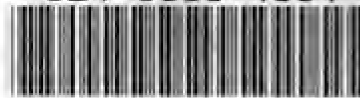
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Do savanna *Acacia*s nodulate as seedlings?

An eco-physiology project towards BSc. Hons. Plant Ecology

Tim Szöke

2006

The nodulation and overall growth performance of seedlings of 5 species of *Acacia* were explored through 3 experiments. *A. karroo*, *A. ataxacantha*, *A. nilotica*, *A. nigrescence* and *A. tortilus* were grown in a pots with 2mM N, 1mM N & 0mM N treatments. The same species were grown in an inoculation vs. no inoculation paired treatment. A grass co-existence field experiment was conducted in which seedlings were grown in grass-dominated plots and grass-free plots. All of the species nodulated, barring only *Acacia ataxacantha*. It was found that the plants grown at 2mM N had a significantly greater biomass than those grown at 1mM N ($p = 0.0000$), but that plants in both of these treatments showed similar ($p > 0.05$) biomass allocations amongst nodules, roots, stems and leaves. Seedlings grown in no-grass plotshad more than 8-fold greater biomass than the acacias grown in grass plots ($p = 0.0001$) due to grass competition. N isotope results show that plants grown in grass dominated plots contain far more N derived from air than plants in cleared plots (average %Ndfa of 65.34 vs. -16.26 & $\delta^{15}N$ of 0.78 vs. 4.74). I conclude that some *Acacia* species do nodulate as seedlings in savannas; and that this ability to host nitrogen fixing rhizobia at the seedling level may be one of the reasons why legumes dominate South African savannas.

Anti-plagiarism Declaration

This project is a compilation of three data sets; acacias were grown in a grass co-existence experiment, a greenhouse and a phytotron. Samson Chimphango grew and harvested the plants that were grown in the greenhouse, and isolated root nodule rhizobia. Mike Cramer, Su van Cauter, and her staff in Hluhluwe-Umpholozi National Park's research camp set up and maintained the grass experiment. I assisted with the harvesting of these plants. The rest of this project is my own; including the growth and harvesting of the plants in the phytotron, and the analysis of the data from the three experiments. Any additional ideas have been appropriately referenced.

Signed

KD SZOK

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Introduction

Savannas occupy 54% of southern Africa (Rutherford 1997) and 12% globally (Scholes & Hall 1996). The complex relationship between grasses and trees in these ecosystems has intrigued ecologists for a long time. Why savanna species are able to co-exist without the grass species succeeding the trees, or vice versa, has been of key interest as the encroachment of woody thickets into grasslands is both an agricultural and a biodiversity conservation issue (Roques *et al.* 2001). Much of the past literature focuses on the interactions between adult acacias and the savanna grasses (e.g. Walter 1971, Stuart-Hill & Tainton 1987, Scholes & Archer 1997) and how climate change may affect this shifting landscape (e.g. Daly et al. 2000), but little has been explored regarding tree seedling-grass interactions.

Walter has published work regarding savannas since the 1930's and later released a compilation of works in 1971. He explains that woody plants are able to co-exist with grasses and shrubs through competition avoidance at the root level. Trees are able to source water and nutrients from lower soil layers than grasses; while grasses are more efficient at taking up available water (and nutrients) in the top layer of soil (Figure 1).

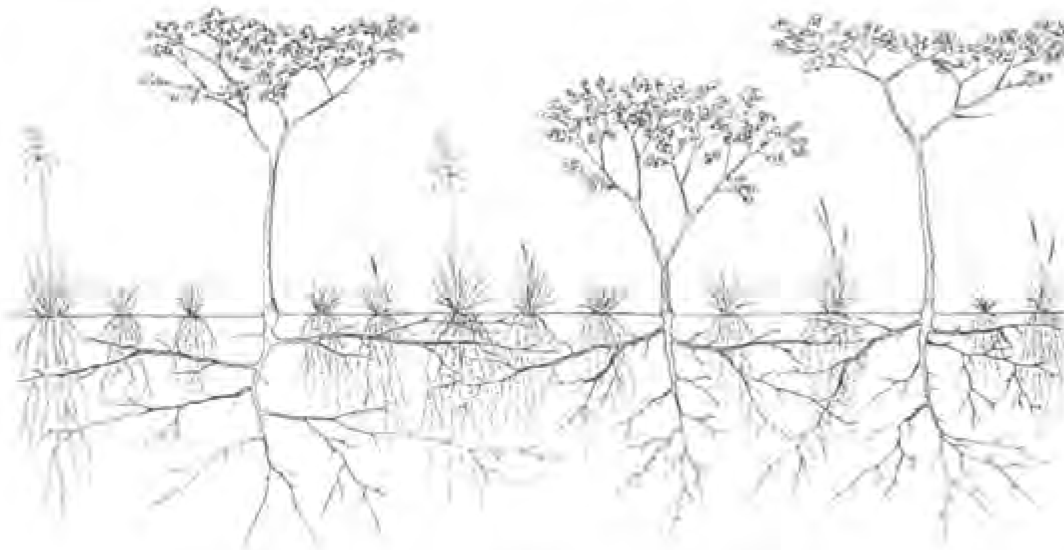

Figure 1. Taken from Walter (1971), where he explains how the trees and grasses in savanna systems avoid root-level competition as they exploit different soil zones.

The dominant woody plants in South African savannas are legumes (*Acacia* species from the family Fabaceae). Legumes are able to nodulate, providing nitrogen fixing

rhizobia with photosynthates in return for nitrogen fixed from the atmosphere (DeFaria *et al.* 1989). This is costly to the plant and often legumes will only nodulate when soil nitrogen is insufficient (Vitousek & Howarth 1991). South African savannas are relatively rich in nitrogen, however, and the reason for legumes being the dominant woody growth-form is unexplained. Bond et al. (2001) grew *A. karroo* and *A. nilotica* in pots with and without grass. They noted that "Root nodules were not present on any of the plants grown without grass".

The majority of the literature does not include the interaction between the grass and the seedlings of the woody plants. Competition avoidance through rooting depth differentiation, as described by Walter (1971) fails to account for young woody plants that are limited to the same soil layer as the surrounding grass. This competition at seedling level may be more pivotal in the spread of the woody layer, or a lack thereof, than competition as adults; as seedling survival determines the future distribution of a species. Balfour (2005) describes an apparent bottleneck being experienced in A*cacia* savanna species. While his thesis is a comprehensive ecological view of the situation, it does not include the impacts of the nitrogen fixation ability of legume-hosted rhizobia.

The focus of this project was to provide answers to the following questions:

1) Are karroo acacias able to nodulate as seedlings; even though they have not been found to nodulate as adults in savanna systems?
2) How is the performance of the seedling affected by inoculation and different nitrogen levels?
3) What effects does grass co-existence have on the acacia seedlings?

After achieving these primary objectives, I hope to support my hypothesis:

Legumes in savannas nodulate as seedlings – a time when nitrogen is limiting.

Methods

A glasshouse experiment was conducted in which seedlings were given two levels of nitrogen. This was done primarily to observe whether the five species are able to nodulate in controlled conditions, as well as to compare levels of overall performance and nodulation between seedlings grown in the two treatments. Seedlings were grown in a phytotron and were not supplied with any nitrogen. It was necessary to grow some plants at 0mM N in order to acquire a 'b-value'. A b-value is the $\delta^{15}N$ of a plant that has had no access to nitrogen other than that fixed by rhizobia in root nodules. This was needed to calculate the percentage of nitrogen fixed from the air (%Ndfa). The same species were grown in Hluhluwe Game Reserve, Kwazulu-Natal, South Africa (28°00'-28°26'S; 31°43'-32°09'E). The 225km^2 park is a typical savanna ecosystem and is an ideal area in which to test the interaction of grass and trees. Plants were grown in grass exclusion plots and grass dominated plots to determine the impact of grass on the acacias' growth and nodulation

Throughout the paper ^{15}N natural isotope abundance ($\delta^{15}N$) is used as an indication of nitrogen fixation (the ratio of $\delta^{15}N$, see Equation 1). Nitrogen is available to plants as ^{14}N and ^{15}N. The heavier isotope is taken up more rapidly and thus high ^{14}N content within a plant is an indication that the plant is taking up N from the soil (Evans 2001), resulting in a higher $\delta^{15}N$. Nitrogen fixing rhizobia convert gaseous N_2 into products comprised of ^{15}N, thus high ^{15}N in plant tissue is a signal that nitrogen fixed from the atmosphere is being absorbed through root nodules.

Equation 1:

$$\delta^{15}N = \left(\frac{R_{sample}}{R_{sandard}} \right) \times 1000‰$$

where $\delta^{15}N$ is the isotope ratio relative to the atmospheric air standard, and R_{sample} and $R_{standard}$ are the molar ratios of ^{15}N to the lighter isotope, ^{14}N. The value for $R_{standard}$ is 0.0036765 (From Box 1, Evans 2001).

Seed Pre-Germination Treatment

The *Acacia* seeds required stimulus before they germinated and three techniques were used. *A. karroo* seeds were left in freshly boiled distilled water for 24hours. *A. tortilis* and *A. ataxacantha* seeds were placed in 98%H_2SO_4 for 30minutes, and *A. nilotica* and *A. nigrescence* seeds were placed in 49%H_2SO_4 for 60minutes; after which they were rinsed with distilled water and left in a flask of distilled water for 24hours. The treated seeds were placed into punnits of 16/30 filter sand. The seeds were placed 2cm below the sand and were covered with an additional 1cm layer of vermiculite. Vermiculite acts as a hydro-insulator and was used to ensure the seeds remained moist.

Glasshouse Experiment

15 seedlings of each of the 5 *Acacia* species were grown (5 plants per species per treatment) in a greenhouse. Once the second set of leaves were unfolded (ca. 3 weeks after the seeds were planted), the plants were removed from their punnits and were placed individually into 17cm diameter pots filled with 16/30 filter sand.

The plants were divided into three treatments:

200mg of soil was added to the pots of 5 plants per species during the replanting. This soil was collected from two areas: one within Hluhluwe and the other from Umfolozi (Hluhluwe's sister nature reserve). This soil was added as an inoculation treatment, in the hope that the rhizobia necessary to induce root nodulation for the five species were present. The 5 plants per species that had been inoculated were fed a 1mM N nutrient solution. 5 plants per species were given a 1mM N nutrient solution, but were not inoculated. The remaining plants were given a 2mM N nutrient solution and were not inoculated. All three treatments received similar nutrient solutions; with N being the only variable (Table 1). 400ml of the nutrient solutions were applied twice per week (Mondays and Fridays). All of the plants were supplied with 400ml distilled water every Wednesday. The pots were flushed with 1000ml of water before the addition of the nutrient solution every Friday. This was done to ensure there was no nutrient build up within the pots and that the specified N supply was maintained.

Table 1. The following volumes of chemicals were added to 10 litres of distilled water to make up the nutrient solution used in the greenhouse experiments. This amount was more than sufficient to provide one treatment of 20 plants with 400ml of nutrient solution. A fresh solution was mixed for each treatment. Note that $NaNO_3$ was only added to the solution given to the nitrogen treatment and that this amount of $NaNO_3$ results in **2 $mM.l^{-1}$ Nitrogen and *1 $mM.l^{-1}$ Nitrogen.

Chemical	g in 1l	ml added per 10l water
$CaCl_2.2H_2$	58.807	100
K_2SO_4	34.851	100
$MgSO_4.7H$	36.97	
NaH_2PO_4	10.453	50
Na_2HPO_4	53.721	
Fe sequestrene	12	16
Long Ashton Micro nutrients:		
H_3BO_3	17.16	50
$MnSO_4.4H_2O$	9.28	
$ZnSO_4.7H_2O$	1.32	
$CuSO_4.5H_2O$	1.65	
$Na_2MoO_4.2H_2O$	0.12	
** $NaNO_3$ (2mM N treatment)	1.6008	16
* $NaNO_3$ (1mM N treatment)	0.8004	8

Phytotron Experiment

Seedlings of each of the 5 *Acacia* species were sown in punnits of 16/30 filter sand, covered with a 1cm layer of vermiculite, and were allowed to germinate in a phytotron – a room with controlled light and temperature regimes. The phytotron was calibrated to a day length of 14 hours and daylight intensity of 800uE. Day temperatures were 25°C and night temperatures were 20°C. Seeds were watered every second day during germination.

As in the previous growth experiment (see Glasshouse Experiment methods), the plants were removed from their punnits and were placed individually into 17cm diameter pots filled with 16/30 filter sand once the second set of leaves were unfolded (ca. 3 weeks after the seeds were planted). The *A. nigresence* seeds were found to

have rotted, thus only 4 of the 5 species germinated. This is likely due to over-watering of seeds that may prefer more arid conditions for establishment. To ensure that the rhizobia were given sufficient opportunity to establish; the roots of the plants were dipped into a rhizobium broth during the replanting and an additional 1ml of this solution was poured into the surrounding sand. Details on the preparation of this inoculum are given below as Appendix 1. A total growth period of 10 weeks was achieved.

The addition of water and nutrients was the same as that of the glasshouse experiment. The ingredients of this solution are listed as Table 1. As with the previous experiment, 400ml of nutrient solutions was applied twice per week (Mondays and Fridays); and of the plants were supplied with 400ml distilled water every Wednesday. The pots were flushed with 1000ml of water before the addition of the nutrient solution every Friday.

Field Experiment

Acacia karroo, A. nilotica, A. tortilus, A. ataxacantha and *A. nigrescence* seeds were grown in a monitored experiment in the research station in Hluhluwe National Park. The study site was originally grass-dominated. This grass was cut down to a height of 5cm using bush cutters. The sight was randomly divided into 20 3x3m plots, which were separated using plastic sheeting that was dug 30cm into the soil. The remaining plant material in half of the plots was removed completely, leaving bare soil. Each plot was further divided into 16 smaller plots – resulting in 20 4x4plant plots (see Appendix 2). Each of these smaller plots was randomly allocated an *Acacia* seedling. The seedlings had been grown in a greenhouse for 3months under similar conditions (An van Cauter pers. comm.). Once the plants were replanted; they were grown in two treatments: half were grown in plots where grass was allowed to grow back, while the other half were grown in plots kept clear of other vegetation through constant maintenance. A watering system supplied the area daily with tap-water via an automated sprinkler system to ensure that competition for water was not a factor. The plants grew in these plots for 13 weeks before the plants were harvested. Figure 2 shows an example of a grass-exclusion plot; while Figure 3 shows a grass-dominated plot.

Figure 2. The corner of a "no-grass plot" in the Hluhluwe growth experiment. Note that the blue plastic is dug down 30cm; separating plots from one another. While grass was excluded from this plot, leaf litter was not removed. The plants were marked using orange stakes.

Figure 3. The edge of a "grass plot" in the Hluhluwe growth experiment. Non-*Acacia*'s were not removed. The plants were marked using orange stakes which are barely visible due to the overgrowth.

Harvests and Analyses

i) Glasshouse and Phytotron Harvests

The plants were divided into leaf material, roots, stems and nodules. All of the samples were dried for 48hours at 70°C. A five-point balance was used to determine the dry weights of the plant organs. The samples were milled and sent for mass spectrometer analyses of N content and $\delta^{15}N$ isotope ratio analyses.

ii) Grass Co-existence Harvests

Three plots of each treatment were harvested for the purpose of my project: Grass harvest 1.1, 1.2, 1.3 and No-grass harvest 1.1, 1.2, 1.3 (see Appendix 2). The plants growing in no-grass plots grew deep roots that could not be dug out without damaging neighbouring acacias. These plants were harvested at the base of the stem. The newest leaves and the oldest leaves were separated. The newest leaves were assumed to be the leaves closest to the tip of the highest branch, but only fully unfolded leaves were sampled. The oldest leaves were assumed to be the most basal leaves on the lowest branches or the stem.

The plants growing in the grass plots were dug up with roots intact as the root systems were shallow enough to access without damaging neighbouring acacias. The dense clay soil was washed off of the roots and the roots, nodules (if present on the roots), newest leaves, oldest leaves and the remaining shoot were separated. Soil was sampled from the first 30cm layer of soil in each plot. A shrub (*Diospyros dichrophylla*, *Ebenaceae*) found growing in the grass plots was harvested to be used as a reference plant in later analyses.

All of the samples (the plant components of both treatments, the *D. dichrophylla*, and the soil) were dried in a drying oven for at least 48hours at 70°C. Shoots (the entire stem and branch system), roots, new leaves, old leaves, and nodules of the plants were weighed. A 2-point scale was used where samples weighed more than 0.5g and a 5-point scale was used to weigh samples less than 0.5g. The samples were milled to powder for the analyses the percentage of C; percentage of N; and C and N isotope ratios using a mass-spectrometer.

Calculating %NDFA required a reference species – a plant similar to the plants being analysed, but does not obtain any N from atmospheric N fixation. As *A. ataxacantha* was found to have no nodules it was decided that it would be a suitable reference species. *D. dichrophylla* found growing in the grass dominated plots and lacks the ability to host N fixing rhizobia. The $\delta^{15}N$ results for the *A. ataxacantha* and were averaged. The %NDFA in the plant tissues was derived from equation 2.

Equation 2:

$$\%NDFA = \frac{\delta^{15}N_{reference} - \delta^{15}N_{sample}}{\delta^{15}N_{reference} - \text{b-value}} \times 100\%$$

Where $\delta^{15}N_{sample}$ is the $\delta^{15}N$ of the tissue in question; δ^{15}Nreference was the average $\delta^{15}N$ of *A. ataxacantha* and *D. dichrophylla*; and the b-value is the $\delta^{15}N$ of a plant of the same species that was supplied with 0mM N.

iii) Statistical Analyses

Both one-way and two-way Analyses of variance (ANOVA's) were performed to compare the interactions of the N supply, inoculation, and grass co-existence on each species. Post-hoc Fisher LSD tests for homogeneous groups were used to cluster statistically similar means (Figures 9a & 9b). The statistical analyses were achieved using the "Statistica 7" software package (Statsoft 2004) and an α of 0.05 was used throughout the statistical analyses.

Results

Are these species able to nodulate

It is clear from all three experiments that *A. karroo*, *A. nilotica*, *A. nigrescence* and *A. tortilis* are able to nodulate; while *A. ataxacantha* consistently produced no nodules. Figure 4 is an example of an *A. karroo* root system that was excavated from a Hluhluwe grass plot; Figure 5 is *A. karroo* seedling from the phytotron; and Figure 6 is a comparable *A. ataxacantha* from the phytotron. Note the absence of nodules on the *A. ataxacantha*. The nodules were considered to be actively fixing as they appeared pink in cross-section (Figure 7).

Figure 4. This the root system of an *Acacia karroo* excavated from plot G3 – a grass dominated plot in the Hluhluwe growth experiment. The grid paper is $1cm^2$. Note the presence of nodules on the root hairs.

Figure 5. This is an *Acacia karroo* from the 1mM N treatment in the phytotron growth experiment. The grid paper is $1cm^2$. Note the presence of nodules clustered on the root hairs; and the slight yellowing in the older leaves – this indicates some nitrogen deficiency.

Figure 6. This is an *Acacia ataxacantha* from the 1mM N treatment in the phytotron growth experiment. The grid paper is 1cm^2. Note the complete absence of nodules; and the yellowing and abscission of the older leaves – an indication of nitrogen deficiency.

Figure 7. Nodules from the *Acacia karroo* shown in figure 5 that have been cut in half. Note the pink hue – an indication of active nitrogen fixing rhizobia presence.

How is the performance of the plant affected by inoculation and different nitrogen levels

The biomasses of the plant components taken from the greenhouse experiment are shown as two stacked bar graphs (Figures 8a & 8b). The statistical tests wher performed on log(x+1)-transformed data as this created a normal distribution of the data set. These results are shown separately as there was a significant ($p = 0.000$) difference between the three treatments. The plants grown without inoculation with 2mM N were significantly ($p = 0.0000$) larger than those grown with 1mM N and no inoculation. Only *A. karroo* and *A. tortilis* grew larger in the inoculation treatment than the no inoculation treatment at 1mM N.

The ratios of the dry mass of Root : Shoot, Leaf : Total mass, and Nodules : Root were plotted as Figures 9a, 9b and 9c respectively, to determine how each species varied its biomass allocations during each of the greenhouse treatments. These graphs suggest that there was no recurring pattern of changing mass allocations common amongst the species or amongst treatments. Once again it is clear that *A. ataxacantha* did not nodulate. The shoot : root ratio and leaf : total dry mass of *A. ataxacantha* was not significantly different to *A. nigrescence* (which did nodulate).

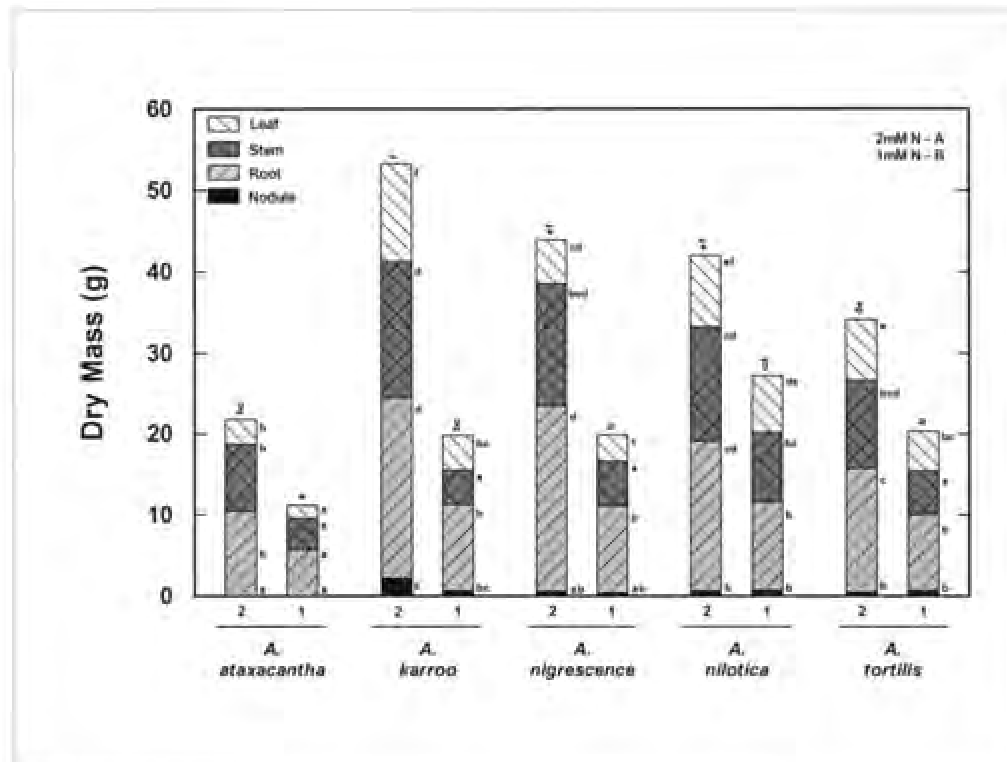

Figure 8a.

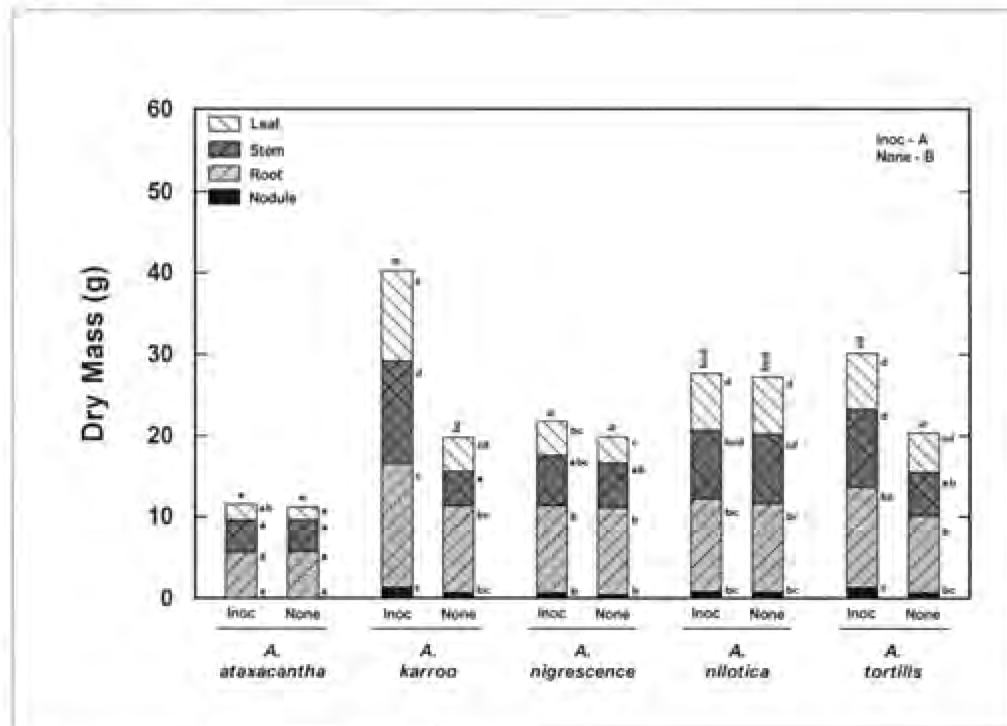

Figure 8b.

Figure 8a & 8b. Dry weights of nodules, roots, stems and leaves of 5 *Acacia* species grown with 3 treatments. 8a shows those grown at 2mM N and 1mM N where no plants were inoculated with N fixing rhizobia. 8b shows those grown with and without inoculation at 1mM N. Dissimilar letters above the bars indicate significant differences between treatments. This was determined using a one-way ANOVA (α=0.05), and subsequent post-hoc LSD tests on log(x+1) transformed data. Different letters next to the bars indicate significant differences in the biomass components amongst treatments. Each component was tested separately. There was a significant difference between the treatments, indicated by the different capital letters. The significance of the interactions between the treatments and the species were 8a) p = 0.012, 8b) p = 0.0116.

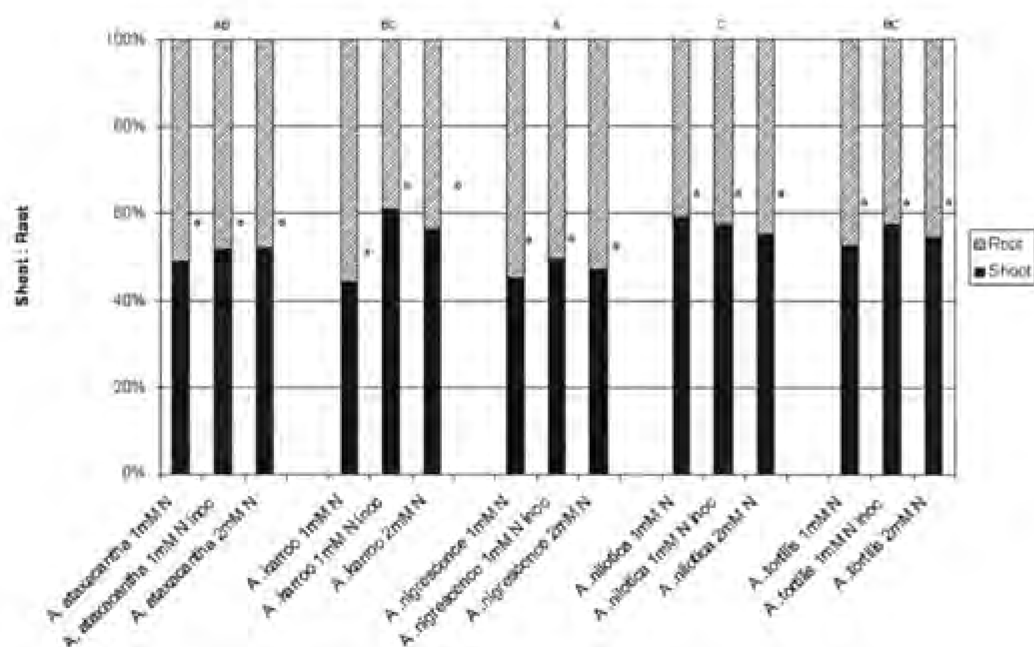

Figure 9a. The ratio of shoot dry mass to root dry mass across the three greenhouse treatments as a percentage. The significance of the interaction between treatments and the species is not significant ($p = 0.2115$). Dissimilar capital letters above the bars indicate significant differences between species. This was determined using a one-way ANOVA ($\alpha=0.05$), and subsequent post-hoc LSD tests. Different lower-case letters next to the bars indicate significant differences in the ratio of biomass components amongst the treatments. Each species was tested separately

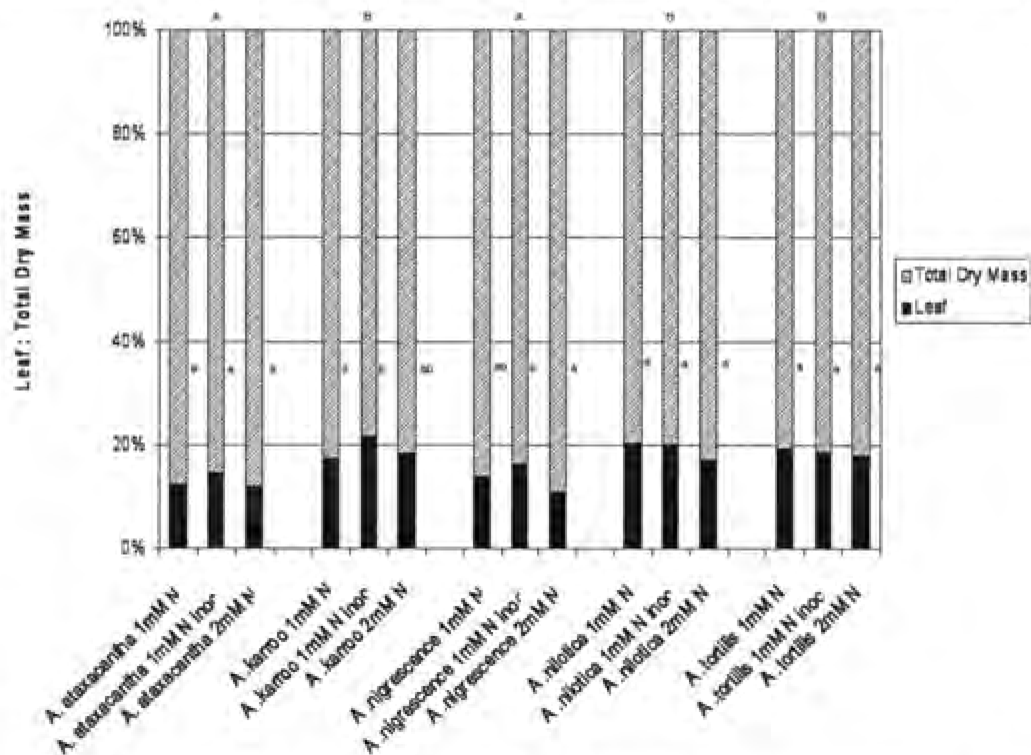

Figure 9b. The ratio of leaf dry mass to total plant dry mass across the three greenhouse treatments as a percentage. The significance of the interaction between treatments and the species is not significant ($p = 0.3747$). Dissimilar capital letters above the bars indicate significant differences between species. This was determined using a one-way ANOVA ($\alpha=0.05$), and subsequent post-hoc LSD tests. Different lower-case letters next to the bars indicate significant differences in the ratio of biomass components amongst the treatments. Each species was tested separately

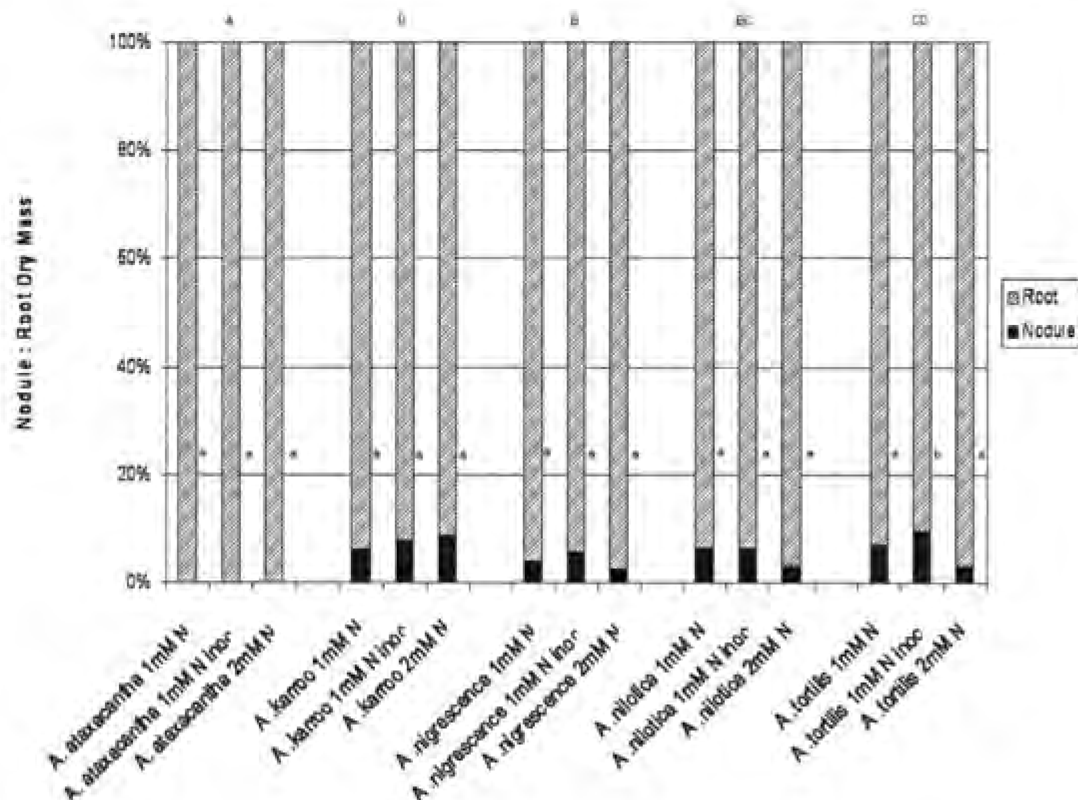

Figure 9c. The ratio of nodule dry mass to root dry mass across the three greenhouse treatments as a percentage. The significance of the interaction between treatments and species is not significant ($p = 0.2835$). Dissimilar capital letters above the bars indicate significant differences between species. This was determined using a one-way ANOVA ($\alpha=0.05$), and subsequent post-hoc LSD tests. Different lower-case letters next to the bars indicate significant differences in the ratio of biomass components amongst the treatments. Each species was tested separately

Table 2 shows that while *A. ataxacantha* did not have a significantly different average %N, the overall mass of N in *A. ataxacantha*s harvested was significantly lower than the rest of the species. It is important to note that the low $\delta^{15}N$ of *A. ataxacantha* is due to the inclusion of all plant components in this analysis (rather than only leaf data as in Tables 3 and 4), but that *A. ataxacantha*s $\delta^{15}N$ is significantly higher than that of the rest of the species sampled. There was no recurring pattern of significantly different $\delta^{15}N$ between treatments within each species. This is supported by the significant interaction term.

Table 2. The nitrogen mass-spectrometer results from the greenhouse growth experiment. The treatment codes are: 1 & 2 denote 1mM N & 2mM N respectively – both without inoculation; and 1 inoc denotes 1mM N with inoculation through the addition of Hluhluwe soil. The capital letters indicate which species have statistically similar means across all treatments; while lower case letters indicate which treatments have statistically similar means within each species. The statistical significance of the interactions between species and treatments are shown as species x treatment. The values are means of whole-plant data weighted according to N content (mg) in each plant component.

	Weighted Whole-Plant $\delta^{15}N$	Weighted Whole-Plant N/g plant	Whole-Plant N (mg)
A. ataxacantha	C	BC	A
1mM N	-0.14 ± 0.13^{a}	1.3 ± 0.02^{a}	11.92 ± 0.56^{a}
1mM N & inoculation	-0.02 ± 0.26^{a}	1.41 ± 0.08^{a}	13.31 ± 1.27^{a}
2mM N	-0.22 ± 0.13^{a}	1.47 ± 0.04^{a}	25.95 ± 1.76^{a}
A .karroo	AB	A	C
1mM N	-0.82 ± 0.15^{b}	1.02 ± 0.07^{a}	18.56 ± 2.6^{a}
1mM N & inoculation	-1.67 ± 0.2^{a}	1.46 ± 0.04^{b}	55.76 ± 6.26^{b}
2mM N	-1.11 ± 0.19^{ab}	1.18 ± 0.12^{a}	56.97 ± 8.02^{b}
A .nigrescence	B	C	B
1mM N	-0.93 ± 0.21^{a}	1.43 ± 0.14^{a}	24.73 ± 3.61^{a}
1mM N & inoculation	-0.86 ± 0.26^{a}	1.53 ± 0.13^{a}	29.48 ± 8.32^{a}
2mM N	-1.14 ± 0.2^{a}	1.35 ± 0.08^{a}	49.67 ± 6.22^{b}
A .nilotica	A	AB	BC
1mM N	-1.19 ± 0.19^{b}	1.15 ± 0.11^{a}	29.47 ± 4.39^{a}
1mM N & inoculation	-1.78 ± 0.18^{a}	1.57 ± 0.05^{b}	40.47 ± 6.95^{a}
2mM N	-1.37 ± 0.22^{ab}	1.1 ± 0.1^{a}	40 ± 1.93^{a}
A .tortilis	B	C	BC
1mM N	-1.43 ± 0.22^{a}	1.32 ± 0.09^{a}	25.37 ± 6.35^{a}
1mM N & inoculation	-1.47 ± 0.12^{a}	1.72 ± 0.13^{b}	47.02 ± 6.21^{b}
2mM N	-0.42 ± 0.34^{b}	1.23 ± 0.04^{a}	38.7 ± 2.09^{ab}
Species x Treatment	0.0056	0.0647	0.0054

What effects does grass co-existence have on the *Acacia* seedlings

The below-ground mass of the plants grown in no-grass plots in Hluhluwe were not excavated, and as such only the above-ground masses of the plants grown with and without grass can be compared. These above-ground masses (those of the stems and leaves) are shown as Figure 10. The *Acacia* seedlings grown in grass-dominated plots were significantly smaller than those grown in no-grass plots. There was no difference

between species within treatments. There was no difference between species ($p = 0.8278$). On average plants grown in no-grass plots had 8.81 times more biomass than those grown in grass-dominated plots.

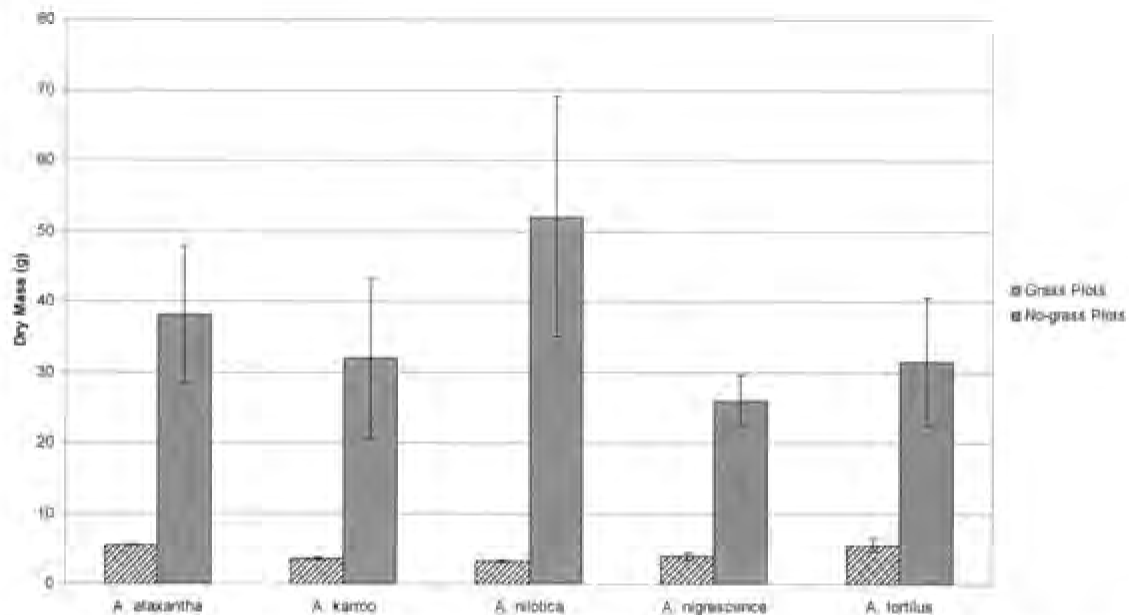

Figure 10. The shoot (leaf + stem) dry masses of each of the 5 species of *Acacia* grown with and without grass in Hluhluwe National Park.. The average shoot mass was significantly ($p = 0.0001$) greater in no-grass plots. Error lines on bars are standard errors.

The %Ndfa was calculated using Equation 2. The b-values for each species are listed as Table 2. Note that *A. ataxacantha*'s $\delta^{15}N$ is listed for reference only, and was not used in any %Ndfa calculations; as no nodules were found on *A. ataxacanthas* from any of the experiments. The average %Ndfa for each species in each of the grass treatments are listed as Table 4, as well as the average $\delta^{13}C$, average $\delta^{15}N$ and average %N. Note that due to the mathematics of Equation 2 it is possible to find a negative %Ndfa result. Table 3 shows that the lack of nodulation in *A. ataxacantha* resulted in a significantly ($p = 0.0000$) higher $\delta^{15}N$ than the other species. Plants grown in grass-dominated plots produced significantly greater %Ndfa values than those from no-grass plots. The lack of significantly different %Ndfa across species is unrealistic, which is shown in the highly significant interaction term.

Table 3. The $\delta^{15}N$ results for the plants grown in the 0mM N phytotron growth experiment. These values were used as b-values as shown in Equation 1 to calculate %Ndfa values, as the only nitrogen available to these plants was that stored in their cotyledons and the nitrogen fixed from the atmosphere. The $\delta^{15}N$ for *A. nigrescence* was calculated as an average $\delta^{15}N$ of *A. karroo*, *A. nilotica*, and *A. tortilis*, as *A. nigrescence* did not germinate.

Species	Average Leaf $\delta^{15}N$
A. ataxacantha	3.66119
A. karroo	0.03946
A. nigrescence	0.01556
A. nilotica	-0.05878
A. tortilis	0.06600

Table 4. The nitrogen and carbon mass-spectrometer results from the Hluhluwe grass experiment. G and N treatments denote the presence or exclusion of grass respectively. The reference results presented are the averages of the data from *A. ataxacantha* ($\delta^{15}N$ = 4.68) and *D. dichrophylla* ($\delta^{15}N$ = 3.73), found in grass dominated plots. The capital letters indicate which species have statistically similar means within a column; while lower case letters indicate which treatments have statistically similar means within each species and column. The statistical significance of the interactions between species and treatments are shown as species x treatment. The values are means of the leaf tissues.

	Leaf $\delta^{13}C$	Leaf $\delta^{15}N$	Leaf N/g plant	Leaf %Ndfa
A. ataxacantha	C	C	B	A
G	-30.12 ± 0.62^a	4.68 ± 1.63^a	2.36 ± 0.14^a	0 ± 0^a
N	-28.47 ± 0.21^b	6.01 ± 0.3^a	3.11 ± 0.12^b	0 ± 0^a
A .karroo	A	A	A	B
G	-30.98 ± 0.02^a	0.27 ± 0.28^a	2.09 ± 0.08^a	94.4 ± 6.7^b
N	-30.65 ± 0.02^a	3.71 ± 0.41^b	2.47 ± 0.03^b	10.67 ± 9.89^a
A .nigrescence	B	B	A	A
G	-31.19 ± 0.01^a	1.27 ± 0.22^a	2.14 ± 0.1^a	68.39 ± 5.29^b
N	-29.59 ± 0.11^b	6.16 ± 0.13^b	2.35 ± 0.07^a	-47.58 ± 2.97^a
A .nilotica	AB	AB	A	AB
G	-30.88 ± 0.18^a	0.57 ± 0.29^a	1.95 ± 0^a	86.58 ± 6.9^b
N	-30.44 ± 0.06^a	5.07 ± 0.38^b	2.45 ± 0.03^b	-22.22 ± 9.25^a
A .tortilis	AB	B	B	A
G	-30.79 ± 0.06^a	0.99 ± 0.77^a	2.35 ± 0.02^a	77.31 ± 18.83^b
N	-30.29 ± 0.04^a	5.06 ± 0.09^b	2.95 ± 0.07^b	-22.19 ± 2.11^a
Species x Treatment	0.0069	0.0805	0.0938	0.0002

Discussion

Are these species able to nodulate?

All of the species were able to nodulate except *A. ataxacantha*. This is not to say that *A. ataxacantha* is unable to nodulate – conditions may have not have facilitated nodulation. The soil brought back from Hluhluwe-Umpholozi as an inoculant may not have contained the rhizobia that *A. ataxacantha* requires for the production of root nodules. The inoculation of the phytotron experiment plants was prepared from the rhizobia present in the soil.

How is the performance of the plant affected by different inoculation and nitrogen treatments?

None of the species produced significantly different masses of nodules amongst the treatments (Figures 8a & 8b). There was no pattern of changing nodule per root dry mass amongst the treatments (Figure 9c). The lack of significant difference between the mass of nodules produced by plants in the 1mM greenhouse treatment that were inoculated with soil versus those that were not (Figure 8b) suggests that rhizobia may have been able to infect the plants that were not inoculated with soil. There were no measures put in place to prevent atmospheric bacteria from reaching the pots.

It is possible that the 200mg of soil, added as the inoculation treatment, affected the outcome of this experiment through the addition of nitrogen and other nutrients. While these resources would have been in small quantities, and therefore not maintained over a long period, the initial boost to the plant may have allowed these plants a "head start" over the plants that were not inoculated with soil. This may be the case with *A. karroo* and *A. tortilis*, but the other species did not seem to be similarly affected (Figure 8b). It is unclear as to whether these species were able to make use of additional nutrients present in the soil while the other species could not.

The possibility that the soil addition fertilised the inoculated plants; and that bacteria could have contaminated neighbouring, un-inoculated plants may result in the 1mM inoculated treatment being imperfect. While these data are useful for comparison, the experiment should be repeated with a more precise method of inoculation and conditions that would prevent the migration of bacteria from pots or the atmosphere that may contaminate un-inoculated pots.

The results of the 2mM N and 1mM N plants that were not inoculated should remain unaffected. All of these plants would have had the same chance of becoming infected by rhizobia. It is clear that the plants grown with 2mM N produced significantly more biomass than those grown with 1mM N (Figure 8a). The lack of significant difference in nodule mass and nodules per root mass seen between the 1mM and 2mM treatment is a pattern common across all of the plant component allocations (Figures 10a-c). While there were significant differences in the %Ndfa and %N amongst the three greenhouse treatments (Table 2), there is no recurring pattern as shown by the insignificant interaction term.

The plants grown in the phytotron showed a yellowing of older leaves (Figures 5 & 6), which is a sign that they were moving nitrogen out of older leaves and reallocating it (probably to newer leaves). This is a sign of nitrogen limitation. The greenhouse and phytotron experiments are not directly comparable due to the different growth conditions and the dissimilar durations of the two experiments, but the patterns of nitrogen limitation in the 1mM treatment is common in both treatments. The fact that the 1mM N plant' showed slower growth rates and early leaf senescence, but no change in biomass allocations, could be due to a lack of plasticity. This was not explored and should be tested further. This nitrogen limitation did not appear to be fatal, and 1mM N seems to be a viable amount for all of the species.

A. ataxacantha showed the most wilting during the phytotron experiment. *A. ataxacantha* is a forest species and may require more water for effective growth than the other species grown during this project. The significantly high N concentrations within the *A. ataxacantha* tissue, together with the insignificantly different mass of N in the *A. ataxacanthas* (Table 3), indicates that the plants were able to maintain a similar amount of nitrogen as the other species, but was forced to store it in higher concentrations. The implications for this are unclear.

The mathematical equation used to calculate %Ndfa (Equation 2) allows for a negative result when the b-values are less than the $\delta^{15}N_{sample}$s; and similarly a result greater than 100% is possible (the equations should not be used when $\delta^{15}N_{reference}$ is less than $\delta^{15}N_{sample}$). This value should therefore be considered relative to the entire data set for biological interpretation. There was no pattern with regards to which

greenhouse treatment resulted in the lowest $\delta^{15}N$ (Table 2). This is supported by a significant interaction term. Within each species, the plants that received the soil inoculation treatment had a significantly higher %N (Table 2), with a lack interaction between treatments and species. This adds further support to a fertilisation effect of the soil inoculation treatment previously mentioned.

What effects does grass co-existence have on the acacia seedlings?

There was a dramatic dwarfing effect of grass on the above ground biomass of all of the *Acacia* species (Figure 9). On average, the plants grown in no-grass plots had more than 8 times the biomass of those grown in grass-dominated plots. While %Ndfa values are not absolute in a biological sense, there is a clear pattern of high N fixing in grass plots and much lower N fixing in no-grass plots (Table 3). All of the plants in the grass plots were found to have a significantly lower %N (Tables 3 & 5). These data indicate significant competition between grass and the acacia seedlings, leading to decreased performance of the acacias.

This competition may be great enough to prevent the establishment of species that are unable to host nitrogen fixing bacteria. While the grass experiment cannot be directly compared to the greenhouse experiment, the patterns seen between the presence of grass and low nitrogen are similar. The grasses and shrubs that grew in the grass-plots severely limited the growth of all of the *Acacia* species through competition (initially and possibly light once the grass overshadowed the acacias). It is unfortunate that we were unable to effectively excavate the entire plants growing in no-grass plots. It is now not possible to either compare root biomasses across the treatments, or calculate patterns of biomass allocations. The %Nfda of plants grown in no-grass plots supports a lack of nodulation where competition is not a factor (e.g. when the acacias are larger and are able to source nitrogen from deeper soils).

The significantly lower $\delta^{13}C$ (Table 4) in the no-grass plots indicates slightly less water-stress, however; as these $\delta^{13}C$ are so negative, there does not seem to be any biological significance to this. Water was not expected to play a major role in this experiment as the plants were watered frequently. Competition for water was not explored, but is likely to play a role at seedling level in natural savannas, as the root systems of both tree seedlings and grasses share the same soil zones. The more

negative $\delta^{13}C$ results from the grass plots correspond to the general impression that the soil in the grass plots was damper. This is likely an effect of the grass lowering evaporation through shading. This shading may also have impacted the tree seedlings as the grass was taller than the seedlings. The grass was cut lower than the seedlings when plots were cleared and the difference in growth rate between grasses and trees suggests that the grass is able to make use of available resources far more effectively than the trees at this stage.

It is interesting to note that while *A. karroo* is the most invasive tree of South African grasslands (O'Connor 1995); it did not show a significantly different growth rate in either the grass- or no-grass plots (Figure 10). The reason for *A. karroo*'s success is unclear from my data and more research is needed.

Conclusion

The effect of grass on the growth of *Acacia* seedlings is severe – much more so than a halving of the nitrogen supply from 2mM N to 1mM N. This is likely due to both competition for nutrients (including nitrogen); and compounding competition for light, as the grass is able to grow taller faster.

Legumes ability to nodulate, and thereby host nitrogen fixing rhizobia, would indeed give a plant competing for nitrogen a significant advantage. I believe that after answering the 3 questions initially stated, I have provided support for the hypothesis that legumes in savannas nodulate as seedlings – a time when nitrogen is limiting. Once the legume is established, and nitrogen is no longer limiting, it would make sense that the adult tree would no longer produce nodules. Trees that are unable to nodulate as seedlings would be unable to escape the tree-grass competitive stage without other adaptations, and would have a much lower chance of establishing within a savanna.

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Appendix 1: ***Growing the Rhizobia Cultures for the 0mM N plants***

As all of the species except *A. ataxacantha* had nodulated during the previous Inoculated vs. 1mM N vs. 2mM N experiment, seedlings were inoculated with the rhizobia from nodules found on the corresponding species previously. Cultures of these nodules had been isolated and kept in at 0°C. These cultures were added to a broth (Table 4) which was buffered to a pH of 6.86 as per Vincent (1970). The mixture was placed into flasks: one flask per species. The solutions were placed in an autoclave to provide the Rhizobia with a sterile environment. A small scraping of the corresponding rhizobium was placed in each of the flasks of sterilised broth. This was done within the confines of a laminar flow chamber, so as to minimise contamination. The bacteria were allowed to mature at 25°C until the liquid in the flask had reached a photometer absorbency reading of 600µm. This was achieved after four days. *A. ataxacantha* seedlings were inoculated with an equal parts mixture of *A. karroo*, *A. tortilis* and *A. nilotica* inoculate as *A. ataxacantha* did not nodulate during the previous growth experiment.

Table 4. The following volumes of chemicals were added per litre of distilled water to make up the nutrient broth in which the cultures used as inoculate. The broth was buffered to a pH of 6.86, after which it was placed into an autoclave. A small scraping of rhizobium culture was added to each flask of sterilised broth.

Chemical	g per 1L water
$K2HPO4$	0.5
$MgSO4.7H2O$	0.2
NaCl	0.1
Mannitol	10
Yeast extract	0.4

Reference:

Vincent, J.M. 1970. *A Manual for the Practical Study of the Root-Nodule Bacteria.* Burgess and Son (Abington) LTD, Berkshire, Great Britain. Ch 1, pp 1-13.