The quantification of biological nitrogen fixation by *Trifolium repens* as affected by soil organic matter

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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. These pressures results from high and increasing population densities, urbanization, industrialization and global warming *(Tate, 1992)*. The holistic aim for sustainable agriculture is attaining the maximum quantity and quality of pasture production with minimal nitrogen (N) input in the form of fertiliser and without 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)*. Therefore, sustainability strives towards being beneficial in the long run, for both humans and the environment. Modern agricultural practices tend to maximise output from the soil in the short term and this deprives the soil from its nutrients. This is a major pitfall for future generations and supplying food for an ever increasing world population is becoming a challenge *(Brockwell et al., 1995)*.

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 seeking of biological alternatives for inorganic forms of fertiliser N has therefore become imperative *(Botha, 2003)*. The incorporation of legumes in pasture systems in the Southern Cape is economically and ecologically promising. ***Trifolium repens*** (white clover) are highly nutritive leguminous species suitable for dairy pasture systems *(Mårtensson and Ljunggren, 1984, Williams, 1987, Ledgard et al., 2001, Mcdonald et al., 2002)*. These species are especially high in organic nitrogenous compounds, making it a promising alternative for inorganic fertilizer-N application *(Mårtensson and Ljunggren, 1984, Abberton et al., 1998)*.

The amount of nitrogen (N) fixed varies widely, depending on the management and environment of the pastures. Diverse management strategies of pastures containing ***Trifolium*** are necessary to maximise the amount of N fixed from the atmosphere. The soil environment should be managed efficiently to be able to utilize the fixed N *(Sprent, 1979)*.

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. Soil organic matter is in turn the main attribute towards soil quality *(Carter, 2002)*. Soil environmental manipulation, particularly soil organic matter, 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. Subsequently profitability of dairy farming systems will be increased. The aim of this study is to quantify the amount of N fixed by ***T. repens*** as affected by the soil organic matter 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)*. The pot trial was conducted under an open structure covered with 50% shade net.

2.2. Soil collection and preparation of pots

Two soils from an Estcourt soil type were identified on the Outeniqua Research Farm *(Soil Classification Working Group, 1991)*. The top 250 mm of the soil profile consists of an orthic A horizon, with a weakly developed structure. The colour of this horizon is grey or dark-grey as it is subject to waterlogging *(Soil Classification Working Group, 1991, Botha, 2003)*. The one soil has a high carbon (C) content (4.25 %C), whilst the other has a low C content (1.29 %C). The two soils were mixed uniformly in the following manner to obtain a steady continuum of soil organic matter (SOM):

	SOIL				
	1	2	3	4	5
High C soil	100%	75%	50%	25%	0
Low C soil	0	25%	50%	75%	100%

Soil was 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. The soils were corrected up to the recommended soil fertility levels for a grass-clover pasture: P >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)*.

2.3. Treatments and sowing

Three treatments were tested on each of the five soils, i.e. a total of 15 treatment combinations:

- ***Trifolium repens cv. Haifa***, seeds inoculated with ***Rhizobium leguminosarum*** bv trifolii, 9 replicates
- ***Trifolium repens cv. Haifa***, seeds not inoculated, 9 replicates
- ***Arctotheca calendula*** (Cape weed), 4 replicates.

Trifolium repens cv. Haifa was grown from seed, the seeds being sown directly in 5.2 liter pots at a density of 5 seeds per pot. After proper establishment of the seedlings, the pots were thinned to two healthy plants per pot.

The pots were arranged in a randomized block design. The replicates were placed in separate rows. The pots were arranged in such a way that all the pots in each row (replicate) will receive the same amount of wind and sunlight. Plants were watered periodically with drippers. The soil moisture status was determined with the aid of tensiometers placed 15 cm into the soil. The soil water potential was kept between -10 and -25 kPa *(Botha, 2002)*.

2.4. Harvesting and analyses

A. calendula served as the non-fixating reference plant. The reference plant was used to quantify biological N fixation with the N difference technique (Equation 1). The total N yield of a non-fixing reference plant (***A. calendula***) is subtracted from the total N yield in the fixing plant system ***(T. repens)*** *(Hart et al., 1994, Carranca et al., 1999)*.

Equation 1

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

A reference plant with similar phenology and growth pattern must be chosen to the extent that is possible. It is assumed that ***A. calendula*** has a similar affinity for N assimilation than ***T. repens*** *(Pate et al., 1994)*. Percentage N derived from the atmosphere (%Ndfa) per unit plant mass is calculated by equation 2.

Equation 2

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

A reference plant with similar phenology and growth pattern must be chosen to the extent that is possible. It is assumed that ***A. calendula*** has a similar affinity for N assimilation than ***T. repens*** *(Pate et al., 1994)*. Percentage N derived from the atmosphere (%Ndfa) per unit plant mass is calculated by equation 2.

The plants were harvested during week twelve after planting. The nine replicates of each treatment of the ***T. repens*** were combined in triplicate. The nodules on each plant were classified as red (active) or white/grey-green (inactive) nodules. Each plant's roots and shoots were dried at 60˚C for 72 hours and milled *(Botha, 2003)*. The total N content was determined by the AgriLASA method *(Aoac International, 2002)*. Soil organic C was determined by the Walkley-Black method *(Walkley, 1935, Chapman and Pratt, 1961, Nelson and Sommers, 1982)*.

2.5. Statistical analyses

The data was analysed according to the described experimental design. The proposed collected data measurements like 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. The 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.

3. RESULTS AND DISCUSSION

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 soils where rhizobial population numbers are low, seedling establishment and vigour is greatly dependent on inoculation of the seed with a species-specific ***Rhizobium*** strain *(Alexander, 1982 , Crush, 1987)*. Indigenous soil rhizobia adapted to specific conditions in the George district (South Africa) is widespread *(Loos, 1963)*. Importance of inoculation in the current situation is questioned. The average %Ndfa for seed inoculated and seed not inoculated was 1.124 % and 1.152 % respectively. Inoculation had no significant effect on the %Ndfa (LSD = 0.1114). It will, however, be wise to keep up with seed inoculation practices, as the diversity and homogeneity of the indigenous rhizobial populations varies widely. The risk of some plants not nodulating will be decreased.

Soil C content did, however, have a significant effect on %Ndfa (Table 1). As soil C content increased, the mean %Ndfa proportionally decreased. The negative correlation between soil C content and %Ndfa is strong (-0.903).

Table 1: The mean %Ndfa, initial and final soil N content as affected by soil C content

Soil C content (%)	Mean %Ndfa	Initial soil N content (g/kg)	Final soil N content (g/kg)
1.29	1.793[a]	0.00[a*]	0.63[a]
2.03	1.335[b]	0.25[b]	1.25[b]
2.77	0.985[c]	0.50[c]	1.70[c]
3.51	0.897[c]	0.75[c]	2.94[d]
4.25	0.680[d]	1.00[d]	3.90[e]
LSD	0.1762	2.060	2.060

*Not detectable

The clover-***Rhizobium*** relationship has evolved to cater for each other's needs. The white clover strongly relies on the availability of N for protein metabolism and DNA synthesis *(Kiers et al., 2003)*. The soil with the lowest C content (1.29%) also had the lowest soil N content. The most atmospheric N has been fixed (1.793 %) in the low C soil. 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 *(West et al., 2002)*. 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 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 (Table 1). In grazing systems where legumes are present and inorganic N is applied, the amount of fixed N is also lower. This is noticed and verified by many researchers *(Mcauliffe et al., 1958, Davies and Evans, 1990, Bohlool et al., 1992, Davies, 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)*. This will subsequently lead to senescence of many nodules and lower N fixation ability of the plant *(Keyser et al., 1992, Slattery et al., 2001)*.

4. CONCLUSION

Inoculation of seeds had no effect on the amount of N fixed. This can be attributed to the fact that rhizobia bacterial populations in the particular soil are large and common. Soil C content has a marked effect on the amount of atmospheric N fixed by ***Rhizobium*** bacteria in nodules. The soil C and N content were inversely related to each other. The correlation of the amount of N derived from the atmosphere and the soil C content is strongly negative. ***T. repens*** growing in an N rich environment has a lower potential to fix N as plant available N is already available. This can be extracted to field application of inorganic N in grass-legume mixed pastures. The ability of the legume to fix N will be decreased.

Management of the soil environment must receive special attention. Future research will not only be on increasing soil quality, but also on rectifying problems caused by our abusive past practices. Soil organic C is the main factor that influences soil quality, which will in turn determine sustainability and profitability. **EJ**

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