The effect of soil carbon on symbiotic nitrogen fixation and symbiotic *Rhizobium* populations in soil with *Trifolium repens* as host plant

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Soil organic carbon (SOC) is the main attribute of high-quality soil. The amount of nitrogen fixed by *Rhizobium* symbiotically with *Trifolium repens* (white clover) is ultimately determined by the quality of the soil environment. The effect of SOC on the total number of symbiotic and saprophytic rhizobia was determined. Subsequently, the amount of nitrogen (N) fixed was assessed by using the N difference technique. Most *Rhizobium* was detected between a SOC content of 2.03% to 3.80% in both inoculated and non-inoculated soils. Inoculation increased the number of rhizobia in soil. Most N was fixed in the soil with the lowest SOC content. Although the amount of N fixed increased as the level of SOC decreased, the efficiency of N fixation decreased proportionally to SOC. Subsequently, more N was rhizodeposited. It was concluded that symbiotic rhizobia introduced by the inoculant were more efficient than free-living rhizobia in soils with higher carbon content, which highlights the importance of inoculation in improving the sustainable production of *T. repens* pastures.

Keywords: colony forming units, inoculation, most probable number, plant infection technique

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). Post-World War II technological advances have led to increased production outputs of inexpensive inorganic nitrogen (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). 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 (Labuschagne 2009) and does not support sustainable production. A holistic aim for sustainable agriculture is the attainment of maximum quantity and quality of pasture production with minimal 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 N fertiliser is not sustainable is that it has become too expensive. Inorganic N fertiliser 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 N fertiliser 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 1987, Michaelson-Yeates *et al.* 1998, Ledgard *et al.* 1999, Ledgard *et al.* 2001, McDonald *et al.* 2002).

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). 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 *Rhizobium* bacteria. Any environmental or managerial constraint can limit growth, symbiotic production, persistence and survival of the bacteria in soil and will, therefore, also affect the amount of N fixed. 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; response to inoculation can be difficult to predict (Thies *et al.* 1991b) and for this reason, many farmers do not inoculate seed before planting. 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).

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 amount of atmospheric N fixed by legumes varies widely, depending on the management of the pastures within the abiotic and biotic soil environmental components. 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 host 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, such as no-till practices aimed to increase soil organic carbon (SOC) levels, may enhance N fixation efficiency. Soil quality have become customary internationally as a science-based means to predict and support sustainability of resources and SOC is, in turn, the main attribute for maintaining soils of a high quality (Carter 2002). Soil environmental manipulation, particularly of SOC, can have a direct effect on the legume plant itself or indirectly affect the rhizobial populations that infect the roots of the legume. 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). Decisions regarding these managerial factors have a major impact on the amount of N fixed. Consequent profitability of dairy farming systems would be increased. Research on efficient management systems to optimise the *T. repens-Rhizobium* symbiosis is lacking. Therefore, more information is required to understand the dynamics and interactions in the environment.

The aim of this study was to determine the total number of symbiotic rhizobia per gramme of soil as affected by SOC and inoculation. Subsequently the success of inoculation was determined by quantification of N fixed by the *T. repens-Rhizobium* relationships.

Materials and Methods

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, conducted under a structure covered with 50% shade net and open sides.

Experimental design

Five soils from an eluvic-prismacutanic soil form (Estcourt form, duplex soil family), with different levels of SOC, 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.

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).

Trifolium repens cv. Haifa seeds were sown in the five different soils. The experiment was a factorial design with nine replications: two levels of inoculation (seeds inoculated with *Rhizobium leguminosarum* bv. *trifolii,* and seeds not inoculated) at five levels of soil organic carbon (1.29%, 2.03%, 2.77%, 3.80% and 4.25% C).

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

Arctotheca calendula (Cape weed) was sown in nine pots which were randomly distributed amongst the other pots as reference plants (see section below: Technique used to quantify N fixation)

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).

Harvesting

Plants were harvested on 15 December 2009, i.e. during the 12th week after planting. Soil was carefully removed from the roots by 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 1. This procedure entailed the scoring of nodules according to size, number and colour (Table 1).

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

Table 1: The visual scoring system to calculate nodulation indices of *Trifolium repens* roots by multiplying a scored value for nodule size (value A), nodule colour (value B) and nodule number (value 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
Nodulation index = A x B x C	

Replicates 1 to 3, 4 to 6 and 7 to 9 were combined to give three sets of composite samples which were mixed thoroughly for soil sampling. Subsamples of 32 ml were taken from the soil, pooled and then refrigerated in previously sterilised, air tight glass sampling bottles for transportation to the microbiological laboratory for analyses.

Plant infection count analysis

Enumeration of symbiotic *Rhizobium* capable of infecting *T. repens* was determined by the plant infection technique used 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). Plastic pouches used for this experiment were obtained from Mega International, St. Paul, Minneapolis. 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 pre-germinated at 26 °C in a dark room for 48 hours.

The pouches were initially filled with 20 ml of an N-free plant nutrient solution (Brockwell 1963, Weaver and Frederick 1972, Somasegaran and Hoben 1985, Weaver and Graham 1994). Seedlings with a similar radical length (between 10 – 20 mm) were transplanted into the pouch. The soil inoculant was prepared by using the soil harvested from the rhizospheres of *T. repens* plants grown in the pots. Each bottle containing the soil inoculant was shaken for five minutes and a ten-fold serial dilution was prepared immediately while the soil was still in suspension. One ml of this dilution was added to each of four replicates from each set of pouches. Pouches were kept in a sterile environmental growth chamber, at 25 °C with a six hour dark period (Weaver and Graham 1994, Briones and Reichardt 1999, Broos *et al.* 2004). Pouches were screened daily for nodule formation. Most probable number (MPN) values were calculated using the method described by Woomer *et al.* 1990 and Briones and Reichardt 1999.

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

A direct quantification technique is necessary to verify the results of the plant infection technique. A representative soil sample of 1 g was added to 9 ml of Ringer's buffer solution. Each bottle was then shaken 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 by adding distilled water to YMA powder, the pH was adjusted to 6.8 ± 0.1, autoclaved and 20 – 30 ml was transferred to 90 mm sterile petri-dishes (plates). Dilutions of 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. 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 were white to somewhat translucent, circular and raised. Colonies that were red, pink or orange, with a distinctive aroma or not circular were unlikely to be *Rhizobium* and were therefore not counted (Weaver and Graham 1994). The colony forming units were subsequently calculated.

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 2). 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).

$$\text{N}_2 \text{ fixed}_{\text{ND}} \text{ (g g}^{-1}) = \text{Total N yield (g g}^{-1})_{T. \ repens} - \text{ Total N yield (g g}^{-1})_{A. \ calendula}$$

$$(2)$$

Percentage N derived from the atmosphere (%Ndfa) per unit plant mass was calculated using Equation 3.

$$\%\text{Ndfa} = \text{N}_2 \text{ fixed}_{\text{ND}} \text{ (g g}^{-1}) \times 100$$

$$(3)$$

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 (Table 1) (Prevost and Antoun 2008).

The total N content in the plant matter was determined by the AgriLASA method (AOAC International 2002). SOC was determined by the Walkley-Black method (Walkley 1935, Chapman and Pratt 1961, Nelson and Sommers 1982).

Statistical analyses

The data were analysed as a randomized block design with two factors, i.e. SOC at five levels and species inoculation at two levels (5 x 2 factorial design). An analysis of variance (ANOVA) with linear contrasts and log transformations was performed using SAS 9.2 (2003 – 2008) for the continuous variables. The assumptions of normality were tested using the Shapiro-Wilk test. The student t-test was conducted at a 5%

significance level to determine least significant differences (LSD) between means. Regression analyses comparing the slopes of the lines for the biomass data was conducted using student t-test at a 5% significance level. Mean biomass per plant for inoculated or non-inoculated seeds was compared using student's t-test (SAS Institute Inc. 2008).

Results

Mean most probable number (MPN) values and colony forming units (CFU) of *Rhizobium* bacteria per gramme of soil are shown in Table 2. The interactive effects was not significant (F-ratio = 1.67, d.f. = 4, P = 0.203).

Table 2: Mean (± SE) most probable number (MPN) values and colony forming units (CFU) of *Rhizobium* bacteria per gramme of soil as well as nodulation indices (optimal maximum value of 18) of *Rhizobium* associated nodules on roots of *Trifolium repens* as affected by soil organic carbon (SOC) content and seed inoculation with *Rhizobium leguminosarum* bv. *trifolii*. Standard error of means (SEM) and P-value of the interaction for each parameter is also indicated.

SOC (%)	Inoculation	MPN (g^{-1}soil)	CFU (g^{-1}soil)	Nodulation index
1.29	No	5.80 ± 0.89	8.86 ± 0.00	8.44 ± 3.06
1.29	Yes	6.85 ± 2.48	10.3 ± 0.00	10.3 ± 3.69
2.03	No	9.38 ± 2.57	9.71 ± 0.30	12.6 ± 0.54
2.03	Yes	7.74 ± 1.32	9.94 ± 0.51	15.7 ± 2.55
2.77	No	7.57 ± 1.08	9.95 ± 0.53	10.2 ± 2.75
2.77	Yes	9.16 ± 1.61	10.6 ± 0.77	13.7 ± 1.26
3.80	No	4.90 ± 0.32	9.02 ± 0.65	13.1 ± 2.50
3.80	Yes	7.50 ± 1.52	9.84 ± 0.64	12.1 ± 0.67
4.25	No	7.32 ± 2.71	9.77 ± 1.08	10.3 ± 1.70
4.25	Yes	6.60 ± 1.17	9.94 ± 0.00	8.28 ± 0.95
SEM		2.621	0.435	4.949

Inoculation had no significant effect (F-ratio = 2.07, d.f. = 11, P = 0.086) on the symbiotic *Rhizobium* numbers in soil (MPN values) within each SOC level (Figure 1). The MPN of symbiotic rhizobia ranged from as little as 78 to over 8 900 bacterial cells per gramme of soil.
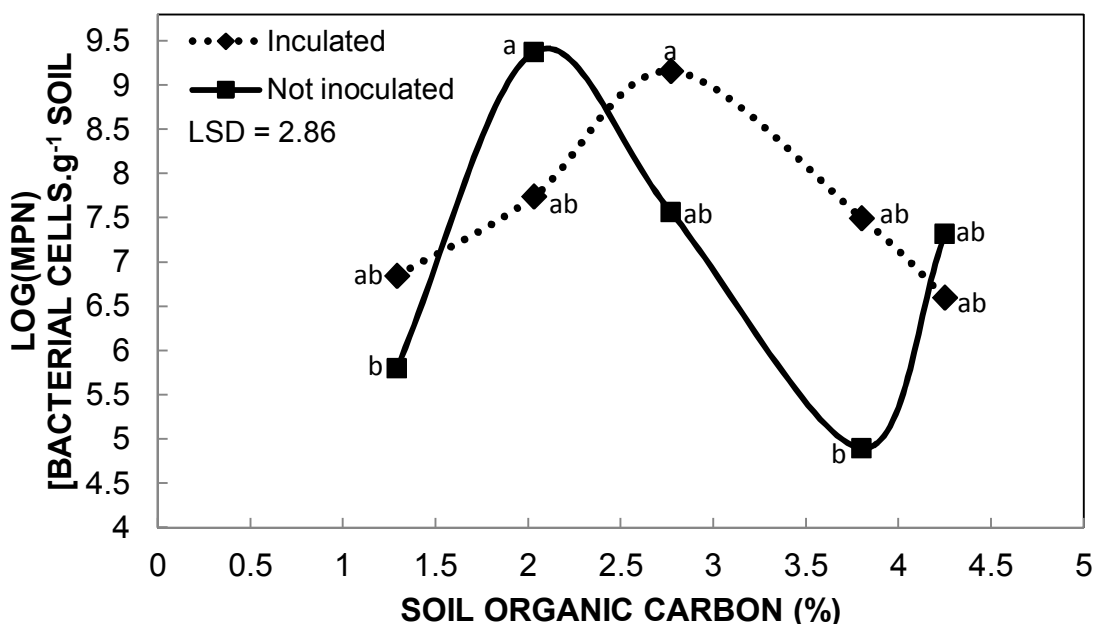

Figure 1: Most-Probable-Number (MPN) values (log transformed) of symbiotic *Rhizobium* bacteria in natural soil at different soil organic carbon (SOC) levels, planted with inoculated or non-inoculated *Trifolium repens* seed.

LSD = Least significant difference (P = 0.05)

MPN values with no common letter (ab) differed significantly (P = 0.05).

At a particular SOC content of ~2.0% the most symbiotic *Rhizobium* was detected from non-inoculated soils and free-living, symbiotic *Rhizobium* predominate. It is interesting to note that the soils containing high levels of SOC (~2.8%) had depressed values of symbiotic *Rhizobium* contrary to belief.

The plate count technique, providing the number of CFU of *Rhizobium* per gramme of soil is shown in Table 2. Figure 2 showed that the total culturable rhizobia were only influenced at low levels of SOC (~1.3%). CFU at higher levels of SOC were not

influenced by inoculation, since these data were statistically similar (F-ratio = 1.27, d.f. = 11, P = 0.341) within SOC levels. These *Rhizobium* colonies are represented by both symbiotic and free-living rhizobia. The data in Figure 2 coincides with data obtained for the symbiotic rhizobia in Figure 1. This, however, differs to the extent that free-living *Rhizobium* is more prevalent in soils with low levels of SOC.
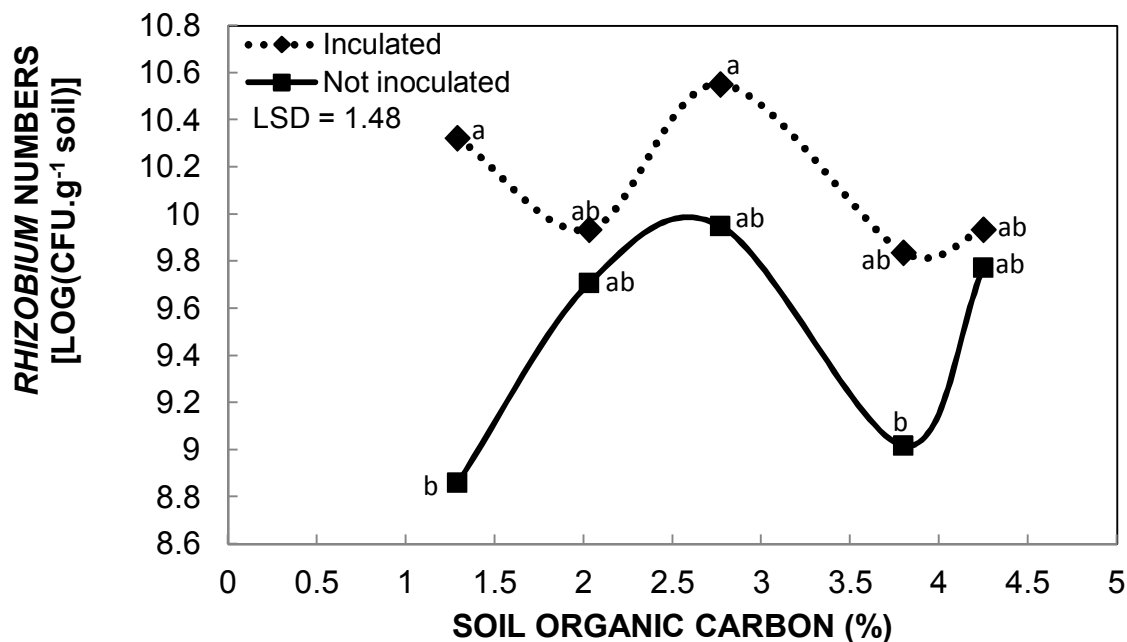

Figure 2: *Rhizobium* (symbiotic and free-living) colony forming units (CFU) per gramme of natural soil (log transformed) at different soil organic carbon (SOC) levels, planted with inoculated or non-inoculated *Trifolium repens* seed.

LSD = Least significant difference (P = 0.05)

CFU values with no common letter (ab) differed significantly (P = 0.05).

Table 2 show the nodulation nodulation indices of *Rhizobium* associated nodules on roots of *Trifolium repens* as affected by soil organic carbon (SOC) content and seed inoculation with *Rhizobium leguminosarum* bv. *trifolii*. Figure 3 represents the actual success of nodulation. It is shown that nodulation was generally similar (F-ratio = 2.99, d.f. = 11, P = 0.019) within SOC levels, except at a SOC level of approximately 2.8% where inoculation had a significant advantage.
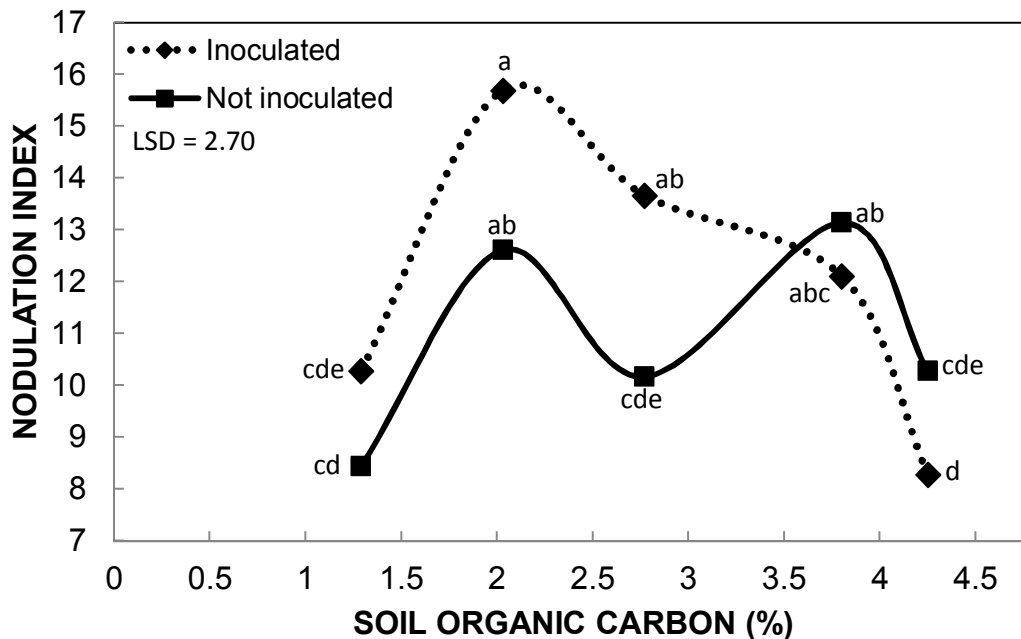
Figure 3: Mean nodulation indices of *Rhizobium* associated root nodules on *Trifolium repens* as affected by soil organic carbon (SOC) content and seed inoculation with *Rhizobium leguminosarum* bv. *trifolii*.

LSD = Least significant difference (P = 0.05)

Index values with no common letter (abcd) differed significantly (P = 0.05).

SOC content had a significant effect on the amount of atmospheric N_2 fixed (%Ndfa) by *Rhizobium* associated root nodules (Table 3). The negative correlation between SOC content and %Ndfa was strong (Pearson correlation coefficient = -0.903). As SOC content increased, the mean %Ndfa proportionally decreased from 1.793% to 0.680% N (Table 3). 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 an N content of 39 g kg^{-1}.

Table 3: The mean (± SE) percentage nitrogen (N) derived from the atmosphere (%Ndfa) by *Trifolium repens-Rhizobium* associated N fixation and final soil N content as at different levels of soil organic carbon (SOC).

SOC content (%)	Mean %Ndfa	Final soil N content (g kg^{-1})
1.29	1.793[a] ± 0.116	6.25[a] ± 0.63
2.03	1.335[b] ± 0.156	12.5[b] ± 1.04
2.77	0.985[c] ± 0.143	17.0[c] ± 2.07
3.51	0.897[c] ± 0.153	29.4[d] ± 1.22
4.25	0.680[d] ± 0.186	39.0[e] ± 1.36
LSD (0.05)	**0.1762**	**2.060**

LSD = Least significant difference (P = 0.05)

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

Mean biomass production was 29.187 g.plant^{-1} and 30.410 g.plant^{-1} for inoculated and non-inoculated soils respectively (t-value = 2.101, d.f. = 11, P < 0.0001). The coefficient of variation (CV) was used to test the precision and reproducibility of the biomass data. The biomass data proved to be highly reproducible since the CV values are low.

The coefficients of linear regression equations for prediction of biomass production from SOC level as affected by inoculation treatment are given in Table 4. The regression equations were highly significant and the coefficients of determination (R^2) were high for both the inoculated and non-inoculated treatments, i.e. 0.971 and 0.979 respectively. The slope of the regression equation for the inoculated seed treatment did not differ significantly (P = 0.108) from than that of the non-inoculated seed treatment, although the intercept of inoculated seeds was lower than the non-inoculated seeds (P = 0.038). There was a significant interaction between SOC and treatment with inoculant (P = 0.014).

Table 4: Coefficients of linear regression equations $y = mx + c$) used to predict biomass production (roots and shoots) of inoculated and non-inoculated *Trifolium repens* from soil organic carbon (SOC) level. The slopes (m) and the intercepts (c) correspond to Figure 4.

	n	m	c	R^2	P-value
Inoculated	45	4.355[a]	16.215[a]	0.979	<0.0001
Not inoculated	45	2.972[a]	21.956[b]	0.971	<0.0001
LSD (0.05)		2.129	4.962		

LSD = Least significant difference (P = 0.05)

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

The linear regression lines to predict biomass production from SOC level for both inoculated and non-inoculated treatments are shown in Figure 4.
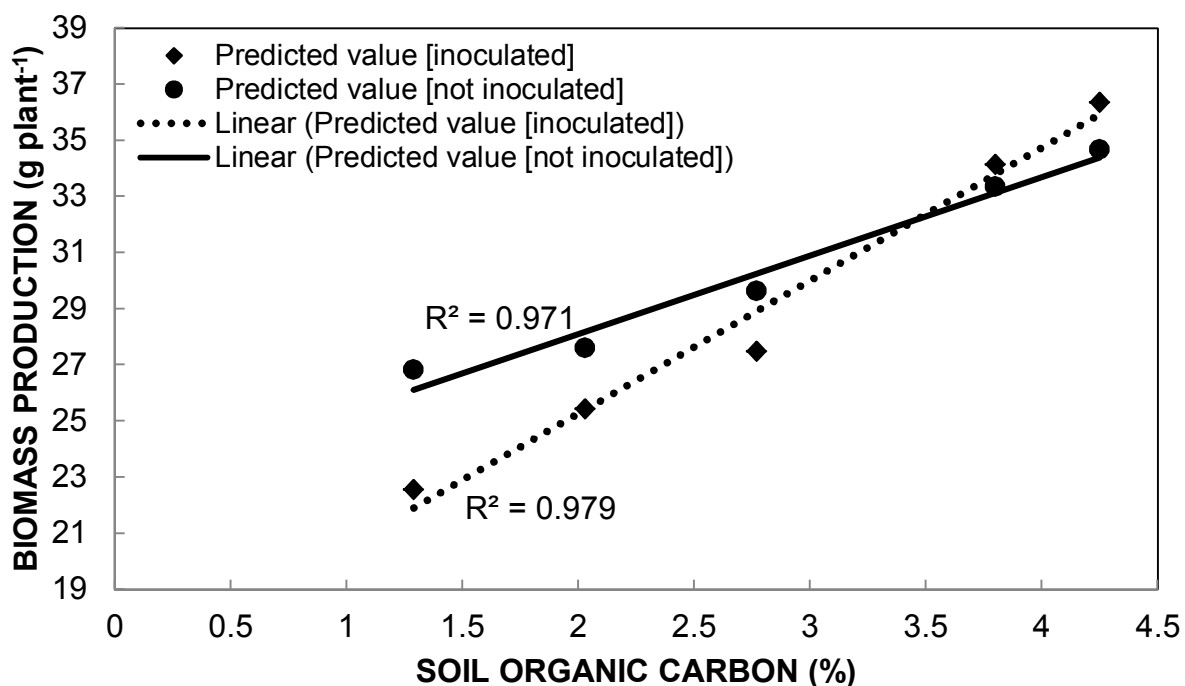

Figure 4: Mean biomass production (dry weight) of *Trifolium repens* roots and shoots as affected by soil organic carbon (SOC) content where seeds were either inoculated with *Rhizobium leguminosarum* bv. *trifolii* or not inoculated.

Discussion

The natural occurrence of *T. repens* in the George area is common and since 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 SOC or treatment with inoculant, emphasising the robustness and adaptability of the genus in different levels of SOC as is supported by Kiers *et al.* (2003). *Rhizobium* and SOC play vital roles in maintenance of soil health by increasing its capacity to function as a living system and to sustain pasture productivity. Soil quality and 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 SOC also contributed to the soil's physical factors related to soil resilience (Bot and Benites 2005, Patra *et al.* 2005). Therefore, the particular soils will likely have a large potential to return to equilibrium after disturbances, being rich in SOC and *Rhizobium*. Research that will determine the optimal level of *Rhizobium* bacteria, the baseline values and population threshold which will enhance or maintain soil health has not been performed and needs to be considered.

According to the success of nodulation for the inoculated and non-inoculated treatments, it appears 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. Lack of legume response to inoculation may be due to limitations in the soil, such as 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 experimental soil was the possible reason for the lack of response to inoculation.

Rhizodeposition of nitrogenous compounds by plant roots growing at high levels of SOC, caused an increase in soil N of more than four times that of the low C soil (Table 3). In low N input grass-clover mixed swards, this will be exceptionally important

as the grasses will be able to utilize this rhizodeposited N (Jørgensen *et al.* 1999, Gylfadóttir *et al.* 2007).

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 (West *et al.* 2002). This also implies that the plants will not divert as much energy to the nodules if SOC is freely available as a source of energy to the microbes as in the case of the soil with the highest SOC content (4.25%), compared with that of the low SOC soil (1.29%). The amount of fixed atmospheric N in *T. repens* plants in the high C soils, was substantially lower. 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). 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.

Even though the N fixation of the plants in the low C soil was the highest, 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 SOC content of 4.25% regardless of inoculation. The mean biomass production of *T. repens* was higher in the non-inoculated, low C content soils as compared to the inoculated low C content soils (Figure 4). Biomass production of *T. repens* was more dependent on the N provided by free-living rhizobia in low C content soils. This contrasted with higher C soils 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.

Conclusion

The MPN value of *Rhizobium* cells per gramme of rhizospheric soil was affected by SOC content. Most symbiotic *Rhizobium* was detected approximately between 2.03% to 3.80% SOC 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 and were more prevalent in soils with low levels of SOC.

The SOC and N contents were directly related to each other. The correlation of the amount of N derived from the atmosphere and the SOC content were strongly negative. SOC content had a significant effect on the amount of atmospheric N fixed by *Rhizobium* bacteria in the nodules. 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 SOC 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.

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 one the most 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.

Acknowledgments

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