UNIVERSITEIT VAN PRETORIA
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THE RELATIONSHIP OF *TRIFOLIUM REPENS* AND *T. AMBIGUUM* WITH HOST-SPECIFIC *RHIZOBIUM* BACTERIA FOR POTENTIAL INCORPORATION INTO SUSTAINABLE, LOW N INPUT PASTURES

by

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Submitted in partial fulfilment of the requirements for the degree MSc (Agric): Pasture Science
In the Faculty of Natural and Agricultural Sciences
University of Pretoria

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April 2010

Dedicated to my parents,

Jan and Hannie Swanepoel

ABSTRACT

THE RELATIONSHIP OF *TRIFOLIUM REPENS* AND *T. AMBIGUUM* WITH HOST-SPECIFIC *RHIZOBIUM* BACTERIA FOR POTENTIAL INCORPORATION INTO SUSTAINABLE, LOW N INPUT PASTURES

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Abstract

Research on efficient management systems to optimise *Trifolium repens-Rhizobium* symbiosis, is lacking in South Africa. The amount of nitrogen (N) fixed by symbiotic rhizobia in root nodules of *T. repens* is ultimately determined by health of the soil environment. Soil organic matter (SOM) is the main attribute that will sustain soil health as it affects the chemical, physical and biological aspects of soil. The aim of this study was to determine the potential of *T. repens* and *T. ambiguum* to sustain low N input pastures. The hypotheses of this study is that SOM can play an important role in ensuring good soil health, which supports the optimum growth and production of *Trifolium* spp. in low N input pastures. Four *Trifolium repens* cultivars were evaluated in field conditions to determine the effect of *Rhizobium* bacteria on the potential of the cultivars to nodulate. The cultivars Grasslands Huia, Haifa, Ladino and Regal were selected for assessment. The thousand-seed-mass (TSM) of each cultivar was measured to determine the quality and viability of the seed; and to determine the interaction between seed mass and nodulation. Mean TSM values of *T. repens* cultivars differed significantly, with Huia having the highest TSM followed by Haifa, Ladino and Regal.

Biomass production was also measured as an indicator of efficiency of nitrogen fixation. The cultivar Huia, with the heaviest seed, showed the highest biomass production. After eight weeks of growth, the nodulation index was determined from the size, number and colour of the bacterially associated root nodules. All plants, regardless of cultivar, formed nodules within eight weeks. It was concluded that TSM had no notable effect on nodulation. Planting date with associated temperature effects and the intrinsic cultivar effect also had no influence on nodulation. It was therefore concluded that nodulation potential of the four cultivars tested was similar in the specific environmental conditions.

The total number of symbiotic rhizobial cells per gram of soil as affected by soil C content and the host plant was also determined. Inoculated and un-inoculated seeds were planted on five soil treatments, each with a different level of soil C. The plant infection technique (most-probable-number/MPN technique) was used to quantify the rhizobial numbers in soil as affected by soil C content and the host plant. The mean MPN-value ranged from 8907 to 78 *Rhizobium* cells per gram of soil for *T. repens* treatments, and 0 to 436 for *T. ambiguum* treatment. Soil C had no effect on the number of *Rhizobium* bacteria present in the soil. Inoculation however, had a significant effect on the MPN value of *T. ambiguum*, but not for *T. repens*. Most symbiotic *Rhizobium* was detected between a soil C content of 2.03% to 3.80% in both inoculated and non-inoculated soils. The spread plate count was used to determine the total number of symbiotic and saprophytic rhizobia. This method was used to quantify both symbiotic and free-living rhizobia.

The effect of different levels of soil C on the amount of atmospheric N fixed was assessed by the N difference technique*. Arctotheca calendula* (cape weed) served as the reference plant in this study, to determine what percentage of N is derived from the atmosphere (%Ndfa). Biomass production was determined and served as the parameter to establish the efficiency of the *Rhizobium* bacteria in the soil. Inoculating seed with host specific rhizobia had no effect on the amount of N fixed. The mean %Ndfa differed significantly between soil organic C treatments with the species *T. repens* but did not differ significantly between soil organic C treatments with *T. ambiguum.* It was concluded from this study that symbiotic rhizobia introduced by inoculant was much more efficient in higher C content soils than free-living rhizobia, which highlights the importance of inoculation in improving the sustainable production of *T. repens* pastures. Although the amount of N fixed increased as the level of soil organic matter decreased, the efficiency of N fixation decreased proportionally. This explains the bigger change in soil N content on soil with a high C content. This study has thus highlighted the importance of soil organic carbon in the host specific *Rhizobium* inoculation sucsess, of *T. repens* low N input pasture systems.

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ACKNOWLEDGEMENTS

This study required collaborate work and support of various people and institutions. I would like to express my sincere gratitude to the following people or institutions:

Dr. Ilse Trautmann, director of Technology, Research and Development Services of the Department of Agriculture Western Cape for supplying the facilities and financial support.

The Outeniqua Dairy Research Trust for the principal funding of the research.

A special tribute is owed to Dr. Philip Botha, co-supervisor and Specialist Agricultural Scientist from the Department of Agriculture of the Western Cape. I am deeply grateful for his ample knowledge in the field of practice, and his willingness to share it with me. I recall with fondness the walks we had on the farm, where he shared much of his field knowledge. His personal support, enthusiastic motivation and encouragement throughout the study were invaluable. Without his guidance and inspiration, this study would not have been possible. The worth of the opportunities created by him will be immense in my career. I am thankful for his patience, especially in teaching me scientific and popular writing, editing and reviewing. I admire the research he has already performed and the influence he has on the direction of future research.

Dr. Wayne Truter, the supervisor from the Department of Plant Production and Soil Science of the University of Pretoria, for assessment, advice and time spent on the dissertation.

Dr. Karen Surridge-Talbot, from the Department of Plant Production and Soil Science of the University of Pretoria, for the role she played as the co-supervisor and the valuable advice on the microbiological aspects of the trial. I am thankful for her factual review and advice on writing the manuscript.

Ilze Fourie, an intern from Nelson Mandela Metropolitan University, for her passionate and hard work. She was of great assistance with the organisation, planning and initiation of the

study, management of the small plots and pots, data collection, data handling and writing of the dissertation.

Alicia van der Merwe, a PhD student from the Department of Plant Production and Soil Science of the University of Pretoria, for her eager assistance, research and advice with the microbiological laboratory work at her own expense. I admire her unconditional support, even when she had no time on hands. I am grateful for the experience that I gained while working with her.

Mr. Hennie Gerber and Mr. Pieter du Plessis, the farm manager and the farm foreman on Outeniqua Research Farm respectively, for assistance and advice on the implementation of the pot trial and shade house, as well as design and construction of the pouch-racks.

The technical team on Outeniqua Research Farm under guidance of Dalena Lombard, by names Abraham Arends, Andrew Petoors, Brian Zulu, Bruce Conradie, Daniël Veldman, Jannie Noorman, John Koeries and Willem Roman for assistance with the practical implementation of the study.

Professor Robin Meeske, Specialist Agricultural Scientist from the Department of Agriculture of the Western Cape, for his advice and giving me the opportunity to become exposed to the industry.

The late Professor Norman Rethman, for the contribution he had on the development and assessment of the research till his passing away.

Dr. Sandra Lamprecht from the Agricultural Research Council's Plant Protection and Research Institute for recommendations and advice on the soil fungal populations.

Me. Mardé Booyse, Genetic and Psychological Biometrician from ARC-Infruitec, for the advice on the design and layout of the pot trial as well performing the statistical analyses.

Dr. Johan Labuschagne, Agricultural Researcher from the Department of Agriculture of the Western Cape for valuable advice during the course of the study.

Heinrich Geyer, a PhD student from the Department of Plant Production and Soil Science of the University of Pretoria, for his assistance in the laboratory on the UP experimental farm.

Carmen Coetzee, MSc (Agric) student from the University of Pretoria, for support and good company at home.

Janke van der Colf, MSc (Agric) student from the University of Pretoria, for suggestions, advice and recommendations during the planning of the trial and the writing-up. She is truly trustworthy and reliable.

Kim Butler and Nobenguni Mboyana, two interns on Outeniqua Research Farm, for the assistance with the planting, data collection and harvesting.

Lihandi van Niekerk, Louise Müller, Annariet Jooste and Dirk Joubert for their assistance in the University of Pretoria's tissue culture laboratory.

The residents of *Die Bulstal* on the University of Pretoria's Experimental Farm for their pleasant hospitality during the period when laboratory work was performed in Pretoria.

Jan and Hannie Swanepoel, my parents, for giving me the opportunity to study and all the sacrifices they made during the course of my studies. I do appreciate their support sincerely. Thank you for the faith you have in me.

Most importantly, I want to thank my Lord Jesus Christ for providing me with the skill and ability to conduct this research. I will always be sincerely grateful for the blessings and His everlasting love from above.

SOLI DEO GLORIA

DECLARATION

I, Pieter Andreas Swanepoel declare that the dissertation, which I hereby submit for the degree MSc(Agric): Pasture Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: ……..

DATE: ……

LIST OF ABBREVIATIONS AND ACRONYMS

°C	Degree Celsius
°Cd	Degree-days
µM	Micromolar
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
B	Boron
C	Carbon
Ca	Calcium
CEC	Cation exchange capacity
cm	Centimeter
Co	Cobalt
CO_2	Carbon dioxide
Cu	Copper
DM	Dry matter
DNA	Deoxyribonucleic acid
e^-	Electron
EC	Electrical conductivity
Fe	Iron
g	Gram
H	Hydrogen
H^+	Proton
H_2	Dihydrogen gas
H_2O	Water
ha	Hectare
K	Potassium
KCl	Potassium chloride
kg	kilogram
Lb	Leghaemoglobin
Mg	Magnesium

mg	Milligram
mm	Millimeter
Mn	Manganese
Mo	Molybdenum
MPN	Most-probable-number
N	Nitrogen
N_2	Dinitrogen gas
N_2O	Nitrous oxide
Na	Sodium
NaCl	Sodium chloride
$-NH_2$	Amide group
NH_3	Ammonia
NH_4^+	Ammonium ion
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
O	Oxygen
OM	Organic matter
P	Phosphorus
PEP	phosphoenolpyruvate
pH	The negative logarithm to the base ten of the hydrogen ion activity in the solution
$pH_{(KCl)}$	pH measured in a solution with KCl
P_i	Inorganic phosphate
PP_i	Inorganic pyrophosphate
RNA	Ribonucleic acid
S	Sulphur
SOM	Soil organic matter
SR	Stocking rate
t	Ton
TSS	Total soluble salts (%)
Zn	Zinc

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$$Nett\ N\ mineralisation = (NH_4^+ + NO_3^-)_{t+1} - (NH_4^+ + NO_3^-)_t$$

Equation 2

$$N_2 + 16\ H_2O + 8\ e^- + 16\ ATP \xrightarrow{\text{nitrogenase enzyme complex}} 2\ NH_3 + 16\ ADP + 16\ P_i + H_2 + 8H^+$$

Equation 3

$$\frac{d[OM]}{dt} = \frac{dP}{dt} - \frac{dB}{dt}$$

CHAPTER 2

Equation 1

$$Establisment\ \% = \frac{nr.of\ seeds\ germinated - nr.of\ seedling\ deaths}{Total\ nr.of\ seeds\ planted} \times 100$$

CHAPTER 3

Equation 1

$$Nodulation\ index = A \times B \times C$$

Equation 2

$$a = \left(\frac{Dilution\ inoculated}{Dilution\ source}\right) \times Actual\ volume\ inoculated$$

Equation 3

$$\frac{a_i p_i}{1 - e^{-a_i x}} + \cdots + \frac{a_n p_n}{1 - e^{-a_n x}} = a_i z_i + \cdots + a_n z_n$$

Equation 4

$$P = \left(\frac{z_i!}{p_i!\,q_i!}\right) \ldots \left(\frac{z_n!}{p_n!\,q_n!}\right) (e^{-a_i x})^{q_i} \ldots (e^{-a_n x})^{q_n} (1 - e^{-a_i x})^{p_i} \ldots (1 - e^{-a_n x})^{p_n}$$

Equation 5

$$Population\ estimate = x\frac{1}{d}$$

Equation 6

$$CF = antilog_{10}\left(2 \times 0.55\sqrt{\frac{log_{10}dr}{n}}\right)$$

Equation 7

$$CFU = \frac{number\ of\ colonies}{Volume\ inoculated} \times \frac{1}{Dilution\ ratio}$$

CHAPTER 4

Equation 1

$$N_2\ fixed_{ND}\ (g.g^{-1}) = Total\ N\ yield\ (g.g^{-1})_{T.\ repens} - Total\ N\ yield\ (g.g^{-1})_{A.\ calendula}$$

Equation 2

$$\%Ndfa = N_2\ fixed_{ND}\ (g.g^{-1}) \times 100$$

CHAPTER 5

Equation 1

$$Nodulation\ index = A \times B \times C$$

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$$\frac{a_1 p_1}{1 - e^{-a_1 x}} + \cdots + \frac{a_n p_n}{1 - e^{-a_n x}} = a_1 z_1 + \cdots + a_n z_n$$

Equation 4

$$P = \left(\frac{z_1!}{p_1!\, q_1!}\right) \ldots \left(\frac{z_n!}{p_n!\, q_n!}\right) (e^{-a_1 x}) q_1 \ldots (e^{-a_n x}) q_n (1 - e^{-a_1 x}) p_1 \ldots (1 - e^{-a_n x}) p_n$$

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Equation 7

$$CFU = \frac{number\ of\ colonies}{Volume\ inoculated} \times \frac{1}{Dilution\ ratio}$$

Equation 8

$$N_2\ fixed_{ND}\ (g.g^{-1}) = \text{Total N yield } (g.g^{-1})_{T.\ ambiguum} - \text{Total N yield } (g.g^{-1})_{A.\ calendula}$$

Equation 9

$$\%Ndfa = N_2\ fixed_{ND}\ (g.g^{-1}) \times 100$$

CHAPTER 1

Prepared according to the guidelines of the African Journal of Range and Forage Science

INTRODUCTION

1. Background

The Southern Cape coastal region is a relatively narrow strip of agriculturally important land, bordered by the Indian Ocean to the south, the Villiersdorp-Botrivier district to the east, the Sonderend-, Lange- and Outeniqua mountain ranges to the north and the Stormsriver to the west (Subramanian *et al.* 2007). The region has a typical temperate climate with a long term average annual rainfall ranging from 500.0 mm in the west to 728.0 mm in the east (ARC 2009).

Common agricultural practices in this region are farming of grains, vegetables, sheep, beef and dairy cattle on planted pastures. Dairy production systems are based mainly on *Pennisetum clandestinum* (kikuyu) pastures oversown with *Lolium multiflorum* (annual ryegrass) and *L. perenne* (perennial ryegrass). Soils in these pastures are sustained by minimum- and no-tillage practices (Botha 2003). This reduces disturbances of the soil structure and decreases the breakdown of soil organic matter (SOM) (Tate 1992). Conservation farming, as in this case, aims at the maintenance of biological, physical and chemical equilibrium that optimises the agro-ecosystem to conserve biodiversity in a sustainable manner. The holistic aim of conservation farming is to achieve a socially, ecologically and economically balanced system (Bot and Benites 2005). Recent trends in research focus on developing a comprehensive understanding of the relationship between soil health, plant function and the impact of human interference on agro-ecosystems. The focus remains sustainable and regenerative farming with minimum inputs. This can be achieved by managing the soil biological activity and increasing the SOM content to conserve soil health and subsequently utilise the benefits of high quality soil (Bot and Benites 2005).

There has been a recent movement in agricultural practices towards lessening costs of fertiliser-nitrogen (N) inputs by incorporating leguminous species, particularly *Trifolium repens* (white clover), into grass pasture systems (Bohlool *et al.* 1992). *Trifolium repens* has a high nutritive value, but produce less above ground biomass when compared to grasses. Therefore, grazing capacity of grass-clover mixed pastures will be lower than that of grass monocultures. In other words, fewer cows grazing per unit area will produce the same amount of milk due to the higher nutritive value of clovers (*Trifolium* spp.), over grasses. Profitability of these pastures will be increased as the input cost of fertiliser-N will be decreased, or even eliminated (Howieson 1995, Botha 2003).

Incorporation of other *Trifolium* species is less common, but may be beneficial for specific functions or under particular environmental conditions. *Trifolium ambiguum* is an example of a *Trifolium* species that performs functional specificity in pastures. It is mainly incorporated into pastures for its persistency under heavy grazing regimes, because of its extensive root system. It does not establish a symbiotic relationship with *Rhizobium* as easily as *T. repens* (Dear and Zorin 1985, Seguin *et al.* 2001, Taylor 2008, Walker 2009). The goal of incorporating *T. ambiguum* is therefore somewhat different to that of *T. repens.* Recent development of host specific *Rhizobium* strains makes the potential of *T. ambiguum* as a species to decrease fertiliser costs unknown (Seguin *et al.* 2001).

2. Problem statement

Maintaining highly productive pastures sustainably in the Southern Cape has become expensive. Recent increases in prices of fertiliser-N and a higher demand for milk production per unit area have intensified pressures on profit margins of the dairy industry (Botha 2003). Future research on alternative sources of N is inevitable and imperative. *Trifolium* spp. are highly nutritious leguminous species suitable for dairy pasture systems (Mårtensson and Ljunggren 1984, Williams 1987b, Ledgard *et al.* 2001, McDonald *et al.* 2002). These species are particularly effective in producing organic nitrogenous compounds, rendering them a promising alternative to inorganic fertiliser-N application (Mårtensson and Ljunggren 1984, Michaelson-Yeates *et al.* 1998). The development of management strategies for pastures containing *T. repens* are necessary to establish and maintain this species in grass pastures in a sustainable manner. Management factors, such as the manipulation of the soil environment,

may enhance the ease of establishment of *T. repens* in grass pasture systems. Soil environmental manipulation, particularly SOM, may have a direct effect on the legume plant itself or may indirectly affect the rhizobial populations that can proliferate saprophytically in the soil or in the root nodules of the legume. Not only must N fixation by legumes be maximised to be an efficient substitute for inorganic N fertilisation of crops and pastures, but the soil environment must be managed efficiently to be able to utilise such N (Sprent 1979). Decisions regarding these factors will have a major impact on the profitability of dairy farming systems.

3. Aim

The aim of this study was to assess the nodulation potential of *Trifolium repens* and *T. ambiguum* by introduced bacteria *(Rhizobium leguminosarum* bv. *trifolii)* from a plant specific inoculant in soil with different levels of SOM. The ability of the introduced rhizobial populations in the soil to successfully nodulate *T. repens* and *T. ambiguum,* as a function of the size of the rhizobial population, will be strongly dependent on the ability of the rhizobial populations to proliferate and be maintained by the soil environment. The population size of the naturally occurring, as well as introduced rhizobial strains, must be assessed. The population size of naturally occurring strains affects the crop response to the applied strains from inoculants and success of efficient N fixation (Bohlool *et al.* 1992). Competitive ability of indigenous strains also plays a role in the success of root-infection by the introduced strain. Larger indigenous population sizes will decrease the ability of the introduced strains to nodulate *Trifolium* spp. successfully (Bohlool *et al.* 1992, Keyser *et al.* 1992). N fixation will be quantified using the N difference technique to compare different treatment-combinations, i.e. between different levels of soil organic matter content and inoculation (Hart *et al.* 1994, Pate *et al.* 1994, Weaver and Graham 1994, Carranca *et al.* 1999). The importance of SOM and inoculation will be stressed in this study. Management of these parameters could subsequently be adapted to obtain and maintain optimal levels of SOM.

This research will contribute towards developing a profitable grazing system for dairy cattle where *Trifolium* spp. can be incorporated into grass pastures in a sustainable manner. Results will lend themselves to the management of soil environmental factors that affect the rhizobial-legume symbiotic relationship, in turn affecting the amount of atmospheric N fixed

(Sprent 1979). By incorporating more atmospheric N into the pasture system, by means of symbiotic N fixation, inorganic N input costs could be reduced. Profitability of dairy-pasture systems will subsequently be boosted. It is also expected that this research could make a valuable contribution to organic farming systems.

The hypotheses of this study is that soil organic matter can play an important role in ensuring good soil health, which supports the optimal growth and production of *Trifolium* spp. in low N input pastures.

4. LITERATURE REVIEW

4.1. Origin, distribution and characteristics of *Trifolium repens*

4.1.1. Taxonomy

Trifolium is a genus containing 250 – 300 species, *Trifolium repens* being agronomically the most important (Van Keuren and Hoveland 1985, Williams 1987a, Lewis *et al.* 2005). Taxonomically *T. repens* L. is classified in the sub-family *Papilionoideae* of the *Fabaceae* family, commonly known as the legume or pod-bearing family (Tainton 2000, Lewis *et al.* 2005). *T. repens* originates from Europe and Asia, but is widespread throughout the world and is even regarded as a weed in certain agricultural practices (Van Wyk and Malan 1988, Lewis *et al.* 2005). This particular species is a perennial, herbaceous, prostrate leguminous species that produces stolons and rhizomes from a primary stem (Figure 1) (Meredith *et al.* 1955, Thomas 1987b). The leaves are glabrous, palmately trifoliolate and leaflets are often characterised by a white V-shaped marking on the adaxial surface (Burdon 1983, Williams 1987b).

4.1.2. Root characteristics

With the exception of some large leaved Ladino-type *T. repens* cultivars, the plant relies initially on a weakly developed taproot system (Figure 2), then, subsequent to stolon propagation, secondary roots develop at nodes and the primary taproot eventually dies, leaving the plant nutritionally dependent on the secondary root system (Tainton 2000). The

secondary root system is weakly developed and, therefore, plants require soils with sufficient moisture (Meredith *et al.* 1955).

4.1.3. Flowering

Numerous flowers are grouped into heads on erect, leafless stalks (Figure 1) (Thomas 1987a, Van Wyk and Malan 1988).

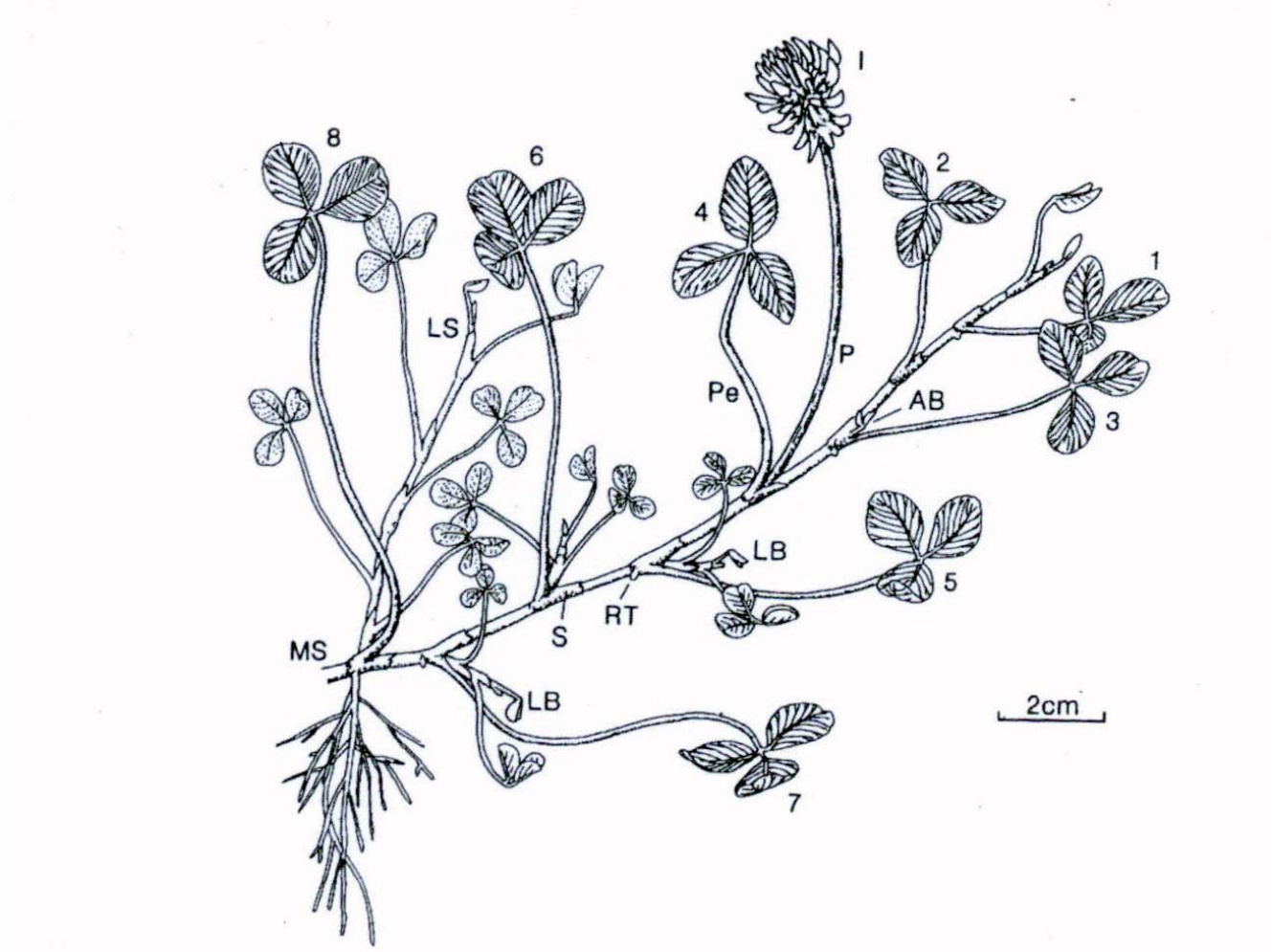

Figure 1: The main stolon (MS) of a *Trifolium repens* plant. The following organs are annotated: AB – apical bud, LB – lateral branches, LS – lateral stolon, S – stipule, Pe – petiole, RT – nodal root primordium, I – inflorescence, P – peduncle. The emerged leaves on the main stolon and the nodes bearing them are numbered 1 to 8 according to descending age (Thomas 1987b).

Initiation of anthesis as a response to a photoperiod is influenced dually by day length and temperature. It is a short-long-day plant, this means that low temperature substitutes for the short day effect and after low temperature stimulation, the plants respond as long day plants (Salisbury and Ross 1992).

4.1.4. Seed development and characteristics

The pods of *T. repens* are oblong and sessile containing three to six heart-shaped seeds, with a thousand-seed-mass between 0.6 g and 0.8 g (Burdon 1983, Thomas 1987a). The size and shape of the seeds are illustrated in Figure 3.

Figure 2: The primary taproot system of a *T. repens* after 10 weeks of development.

The quantity of stored nutrients in the endosperm has a major impact on the mass of ripe seeds. Sub-optimal managerial practices lead to lower endosperm production, resulting in seeds with low thousand-seed-masses (Thomas 1987a). Larger *T. repens* seeds result in a

greater emergence rate than for smaller seeds, with growth in the early stages of development also greater for larger seeds as the seedling still relies on the stored nutrients from the endosperm (Harris 1987). Lighter seeds result in an extended time before the seedling shows signs of nodules (Table 1). The lack of interaction between N source and the seed size indicates that the early growth of all seed sizes was not met by symbiotic N fixation (Table 1) (Mytton 1973). Early growth differences due to seed size can not be extrapolated to production differences in mature plants (Harris 1987).

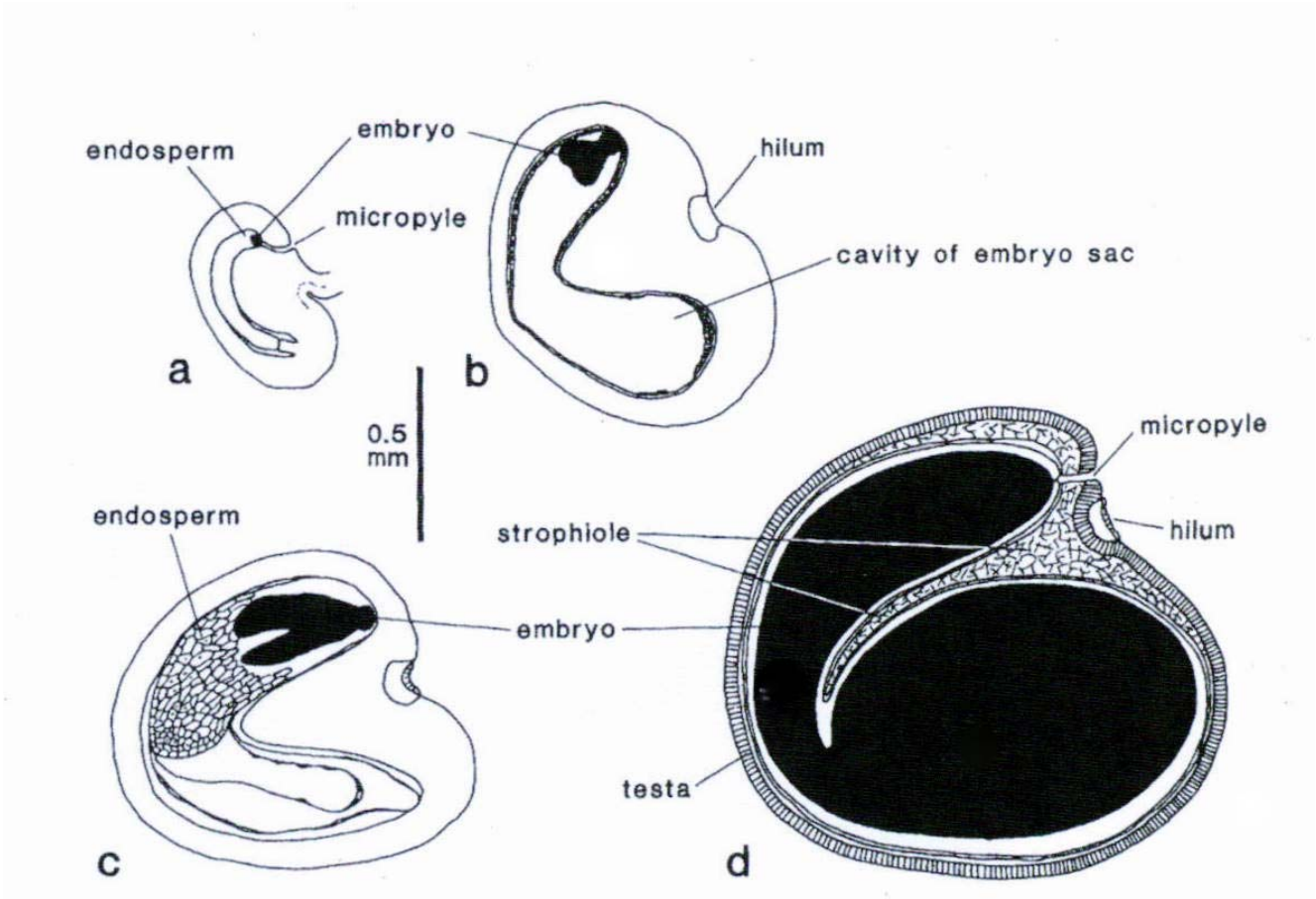

Figure 3: Longitudal sections of the successive stages of ovule development to a mature seed (a to d) (Thomas 1987a).

Table 1: The total dry matter (DM) production of *T. repens* seedlings after 30 days and days to nodulate as affected by seed weight (Mytton 1973).

	Light seed (0.36 – 0.45 mg)	*Intermediate seed (0.56 – 0.65 mg)*	*Heavy seed (0.76 – 0.85 mg)*
Days to nodulate	8.93	7.57	7.61
Reliant on N fixation only	783	1400	1603
Reliant on N fixation and fertilisation	1436	2011	2303

4.1.5. Nutritive value

The plant forms dense colonies frequently as a companion crop with grasses and other plant species. It is highly palatable and nutritionally superior to grasses, especially in calcium (Ca), phosphorus (P), magnesium (Mg), copper (Cu) and cobalt (Co). The voluntary dry matter intake of sheep and cattle are 20% more than those compared with grass only, and the ruminal passage rate of particles is more rapid (McDonald et al. 2002). This makes incorporation of *T. repens* particularly suitable for introduction into grass pasture systems.

4.1.6. Breeding of cultivars

Breeders of *T. repens* cultivars have attempted to primarily select for traits that will improve the production of grazing animals. Among the important traits that have been pursued were yield, competitive ability, persistence, complementarities with companion grasses, intrinsic quality and anti-quality factors, compatibility with *Rhizobium* bacteria and disease or pest resistance (Williams 1987b).

Trifolium spp. are widely distributed in the world. As a result adaptive genetic and ecophysiological diversity in traits are profuse. This provides ample genetic variation that is available for plant breeding and genetic manipulation. By 1988, more than 230 cultivars had been developed from multiple countries, each bred specifically to strongly fit the relative target environment (Williams 1987a, Caradus *et al.* 1989). The four applicable *T. repens* cultivars used in this study, their origin and breeding purpose are discussed:

- **Grasslands Huia** was bred specifically for increased productivity and persistency in New Zealand from indigenous ecotypes and released in 1964. It has medium to small leaves with long petioles and branches densely. Growth and production is throughout the year under South African conditions, but winter growth is slow (Williams 1987a).
- **Haifa** is more stoloniferous than other cultivars and was developed in Australia from material collected in Israel. The leaves are large and dense and plants do not grow tall. It is adapted to heat and low rainfall conditions, grows actively in winter and flowering is early in the season (Williams 1987a).
- **Regal** was bred in the USA from five clones originating in South-Eastern USA. It was released in 1962. The breeding purpose was to develop a high yielding cultivar adapted to summer conditions and enhanced persistency. It is acyanogenic and resistant to *Ditylenchus dipsaci* (Williams 1987a).
- **Ladino** is an ecotype originating in the Po river valley in Italy. It was first observed in 1834 – 1845 and has since spread throughout the world. Numerous cultivars have been bred from Ladino. Its large leaves and thick stems render it exceptionally productive. The tap-root is also more prominent compared to other smaller leaved *T. repens* cultivars or varieties. It is tolerant of a wide range of climates, but lacks persistency under a grazing regime. It is nearly acyanogenic and is resistant to *Ditylenchus dipsaci* (Williams 1987a).

4.2. Trifolium ambiguum M. Bieb.

T. ambiguum (Kura clover) originated in the Caucasian region of Russia and therefore, is occasionally referred to as Caucasian clover (Walker 2009). It is a perennial, rhizomatous plant with slower growth and establishment rates compared to *T. repens* (Townsend 1985, Walker 2009). Despite these adverse characteristics, the potential of *T. ambiguum* as a pasture species has recently generated much interest. The species has a large root and rhizome system which differentiates it from other *Trifolium* species (Figure 4) (Taylor 2008). The outstanding root system makes persistency of the species in grass pastures exceptional under heavy grazing regimes, regardless of climatic environment or altitude (Dear and Zorin 1985, Seguin *et al.* 2001, Walker 2009).

The main setback of incorporating *T. ambiguum* into grass pastures is that nodule occurrence is rare and atmospheric N fixation is subsequently low. Successful infection of this species by *Rhizobium* was only achieved in 1954 (Taylor 2008). Commercial inoculants have since been developed, but response to inoculation remains poor (Seguin *et al.* 2001). The aim of incorporating this species is somewhat different than that of other *Trifolium* spp., as the application of fertiliser-N is still necessary to maintain high quality herbage in grass pasture systems (Seguin *et al.* 2001).

Figure 4: The root system of *T. ambiguum* with various developed rhizomes.

4.3. The Nitrogen cycle

Dinitrogen-gas (N_2) is the most abundant gas and makes up approximately 78% of atmospheric gasses (Brady and Weil 2002). Nitrogen is a fundamental component for all life forms and is usually the first limiting nutrient in food production systems. Technological development of inorganic fertiliser-N for application in natural and agricultural systems has resulted in a drastic increase in the amount of global N cycling between water, soil and the atmosphere, leading to the green revolution (Bohlool *et al.* 1992, Bot and Benites 2005). According to Gruber and Galloway (2008), the optimum amount of N in food production systems has not yet been reached and even higher global application rates of N will increase production of crops yet further.

The dynamics of the soil N cycle are complex. It has many input and output pathways as well as undergoing seasonal fluxes of N content (Brady 1974). The N cycle can be divided into three sub-cycles in prevalent spheres, namely the atmosphere (air), the hydrosphere (water) and the pedosphere (soil). Detailed description of the atmospheric and hydrospheric N cycles exceeds the objectives of this literature review, and only the pedospheric N cycle will be discussed.

4.3.1. Divisions of the pedospheric N cycle

Nitrogen from the atmosphere can be incorporated into soil by biological or industrial N fixation (fertilisers), animal manures or residues, plant wastes, dissolved N in rainwater and by soil aeration (Figure 5) (Brady 1974).

Atmospheric N_2 is taken up by bacteria capable of biological N_2 fixation and converted into functional organic nitrogenous compounds required in their biological life cycles (Figure 5). The *Rhizobium* genus is the most important N fixating genus (Sprent and James 2007). These bacteria may be symbiotic or non-symbiotic (saprophytic) (Keyser et al. 1992). Industrial N fixation and deposition, or emissions of this process are a major influx of N into the pedosphere and hydrosphere (Gruber and Galloway 2008). Inorganic fertiliser-N is produced from fossil-fuels or by the Haber-process (Bohlool et al. 1992).

Most of the N in soil is immobilsed in the organic N pool. This pool of N is not available to plants for assimilation into tissues (Brady and Weil 2002). Tainton (2000) reported that 98% of the 1 to 5 $t.ha^{-1}$ N found in Kwazulu-Natal (South Africa) topsoil is bound to SOM and is, therefore, not directly available for plant uptake. For this immobilised N to become plant-available the amide functional groups ($–NH_2$) of the bound organic molecule need to be broken down (mineralised) to inorganic N compounds such as nitrates (NO_3^-) or ammonia (NH_3).

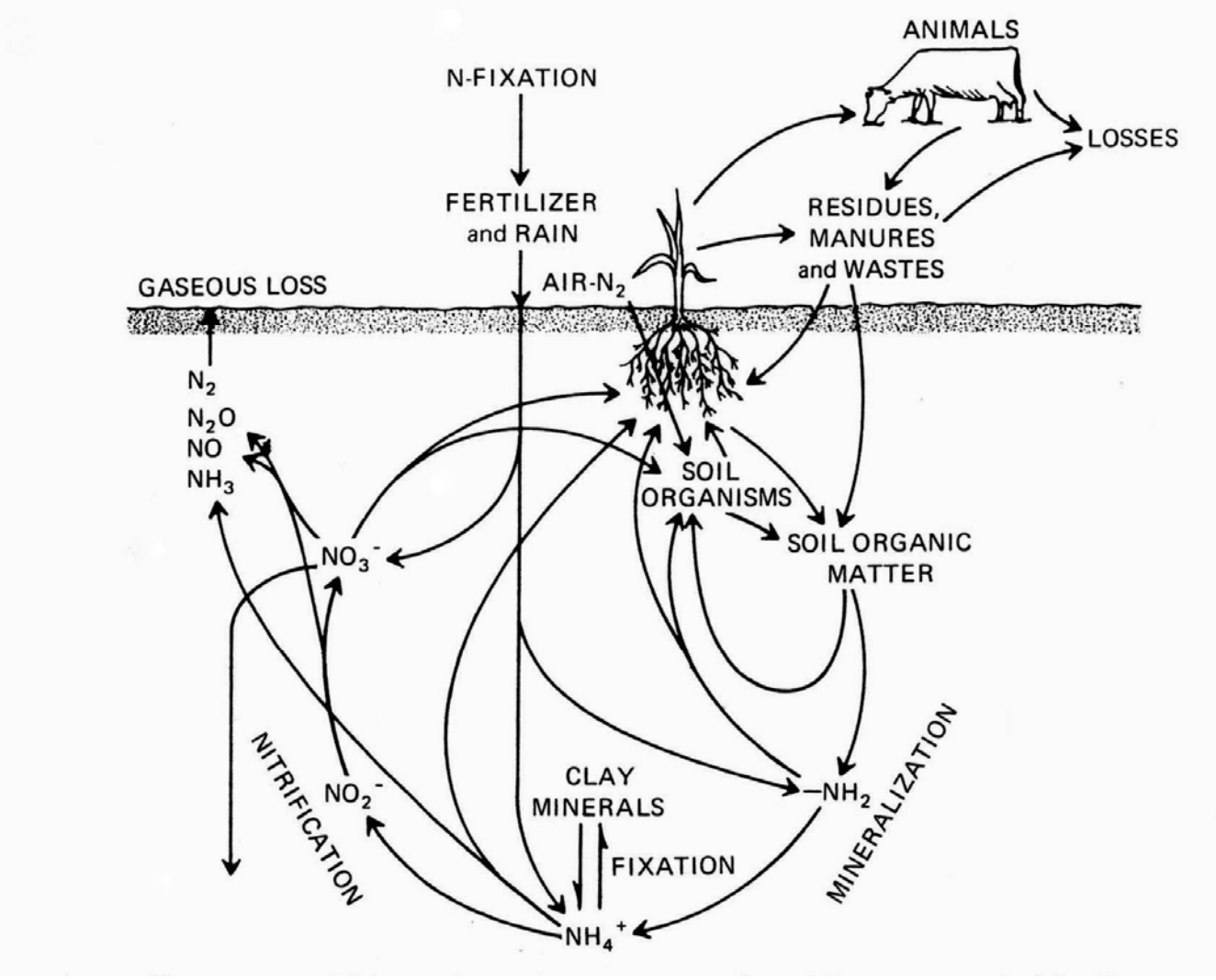

Figure 5: A basic depiction of the pedospheric N cycle (Brady 1974).

Nett rate of mineralisation is determined by equation 1 (Postgate 1973).

$$Nett\ N\ mineralisation = (NH_4^+ + NO_3^-)_{t+1} - (NH_4^+ + NO_3^-)_t \quad (1)$$

A negative value will indicate a nett immobilisation of N. The process, called ammonification, refers to microbial conversion of organic-N into ammonia. In effect, it occurs under oxidising conditions in all ecosystems where organic matter decay is occurring. Autolysis, decomposition and putrefaction of organic matter from animal manure or residues, plant wastes and bacterial matter are the principle forms of ammonification in soil. Ammonia can be oxidised to nitrites (NO_2^-) and then nitrates by nitrifying bacteria (*Nitrosomonas* and *Nitrobacter*) by a process called nitrification. Denitrification bacteria convert nitrates back into gaseous nitrous oxide (N_2O) and N_2 that escapes into the atmosphere, which are in grazing systems an undesirable loss of soil N. The rate of denitrification is mainly dependent on soil aeration and presence of nitrates. Because N is usually the first limiting factor for plant growth the state of the N cycle has a strong effect on the productivity of plants (Hart *et al.* 1994). The input sources of available soil N is indicated in Figure 6.

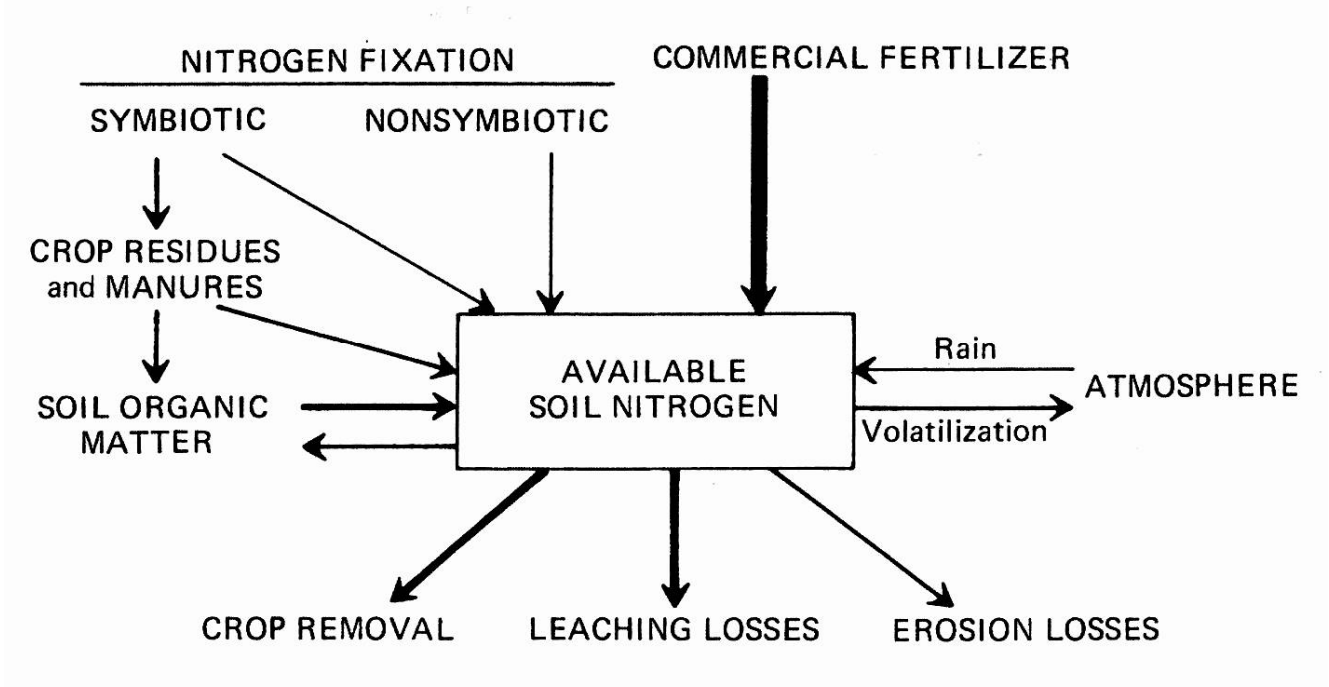

Figure 6: Inputs and output pathways of available soil nitrogen (Brady 1974).

Effectiveness of production in agricultural systems is dependent on the rate of N turnover, which is determined by the availability soil moisture and competition for inorganic N. (Postgate 1973, Høgh-Jensen and Schjoerring 1997, Gruber and Galloway 2008). The N cycle is closely coupled to the carbon and phosphorous cycles (Gruber and Galloway 2008).

4.4. The role of nitrogen in plant metabolism

Nitrogen is a vital and primary nutrient element in plant physiology. Primary nutrients are required in relatively large quantities by the plant for growth and development (Misstofvereniging van Suid Afrika 2007). The metabolism of N includes anabolism and catabolism of amino acids, porphyrins and nucleotides. Proteins, such as enzymes, coenzymes and hormones, are chains of combined amino acids that have a carbon skeleton and amide functional groups originating from ammonia (Campbell and Farrel 2006). Ammonia in plant cellscan become toxic as it inhibits ATP formation in mitochondria and chloroplasts. To prevent toxicity, the plant needs to incorporate it as quick as possible into organic compounds. All ammonia is first converted into the amide group (-NH_2) of glutamine, a particularly important plant amino acid as it is an important product for synthesis of various other amino acids and a principle form of N storage (Figure 7) (Salisbury and Ross 1992). Glutamic acid, aspartic acid, asparagine and oxaloacetic acid can subsequently be formed from glutamine. Aspartic acid is another important plant amino acid because it performs practically the same function as glutamine.

4.5. The legume-*Rhizobium* symbiotic relationship

4.5.1. Taxonomical specificity of rhizobia

The *Fabaceae* family is well known for forming a host-specific symbiotic relationship with *Rhizobium* bacteria, it is divided into three subfamilies viz. *Caesalpinioideae, Mimosoideae* and *Papilionoideae.* Nodules are rarely formed in the *Caesalpinioideae* subfamily and commonly so in *Mimosoideae*. Most of the agriculturally important species are part of the *Papilionoideae* subfamily where nodulation is also common (Sprent and James 2007). All species of the *Rhizobium* genus fix N_2 in symbiosis with legume roots (Sprent 1979). Under exceptional conditions, such as in soils with a high soil N content, legumes do not rely on

symbiosis for N availability. However, rhizobia and legumes have co-evolved to be companions and developed a relationship where the one partner is often the reason for the occurrence of the other (Woomer *et al.* 1988a). As in all other species that potentially form nodules, *Trifolium* spp. are nodulated by a host specific *Rhizobium* species. This symbiotic relationship is delicately balanced, the host plant supplies the bacteria with their nutritional and environmental needs in exchange for providing a plant-available source of N (Sprent 1979, Bohlool *et al.* 1992).

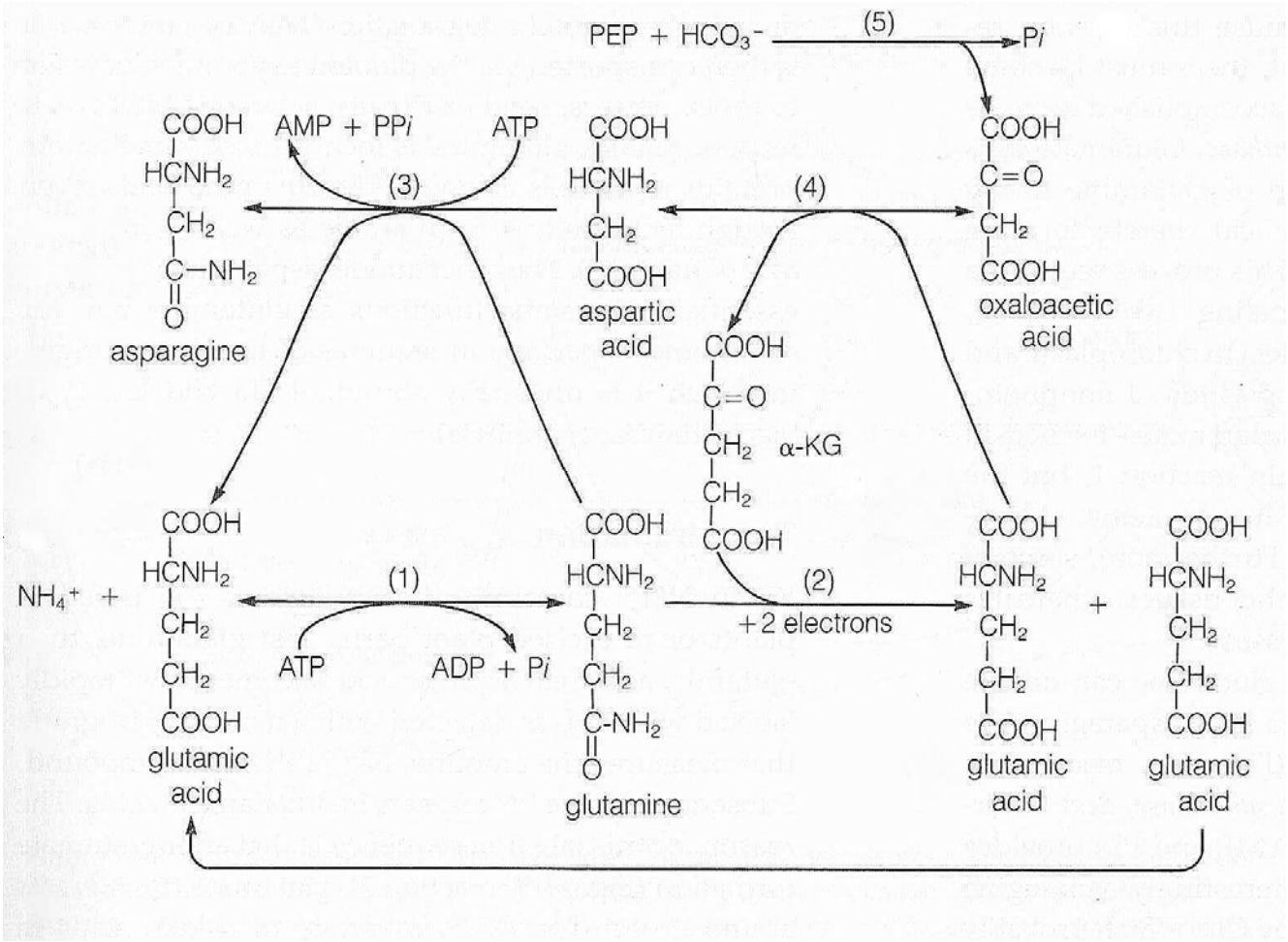

Figure 7: NH_4^+ is converted by bacteria into the amide group ($-NH_2$) of glutamine (reaction 1). It can subsequently be converted to glutamic acid, aspartic acid, asparagine and oxaloacetic acid (reactions 2, 3 and 4) (Salisbury and Ross 1992).

Even though the function of N in DNA, RNA, purine and pyrimidine synthesis is vital for plant growth, a complete discussion of the topics is beyond the scope of this literature review.

Rhizobium leguminosarum bv. *trifolii,* as classified according to the present classification system, is the rhizobial strain associated with *Trifolium* spp. (Somasegaran and Hoben 1985, Brockwell *et al.* 1995, Bot and Benites 2005). These bacteria are microaerophilic N fixers within root nodules (Kiers *et al.* 2003). Microaerophilic bacteria is a diverse and ubiquitous group of bacteria, specialised for growth in oxygen limited environments (Ludwig 2004).

Host-specificity is also regulated by the expression of particular genes. The *nod* gene, specifically *nodD*, on the rhizobial plasmid is responsible for specificity of the rhizobial nodulation on its host legume (Brockwell *et al.* 1995). From the host plant, aromatic compounds, such as flavonoids, activate nodulation by prompting expression of *nod* genes (Brockwell *et al.* 1995, Mathesius *et al.* 2000).

4.5.2. Ecological phases of rhizobial dynamics from soil to plant

Establishment of a symbiotic relationship depends on a chain of events from host-*Rhizobium* detection and recognition, infection, nodule formation to differentiation of rhizobia in nodules. Keyser *et al*. (1992) divided the ecological dynamics of rhizobia into three phases.

The first phase is the **saprophytic phase** where free-living rhizobia in soil form part of the soil microbial biomass like any other soil bacteria independent from a host organism (Woomer *et al.* 1988a). In this phase the bacteria obtain nutrients from decaying organic matter originating mainly from plants. Therefore, soil rhizobial numbers can be positively correlated to soil nutritive parameters, viz. total soil N, available S and K and P, TSS, EC and pH (Riffkin *et al.* 1999). The host plant is of fundamental importance, since plants supply suitable rhizospheric conditions and are a source of nutrients through rhizodeposition. This topic is discussed in detail in section 6.4. Spatial distribution of rhizobia, micro-habitat and microbe interaction are factors occasionally more important than nutritive factors (Keyser *et al.* 1992).

In the **infective phase**, a suitable host plant's root system is infected with species specific rhizobia that live symbiotically within it. Rhizobia are stimulated by host plant secretions, such as mucigel, a substance secreted by the host for protection to aid in binding of the root hairs to soil particles. Rhizobia are often adsorbed onto the root hairs by the mucigel (Sprent 1979). The rhizobia generally enter the root hair of the host by forming an infection thread, which

leads to a deformation of the root hair, a process referred to as root-hair curling (Somasegaran and Hoben 1985). The infection thread grows between the root hair cells until it reaches the root cortical cells that divide anticlinally once the infection threads penetrates the cell walls (Sprent 1979). Bacteria released from the threads multiply and differentiate into active N fixing bacteroides (O'Hara *et al.* 1988, Chen *et al.* 2003). The nodule meristem continues to divide until nodulation becomes apparent. Rhizobia are physically encompassed by the host plant tissue, but remain separate from host plant cells by means of a peribacteroid or symbiosome membrane (O'Hara *et al.* 1988, Madigan *et al.* 2000). The infective phase is perhaps the most sensitive phase as the rhizobia are subject to mineral supply within the rhizosphere (O'Hara *et al.* 1988, Keyser *et al.* 1992). Only a small proportion of infected root hairs give rise to nodules (Sprent 1979). Two other methods of rhizobial entry into the host have been identified, but are less common. The rhizobia can enter *T. repens* via wounds and openings in the root epidermis, or through undamaged epidermal tissue of the roots (Sprent and Faria 1988, Mathesius *et al.* 2000). However, infection of mature roots is uncommon (Mathesius *et al.* 2000).

The third phase is reached when the rhizobia have differentiated in the nodule and are fully functional as N fixers. Once the bacteria have transformed to bacteroides inside the nodule, they are entirely covered by host plant tissue. The bacteroides are then fully dependent on the host plant for their nutritional demands and finally enter the **symbiotic phase.**

Rhizobia have the ability to store some nutrients, at least for a few generations. However, nutrients accumulated by rhizobia will not be sufficient to sustain nodule growth and development (O'Hara *et al.* 1988). The rhizobia are dependent on the host plants for a continuous supply of compounds synthesised from photosynthesis. In exchange the host plant benefit from the relationship by utilising the reduced N and incorporating this essential element into plant tissues. Symbiotic bacteria are intermediaries in converting N_2 to a plant available source of N. The contribution of fixed N into the soil by free-living rhizobia is relatively small compared with the amounts fixed symbiotically (Bohlool *et al.* 1992).

4.5.3. Biochemistry of N fixation

Atmospheric N_2-gas is strongly bound by a triple intramolecular bond and is therefore, a very stable molecule, unavailable to plants (Kotz *et al.* 2003). Rhizobia mediate the chemical reaction that break this bond by providing energy by means of the nitrogenase enzyme complex to destabilise the bonds and reduce the molecule to ammonia (Sprent 1979). This enzyme consists of two metalloprotein components. The Fe-S-protein component, also called dinitrogenase reductase, couples adenosine triphosphate (ATP) hydrolysis to electron transfer. The Fe-Mo-protein, or dinitrogenase, offers the active site for N_2 reduction (Sprent 1979, Lowe *et al.* 1980, Salisbury and Ross 1992, Campbell and Farrel 2006). ATP supplies the energy to break the N_2 triple bond and is dephosphorylated to adenosine diphosphate (ADP) and an inorganic phosphate ion (P_i). The half-reaction (Equation 2) of reduction of N_2 to ammonia in nature is called biological N fixation (Salisbury and Ross 1992, Campbell and Farrel 2006). The inputs of this process are N_2-gas, water, electrons and photosynthetic carbohydrate energy that is supplied by the plant in the form of ATP. The first stable product is ammonia (Salisbury and Ross 1992, Madigan *et al.* 2000).

$$N_2 + 16\ H_2O + 8\ e^- + 16\ ATP \xrightarrow{\text{nitrogenase enzyme complex}} 2\ NH_3 + 16\ ADP + 16\ P_i + H_2 + 8H^+ \quad (2)$$

The bacteroid and the plant can assimilate this ammonia into organic forms. The export products are in the form of amides and ureides (Figure 8) (Madigan *et al.* 2000). Interestingly, acetylene reduction to ethylene is also mediated by dinitrogenase reductase and dinitrogenase (Salisbury and Ross 1992, Madigan *et al.* 2000). Acetylene reduction assays are an indirect estimate of N fixation, and are used to measure the activity of the nitrogenase complex (Boddey *et al.* 2000).

Enzymes, like all other proteins, can be denatured in several ways (Campbell and Farrel 2006). Rhizobia, being microaerophilic, must be adapted to alter the oxygen-rich environment around them as oxygen denatures the nitrogenase enzyme complex to a state in which it is permanently damaged (Postgate 1973, Zahran 1999). A small and steady supply of oxygen to the bacteroides must be controlled since this is necessary for ATP synthesis.

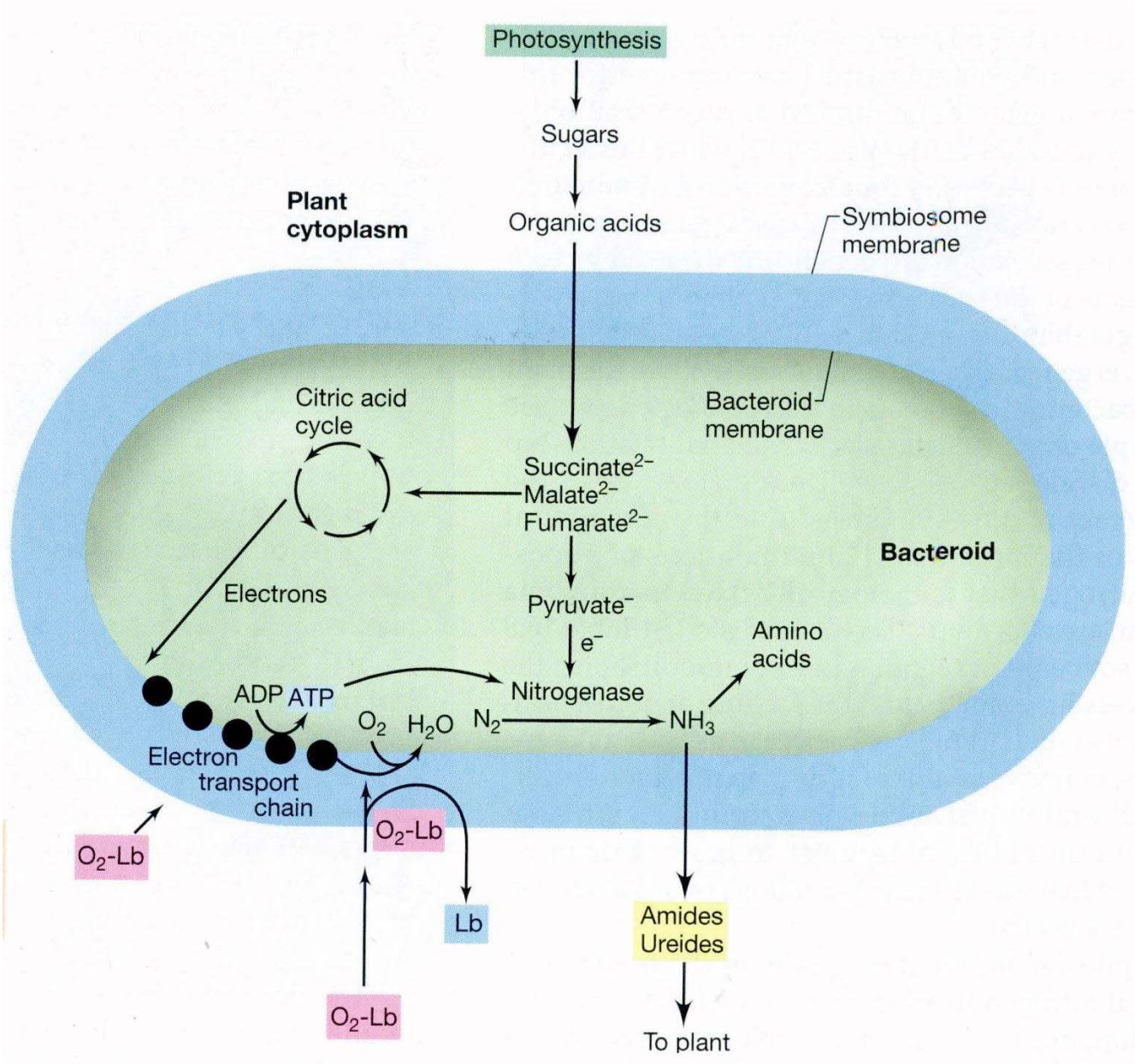

Figure 8: A representation of the chemical and metabolic reactions in the bacteroid. The influx and efflux products are indicated. Lb = Leghaemoglobin (Madigan *et al.* 2000)

Leghaemoglobin is an oxyphilic pigment inside root nodules that is in part responsible for the regulation of a uniformly low oxygen concentration (Sprent 1979). It is structurally and functionally similar to myoglobin found in animal cells, and has a high affinity to bind oxygen. It is high enough that the flux of oxyhaemoglobin exceeds that of free oxygen in the nodule by a factor of 10^4 to 10^5 (Sprent 1979). Leghaemoglobin prevents high intranodular

concentrations of oxygen and provides protection against nitrogenase degradation (Postgate 1973). Plant cortical cells surrounding vascular bundles and cells containing rhizobia also play a major role in keeping the oxygen levels low (Salisbury and Ross 1992).

Once N_2 is reduced to ammonia, it has to be transported out of the bacteroides before it can be metabolised and assimilated into host plant tissues. Ammonia is converted in the bacteroides to ureides (allantoin and allantoic acid) and asparagine. The ureides are degraded in plant tissues back to ammonia that is used for amino acid and protein synthesis. Excessive N in the shoot tissues is transported back to the roots by phloem (Salisbury and Ross 1992).

4.5.4. Factors affecting rhizobial dynamics and N fixation in the rhizosphere and nodules

Management of legumes is multifaceted and generally difficult to optimise due to the nutritional and environmental requirements of the host plant and of the micro-symbiont. Sprent (1979) identified four general factors affecting the legume-*Rhizobium* relationship:

- Phytosynthates produced during photosynthesis must be in sufficient supply for nodule metabolism. Factors affecting plant growth will influence production and supply of these compounds, which must then be transported effectively to the nodules.
- Nodules must be able to respire effectively.
- Conditions affecting N fixation must be optimal.
- Rhizobial synthates (N compounds) must finally be transported and redistributed throughout the plant.

It has been observed in many instances that introduced rhizobia from an inoculant fail to multiply and persist in soil (Lawson *et al.* 1987). Any environmental or managerial constraint can limit multiplication, population size, persistence and survival of the rhizobia and, therefore, the plant-bacterial symbiotic production. Management of the soil environment to prevent a loss in potential symbiotic production, the most limiting constraint needs to be

ameliorated. Potential symbiotic production will increase until the next factor becomes limiting (Bohlool *et al.* 1992).

The interactions of microbes with and within their environment are complex. Even though extensive reviews have been done in this particular subject, a complete understanding of the interaction is difficult. Several environmental and managerial factors will be reviewed:

4.5.4.1. The host plant effect and nutritional factors

The rhizosphere environment is the region in the soil immediately adjacent to the plant root surface (Van der Watt and Van Rooyen 1995). Microbial populations in the rhizosphere can be two to ten times (Brady and Weil 2002) or even up to 500 times (Bot and Benites 2005) higher than in the bulk soil. Environmental conditions have a strong influence on the rhizosphere and plant factors influence the region significantly. Rhizodeposition is the process by which organic and inorganic compounds are transferred from the plant roots to the soil. This can be either by means of root exudates or root breakdown (Gylfadóttir *et al.* 2007). The abundance and composition of microbial populations in the rhizosphere are supported by rhizodeposition as this is a nutrient source available for microbe proliferation (Van der Watt and Van Rooyen 1995, Brady and Weil 2002). Therefore, rhizobial abundance is highly reliant on the occurrence of a host plant and numerous linear regressions predicting the quantity of rhizobia occurring naturally in soil in Hawaii have been developed with a high level of significance (Table 1). These models predict the densities of rhizobia in soil (Woomer *et al.* 1990a). Vegetation characteristics, such as *P. clandestinum* mat thickness, also influence the soil rhizobial numbers as strong correlations were obtained between rhizobial abundance, host plant cover and decreased plant mat thickness (Woomer *et al.* 1990a).

Woomer *et al*. (1988a) and Woomer *et al*. (1990a) concluded from the regressions that the abundance of *Rhizobium* in the saprophytic phase was dependent on the presence of a host plant. *T. repens* establishment frequently fail when incorporated into grass systems and it can not be ascribed to the failure of the rhizobia to persist in the soil, but rather to above-ground competitive effects.

Table 2: Multiple regression models for *Rhizobium leguminosarum* bv. *trifolii* numbers in Hawaiian soil (Woomer *et al.* 1988a, Woomer *et al.* 1990a).

Regression	R^2	P-value
2.9 + 0.0005 *T. repens* (kg ha^{-1})	0.78	< 0.05
0.005 *T. repens* (kg ha^{-1}) + 0.73 (pH) – 1.3	0.84	< 0.01
0.17 + 0.08 *T. repens* (%) + 0.47 (pH)	0.80	< 0.05
1.50 + 0.06 *T. repens* (%) + 0.35 (pH) – 0.04 *P. clandestinum* grass mat (cm)	0.80	< 0.05
2.07 + 0.056 (% legumes)	0.75	< 0.01
1.45 + 0.002 (Mean annual rainfall)	0.82	< 0.001
1.33 + 0.030 (% legumes) + 0.0013 (Mean annual rainfall)	0.89	< 0.001
0.48 + 0.033 (% legumes) + 0.0016 (Mean annual rainfall) + 0.028 (total extractable bases)	0.95	< 0.001

Photosynthetic activity directly affects the quantity and composition of root exudates. Any external factor that enhances photosynthetic ability of the host plant, such as availability of nutrients and water, solar radiation, defoliation etc., will influence the quantity and diversity of associated rhizospheric bacteria (Lawson *et al.* 1987, Keyser *et al.* 1992). This is reinforced by the fact that N fixation is highest in early afternoon, due to the rapid transportation of carbohydrates from the leaves to the roots at this time of day (Salisbury and Ross 1992). This also puts more strain on a nodulated host plant to respire at a higher rate compared with plants that only rely on soil N uptake (Michaelson-Yeates *et al.* 1998).

Other intrinsic plant factors that may have an influence on nodulation or rhizobial abundance in soil have not been clearly identified. It is suggested that an antibiotic substance in the seed coats of *T. repens*, *T. subterranean* and *Medicago sativa* may be partially responsible for inhibition of nodule bacteria (Thompson 1960).

Bacteria require a balanced nutrient supply to proliferate in soil. Essential nutrient elements for successful N fixation from a symbiotic relationship are carbon (C), hydrogen (H), oxygen (O), nitrogen (N), phosphorus (P), sulphur (S), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), molybdenum (Mo), boron (B), chloride (Cl), nickel (Ni) and cobalt (Co) (O'Hara *et al.* 1988). The requirement level

and function of each of these nutrient elements necessary for the plant-rhizobial relationship is shown in Table 2. Amelioration of any plant nutrient deficiency in soil can lead to enhanced rhizobial colonisation of the rhizosphere (Brockwell *et al.* 1995).

Table 3: Nutrient requirements and function of *Rhizobium* and *Bradyrhizobium*. Compiled from O'Hara *et al*. (1988) and Salisbury and Ross (1992).

Nutrient element	***Requirement level***	***Comments***	***Function***
P	0.006 - 0.5 µM	Significant strain differences. Storage capacity of 1 – 2% P (DM)	Growth and development Metabolism Nucleotides Membrane activity Energy metabolism
S	3.1 – 20 µM	---	Amino acid metabolism Vitamins (thiamin, biotin) Respiration and fatty acid metabolism: Coenzyme A
K	Linear response up to 0.006 mM	---	Activator of enzymes Regulate osmotic potential
Mg	100 µM	Divalent cation requirement of 0.5 mM	Energy metabolism Enzymes Metabolism, Nucleotides

Table 3 (continued)

Nutrient element	*Requirement level*	*Comments*	*Function*
Ca	16 – 31 µM. 300 µM when P-levels are low	See above mentioned comment. Rhizobia are not Ca sensitive organisms	Cell wall integrity Regulator of growth Growth of infection thread Aid in root hair infection Enzymes
Fe	5 – 200 µM	Scavenge for Fe by means of ligands and siderophores	Enzymes Proteins Metabolism
Mn	0.1 – 10 µM	---	Enzyme activator Photosynthetic split of water
Zn	0.1 – 1.0 µM	---	Enzymes
Co	0.002 – 0.1 µM	---	Synthesis of Vitamin B_{12} (Cobalamin)
Mo	No quantative data available	---	Breakdown of Purines Enzyme: Nitrate reductase – Mo-Fe protein complex Abscisic acid (Hormone)
Cu	No quantative data available	---	Proteins Enzyme: Cytochrome oxidase
B	---	Non essential for rhizobia, but essential for host plant	Proposed biochemical and physiological function
Ni, V	---	Possibly beneficial	Ureide metabolism, especially important in legumes

4.5.4.2. Inorganic fertiliser-N

It is a well documented fact that high concentrations of inorganic and organic soil N decreases nodulation success and, therefore, also decreases the proportion of N fixed

(McAuliffe *et al.* 1958, O'Hara *et al.* 1988, Davies and Evans 1990, Thies *et al.* 1991, Bohlool *et al.* 1992, Hogh-Jensen and Shoerring 1994, Brockwell *et al.* 1995, Harris and Clark 1996, Høgh-Jensen and Schjoerring 1997, Ledgard *et al.* 1999, Seneviratne *et al.* 2000, Herrmann *et al.* 2001, Ledgard *et al.* 2001, Abbasi and Khan 2004). Ledgard *et al.* (2001) reported that the two key factors determining clover content in grass pastures and N fixation is grazing management (stocking rate, SR) and N supply. There was no significant effect of fertiliser-N application on N concentration in *T. repens* plants (Table 3). At a low rate of fertiliser application, the higher symbiotic rate of fixation completely compensated for a low mineral N input. In other words, the proportion of atmospheric N fixed in total herbage N content decreased with increasing levels of N fertilisation.

Table 4: Effect of N fertilisation and stocking rate (SR) on clover production, total clover N and N fixation (Ledgard *et al.* 2001).

	0N, Low SR	*200N, Low SR*	*400N, Low SR*	*400N, High SR*	**SED**
Clover production (kg.N.ha^{-1}.yr^{-1})	2494	1964	997	1425	265
Clover % N	4.60	4.68	4.70	4.66	NS
Total clover N (kg.N.ha^{-1}.yr^{-1})	116	93	48	66	12
Proportion of total clover N from N fixation (%)	76.7	63.7	48.3	42.9	3.0
Fixed N in herbage (kg.N.ha^{-1}.yr^{-1})	90.7	58.0	22.8	31.4	9.0

0N, 200N and 400N = No N application, 200 kg N.ha^{-1}.yr^{-1} and 400 kg N.ha^{-1}.yr^{-1}

SED = Standard error of difference; NS = Not significant

In the treatment where no fertiliser-N was applied, the proportion of herbage-N that was derived from the atmosphere was 76.7%. This proportion was less than 50% in the treatment where 400 kg N.ha^{-1}.yr^{-1} was applied. This is due to a substitution effect of N uptake of fertiliser-N, rather than fixed N. This substitution is a transient effect and N fixation activity resumes once soil N levels are depleted (Harris and Clark 1996). Ledgard *et al.* (2001) also found that the annual amount of N fixed varied greatly between years, although the proportion of fixed N in the 0N treatment always remained the highest (between 47 and 125 kg N.ha^{-1}.yr^{-}

^{1}) compared with the 400N treatment (between 11 and 69 kg N.ha^{-1}.yr^{-1}). Annual N_2 fixation decreased with 0.27 kg N per kg fertiliser-N applied. A similar data pattern was obtained by Ledgard *et al.* (1999) in a long term trial conducted on dairy pastures in New Zealand. In grass-clover mixed swards, the competitive ability of the grasses was usually greater than that of the clovers. The proportion of clovers in the botanical composition of the pasture decreased with increasing levels of fertiliser-N application (Harris and Clark 1996, Ledgard *et al.* 2001).

Fertiliser-N application not only has a substitution effect, but it also changes the morphology of the clover plant, which may indirectly reduce the N fixation potential (O'Hara *et al.* 1988, Harris and Clark 1996). It has been reported that clover plants had shorter and fewer stolons at a fertiliser application rate of 400 kg N.ha^{-1}.yr^{-1} compared with the control treatment (Ledgard *et al.* 2001). It was suggested that morphology of the legume in a grass-legume mixed sward has a pronounced effect on the persistence of the legume in the pasture (Woomer *et al.* 1990a).

Nodule number, growth rate, mass per plant and per unit root weight also decreased with increased levels of fertiliser-N application (Harris and Clark 1996, Michaelson-Yeates *et al.* 1998, Ledgard *et al.* 2001). The magnitude of the effect that nitrate application had on nodule growth rate was in some respect also related to the genetics of the plant (Ledgard *et al.* 2001). Even though nodulation number, growth rate and mass were limited by high levels of soil N, nodulation was seldom prevented (Woomer *et al.* 1990a, Michaelson-Yeates *et al.* 1998). In soybean trials conducted in Sri Lanka, fertilisation resulted in markedly greater plant yields, but there was no significant variation in nodule dry weight. It has also been found that level of fertiliser application did not have any effect on the indigenous rhizobial strains in the soil (Seneviratne *et al.* 2000).

4.5.4.3. Interaction with other microbes

The interactions between different microorganisms and the host plant is complex. It is a function of competition between one or more microorganism species or populations in close association, host plant and other plants. Interactions often affect plants in a synergistic manner (Grayer and Kokubun 2001, Slattery *et al.* 2001). Certain microorganisms improve

the root health and quality of the rhizospheric environment, while others are detrimental to root growth (Keyser *et al.* 1992).

Indigenous organisms in the rhizosphere may compete for the same resources and space that the rhizobia require. Successful establishment and effective performance of introduced rhizobia can be difficult when many microbes are present in close proximity (O'Hara *et al.* 1988, Bohlool *et al.* 1992).

4.5.4.4. Bacteriophages

Bacteriophages are another biotic factor that needs to be considered in the soil environment. These viruses that specifically infect bacteria were discovered independently by Twort and d'Herelle (Somasegaran and Hoben 1985, Campbell and Farrel 2006). The virus injects its genetic material into the host bacterial cell, using the host cell's apparatus, and replicates within the cell. Once the virus has proliferated enough to cause the cell to die and lyse, multiple copies of the virus are released to the surrounding environment (Somasegaran and Hoben 1985). Rhizobiophages are bacteriophages that specifically use rhizobia as host cells. Rhizobiophages can diminish rhizobial persistency in soil. This can directly influence bacterial population sizes in the soil through cell lysis either inside the nodule or in the soil (Loos 1963). The presence of bacteriophages may also drive the evolution of rhizobia in a direction where bacteriophage resistant lysotypes will be more profuse (Loos 1963, Lawson *et al.* 1987).

4.5.4.5. Biocides

Currently, increasing pressures to produce more food per unit area forces agriculturalists to incorporate a biocide application schedule to prevent or minimise economic losses caused by pests and diseases. Biocides important in agriculture include herbicides, fungicides, insecticides, rodenticides and nematocides. Application of biocides exerts various stresses on non-target organisms in the vicinity. A number of biocides have been reported to inhibit rhizobial proliferation in soil (Slattery *et al.* 2001, Brady and Weil 2002). The specific fate of each type of biocide will be determined in large by the chemical structure of the active ingredient and carriers. The rate of biocide degradation is affected by a range of edaphic and climatic factors (Brady and Weil 2002). A slow rate of degradation has an effect on the degree of inhibition of rhizobial proliferation and persistency. There have been some reports

that some pesticides may even enhance the activity of N-fixing bacteria. In these cases, the pesticide reduces the activity of competitors for substrates and space, thus the rhizobia benefits from the situation (Brady and Weil 2002). Taking all the environmental, plant, microbial and chemical factors of pesticides into account, their impact becomes difficult to predict. A thorough evaluation of soil and climatic properties is necessary to predict at least to some degree, the potential impact of the biocide as it is applied and subsequently to adopt a management programme that will take rhizobial persistence into consideration.

4.5.4.6. Climatic factors and soil moisture status

There are generally dual opinions on the effect that climatic factors have on rhizobia in the rhizosphere. Some researchers' view is that external climatic factors may not directly affect soil rhizobial populations, but would rather affect growth and production of the host plant. This will in turn affect the rhizobial abundance in soil (Lawson *et al.* 1987, Woomer *et al.* 1988a). Direct correlations of rhizobial population sizes to other external factors such as soil moisture, soil type and temperature are not clearly distinguished from the plant effect. However, the fact that climatic extremes in soil will affect rhizobia cannot be ignored. Most agriculturally important regions do not have such cases of climatic extremes and, therefore, less emphasis is placed on the direct influence of climatic factors on soil rhizobia.

Lawson *et al.* (1987) developed a regression in which rhizospheric rhizobial populations are related to host plant height and solar radiation. The quantity of N fixed is strongly related to the physiological state of the plant. Therefore, the host plant is considered as the dominant partner in the relationship (Brockwell *et al.* 1995). The abundance of rhizobia and quantity of N fixed also co-vary with rainfall (Palmer and Iverson 1983, Woomer *et al.* 1988a). Rhizobial populations decrease as the ambient temperature increases and the soil moisture decrease which is often the cause of varying population sizes over seasons (Slattery *et al.* 2001). Most rhizobia have an optimal growth temperature of between 28°C and 31°C (Zahran 1999), however, in temperate regions the soil temperatures seldom reach this high. *T. repens* has an optimal temperature range of 15°C to 25°C (Botha 2003), but because the plant is the dominant partner in the relationship soil temperatures are nevertheless closer in the host plant's optimal temperature growth range. The complexity of the interaction between different external factors is emphasised by survival of rhizobia in high soil temperatures being favoured

by aggregated soil and dry conditions, rather than moist conditions (Zahran 1999). Thus, a specific temperature will have different effect on rhizobia in different soil types. Low soil temperatures favour *T. repens* growth and absorption of N when the clover plant relies on uptake of inorganic soil N (Ledgard *et al.* 2001).

Low soil water content adversely affects nodule initiation, growth and activity (Sprent 1979). Nodules are more sensitive to low soil water potential than the roots themselves (Brockwell *et al.* 1995). N fixation can directly be inhibited by low water potential inside the nodule. Export and import of products within the nodule is then depressed (Sprent 1979). To the contrary, waterlogged conditions reduce root and nodule gaseous exchange. ATP production is limited in conditions where soil is water saturated due to the inability to gain oxygen (Brockwell *et al.* 1995).

4.5.4.7. Soil pH (acidity and alkalinity)

Acidic soil conditions especially occur in higher rainfall areas. Acidification is a major chemical imbalance common in agricultural soil these areas, leading to impeding the production of legumes and rhizobia. *T. repens* requires soil at an optimal $pH_{(KCl)}$ of 7, with a lower limit of 5 (Botha 2003).

Slattery *et al.* (2001) summarised the effects of pH on the symbiotic relationship and are supported by Keyser *et al.* (1992) and Ballard *et al.* (2002):

- Soil pH of 5 to 6, *nod* genes are suppressed;
- Soil pH of 4 to 5, multiplication, distribution, infection and nodulation of rhizobia will be limited. The infective phase is the most sensitive phase towards acidic conditions, if rhizobia fail to attach to the roots then root hair infection fails.
- Soil pH < 4 will decrease rhizobial numbers in soil and they will fail to persist.

The absence of rhizobia in strongly alkaline soil may be more a factor of the host plant being absent in these soils (Ballard *et al.* 2002). Factors, related to acidic soil - such as aluminium and manganese toxicity and low calcium and phosphorous availability - limit effective N fixation (Woomer *et al.* 1988a, Bohlool *et al.* 1992, Slattery *et al.* 2001).

Clear differences based on acid soil sensitive and tolerant rhizobial strains were found which stress the importance of decisions regarding most suitable strains (Watkin *et al.* 2000).

Rectifying the soil pH by application of lime is not always a simple solution as this may cause major electrolyte imbalances (Brockwell *et al.* 1995).

4.5.4.8. Sodicity, salinity and osmotic stress

Soil salinity poses a serious threat to agiculture in more arid regions, compared with soil acidification, in higher rainfall areas. Saline conditions clearly alter the morphology of plants in general (Zahran 1999). The degree of success of nodulation is a factor of the plants ability to tolerate high NaCl conditions in saline soil. High concentrations of NaCl in the rhizosphere impede infection by hampering the attachment of the rhizobia to the roots (Keyser *et al.* 1992, Slattery *et al.* 2001). Crops and cultivars vary in tolerance of saline and sodic soils and the response of the plant depends on its edaphic, climatic and physiological state (Zahran 1999). Successful N fixation in saline and sodic soil requires co-selection of a suitable salt-tolerant legume crops and rhizobial strains (Zahran 1999).

4.5.4.9. The animal factor – grazing, manure and trampling

Defoliation by grazing cause senescence of roots and organic matter become available as an energy source for microbe proliferation. Root exudates may also increase after grazing. Grazing has shown to have three effects on microbes involved in soil N cycling (Patra *et al.* 2005):

1. Microbial enzyme activities are promoted. This can be explained by higher availability of carbon for mineralisation by microbes.
2. Size of bacterial population size increase. The microbial biomass is higher in frequently disturbed soil, compared with no disturbance. Cessation of grazing leads to a significant drop in the most-probable-number (MPN) within microbial biomass. Large amounts of nitrogenous compounds from faeces and urine may hamper N fixation for up to two months since rhizobia are sensitive to high concentrations of N (Høgh-Jensen and Schjoerring 1997, Riffkin *et al.* 1999).
3. Composition of bacterial and fungal populations is altered. Urine and faecal inputs are sources of concentrated nitrogenous compounds and electrolytes. Different microbial species respond differently to faecal and urinal compounds. Some are favoured by these conditions, while others are discriminated against.

Short grazing of plants may lead to increased soil surface temperatures and be detrimental to stolon growth and development. This influences the effect which the host plant has on saprophytic and symbiotic bacteria (Ledgard *et al.* 2001). Intensive stocking rates may damage plants and cause senescence of roots, making more carbon available for mineralisation (Harris and Clark 1996, Ledgard *et al.* 2001).

4.6. Effective and ineffective nodules

Nodule effectiveness may differ between species, plants or even within the same plant (Postgate 1973). Geneticists have succeeded in increasing nodule tissue of legumes, i.e. size and number of nodules, that develop, but plant yields changed little. This suggests that efficiency of N fixation decreases per unit nodule tissue. Since nodule number is inversely related to nodule size, it is suggested that plants with fewer nodules are more effective than plants with many and larger nodules. The hypothesis is, that a plant with few nodules will divert enough resources to the nodules, to allow more efficient bacterial metabolism (Crush 1987, Hart 1987). For this reason nodule respiration coincides with plant photosynthesis. During photosynthetically active light periods, plant nutrient compounds are diverted to sinks, providing energy for the rhizobia (Hart 1987). Plant sanctions are the process by which plants preferentially supply more photosynthetic resources to nodules that are fixing more atmospheric N. This also implies that the plants will not divert as much energy to the nodules if soil N is freely available (West *et al.* 2002, Kiers *et al.* 2003).

It has been found that plants are more productive when they are inoculated with rhizobia from their own nodules, this implies that plants may have a selective mechanism that opts for the rhizobial strain most effective in fixing N (Crush 1987).

Nodule colour is an indication of the activity of the nodule, since the pigment leghaemoglobin, which is required for active N fixation, has a pink-red colour. Ineffective nodules have a white colour because the leghaemoglobin content is low or the pigment is absent (Prevost and Antoun 2008). White nodules are merely a strain on the plants' resources and are nothing more than parasitic (Sprent 1979).

4.7. Rhizobial diversity and effectiveness of different rhizobial strains

4.7.1. Genetic diversity and evolutionary trend

Several factors that may have a direct or indirect effect on rhizobial populations and genetic diversity, effectiveness of N fixation and which differ between strains have been suggested by Slattery *et al.* (2001):

- Ability to colonise and survive in soil.
- Tolerance of environmental limitations.
- Competitiveness against native rhizobia occurring in soil.
- Ability to form nodules effectively to fix N efficiently.

4.7.2. Saprophytic competence

Saprophytic competency, also termed persistency, is often the measure for selection of adapted rhizobial strains (Woomer *et al.* 1988a). Introduced rhizobial strains often fail to persist in soil as they are subject to combinations of various environmental factors and competitive effects with other microbe populations (Latch and Skipp 1987, Woomer *et al.* 1990a, Bohlool *et al.* 1992). These strains are likely to be more efficient in N fixation, but commonly fail to persist in soils with naturally occurring rhizobia that are better adapted to the local conditions and therefore out-compete them. The ecological interaction of rhizobia within their environment must be understood in order to select a suitable strain for effective N fixation and persistency in the soil (Woomer *et al.* 1988a). Various studies have shown that infection of *Trifolium spp.* in year two after introduction of a rhizobial strain in year one varied between 0 and 80%. Loss of viability can be attributed to many interrelated environmental aspects. In Australia a problem, referred to as “second-year clover mortality”, occurred. This was as a result of the high soil temperatures and low water potential. The rhizobia failed to persist in the saprophytic stage under these stressed conditions (Alexander 1982, Brockwell *et al.* 1995). It was also observed in Kwazulu-Natal, South Africa and was caused by the taproot dying off and shallow secondary roots not being able to supply plants with sufficient nutrients to survive harsh conditions. It can also be caused by very low pH soils inhibiting root penetration due to high aluminium levels. A cultivar, *T. repens* cv. Dusi was bred which produced secondary taproots and survived for much longer periods (Smith 1988).

Saprophytic competency commonly differs between strains. However, endurance in soil as a genetic trait is not always desirable, as this will limit the chance of introducing other more effective rhizobial strains into the soil at a later stage. Strains with higher saprophytic competence are likely to be numerically dominant in soil (Alexander 1982, Brockwell *et al.* 1995).

4.7.3. Symbiotic N fixation

The amount of N fixed by *T. repens* varies widely depending on interrelated soil, climate and management factors, ranging from 0 to more than 680 kg N ha^{-1} $year^{-1}$ (Crush 1987, Harris 1987, Aarts *et al.* 1992, Carranca *et al.* 1999, Ledgard *et al.* 1999, Herrmann *et al.* 2001, Abbasi and Khan 2004, Dahlin and Mårtensson 2008). Legume N requirements cannot be met solely from atmospheric N fixation, especially under conditions with low soil N content (Seneviratne *et al.* 2000). This is reinforced by the fact that *T. repens* herbage yield is usually greater when fertiliser-N is applied in *T. repens* monoculture stands than in situations where the plants completely rely on biological N fixation (Harris and Clark 1996). The percentage N derived from the atmosphere (%Ndfa) is primarily related to total soil N and P (Riffkin *et al.* 1999). It is suggested that an observed reduction in %Ndfa where fertiliser-N is applied is offset by the increase in *T. repens* yield and there is, therefore, a positive association between N in soil and %Ndfa (Riffkin *et al.* 1999). To the contrary, when fertiliser-N is applied to grass-clover mixed swards, clover herbage yield is less than it would have been in a monoculture. This is due to the competitive advantage for N uptake that grasses have over clover and which, therefore, limits clover production. Application of N to a grass-clover mixed sward will favour the grass' relative growth advantage until the soil N levels become low and the clover component will increase (Harris and Clark 1996, Riffkin *et al.* 1999, Elgersma *et al.* 2000, McCallum *et al.* 2000, Ledgard *et al.* 2001, Botha 2003). This cyclical pattern of annual clover component has an amplitude of 3 to 5 years (Ledgard *et al.* 2001). Environmental effects must be optimally balanced in order for *Trifolium-Rhizobium* symbiosis to fix N at full potential.

There is generally a strong correlation between shoot weight and nodule weight. Shoot weight is, therefore, often used as an indicator of rhizobial strain effectiveness (Somasegaran and Hoben 1985). N fixation ability is strongly correlated with nodule morphology as well as

plant morphology (Varin *et al.* 2009). Nodule morphology is affected by the size of the nodule, the viability of nodule content and the extent of development of the bacteroides (Brockwell *et al.* 1995).

4.7.4. N fixation by free-living rhizobia

Apart from rhizobia, many other bacterial species are able to fix N saprophytically (Yates and Eady 1980). Cyanobacteria fixed N in rice crops at a rate of 10 to 80 kg $N.ha^{-1}$ per crop and this number varied in sugarcane plantations between 20 and 160 kg $N.ha^{-1}$ per crop (Bohlool *et al.* 1992). However, the *Rhizobium* genus remains economically the most important for agriculture (Child 1980, Palmer and Iverson 1983). Rhizobia do not rely solely on host plants for N fixation, they fix N asymbiotically implying that they have all the genetic material necessary for completion of the process (Child 1980). These bacteria are, like bacteroides in nodules, micro-aerophyllic. In order for efficient N fixation to take place the soil environment must be suitable for microbe proliferation, with only small amounts of oxygen available to rhizobia. Free-living bacteria are also subject to competition for nutrients with other bacteria in their vicinity (see section 6.4) (Keyser *et al.* 1992).

Even though rhizobia in the soil are likely to be free-living, the presence of a host plant remains one of the biggest contributors towards maintaining rhizobial abundance. Table 2 shows regression models of number of free-living rhizobia with major factors affecting their abundance in soil. Free-living bacteria export fixed N, mostly in the form of ammonia, which is beneficial for plants as it is a plant-available source of N (Child 1980).

4.7.5. Interaction between free-living and introduced rhizobia – interstrain competition and antagonistic effects

Success of inoculation is a direct response to the indigenous rhizobial population numbers (Thies *et al.* 1991, Riffkin *et al.* 1999). Thies *et al.* (1991) showed that where there are less than 10 indigenous rhizobial cells per gram of soil the plant yield was increased by 85%. Poor establishment of *T. repens* may be a result of large microbial populations in the soil leading to a strong competitive ability of other microbes on an ecological basis. Antagonists of *Rhizobium leguminosarum* bv. *trifolii* are found to be common in soils of George in the

Western Cape Province, South Africa. 16.3% of all bacteria and actinomycetes in these soils exhibited antagonistic effects on rhizobia in pure culture (Hattingh 1965).

4.8. Plant infection count (Most-Probable-Number/ MPN method)

Quantification of rhizobial populations from the many microbial populations present in soil is a challenge, especially in artificial culture media. A method, referred to as the plant infection count, has been developed that estimates the most-probable-number (MPN) of symbiotic rhizobia present in the inoculant or soil (Weaver and Frederick 1972, Somasegaran and Hoben 1985). This dilution method estimates the density of organisms in a liquid, without a direct count. It is based on the theory of probability to certain assumptions (Cochran 1950). There are three applicable assumptions for this study:

- Rhizobia are randomly distributed throughout the liquid-medium.
- Each sample will always exhibit growth whenever it contains one or more organisms.
- The organisms will not replicate in the sample (Cochran 1950).

Legume plants are grown aseptically and serial dilutions of the investigated source of rhizobia are added to an N-free growth medium. A single viable symbiotic rhizobial cell being present in the source may lead to nodulation. A pattern of positive growth pouches at different dilution levels gives a code to calculate the number of organisms in the original suspension (Brockwell 1963, Somasegaran and Hoben 1985, Scott and Porter 1986, Weaver and Graham 1994).

Minimum generation time for *Rhizobium trifolii* in a synthetic medium is five hours. However, it has been proven that rhizobia cannot reproduce in growth media during the preparation of serial dilutions and media, even though preparation of the diluents takes longer than five hours. This supports accurate measurement of the total rhizobial population number within the source as it excludes one of the assumptions (Weaver and Frederick 1972, Somasegaran and Hoben 1985, Woomer *et al.* 1990b).

Estimates of rhizobial numbers obtained from plate counting techniques differ from results obtained from plant infection techniques by 89% to 131%. When test plants are grown in vermiculite, with little rhizosphere effects, the rhizobial population count was overestimated by 39% with use of the plant infection count. The results are reproducible and correlate well with

culture-based plate counting methods for rhizobial populations (Brockwell 1963). The reliability of the MPN technique is ascertained (Woomer *et al.* 1988b, Woomer *et al.* 1990b).

4.9. Dynamics of soil organic matter

4.9.1. The carbon cycle

Carbon has four electrons on its outer orbital. These electrons can form four single bonds, a double bond and two single bonds or two double bonds. This gives carbon the ability to form an infinite amount of compounds in combination with more carbon atoms and other elements. Carbon forms, by definition, the backbone of all organic compounds and is, therefore, also the foundation of all life forms (Brady and Weil 2002, Kotz *et al.* 2003). In the global carbon cycle, five predominant spheres are integrally linked by carbon fluxes (Lal *et al.* 1997):

- Atmosphere
- Pedosphere
- Biosphere
- Lithosphere
- Hydrosphere

The pedosphere is the interception between all the spheres. It plays a major role in production of food and fibre (Lal *et al.* 1997). The aboveground biosphere is most obvious and carbon sources in this sphere are aesthetically the most important. These sources rely strongly on the less noticeable belowground sources of carbon within the pedosphere. The earth's soil contains an estimated 30×10^{14} kg organic carbon (Tate 1992). Due to the large-scale crises presented as a result of global warming, efficient management strategies must be adopted to decrease the efflux of CO_2 from the pedosphere to the atmosphere and facilitate increases in soil organic carbon. This may, in some respects, alleviate the strain of increased atmospheric CO_2-levels (Janzen *et al.* 1997).

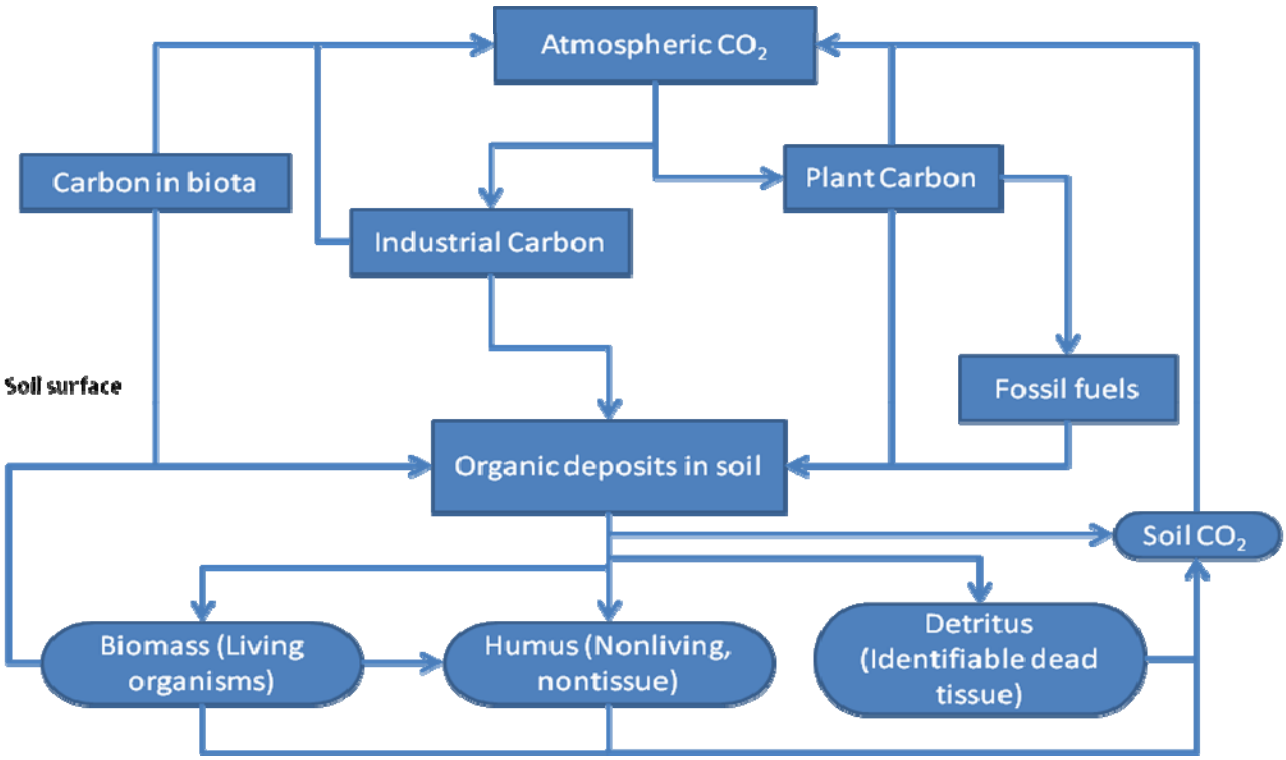

Figure 9: A basic depiction of the carbon cycle and forms of soil organic matter (Compiled from Tate, 1992; Brady and Weil, 2002).

The organic matter entering the soil primarily has three fates (Figure 9). It can be broken down and converted to CO_2, it can be assimilated into biota or it can remain in soil as a more stable source of organic matter (Tate 1992).

4.9.2. Spatial patterns in soil carbon pools

The primary source of organic matter sequestration is photosynthetic plants (Tate 1992). Total SOM is the sum of several different carbon pools in the soil. The model described by Brady and Weil (2002) divides SOM into three independent fractions, namely the active, slow and passive fractions. Organic matter in the active fraction has a C:N ratio of between 15:1 – 30:1 and has a short half-life. This provides readily available nutrients for plants and soil microbes and is responsible for most of the beneficial effects of soil quality. The passive fraction of SOM is extremely stable, and autolysis is very slow. This fraction contributes to the cation exchange capacity (CEC) of the soil, acting like colloidal clay particles. The slow

fraction is intermediate between the active and passive fractions and half-lives are typically measured in decades (Brady and Weil 2002).

Often soil disturbance leads to a rapid breakdown of SOM in soil. This is because of the fine and complex balance in chemically and physically protected and rapidly metabolisable organic matter pools found within the soil of a steady state ecosystem. Conservation of SOM can be achieved by either supplying the soil with carbon at a rate equal to the rate of decomposition and consumption of carbon, or by minimising the losses of the current soil organic carbon content. Control of soil erosion, minimum or no- tillage systems, crop rotations, addition of animal manure and application of fertilisers contribute to maintaining a high SOM content (Hendrix *et al.* 1992, Elgersma and Hassink 1997). Elgersma and Hassink (1997) showed that the inclusion of *T. repens* into pastures enhances the quality of SOM by rapidly increasing the active fraction of SOM. Debris from roots and shoots decomposes and a lower C:N ratio results. The N is then mineralised to ammonium which may then in turn be nitrified to nitrates.

4.10. Soil organic matter as a nutrient source

Soil organic matter serves as a nutrient reservoir and gives rise to a steady supply of nutrients, moisture and energy that provides a suitable environment for bacterial proliferation and plant growth (Bohlool *et al.* 1992, Tate 1992, Brady and Weil 2002). This is reinforced by the fact that there is a higher MPN of soil microbial biomass under a grazing regime, when compared with grazing cessation. The disturbance of the grasses by defoliation, by means of grazing, leads to a breakdown of grass roots and a supply of labile organic matter to be available for soil microbe proliferation (Patra *et al.* 2005). SOM is a reservoir for potential energy, but is not all readily available as a nutrient source (Tate 1992). The rate at which nutrients become available for plant and microbe utilisation depends on three aspects:

- Factors affecting mineralisation of organic carbon sources.
- Factors affecting rate of organic matter accumulation.
- Carbon inputs into soil (Tate 1992).

The rate at which nutrients become available can be illustrated by equation 3:

$$\frac{d[OM]}{dt} = \frac{dP}{dt} - \frac{dB}{dt} \quad (3)$$

Where OM is the rate of organic matter accumulation, P is rate of organic matter inputs and production and B is rate of organic matter breakdown. At the optimal SOM level, management must be such that $\frac{d[OM]}{dt} = 0$ (Tate 1992). The SOM pool becomes a nett nutrient sink if $\frac{dP}{dt} > \frac{dB}{dt}$ and a nett nutrient source when $\frac{dP}{dt} < \frac{dB}{dt}$ (Tate 1992). Soil with high organic matter content, i.e. soil C > 4%, contains a considerable amount of energy. Only some of this energy can be used by microbes and plants, the rest is dissipated as heat (Brady and Weil 2002). The processes whereby nutrients are transferred between plants, microbes and soil are referred to as biogeochemical cycles, referred to in the section 10.1 (Tate 1992).

The labile or passive organic fraction of organic matter in soil is the basic nutrient source of N, S and P (Tate 1992, Brady and Weil 2002). The major constituents of the passive fraction of organic matter are plant residues, microbial biomass, roots and root exudates. When SOM is in short supply, bacteria utilise soil nitrates and incorporate them into their cell material making them unavailable to plants (immobilisation of N). Ionisation of mineral elements is a prerequisite for assimilation into microbial or plant biomass. SOM has the potential to bind metal ions that may reduce the potential for metal toxicity in plants (Postgate 1973, Høgh-Jensen and Schjoerring 1997).

4.11. Factors and practices affecting level and quality of soil organic matter

4.11.1. Tillage or soil disturbance

Physical condition of the soil can be manipulated to affect the rate at which nutrients become available to plants and bacteria (Tate 1992). More organic matter is found in surface layers of a soil profile in no-till and minimum-till systems compared with conventional tillage systems. SOM content declines exponentially in disturbed soils and frequently cultivated pastures (Portela *et al.* 2006). Bacteria responsible for breakdown of the organic matter are limited physically by their location. Tillage exposes organic matter and microbes to air and moisture,

which in turn increases the rate of mineralisation of organic material and N turnover (Bot and Benites 2005). This decline after cultivation rapidly leads to lower soil fertility, weakened soil structure and, therefore, also lower water holding capacity and biological activity. The soil physical condition is destabilised and this will decrease future availability of a plant available N source (Tainton 2000).

4.11.2. Temperature and moisture

Biochemical reactions need energy to proceed, thus heat will increase the rate of enzyme-mediated reactions at organic matter decomposition and N fixation will consequently increase (Campbell and Farrel 2006). Therefore, the rate of carbon cycling is faster in the tropics compared with colder areas. For every 8 to 9°C increase in mean annual temperature, reaction rates are double (Bot and Benites 2005). The C:N ratio also shifts from a higher to a lower ratio, when one moves into cooler climatic conditions (Brady and Weil 2002). As all biological activities require air and moisture to proliferate, wet and dry extremes will limit them. In pasture production systems 60% of soil pores must be filled with water. Wet-dry cycles will increase availability of organic matter for microbial breakdown (Bot and Benites 2005).

4.11.3. Mineral elements and fertilisers

Any soil mineral constraint, that will cause a lower microbial biomass in soil, will also decrease mineralisation rate of carbon compounds in soil. Amelioration of deficiencies will enhance crop growth and in turn increase soil organic matter input (Janzen *et al.* 1997).

4.11.4. Soil physical properties, topography and aspect

The main factors affecting soil physical properties are texture, clay content, CEC and organic carbon. Organic compounds have pore characteristics which improve the capacity to absorb and hold water. Organic compounds also form microaggregates in soil, beneficial to the soil structure and that aids in resistance against stresses arising from traffic, wetting and abrasion (Kay 1997).

Soil in valleys is often higher in organic matter, this is due to the physical transportation of material down slopes by water runoff. Another factor that contributes is that it is often wetter in valleys and vegetation is more lush and dense, adding to the organic matter turnover. North facing slopes in the southern hemisphere are drier and hotter, which cause a more rapid breakdown of organic material (Bot and Benites 2005).

4.12. Management of soil organic matter content and quality control

Management to improve carbon sequestration must be adapted in such a way that it has at least one of the following effects:

- Increases production of organic carbon in soil.
- Increases the amount of organic carbon that is incorporated into soil.
- Decreases the rate of breakdown of organic carbon compounds in soil (Janzen *et al.* 1997).

In grazing systems, where the above-ground biomass is removed, the biggest contribution to SOM is root turnover (Peoples and Baldock 2001, Gylfadóttir *et al.* 2007). Peoples and Baldock (2001) found that leguminous organic matter decomposed and mineralised faster than non-leguminous plants. However, a stable and slow N cycle affects future N availability in agricultural soil. In Brazilian Amazon, greater concentrations of SOM result in higher rates of N fixation, but this relationship was only true under forest biomes (Neill *et al.* 1997). In areas where this forest was cleared for pastures, N fixation rates were uniformly low regardless of soil type or texture (Neill *et al.* 1997).

Organic matter accumulates under normal conditions in natural topsoils (Soil Classification Working Group 1991). This accumulation is more rapid in minimum- and no-tillage systems, when these are adopted there is some redistribution of organic carbon pools within soil. The passive fraction of SOM and microbial biomass increases rapidly in these systems, leaving the land fallow every few years will also help preserving the organic matter (Janzen *et al.* 1997).

Plants will not be as reliant on organic matter pools when nutrient fertilisation is applied to alleviate any deficiencies. Enhanced plant growth ensures a greater flow of carbon from the atmosphere to the soil, this also increasing the soil's potential to store carbon. Root growth, size of microbe populations in the rhizosphere, nutrient cycling and nutrient availability are vitally linked, affecting soil health and, therefore, plant growth. It has been suggested that the application of N may increase the rate of decomposition (Janzen *et al.* 1997). The amount of soil organic carbon and N is controlled by the incorporation of carbon into the soil and not by input of N (Elgersma and Hassink 1997). Soil microbes require a balanced nutrient supply to function optimally, carbon and N being no exception. The average optimal ratio of C:N for soil microbes is about 8:1 (Brady and Weil 2002). Most soils have a C:N ratio much higher than 8:1 and as a result the rate of mineralisation of carbon accelerates when N becomes more available in the soil. In other words, the rate of decomposition of organic matter will increase as the C:N ratio decreases (Henriksen and Breland 1999).

Adding organic matter to soil, with a higher C:N ratio than the optimal, will cause the microbes to deplete soil N (negative N period). Mineralisation of N will predominate in soil with a relatively low C:N ratio, i.e. less than 25:1. With ratios higher than 30:1, prolonged immobilisation will prevail. Clover roots and herbage have a C:N ratio of 12 to 20:1, grass roots fertilised with 350kg N ha^{-1} $year^{-1}$ 25 to 30:1 and unfertilised grass roots 40 to 50:1 (Table 5). However, the C:N ratio of a well managed pasture should remain around 10:1 (Tainton 2000). Intensive utilisation practices may exceed the balance between the rate of autolysis of organic matter and organic matter input into soil and, therefore, the SOM content will decline, resulting in a decline of soil N as well (Tainton 2000).

With recent pressures on farmers to produce more per unit area, sustaining soil quality is an essential method for more efficient and sustainable production. Soil quality is defined by Karlen *et al.* (1997) as "*the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality and support human health and habitation* (Karlen *et al.* 1997)*."* Organic matter plays vital roles in almost all aspects of soil quality since it affects soil physical, chemical and biological processes (Seybold *et al.* 1998). Figure 10 shows the beneficial effects that SOM has on soil quality.

Table 5: Typical C:N ratios of organic substances associated with soils (adapted from Tainton 2000, Brady and Weil 2002).

Organic substance	**C:N ratio**
Clover	12-20:1
Mature Lucerne hay	25:1
Young Lucerne hay	13:1
Grass roots (fertilized with 350kg N ha^{-1} $year^{-1}$)	25-30:1
Grass roots and herbage (low fertiliser rate)	40-50:1
Soil bacteria	5:1
Sawdust	400:1

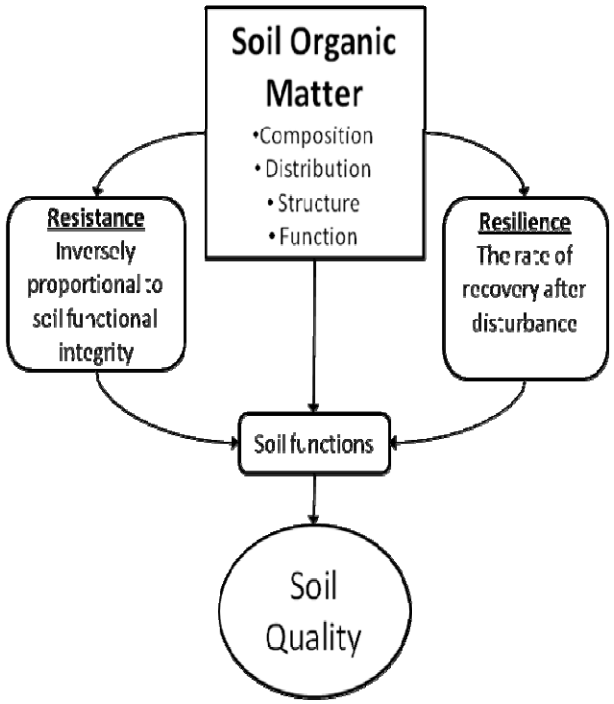

Figure 10: The relationship between soil organic matter and soil quality (Seybold *et al.* 1998).

4.13. Directions in agriculture

There are currently immense societal pressures on agriculturalists to farm not only in a sustainable manner, but also to keep the production per unit area high. These pressures

results from high and ever-increasing population densities, urbanisation, industrialisation and global warming (Tate 1992). Inorganic fertiliser N production costs are heavily dependent on the price of fossil-fuels, with the current oil price crises the manufacturing process of inorganic N has become increasingly expensive (Bohlool *et al.* 1992). Seeking biological alternatives for inorganic forms of fertiliser-N has become inevitable and imperative. Incorporation of legumes in pasture systems is economically and ecologically promising. Not only must symbiotic N fixation of legumes be maximised to be an efficient substitute for inorganic fertilisation of crops and pastures, but the efficient management of utilisation of the fixed N is just as important. A holistic aim for sustainable agriculture is attaining the maximum quantity and quality of pasture production with minimal N input in the form of fertiliser and exclusive of N pollution of environmental resources. Human health can potentially also suffer from excessive and long term application of N. Methaemoglobinemia, cancer, pulmonary and respiratory diseases are some of the adverse effects of inorganic N on human health (Bohlool *et al.* 1992). Modern agricultural practices tend to maximise output from the soil in the short term and this is a major pitfall for future generations and supply of food to an ever increasing world population.

Sustainable agriculture has many constraints. Much of the existing knowledge is difficult, and sometimes even impossible, to apply and manage in practice. Extensive research has been done on symbiotic N fixation. However, carrying the knowledge over to farm-level, especially in developing countries has been poor (Bohlool *et al.* 1992). Education and training of extension officers and farmers is time-consuming and expensive, while knowledgeable educators are scarce.

Advanced technology in biochemistry, plant breeding and genetics has made it possible to change the genetic makeup of organisms. This is limiting in the sense that it is difficult to predict the outcome and interaction of the specific modified gene in a selected environment (Bohlool *et al.* 1992). Management of soil must receive specific attention, as any one limiting factor will prevent the crop from reaching its potential yield. Future research will not only be on the improvement of existing practices but also on rectifying problems caused by our abusive past practices, waste management and soil reclamation.

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CHAPTER 2

Prepared according to the guidelines of the African Journal of Range and Forage Science

NODULATION POTENTIAL OF FOUR *TRIFOLIUM REPENS* CULTIVARS UNDER FIELD CONDITIONS

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Abstract

Four *Trifolium repens* cultivars were evaluated in field conditions to determine the effect of *Rhizobium* bacteria on the potential of these cultivars to nodulate. Nodulation of *Trifolium repens* (white clover) is often poor, because of sub-optimal environmental conditions or absence of adequate host specific rhizobia. Thus, the cultivars Grasslands Huia, Haifa, Ladino and Regal were selected for this study, as these are the most popular cultivars available in South Africa. The thousand-seed-mass (TSM) of each cultivar was measured to determine the quality and viability of the seed; and to determine the interaction between seed mass and nodulation. Mean TSM values of *T. repens* cultivars differed significantly, with Huia having the highest TSM followed by Haifa, Ladino and Regal. Biomass production was also measured as an indicator of efficiency of nitrogen fixation. The cultivar Huia, with the heaviest seed, showed the highest biomass production. After eight weeks of growth, the nodulation index was determined from the size, number and colour of the bacterially associated root nodules. All plants, regardless of cultivar, formed nodules within eight weeks. It was concluded that TSM had no notable effect on nodulation. Planting date with associated temperature effects and the intrinsic cultivar effect also had no influence on nodulation. It was therefore concluded that nodulation potential of the four cultivars tested was similar in the specific environmental conditions.

Keywords: establishment, nodulation index, rhizobia, thousand-seed-mass (TSM), white clover

1. Introduction

Trifolium repens (white clover) has long been recognised as one of the world's most outstanding and valuable forage species, especially for dairy production systems (Williams 1987a, Michaelson-Yeates *et al.* 1998, Ledgard *et al.* 2001, McDonald *et al.* 2002). The incorporation of *T. repens* in grass pasture systems has a pronounced effect on nitrogen (N) dynamics within such a system. This is as a result of the atmospheric N_2 fixation ability resulting from a symbiotic relationship with the bacterial genus *Rhizobium* that is supported by the plant within root nodules. However, a major problem is as N content of soil increases, by means of rhizodepostion, N fixation decreases (Rys and Mytton 1985). The competitive ability of companion grasses also increase and the *T. repens* plants are overshadowed until they eventually diminish into the system (Botha 2003).

Trifolium spp. are found all over the world, which results in a wide range of adaptive genetic and ecophysiological diversity of traits. This provides ample genetic variation that is available for plant breeding and genetic manipulation. In 1988, more than 230 cultivars were developed from multiple countries, each bred specifically to exist in the target environment. Many more cultivars have been developed to date (Williams 1987a, Caradus and Woodfield 1998). Breeders of *T. repens* cultivars have attempted to select primarily for traits that will improve the phytomass production for grazing animals. Yield, competitive ability, persistence, complementarities with companion grasses, intrinsic quality and anti-quality factors, compatibility with *Rhizobium* bacteria and disease or pest resistance are some important traits that have been pursued (Williams 1987a).

Clover plants under optimal management practices must be well nodulated and capable of fixing satisfactory amounts of atmospheric N to sustain productivity and maximise quality (Gylfadóttir *et al.* 2007). Nodulation of *T. repens* is, however, often poor, since cultivar selection and breeding may have had an influence on this potential in the plants (Michaelson-Yeates *et al.* 1998). Plant factors, such as root morphology, which can be altered by breeding, may also have a pronounced indirect effect on the potential of the plant to form nodules. Nodal roots are formed to replace the seedling taproot. Short stolon internodes give rise to roots that are more suitable for nodule development (Williams 1987a, Caradus *et al.* 1989). The degree of genetic variation, which may affect the extent of root morphological

manipulation and the response of selection for adaptive traits, is extensively described by Caradus and Woodfield (1998).

Assessment of nodule size, colour and number gives an indication of the success of root-infection by species specific *Rhizobium* bacteria (Prevost and Antoun 2008). The cultivar-effect of nodulation on the most popular cultivars in South Africa has, however, not yet been assessed. The aim of this study was to evaluate the degree of nodulation of *T. repens* cultivars Grasslands Huia, Haifa, Regal and Ladino.

2. Materials and methods

2.1. Experimental field trial site

This study was carried out on Outeniqua Research Farm near George, Western Cape, South Africa (Altitude 201 m, 33 58'38" S and 22 25' 16" E) (Botha *et al.* 2009). The area has a temperate climate with a long term average annual rainfall of 728 mm. Rainfall is evenly distributed throughout the year (ARC 2009). The area is characterised by an Estcourt soil type (Soil Classification Working Group, 1991). The top 250 mm of the soil profile consisted of an orthic-A horizon with a weakly developed structure. The colour of this horizon was grey or dark-grey, since it was subjected to waterlogging. This horizon has a loamy texture, an organic carbon content of 2.16% and a $pH_{(KCl)}$ of 5.7. The top horizon is followed by an E-horizon and subsequently a prismacutanic B horizon (Soil Classification Working Group 1991, Botha 2003).

2.2. Experimental design

The first component of the study entailed the determination of the thousand-seed-mass (TSM) of each cultivar by randomly selecting, counting and weighing 1000 seeds in the laboratory. This process was replicated seven times for each cultivar for accuracy, to determine the quality and viability of the seed. Secondly, germination percentage was determined by placing 100 seeds in nine replicates for each cultivar on moist filter paper, in a dark cabinet for two weeks.

The third component of this study entailed a field trial evaluating the four selected cultivars. The site was prepared in such a way that all weeds were eradicated by applying

glyphosate based herbicide. Soil was tilled with a disk harrow to level the area to form a fine and firm seedbed. The layout was a randomized block design with 16 plots in each of the three blocks. Each plot measured 50 cm x 50 cm and was equally divided into 100 small blocks of 5 cm x 5 cm each. One seed was planted per block, i.e. 5 cm x 5 cm apart from each other. Each seed was planted at a depth of approximately 3 mm. The seeds were inoculated prior to planting with a host specific inoculant, containing *Rhizobium leguminosarum* bv. *trifolii*. Each treatment was planted in triplicate on 17 July 2009, 7 August 2009, 28 August 2009 and 18 September 2009. Plants were watered periodically by using a permanent irrigation system, allowing soil moisture status to be determined with the aid of tensiometers placed at 15 cm in the soil. The soil water potential was kept between -10 and -25 kPa (Botha 2002).

Seedlings of each cultivar in the field were counted weekly from the day of emergence to determine the trend in seedling emergence over time. Each plant was monitored and accounted for to be able to determine whether or not it had germinated or died post-germination.

2.3. Cultivars

Four different *T. repens* cultivars were evaluated in the study. These cultivars selected were of the most common cultivars available in South Africa, i.e. Grasslands Huia (referred to as Huia), Haifa, Ladino and Regal.

2.4. Measurements and data analyses

The following measurements with respect to the aforementioned procedures are as follows:

The establishment percentage over ten weeks of each plot was calculated using Equation 1.

$$Establisment\ \% = \frac{nr.of\ seeds\ germinated - nr.of\ seedling\ deaths}{Total\ nr.of\ seeds\ planted} \times 100 \quad (1)$$

The plants were harvested during week ten after germination. Each plot (50 x 50 cm), was removed with a spade to the depth of 20 cm. Soil was carefully removed from the roots rinsing them with water (Somasegaran and Hoben 1985). Care was taken not to damage or remove nodules from the roots. Thereafter, the nodulation index was calculated as described by Prevoust and Antoun (2008) using Equation 2. This procedure entailed the scoring of nodules according to size, number and colour.

$$Nodulation\ index = A \times B \times C \quad (2)$$

Table 1: Value table for calculating nodulation indices by multiplying value A, B and C (Prevost and Antoun 2008).

Nodule size	**Value A**
Small	1
Medium	2
Large	3
Nodule colour	**Value B**
White	1
Pink	2
Nodule number	**Value C**
Few	1
Several	2
Many	3

The average nodulation index was calculated as a pooled sample of the plants in each plot. There was potentially a maximum of 100 plants (replicates) per plot. The roots and shoots were dried separately at 60 ˚C for 72 hours to determine the shoot production, root production and root-to-shoot ratio (Botha 2003).

2.5. Statistical analyses

The data was analysed according to the described experimental design. The proposed collected data measurements such as TSM, establishment and germination percentages are continuous variables and therefore an analysis of variance was performed using SAS 9.2

(2003 – 2008). The GLM model was used for the analysis of variance. Assumptions of normality were tested to determine the significant difference between means. The student t-test was conducted at a 5% significance level. Ordinal data, as in the case of nodulation index, was analysed by a chi-square analyses (SAS Institute Inc. 2008).

3. Results and discussion

From the first component of the study it was noted that the TSM means of *T. repens* cultivars differed significantly (P-value < 0.05) (Table 2). Huia had the highest TSM followed by Haifa, Ladino and Regal. With respect to the evaluation of cultivar's germination percentages, the data as reflected in Table 2 indicates that germination was good among all cultivars especially Huia. It was noted that germination percentage correlated well with TSM of the selected cultivars, except for Regal. These significantly different TSM's are due to the fact that seed weight is influenced by many interrelated environmental, managerial and intrinsic genetic factors (Harris 1987, Thomas 1987). Management and particular environments, where the seeds are produced, have a diverse influence on the seed weight (Thomas 1987). The differences in seed weight cannot be ascribed to only the cultivar-effect, but also to specific management of the cultivation site during seed production.

It was evident from the field trial results that TSM had no significant effect on nodulation, but did affect the biomass production. The cultivar Huia, having the heaviest seed, had the greatest biomass production of the four cultivars after ten weeks. The TSM was also consistent with establishment of seedlings up to ten weeks after germination. Although it has been reported that TSM had a significant effect on early plant size, development rate, nodulation rate and N fixation (Mytton 1973), this was not the case in this study. The effect of TSM on nodulation rate, as reported by Mytton (1973), may have been an indirect effect on plant size and vigour, rather than a direct effect of seed weight (Connolly *et al.* 1969, Williams 1987a). Mean nodulation was similar in this study, because three of the four cultivars had similar biomass productions. The potential of plants to nodulate are strongly correlated with traits affecting superior growth rates. Genetic factors are unlikely to have a direct and sure effect on nodulation (Rys and Mytton 1985). It has also been reported that seed having a low TSM had slower nodulation in growth tube cultures, but had no significant effect in larger

containers (Crush 1987). This may contribute to the reason why no differences in nodulation were observed under these field conditions.

Table 2: The mean thousand-seed-mass (TSM), establishment %, biomass production and nodulation indices of each of the four *T. repens* cultivars

Cultivar	TSM (g)	Germination (%)	Establishment (%)	Biomass production (g)	Mean nodulation index
Huia	0.709[a]	95.60[a]	77.09[a]	12.55[a]	12.98[a]
Haifa	0.653[b]	87.74[b]	73.50[ab]	4.53[b]	11.09[a]
Ladino	0.619[c]	74.98[c]	68.08[ab]	6.64[b]	10.32[a]
Regal	0.578[d]	89.83[b]	60.59[b]	5.11[b]	7.44[a]
LSD (0.05)	**0.0087**	**3.510**	**16.871**	**5.036**	**7.654**

LSD = Least significant difference (P-value < 0.05)

[abc]Means with no common superscript differed significantly (P-value < 0.05).

Table 2 highlights the results obtained from the field trial in conjunction with the data obtained from laboratory tests during the study. Seedling emergence and cultivar production was evaluated for the four selected cultivars, and the differences in results can be ascribed to various external and environmental factors. For seedling emergence, daily temperatures play an imperative role.

Weather data collected, it was noted that the daily maximum temperatures increased slightly during the course of the trial. The rate of change of minimum temperature over time was slightly higher than that of the maximum temperature (Figure 1). The optimum temperature for *T. repens* growth is 24°C, however, it is able to grow at ambient temperatures as low as 8 – 9°C (Hart 1987, Moot *et al.* 2000). The base ambient temperature of *T. repens* is 2.5°C (Moot *et al.* 2000), and can tolerate maximum temperatures as high as 35°C (Hart 1987). The effect of temperature in the Southern Cape of South Africa is normally not a limiting factor for nodulation. However, low temperatures cause pronounced effects in time of nodule appearance and development (Lira Junior *et al.* 2005), but the variation of minimum and maximum air temperatures during the trial remained within the critical range for optimal growth of the plants (Figure 1) (ARC 2009).

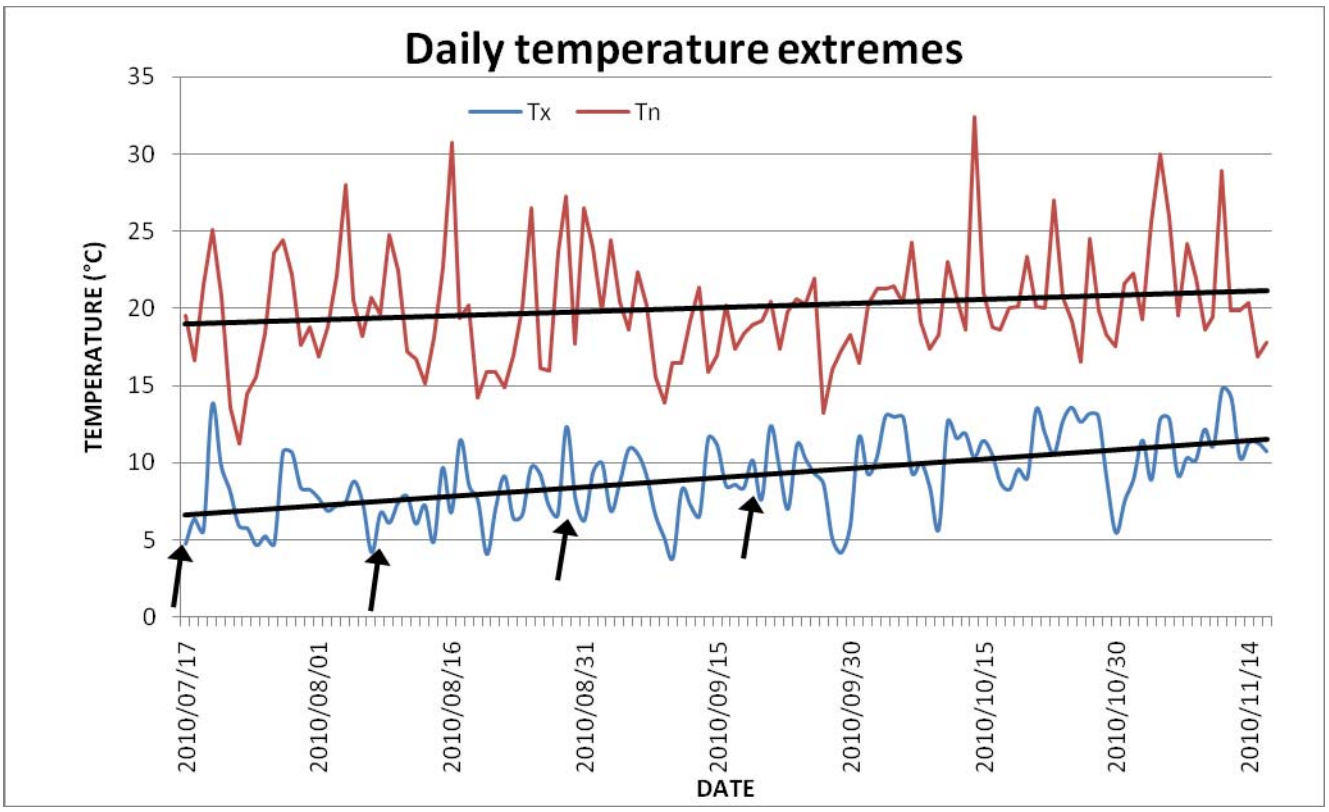

Figure 1: The daily temperature extremes during the time period covered by the trial on Outeniqua Research Farm (Tx = minimum daily temperature, Tn = maximum daily temperature). The planting dates are indicated with black arrows.

All plants, regardless of cultivar and planting date, formed nodules within eight weeks. Naturalised *Rhizobium leguminosarum* bv. *trifolii* are robust, persistent and widespread in the soil of the George region and nodulation of *T. repens* without inoculant application is common (Loos 1963, Swanepoel *et al.* 2010a). Nodules were well developed and success of nodulation was good, as indicated by nodulation indices that ranged between cultivars from 7.44 to 12.98 (Table 2). The nodulation indices of the four cultivars, did however, not differ significantly (P-value < 0.05). Pasture systems containing *T. repens* with nodulation indices as high as these, are considered well managed with a good soil health, because saprophytic competency of adapted rhizobia will ultimately determine the success of *T. repens* in grass-clover pastures (Keyser *et al.* 1992, Watkin *et al.* 2000). Crush (1987) reported that host-genotype-*Rhizobium*-strain interaction is strong. *Trifolium* spp. will select, from heterogeneous populations, those *Rhizobium* strains with which it can form an effective symbiotic

relationship, therefore, cultivar differences in nodulation were reported (Crush 1987). In this study, however, no significant differences in nodulation indices between cultivars were observed (Table 2), and may have been an effect of vigorous plant growth, homogenous *Rhizobium* populations in soil, and optimal growing conditions.

To illustrate the intrinsic cultivar effect on nodulation, the data presented in Figures 2 and 3 are of relevance. A significant chi-square analysis was performed for all cultivars to illustrate the intrinsic cultivar effect on nodulation (P-value < 0.0001, Figure 2). The number of observations presented in Figure 2 and 3, is with reference to the number of plants classed by the nodulation index.

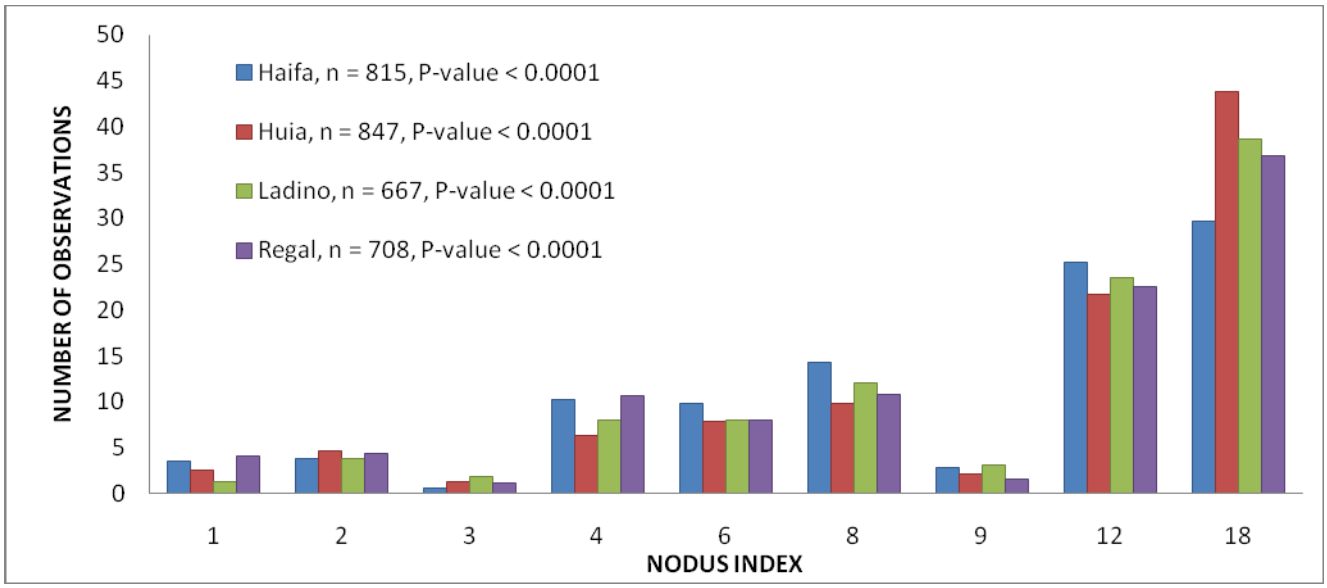

Figure 2: The effect of cultivar on the distribution of nodulation index results.

With reference to the procedure to determine nodulation index, which entails the scoring of nodules according to size, number and colour, it was noted that most plants fell into higher classes of the nodulation index. The higher the nodulation indices the healthier the nodulation. In a *T. repens* pasture system, it is required that plants have a high nodulation index. The test of association showed that nodulation indices reacted in a similar way for all cultivars, and no cultivar was superior in nodulation to any other.

Another important factor, which can have an effect on *T. repens* nodulation, is the time at which the species were planted. A significant chi-square analysis was performed for all

planting dates, except for 18 September 2009 (Figure 3). The test of association showed that the nodulation indices reacted in a similar manner for all planting dates. Although, no significant differences in nodulation index between planting dates were observed there was a tendency for spring plantings to record lower values, than winter plantings. This may have been an effect of the small differences in thermal time between the four planting dates (planting dates 1 to 4 had thermal times of 700.5, 723.4, 766.6 and 779.6°Cd respectively) (ARC 2009, Walker 2009).

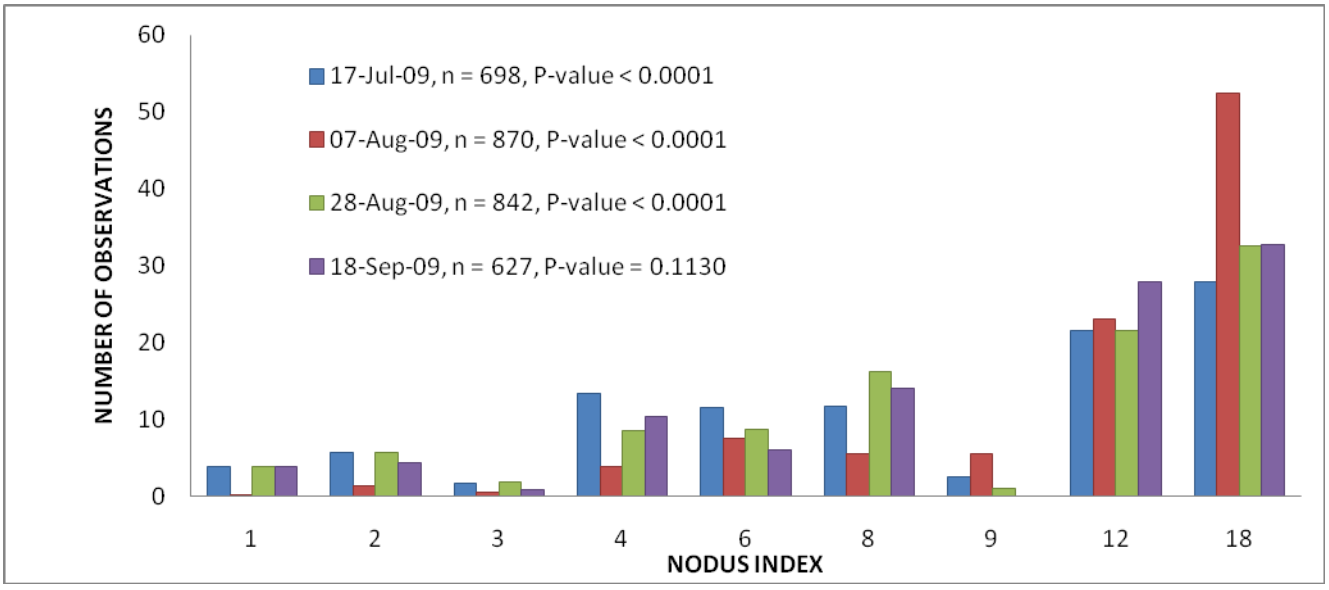

Figure 3: The effect of planting date on the distribution of nodulation indices as result of chi-square analyses.

4. Conclusion

The rhizobial nodulation potential of the four *T. repens* cultivars tested was similar under prevailing environmental conditions. This is possibly attributed to the effect of vigorous plant growth, homogenous *Rhizobium* populations in soil, and optimal growing conditions. Although the TSM means of the *T. repens* cultivars differed significantly, the differences in seed weight cannot be entirely ascribed to the cultivar-effect. The cultivar Huia, with the heaviest seed, had the greatest biomass production of the four cultivars.

Nodules were well developed and the success of nodulation was acceptable for all cultivars. Nodulation of the four selected cultivars was similar, and was not significantly

influenced by TSM and establishment percentage. Pasture systems containing *T. repens* with nodulation indices as high as was obtained in this study, are considered to be well managed.

No significant differences in nodulation indices between cultivars or planting date were observed, possibly due to the small differences in thermal time of the four planting dates. The test of association showed that the nodulation index reacted in a similar manner for all planting dates. The nodulation effects of indigenous and introduced *Rhizobium* strains were similar for all cultivars.

The potential of the cultivars to fix N efficiently must, however, still be investigated. In soils where rhizobia are either scarce or inefficient, inoculation with better suited rhizobia is necessary. Eventually the inefficient indigenous strains can be replaced by highly efficient rhizobia able to fix nitrogen at minimum expense. Therefore, it is concluded that the nodulation potential of the four *T. repens* cultivars was not significantly different under these environmental field conditions.

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CHAPTER 3

Prepared according to the guidelines of the African Journal of Range and Forage Science

THE EFFECT OF SOIL CARBON ON SYMBIOTIC *RHIZOBIUM* POPULATIONS IN SOIL WITH *TRIFOLIUM REPENS* AS HOST PLANT

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Abstract

Research on efficient management systems to optimise *Trifolium repens* - *Rhizobium* symbiosis is lacking in South Africa. Soil organic matter is the main attribute towards managing soils with a high quality. The amount of nitrogen fixed by symbiotic rhizobia in root nodules of *T. repens* is ultimately determined by health of the soil environment. The aim of this study was to determine the total number of symbiotic and free-living rhizobial cells per gram of soil as affected by soil carbon (C), and the host plant. Inoculated and non-inoculated *T. repens* seeds were planted on five soil treatments, each with a different level of soil C. The most probable number (MPN) technique was used to quantify symbiotic rhizobial numbers capable of nodulating *T. repens* in soil as affected by soil C and the host plant. The plate count technique was used to determine the total number of symbiotic and free-living rhizobial bacteria in soil. The MPN technique aids in predicting the need to inoculate with symbiotic rhizobial bacteria, which were detected in all soils. The mean MPN-values obtained in this study did not differ significantly. Most symbiotic *Rhizobium* was detected between a soil C content of 2.03% to 3.80% in both inoculated and non-inoculated soils. Soil C and inoculation had no effect on the amount of these bacteria present in the soil, however, inoculation increased the number of rhizobia in soil, but did not have an effect on the success of nodulation.

Key words: *Rhizobium*, inoculation, MPN technique, spread plate count

1. Introduction

Rhizobium is one of the most important bacterial genera occurring in agricultural soil (Sprent 1979). Rhizobia and legumes are the two species that have co-evolved in a symbiotic relationship where the one species is often the reason for occurrence of the other (Woomer *et al.* 1988). In this relationship, plant-unavailable atmospheric dinitrogen (N_2) is fixed and transformed into organic nitrogenous compounds, available for plants. Subsequent to the current oil-price crisis, inorganic fertiliser-nitrogen (N) prices have elevated and the value of leguminous species in agriculture was emphasised.

A worldwide decline of *Trifolium* spp. (clover species) was observed in 1909 when the Haber-Bosch process was developed to fix atmospheric N_2 industrially (Bohlool *et al.* 1992, Taylor 2008). Post-World War II technological advances have led to increased production outputs of inexpensive inorganic N fertiliser for application in natural and agricultural systems. This resulted in a drastic increase in yield of agricultural important crops, leading to the green revolution. This has, however, also led to dangerous levels of N in waters, soils and the atmosphere (Bohlool *et al.* 1992, Bot and Benites 2005). South African farming systems are continually changing to conform to the needs of society. In the twentieth century, progressively more emphasis is placed on decreasing levels of N fertiliser application in South African farming systems. The deleterious effects that the inorganic N fertiliser has on the environment are considered to be no longer acceptable (Taylor 2008). Development of management strategies to incorporate leguminous crops into grass pasture systems currently receive special attention as they have the potential to sustain low N input grass pastures (Brockwell *et al.* 1995, Taylor 2008).

Trifolium repens (white clover) is, under most circumstances, dependent on symbiosis with rhizobia to provide a plant-available source of N. Management of *T. repens* in pastures is multifaceted and generally difficult to optimise due to nutritional and environmental requirements that must be dually met, i.e. that of the host plant and the rhizobial bacteria. Any environmental or managerial constraint can limit growth, symbiotic production, persistence and survival of the bacteria in soil. It has been observed in many instances that introduced rhizobia from an inoculant fail to persist in soil due to interrelated environmental and plant intrinsic factors (Lawson *et al.* 1987, Thies *et al.* 1991a, Keyser *et al.* 1992, Brockwell *et al.*

1995). Determining the need for inoculation can be an important management factor for consideration in profitable *T. repens* pastures. However, response to inoculation can be difficult to predict (Thies *et al.* 1991b). The ecological interaction of rhizobia within their environment must be understood in order to select a suitable strain for effective N fixation and persistency in the soil (Woomer *et al.* 1988). Research on efficient management systems to optimise the *T. repens-Rhizobium* symbiosis is thus lacking. Therefore, more information is required to understand the dynamics and interactions in the environment.

Quantification of rhizobial populations from the many background microorganisms in soil is a challenge, especially using culture-based techniques. The plant infection technique, fundamentally similar to the most probable number (MPN) assay, is used to estimate microbial populations in natural soil samples (Weaver and Frederick 1972, Somasegaran and Hoben 1985). Most probable number assays are simple measures that aid in predictions for inoculation with a host specific rhizobial inoculant.

The occurrence of one species in a symbiotic relationship is often the reason for the occurrence of the other, large numbers of soil rhizobia will ensure a healthy environment for *T. repens* propagation (Woomer *et al.* 1988a, Woomer *et al.* 1990a). Inoculation practices attempt to increase soil *Rhizobium* numbers directly surrounding the seedling's growing environment. Success of inoculation is a direct response to the indigenous rhizobial population numbers (Thies *et al.* 1991a). Rhizobia must be prevalent enough in the soil to ensure success in sustainability of legumes in the grass systems (Slattery *et al.* 2001). The aim of this study was to determine the total number of symbiotic rhizobia per gram of soil as affected by soil C and inoculation. Subsequently the success of inoculation was determined.

2. Materials and Methods

2.1. Experimental site

The study was carried out on Outeniqua Research Farm near George, Western Cape, South Africa (Altitude 201m, 33 58'38" S and 22 25' 16" E) (Botha *et al.* 2009). The area has a temperate climate with a long term average annual rainfall of 728 mm, evenly distributed throughout the year (ARC 2009). This study consisted of a pot trial, which was conducted under a structure covered with 50% shade net having open sides.

2.2. Experimental design

Five soils from an Estcourt soil type (sandy loam), with different levels of soil C, were identified on the Outeniqua Research Farm (Soil Classification Working Group 1991, Botha 2003). This is the soil type indicative of the soils in the Southern Cape region. Soil C was determined by the Walkley-Black method (Walkley 1935, Chapman and Pratt 1961, Nelson and Sommers 1982). The soil C contents were 1.29%, 2.03%, 2.77%, 3.80% and 4.25% C.

Soils were analysed for magnesium (Mg), calcium (Ca), potassium (K), sodium (Na), phosphorous (P), copper (Cu), zinc (Zn), manganese (Mn), sulphur (S), boron (B) and soil pH(KCl). The fertility status of each of the five soil treatments were corrected up to the recommended soil fertility levels for a grass-clover pasture namely P (citric acid) > 30 ppm, K 80-100 ppm, S > 11 ppm, Cu > 1.0 ppm, Zn > 1.0 ppm, Mn 10-15 ppm and pH(KCl) of 5 – 5.5 (Botha 2003).

With respect to the treatments applied, there were two treatments, replicated nine times, tested on each of the five soils, i.e. a total of 10 treatment combinations of:

- *T. repens* cv. Haifa, seeds inoculated with *Rhizobium leguminosarum* bv. *trifolii*
- *T. repens* cv. Haifa, seeds subject to indigenous rhizobia only (not inoculated)

Trifolium repens cv. Haifa was selected as the best cultivar from a different study (Swanepoel *et al.* 2010b), which was grown from seed sown directly into the pots (diameter: 160mm, height: 220mm) at a density of five seeds per pot. After establishment of seedlings, pots were thinned out to two healthy plants per pot.

Pots were arranged in a randomised block design and replicates were placed in separate rows. Pots were arranged in such a way that all the pots in each row received a similar amount of wind and sunlight. Plants were watered by means of drip irrigation and the soil moisture status was determined with the aid of tensiometers placed at 15 cm in the soil of each treatment. Soil water potential was kept between -10 and -25 kPa (Botha 2002).

2.3. Techniques used to quantify Rhizobium populations

Plants were harvested in the 12th week after planting. Soil was carefully removed from the roots rinsing them with water (Somasegaran and Hoben 1985). Care was taken not to

damage or remove nodules from the roots. The nine replicates of each treatment-combination were combined to give three sets of three and mixed thoroughly for soil sampling. Thereafter, the nodulation index was calculated as described by Prevoust and Antoun (2008) using Equation 1. This procedure entailed the scoring of nodules according to size, number and colour (Table 1).

$$Nodulation\ index = A \times B \times C \quad (1)$$

Table 1: Calculation of nodulation indices by multiplying value A, B and C (Prevost and Antoun 2008)

Nodule size	**Value A**
Small	1
Medium	2
Large	3
Nodule colour	**Value B**
White	1
Pink	2
Nodule number	**Value C**
Few	1
Several	2
Many	3

Subsamples of 32 ml were taken from the rhizosphere soil, pooled together, and then refrigerated in previously sterilised, air tight glass sampling bottles for transportation to the microbiological laboratory for analyses.

2.3.1. Plant infection count analysis

Plastic pouches used for this experiment were obtained from Mega International, St. Paul, Minneapolis (Figure 1). These pouches are specifically designed to be an inexpensive and space saving alternative to Leonard jars (Toomsan *et al.* 1984, Somasegaran and Hoben 1985). Seeds were surface sterilised by immersion in 5% sodium hypochlorite for ten minutes. They were subsequently rinsed four times with sterile distilled water placed on filter paper in a

sterile petri-dish for pre-germination at 26°C in a dark room for 48 hours. For convenience, the germinated seedlings were placed in a refrigerator for a maximum of 5 days or until transplantation.

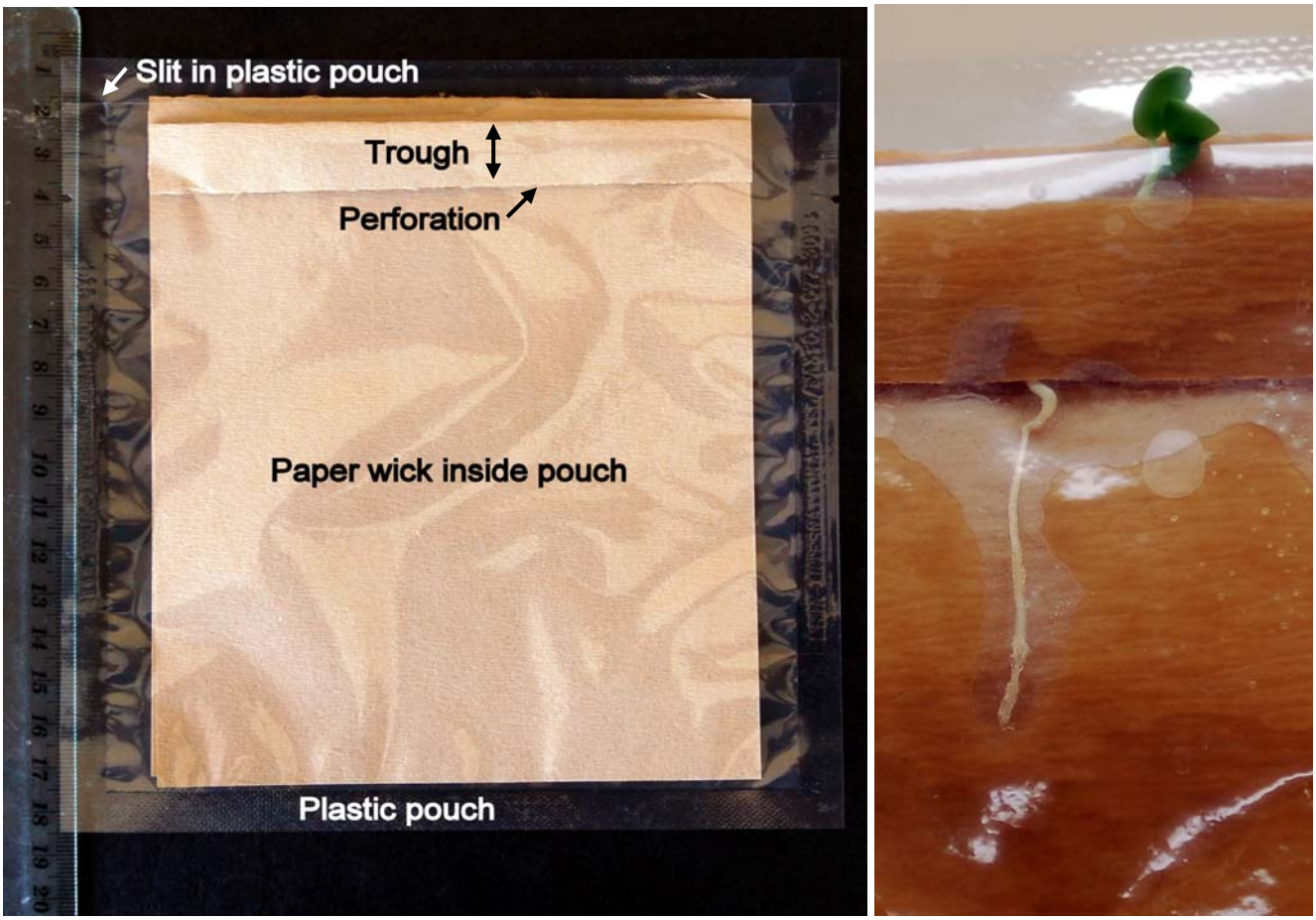

Figure 1: Plastic cyg™ seed germination pouch from Mega International (right). Seedling root development can easily be screened (left).

They were filled initially with 20 ml of an N-free plant nutrient solution (Brockwell 1963, Weaver and Frederick 1972, Somasegaran and Hoben 1985, Weaver and Graham 1994). The solution contained the following nutrients (Somasegaran and Hoben 1985):

- Cobalt chloride - $CoCl_2.6H_2O$, 0.004 mg
- Boric acid - H_3BO_3, 2.86 mg
- Manganese chloride - $MnCl_2.4H_2O$, 1.81 mg
- Hydrated zinc sulphate – $ZnSO_4.7H_2O$, 0.22 mg
- Hydrated copper sulphate - $CuSO_4.5H_2O$, 0.08 mg

- Sodium molybdate – $NaMoO_4$, 0.1211 mg
- Hydrated manganese sulphate - $MgSO_4.7H_2O$, 492.96 mg
- Dipotassium hydrogen phosphate - K_2HPO_4, 1474.18 mg
- Potassium dihydrogen phosphate - KH_2PO_4, 600.09 mg
- Calcium chloride - $CaCl_2$, 110.99 mg
- Hydrated ferric citrate - $FeC_6H_5O_7.H_2O$, 5.00 mg
- Peptone - 1.0 g
- Distilled water – 1 L

Seedlings with a similar radical length (between 10 – 20 mm) were transplanted into the pouch by placing the radical through a perforation in the trough with sterile forceps (Figure 1) (Somasegaran and Hoben 1985). The inoculant was prepared by using the soil harvested from the rhizospheres of *T. repens* plants grown in the pots. A representative soil sample of 1 g was added to 9 ml of Ringer's buffer solution in an autoclaved glass bottle along with 30 sterilised glass beads of similar size. The buffer solution contained the following (Somasegaran and Hoben 1985):

- Dipotassium hydrogen phosphate - K_2HPO_4, 1.21 g
- Potassium dihydrogen phosphate - KH_2PO_4, 0.34 g
- Peptone - 1.0 g
- Distilled water - 1 L

The pH of the buffer solution was brought to 7.0 ± 0.1 by using 1M KOH or HCl. Each bottle containing the soil was shaken vigorously by hand for five minutes and a ten-fold serial dilution was prepared immediately while the soil was still in suspension. One milliliter (ml) of the dilution was added to each of four replicates from each set. MPN counts were made by using four replications and tenfold serial dilutions with a control for each set containing N-free nutrient solution and 1 ml of buffer solution. For this study to have any significance, the uninoculated controls must be negative. All the work was performed in a sterile environment (laminar flow cabinet). Instruments used were sterilised by immersion into 99% ethanol and flaming (Weaver and Frederick 1972, Somasegaran and Hoben 1985, Weaver and Graham 1994).

Pouches were placed on a rack, built from a wooden frame and galvanised wire. It was kept in a sterile environmental growth chamber, at 25 °C with a six hour dark period (Weaver and Graham 1994, Broos *et al.* 2004). Pouches were screened daily for nodule formation. The nutrient solution was replenished as necessary (Somasegaran and Hoben 1985, Weaver and Graham 1994).

Only the absence or presence of nodules bears significance in this study and quantity of nodules is not applicable. The number of plants positive (nodules present) was recorded for each set. MPN values were calculated by the following series of formulae (Equations 2 to 6) (Woomer *et al.* 1990, Briones and Reichardt 1999):

1. Actual volume inoculated

$$a = \left(\frac{Dilution\ inoculated}{Dilution\ source}\right) \times Actual\ volume\ inoculated \qquad (2)$$

Where a = Succeeding volume inoculated

2. Halvorson and Ziegler's general MPN equation

$$\frac{a_i p_i}{1 - e^{-a_i x}} + \cdots + \frac{a_n p_n}{1 - e^{-a_n x}} = a_i z_i + \cdots + a_n z_n \qquad (3)$$

Where a_i = Succeeding volume inoculated,
p_i = Number of positive pouches in the i^{th} set
x = MPN-value
z_i = Number of tubes in the i^{th} set
n = Number of samples

3. The probability value provides an estimate of frequency of a certain combination for a probable number.

$$P = \left(\frac{x_i!}{p_i!\,q_i!}\right)\ldots\left(\frac{x_n!}{p_n!\,q_n!}\right)\left(e^{-a_i x}\right)^{q_i}\ldots\left(e^{-a_n x}\right)^{q_n}\left(1-e^{-a_i x}\right)^{p_i}\ldots\left(1-e^{-a_n x}\right)^{p_n} \quad (4)$$

Where P = Probability value

a_i = Succeeding volume inoculated

p_i = Number of positive pouches in the i^{th} set

q_i = Number of negative pouches in the i^{th} set

x = MPN-value

n = Number of samples

4. The population estimate

$$Population\ estimate = x\frac{1}{d} \quad (5)$$

x = MPN

d = the single dilution source basing the values for the volumes inoculated.

5. Calculation of the 95% confidence intervals

$$CF = antilog_{10}\left(2 \times 0.55\sqrt{\frac{log_{10}dr}{n}}\right) \quad (6)$$

Where

dr = dilution ratio

Microsoft Excel® was used to solve the equations (Briones and Reichardt 1999, Microsoft Office 2007).

2.3.2. Quantification of culturable (free-living and symbiotic) rhizobia using the plate count method

A representative soil sample of 1 g was added to 9 ml of Ringer's buffer solution (as outlined above) in an autoclaved glass bottle containing 30 glass beads of similar size. Each bottle was then shaken vigorously by hand for 5 minutes and an eight-fold serial dilution was prepared immediately while the soil was still in suspension. Yeast mannitol agar (YMA) amended with Congo red dye was prepared as prescribed by the manufacturers, autoclaved and 20 – 30 ml was transferred to 90 mm sterile petri-dishes (plates). This was allowed to set at room temperature for at least 2 hours, thereafter the plates were inverted and refrigerated until further use. Test tubes containing the serial dilution were shaken by a vortex. Dilutions 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} were plated out by spreading 100 µl of the suspension on each plate of YMA, in four replicates using a hockey stick. Plates were inverted and incubated at 25 °C for 4 days in a dark cabinet. After incubation, colonies were counted as described by Somasegaran and Hoben (1985). Colonies counted must be white to somewhat translucent, circular and raised and may produce significant amounts of extracellular mucilage polysaccharides. Colonies that are red, pink or orange, with a distinctive aroma and/or not circular, are unlikely to be *Rhizobium* (Weaver and Graham 1994). The colony forming units were calculated by equation 7.

$$CFU = \frac{number\ of\ colonies}{Volume\ inoculated} \times \frac{1}{Dilution\ ratio} \quad (7)$$

2.4. Statistical analyses

The data were analysed according to the described experimental design. An analysis of variance was performed using SAS 9.2 (2003 – 2008) for the continuous variables. The GLM model was used for the analysis of variance. The assumptions of normality were tested to determine significant difference between means and the student t-test was conducted at a 5% significance level (SAS Institute Inc. 2008).

3. Results and discussion

In this study, for the enumeration of symbiotic *Rhizobium* capable of infecting *T. repens,* the plant infection technique was chosen to estimate the number of viable symbiotic *Rhizobium* cells present in the rhizospheric soil. This is the only means available to estimate *Rhizobium* numbers in soil samples with heterogenous background bacteria present (Toomsan *et al.* 1984, Scott and Porter 1986, Woomer *et al.* 1990b). A direct quantification technique is necessary to verify the results of the plant infection technique, but direct techniques for samples with background microorganisms are not available, rendering statistical validation, within the plant infection technique, necessary. Probability values (P-values) for each pattern of positive pouches in a dilution series were produced (Cochran 1950, Scott and Porter 1986).

The plant infection technique produced a MPN value of rhizobial cells per gram of soil. This technique highlighted the presence of symbiotic *Rhizobium*, and not free-living *Rhizobium*. The MPN values with associated P-values greater than 0.05 were not statistically interpretable, thus 66.7% of MPN values were considered to be significant (P-value < 0.05). The MPN values with associated P-values greater than 0.05 and confidence intervals are indicated in Table 2. The control treatment values are not shown since they were negative, i.e. a MPN value of zero.

The MPN of symbiotic rhizobia ranged from as little as 78 to over 8900 bacterial cells per gram of soil (Table 2). These values, however, are not statistically different, but statistically significant. It is evident that inoculation did have an effect on the *Rhizobium* populations (Figure 2). The natural occurrence of *T. repens* in the George area is common and the rhizobial population density is highly correlated with presence of the particular host legume (Keyser *et al.* 1992). *Rhizobium leguminosarum* bv. *trifolii* is also found to be widespread in the soil of the George district (Loos 1963).

Rhizobium was detected in all soils, regardless of level of soil C or treatment with inoculant, emphasising the robustness and adaptability of the genus in different levels of soil C as is supported by Kiers *et al.* (2003). *Rhizobium* and soil C play vital roles in maintenance of soil health by increasing its capacity to function as a living system and to sustain pasture productivity. Soil health deals with integrated management practices to improve productivity in an economically and environmentally compatible manner (Barabasz *et al.* 2002). It has been proposed for *Rhizobium* to be a viable and accurate indicator of the soil health status (Van

Bruggen and Semenov 2000, Nielsen and Winding 2002). Microorganisms, especially *Rhizobium* in association with SOM also contributed to the soil's physical factors related to soil resilience (Bot and Benites 2005, Patra *et al.* 2005). Thus, the particular soils will likely have a large potential to return to equilibrium after disturbances, being rich in soil C and *Rhizobium*.

Table 2: Most-Probable-Number (MPN) of symbiotic *Rhizobium* as affected by soil C content and seed inoculation.

Soil C content (%)	Inoculation	Mean MPN-value	95% Confidence interval	
			Upper limit	Lower limit
1.29	**No**	**81[a]**	309	21
1.29	**Yes**	**198[a]**	701	56
2.03	**No**	**715[a]**	2719	188
2.03	**Yes**	**4232[a]**	13948	1113
2.77	**No**	**556[a]**	2116	146
2.77	**Yes**	**8907[a]**	33864	2343
3.8	**No**	**138[a]**	678	30
3.8	**Yes**	**4330[a]**	16091	1396
4.25	**No**	**78[a]**	298	21
4.25	**Yes**	**1190[a]**	4523	313
LSD (P<0.05)[1]		9577		

[1]LSD = Least significant difference of the mean MPN values of treatment-combinations. This P-value provides the significance level (0.05) of the test statistic.

[abc]Means with no common superscript differed significantly (P-value < 0.05).

In Figure 2 it is clear from the data presented that inoculation had an effect on the number of symbiotic rhizobia per gram of soil, even though not being statistically significant, but this can be ascribed to the variation of results caused by the technique, rather than the treatments themselves. It is evident from the data presented in Figure 2 that at a particular soil C content of approximately between 2.03% to 3.80%, the most symbiotic *Rhizobium* was detected from either inoculated or non-inoculated soils. It is interesting to note that the soils containing the

highest soil C content had depressed values of symbiotic *Rhizobium* contrary to belief. This general observation is, however, relevant to the Estcourt soil type being evaluated.

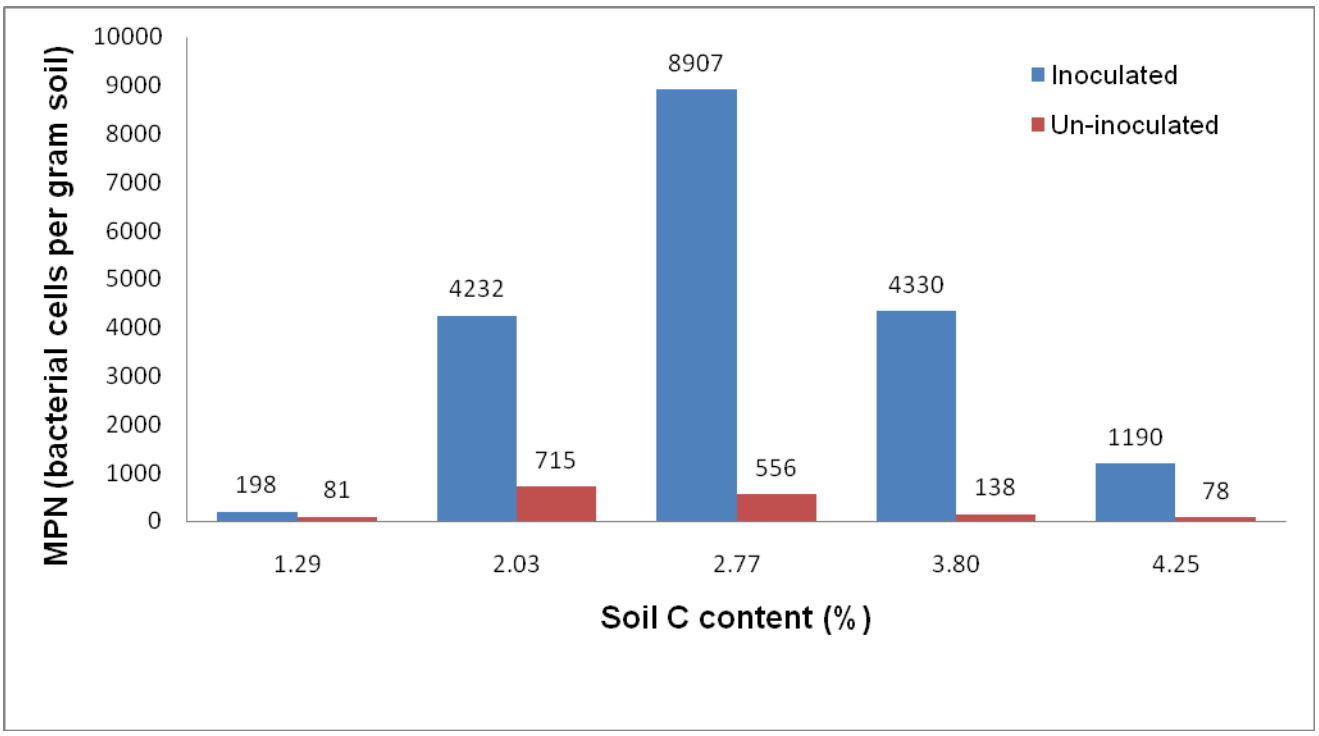

Figure 2: Most-Probable-Number (MPN) values as affected by soil C content and seed inoculation.

The plate count technique provided data which emphasises that the total culturable rhizobia were not drastically influenced by the different levels of soil C (Table 3). This supports the findings of Brockwell (1963), Weaver and Frederick (1972). These *Rhizobium* colonies are represented by both symbiotic and free-living rhizobia.

The data in Figure 3 coincides with data obtained for the symbiotic rhizobia in Figure 2. This data, however, differs to the extent that free-living *Rhizobium* is more prevalent in extreme soil C content soils. It is also noted that a similar soil C content threshold exists between 2.03 and 3.80% C, where free-living *Rhizobium* predominate. It is deducted from this data that free-living rhizobia have a lower potential to infect *T. repens*, than that of the introduced symbiotic rhizobia. *Trifolium repens* is not necessarily host specific to these free-living rhizobia.

Table 3: *Rhizobium* colony forming units as quantified by the plate count method (symbiotic and free-living) and affected by soil C content and seed inoculation.

Soil C content (%)	Inoculation	Log_{10}(Plate count) CFU
1.29	No	8.86[a]
1.29	Yes	-
2.03	No	9.35[a]
2.03	Yes	9.93[a]
2.77	No	9.35[a]
2.77	Yes	10.26[a]
3.8	No	9.02[a]
3.8	Yes	10.21[a]
4.25	No	8.59[a]
4.25	Yes	9.93[a]
LSD (P<0.05)		1.870

LSD = Least significant difference of the mean MPN values of treatment-combinations.

[abc]Means with no common superscript differed significantly (P-value < 0.05).

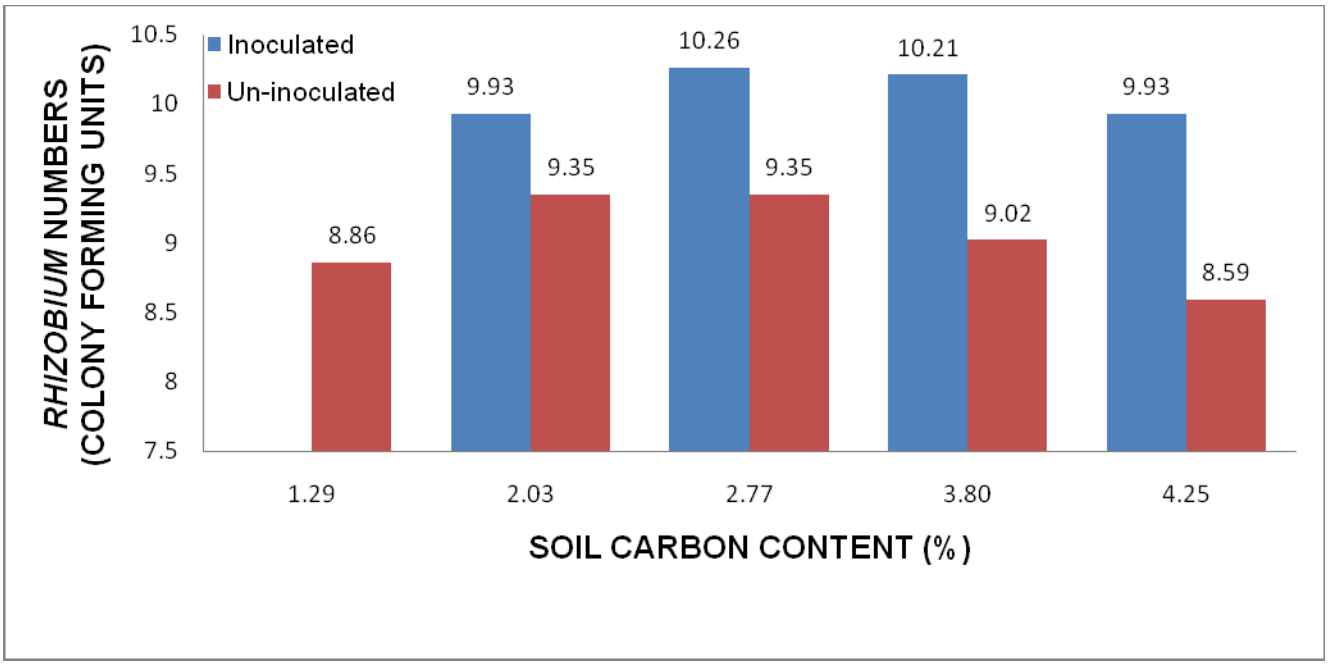

Figure 3: *Rhizobium* colony forming units as affected by soil C content and seed inoculation.

Table 4 represents the actual success of nodulation. It is shown that nodulation was generally similar and unaffected by soil C content of this Estcourt soil type.

Table 4: Mean nodulation index as affected by soil C content and seed inoculation.

Soil C content (%)	Inoculation	Mean Nodulation index
1.29	No	7.3[b]
1.29	Yes	11.6[ab]
2.03	No	12.0[ab]
2.03	Yes	15.7[a]
2.77	No	13.0[ab]
2.77	Yes	13.0[ab]
3.8	No	13.1[ab]
3.8	Yes	12.4[ab]
4.25	No	11.5[ab]
4.25	Yes	8.3[b]
LSD (P<0.05)		6.34

LSD = Least significant difference of the mean MPN values of treatment-combinations.

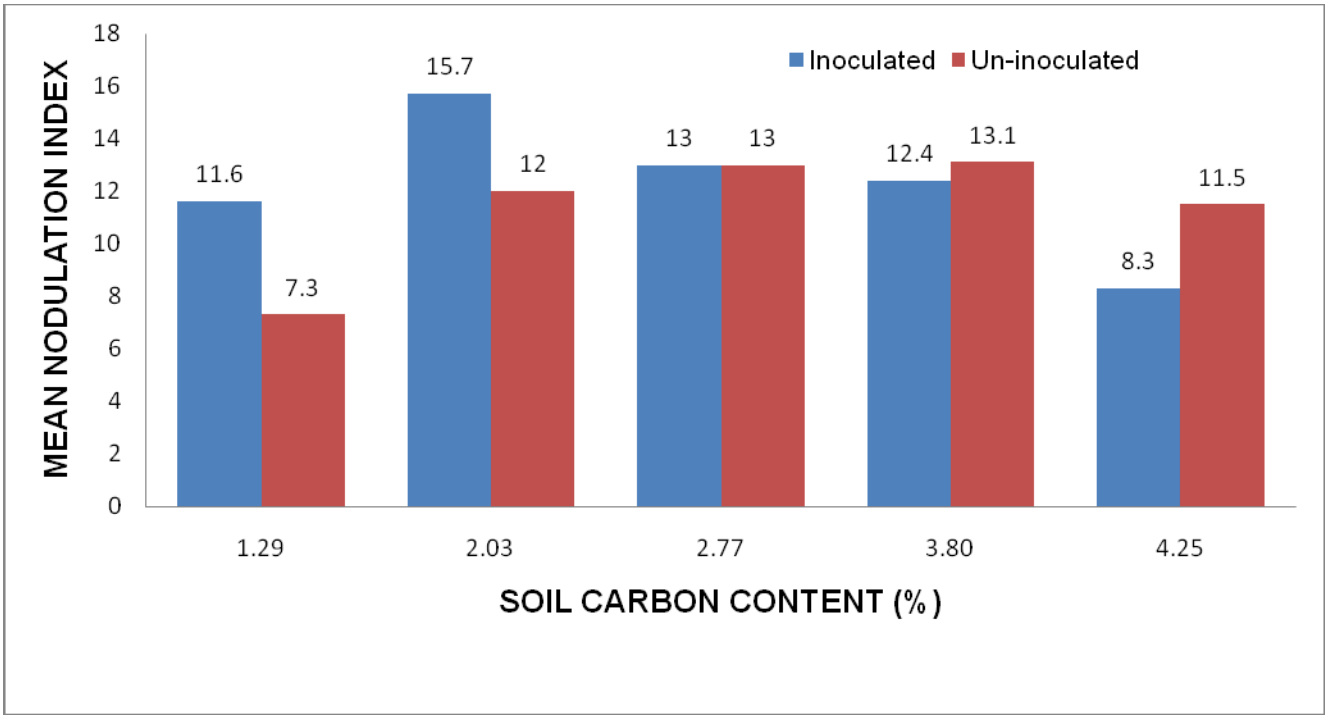

Figure 4: Mean nodulation indices of *T. repens* as affected by soil C content and seed inoculation.

Figure 4 illustrates that there was no significant difference in nodulation between inoculated versus non-inoculated treatments, irrespective of the different soil C contents.

Because soil C content had no significant effect on the MPN values, the data was pooled to give a mean value for inoculation treatments. The effect of inoculation on the rhizobial numbers quantified by the plant infection technique (MPN), showed that the MPN value for inoculated and non-inoculated plants were 3757 and 270 rhizobial cells per gram of soil, respectively.

The plate count technique, however, indicated that inoculation had a statistically significant effect on the *Rhizobium* colonies (free-living and symbiotic) of the soil. The inoculated soils had a plate count of 10.08 CFU per gram of soil, as compared to the non-inoculated soils with a plate count value of 9.03 CFU per gram of soil.

The mean nodulation indices for inoculated plants and non-inoculated plants were 12.16 and 11.30, respectively. Nodulation indices did not differ significantly between inoculation treatments, which show that inoculation increased the number of rhizobia in soil, but did not have an effect on the success of nodulation.

Lack of legume response to inoculation may be due to limitations in the soil, such as non-vigorous plant growth, high indigenous rhizobial numbers or highly effective indigenous strains and the availability of mineral N in the soil (Keyser *et al.* 1992, Turk *et al.* 1993, Brockwell *et al.* 1995). The high free-living *Rhizobium* numbers in the particular soil was the possible reason for the lack of response to inoculation.

4. Conclusion

The MPN value of *Rhizobium* cells per gram of rhizospheric soil was not affected by soil C content. Inoculation, however, had an effect on the MPN values, even though not being statistically significant. Most symbiotic *Rhizobium* was detected approximately between 2.03% to 3.80% C from either inoculated or non-inoculated soils. Inoculation increased the number of rhizobia in soil, but did not have an effect on success of nodulation. Free-living *Rhizobium* had a lower potential to infect *T. repens* than that of introduced rhizobia.

Researchers in the Southern Cape of South Africa need to give innovative attention to soil health and resilience, as the current high N input pasture systems are not sustainable. *Rhizobium leguminosarum* bv. *trifolli*, being an indicator of soil health, is a common and

beneficial bacterial species in pasture soils in the area of George, South Africa. This *Rhizobium* species is robust and adaptable under many soil conditions. Introduction of rhizobia by means of inoculation of seed, may therefore be beneficial, since indigenous strains might form nodules, but can still be ineffective in supplying the plant with N. On a regional basis, the plant infection technique can be used to help identify the areas where inoculation is likely to result in improved productivity, but requires a thorough assessment on the N fixing efficiency of indigenous and introduced *Rhizobium*, as well as temporal and spatial variability of rhizobial populations.

Soil organic matter is the most important contributor to soil health, and rhizobia are accepted as important biological indicators of a healthy soil. The health of the soil, cannot, however, be solely estimated by means of rhizobial counts, but other indicators need to be assessed in conjunction with these rhizobial indicators.

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CHAPTER 4

Prepared according to the guidelines of the African Journal of Range and Forage Science

QUANTIFICATION OF BIOLOGICAL NITROGEN FIXATION BY *TRIFOLIUM REPENS* AS AFFECTED BY SOIL CARBON

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Abstract

Soil organic matter (SOM) is one of the main attributes of soils with a high quality. The amount of nitrogen (N) fixed by symbiotic rhizobia in root nodules of *Trifolium repens* (white clover) is ultimately determined by the health of the soil environment. Soil carbon (C) was measured as an indicator of SOM. The effect of different levels of soil C on the amount of atmospheric N fixed by *T. repens* was assessed by using the N difference technique with *Arctotheca calendula* (Cape weed) used as the reference plant. Soils with different levels of organic C, i.e. 1.29, 2.03, 2.77, 3.51 and 4.25% were used in this study. The mean percentage N derived from the atmosphere (%Ndfa) differed significantly between soil C treatments, with the highest %Ndfa (1.79%) in the soil with the lowest C content. Biomass production was determined and served as a parameter for efficiency of the *Rhizobium* bacteria in soil. Although the amount of N fixed increased as the level of soil C decreased, the efficiency of N fixation decreased proportionally to soil C. Subsequently more N was rhizodeposited into the soil. It was concluded from this study that symbiotic rhizobia introduced by inoculant was much more efficient in higher C content soils than free-living rhizobia, which highlights the importance of inoculation in improving the sustainable production of *T. repens* pastures.

Keywords: Nitrogen, inoculation, *Rhizobium*, soil carbon content

1. Introduction

There are currently immense societal pressures on agriculturalists to farm not only in a sustainable manner but also to keep the production per unit area high because of limited land, high and increasing population densities, urbanisation, industrialisation and global warming (Tate 1992). Modern agricultural practices tend to maximise outputs in the short term and this deprives the soil of nutrients, because of a greater nutrient efflux from the soil than influx. This is a major problem confronting future generations, while supplying food for an ever increasing world population (Brockwell *et al.* 1995). Sustainability in agriculture is defined by Bohlool *et al*. (1992) as "successful management of resources to satisfy changing human needs while maintaining or enhancing the quality of the environment and conserving resources". Current recommended fertilisation rates in the Southern Cape of South Africa are too high and does not support sustainable production (Labuschagne 2009). A holistic aim for sustainable agriculture is the attainment of maximum quantity and quality of pasture production with minimal nitrogen (N) input in the form of fertiliser and without N pollution of environmental resources.

One of many reasons why the current recommended application rate of fertiliser N is not sustainable is that it has become too expensive. Inorganic fertiliser-N production costs are heavily dependent on the price of fossil-fuels (Bohlool *et al.* 1992). With the current oil price crisis, the manufacturing process of inorganic N has become increasingly expensive. The search for biological alternatives to inorganic forms of fertiliser-N for dairy pasture systems in the Southern Cape of South Africa has become imperative (Botha 2003). The incorporation of legumes, especially *Trifolium repens* (white clover), in pasture systems are economically and ecologically promising because they form a symbiotic relationship with atmospheric N_2 fixing *Rhizobium*, rendering them nutritious and especially high in organic nitrogenous compounds (Williams 1987a, Michaelson-Yeates *et al.* 1998, Ledgard *et al.* 1999, Ledgard *et al.* 2001, McDonald *et al.* 2002).

The amount of atmospheric N fixed by legumes varies widely, depending on the management and environment of the pastures. Factors affecting plant growth will influence production of energy products derived from photosynthesis that must be in sufficient supply for the symbiotic *Rhizobium* metabolism in root nodules. Nodules must also be able to respire effectively to maximise symbiotic N fixation. Conditions affecting N fixation must be optimal so

that rhizobial synthates can finally be transported and redistributed throughout the plant. Diverse management strategies of pastures containing *T. repens* are necessary to maximise the amount of N fixed from the atmosphere. The soil environment should also be managed efficiently to be able to promote N fixation (Sprent 1979). Thus, managerial factors to improve the soil quality may enhance N fixation efficiency. Soil quality is defined as "the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality and support human health and habitation" (Karlen *et al.* 1997).

Soil quality is the key element in sustainable agriculture and soil organic matter (SOM) is, in turn, the main attribute for maintaining soils of a high quality (Carter 2002). Soil environmental manipulation, particularly of SOM, can have a direct effect on the legume plant itself or indirectly affect the rhizobial populations that infect the roots of the legume. Decisions regarding these managerial factors have a major impact on the amount of N fixed. Consequent profitability of dairy farming systems would be increased. The aim of this study was to quantify the amount of N fixed by *T. repens* as affected by soil carbon (C) content.

2. Materials and Methods

2.1. Experimental site

The study was carried out on Outeniqua Research Farm near George, Western Cape, South Africa (Altitude 201m, 33 58'38" S and 22 25' 16" E) (Botha *et al.* 2009). The area has a temperate climate with a long term average annual rainfall of 728 mm, evenly distributed throughout the year (ARC 2009). This study consisted of a pot trial, which was conducted under a structure covered with 50% shade net having open sides.

2.2. Experimental design

Five soils from an Estcourt soil type (sandy loam), with different levels of soil C, were identified on the Outeniqua Research Farm (Soil Classification Working Group 1991, Botha 2003). This is the soil type representative of the soils in the Southern Cape region. The soil C contents were 1.29%, 2.03%, 2.77%, 3.80% and 4.25% C.

Soils were analysed for magnesium (Mg), calcium (Ca), potassium (K), sodium (Na), phosphorous (P), copper (Cu), zinc (Zn), manganese (Mn), sulphur (S), boron (B) and soil pH(KCl). The fertility status of each of the five soil treatments were corrected up to the recommended soil fertility levels for a grass-clover pasture namely P (citric acid) > 30 ppm, K 80-100 ppm, S > 11 ppm, Cu > 1.0 ppm, Zn > 1.0 ppm, Mn 10-15 ppm and pH(KCl) of 5 – 5.5 (Botha 2003).

With respect to the treatments applied, there were two treatments, replicated nine times, tested on each of the five soils, i.e. a total of 15 treatment combinations of:

- *T. repens* cv. Haifa, seeds inoculated with *Rhizobium leguminosarum* bv. *trifolii*
- *T. repens* cv. Haifa, seeds subject to indigenous rhizobia only (not inoculated)
- *Arctotheca calendula* (Cape weed)

Trifolium repens cv. Haifa was selected as the best cultivar from a different study (Swanepoel *et al.* 2010b), which was grown from seed sown directly in the pots (diameter: 160mm, height: 220mm) at a density of five seeds per pot. After establishment of seedlings, pots were thinned out to two healthy plants per pot.

Pots were arranged in a randomised block design and replicates were placed in separate rows. Pots were arranged in such a way that all the pots in each row received a similar amount of wind and sunlight. Plants were watered by means of drip irrigation and the soil moisture status was determined with the aid of tensiometers placed at 15 cm in the soil of each treatment. Soil water potential was kept between -10 and -25 kPa (Botha 2002).

2.3. Technique used to quantify N fixation

A reference plant with similar phenology and growth pattern as *T. repens* was chosen and it was assumed that *A. calendula* has a similar affinity for N assimilation as *T. repens* (Pate *et al.* 1994). *A. calendula* thus served as the non N-fixing reference plant. This reference plant was used to quantify biological N fixation with the N difference technique (Equation 1). The total N yield of *A. calendula* was subtracted from the total N yield in the N-fixing plant system (*T. repens*) (Hart *et al.* 1994, Carranca *et al.* 1999).

$$N_2 \text{ fixed}_{ND} \ (g.g^{-1}) = \text{Total N yield} \ (g.g^{-1})_{T.\ repens} - \text{Total N yield} \ (g.g^{-1})_{A.\ calendula}$$

(1)

Percentage N derived from the atmosphere (%Ndfa) per unit plant mass was calculated using Equation 2.

$$\%\text{Ndfa} = N_2 \text{ fixed}_{ND} \ (g.g^{-1}) \times 100$$

(2)

The plants were harvested during the 12th week after planting. The symbiotic effectiveness was measured as biomass weight (dry matter). Each plant's roots and shoots were dried at 60 ˚C for 72 hours, weighed and milled as described by Botha (2003). The same plants that were grown to determine symbiotic effectiveness were also used to assess nodulation using a categorical scoring system (Prevost and Antoun 2008).

The nine replicates of each treatment were combined to give three sets of three. The total N content in the plant matter was determined by the AgriLASA method (AOAC International 2002). Soil C was determined by the Walkley-Black method (Walkley 1935, Chapman and Pratt 1961, Nelson and Sommers 1982).

2.4. Statistical analyses

The data were analysed according to the described experimental design. An analysis of variance was performed using SAS 9.2 (2003 – 2008) for the continuous variables. The GLM model was used for the analysis of variance. The assumptions of normality were tested to determine significant difference between means and the student t-test was conducted at a 5% significance level (SAS Institute Inc. 2008).

3. Results and discussion

It is clear from Table 1 that soil C content had a significant effect on the amount of atmospheric N_2 fixed (%Ndfa). The negative correlation between soil C content and %Ndfa was strong (Pearson correlation coefficient = -0.903). As soil C content increased, the mean %Ndfa proportionally decreased from 1.793% to 0.680% N (Table 1).

Even though the plants growing in the low C soil fixed the most atmospheric N, the soil N content was 6.25 g kg^{-1} soil in comparison to the high C soil which had a N content of 39 g kg^{-}

[1]. Therefore, plants growing in the high C soil, caused an increase in soil N of more than 4 times that of the low C soil (Table 1). In low N input grass-clover mixed swards, this will be exceptionally important as the grasses will be able to utilise only this rhizodeposited N.

Table 1: The mean percentage N derived from the atmosphere (%Ndfa), final soil N content as affected by soil C content

Soil C content (%)	**Mean %Ndfa**	**Final soil N content (g kg^{-1})**
1.29	1.793^{a}	6.25^{a}
2.03	1.335^{b}	12.5^{b}
2.77	0.985^{c}	17.0^{c}
3.51	0.897^{c}	29.4^{d}
4.25	0.680^{d}	39.0^{e}
LSD (0.05)	**0.1762**	**2.060**

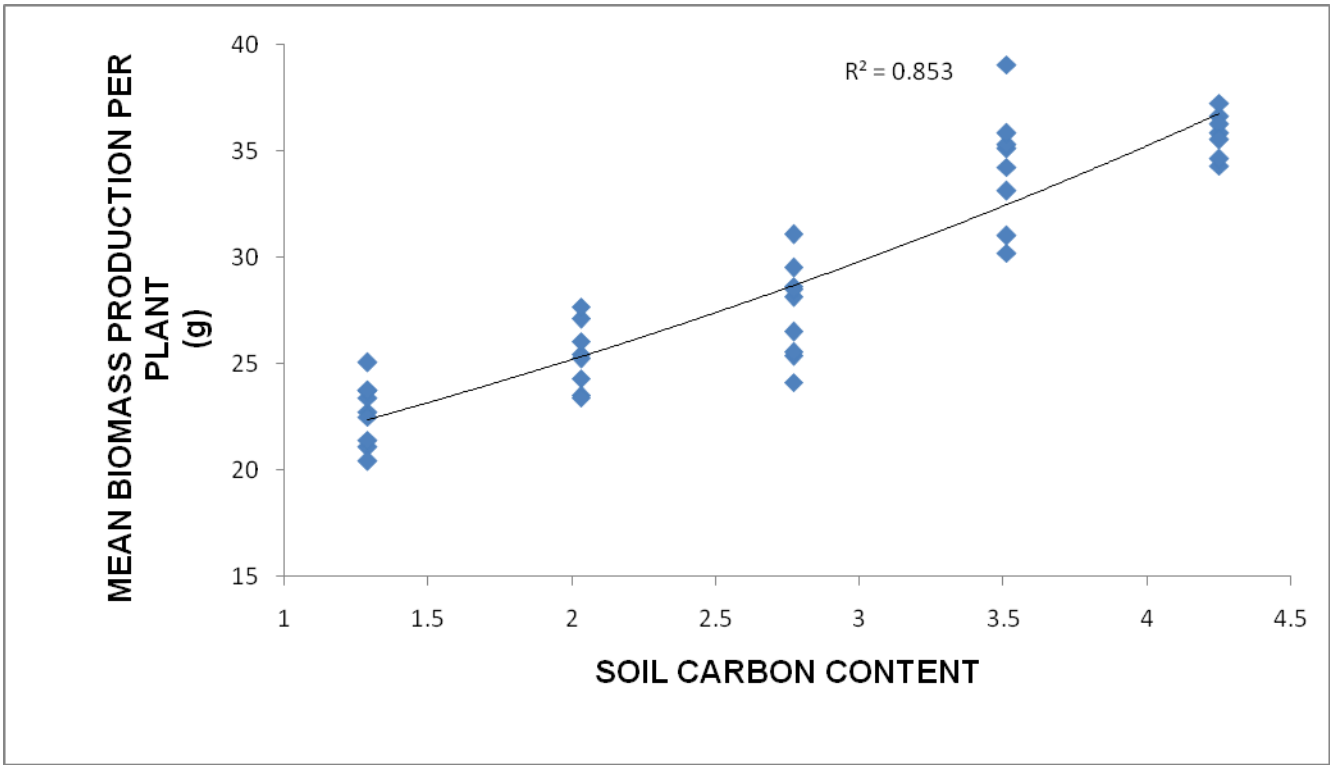
LSD = Least significant difference (P-value = 0.05)

^{abc}Means with no common superscript differed significantly (P-value < 0.05).

Plant sanctions are the process where plants preferentially supply more photosynthetic resources to bacterial root nodules that are fixing more atmospheric N than to other nodules. The aim of this process is to improve nodule efficiency. This also implies that the plants will not divert as much energy to the nodules if soil C is freely available as a source of energy to the microbes as in the case of the soil with the highest C content (4.25%), compared with that of the low C soil (1.29%). The amount of fixed atmospheric N in *T. repens* plants in the high C soils, was substantially lower (Table 1). This will subsequently lead to senescence of many nodules and soil N content will increase by rhizodeposition (Keyser *et al.* 1992, Slattery *et al.* 2001). It is deducted from literature that the possible reason for lower soil N content of low C soil is as a result of more plant energy being available for plant growth, rendering biomass production more efficient. Biomass production was the parameter used to measure efficiency of N fixation.

The interaction of soil C and biomass production, where seeds were inoculated, was significantly higher regardless of treatment with inoculant (P-value < 0.05) (Figures 1 and 2).

Even though the N fixation of the plants in the low C soil was the highest (Table 1), the biomass production of the specific plants remained the lowest. The plants in the low C soil were thus greatly dependent on the rhizobia for a source of N by fixation. In exchange, the plants divert much of the photosynthetic energy to the nodules that could have been used otherwise for growth and production. Efficiency of N fixation remained highest in the soil with a C content of 4.25% regardless of inoculation.

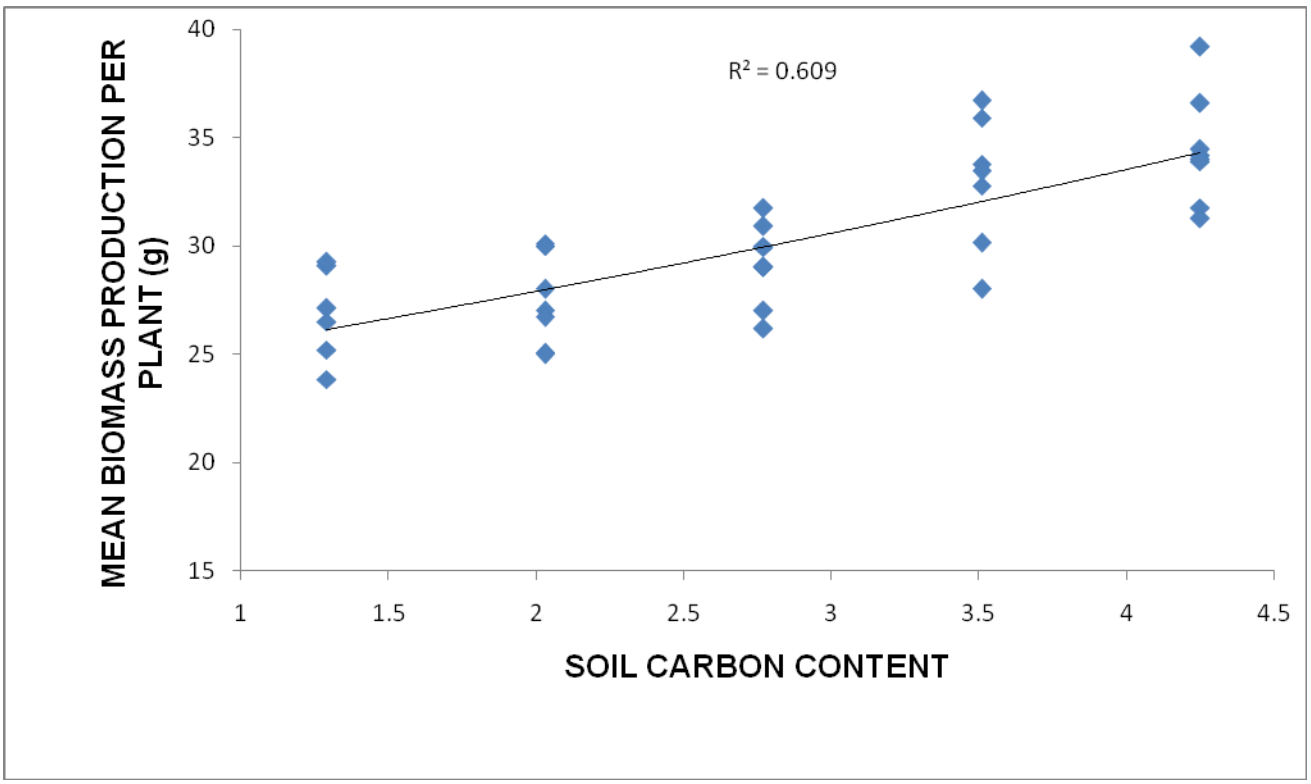


[abcde]Means with no common superscript differed significantly (P-value < 0.05).

Figure 1: Mean biomass production (dry weight) as affected by soil C content where seeds were inoculated with *Rhizobium leguminosarum* bv. *trifolii*.

It is evident from Figures 1 and 2 that the mean biomass production of *T. repens* was much higher in the non-inoculated, low C content soils as compared to the inoculated low C content soils. These results illustrate that biomass production of *T. repens* was more dependent on the N provided by free-living rhizobia in low C content soils (Figure 2). It is deemed to be opposite situation as illustrated in Figure 1 where the biomass production of *T. repens* was

more dependent on the N provided by the more efficient symbiotic rhizobia introduced by inoculation in the higher C content soils.



[abcde]Means with no common superscript differed significantly (P-value < 0.05).

Figure 2: Mean biomass production (dry weight) as affected by soil C content where seeds were only subject to indigenous, free-living *Rhizobium* bacteria (not inoculated).

4. Conclusion

The soil C and N contents were directly related to each other. The correlation of the amount of N derived from the atmosphere and the soil C content were strongly negative. Soil C content had a significant effect on the amount of atmospheric N fixed by *Rhizobium* bacteria in the nodules. The low C content soils had high rates of N fixation, but low biomass production, rendering efficiency of N fixation low. The data obtained on the efficiency of inoculants in different C content soils, concludes that symbiotic rhizobia introduced by inoculant was much more efficient in higher C content soils than free-living rhizobia, which highlights the importance of inoculation in improving the sustainable production of *T. repens* pastures.

Conditions affecting N fixation must be optimal so that fixed N can be transported and redistributed throughout the plant. Diverse management strategies to increase soil C content of pastures containing *T. repens* are necessary to maximise the efficiency of N fixed and rhizodeposition. The environment in which mixed pastures are grown should also be managed efficiently so that the grass component is able to utilise the N fixed by the legume component of these mixed pastures.

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CHAPTER 5

Prepared according to the guidelines of the African Journal of Range and Forage Science

THE EFFECT OF SOIL CARBON ON THE POTENTIAL RHIZOBIAL INFECTION, NODULATION AND NITROGEN FIXATION OF *TRIFOLIUM AMBIGUUM*

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Abstract

***Trifolium ambiguum* (Kura clover) is a clover species neither commercially available nor naturally found in South Africa. This genus has the potential to form a symbiotic relationship with atmospheric nitrogen (N) fixing *Rhizobium* bacteria that form nodules on the host plant's roots. Research on efficient management systems to optimise the *T. ambiguum-Rhizobium* symbiosis is lacking. The main setback of incorporating *T. ambiguum* into grass pastures is that nodule occurrence is rare and atmospheric N fixation is subsequently low. This study aimed to assess the potential of *T. ambiguum* to be infected by host specific *Rhizobium* spp., fix N efficiently and maintain productivity with zero N inputs. The plant infection technique was used to determine the total number of *Rhizobium* bacteria per gram of soil, able to infect the roots of *T. ambiguum.* The results show that indigenous host specific *Rhizobium* bacterial populations in soil were low. A commercial inoculant was applied, but infection response was poor, especially in soils with a C content higher than approximately 2.77%. Nitrogen fixation was low for *T. ambiguum* as compared to that found in *T. repens*. However, inoculation had a significant effect on nodulation indices, but not on soil *Rhizobium* numbers. The aim of incorporation of *T. ambiguum* into low N input grass systems must, therefore, be somewhat different to that of other *Trifolium* spp., because it was evident from this study that this species does not have the capacity to fixate large amounts of atmospheric N due to the symbiotic relationship with *Rhizobium*. Further research needs to be conducted to justify these preliminary findings.**

Key words: Nodulation, *Rhizobium*, nodulation index, inoculation

1. Introduction

Trifolium ambiguum (Kura clover) is a perennial, rhizomatous legume species originating from Russia. The most important agronomic benefits of this species are its extensive root-rhizome system, winter-hardiness, resistance to several pests and the production of nectar-rich flowers that are attractive to bees (Parker and Allen 1952, Allinson *et al.* 1985, Seguin *et al.* 2001, Walker 2009). However, it exhibits about half the rate of growth and development than that of *T. repens. Trifolium ambiguum* has a much larger root mass which makes it particularly suitable for incorporation into grass pastures, since its persistency is excellent, despite frequent and heavy defoliation (Dear and Zorin 1985).

Trifolium spp. are known to form a symbiotic relationship with host specific *Rhizobium* bacteria that form nodules on the roots of the plant, where atmospheric dinitrogen (N_2) is fixed into plant available sources of nitrogen (N) (Tate 1992). The downside of *T. ambiguum,* however, is that nodule occurrence on the roots is infrequent and subsequently renders atmospheric N fixation low (Parker and Allen 1952, Allinson *et al.* 1985). Because of this, Seguin *et al*. (2001) suggested that N fertilisation must be applied to sustain high plant production of a good quality in grass pasture systems. This could, however, potentially increase weed competition and possible eutrophication pollution of the environment.

No breeding programmes have yet been conducted to increase the potential to form nodules (Taylor 2008). Breeding of *T. ambiguum* is concentrated mainly on plant growth characteristics. The potential of this species to sustain low N input pastures is questioned as it has limited measurable effects from N fixing available inoculants (Seguin *et al.* 2001). The aim of this study was to assess the potential of *T. ambiguum* to be infected by host specific *Rhizobium* species, nodulate successfully and fix N efficiently for potential incorporation into low N input grass pastures. In addition to this, research conducted by Swanepoel *et al.* (2010b) has shown that soil C has a significant effect on inoculation of *T. repens.* This, however, has not been established for *T. ambiguum* and will be an objective of this study.

2. Materials and Methods

2.1. Experimental site

The study was carried out on Outeniqua Research Farm near George, Western Cape, South Africa (Altitude 201m, 33 58'38" S and 22 25' 16" E) (Botha *et al.* 2009). The area has a temperate climate with a long term average annual rainfall of 728 mm, evenly distributed throughout the year (ARC 2009). This study consisted of a pot trial, which was conducted under a structure covered with 50% shade net having open sides.

2.2. Experimental design

Five soils from an Estcourt soil type (sandy loam), with different levels of soil C, were identified on the Outeniqua Research Farm (Soil Classification Working Group 1991, Botha 2003). This is the soil type representative of the soils in the Southern Cape region. The soil C contents were 1.29%, 2.03%, 2.77%, 3.80% and 4.25% C.

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With respect to the treatments applied, there were two treatments, replicated nine times, tested on each of the five soils, i.e. a total of 15 treatment combinations of:

- *T. ambiguum*, seeds inoculated with a host specific *Rhizobium* strain
- *T. repens* cv. Haifa, seeds subject to indigenous rhizobia only (not inoculated)
- *Arctotheca calendula* (Cape weed)

Trifolium ambiguum was selected as the best cultivar from a different study (Swanepoel *et al.* 2010b), which was grown from seed sown directly in the pots (diameter: 160mm, height: 220mm) at a density of five seeds per pot. After establishment of seedlings, pots were thinned out to two healthy plants per pot.

Pots were arranged in a randomised block design and replicates were placed in separate rows. Pots were arranged in such a way that all the pots in each row received a similar

amount of wind and sunlight. Plants were watered by means of drip irrigation and the soil moisture status was determined with the aid of tensiometers placed at 15 cm in the soil of each treatment. Soil water potential was kept between -10 and -25 kPa (Botha 2002).

2.3. Techniques used to quantify Rhizobium populations

Plants were harvested in the 12th week after planting. Soil was carefully removed from the roots rinsing them with water (Somasegaran and Hoben 1985). Care was taken not to damage or remove nodules from the roots. The nine replicates of each treatment-combination were combined to give three sets of three and mixed thoroughly for soil sampling. Thereafter, the nodulation index was calculated as described by Prevoust and Antoun (2008) using Equation 1. This procedure entailed the scoring of nodules according to size, number and colour (Table 1).

$$Nodulation\ index = A \times B \times C \quad (1)$$

Table 1: Calculation of nodulation indices by multiplying value A, B and C (Prevost and Antoun 2008)

Nodule size	**Value A**
Small	1
Medium	2
Large	3
Nodule colour	**Value B**
White	1
Pink	2
Nodule number	**Value C**
Few	1
Several	2
Many	3

Subsamples of 32 ml were taken from the rhizosphere soil, pooled together, and then refrigerated in previously sterilised, air tight glass sampling bottles for transportation to the microbiological laboratory for analyses.

2.3.1. Plant infection count analysis

Plastic pouches used for this experiment were obtained from Mega International, St. Paul, Minneapolis (Figure 1). These pouches are specifically designed to be an inexpensive and space saving alternative to Leonard jars (Toomsan *et al.* 1984, Somasegaran and Hoben 1985). *Trifolium ambiguum* seeds were surface sterilised by immersion in 5% sodium hypochlorite for ten minutes. They were subsequently rinsed four times with sterile distilled water placed on filter paper in a sterile petri-dish for pre-germination at 26°C in a dark room for 48 hours. For convenience, the germinated seedlings were placed in a refrigerator for a maximum of 5 days or until transplantation.

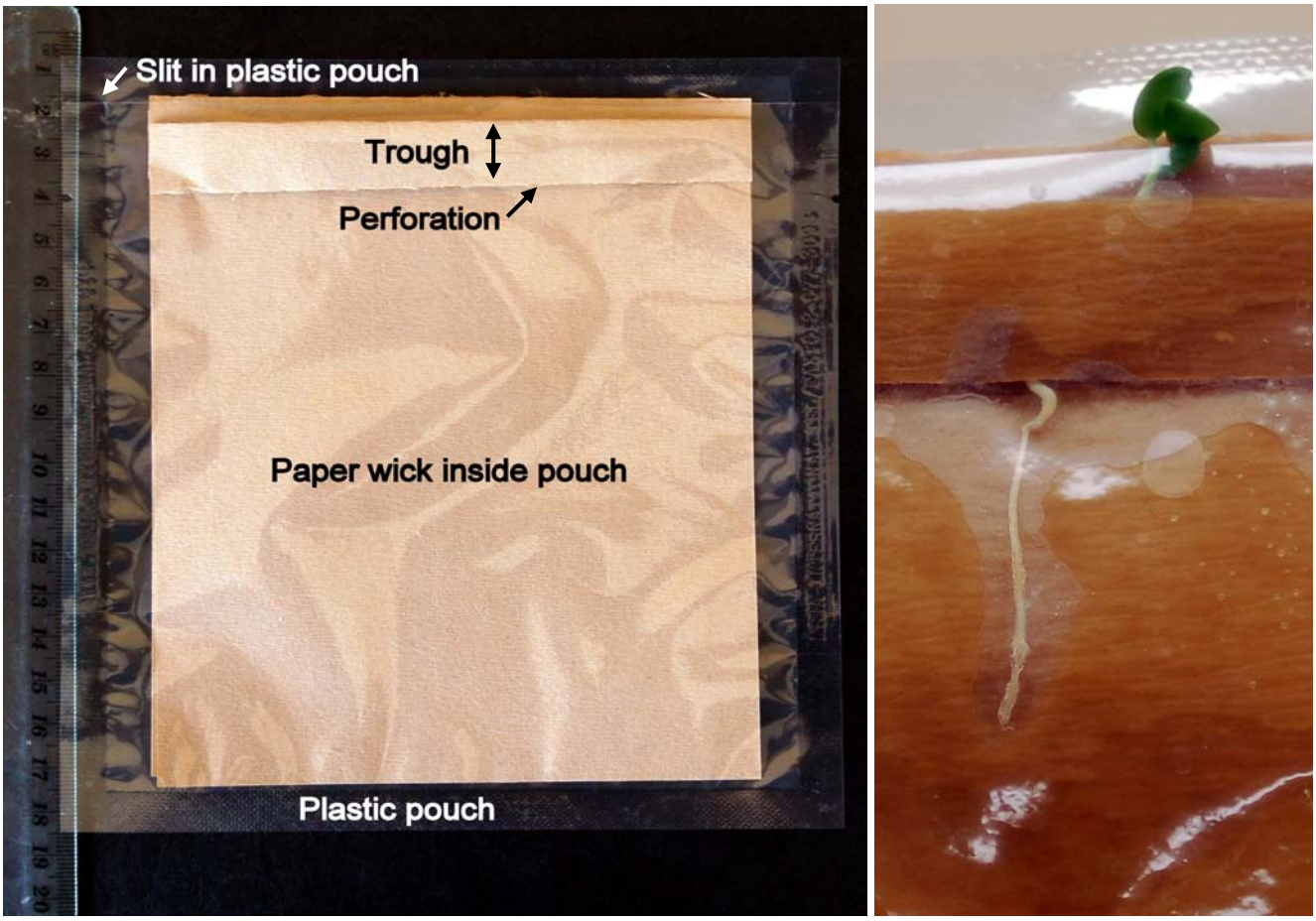

Figure 1: Plastic cyg™ seed germination pouch from Mega International (right). Seedling root development can easily be screened (left).

They were filled initially with 20 ml of an N-free plant nutrient solution (Brockwell 1963, Weaver and Frederick 1972, Somasegaran and Hoben 1985, Weaver and Graham 1994). The solution contained the following nutrients (Somasegaran and Hoben 1985):

- Cobalt chloride - $CoCl_2.6H_2O$, 0.004 mg
- Boric acid - H_3BO_3, 2.86 mg
- Manganese chloride - $MnCl_2.4H_2O$, 1.81 mg
- Hydrated zinc sulphate – $ZnSO_4.7H_2O$, 0.22 mg
- Hydrated copper sulphate - $CuSO_4.\ 5H_2O$, 0.08 mg
- Sodium molybdate – $NaMoO_4$, 0.1211 mg
- Hydrated manganese sulphate - $MgSO_4.7H_2O$, 492.96 mg
- Dipotassium hydrogen phosphate - K_2HPO_4, 1474.18 mg
- Potassium dihydrogen phosphate - KH_2PO_4, 600.09 mg
- Calcium chloride - $CaCl_2$, 110.99 mg
- Hydrated ferric citrate - $FeC_6H_5O_7.H_2O$, 5.00 mg
- Peptone - 1.0 g
- Distilled water – 1 L

Seedlings with a similar radical length (between 10 – 20 mm) were transplanted into the pouch by placing the radical through a perforation in the trough with sterile forceps (Figure 1) (Somasegaran and Hoben 1985). The inoculant was prepared by using the soil harvested from the rhizospheres of *T. ambiguum* plants grown in the pots. A representative soil sample of 1 g was added to 9 ml of Ringer's buffer solution in an autoclaved glass bottle along with 30 sterilised glass beads of similar size. The buffer solution contained the following (Somasegaran and Hoben 1985):

- Dipotassium hydrogen phosphate - K_2HPO_4, 1.21 g
- Potassium dihydrogen phosphate - KH_2PO_4, 0.34 g
- Peptone - 1.0 g
- Distilled water - 1 L

The pH of the buffer solution was brought to 7.0 ± 0.1 by using 1M KOH or HCl. Each bottle containing the soil was shaken vigorously by hand for five minutes and a ten-fold serial

dilution was prepared immediately while the soil was still in suspension. One milliliter (ml) of the dilution was added to each of four replicates from each set. MPN counts were made by using four replications and tenfold serial dilutions with a control for each set containing N-free nutrient solution and 1 ml of buffer solution. For this study to have any significance, the uninoculated controls must be negative. All the work was performed in a sterile environment (laminar flow cabinet). Instruments used were sterilised by immersion into 99% ethanol and flaming (Weaver and Frederick 1972, Somasegaran and Hoben 1985, Weaver and Graham 1994).

Pouches were placed on a rack, built from a wooden frame and galvanised wire. It was kept in a sterile environmental growth chamber, at 25 °C with a six hour dark period (Weaver and Graham 1994, Broos *et al.* 2004). Pouches were screened daily for nodule formation. The nutrient solution was replenished as necessary (Somasegaran and Hoben 1985, Weaver and Graham 1994).

Only the absence or presence of nodules bears significance in this study and quantity of nodules is not applicable. The number of plants positive (nodules present) was recorded for each set. MPN values were calculated by the following series of formulae (Equations 2 to 6) (Woomer *et al.* 1990, Briones and Reichardt 1999):

1. Actual volume inoculated

$$a = \left(\frac{Dilution\ inoculated}{Dilution\ source}\right) \times Actual\ volume\ inoculated \qquad (2)$$

Where a = Succeeding volume inoculated

2. Halvorson and Ziegler's general MPN equation

$$\frac{a_i p_i}{1 - e^{-a_i x}} + \cdots + \frac{a_n p_n}{1 - e^{-a_n x}} = a_i z_i + \cdots + a_n z_n \qquad (3)$$

Where a_i = Succeeding volume inoculated,

p_i = Number of positive pouches in the i^{th} set

x = MPN-value

z_i = Number of tubes in the i^{th} set

n = Number of samples

3. The probability value provides an estimate of frequency of a certain combination for a probable number.

$$P = \left(\frac{z_i!}{p_i!\,q_i!}\right)\ldots\left(\frac{z_n!}{p_n!\,q_n!}\right)\left(e^{-a_i x}\right)^{q_i}\ldots\left(e^{-a_n x}\right)^{q_n}\left(1-e^{-a_i x}\right)^{p_i}\ldots\left(1-e^{-a_n x}\right)^{p_n} \quad (4)$$

Where P = Probability value

a_i = Succeeding volume inoculated

p_i = Number of positive pouches in the i^{th} set

q_i = Number of negative pouches in the i^{th} set

x = MPN-value

n = Number of samples

4. The population estimate

$$Population\ estimate = x\frac{1}{d} \quad (5)$$

x = MPN

d = the single dilution source basing the values for the volumes inoculated.

5. Calculation of the 95% confidence intervals

$$CF = antilog_{10}\left(2 \times 0.55\sqrt{\frac{log_{10}dr}{n}}\right) \quad (6)$$

Where

dr = dilution ratio

Microsoft Excel® was used to solve the equations (Briones and Reichardt 1999, Microsoft Office 2007).

2.3.2. Quantification of culturable (free-living and symbiotic) rhizobia using the plate count method

A representative soil sample of 1 g was added to 9 ml of Ringer's buffer solution (as outlined above) in an autoclaved glass bottle containing 30 glass beads of similar size. Each bottle was then shaken vigorously by hand for 5 minutes and an eight-fold serial dilution was prepared immediately while the soil was still in suspension. Yeast mannitol agar (YMA) amended with Congo red dye was prepared as prescribed by the manufacturers, autoclaved and 20 – 30 ml was transferred to 90 mm sterile petri-dishes (plates). This was allowed to set at room temperature for at least 2 hours, thereafter the plates were inverted and refrigerated until further use. Test tubes containing the serial dilution were shaken by a vortex. Dilutions 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} were plated out by spreading 100 µl of the suspension on each plate of YMA, in four replicates using a hockey stick. Plates were inverted and incubated at 25 °C for 4 days in a dark cabinet. After incubation, colonies were counted as described by Somasegaran and Hoben (1985). Colonies counted must be white to somewhat translucent, circular and raised and may produce significant amounts of extracellular mucilage polysaccharides. Colonies that are red, pink or orange, with a distinctive aroma and/or not circular, are unlikely to be *Rhizobium* (Weaver and Graham 1994). The colony forming units were calculated by equation 7.

$$CFU = \frac{number\ of\ colonies}{Volume\ inoculated} \times \frac{1}{Dilution\ ratio} \quad (7)$$

2.4. Quantification of nitrogen fixation

A reference plant with similar phenology as *T. ambiguum* was chosen and it was assumed that *A. calendula* has a similar affinity for N assimilation as *T. ambiguum* (Pate *et al.* 1994). *A. calendula* thus served as the non N-fixing reference plant. This reference plant was used to quantify biological N fixation with the N difference technique (Equation 1). The total N yield of *A. calendula* was subtracted from the total N yield in the N-fixing plant system (*T. ambiguum*) (Hart *et al.* 1994, Carranca *et al.* 1999).

$$N_2 \text{ fixed}_{ND}\ (g.g^{-1}) = \text{Total N yield}\ (g.g^{-1})_{T.\ ambiguum} - \text{Total N yield}\ (g.g^{-1})_{A.\ calendula} \quad (8)$$

Percentage N derived from the atmosphere (%Ndfa) per unit plant mass was calculated using Equation 2.

$$\%\text{Ndfa} = N_2 \text{ fixed}_{ND}\ (g.g^{-1}) \times 100 \quad (9)$$

The plants were harvested during the 12th week after planting. The symbiotic effectiveness was measured as biomass weight (dry matter). Each plant's roots and shoots were dried at 60 ˚C for 72 hours, weighed and milled as described by Botha (2003). The same plants that were grown to determine symbiotic effectiveness were also used to assess nodulation using a categorical scoring system (Prevost and Antoun 2008).

The nine replicates of each treatment were combined to give three sets of three. The total N content in the plant matter was determined by the AgriLASA method (AOAC International 2002). Soil C was determined by the Walkley-Black method (Walkley 1935, Chapman and Pratt 1961, Nelson and Sommers 1982).

2.5. Statistical analyses

The data were analysed according to the described experimental design. An analysis of variance was performed using SAS 9.2 (2003 – 2008) for the continuous variables. The GLM model was used for the analysis of variance. The assumptions of normality were tested to

determine significant difference between means and the student t-test was conducted at a 5% significance level (SAS Institute Inc. 2008).

3. Results and discussion

In this study, for the enumeration of symbiotic *Rhizobium* capable of infecting *T. ambiguum,* the plant infection technique was chosen to estimate the number of viable symbiotic *Rhizobium* cells present in the rhizospheric soil.

The plant infection technique produced a MPN value of rhizobial cells per gram of soil. This technique highlighted the presence of symbiotic *Rhizobium*, and not free-living *Rhizobium*. The MPN values with associated P-values greater than 0.05 were not statistically interpretable, thus 83.3% of MPN values were considered to be significant (P-value < 0.05). The MPN values with associated P-values greater than 0.05 and confidence intervals are indicated in Table 2. The control treatment values are not shown since they were negative, i.e. a MPN value of zero.

The MPN of bacterial cells per gram of soil ranged from 0 to 436.44 bacterial cells, this was low when compared with *T. repens* on the same treatments (Swanepoel *et al.* 2010c). Soil where no host specific rhizobia were detected had the highest treatment level of soil C (4.25%), indicating the lack of rhizobial persistency in these soils, compounded by the fact that these seeds were also inoculated with a host specific *Rhizobium*. It was, however, expected that population densities would be low or even absent, since *Rhizobium* population density is highly correlated with presence of its particular host legume (Keyser *et al.* 1992), and this plant species is not present in the region.

Table 2: MPN values as affected by soil C content and inoculation.

Soil C content (%)	Inoculation	Mean MPN	95% Confidence interval	
			Upper limit	Lower limit
1.29	Yes	52.0[b]	262.6	20.89
1.29	No	23.0[b]	197.5	13.48
2.03	Yes	51.3[b]	194.9	18.17
2.03	No	26.5[b]	301.9	13.67
2.77	No	436.4[a]	1659.3	114.79
2.77	Yes	125.1[b]	475.7	32.91
3.8	No	97.8[b]	371.9	28.43
3.8	Yes	73.7[b]	420.5	25.73
4.25	No	113.0[b]	452.8	29.09
4.25	Yes	0[b*]		
LSD (0.05)[1]		205.80		

* = Not detected

[1]LSD = Least significant difference of the mean MPN values of treatment-combinations. This P-value provides the significance level (0.05) of the test statistic.

[abc]Means with no common superscript differed significantly (P-value < 0.05).

In Figure 2 it is clear from the data presented that inoculation had an effect on the MPN values when the soil C content was lower than approximately 2.77% C. Legume response to inoculation is therefore dependent on factors in the soil, such as plant growth, indigenous rhizobial numbers, effectiveness of indigenous strains, competitive ability with other soil microorganisms and availability of mineral N in the soil (Keyser *et al.* 1992, Turk *et al.* 1993, Brockwell *et al.* 1995). The lack of saprophytic competency of symbiotic *Rhizobium* in soils where the C content is high, is possibly due to the lower ability of these rhizobia to compete with other microorganisms that are common in these soils.

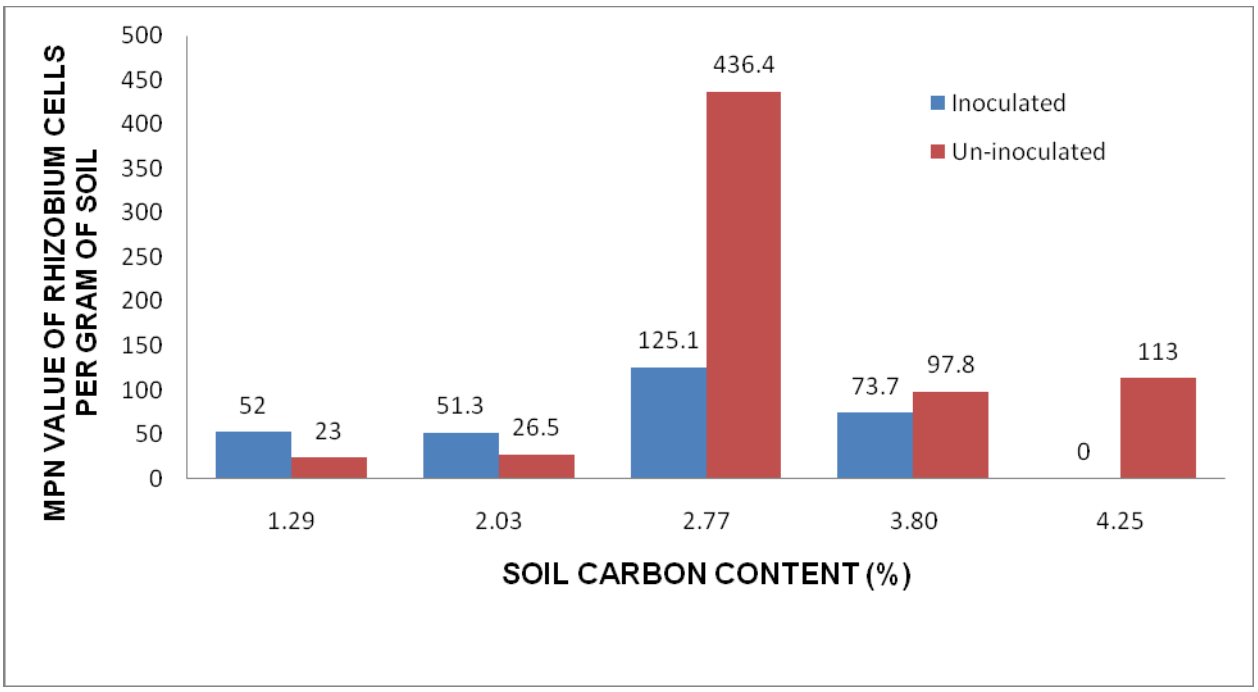

Figure 2: Most-Probable-Number (MPN) values as affected by soil C content and seed inoculation.

It is evident from the data of this study presented in Figure 2, that indigenous free-living *Rhizobium,* proliferate at a particular soil C content of approximately 2.77% or higher, and that most symbiotic *Rhizobium* introduced by inoculation, proliferate in soils where the C content is lower than approximately 2.77% C. It was also noted from this data that the introduced *Rhizobium* completely failed to persist in the high C content soil.

Thies *et al.* (1991b) showed that where there are less than ten native rhizobial cells per gram of soil, the clover plant yield was increased by 85% when inoculated with host specific *Rhizobium* strains and, therefore, a marked increase in production can be anticipated if these seeds are to be inoculated (Thies *et al.* 1991b). In soils where rhizobial population numbers are low or absent, seedling establishment and vigour is greatly dependent on inoculation of the seed with a host specific *Rhizobium* strain (Alexander 1982, Crush 1987). However, even when seeds are inoculated, a sufficient number of *Rhizobium* bacteria must be present in close vicinity of the legume seed to be able to effectively infect the roots (Keyser *et al.* 1992, Brockwell *et al.* 1995, Tainton 2000). In this study inoculation did not increase the number of

soil rhizobia, as measured by both the plant infection technique and the plate count method (Figure 2).

The plate count technique provided data which emphasises that the total culturable rhizobia were not drastically influenced by the different levels of soil C (Table 3). This supports the findings of Brockwell (1963), Weaver and Frederick (1972). These *Rhizobium* colonies are represented by both symbiotic and free-living rhizobia.

Data where total number of rhizobia (free-living and symbiotic) was quantified (Figure 3), did not coincide with data obtained for symbiotic rhizobia in Figure 2, indicating that few free-living rhizobia are able to infect the roots of *T. ambiguum*.

Table 3: *Rhizobium* colony forming units as quantified by the plate count method (symbiotic and free-living) and affected by soil C content and seed inoculation.

Soil C content (%)	Inoculation	Log_{10}(Plate count) (CFU)
1.29	Yes	11.36^{a}
1.29	No	11.14^{ab}
2.03	Yes	9.75^{c}
2.03	No	11.30^{a}
2.77	No	10.60^{b}
2.77	Yes	10.88^{ab}
3.8	No	11.29^{a}
3.8	Yes	11.32^{a}
4.25	No	9.61^{c}
4.25	Yes	9.21^{c}
LSD (0.05)		0.627

LSD = Least significant difference of the mean MPN values of treatment-combinations.

^{abc}Means with no common superscript differed significantly (P-value < 0.05).

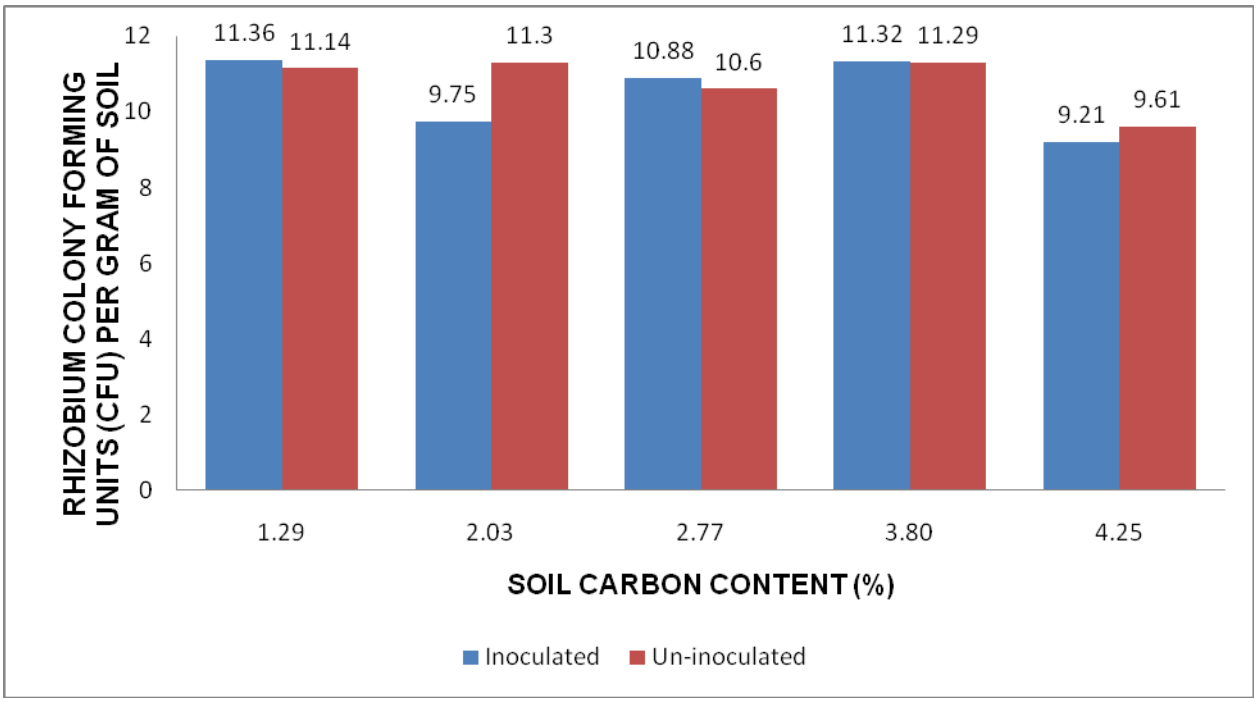

Figure 3: *Rhizobium* colony forming units as affected by soil C content and seed inoculation.

Table 4 represents the actual success of nodulation. Nodulation indices for both inoculation treatments were very low compared with that of *T. repens* undergoing the same treatment-combinations. For comparative purposes, the mean nodulation index of *T. repens* was 12.16 for the treatment where seeds were inoculated and 11.30 in the treatment where seeds were not inoculated. If the assumption can be made that soil C content had no significant effect on any parameter or interaction of parameters (Swanepoel *et al.* 2010a) this latter statement holds truth. To increase the number of introduced host specific rhizobia in the soil successfully, the number of rhizobia in the inoculant must be high and the inoculant application procedures must be well managed (Brockwell *et al.* 1995).

The mean nodulation index did, however, show a marked increase when seeds were inoculated with host specific *Rhizobium*. Figure 4 illustrates that there was a significant difference in nodulation between inoculated versus non-inoculated treatments for *T. ambiguum*. It is shown that nodulation was more successful in the lowest C content soil (1.29% C).

Table 4: Mean nodulation index as affected by soil C content and seed inoculation.

Soil C content (%)	Inoculation	Mean Nodulation Index
1.29	Yes	9.75[a]
1.29	No	2.50[bc]
2.03	Yes	4.00[b]
2.03	No	1.25[c]
2.77	Yes	4.25[b]
2.77	No	1.63[c]
3.8	Yes	3.89[b]
3.8	No	1.53[c]
4.25	Yes	3.08[bc]
4.25	No	1.47[c]
LSD (0.05)		1.99

LSD = Least significant difference of the mean MPN values of treatment-combinations.

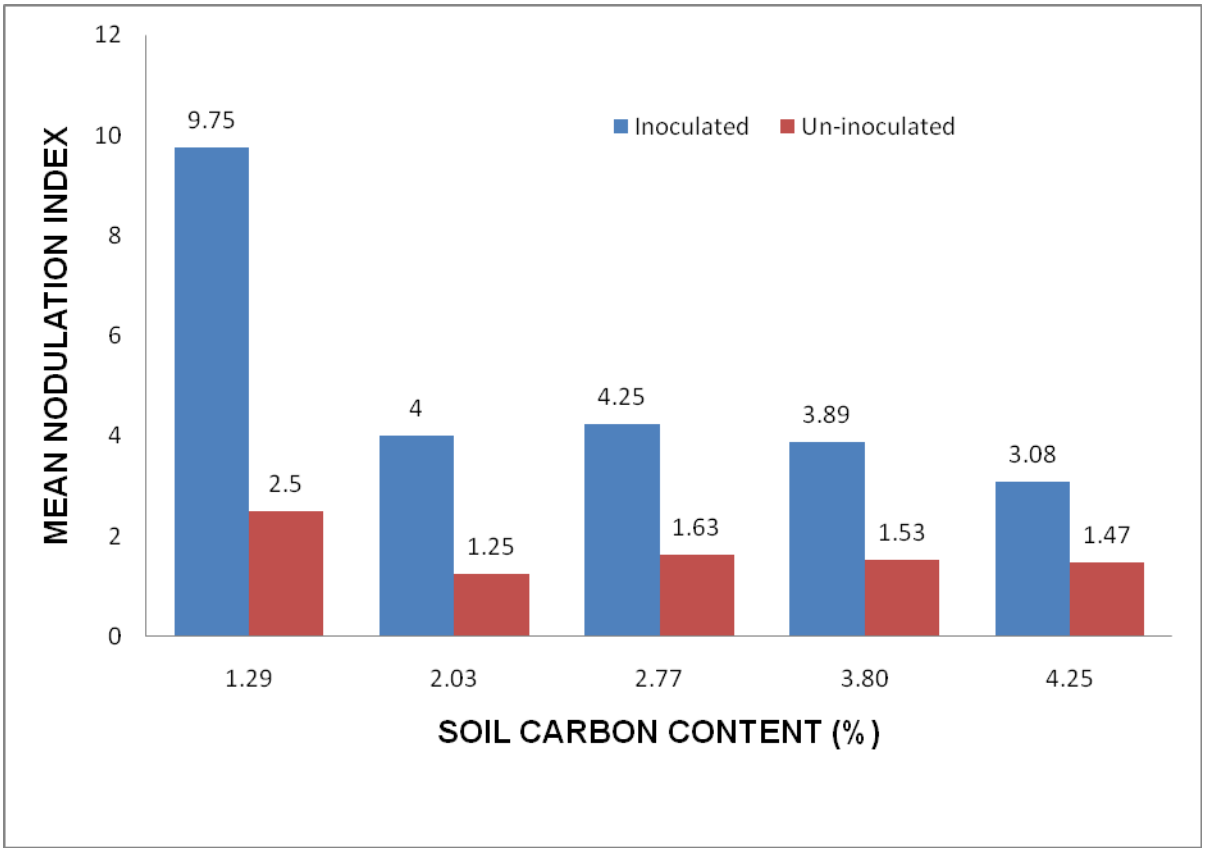

Figure 4: Mean nodulation indices of *T. repens* as affected by soil C content and seed inoculation.

It was visually observed that most nodules were formed at the root crown of *T. ambiguum*. This is the area where rhizobia were introduced from the inoculant on the seed. The rhizobia failed to spread throughout the entire rhizospheric soil body.

It is clear from Table 5 that soil C content had no significant effect on the amount of atmospheric N_2 fixed (%Ndfa) with *T. ambiguum* as host plant. Even though the plants growing in the low C soil fixed equal amounts of atmospheric N, the soil N content was 30.2 g kg^{-1} of the high C content soil in comparison, to the low C content soil which had a N content of only 7.5 g kg^{-1}. In low N input grass-clover mixed swards, this will be exceptionally important as the grasses will be able to utilise only this rhizodeposited N.

Table 5: The mean %Ndfa, final soil N content as affected by soil C content

Soil C content (%)	Mean %Ndfa	Final soil N content (g kg^{-1})
1.29	1.3667[a]	7.500[a]
2.03	1.2600[a]	10.25[a]
2.77	0.6775[a]	16.00[b]
3.51	1.1150[a]	22.70[c]
4.25	1.0350[a]	30.20[d]
LSD (0.05)	**0.7494**	**0.0401**

[abc]Means with no common superscript differed significantly (P-value < 0.05).

4. Conclusion

The plant infection technique revealed that the indigenous host specific rhizobial populations that are able to infect *T. ambiguum*, are low or absent in soils of the George region of South Africa. *T. ambiguum* is not a naturalised species of the region and, therefore, the indigenous *Rhizobium* numbers in the soil were low, especially in lower soil C content soils. Indigenous, free-living *Rhizobium* proliferated at a particular soil C content of approximately 2.77% or higher, and the most symbiotic *Rhizobium* that was introduced by inoculation, proliferated in soils where the C content was lower than approximately 2.77% C. This can be ascribed to the competitive effect between rhizobia and other soil microorganisms. Introduced *Rhizobium*

completely failed to persist in the high C content soil. However, nodulation response was significantly better with inoculation treatments, even though the nodulation indices were low regardless of soil C content. This illustrates that introducing host-specific *Rhizobium* strains by inoculation of seed, will support the growth of indigenous *Rhizobium* numbers in the soil. Even though *T. ambiguum* has shown a nodulation response, the saprophytic competency of the *Rhizobium* in the soil was low as the populations are not adapted to the particular environment. The importance of inoculating *T. ambiguum* seed was highlighted in this study. It was also concluded that soil C content had no significant effect on the amount of atmospheric N_2 fixed (%Ndfa).

The major failure of this species to meet the optimum nodulation rate to fix N for sustainable low N input pasture systems, makes it unsuitable as a species to decrease N fertiliser inputs in dairy production systems in the Southern Cape region of South Africa. It is therefore recommended that further research needs to be conducted to justify these preliminary findings.

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CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

Nodulation potential of the four cultivars tested (Huia, Haifa, Regal, Ladino) was similar in specific environmental conditions. The TSM means of the *T. repens* cultivars differed significantly between all four cultivars, but only affected the cultivar Huia, having the heaviest seed and the greatest biomass production. The test of association showed no significant differences in nodulation index between cultivars or planting dates. When farmers need to decide which cultivar to plant, it should not be based on the potential of the plant to nodulate, as there were no significant differences between cultivars. These decisions should rather be based on the traits that render the plant most adapted to the specific environment. Vigorous plant growth which is also determined by many other different factors will ensure healthy plant nodulation. Farmers can, though, take TSM into consideration as this will ensure higher biomass production earlier in the plants life. The differences in seed weight cannot entirely be ascribed to the cultivar-effect, but harvesting and management factors will also affect TSM. The potential of these cultivars to fix N efficiently must, however, still be investigated. In soils where rhizobia are either scarce or inefficient, inoculation with adapted rhizobia is deemed necessary. Eventually the inefficient inoculant strains can be replaced by highly efficient rhizobia to fix nitrogen at a minimum expense.

Quantification of *Rhizobium*, especially in samples where background bacteria and other microorganisms are present, is limited to only a few methods. Direct counts can only be performed in pure cultures of rhizobia, using optical density measurements with a microscope (Somasegaran and Hoben 1985). A viable count is accomplished by plating methods (pour-, spread- and drop plate counts). The plant infection technique is an indirect method fundamentally similar to the MPN technique (Cochran 1950, Toomsan *et al.* 1984, Somasegaran and Hoben 1985, Woomer *et al.* 1990). Choice of method is dependent on the type, number and composition of samples. In this study, the plant infection technique was chosen to estimate the number of viable symbiotic *Rhizobium* cells present in the rhizospheric soil as it is the only means available to estimate *Rhizobium* numbers in soil samples with

heterogenous background bacteria present (Toomsan *et al.* 1984, Scott and Porter 1986, Woomer *et al.* 1990b). A direct quantification technique is necessary to verify the results of the plant infection technique, but direct techniques for samples with background microorganisms are not available, rendering statistical validation, within the plant infection technique, necessary.

Probability values (P-values) for each pattern of positive pouches in a dilution series were produced (Cochran 1950, Scott and Porter 1986). MPN values with associated P-values greater than 0.05 were not statistically interpretable. Variation between results of MPN values of soil where *T. repens* grew, was very high, i.e. a least significant difference (LSD) of 9577.3. This was likely to be the cause of no statistical differences between means of treatment-combinations, rather than that of the treatments themselves. The sensitivity of the technique must be increased in future to prevent this variation. Each set consisted of four replicates and a control and it is recommended to increase the number of replications in each set to at least 10. A ten-fold serial dilution (10^{-1} to 10^{-10}) for each treatment-combination was prepared, but positive pouches were only observed up to 10^{-6}. If laboratory capacity is limited, and only a certain number of pouches can be accommodated, it is preferred to prepare dilutions up to 10^{-6} or 10^{-7} and have more replicates. Estimating *Rhizobium* population counts by MPN using Microsoft Excel® worked well, and would be recommended above using Tables with codes (Somasegaran and Hoben 1985, Scott and Porter 1986, Woomer *et al.* 1988b).

The spread plate count is often used to verify the results of the plant infection technique even though it was shown that it is not suitable for samples that are contaminated with other microorganisms. The lack of aptness of this technique is mainly due to the difficultly of distinguishing *Rhizobium* colonies from other commonly occurring contaminants that also do not take up Congo red dye (Figure 1) (Weaver and Frederick 1972, Toomsan *et al.* 1984, Somasegaran and Hoben 1985). The results of the two methods used in this study were strongly correlated. These findings were supported by Brockwell (1963), Weaver and Frederick (1972). These controversial reports show that the variability of results depends on test conditions.

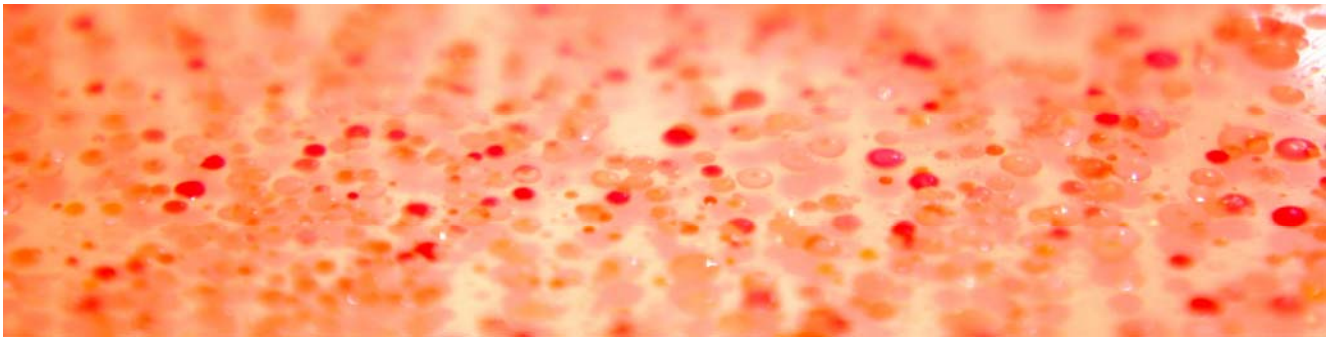

Figure 1: A yeast mannitol agar plate amended with Congo red dye (dilution 1 x 10^{-3}). Distinguishing *Rhizobium* colonies from other commonly occurring bacteria that also do not take up Congo red dye is difficult. The colour of colonies ranges from translucent/white to pink, orange and bright red.

If plate count techniques are used in companion with the plant infection technique, it is suggested to prepare the samples in parallel for both methods by preparing excess diluents in order to plate out the exact diluents for yeast mannitol agar plating and the plant infection technique (Scott and Porter 1986).

Researchers in the Southern Cape of South Africa need to give innovative attention to soil health and resilience, as the current high N input pasture systems are not sustainable. Soil organic matter is the most important factor influencing soil health and rhizobia is an equally important biological indicator of a healthy soil. The health of the soil, cannot, however, be solely estimated by means of rhizobial counts, but other indicators need to be assessed in collaboration with the rhizobial indicators. *Rhizobium leguminosarum* bv. *trifolli* is also robust and adaptable in the soil of the George, South Africa region. The MPN values ranged from 78 to 8907 bacterial cells per gram of soil, but was not affected by soil C.

The MPN assay of soil where *T. ambiguum* was grown, revealed that the indigenous host specific rhizobial populations in soil of the George, South Africa region are low or absent. The MPN values ranged from 0 to 436.4 bacterial cells per gram of soil, and no specific trend with soil C treatment was observed. *T. ambiguum* is not a naturalised species of the region and, therefore, the indigenous *Rhizobium* numbers in soil was low.

The plant infection technique (MPN technique) can be used as a tool to help identify the areas where inoculation is likely to result in improved productivity. *Rhizobium leguminosarum*

bv. *trifolii* was common in the specific soil, however, the response of *T. repens* plants to inoculation was lacking since no significant effect on the MPN of rhizobia per gram of soil was observed, but there was a significant interaction between soil rhizobial numbers from the spread plate count and inoculation treatments. The LSD's between treatment-combination means of the MPN data were high and the fact that no differences were observed may have been more an effect of the variability of the method rather than the true effect of soil C and inoculation. Nodulation indices did not differ significantly between inoculation treatments. It is recommended to persist with inoculation procedures of *T. repens* seeds, even though no response from inoculation was noticed in the MPN results.

Inoculation of *T. ambiguum* seeds had no effect on the *Rhizobium* population sizes in rhizospheric soil as indicated by both spread plate count and MPN methods. However, nodulation response was significantly better with inoculation treatments. This illustrates that introducing host-specific *Rhizobium* strains by inoculation of seed will build on low indigenous *Rhizobium* numbers in the soil. Even though *T. ambiguum* had shown a nodulation response, the saprophytic competency of the *Rhizobium* in the soil was low as the populations are not yet adapted to the particular environment. The importance of inoculating *T. ambiguum* seed is stressed from the results found and observations made in this study.

Spatial and temporal variability of *Rhizobium* populations in pasture soils were not included in this study and may lead to variability in response to inoculation. Further assessment of the host specific *Rhizobium* numbers as affected by seasonal changes and soil environmental dissimilarity are required before accurate conclusions can be drawn on the necessity of inoculation procedures.

Conclusions drawn directly from in-field situations may be precarious as the controlled soil environment in pots may be greatly different from that of field soil. Pot studies poorly predict biomass production and persistence of *T. repens* in-field (Caradus *et al.* 1989) and it may, therefore, be logical to accept that pot studies poorly predict the true situation in field, even though it is the same soil used.

It could also be argued that soil C in this temperate zone, with a minimum of 1.29% C and a maximum of 4.25% C, ought not to be critical, but soil C values which are much lower in subtropical areas (e.g. < 0.2%) might be critical.

The soil C and N contents were directly related to each other. The correlation of the amount of N derived from the atmosphere and the soil C content is strongly negative. Soil C content had a marked effect on the amount of atmospheric N fixed by *Rhizobium* bacteria in nodules of *T. repens* plants. The low C soils had high rates of N fixation, but low biomass production, showing low efficiency of N fixation. *Trifolium repens* growing in an environment with N present, had a lower potential to fix N as soil N was already plant available and plants were less dependent on N fixation for N metabolism and protein synthesis. A state of equilibrium is approached as less ureides are exported from the nodules to the plant shoots that lead to lower rates of N fixation (Madigan *et al.* 2000). This can be applied to field situations of inorganic N in grass-legume mixed pastures. The ability of the legume to fix N will be decreased subsequent to inorganic fertiliser-N application. Inoculation of seeds had no effect on the amount of N fixed or on biomass production. This can be ascribed to the fact that rhizobial populations in the particular soil were large.

Management of the soil environment must receive special attention. Future research will not only be on increasing or sustaining soil quality by building SOM. This will lead to more efficient plant production. Soil organic matter is the main factor that influences the maintenance of a high soil quality, which will in turn, determine sustainability of the soil and the profitability of pasture systems.

Trifolium ambiguum is a species not yet commercially available in South Africa and therefore, indigenous host specific *Rhizobium* populations in the soil were low or absent. Inoculation had no significant effect on the amount of N fixed. The major failure in these species to meet the optimum nodulation rate to fixate N for sustainable low N input systems, makes it unsuitable as a species to decrease N fertiliser inputs in dairy production systems in the Southern Cape region of South Africa.

The N difference technique underestimated N fixation compared with isotopic methods (natural abundance and ^{15}N dilution techniques) when only above ground biomass was analysed (Carranca *et al.* 1999). However, other researchers have found good agreement between the isotopic and N difference techniques (Legg and Sloger 1975, Talbot *et al.* 1982, Boller and Nosberger 1987). If only shoots are analysed, it is assumed that total N in roots and shoots is equal, however, the roots and nodules may contain a relatively large amount of fixed N and it is therefore suggested to analyse total plant biomass when the N difference

technique is used. The N difference method is extremely simple and offers an good alternative to isotopic techniques or the acetylene reduction assay, especially if financial aid is limited (Mårtensson and Ljunggren 1984).

In conclusion good results have been obtained on the efficiency of N fixing *Rhizobium* and the importance thereof in sustainable low N input pasture systems. This research however, has led to many more questions and it is recommended that such research continue, and that attention should be given to the interrelationships of these *Rhizobium* species with there hosts in other environmental conditions.

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CHAPTER 7

SUMMARY

The current recommended rate of fertiliser-N application for pastures in the Southern Cape of South Africa is too high to sustain pasture systems economically. Therefore, current agricultural practices need to focus on maintaining soil health and reducing fertiliser-N input costs by incorporating leguminous N-fixating species, particularly *Trifolium repens*, into grass pasture systems. Soil organic matter (SOM) is an important factor for maintaining soil health as it affects the biological, chemical and physical aspects of soil. Incorporation of other *Trifolium* spp. is less common, but may be beneficial for specific functions or under particular environmental conditions. *T. ambiguum* is mainly incorporated into pastures for its persistency under heavy grazing regimes. However, it does not establish a symbiotic relationship with *Rhizobium* as easily as *T. repens* does. The goal of incorporating *T. ambiguum* must be somewhat different than to of *T. repens* because the potential of *T. ambiguum* as a species to decrease fertiliser costs of kikuyu-ryegrass pastures is unknown, because host specific *Rhizobium* spp. have only recently been developed. Choice of species and soil management practices will have a major impact on the profitability of dairy farming systems. The aim of this study was to assess the nodulation potential of *T. repens* and *T. ambiguum* to decrease fertiliser-N input costs in sustainable pasture systems. The ability of the introduced rhizobial populations in the soil to successfully nodulate *T. repens* and *T. ambiguum,* as a function of the size of the rhizobial population, will be strongly dependent on the ability of the rhizobial populations to proliferate and be maintained by the soil environment. The population size of the naturally occurring and introduced rhizobial strains, were assessed and N fixation was quantified using the N difference technique to compare different treatment-combinations, i.e. between different levels of soil C content and inoculation.

The assessment of nodulation potential of four *T. repens* cultivars (Huia, Haifa, Regal, Ladino) revealed that all plants, regardless of cultivar and planting date, formed nodules after eight weeks. Naturalised rhizobia are robust and widespread in the soil of the George region, and nodulation of *T. repens* with or without inoculant application was common. The cultivar

Huia, had the highest TSM, and the highest biomass production. There were no consistent differences between nodule indices of any of the four cultivars. Therefore, TSM alone had no notable effect on nodulation. The TSM was consistent, however, with the establishment of seedlings up to ten weeks after germination. The TSM also had a significant effect on early plant size and development rate, but not on nodulation. A significant chi-square analysis was performed for all cultivars and planting dates and the tests of association showed that the nodulation index reacted similarly for all cultivars and planting dates. When a farmer needs to decide which cultivar to plant, it should not be based on the potential of the plant to nodulate but rather be based on the adapted production traits for the specific environment.

The plant infection technique was used to quantify microbial populations in rhizospheric soil of different treatment-combinations. The objective of this trial was to quantify the total number of symbiotic rhizobial cells per gram of soil as affected by SOM and host plant. The effect inoculation of the host plant seeds had on the *Rhizobium* numbers in rhizospheric soil was also assessed.

Rhizobium leguminosarum bv. *trifolii* was detected in all soils, regardless of level of soil C or treatment with inoculant, emphasising the robustness and adaptability of *Rhizobium* in different levels of SOM. *Rhizobium* and SOM can play vital roles in the maintenance of soil health by increasing its capacity to function as a living system and sustain pasture productivity. *Rhizobium* in association with SOM also contributes to the soil's physical factors related to soil resilience. These soils will likely have a large potential to return to equilibrium after disturbances. Researchers in the Southern Cape of South Africa need to give innovative attention to soil health and resilience as the current high N input pasture systems are not sustainable.

The MPN assay is a simple measure that aids in predictions of the need to inoculate with a host specific *Rhizobium* inoculant. Response to inoculation was lacking and may have been caused by limitations in the soil, such as poor plant growth, high indigenous rhizobial numbers or highly effective indigenous strains and availability of mineral N in the soil. This study showed that nodulation indices did not differ significantly between inoculation treatments, and therefore inoculation practices by farmers in the area are, however, not discouraged by these results. Even though the indigenous rhizobial population in the particular soil is large, the spatial and temporal variability is not included in the assessment and may considerably alter

the response. Apart from this, rhizobia in commercial inoculants are selected to be highly effective and may, therefore, be superior in their N fixing ability compared with indigenous strains.

As soil C content increased, the mean amount of atmospheric N_2 fixed (%Ndfa) by *T. repens* decreased proportionally. The largest amount of atmospheric N that had been fixed (1.79%) was from plants in the low C soil treatment. This can be ascribed to the greater dependence of the plant on the fixed N as very little soil N is available. As the fixed N is exported out of the nodules to the plant, the rhizobia need to fix more N in an attempt to maintain the nutrient equilibrium inside the nodule. The plant, in turn, supplies energy for the fixation process derived from photosynthesis. Plant sanctions are the process where plants preferentially supply more photosynthetic resources to nodules that are fixing more atmospheric N. The aim of this process is to improve nodule efficiency. This also implies that the plants will not divert as much energy to the nodules if soil N is freely available as in the case of the soil with the highest C content (4.25%). The amount of fixed atmospheric N in these plants is substantially lower. By means of rhizodeposition, the soil N content of the high C soil (4.25% C) increased more, compared with the low C soil (1.29% C). Even though the N fixation of the plants in the low C soil was the highest, the production of the specific plants remained the lowest. The plants in the low C soil were greatly dependent on the rhizobia for a source of N by fixation. In exchange, the plants divert much of the photosynthetic energy to the nodules that could have been used otherwise for growth and production. Efficiency of N fixation remained highest in the soil with a C content of 4.25%.

Relatively low numbers of *Rhizobium* that are able to form symbiosis with *T. ambiguum* was detected in the soils, except for one soil treatment (4.25%). It was, however, expected that the *Rhizobium* population densities would be low or even absent because *Rhizobium* density is highly correlated with presence of its particular host legume, being previously absent in the particular soil. Inoculation of *T. ambiguum* seed prior to planting was successful. The mean nodulation index has shown a marked increase with inoculation, but was low compared to that of *T. repens* on the same treatment-combinations. Response to inoculation is dependent on various aspects, such as plant growth, indigenous rhizobial numbers, effectiveness of indigenous strains, and availability of mineral N in the soil. Few of the host specific bacteria was present in these soils and this attributes to the fact that there

was a large response from inoculation. Inoculation and soil C content had no significant effect on the %Ndfa, nevertheless, the importance of inoculation of *T. ambiguum* seed with a host specific *Rhizobium* inoculant was emphasised by the results from the MPN assay.