Nodulation potential of four *Trifolium repens* cultivars under field conditions

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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 1987, 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 1987, 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 1987).

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 (Abberton *et al.* 1998). Plant factors, such as root morphology, which can be altered by breeding, may also have an 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 1987, 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.

Materials and methods

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 occasional waterlogging. The chemical status of the top 100mm of soil in the study area is shown in the soil analysis report in Table 1. The top horizon is underlayed by an E-horizon and subsequently a prismacutanic B horizon (Soil Classification Working Group 1991, Botha 2003).

Insert Table 1

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 of each cultivar on moist filter paper in a petridish. The petridishes were placed in a dark cabinet for two weeks. This was replicated four times.

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 (3L ha^{-1}). Soil was tilled with a disc harrow to level the area and was rolled with a Cambridge landroller to form a fine and firm seedbed. The layout was a randomised block design with 16 plots that includes four cultivars replicated four times with one seed planted per 25 cm^2. Each plot measured 500 mm x 500 mm and was equally divided into 100 small blocks of 50 mm x 50 mm each. 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 150 mm 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 record germination percentage and plant survival.

Cultivars

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

Measurements and data analyses

The following measurements were recorded:

The establishment percentage of each plot was calculated over a ten week period using Equation 1.

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

The plants were harvested during week ten after germination as this was sufficient time for proper nodulation (Thomas 1987), and stolon growth initiated after ten weeks rendering recognition of individual plants difficult in the plots. Each plot (500 x 500 mm), was removed with a spade to the depth of 200 mm. 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 2. This procedure entailed the visual scoring of nodules according to size, number and colour (Table 2).

$$Nodulation\ index = A \times B \times C \tag{2}$$

Insert Table 2

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 plants were dried at 60 ˚C for 72 hours to determine the biomass production (Botha 2003).

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).

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 3). Huia recorded 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 3 indicate that germination was good among all cultivars, especially Huia. It was noted that germination percentage compared well with TSM of the selected cultivars, except for Regal. These significantly different values for TSM are due to the fact that seed mass 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 mass (Thomas 1987). The differences in seed mass cannot be ascribed to only the cultivar effect, but also to specific management of the cultivation site during seed production.

Insert Table 3

It was evident from the field trial results (Table 3) that TSM had no significant effect on nodulation, but did affect the biomass production. The cultivar Huia, having the highest TSM, resulted in the highest 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 mass (Connolly *et al.* 1969, Williams 1987). 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 4 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 environmental factors. For seedling emergence, daily temperatures played an imperative role (Harris 1987), however, because temperature readings during the study period were representative to those of the long term average (39.5 years), evident from Table 4, the guidelines in this article can be used as general guidelines for the Southern Cape region.

Insert Table 4

From 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 (4.96°C) was slightly higher than that of the maximum temperature (2.04°C) (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 in bean, lentil and pea (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

All plants, regardless of cultivar and planting date, formed nodules within ten weeks. Naturalised *Rhizobium leguminosarum* bv. *trifolii* is 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.* 2010). 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 4). The nodulation indices of the four cultivars did 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 *Rhizobium* 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 4), 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 showing a significant chi-square analysis for all cultivars (P-value < 0.0001, Figure 2). The number of observations presented in Figures 2 and 3 is with reference to the number of plants classed by the nodulation index.

Figure 2

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. No significant differences in nodulation index between planting dates were observed. 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

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 mass cannot be entirely ascribed to the cultivar effect. The cultivar Huia, with the highest TSM, 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 were 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 nitrogen 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 the environmental conditions that prevailed during the study.

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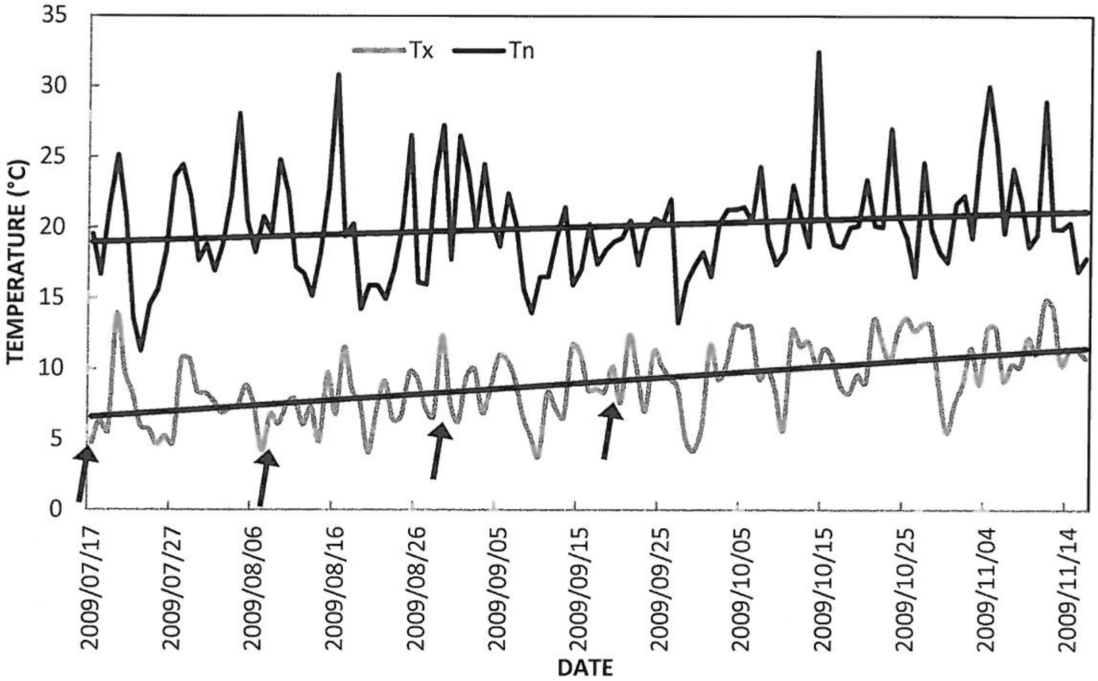

Figure 1: The daily temperature extremes during the time period covered by the trial on Outeniqua Research Farm (Tx = minimum daily temperature, Tn = maximum daily temperature). The planting dates are indicated with black arrows.

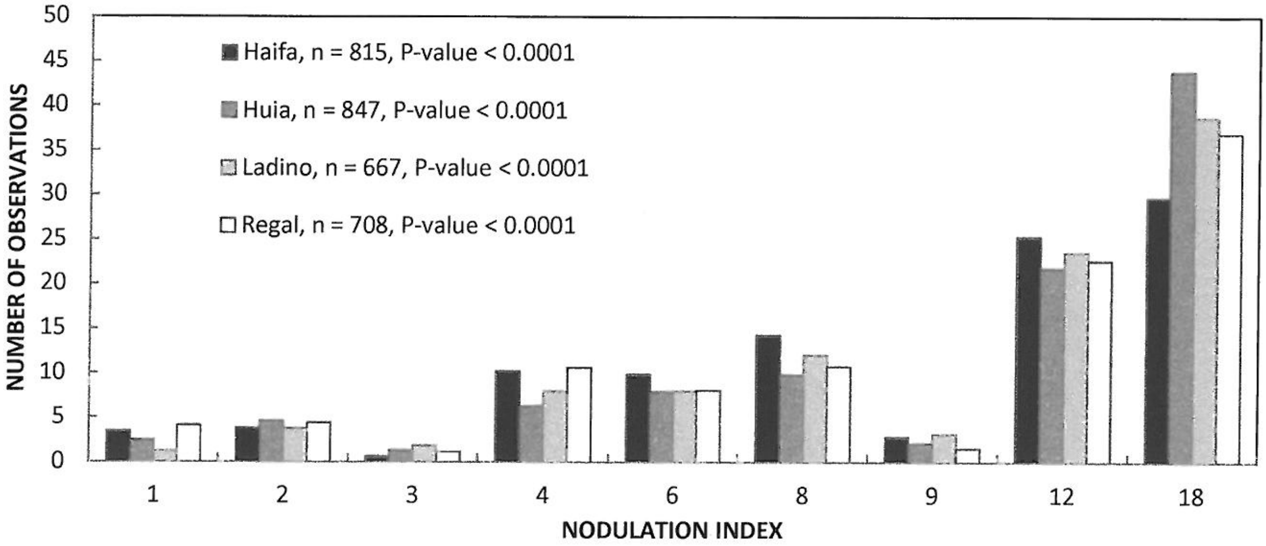

Figure 2: The effect of cultivar on the distribution of nodulation index results.

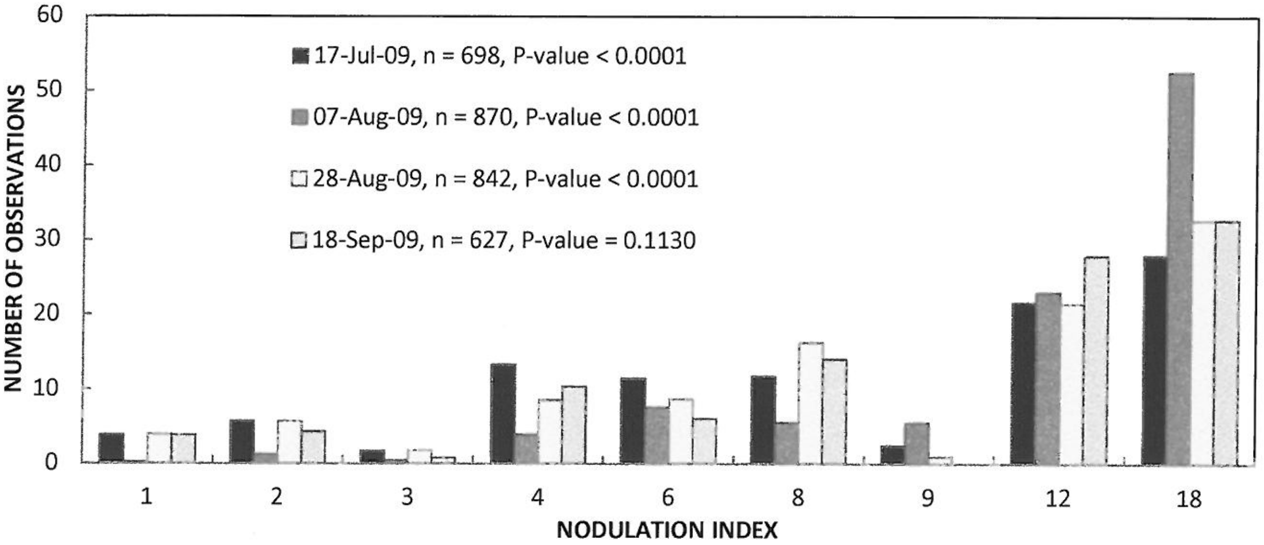

Figure 3: The effect of planting date on the distribution of nodulation indices as result of chi-square analyses.

Table 1: Soil analysis report of the top 100mm of the study area

Parameter	Result
Texture	Loam
pH(KCl)	5.7
Calcium (cmol(+).kg^{-1})	6.16
Potassium (mg.kg^{-1})	80
Magnesium (cmol(+).kg^{-1})	1.24
Sodium (mg.kg^{-1})	125
Phosphorus (citric acid) (mg.kg^{-1})	108
Copper (mg.kg^{-1})	1.01
Zinc (mg.kg^{-1})	4.26
Manganese (mg.kg^{-1})	12.37
Boron (mg.kg^{-1})	0.37
Carbon (%)	2.16
Sulphur (mg.kg^{-1})	3.60

Table 2: 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
Nodulation index = Value A x B x C	

Table 3: The mean thousand-seed-mass (TSM) (g), germination %, establishment %, biomass production (g), 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}
CV	**1.218**	**3.380**	**26.141**	**18.234**	**10.768**
LSD (0.05)	**0.0087**	**3.510**	**16.871**	**5.036**	**7.654**

CV = Coefficient of variation

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

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

Table 4: The long term average monthly minimum (T_n) and maximum temperatures (T_x) (°C), compared to the average monthly minimum and maximum temperatures (°C) during the study period

	Jul	Aug	Sep	Oct	Nov
Long term average T_x (°C)	18.53	18.44	19.1	20.2	21.61
Average monthly T_x (°C) in study period	19.37	19.79	18.84	20.76	22.58
Long term average T_n (°C)	7.26	7.48	8.56	10.39	12.02
Average monthly T_n (°C) in study period	8.53	7.51	8.51	10.69	11.8