 20 parent strain exhibited one band of 1,150 bp (Figure 7.3). Although some of the PCR products from the bacteria isolated from the nodules of plants inoculated with strain H 20 also contained a band of this size (lanes 1, 6, 7, 8, 10), none contained just a single band matching the parent strain. Lane 9 produced a single band of 600 bp. Lanes 6, 7 and 10 contained three bands of 1,150, 800 and 500 bp long. Lanes 1 and 8 contained 4 bands of 1,150, 800, 600 and 500 bp. Lanes 13, 14, 15 did not produce any bands. Lanes 2, 3, 4, 5 produced 1 band of 500 bp. In total, 4 different banding patterns were observed.

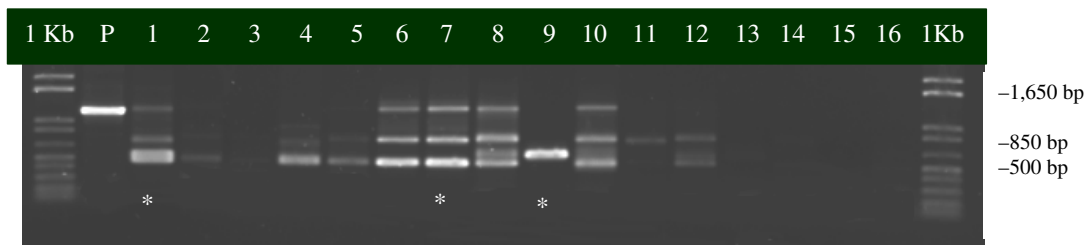


Figure 7.3. The PCR products obtained with primers fC and rD from nodules of plant inoculated with strain H 20. From left to right: 1Kb Plus DNA Ladder™ (Invitrogen), Parent DNA (P), lanes 1 to 7 represent individual nodules of cv. Scylla and lanes 8 to 15 cv. T-49, 16 negative control, 1Kb Plus DNA Ladder™ (Invitrogen). (Banding patterns present: Lane 9 = A, lane 7 = D, lane 5 = E, lane 8 = C). **Asterisk** represents fC and rD amplicons selected as a representative of the nodule inhabitants

The size and number of the bands generated by bacteria isolated from nodules recovered from plants inoculated with isolate PhP 17 (Figure 7.4) showed four different patterns. The parent (P) produced a band of approximately 1,150 bp which was equivalent to the largest band produced by some of the nodule recovered isolates. Lane 4 also produced one band which was 600 bp, while lanes 1, 5 and 13 exhibited two bands, the smallest of 600 bp and the largest of 800 bp. Lanes 7, 9, 10 and 11 produced 3 distinct bands of 1,150, 800 and 600 bp in length. Lanes 2, 3, 6, 8, and 12 produced 4 bands of 1,150, 800, 600 and 500 bp.

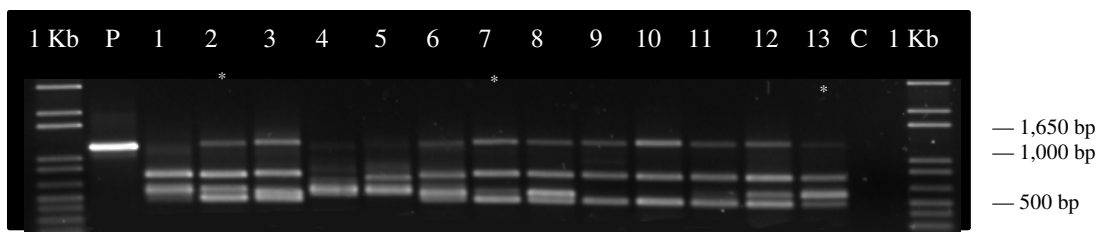


Figure 7.4. The PCR products obtained with primers fC and rD from nodules of plant inoculated with strain PhP 17. From left to right: 1Kb plus DNA Ladder™ (Invitrogen), Parent DNA (P), lanes 1 to 6 represent individual nodules of cv. Scylla and lanes 7 to 13 cv. T-49, C is a negative control, and 1Kb plus DNA Ladder™ (Invitrogen). (Banding patterns present: lane 10 = D, lane 5 = B, lane 4 = A, Lane 2 = C). **Asterisk** represents fC and rD amplimers selected as a representative of the nodule inhabitants

Figure 7.5 shows the PCR results from nodules of RCR 3644, where eight nodules were tested from the two bean cultivars. The parent strain (P) gave 3 distinct bands one of which (800 bp) was observed in the screened nodules. The remaining two bands were not observed in any of the screened nodules and were 600 and 400 bp long respectively. Lanes 1, 2 and 3 from cv Scylla gave 3 bands of 1,150, 800 and 500 bp and lanes 4, 5, 6, and 7 from DNA isolated from cv T-49 had a single band of 500 bp (Figure 7.5) plus a faint band at 800 bp and possibly another at 1,150 bp.

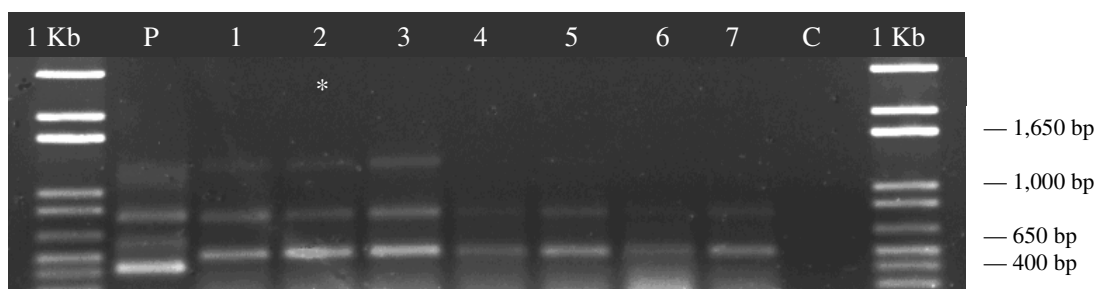


Figure 7.5. The PCR products obtained with primers fC and rD from nodules of plant inoculated with strain RCR 3644. From left to right: 1Kb Plus DNA Ladder™ (Invitrogen), Parent DNA (P), lanes 1 to 3 represent individual nodules of cv. Scylla, and lanes 4 to 7 represent individual nodules of cv. T-49, C is a negative control, and 1Kb Plus DNA Ladder™ (Invitrogen). (Banding patterns present: lane 3 = D, lane 7 = E). **Asterisk** represents fC and rD amplimers selected as a representative of the nodule inhabitants

The size and number of bands generated by bacteria isolated from nodules collected from plants inoculated with isolate PRF 81 are shown in (Figure 7.6). All of the nodules gave fragments dissimilar to that of the parent which was 1,150 bp long. Lanes 1, 2, 3, 4, 5, 8, 10 and 12 all produced 2 clear bands approximately 500 and 800 bp in length. Lane 9

gave a band of 1,000 bp and broad bands around 600 and 500 bp which were indistinguishable. Lanes 6, 7 and 11 had 2 bands of 800 and 600 bp.

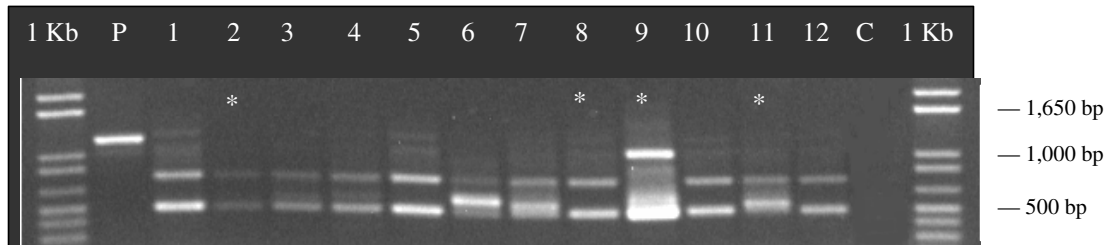


Figure 7.6. The PCR products obtained with primers fC and rD from nodules of plant inoculated with strain PRF 81. From left to right: 1Kb plus DNA Ladder™ (Invitrogen), Parent DNA (P), lanes 1 to 6 represent individual nodules of cv. Scylla and lanes 7 to 12 cv. T-49, C is a negative control, and 1Kb plus DNA Ladder™ (Invitrogen). (Banding patterns present: lane 1 = D, lanes 7 = C, lane 9 = Y(indistinguishable)). **Asterisk** represents fC and rD amplimers selected as a representative of the nodule inhabitants.

Amplification with fC and rD for nodules collected from plants inoculated with isolate UK 2 exhibited a variety of bands (Figure 7.7). The parent isolate (P), showed a band of 1,100 bp in length. Lanes 1, 4 and 10 exhibited 4 bands of 1,150, 850, 600 and 500 bp long. Lanes 2, 3, 5, 6, 7, 8, 9 and 12 showed 3 bands of 1,150, 800 and 500 bp. Lanes 11 and 13 showed three bands at 600, 800, and 1000 bp while lane 14 showed a single band of 500 bp (Figure 7.7).

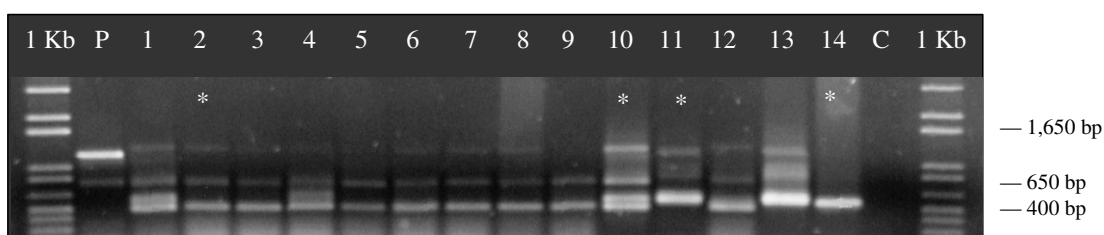


Figure 7.7. The PCR products obtained with primers fC and rD from nodules of plant inoculated with strain UK 2. From left to right: 1Kb Plus DNA Ladder™ (Invitrogen), (P) Parent DNA, lanes 1 to 6 represent individual nodules from cv. Scylla and lanes 7 to 14 cv. T-49, C is a negative control, and 1Kb Plus DNA Ladder™ (Invitrogen). (Banding patterns present: lane 1 = C, lane 2 = D, lane 11 = B and lanes 14 = A). **Asterisk** represents fC and rD amplimers selected as a representative of the nodule inhabitants

Nodules from uninoculated control plants also showed variable band patterns (Figure 7.8). There was a band of 650 bp in lane 1, while lanes 2, 3, 4, 5, 7, 11 and 12 all showed one band of 500 bp. There were two bands of 600 and 500 bp long in lanes 6, 8 and 9. Lane 10 had 3 bands of 800, 600 and 500 bp.

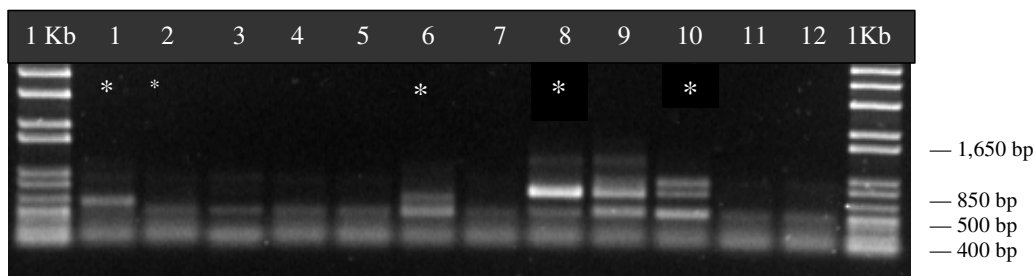


Figure 7.8. The PCR products obtained with primers fC and rD from nodules of the uninoculated control plants. From left to right: 1Kb Plus DNA Ladder™ (Invitrogen), lanes 1 to 6 represents individual nodules of cv. Scylla and lanes 7 to 12, cultivar T-49 and 1Kb Plus DNA Ladder™ (Invitrogen). (Banding patterns present: lanes 12 = E, lane 8 = B and lane 10 = likely to be C). **Asterisk** represents fC and rD amplimers selected as a representative of the nodule inhabitants

By comparing banding patterns obtained across gels, isolates and nodules, one indistinguishable and five main distinct patterns were observed (Table 7.4). A single strong band of 600 bp was designated A, compared to E which was a single weak band of 500 bp. Triple banding of 1,150, 850 and 500 bp was assigned D although the 1,150 bp

was sometimes weak or absent. Designation B had 2 bands at 600 and 800 bp and occasionally a third at 1,000 bp. Designation C had 4 bands at 500, 600, 800 and 1,150 bp. The indistinguishable pattern was assigned the letter Y.

Table 7.4. The main banding patterns generated using primers fC and rD from nodules collected from the 2004-05 field experiment.

Code	Banding patterns identified	Size(s) of ITS fragment(s) base pairs (bp)
A	1 strong single band	600
B	2 bands (sometime a third at 1000 bp)	600, 800
C	4 bands (double bottom weak top)	500, 600, 800, 1,150
D	Triple bands (sometimes 1,150 bp faint or absent)	500, 800, 1,150
E	1 band (weak single)	500
Y	indistinguishable	

7.3.2.1 Identification and confirmation of isolates and sequence analysis

To assign a species identity to the bacteria isolated from the nodules, the rDNA from the isolates needed to be sequenced. However, the fC/rD product, with its multiple bands, was unsuitable for DNA sequencing. Therefore, the isolates were amplified with primers F27 and R1494 which produced a single band of approximately 1,500 bp suitable for DNA sequencing. The band corresponded to most of the 16S rDNA region. As shown in (Figure 7.9) all isolates recovered from nodules and parental strains produced single bands with this primer pair. The amplimers produced using primers F27 and R1494 on nodules DNA and original parent isolates were indistinguishable from each other.

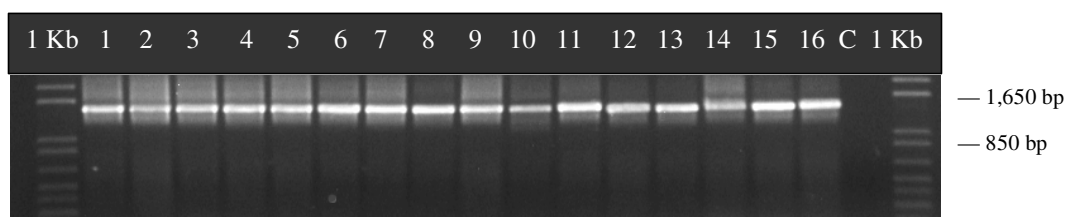


Figure 7.9. The PCR products obtained with primers F27 and R1494 in preparation for sequencing. From left to right 1Kb Plus DNA ladder™ (Invitrogen), 1 to 16 are representative nodules that had produced patterns A, B, C, D, and E with the fC and rD primers, C – control and another 1Kb Plus DNA Ladder™ (Invitrogen) patterns A (lane 11), B (lanes 1, 2, 7 and 8), D (lanes 3, 4, 5) E (lane 6 and 9) Y (lane 10). Lanes 12-16 was PCR product from the parent isolates UK 2, PRF 81, RCR 3644, H 20 and PhP 17 respectively.

The 16S rRNA sequences of the isolates purified from nodules of field grown beans consisted of approximately 500 bp and were generated using the sequencing primer F968. These sequences were representative of the banding patterns generated by primers fC and rD (Table 7.4). A search of the BLASTn function in GenBank (<http://www.ncbi.nlm.nih.gov/BLAST>) revealed species designations as shown in Table 7.5. The sequences from nodules of field grown beans did not match any *Rhizobium* sequence lodged in GenBank. Four of the selected isolates were between a 94 to 99 % match for *Pseudomonas* species (Table 7.5). Of the selected sequences submitted to GenBank, two did not match any of the sequences present. The identities of the parent strains sequenced with primers F27 and R1494 (Table 7.5) were similar to those obtained with primers fC and rD (Table 7.3). Isolates UK 2, H 20 and PhP 17 identities were consistent with those generated by sequencing of the amplicons generated by the fC and rD primers. No match was found for strain RCR 3644. The sequence of PRF 81 was identified as *Rhizobium hainanense* (97 %) using primers fC and rD (Table 7.3); while with primers F27 and R1494 (Table 7.5) was identified as *Agrobacterium rhizogenes*. The presence of species matches from organisms inhabiting the nodules that were not consistent with known nodulating species led to the authentication component of the study. This was undertaken to verify whether the recovered isolates would nodulate their original host after reinoculation.

Table 7.5. Identification of isolates recovered from the nodules of field grown beans and the parents strain amplified with primers F27 and R1494 obtained from Genbank search used in this study

Isolates selected from treatment	Sequence length providing GenBank match (bp)	Species	Identity (%)	E value
Control	511 (E)	<i>Pseudomonas chlororaphis</i> (AY509898*)	99	0.00
	511 (E)	Endophyte bacterium (AY842149*)	99	0.00
	511 (E)	Uncultured bacterium clone (AY838488)*	99	0.00
Control	95	-	-	-
PhP 17	546 (D)	<i>Pseudomonas</i> sp. (AY303253*)	94	0.00
	542 (D)	<i>Pseudomonas</i> sp. (AY331373*)	94	0.00
UK 2	439 (C)	<i>Pseudomonas</i> sp. (DQ219370*)	98	0.00
PRF 81	441 (B)	-	-	-
Parent strains				
RCR 3644	600	-	-	-
PhP 17	518	<i>Rhizobium leguminosarum</i> (U73208*)	99	0.00
PRF 81	506	<i>Agrobacterium rhizogenes</i> (AY626390*)	99	0.00
		<i>Rhizobium tropici</i> (AY117624*)	99	0.00
UK 2	523	<i>Agrobacterium tumefaciens</i> (AY626383*)	99	0.00
H 20	501	<i>Rhizobium tropici</i> (AY166841*)	99	0.00

(E, D, C, B) represented band pattern from Table 7.4.

* accession number in parenthesis,

The fC and rD amplimers produced by the sequenced isolates are marked on the respective Figures 7.3 - 7.8 on the gel with an * on the gel lanes.

7.3.3 Part 3

7.3.3.1 Authentication of nodule isolates

A pilot study was undertaken and the control failed to nodulate the beans (results not shown). Due to time constraints the study was not repeated.

7.4 Discussion

7.4.1 Summary of results

In this study, analysis by sequencing of the rDNA region of the bacteria from the nodules of field grown beans was done to identify their inhabitants. Nodule recovered isolates exhibited similar phenotypic qualities as the parent isolates when grown on bacteria specific agar plates. Increased numbers of nodules in treated plots implied a successful outcome to the inoculation process but PCR amplification and sequencing of the 16S rRNA of nodule derived DNA did not match that of the original inoculated isolates. The recovered nodule inhabitant was identified and a species assigned using BLASTn matches with the GenBank database. The results are in accordance with the assumption that *Phaseolus vulgaris* is a non selective host for nodulation and supports the suggestion of cohabitation during nodule initiation. Possible novel symbiotic associations were identified which require further evaluation.

7.4.2 Part 1

Bacterial growth obtained by culturing original isolates on Rhizobia Defined Media (RDM) (Appendix 2A) was consistent in appearance, except for isolates 3, 11 and 12 (Plate 7.1). These three isolates appeared dry and lacked the extracellular polysaccharide slime (Humphry *et al.*, 2001) of the other isolates. Variation in appearance is consistent with other studies that have cultured *Rhizobium* on RDM. For example, dry and wet colonies of *Rhizobium* grown on media were reported by Jain *et al.* (2002) working with five isolates of *Rhizobium ciceri*. All the isolates were screened for acid production. After monitoring for 4 d at 28 °C, the RDM media changed from blue green to yellow green. This colour change was recorded as positive for acid production. The colour change is characteristic of fast growing, acid producing *Rhizobium* (Martinez-Romero *et al.*, 1991; Young *et al.*, 2001; Hung *et al.*, 2005; Leblanc *et al.*, 2005), although it is not a universal or conclusive property as alkali-producing *Rhizobium* isolates (Hernandez and Focht, 1984) and acid-producing *Bradyrhizobium* isolates (Moreira *et al.*, 1993) have been reported. The rate of colony growth also confirmed them as fast-growing bacteria over 3-4 d of incubation (Young *et al.*, 2001) (data not shown).

The majority of the original isolates were from environments unlike that of Canterbury. Large fluctuations in day/night temperature and strong dry windy conditions,

as encountered in Canterbury during the recommended bean sowing season (McKenzie, 1989) are unlike conditions in Colombia where the strain CIAT 899 was isolated (Laguerre *et al.*, 1997). These strong dry winds and fluctuating day/night conditions, unlike those in Colombia, could have negatively affected the ability of CIAT 899 to adapt and multiply in Canterbury soils. However, other research has shown that this strain gives good nodulation in a number of bean growing areas with varying environmental conditions such as France, West Africa (Senegal and Gambia) and Brazil (Laguerre *et al.*, 1997; Diouf *et al.*, 2000; Mostasso *et al.*, 2002).

Strains H 20, H 12 and PRF 81 were isolated in the Cerrados region of Brazil which is characterised by environmentally inhospitable conditions such as long periods of water stress, high temperatures (> 40 °C), low pH (< 5.0), aluminium toxicity and low soil P levels (Hungria and Vargas, 2000). These isolates are currently used as commercial inoculants in Brazil (Hungria *et al.*, 2000). Strains UK 1, UK 2, UK 3 and UK 4 were obtained from England but were originally isolated from soils of the Jordan valley, Jordan (Tamimi, 2002). Their commercial potential as inoculants is currently being evaluated (Tamimi, 2002). Obtained from Australia, CC 551 and RCR 3644 are commercially available and have been field tested in Australia and in Canterbury, New Zealand (Dapaah *et al.*, 1995), with variable results ranging from sporadic to profuse nodulation in experimental sowings. The French derived isolates (Table 7.1) were from bean nodules in soils around Dijon, France. It has been reported that these isolates can nodulate beans in commercial production under varying environmental conditions (Laguerre *et al.*, 1993b; Laguerre *et al.*, 1994).

The literature shows that isolates from diverse climates can nodulate beans in countries other than those they originated from (Martinez-Romero *et al.*, 1991; Laguerre *et al.*, 1993b; Amarger *et al.*, 1994). For example, *Rhizobium tropici* is of tropical origin and was originally isolated from the bean growing area of the Americas (Martinez-Romero *et al.*, 1991), a centre of common bean diversity (Gepts and Debouck, 1991). The isolation and documentation of *Rhizobium tropici* present in the nodules of beans grown in France (Amarger *et al.*, 1994) supports the proposal that these novel strains, from different climates and environments, are capable of nodulating beans under the production and environmental conditions of Canterbury. The success of soybeans in Brazil was due to the successful introduction of legume inoculating rhizobia (Hungria and Bohrer, 2000). This

also indicates that foreign *Rhizobium* can nodulate legumes (beans) under soil and climatic conditions other than those in which they were isolated.

Following preliminary culturing, molecular methods were used to identify parent isolates to the species level. Primers fC and rD, described by Bala *et al.* (2002) were used to amplify DNA extracted from the parent isolates. Primers fC and rD were derived from 16S and 23S rRNA conserved sequences (Navarro *et al.*, 1992) and used in identification of the genetic diversity of rhizobia nodulating *Sesbania sesban* in African soils (Bala *et al.*, 2002). Bala *et al.* (2002) obtained good results with primers fC and rD in documenting rhizobia occurrence and diversity in African soils. They reported a variety of amplification results using these primers, including the generation of single and multiple bands. The size and number of fragments generated by Bala *et al.* (2002) varied within and between soil type and environment. The sizes of single bands were either 500 or 1,000 bp or multiple bands between these ranges. Fragment sizes varied from 1,100 to 500 bp (Bala *et al.*, 2002). This is consistent with the range of 1,200 and 400 bp obtained from the parent strains in this work. The species identified with these primers were distributed across all major phylogenetic branches and included *Rhizobium*, *Agrobacterium* and *Mesorhizobium* (Bala *et al.*, 2002). The correlation between the results in this study and those of Bala *et al.* (2002) and the broad application of these primers support the use of primers fC and rD in identification of bean nodule inhabitants.

In this work, a single amplicon was predominantly produced using primers fC and rD (Bala *et al.*, 2002) for the parent strains. However, parent strains PRF 81 and RCR 3644 gave multiple bands. A possible reason for the presence of multiple bands is that the colonies used for DNA extraction were not pure. However, this is unlikely, as repeated streaking of each isolate on growing media (agar) ensured that only a single (pure) colony was used for genomic DNA extraction. In addition, as stated by Bala *et al.* (2002), these primers are capable of amplifying a wide variety of bacterial taxa (for example *Agrobacterium*, *Pseudomonas*, *Mycobacterium*, *Arthrobacter*) (Weisburg *et al.*, 1991) often giving quite variable banding patterns as seen in this study. This facilitated their use here to detect the rhizobia recovered from nodules of field grown common bean in Canterbury, as they can also amplify a variety of endemic strains and species.

Identification of these isolates by GenBank comparison with rDNA regions confirmed the isolates as either *Rhizobium tropici*, *R. leguminosarum* bv. *phaseoli* or

trifolii, *R. rhizogenes*, *R. hainanense* or *Agrobacterium tumefaciens* (Table 7.3). Only strain H 441 (99 %) and CIAT 899 (99 %) matched the identification sent by the supplier which was *Rhizobium leguminosarum* bv. *phaseoli* and *Rhizobium tropici* respectively. The 93 % match of CARA 72 as *Rhizobium rhizogenes* and the 97 % match of PRF 81 as *Rhizobium hainanense*, both supplied as *Rhizobium tropici* confirms the taxonomic closeness of these organisms (Stackebrandt and Goebel, 1994; Cummings *et al.*, 2001). The deposition of *Rhizobium tropici* in GenBank as a nodulating isolate for bean has been documented (accession number U89832 *R. tropici* IIB and X76233.1 *R. tropici* IIA) (Chueire *et al.*, 2003). *Rhizobium hainanense* is documented as a species of root nodulating bacteria of prairie legumes (Graham, 2005). Based on fatty acid analysis and phylogenetic data, there is great similarity among *Rhizobium hainanense*, *Rhizobium tropici* and *Agrobacterium* biovar 2 (Tighe *et al.*, 2000).

Twenty-five percent of the original isolates were closely affiliated to the genus *Agrobacterium*. Strain CARA 56 was supplied as *Rhizobium tropici* but sequenced and identified (99 %) as *Agrobacterium tumefaciens*. *Agrobacterium* were not morphologically distinguishable in this study. Growth of the isolates on RDM was similar and differences could not be visually distinguished. Similar colony morphology, growth in different media and ribosomal sequences have been reported for *Rhizobium* (Martinez-Romero, 1994; Mhamdi *et al.*, 1999). The exact position of the genus *Agrobacterium* on the phylogenetic tree of *Rhizobium* is yet to be confirmed by means of full 16S rRNA sequencing and DNA-DNA homologies (Graham *et al.*, 1991; Stackebrandt and Goebel, 1994). The similarity between rhizobia and agrobacteria has long been known (Young *et al.*, 2001; Bala *et al.*, 2002; Young *et al.*, 2003) and the ability of *Agrobacterium* to form nodules and fix nitrogen has occurred in *Agrobacterium* mutants which were supplied with the symbiotic rhizobia plasmids (Martinez *et al.*, 1987; Brom *et al.*, 1988). Additionally, both pathogenic and nodulating bacteria belonging to the genera *Agrobacterium*, *Rhizobium* and *Sinorhizobium* are reported as being interspersed on sub-branches of 16S or 23S rDNA phylogenetic trees (de Lajudie *et al.*, 1998; Terework *et al.*, 1998).

7.4.3 Part 2

Field grown common bean inoculated with competitive and effective *Rhizobium* isolates could be expected to have these isolates in their nodules, particularly when the beans are sown in fields that are low in native or indigenous *Rhizobium* or in fields that

have not been sown with beans for a number of years, as was the case in this study. Growth of recovered, nodule-derived isolates on RDM was similar to the original parent isolates. Individual colonies were clear or white in colour, and were round shaped with smooth edges (Leblanc *et al.*, 2005). The consistency and robustness of these organisms was noted after repeated streaking to obtain single colonies for DNA isolation. This characteristic alone was not a clear criterion to identify them as the original isolates which had been used in the inocula but gave reason to assume that the cultures were strains of rhizobia-type bacteria (Somasegaran and Hoben, 1994).

Rhizobium species appear visually similar when grown on isolation plates, and only differed in their generation time (3-5 d fast growers and 7-10 d slow growers) (Vincent, 1970). In addition, most soils in which beans are grown are known to contain nodule forming bacteria (Graham, 1981) which are very competitive and can outcompete the inoculant strain during nodulation (van Rhijn and Vanderleyden, 1995; Vlassak and Vanderleyden, 1997; Graham *et al.*, 2003). Thus, although the isolates recovered from field grown nodules exhibited similar characteristics on RDM as the original parent strains, certification of the isolate derived from the nodule of the field grown beans could not be guaranteed (Graham, 1981). This led to DNA extraction from these isolates (Section 7.2.2.1), and the use of molecular methods to identify them.

The extraction of DNA from nodules of field grown beans by the Chelex method was successful as with the parent isolates. However, amplification with the fC and rD primers gave unexpected banding patterns that were different from the inoculated strain. Thus, the PureGene method was used to ensure the DNA was of high quality and gave sufficient DNA for multiple PCR reactions. When the PureGene DNA was amplified with primers fC and rD the band pattern still did not match that of the parental isolate (Figure 7.2 to Figure 7.6). Of all the bacteria extracted from the 88 nodules tested in this experiment an amplicon of a similar size to the parent strains was never observed.

Using primers fC and rD to amplify the genomic DNA extracted from the field grown nodules gave multiple bands when visualised on 1 % agarose gel (Figure 7.3) through (Figure 7.8). Multiple bands of varying sizes were also reported for single isolates by Bala *et al.* (2002) from legume nodulating rhizobia in African soils. The isolates were identified as either *Agrobacterium tumefaciens* and/or *Rhizobium* spp. based on 16S rRNA sequence similarity of 750 bp (Bala *et al.*, 2002). However, multiple bands on a gel could

also be an indication of multiple species and/or strains. However, as in the previous experiment, the use of repeated colony streaking and single colony selection was used to avoid this possibility.

The banding patterns were variable in both fragment size and intensity (Figure 7.3) to (Figure 7.8). A comparative visual analysis of the gels, summarised in (Table 7.4) gives the five main banding patterns from field recovered isolates when amplified with primers fC and rD. The difference in band intensity in bands from the field grown nodules on the gels (Figure 7.3) to (Figure 7.8) could be attributed to subtle variation in the purity of the DNA used for amplification. However, all cultures were extracted using the PureGene kit and diluted to $10 \text{ ng } \mu\text{l}^{-1}$ prior to adding to the PCR reaction. It is possible, that inhibitors were co-purified and that this altered the reaction kinetics in some instances, leading to faint or absent bands.

Five banding patterns were obtained in this study. This wide range of patterns from two cultivars confirms that common bean can be a source of large rhizobial diversity and is consistent with the suggestion that common bean is a promiscuous host (Graham, 1981; Hungria *et al.*, 2000). Several reports of cultivar by isolate interaction are reported in the literature (Chaverra and Graham, 1992; Rodriguez-Navarro *et al.*, 1999; Santalla *et al.*, 2001a). The predominant pattern seen in both bean cultivars was D (Table 7.4). Patterns A, B, C, D, and E were well represented in cv. Scylla. Pattern A was not seen in cv. T-49 but B, C, D and E were, as was pattern Y, which was not found in Scylla. It is surprising that only three band patterns were observed from control plots. If the nodules occupants were all indigenous to the growing areas, it could be expected that all five band patterns would be found in control plots, but this was not the case. However, band pattern E was common to both cultivars in control plots. It is possible that some of the isolates recovered, preferentially co-inhabited with the parent strains. Bacteria species other than rhizobia are known to inhabit legumes nodules (Sturz *et al.*, 1997) and many studies have shown that this is due to simultaneous infection (Grimes and Mount, 1984; Bolton *et al.*, 1990; Misko and Germida, 2002). Further evidence of cohabitation in nodules with rhizobia was found in rescue studies where different bacterial (rhizobial) progeny was found in the same nodule, even in the infection thread of legumes inoculated with a specific nodulating bacteria (Stuurman *et al.*, 2000).

Subsequent amplification of the genomic DNA with universal bacterial primers F27 and R1494 gave a single PCR product (Figure 7.9) and DNA sequencing of this PCR product gave one sequence, confirming that a single bacterial species had been cultured for genomic DNA extraction. Primers F27 and R1494 were designed to amplify the 16S rRNA region and did not encompass the highly variable ITS regions. The 16S rDNA region was used because of its high information content, conserved nature and universal distribution (Lane *et al.*, 1985). It is a simple, rapid method of identification (Laguerre *et al.*, 1994; Laguerre *et al.*, 1997) which is commonly used to examine phylogeny and in grouping of new isolates in rhizobial studies (Haukka *et al.*, 1996; Tan *et al.*, 1999). The 16S rRNA sequence analysis has also been useful in demonstrating rhizobial diversity (Haukka *et al.*, 1996; Moreira *et al.*, 1998; McInroy *et al.*, 1999). The 16S rRNA sequence can be readily compared to corresponding sequences from other isolates. However, resolving power between closely related species and strains is limited. This makes it the best tool for determining which genus an isolate belongs to and the most likely species to which it is closely related (Cummings *et al.*, 2001). No gene has shown such broad applicability over all taxonomic groups, as the 16S rRNA gene. It is an ideal candidate to identify unknown organisms where there is no prior knowledge (Jensen *et al.*, 1993; Gurtler and Stanisich, 1996; Clarridge III, 2004).

World bacterial diversity is generally unknown, because so little of the diversity has been identified. It is estimated that there are 10^9 bacterial species (Dykhuisen, 1998). The International Committee on Systematic Bacteriology currently recognises 5,236 bacterial species, each of which has been cultured and characterised (Cohan, 2001; Topp, 2003). The estimation of bacterial diversity is limited to culturable species known to microbiologists (Dykhuisen, 1998). The nodulation of plants in control plots implies the presence of endemic nodule inhabiting species. Nodules formed on control plants were not as large as those on inoculated plants and lacked the intense pink colour seen in nodules from the inoculated treatments. Over the duration of the experiment period, control plants were not as well nodulated as inoculated plants. At 70 DAS, when nodules were collected for analysis, control plants had averaged 2 nodules plant⁻¹ compared with the best nodulated treatment which had approximately 75 nodules plant⁻¹. These results confirm the effectiveness of the inoculated isolates in inducing nodulation although this result was not confirmed by molecular analysis. However, as only 2 % of all nodules produced were evaluated this could have affected the outcome.

Adequate sterilisation is necessary to ensure that organisms recovered after plating from field grown nodules are those from inside the nodules. Surface sterilisation is one of the most frequent and easily applied methods of sterilisation reported in the literature (Vincent, 1970; Somasegaran and Hoben, 1994; Hungria *et al.*, 2003; Singh *et al.*, 2006). Surface sterilisation of the nodules with a 50:50 solution of 6 % hydrogen peroxide and 95 % ethyl alcohol (modified from (Vincent, 1970)) as used in this experiment should have eliminated any contaminants from the outer surface of the nodules before culturing and isolation. A higher concentration (6 %) of hydrogen peroxide was used compared to the recommended 3-5 % (Vincent, 1970) and immersion time was reduced from 3-4 to 1-2 minutes. This immersion period was thought to be adequate to surface sterilise nodules, eliminating contaminating organisms. Thus isolates recovered on the agar were more than likely endophytic in the nodules. However, contamination cannot be completely excluded.

Biological nitrogen fixation is only one of outcomes of the soil organisms in the *Rhizobium*/legume symbiosis. The diversity of organisms present in agricultural soils (Laguerre *et al.*, 1993b; Martinez-Romero, 2003) warrants that organisms isolated from the nodules of field grown legumes should be checked to verify their identity. Numerous bacterial species are known to inhabit nodules of nodulated crops. A recent review on bacterial endophytes of agricultural crops identified *Pseudomonas* as one of the most commonly isolated bacterial genera from various plant parts (Hallmann *et al.*, 1997). The isolates recovered from the common bean nodules in this experiment were identified predominantly as *Pseudomonas*. Various other organisms; *Agrobacterium*, *Azospirillum*, *Azobacter* and *Bacillus* have been found associated with rhizobia in legume nodules and have the ability to stimulate root growth and nodulation (Mhamdi *et al.*, 2005). Evidence of the importance of such beneficial bacteria in the colonisation of root nodules to enhance both nodule growth and bacteroid differentiation was recently documented for *Streptomyces lydicus* (Tokala *et al.*, 2002).

Root nodulating bacteria are tentatively grouped according to their rate of growth on a specific agar (Graham *et al.*, 1991; Somasegaran and Hoben, 1994), *Rhizobium* spp. include very fast, fast and intermediate (acid-producing) types; and *Bradyrhizobium* spp. include the very slow, slow and intermediate (alkali-producing) types (Jordan, 1984; Odee *et al.*, 1997). It is possible that among root inhabiting bacteria, one species could out-compete another on agar, allowing the faster growing isolate to mask the presence of the

inoculated parent strain in the isolation process. Dual occupants in nodules of trap plants *Acacia* sp., *Sesbania sesban*, *Phaseolus vulgaris* and *Vigna unguiculata* are known to occur (Odee *et al.*, 2002). In woody legumes, double occupancy accounted for 6.6 % of the isolates recovered from nodules. This was possibly an underestimate due to the possible masking effect of fast growing over slow growing types.

Pseudomonads are among the most abundant culturable, rhizosphere organisms, and can utilise a wide range of low molecular mass and complex compounds as energy sources (Misko and Germida, 2002). Pseudomonads, as identified in the recovered field nodules, are generally quick to adapt to nutrient availability in their environment (Misko and Germida, 2002) and this could account for their presence in the nodules. Accordingly, with the readily and easily available carbon source (glucose) in the inoculation broth and the RDM, it is expected that the pseudomonads could easily dominate the rhizobia isolate in the inoculant in the field. The inoculant used was prepared with TY broth which presented a ready source of nutrients (glucose) for the inoculant strain as well as for the indigenous population. This could have induced an explosion in growth of the local population at inoculation when the inoculant isolate would be adapting to the new soil environment. An increased population of indigenous isolates in the region of seed germination would cause great competition for nodulating sites (Graham, 1981) which could account for these strains being preferentially present in these experiments. The results suggest the need for continued evaluation and further examination of the isolates collected in these experiments.

The nodules evaluated were chosen as a representative sample of the nodules produced. Of approximately 5,000 nodules collected at 70 DAS only 88 were evaluated for the presence of the nodulating organism. It is possible that the nodules evaluated were not truly representative of the nodulating organisms. Evaluation of all the nodules produced would have been necessary to give a complete indication of the nodule inhabitants. However, this would have been expensive, time consuming and impossible with the available resources. The presence of other bacteria in nodules could be an indication of the competitive ability of the indigenous soil bacterial population to out-compete the inoculant isolates in this experiment. The ability of indigenous populations to out-compete inoculant isolates has been frequently reported (Graham, 1981; Peoples *et al.*, 1995c; Sadowsky and Graham, 1999; Sessitsch *et al.*, 2002; Graham *et al.*, 2003).

This study confirms the promiscuous ability of *Phaseolus vulgaris* (Graham, 1981; Michiels *et al.*, 1998; Franco *et al.*, 2001). These findings are supported by the presence of organisms capable of nodulating common beans in some fields of the Lincoln University, Horticultural Research Unit (HRU) represented by plants nodulating in control plots. This is supported by the work of Dapaah (1995), working with pinto beans (also *Phaseolus vulgaris*) in the same environment, where plants from both control and inoculated plants were well nodulated. The possibility of nodulation due to the presence of *Rhizobium* on the seed cannot be ruled out, as seed used in the field trial was not surface sterilised. To explain the incidences of nodulation without inoculation, Diouf *et al.* (2000) working in West Africa, and Grange and Hungria (2004) in Brazil, proposed the seed as a carrier of high numbers of bacteria, including viable rhizobia cells, capable of effecting nodulation. To demonstrate the natural occurrence of *Rhizobium* on *Phaseolus vulgaris* seed and explain the worldwide dissemination of *Rhizobium* organisms, Perez-Ramirez *et al.* (1998) recovered rhizobia from common bean seed and subsequently obtained nodulation after re-inoculation of bean plants.

This work attempted to identify the nodulating agent after inoculant was applied to field grown beans using molecular technology. The use of 16S rRNA sequencing clearly showed its importance as an evaluation tool in bacterial identification as the technique clearly identified the parental strains (Table 7.1) as *Rhizobium* and identified the recovered species in root nodules of field grown beans as bacteria (Table 7.5). Most of the isolates were identified as *Pseudomonas* spp. (66 %), 16.7 % were an endophytic bacterium and an uncultured bacterium respectively. Recent reports have shown that rhizosphere endophytic bacteria (e.g. *Pseudomonas*) can favourably affect plant growth and yield in commercially grown crops (Cocking, 2003). Possible explanations for the apparent inconsistency between the inoculated and recovered isolates include: masking of the inoculant strain by the identified *Pseudomonas* spp., possible inability of the inoculant strain to adapt and colonise the rhizosphere thus decreasing nodulation efficacy, and inadequate surface sterilisation of the nodules, resulting in an explosion in the growth of *Pseudomonas* spp. on the agar plates. These findings need further exploration and given the importance of the legume/*Rhizobium* symbiosis to the New Zealand pastoral landscape this highlights the need for resources to be channelled to research into the identification of organisms associated with legumes in New Zealand soils.

7.4.4 Part 3

Given that the bacterial isolates recovered from common bean nodules were neither the parent strains nor any other recognised nodulating species, an attempt was made to confirm whether the newly identified isolates were capable of inducing nodulation. The use of an *in vitro* plant nodulation test (Somasegaran and Hoben, 1985) (Section 7.3.3.1) to assess the capability of bacterial isolates to nodulate the bean cultivars Scylla and T-49 in the laboratory was unsuccessful. The control used in the plant nodulation test, strain RCR 3644, which is the recommended strain for common beans in New Zealand, gave sporadic nodulation in this study. Strain RCR 3644 gave good nodulation in a greenhouse but attempts to recover the isolate from nodules of field grown beans were unsuccessful. The lack of nodulation in the control (RCR 3644) resulted in a nodulation failure in the *in vitro* plant nodulation assay, which could not be repeated due to time constraints.

The failure of the parent isolates to nodulate their original host cultivars in the laboratory could be due to a loss of the symbiotic gene during storage and subculturing (Mhamdi *et al.*, 1999; Mhamdi *et al.*, 2002). Alternatively, it may have been due to difficulties with the experimental set up. Nodulation (plant) tests are widely reported in the literature for the classification of rhizobia as a function of their symbiotic properties (Vincent, 1970; Somasegaran and Hoben, 1994). Plant tests are time and space consuming and can only provide a partial characterisation of the host spectrum (Lortet *et al.*, 1996). It is therefore possible that the result of the nodulation test was due to overall constraints in the procedure.

The loss of nodulating ability in *Rhizobium* during culturing and storage has frequently been reported in the literature. Originally, symbiotic bacteria were reported to lose their symbiotic gene in laboratory experiments (Laguerre *et al.*, 1993a). Loss of infectivity in rhizobial strains during storage has also been reported (Sutherland *et al.*, 2000). A wide diversity of non-symbiotic bacteria have been reported in soils (Segovia *et al.*, 1991) with the potential to be mixed, in association with symbiotic bacteria, in legume nodules (de Lajudie *et al.*, 1999). It is possible that during initial culturing of the field nodulated isolates, other organisms in the nodules suppressed growth of the inoculated isolate and grew faster on the media used. A similar reason was put forward by Frohlich and Konig (1999) who worked on the rapid isolation of single microbial cells from mixed natural and laboratory populations. They proposed the use of a micromanipulator to

prevent culturing mixed populations and to promote isolation of pure single colonies in microbial studies.

Previous work on identification of *Rhizobium* isolates was based on phenotypic features covering utilisation of sole carbon and nitrogen sources, pH ranges for growth rates (Selenska-Pobell, 1994; Somasegaran and Hoben, 1994) and some biochemical methods (Cummings *et al.*, 2001). Since the work of Woese (1987) a number of authors have reported the use of molecular biology in legumes for identification of nodule occupying organisms (Wilson, 1995; Gurtler and Stanisich, 1996; Haukka *et al.*, 1996; Aguilar *et al.*, 1998; Young *et al.*, 2004). In these studies the use of molecular analysis in beans resulted in the recovery of isolates other than those used in the inoculant. It must be noted here that as only 2 % of nodules were tested for the nodulating organism, and the selected nodules were usually the largest nodules, these factors combined may have biased the sampling protocol. Literature on the use of molecular analysis in bean trials in France (Laguerre *et al.*, 1993b; Amarger *et al.*, 1994; Amarger *et al.*, 1997; Laguerre *et al.*, 2001) and Brazil (Hungria *et al.*, 1999; Hungria *et al.*, 2000; Chueire *et al.*, 2003; Cloud *et al.*, 2004; Grange and Hungria, 2004) resulted in the recovery of the inoculated organism from the nodules which contrasts with the results of this study. This work appears to be the only documented use of molecular analysis for the identification of bean nodulating organisms in New Zealand. The approach offers great potential for the identification and documentation of legume nodulating bacteria not only in crop plants but also in pasture and native legumes which are a common feature of the New Zealand landscape.

7.5 Conclusions

1. Polymerase chain reaction and sequencing of the 16S rRNA is a quick, reliable means of identifying *Rhizobium* bacteria and should be an integral part of future agronomic work into the legume/*Rhizobium* symbiosis.
2. Fields need to be surveyed for bean nodulating organisms and a collection of these organisms should be kept for further evaluation. This information could be used as an indicator as to which fields are most likely to yield successful results from *Rhizobium* inoculation.
3. The competitive ability of inoculant strains needs to be evaluated in the local environment to ensure their effectiveness in nodulation before being recommended for wide scale commercial use.
4. Although contamination is possible, these results suggest that it is possible that organisms other than *Rhizobium* species may be found in common bean nodules.

CHAPTER 8

General Discussion

8.1.1 Inoculation and nodulation

Field inoculation with selected *Rhizobium* species is a world wide, common agricultural practice, used to improve the yield of legume crops based on the ability of the bacteria species to infect the legume root system eliciting nitrogen-fixing nodules (Burgos *et al.*, 1999). When legumes are inoculated, the norm is to apply the appropriate rhizobia at seeding. This practice presents some challenging, and often insurmountable problems (Graham, 1981; Sadowsky and Graham, 1999). Faced with a new and potentially hostile environment, the applied inoculant often fails to increase plant productivity (Buttery *et al.*, 1997; Catroux *et al.*, 2001; Deaker *et al.*, 2004). The behaviour of the inoculant strain depends on the number of nodules formed, their effectiveness in N fixation, and their ability to survive in the root ecosystem (Kyei-Boahen *et al.*, 2002; Kyei-Boahen *et al.*, 2005).

Exploration of the N fixing potential of common bean is hampered by the unreliability of the association with its symbiont, *Rhizobium* spp (Hungria *et al.*, 2000; Sessitsch *et al.*, 2002). Common bean productivity is markedly affected by environmental factors such as soil acidity, extreme temperatures, water availability and chemical components of the soil especially combined N (Popescu, 1998; Hungria and Vargas, 2000; Graham *et al.*, 2003). These symbionts may contribute to the N economy of the crop, thus reducing the requirements for fertiliser N and would be especially important in organic agriculture and for small-scale farmers who cannot afford expensive fertiliser applications.

There is no reliable estimate of the area of pulse crops in New Zealand that are inoculated or are affected annually by inoculation failure, but when it occurs it can be financially harmful for individual producers. Environmental conditions and management practices influence the frequency of inoculation failure (Deaker *et al.*, 2004). The primary environmental factor in inoculation failure is dry seed bed conditions (Hungria and Vargas, 2000; McConnell *et al.*, 2002; Hungria *et al.*, 2003). This is a common occurrence on the Canterbury plains.

Inoculation response over the two seasons evaluated was variable. A lack of response (no nodulation) as experienced in the first season is regularly reported (Ramos and Boddey, 1987; Hungria *et al.*, 1999). Dry windy conditions as experienced during sowing could have produced warm, dry soil that desiccated the inoculant bacteria reducing their viability and ability to survive until the seed germinated to effect nodulation. Nodulation failure due to the desiccation of inoculant bacteria, before root infection, has been reported (Chatel and Parker, 1973b, a; Evans *et al.*, 1980; Kremer and Peterson, 1982). The legume rhizosphere is a complex environment where individual rhizobial strains must compete with one another to survive and successfully nodulate the legume root system (Stephens and Rask, 2000). Faced with a new and potentially hostile environment, applied inoculants often fail to effect nodulation. As nodulation was not seen, and most fields reportedly contain indigenous rhizobia it is proposed that the field was devoid of rhizobia capable of nodulating common bean or that the inoculant bacteria used were dead or died before nodulation was achieved.

In contrast, in the second field experiment, effective nodulation was observed, even on uninoculated plants. Nodulation of uninoculated plants suggest the presence of indigenous nodulating organisms. Many indigenous organisms are considered inefficient N fixers but can out compete the inoculant strain selected for enhanced N fixing potential (Thies *et al.*, 1991; Triplett and Sadowsky, 1992). Inoculation rates of more than 1,000 times the estimated number of indigenous rhizobia have been reported needed to achieve more than 50 % nodule occupancy by the inoculant strain (Thies *et al.*, 1991). However, this did not guarantee yield improvement. This inherent competitive ability among bacteria for inoculation sites has been attributed to anti-rhizobial compounds which present another challenge for researchers working on the *Rhizobium*-legume symbiosis (Twelker *et al.*, 1999). The competitive ability of indigenous over inoculant strains has caused some authors to advocate mixed inoculant strain formulations (Sutherland *et al.*, 2000; Kyei-Boahen *et al.*, 2005).

In some countries, for example Western Canada (Kyei-Boahen *et al.*, 2005) and the United States (Graham, 2005), only a single inoculant is used, while in Brazil (Hungria *et al.*, 2000), it is more common to inoculate with a mixture of several strains. Few studies have compared the benefits and disadvantages of these two approaches. However, Sutherland *et al.* (2000) suggested that inoculation with a single strain could be risky

because the ability of a particular strain to establish an effective symbiosis varies within a wide range of environmental factors and, as a result, one strain may not be effective under all environmental conditions. Mixed strain inoculants however, could contain bacteriocins and antibiotic-producing strains that could suppress the growth of sensitive strains in the mixture or in the plant rhizosphere (Schwinghamer and Brockwell, 1978). Under favourable growing conditions no apparent advantage was found when using either single or multi-strain inoculants but under adverse conditions multi-strain formulations may reduce the risk of inoculation failure (Kyei-Boahen *et al.*, 2005).

More than a century after its discovery, the enhancement of biological N fixation capacity of legumes is still a major agronomic goal (Vasquez-Arroyo *et al.*, 1998). It is essential that more efficient strains are isolated under field conditions where beans are grown. It is well known that the strains selected as good inoculants in greenhouse experiments do not always behave in the same way after release into the field (Vlassak and Vanderleyden, 1997). This point was reinforced by the results of the greenhouse trial where all the isolates tested nodulated cv. Scylla, confirming the nodulating ability of the strains.

Numerous *Rhizobium* strains, capable of high N fixation with their target host, have been identified (Odee *et al.*, 2002; Howieson and Ballard, 2004). In spite of this, attempts to increase legume yield in agricultural fields by inoculation with these superior strains have often failed (Brockwell and Bottomley, 1995; Brockwell *et al.*, 1995). This may be due, in part, to inappropriate inoculation technology (Date, 2000; Lupwayi *et al.*, 2000), but it is often caused by the inability of the inoculant strain to compete with indigenous rhizobia for nodule formation on the host plant. The need to adapt to the soil habitat, compete with indigenous populations of rhizobia to colonise the roots, to form nodules, and to avoid the negative effect of native organisms, affects survival of applied inoculants (Vlassak and Vanderleyden, 1997).

This work showed that the formulation and delivery system could have affected the nodulation outcome. Formulation inadequacies are often the most common barriers to the commercialisation of legume inoculants (Smith, 1992; Stephens and Rask, 2000). Under laboratory conditions, micro-organisms may function optimally but formulating that organism for the end user to ensure equivalent results in the field is difficult (Stephens and Rask, 2000). The inoculant formulation and delivery system should allow for a high

numbers of viable cells to be applied to the seed or soil, as this is the most vulnerable phase of the transfer (Date, 2001).

Liquid inoculation, as used in the second experiment, supplied bacteria which were actively growing which could have reduced the transfer shock of the bacteria when compared to the first experiment, when a peat-based inoculant was used. The survival ability of rhizobia on seed was shown to be affected by carrier type and exposure to hot dry conditions (Kremer and Peterson, 1982). Liquid inoculants have been shown to give equivalent results to peat-based inoculants, are easier to handle, but have limited shelf life. Cool temperature storage for the inoculants increase handling and end user costs (Hynes *et al.*, 1995; Stephens and Rask, 2000). In spite of the environmental and management factors which are reported to affect and contribute to inoculation failure (Graham, 1981; Vlassak and Vanderleyden, 1997; Hungria and Vargas, 2000), too often human error contributes to inoculation failure through improper storage or application of the inoculant, mechanical failure of inoculant delivery, or the inadvertent selection of the wrong rhizobial strains (McConnell *et al.*, 2002).

8.1.2 Growth, TDM and Seed yield

Several studies have shown variable ranges in nodulation (Moawad and Abd El-Rahim, 2002; Rasposeiras *et al.*, 2006), growth (Deibert and Utter, 2002; Thomas *et al.*, 2004) and yield (Redden and Herridge, 1999; Hungria *et al.*, 2003) with legume inoculation. The evaluation of 16 bacterial isolates in the greenhouse support the finding of the above authors. In the greenhouse study, cv. Scylla gave positive and variable responses to inoculation with the different isolates under sterile growing conditions. Good nodulation was a precursor to high plant dry weight (Table 3.10) and (Table 3.11). Variation in plant growth among plants in a treatment was high. Shoot dry weight response was small, but significant; indicating that enhanced plant growth was due to the nodulation effect of the isolates. The response to inoculation shows the presence of a strain by cultivar association, as reported by Moawad and Abd El-Rahim (2002); Moawad *et al.* (2004) and Kyei-Boahen *et al.* (2005).

In the greenhouse, there was a strong relationship between shoot growth and pod yield plant⁻¹. Inoculation and N, applied individually, increased SDM and pod yield plant⁻¹. In the field in 2004-05, green pod yield of inoculated plants was significantly ($p < 0.05$)

higher than in the uninoculated treatment but SDW was not affected by inoculation. Introducing the strain/cultivar association to the field presented another variable to the relationship. The soil environment is known to influence the strain/legume association (Graham, 1981; Hungria and Vargas, 2000; Moawad and Abd El-Rahim, 2002). This probably accounted for the variable response to inoculation in the field in the second experiment. Evaluation of the legume/*Rhizobium* symbiosis under sterile conditions is a valuable first step in the selection of effective legume nodulating bacteria for field evaluation. However, evaluation under sterile conditions is only a guide to selection, as field evaluation introduces variables (such as high available soil N, low available P, soil salinity or micro nutrients deficiency) which affect both macro and micro symbiont and hence nodulation, N₂ fixation and legume yield (Graham, 1981; Hungria and Vargas, 2000; Moawad *et al.*, 2004).

Common bean is a native to the semitropical regions of Central America (Kemp, 1973) and requires moderately high temperatures for optimum germination and growth (Austin and Maclean, 1972a). The growth of beans is severely limited by temperatures below 15 °C (Kemp, 1973). It is proposed that low temperature is a major factor limiting common bean production in temperate zones. In these experiments, temperatures below a base temperature of 10 °C affected stages in the growth of the bean crop. The effect of low temperatures in both seasons, including a frost in 2003-04, affected the relationship between total dry matter (TDM) accumulation and cumulative intercepted PAR (Figure 5.8 and Figure 5.9). The temperatures leading up to the period when TDM showed a minimal decrease (50 DAS) were on average between 3 and 10 °C, which is well below the 20 °C required for good bean growth (Dale, 1964; Kemp, 1973; Farlow, 1981).

These low temperatures could have affected bean plant growth rate by damaging or destroying the terminal growing points and affecting leaf area and thus partitioning of dry matter (DM) between leaves and the rest of the plant (Kemp, 1973). The effect of temperature in beans was found to be related to cultivar, and where it was developed (Kemp, 1973). Working with four bean cultivars, developed under different growing conditions, Kemp (1973) found that the cultivar that was developed under the moderate temperature conditions of Idaho appeared to be less tolerant of low temperatures than those developed under the cooler continental conditions of the Canadian prairies. The cultivar developed under the more maritime conditions of Scotland appeared to be intermediate in

its tolerance to temperature extremes, while a cultivar developed in Western Europe was highly tolerant of low temperatures. The results of the experiment show that cultivars selection is an important management decision when deciding to grow common beans in Canterbury.

In Canterbury, with its narrow window for production of common bean, matching the crop life cycle to the length of the growing season is an important management decision. This is probably one of the most important factors affecting the partitioning of biomass and the balance between vegetative and reproductive growth and is seen as a critical aspect determining HI and seed yield (Richards, 1991; Loss and Siddique, 1994). The results of this study showed that cultivar, irrigation and inoculation positively affected TDM. The effect of cultivar on DM production of *Phaseolus vulgaris* was reported by Deibert and Utter (2002); Moawad and Abd El-Rahim (2002) and Moawad *et al.* (2004). The accumulation of more DM in later maturing bean cultivars was attributed to their interception of more photosynthetically active radiation as a result of the longer growth period (San Jose *et al.*, 2004). In addition, environmental factors such as frost (as discussed earlier) can have a negative effect on DM accumulation and hence yield as seen in these experiments.

Cultivar and fertiliser affected maximum TDM (2003-04), which resulted from average growth rates of 7.06, 4.84, 6.76, 5.82 g m⁻² of TDM for Scylla, T-49, applied N and no applied N respectively. The greater DM production of Scylla, in both seasons, was associated with increased intercepted PAR (Table 5.2). In 2004-05, low air temperatures experienced early in the growing season resulted in plants accumulating an average of 0.24 g m⁻² MJ⁻¹, which increased to 2.26 g m⁻² MJ⁻¹ over the second part of the growing period when the temperature increased above the base temperature of 10 °C.

Dry matter accumulation between two phenological stages is characterised by the growth rate (Cantarero *et al.*, 1999). The growth rate results from the amount of daily incident radiation that is intercepted by the canopy and the efficiency of the crop to convert it into DM, as well as the length of the phase (Cantarero *et al.*, 1999). In addition, night temperatures, as seen in these experiments, also affect crop development rate (Bonhomme *et al.*, 1994). The higher DM production in Scylla was associated with increased intercepted PAR but a decrease in RUE from 1.18 g DM MJ⁻¹ PAR (T-49) to 1.02 g DM MJ⁻¹ PAR (Scylla) and 1.33 g DM MJ⁻¹ PAR (T-49) to 1.29 g DM MJ⁻¹ PAR (Scylla) in

2003-04 and 2004-05, respectively. In both seasons, the lower RUE for Scylla was associated with a higher seed yield and 100 seed weight. High intercepted PAR and TDM do not necessarily reflect economic yield which to some extent depends on DM partitioning (Coulson, 1985). The results suggest Scylla is better than T-49 at DM partitioning, although in this study T-49 developed leaf area faster than Scylla. This highlights the need for continual assessment of new bean cultivars for their adaptability to different growing conditions.

The production of high amounts of DM, by irrigated plants, was associated with higher intercepted PAR and an increase in RUE of 0.18 g DM MJ⁻¹ PAR over the 1.02 g DM MJ⁻¹ PAR of unirrigated plants. Increased DM in irrigated plants over unirrigated plants has been reported in other grain legumes, in Canterbury (Anwar, 2001) and Australia (Thomas and Fukai, 1995). The lower RUE of the unirrigated crop was likely due to a reduced rate of photosynthesis as a result of stomatal closure and high canopy temperature which increased respiration.

Increased seed yield (2003-04) in irrigated plants was a result of increased numbers of pods plant⁻¹ and seeds pod⁻¹. The 100 seed weight averaged 26 g in both irrigated and unirrigated plants (Chapter 6). In 2004-05, the full experimental area was irrigated and the average pods plant⁻¹ and seeds pod⁻¹ increased from 7.2 (2003-04) to 11.6 (2004-05) and 2.9 (2003-04) to 3.5 (2004-05) respectively, while 100 seed weight decreased from 26.1 g (2003-04) to 22.9 g (2004-05). Higher pod retention possibly gave more pods plant⁻¹ (2004-05). The increase in pods plant⁻¹ from 7.2 (2003-04) to 11.6 (2004-05) could have been due to increased TDM production (Chapter 6). The higher TDM could have provided more assimilates to fill a higher percentage of the seeds produced in irrigated plants. A similar response, in common beans, to irrigation was reported by Krishnamurty *et al.* (1999). However, harvest of the crop before the complete reallocation of assimilates from source (leaves) to sinks (seed) was completed could partially explain the lower 100 seed weight in the second year of this study.

The effect of water stress on seed yield and other morphological traits (loss of leaf area, reduced size of younger leaves and inhibition of leaf expansion) is well documented (Ramirez-Vallejo and Kelly, 1998; Boutraa and Sanders, 2001a). Water stress is reported to be a large contributor to reduced seed yield and yield components, as shown in this study. When water is in short supply, assimilate is redirected to roots (Boutraa and

Sanders, 2001b). This assimilate redistribution reduces the supply to developing pods and seeds during the reproductive stage. This leads to reduced pods and seed numbers and subsequently reduced yield (Pena-Cabriaes and Castellanos, 1993; Boutraa and Sanders, 2001b).

The effect of inoculation on bean yield was variable (Chapter 6). Similar results have been reported worldwide (Hardarson, 1993; Hungria *et al.*, 2003). The presence of nodules on plants does not necessarily mean that sufficient N₂ is being fixed for maximum benefit to the host plant (Slattery *et al.*, 2004). In this study there was a significant difference ($p < 0.05$) in green pod yield in response to different inoculant strains (Table 6.5). Recommendations for green bean production suggest 75-150 kg N ha⁻¹ is required for maximum yield (Redden and Herridge, 1999). Application of 150 kg N ha⁻¹ or inoculation (CC 511 and RCR 3644) did not affect green pod yield in 2003-04. In comparison, in 2004-05, inoculation with different *Rhizobium* strains affected green pod yield. Wange *et al.* (1996) reported obtaining increased nodulation, DM and higher green pod yield in French beans with the application of *Rhizobium phaseoli*. This result was based on the application of 1000 g of inoculant ha⁻¹ and 20 kg P ha⁻¹. In this study increased green pod yield was achieved with only the application of *Rhizobium* inoculant, this suggests a positive influence of inoculation on green pod yield. The study also highlights the importance of introducing superior strains of rhizobia to ensure adequate N₂-fixation for maximum growth and yield of the host plant (Thies *et al.*, 1991).

The variable response to inoculation, as reported here, is usually attributed to environmental and genetic factors (Rodriguez-Navarro *et al.*, 1999; Hungria *et al.*, 2003). The variable response (nodules, TDM and seed yield) of the *Rhizobium* isolates with the bean cultivars used seems to imply varying levels of cultivar/isolate compatibility (Redden *et al.*, 1990). A similar idea was proposed by Dapaah (1997), working with pinto beans in Canterbury. These results highlight the need for further cultivar/strain evaluations under varying environmental conditions to identify the factors involved. These factors are reported to be complex (Hungria and Vargas, 2000; Amarger, 2001; Peoples *et al.*, 2002; Graham *et al.*, 2003) but their resolution could provide mechanisms for improved crop productivity.

There are few reports of positive seed yield response to bean inoculation (Vargas *et al.*, 2000; Hungria *et al.*, 2003), while there are frequent reports of failure in the literature

(Buttery *et al.*, 1987; Ramos and Boddey, 1987). Hungria *et al.* (2003) reported yields comparable to that of control plants receiving 60 kg N ha⁻¹. Yield variability, associated with inoculation, has caused farmers to doubt the importance of inoculation as a management tool. The results of this study show that green pod yield in common beans in Canterbury can be increased with the right bean cultivar/strain combination, although more work is needed to support these findings. Seed yield of inoculated plants was increased over uninoculated plants in 2004-05. However, the premature harvest of the plants for seed could have affected a significant response of seed yield to inoculation. In this study, the importance of testing 'new' *Rhizobium* strains on common beans has been highlighted. This supports previous work on common beans in Brazil (Vargas *et al.*, 2000; Hungria *et al.*, 2003).

8.1.3 Isolation and characterisation

In this study the agronomic implication of the legume/*Rhizobium* symbiosis was investigated and the nodule inhabiting organism was assessed by application of molecular techniques. Analysis of rDNA showed that nodules of field grown beans can be inhabited by organisms other than root nodulating and N fixing *Rhizobium* bacteria. These results confirm both the promiscuous behaviour of the host plant (bean) and the level of diversity of micro-organisms that remains to be characterised in New Zealand soils.

Only two percent of the nodules produced were evaluated for the nodulating organisms and this could have biased the findings reported here. Evaluation of all of the nodules would have given a clearer picture of the nodulating organisms. However, this would have been too costly and time consuming considering the constraints of this study. Using a non selective media would have allowed the growth of organisms other than bacteria. Multiplying single colonies of all available micro-organism growth on the media would have given a better indication of the nodule inhabiting organisms. However, characterisation would have been too costly. The use of Rhizobia Defined Media (RDM) was more specific allowing only bacterial growth and this affected the growth of other organisms on the media. The identification of *Pseudomonads* and not *Rhizobium* confirms the ability of these bacteria to grow on the media but does not exclude the possibility that *Rhizobium* were present in the nodules.

Some *Pseudomonads* produce a toxin that specifically inhibits winter wheat (*Triticum aestivum* L.) root growth and the growth of several micro-organisms (Bolton *et al.*, 1990). When pea (*Pisum sativum* L.) was inoculated with *Rhizobium leguminosarum*, and a toxin-producing *Pseudomonas* sp., after 10 days, the pea roots were colonised more rapidly by the *Pseudomonas* sp. and in greater numbers than by *Rhizobium leguminosarum* (Bolton *et al.*, 1990). The result was attributed to the toxic effect of the *Pseudomonas* sp. on the *Rhizobium leguminosarum*. The inability to find the inoculant *Rhizobium* strains, after culturing the nodule inhabitants on RDM, could possibly be explained by a similar mode of action of the *Pseudomonads* found on the inoculant strains in this study. Subsequent work should involve the evaluation of these *Pseudomonads* for their toxic properties in association with the *Rhizobium* isolated used in the inoculants for further confirmation.

The detection of bacteria other than the *Rhizobium* spp. from the nodules of surface sterilised nodules has to a large extent been dismissed (Vincent, 1970; Blauenfeldt *et al.*, 1994). However, increased nodulation and growth in a wide variety of legumes has been shown with simultaneous infection with rhizobia and rhizosphere bacteria (Bolton *et al.*, 1990; Hallmann *et al.*, 1997; Sturz *et al.*, 1997). In this work, nodule infection by *Pseudomonads* gave increased TDM at final harvest in 2004-05 when compared to 2003-04 when there was no observed nodulation. The plant growth promoting effect of rhizobacteria strains particularly those of the genera *Bacillus* and *Pseudomonas* is known (Shishido *et al.*, 1999). The beneficial and positive effect of these organisms on the *Rhizobium*-legume symbiosis has also been indicated in several studies (Marek-Kozaczuk *et al.*, 2000; Kumar *et al.*, 2001). Enhanced growth and N fixation has been reported in western Canadian lentils (*Lens culinaris* Medik.) and pea cultivars both under field and laboratory conditions (Chanway *et al.*, 1989). Enhanced nodule weight, root length, shoots biomass and total N was reported in chickpea grown in sterile jars, or in pot culture with cohabitation in the nodules (Parmar and Dadarwal, 1999). The production of increased levels of flavonoid like compounds by the rhizobacteria was proposed to have triggered plants to be more susceptible to rhizobial infection and thus improved plant productivity. The increased DM production with inoculation and nodulation in the 2004-05 experiment could be the result of this proposed mechanism. Further work is required to explore the physiology and ecology of the isolated organisms to confirm the production of these flavonoid like compounds.

In this study, the bacterial organisms *Pseudomonads* were isolated from the nodules of field grown beans. Various reports have documented the isolation of other soil organisms other than rhizobia from legume nodules (de Lajudie *et al.*, 1999; Tokala *et al.*, 2002). As in this study Valverde *et al.* (2003) reported an organism other than rhizobia in nodules of common bean. Although the organism (*Herbaspirillum lusitanum*) was a N fixing species, the biological significance of these associations are not fully understood and need further investigation (Mrabet *et al.*, 2006). To support the presence of bacteria other than rhizobia in the root, Albareda *et al.* (2006) reported that plant growth promoting rhizobacteria were generally better colonisers than specific rhizobial species in bean and soybean. This possibly explains the presence and dominance of *Pseudomonas* in the roots and on the isolation media in this study.

The isolation of *Pseudomonads* from sterilised nodules of field grown bean suggests an association between bean plant, *Rhizobium* and *Pseudomonads*. Similar reports of *Agrobacterium* in nodules of common bean were explained as the bacterium having the ability to colonise pre formed nodules (Mhamdi *et al.*, 2005), or by a mechanism which is still unknown. This could also be an explanation for the presence of *Pseudomonads* in nodules in this study. These reports suggest a complex association between legumes and nodule inhabiting organisms other than the *Rhizobium*/legume association which is responsible for N₂ fixation.

Although the nodules were sterilised, contamination cannot be fully ruled out. The results of this study show that co-habitation in the bean nodules probably caused increased TDM, nodules plant⁻¹ and yield. The variations in growth, nodulation and yield observed with the different inoculant strains used in the second season indicate some specificity in response between *Rhizobium* isolate and *Pseudomonas*. Against the background of years of research in the legume/*Rhizobium* symbiosis, the results of this study show that there is still much to be learnt about the common bean/*Rhizobium* symbiosis on nodulation and plant growth. Further investigation is needed to make conclusive recommendation as to whether these findings can add benefit to the bean/*Rhizobium* symbiosis, which is known for its low N₂ fixation (Hungria *et al.*, 2003).

8.2 Conclusions

Both field and greenhouse nodulation were improved with *Rhizobium* inoculation; nodulation depended on both the macro and micro symbiont. Continued research on the effectiveness of rhizobia is needed, as the symbiosis is often not optimal.

The good symbiotic performance of imported rhizobial strains encourages the identification of new competitive and efficient *Rhizobium* strains for bean crops in temperate regions.

Cultivar selection should be a major component of future work related to the bean/*Rhizobium* symbiosis in Canterbury, as cultivar affected leaf area index, TDM, radiation use efficiency, nodules plant⁻¹ and seed yield

Climatic conditions (cold temperatures) have a severe effect on growth and development of common bean and great care must be taken in cultivar selection with an emphasis on cold tolerance for production in Canterbury.

Molecular methods including rDNA analysis are an effective and rapid means of identification and characterisation of plant-associated bacteria.

Organisms that are capable of co-habitation with rhizobia in nodules of field grown legumes are a feature of the legume/*Rhizobium* symbiosis.

8.3 Recommendations for future research

There is a need for further research in the following areas:

Strain characterisation studies to understand the ecology of rhizobia in soil. These studies would be geared towards strain competitiveness and nodule occupancy and placement of the inoculum in areas to increase nodulation and N fixation. This would aid in the better management of the host/rhizobia system.

Detailed ecological studies and fields surveys to determine the diversity of indigenous organisms present which are capable of nodulating specific legumes, their N₂ fixing ability evaluated and classified based on the host plants they nodulate.

Complete evaluation of nodule occupants and the relationships of these organisms to plant growth, development and yield.

Continuous evaluation of available *Rhizobium* isolates and emerging bean cultivars is necessary to fully ascertain the capability of this symbiosis for the production of this crop in Canterbury. The effectiveness of the two *Rhizobium* strains (CC 511 and RCR 3644) recommended for beans in New Zealand needs to be re-examined.

The evaluation of different strains of *Rhizobium* individually and in mixtures, inoculation types and different inoculum placements to enhance nodule formation on the entire root system is needed.

The development of efficient and improved inoculants and delivery systems, with good shelf life and low cost. These would reduce the impact of harsh biotic and abiotic conditions encountered by the inoculant strains after manufacture and before nodulation. An example is the use of technology such as PulseSignal which was developed in Canada to promote earlier nodule formation under low soil temperatures.

Bacterial strain evaluation should be combined with bean breeding programmes so that selected cultivars would be developed in collaboration with their associated inoculant strain(s) to reduce the dependence of these cultivars on commercial fertiliser which are becoming an increasing environmental concern.

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Appendices

Appendix 1A

Maturity Criteria for green bean

Randomly select 25 bean plants from a plot and pick the biggest beans from these plants

Remove the seed from the centre of each of the 25 bean pods

Line the seeds up end-to-end along a ruler and measure the total length of the 25 seeds.

i.e. (OOOOOOOOOOOOOOOOOOOOOOOOOOOO)

Divide the total length of the seed by 25 to get the average length of each seed.

Carry out practice daily or every second day.

At an average seed length of approximately 11 mm the crop is ready for harvest

Appendix 1B

Marketable (Processing) Beans

Beans are deemed marketable for processing, if they have attained a length of greater than 40 mm and a diameter greater than 4 mm. Beans less than 40 mm in length and/or less than 6 mm in diameter are graded out.

Appendix 2A

Formula for Rhizobia Defined Media (Glucose RDM or Mannitol RDM) (Ronson *et al.*, 1984)

Stock solutions

Salts:

MgSO ₄ .7H ₂ O (Magnesium sulphate heptahydrate)	1.25 g
CaCl ₂ .2H ₂ O (Calcium chloride dehydrate)	1 g
Na ⁺ EDTA (Ethylene diaminetetra acetic acid)	0.75 g
FeCl ₃	0.33 g
NaCl (Sodium chloride)	10 g
Deionised H ₂ O	500 ml

Trace elements:

ZnSO ₄ .7H ₂ O (Zinc sulphate heptahydrate)	0.03 g
Na ₂ MoO ₄ .2H ₂ O (Sodium molybdate)	0.4 g
H ₃ BO ₃ (Boric acid)	0.5 g
MnSO ₄ .4H ₂ O (Manganese sulphate)	0.4 g
CaSO ₄ .5H ₂ O (Calcium sulphate pentahydrate)	0.04 g
CoCl ₂ .6H ₂ O (0.2g/l) [0.02g/100ml-200ul/l]	1 ml
Deionised H ₂ O	2000 ml

Vitamins:

Thiamine HCL	0.05 g
Ca Pantothenate	0.1 g (Store at 4°C in the dark)
Biotin*	1 ml
Deionised H ₂ O	to 50 ml

* dissolve fully (with heat) 1mg Biotin in 1 ml deionised H₂O prior to adding to remaining ingredients

Filter sterilise using a sterile 50 ml syringe and sterile 0.45µm filters. Draw up into syringe and filter 20 ml aliquots into pre-autoclaved McCartney bottles.

Bromothymol blue (BTB):

Bromothymol blue	0.4 g
Deionised H ₂ O	200 ml

Ammonium chloride:

NH ₄ Cl	3.6 g
Deionised H ₂ O	200 ml

Phosphates:

K ₂ HPO ₄ (Dipotassium hydrogen phosphate)	20g
KH ₂ PO ₄ (Potassium dihydrogen orthophosphate)	20g
Deionised H ₂ O	200 ml

Autoclave at 121°C for 15 minutes

Carbon source:

either Glucose	20g
Deionised H ₂ O	100 ml
or Mannitol	50g
Deionised H ₂ O	500 ml

Autoclave at 121°C for 15 minutes

Antifungal agent:

Quintozene (pentachloronitrobenzene)	0.05g
Dimethylformamide	1000 ml

2. Media make-up

1. For 1 litre of media use 882 ml for MRDM or 942 ml for GRDM of deionised water:
Add:

Salt solution	10 ml
BTB	10 ml
NH ₄ Cl	6 ml
Trace element solution	1 ml
CoCl ₂	200 µl

2. Add 0.1 g L-Histidine and dissolve

3. Adjust pH to 6.5-7 if required using 2M KOH

4. Add 15 g Agar and dissolve

5. Autoclave at 121 °C for 15 minutes

6. Hold at 55 °C in water bath and add other ingredients. NB: if media is allowed to cool too far it will solidify when other ingredients are added

7. Add carbon source:

either Glucose stock solution	20 ml
Mannitol stock solution	80 ml

8. Add 10 ml phosphate stock solution

9. Add 1 ml Vitamin stock solution

10. Add antifungal agents

 Quintozene stock solution* 0.1 ml

11. Pour plates quickly after removing molten agar from water bath.

* NB: If isolating rhizobia from soil it's recommended that other anti fungal agents are incorporated in addition to Quintozene:

e.g. Cycloheximide at 50-100 mg/litre of media. Nystatin at 100 mg/litre of media. Both dissolved in water.

Appendix 2B

Formula for Tryptone Yeast (TY) medium and broth (Beringer, 1974; Somasegaran and Hoben, 1994).

Tryptone	5g
Yeast extract	3g
CaCl ₂	0.87g
Technical agar	15g (omit if making TY broth)
Deionised water	1litre

Adjust the pH to 6.8-7.2 with NaOH or HCL, aliquot into culture vessels and autoclave at 121 °C for 15-20 minutes.

Appendix 2C

Formula for Seedling Agar (Broughton and Dilworth, 1971).

Stock Solutions:

Solution 1:

147.05 gm CaCl₂.2H₂O (Calcium chloride dihydrate)
500 ml d. H₂O

Solution 2:

68.05 gm KH₂PO₄ (Potassium dihydrogen orthophosphate)
500 ml d. H₂O

Solution 3:

3.35 gm FeC₆H₅O₇ (Ferric citrate)
61.65 gm MgSO₄.7H₂O (Magnesium sulphate heptahydrate)
43.50 gm K₂SO₄ (Potassium sulphate)
0.169 gm MnSO₄.H₂O (Manganese sulphate)
500 ml d. H₂O

Solution 4:

0.494 gm H₃BO₃ (Boric acid)
0.576 gm ZnSO₄. 7H₂O (Zinc sulphate heptahydrate)
0.2 gm CuSO₄. 5H₂O (Cupric sulphate pentahydrate)
0.112 gm CoSO₄. 7H₂O (Cobalt sulphate heptahydrate)
0.096 gm Na₂MoO₄. 2H₂O (Sodium molybdate)
2 L d. H₂O

To make up Seedling Agar:

0.5 ml each of solutions 1, 2, 3, and 4.
1 L d. H₂O
100 gm bacteriological agar

Mix well and autoclave at 121°C for 15 minutes.

Appendix 2D

Formula for Water Agar (Brown, 1924).

To make water agar

22.5 gm of plain agar
1 L of d. H₂O

Appendix 3

Growth habit classification and description of common bean (Schwartz *et al.*, 2005).

Growth Habit	Description ^a
Type I	* Habit determinate Terminal bud reproductive Stem and branches erect or prostrate Terminal guide absent or small Pods distributed along the length of the stem
Type II	* Habit indeterminate Terminal bud vegetative *Stem and branches erect Terminal guide absent or medium Pods distributed along the length of the stem

Type III	Habit indeterminate
	Terminal bud vegetative
	*Stem and branches prostrate with little or on climbing ability
	Terminal guide small or long
	*Pods distributed mainly on the basal portion of the stem
Type IV	Habit indeterminate
	Terminal bud vegetative
	Stem and branches twining with strong climbing ability
	Terminal guide long or very long
	*Pods distributed along the length of the stem or mainly in the upper portion

^a Key characteristics of the growth habit classification are marked by an asterisk

Stage number	Stage	GENERAL DESCRIPTION (Vegetative stages)
V1		Completely unfolded leaves at the primary (unifoliate) leaf node
V2	1	First node above primary leaf node, Count when leaf edge no longer touch
V3	2	Three nodes on the main stem including the primary leaf node. Secondary branching begins to show from branch of V1
V(n)		n nodes on the main stem, but with blossom clusters still not visibly opened
	3	V5 Bush (determinate) plants may begin to exhibit blossom and become stage R1
		V8 Vine (indeterminate) plants may begin to exhibit blossom and become stage R1

Stage number	Stage	BUSH DESCRIPTION (Reproductive stage)
R1	3	One blossom open at any node
R2		Pods 1.27 mm at first blossom position. Usually node 2 to 3
R3		Pods 2.5 mm at first blossom position. Secondary branching at all nodes, so plant is becoming denser but not taller. ½ bloom.
R4		Pods 8 mm in length. Seed not discernible. Bush dry types may be shorter
R5	4	Pods 13-15 mm maximum length. Seeds discernible to feel in garden varieties, Bush dry; pods 8-10 mm. Seed discernible
R6		Seed at least 0.6 mm over long axis
R7	5	Oldest pods have developed seeds. Other parts of plant will have full length pods with seeds almost as large as first pods. Pods will be developing over the whole plant
R8		Leaves yellowing over half of plant, very few small pods and these in axils of secondary branches, small pods may be drying. Point of maximum production has been reached
R9		Mature, at least 80 % of the pods showing yellow and mostly ripe. Only 40 % of leaves still green colour

Appendix 4

Protocol for serial dilutions (Somasegaran and Hoben, 1985).

- Streak bacteria onto a fresh agar plate and incubate so that a single young actively growing colony is ready at time of inoculation.
- Prepare broth (specific to bacterial species) in flasks leaving enough space for good aeration (ie. 100 ml in a 250 ml flask). Seal with foam or cotton wool bungs, cover with foil and autoclave. Leave to cool.
- Either:
Aseptically (in flow hood) pick a single distinct colony from the plate using a sterile loop and add to the broth (take care not to contaminate the flask).
- Or:
Aseptically pick a single distinct colony and use to inoculate a small amount of pre-prepared broth (i.e. 10 ml in a 50 ml tube). Grow for 24 h and then use 1 ml to inoculate the flasks.

The latter will result in a faster growing culture as the lag phase will be shorter due to the cells already growing in the broth media.

The broth culture is 10^0 ; first dilution is 10^{-1} .

Dilution series:

- Take 100 ul from well mixed broth culture.
- Add to 900 ul sterile distilled water (SDW).
- Mix thoroughly.
- Take 100 ul from 10^{-1} dilution and add to 900 ul SDW to give 10^{-2} .
- Mix thoroughly.
- Repeat until required dilution is reached.

Use a new pipette tip for each dilution. Easiest way to mix samples is to pipette sample into water and then keeping tip in the solution, pipette up and down a few times, and take care not to suck up sample into the pipette body. You can use any volume as long as it produces the required dilution (i.e. 50 ul in 450 ul).

Dilution Plating:

- Pipette a 100 ul sample from weakest dilution onto an agar plate.
- Use a sterile spreader to lightly spread the sample as evenly as possible across the whole plate.
- Leave to dry (lid on) and seal with gladwrap.
- Place in incubator upside down (this stops water dripping onto the surface of the plate if any condensation forms).

To speed things up pipette the sample onto a few plates at a time (be careful not to do so many that the sample dries before being spread, 4-6 plates is usually enough). You can use

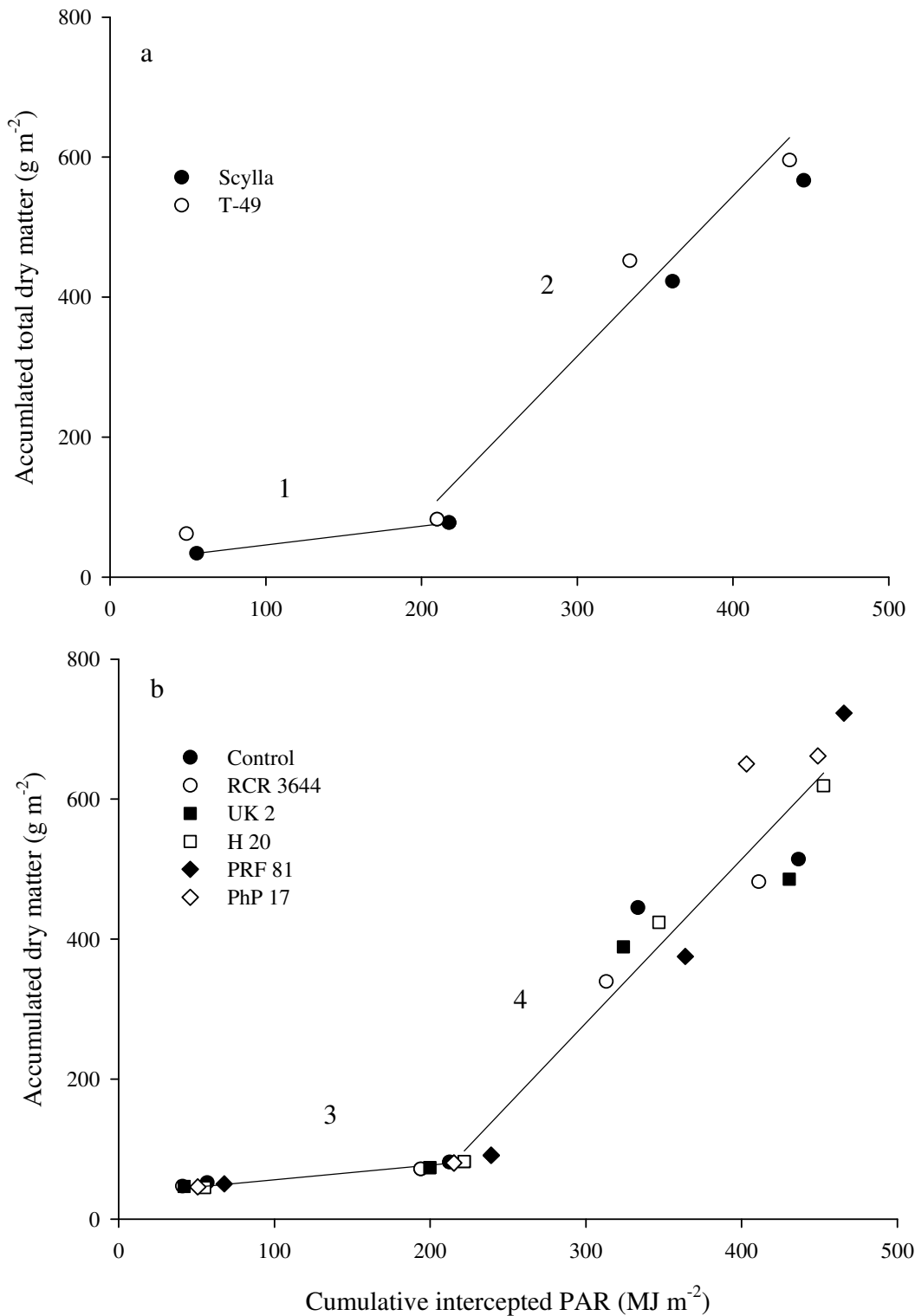
the same tip (and spreader) when going from a weak dilution to a more concentrated one. Flame the spreader at regular intervals to prevent spread of any contaminants but wait for the spreader to cool before spreading as heat can kill the bacteria.

Check the plates daily and count when the colonies are all visible and not too small, take care that they don't get too big and merge into each other. Count the dilution plates that have 30 to 300 colony forming units (cfu's) on them. If you're unsure that all the cfu's are visible count the same plate over a number of days until the count is stable.

Spectrophotometry

- Turn the spectrophotometer on and wait for it to warm up.
- Set absorbance to 600 nm for bacteria.
- Take a 1-2 ml aliquot (depending on cuvette size) of control broth (no bacteria) and pipette into the cuvette.
- Place into the spectrophotometer taking care not to touch the clear sides at any time. Ensure the cuvette is in the correct orientation, the light must pass through the clear sides.
- Close the lid and take the reading.
- Set the spectrophotometer to zero.
- Tip the broth out of the cuvette and wash with distilled water. Tap upside down on tissue to get the water out.
- Pipette 1-2 ml aliquot of broth culture into the cuvette and read at 600 nm.
- Discard broth in bleach solution or container to be autoclaved and wash cuvette.
- Repeat with the other samples.
- Use a fresh tip for each sample and discard tips and cuvettes in an autoclave bag or soak in a bleach solution before putting in bin.

Appendix 5



The relationship between cumulative intercepted PAR (MJ m^{-2}) and accumulated total dry matter (g m^{-2}) during crop growth for common beans in Canterbury in 2004-05

$$1: y = 19.0 + 0.27x \quad (r^2 = 1) \quad 2: y = -371.2 + 2.29x \quad (r^2 = 0.96)$$

$$3: y = 35.7 + 0.21x \quad (r^2 = 1) \quad 4: y = -421.4 + 2.24x \quad (r^2 = 0.99)$$