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Grass competition induces N₂ fixation in some species of African *Acacia*

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Summary

1. Indigenous species of *Acacia* are common in African savannas that have N-rich soils. This raises doubt regarding the extent of plant dependence on N₂-fixation. Why would *Acacia* spp. enjoy an advantage over other tree species on N-replete soils?

2. We tested the hypothesis that competition by grass for nutrients would induce increased nodulation of *Acacia karroo*, *A. nilotica*, *A. tortilis* and *A. nigrescens* seedlings that would enable them to survive better than a non-nodulating congeneric species (*A. ataxacantha*). A glasshouse pot experiment was conducted to determine the capacity of the *Acacia* spp. to nodulate. The *Acacia* spp. were also grown in a randomized field plot experiment in Hluhluwe-iMfolozi Park with and without grass coexistence, and biomass accumulation and $\delta^{15}\text{N}$ values were determined. We also sampled a range of legume and non-legume saplings from the Hluhluwe-iMfolozi Park for $\delta^{15}\text{N}$ values.

3. In the pot experiment all species, except *A. ataxacantha*, which is a forest margin species, nodulated and consequently had lower $\delta^{15}\text{N}$ isotope values than *A. ataxacantha*. In the field experiment the $\delta^{15}\text{N}$ values of the plants grown with grass were significantly lower ($\delta^{15}\text{N} = 0.77 \pm 0.08\text{‰}$) than those grown without grass ($\delta^{15}\text{N} = 5.0 \pm 0.16\text{‰}$) for all species, except *A. ataxacantha*. The $\delta^{15}\text{N}$ isotope abundances of field-collected leaves of legume saplings were found to be significantly lower than those of non-legume species (legume $\delta^{15}\text{N} = 0.98 \pm 0.32\text{‰}$, non-legume $\delta^{15}\text{N} = 2.15 \pm 0.32\text{‰}$).

4. These data confirm that *A. karroo*, *A. nilotica*, *A. tortilis* and *A. nigrescens* seedlings are capable of nodulating, and do so in their native habitat. The decreased $\delta^{15}\text{N}$ in plants grown with grass indicates that N₂ fixation was strongly enhanced by competition with grass for N.

5. N₂ fixation may thus be an important attribute allowing legume tree seedlings to survive competition with grass through a critical period when tree roots and grass roots must compete for nutrients.

Key-words: ¹⁵N isotope, African *Acacia*, competition, grass, natural abundance, nitrogen fixation, nodule, savanna, tree-grass competition

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Introduction

Savannas occupy 54% of southern Africa (Rutherford 1997), and 12% of global land area (Scholes & Hall 1996). They are a species-rich complex containing many leguminous and non-leguminous species, especially

grasses. Leguminous trees are an important component of the subtropical savannas (Midgley & Bond 2001) and are proliferating in many areas formerly dominated by grasses in a process known as ‘bush encroachment’ (O’Connor 1995; Hudak & Wessman 2001; Hudak *et al.* 2003). Crews (1999) argued that the presence/absence of legumes is primarily dependent on the biogeography and the availability of legume species in the flora. The predominance of tree-legumes in savannas and the fact that bush encroachment is often associated with

legumes may thus merely be a consequence of their general richness and importance in African savannas. However, in this context, ecological explanations for understanding the colonization and activity of N₂ fixers are also appropriate (Crews 1999). Of the c. 1200 species of *Acacia*, the majority investigated (204 out of 216; 94%) are known to nodulate (DeFaria *et al.* 1989). However, some *Acacia* species in the subgenus *Aculeiferum* section *Monacantha* have been consistently found not to nodulate (Harrier *et al.* 2000).

The presence/absence of legumes from ecosystems has been suggested to be linked to (i) the energetic costs of N₂ fixation, (ii) limitation of N₂ fixation by other nutrients (e.g. P), and (iii) 'other' physical and ecological factors (Vitousek & Howarth 1991). The ability of leguminous trees to fix N₂ would seem to put legumes at an advantage. However, the tree-legume species in savanna systems do not always fix N₂ (Aranibar *et al.* 2003). The high energetic costs of N₂ fixation may favour the utilization of combined N by legumes, if available, over fixation of N₂ (Crews 1999). The implication of the energy-intensive nature of N₂ fixation is that legumes do not perform well in low-light circumstances (e.g. shade) where non-legumes out-compete legumes (Vitousek & Howarth 1991). The lack of N₂ fixation by tree-legumes may also be due to sufficient combined N in the savanna soils (Aranibar *et al.* 2003). It is generally accepted that nodulation by legumes is reduced with increased availability of soil N, although this may be a more popular belief than is warranted by the data. For example, N in the form of NH₄⁺ seems to have only a weak suppressive effect on nodulation (Sprent 1999) or may even stimulate nodulation (Crews 1999). N₂ fixation by legumes is often thought to be especially sensitive to P limitation. For example, Crews (1993) showed that alfalfa N₂ fixation capacity increased with P addition rates. However, the data showing that N₂ fixation is especially sensitive to limited P are inconsistent (Hartwig 1998). Phosphorus is certainly required for nodule metabolism (e.g. Tang *et al.* 2001), but apparently no more so than for other aspects of plant metabolism (Sprent 1999). For instance, the Australian tree legume, *Acacia mangium*, has similar requirements for P whether it relies on N₂ fixation or urea for N (Ribet & Drevon 1996). Nevertheless, P deficiency is likely to constrain the success of fast growing species not adapted to nutrient conservation.

Although both grazing and frequent fires (Aranibar *et al.* 2003; Fynn *et al.* 2003) may remove N from the ecosystem, soils from these savanna systems seem to have sufficient total N for tree growth. The nitrogen lost through fire or grazing may be replenished by biological nitrogen fixation, particularly by legume tree species (Högberg 1986a). There is much evidence that grasses limit tree seedling establishment through competition for light, water and nutrients and by exposing tree seedlings to the hazards of fire (Scholes & Archer 1997). It has long been thought that trees are able to coexist with grasses and shrubs through competition

avoidance at the root level (Walter 1971; Walker & Noy-Meir 1982). Trees are assumed to be able to obtain 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. This neat scheme is confounded by the fact that tree and grass roots both explore the upper soil layers, and, while some studies show that tree roots do extend deeper than grass roots, others do not (see Higgins *et al.* 2000; Sankaran *et al.* 2004).

Root niche differentiation is not possible for tree seedlings germinating in a grass-dominated environment where the seedlings will be competing directly with grasses for water and nutrients. Higgins *et al.* (2000) showed that the failure of trees to dominate in savannas could be explained by poor seedling establishment or, where fires are frequent, slow sapling growth rates. One implication is that woody plants that are better able to compete with grasses for resources in the establishment phase should be more common in savannas than weaker competitors. The ability of tree seedlings to fix nitrogen may confer growth advantages in the presence of grass competition, favouring leguminous over non-leguminous woody species. Bond *et al.* (2001) grew *A. karroo* and *A. nilotica* in pots with and without grass. They noted that root nodules were only present on plants grown with grass. In this study, we investigated whether leguminous trees are more plentiful than non-legume trees in the savanna areas of Hluhluwe-iMfolozi Park (Kwazulu-Natal, South Africa) and tested the hypothesis that leguminous tree seedlings could survive competition with grass for nutrients by nodulating. To do this we grew seedlings of five *Acacia* spp. in sand-filled pots in a glasshouse to establish whether they could nodulate. We measured δ¹⁵N values of the same five *Acacia* spp. grown in a field experiment with and without grasses. We also measured the δ¹⁵N values of saplings of a range of legume and non-legume species growing in Hluhluwe-iMfolozi. The use of the δ¹⁵N technique is subject to some caveats, as it may not provide accurate quantitative estimates of N₂ fixation (Högberg 1997; Evans 2001; Robinson 2001; Dawson *et al.* 2002). Furthermore, the 'reference' plants must have similar root distributions and temporal patterns of nitrogen uptake and preferences for inorganic and organic nitrogen (Högberg 1997). To address this limitation we used the non-nodulating *Acacia ataxacantha*, grown under identical circumstances to the other legumes, as a reference plant in both the field and the pot experiments. δ¹⁵N values are determined by the integration of the entire soil- and plant-nitrogen cycle (Evans 2001; Robinson 2001). The pot and field experiments reported here had very similar conditions between treatments so that N-cycling was unlikely to differ. Furthermore, in field collected material we used a very large number of non-fixing tree species of similar life form to the legumes as a 'reference group'. We concluded that seedlings grown in the presence of grass rely extensively on N₂ fixation as a source of N.

Methods

GLASSHOUSE EXPERIMENT

A. karroo Hayne seed were soaked in freshly boiled distilled water for 24 h. *A. tortilis* (Forsk.) Hayne and *A. ataxacantha* DC. seeds were placed in 98% H₂SO₄ for 30 min and *A. nilotica* L. and *A. nigrescens* Oliver seeds were placed in 49% H₂SO₄ for 60 min. After this the seeds were rinsed with at least six changes of water and soaked in water for 24 h. Sixteen seedlings of each of the five *Acacia* species were germinated in vermiculite in a glasshouse and transplanted to 0.17 m diameter pots filled with filter sand. During replanting, soil (200 g) collected from a savanna site in Hluhluwe-iMfolozi Park was added to the pots of six plants per species as an inoculation treatment to introduce native *Rhizobia* spp. and induce root nodulation of the five *Acacia* species. The plants were supplied with 400 mL Long Ashton nutrient medium (Hewitt 1966) modified to contain either 1 or 2 mM NaNO₃ (pH 6.5) twice weekly. The pots were leached with 1 L weekly and watered as necessary. Plants were harvested 150 days after transplanting and separated into leaves, stems, roots and nodules, weighed, dried at 80 °C for 48 h, re-weighed and milled in a Wiley mill using a 0.5 mm mesh (Arthur H Thomas, Philadelphia, PA, USA). The milled material was analysed for tissue N concentration and δ¹⁵N values through mass spectrometry.

FIELD EXPERIMENTS

Seeds of *Acacia karroo*, *A. nilotica*, *A. tortilis*, *A. ataxacantha* and *A. nigrescens* were scarified with sand paper, soaked in hot water for 24 h and then grown in a shade house in Hluhluwe-iMfolozi Park for 3 months (September to December) in potting soil. Seedlings were planted out on the 17 December 2005. The study site (S28.07696, E32.04103) was an area of mixed grass that was divided into 20 plots measuring 3 × 3 m that were separated from each other by burying a heavy plastic sheet to a depth of 0.5 m. The 20 plots were randomly assigned to treatments with grasses present or removed. In plots with grass, the grass was cut to a height of 0.05 m before planting while in the 'no grass' plots the grass was eliminated by digging out the grass and grass roots. Seedlings were planted 0.75 m apart with 25 seedlings randomly assigned to each plot. The seedlings were selected to ensure that initial sizes of individuals of a species were similar. The plots with no grass were regularly weeded to keep out plants other than the *Acacia* seedlings. An irrigation system was installed to ensure regular watering to prevent water limitations.

The stem length of the plants was measured thrice (33, 112 and 261 days) after transplanting. After 112 and 261 days, three replicate plots of both the 'grass' and 'no grass' treatments were harvested for biomass determinations and leaf analyses. Remaining plants

were pruned back to ground level to determine their capacity to resprout. This resprout growth was harvested after an additional 141 days. The leaves of plants harvested after 112 days were divided into young and mature. The leaf tissue samples were dried at 80 °C for 48 h, weighed, milled to powder and the N concentration (% DW), δ¹⁵N and δ¹³C values measured using a mass-spectrometer. Samples from the top 0.3 m of soil were collected from each plot after 112 days and analysed for pH and N, C, P, K, Na, Mg, Ca and also δ¹⁵N.

FIELD-COLLECTED MATERIAL

The number of trees of each species larger than 1 m tall was counted in 10 different 0.64-ha plots distributed through the savanna regions of Hluhluwe-iMfolozi Park during 2000. During 2005, legume saplings in these savanna regions were excavated to search for nodules on the roots. The occurrence of nodules was scored and the proportion of individual trees of a species with nodules calculated. Leaf samples of a number of legume and non-legume tree species were collected in March/April 2005 and 2006. The youngest fully expanded leaves were collected from six individuals of each species, bulked, and milled for analysis (see Appendix S1 in Supplementary Material for species list). The leaf material was subjected to mass-spectrometer analysis for leaf N, δ¹⁵N and δ¹³C values.

SOIL ANALYSIS

The soil was dried at 80 °C for 48 h and then sieved (1 mm mesh). Soil pH was determined by shaking 2 g soil in 20 mL 1 M KCl at 180 r.p.m. for 60 min, centrifuging at 10 000 g for 10 min and measuring the supernatant pH. Soil nitrogen was determined by digestion with a FP-528 Nitrogen Analyser (Leco Corporation, St Joseph, USA). Soil was prepared for P analysis by extracting 6.6 g soil in Bray II solution (Bray & Kurtz 1945) before filtering and analysing using ICP-AES (Varian Vista MPX, Melbourne, Australia). Exchangeable cations were displaced from 10 g soil with 25 mL of 0.2 M ammonium acetate. The samples were filtered through Whatman no. 2 and made to 200 mL and K, Na, Ca and Mg measured using ICP-AES analysis.

MASS SPECTROMETER DETERMINATIONS

The oven-dried plant components were milled in a Wiley mill using a 0.5 mm mesh and 2.100–2.200 mg of the leaf sample weighed into a tin capsule (Elemental Microanalysis Ltd, Okehampton, UK). Dried soil samples were sieved (0.5 mm mesh) and c. 40 mg weighed into a tin capsule. The samples were combusted in a Thermo Flash EA 1112 series elemental analyser and the gasses fed into a Delta Plus XP isotope ratio mass spectrometer (Thermo Electron Corporation, Milan, Italy).

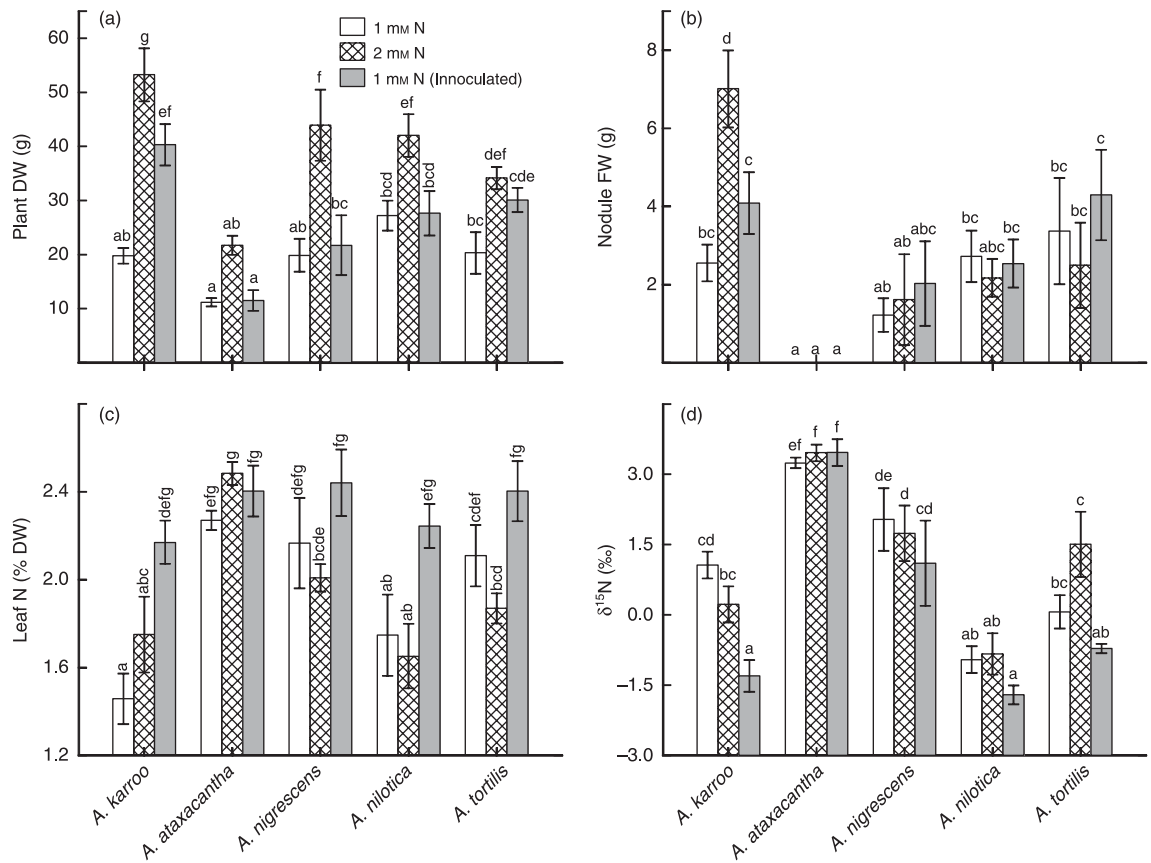


Fig. 1. (a) Plant dry weight, (b) nodule fresh weight, (c) leaf N concentration, and (d) $\delta^{15}\text{N}$ values in leaves of five *Acacia* species with different levels of N and inoculation. Bars and errors represent mean \pm SE and letters indicate significant differences between species and treatments (two-way ANOVA, factors species and N concentration/inoculation followed by Fisher *post hoc* LSD, $P < 0.05$). There were significant differences between species for plant DW ($F_{4,89} = 16.5$, $P < 0.01$), nodule FW ($F_{4,89} = 13.5$, $P < 0.01$), leaf N concentration ($F_{4,89} = 10.6$, $P < 0.01$) and leaf $\delta^{15}\text{N}$ values ($F_{4,89} = 45.1$, $P < 0.01$). There were significant differences between treatments for plant DW ($F_{2,89} = 37.1$, $P < 0.01$), leaf N concentration ($F_{2,89} = 14.6$, $P < 0.01$) and leaf $\delta^{15}\text{N}$ ($F_{2,89} = 8.1$, $P < 0.01$).

Two in-house, and one IAEA, standards were used to calibrate the results. The carbon isotopic ratio of a sample was expressed as $\delta^{13}\text{C}$ (Ehleringer & Rundel 1989) and the nitrogen isotopic ratios as $\delta^{15}\text{N}$ (Evans 2001). Tissue total C and N were expressed as a percentage of dry weight. The proportion of N derived from N_2 fixation was calculated by using either *A. ataxacantha* or non-legume species as a reference. The contribution of N_2 fixation to the plant N budget was calculated from:

$$\text{percentage N from fixation} = \frac{(\delta^{15}\text{N}_{\text{reference}} - \delta^{15}\text{N}_{\text{sample}})}{\times 100/\delta^{15}\text{N}_{\text{reference}}}$$

where $\delta^{15}\text{N}_{\text{sample}}$ was the $\delta^{15}\text{N}$ of the *Acacia* spp., and $\delta^{15}\text{N}_{\text{reference}}$ was the average $\delta^{15}\text{N}$ of the *A. ataxacantha* non-legume species.

Results

POT EXPERIMENT

Biomass accumulation was significantly ($P < 0.05$) enhanced by supplying the plants with 2 mM N compared with 1 mM N in all the species except *A.*

ataxacantha (Fig. 1). The biomass accumulation of inoculated *A. karroo* was greater ($P < 0.05$) than that of equivalent plants receiving 1 mM N, but without inoculant (Fig. 1a). However, inoculation did not alter biomass accumulation in the other species. Nodules were found in all the treatments and species, except in *A. ataxacantha* (Fig. 1). Supply of 2 mM N increased the nodule fresh weight in *A. karroo*, but not in the other species where nodulation was not affected by the different N treatments. The concentration of nitrogen in the leaf tissue of the inoculated plants receiving 1 mM N was significantly (*post hoc* Fisher LSD, $P < 0.05$) higher than that of plants that received either 1 or 2 mM N without inoculation for all species except *A. ataxacantha* (Fig. 1). However, $\delta^{15}\text{N}$ values of leaves of 1 mM N inoculated *A. karroo* plants were significantly (*post hoc* Fisher LSD, $P < 0.05$) lower than those fed with 1 or 2 mM N, but remained unchanged in the other species (Fig. 1).

FIELD EXPERIMENT

The soils in the 'grass' and 'no grass' plots were statistically indistinguishable from each other, apart from a higher soil EC and Bray II P in the 'no grass' plots

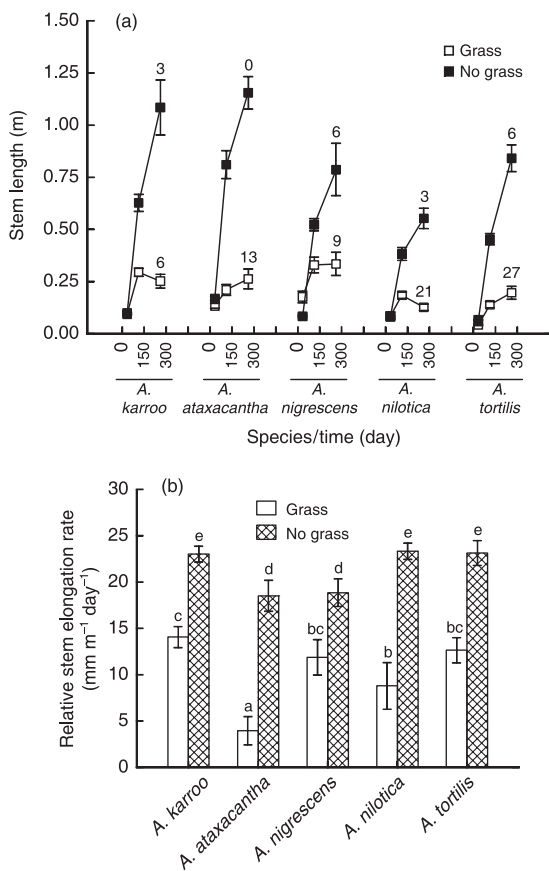


Fig. 2. (a) Main stem length above ground level of five *Acacia* spp. sampled 33, 112 and 261 days after transplanting to the cut ('Grass') and cleared ('No grass') plots. The points and error bars represent the mean \pm SE ($17 < n < 31$). The percentage mortality after 261 days is indicated on the graph. (b) Relative stem elongation rates were calculated from the stem length between 33 and 112 days. Bars and errors bars represent mean \pm SE and letters indicate significant (two-way ANOVA, factors species and presence/absence grass, followed by Fisher *post hoc* LSD, $P < 0.05$) differences between species and treatments. There was a significant interaction between the presence/absence of grass and species ($F_{4,253} = 2.46$, $P = 0.045$).

(Table S2). The higher EC in the 'no grass' plots was probably the cumulative consequence of small differences in measured P, Na, K, Ca, Mg, and possibly other elements not measured. The lack of differences in the concentration of N in the soil and of the soil $\delta^{15}\text{N}$ values between 'no grass' and 'grass' plots allowed meaningful comparison of the N_2 fixation capacity of the *Acacia* spp. between the two treatments.

The *Acacia* spp. in the 'no grass' plots grew much larger than those in the presence of grass (Fig. 2). The *Acacia* spp. in the 'grass' plots were rapidly over-taken by the grasses. *A. karroo*, *A. nilotica* and *A. nigrescens* did not grow between the 112 and 261 days sample periods. Dead plants were not included in the stem length measures; however, a number of plants died, especially in the 'grass' plots. After 112 days, 6.3% of the plants in 'grass' plots had died compared with 2.5% in the 'no-grass' plots. After 261 days, 15.0% of plants

in 'grass' plots had died compared with only 3.8% in the 'no-grass' plots. Because of variable initial sizes of the different species of *Acacia* transplanted, we calculated the relative rate of stem elongation from the natural logarithms of the stem lengths at the 33 and 112 days measurements (Fig. 2). The relative rates of stem elongation were lower for *A. ataxacantha* both with and without grass than for the other species.

Overall, there was a 12% lower concentration of N in mature leaf tissue than in young leaf tissue of the *Acacia* spp. (Fig. S3). This was mostly due to lower N concentration in the mature than in the young leaves of *A. nigrescens*, *A. tortilis* and *A. ataxacantha*. In contrast, the $\delta^{15}\text{N}$ values were not significantly different between young and mature leaves for the *Acacia* spp. (Fig. S3). Overall, the *Acacia* spp. had significantly higher leaf N concentrations when grown without grass (Fig. 3). At the first harvest (112 days), *A. ataxacantha* and *A. tortilis* had the highest leaf N concentrations in the absence of grass, while in the presence of grass, the leaf N concentrations of the *Acacia* spp. were lower, but followed the same pattern between species as those grown without grass. At the second harvest (261 days) the leaf N concentration was higher than at the first harvest and the differences between the 'grass' and 'no grass' treatments were smaller. The leaf $\delta^{13}\text{C}$ values of the *Acacia* spp. grown with grass were higher than those from the 'no grass' (Fig. 3). The leaf $\delta^{15}\text{N}$ values of the *Acacia* spp. grown with grass were significantly lower than those from the 'no grass' plots (Fig. 3). In the 'grass' plots *A. ataxacantha* had the highest leaf $\delta^{15}\text{N}$ values while the other *Acacia* spp. had statistically similar $\delta^{15}\text{N}$ values. The leaf $\delta^{15}\text{N}$ values were significantly higher at the second harvest than at the first. Overall, the leaf N concentrations of the resprout growth were similar to those of the initial growth (112 and 261 days). There were no significant differences in leaf N between plants from the 'grass' and 'no-grass' plots. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the resprouting 'grass' plants were lower than those of previous harvests and also lower than those of 'no-grass' resprouting plants.

We used the non-nodulating *A. ataxacantha* as a reference species for calculating the extent of N_2 fixation. The contribution of N_2 fixation to the N budget of resprout growth was not calculated as none of the *A. ataxacantha* resprouted in the presence of grass. The calculated proportion of N derived from N_2 fixation was high (83% across the four species) in the leaves of plants grown for 112 days, particularly in the presence of grass (Fig. 4). This proportion of N derived from N_2 fixation dropped to 46% after 261 days. For the plants grown without grass, the proportion of N derived from N_2 fixation remained at 17% for both harvests.

FIELD-COLLECTED MATERIAL

Twenty-four per cent of the tree species greater than 1 m tall in these sites were legumes (Table S4). However, 78% of the individual trees per hectare were legumes.

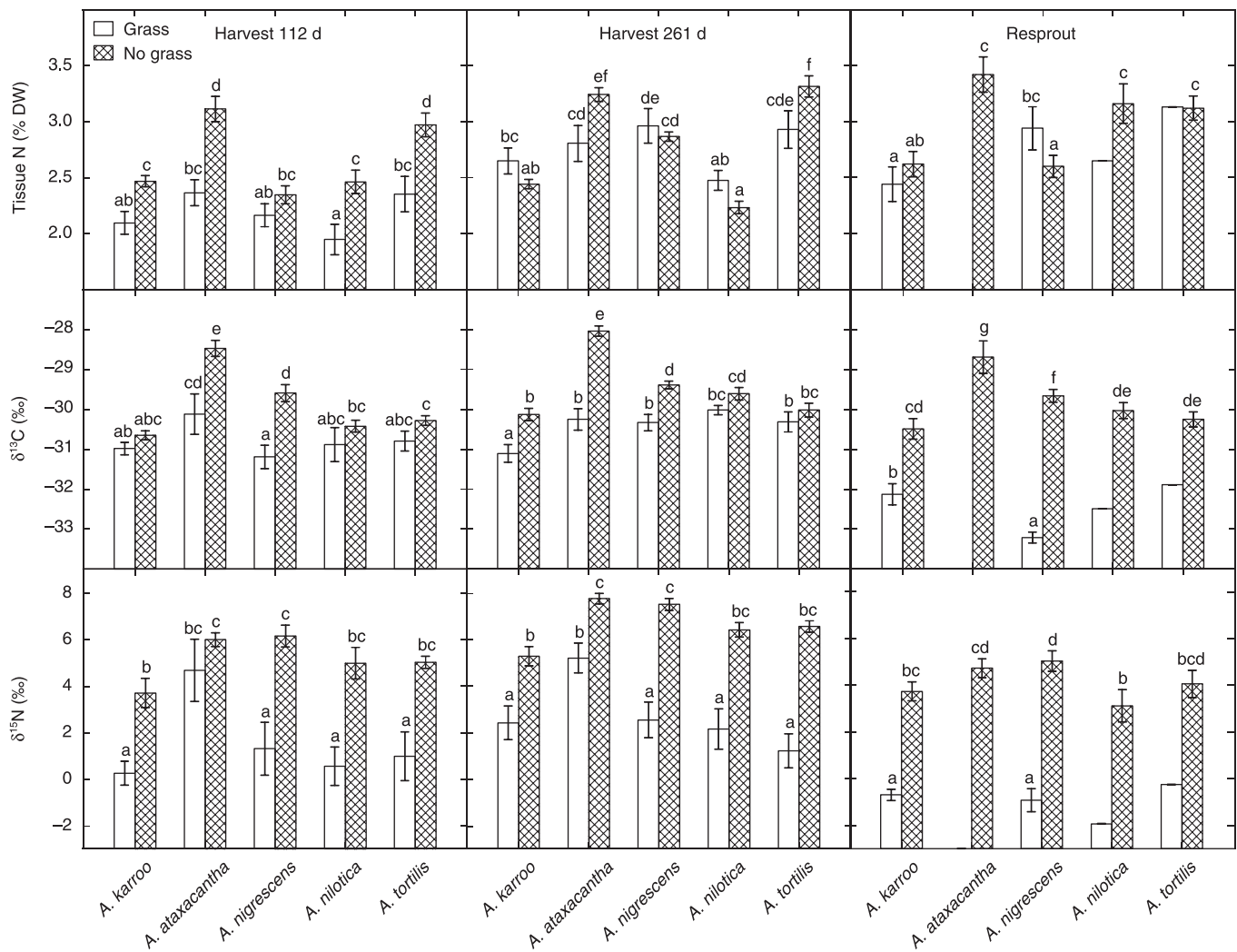


Fig. 3. Comparison of the leaf N concentrations, leaf $\delta^{15}\text{N}$ and leaf $\delta^{13}\text{C}$ for five *Acacia* spp. grown with and without grass and harvested 112 (left) or 261 days (right) after transplanting or after 141 days of resprout growth after plants had been cut back at 261 days. Bars and errors represent mean \pm SE and letters indicate significant differences between species and treatments (two-way ANOVA, Fisher *post hoc* LSD, $P < 0.05$). *Post hoc* tests for the two harvest times (112 and 261 days) were conducted separately for simplicity of representation. Three-way ANOVA (factors: species, presence/absence grass, harvest time) revealed significantly greater N concentrations ($F_{1,240} = 30.0$, $P < 0.01$), $\delta^{15}\text{N}$ ($F_{1,240} = 190.5$, $P < 0.01$) and $\delta^{13}\text{C}$ ($F_{1,240} = 93.6$, $P < 0.01$) in the leaves of plants grown without grass than in those grown with grass. There was statistically significant interaction between the presence/absence of grass and species for N concentration ($F_{4,240} = 5.58$, $P < 0.01$), $\delta^{15}\text{N}$ ($F_{4,240} = 12.61$, $P = 0.03$) and $\delta^{13}\text{C}$ ($F_{4,240} = 9.3$, $P < 0.01$). The time of sampling had a significant consequence for N concentration ($F_{1,240} = 53.9$, $P < 0.01$), $\delta^{15}\text{N}$ ($F_{1,240} = 16.6$, $P = 0.03$) and $\delta^{13}\text{C}$ ($F_{1,240} = 8.7$, $P < 0.01$), but interactions between the presence/absence of grass and sample time were only significant for N concentration ($F_{1,240} = 19.0$, $P < 0.01$). There were no significant three-way interactions.

Thus the density of legume trees was much greater than that of the non-legumes, despite the fact that there were more non-legume species. Similar patterns were found when trees less than 1 m tall were included in the analysis. A number of saplings (< 1.3 m height) were found to have nodules on their roots (Table 1). As only a limited number of trees could be excavated, the lack of nodules does not necessarily indicate that the tree species are not capable of producing nodules.

The leaves of a large number of legume and non-legume species were sampled from Hluhluwe-iMfolozi Park for leaf N concentration and $\delta^{15}\text{N}$ determination. The mean tissue N concentration of the legumes was 23% greater and the $\delta^{15}\text{N}$ values 54% smaller than

those of the non-legumes (Table 2). This translated to a 55% contribution of N_2 to the N budget of the legume species. We have not listed the individual contributions of N_2 fixation to the N budgets of the legume species as the number of replicate bulked samples of each of the species was low. The $\delta^{15}\text{N}$ values were positively correlated with leaf N for both the legumes ($r = 0.61$, $P < 0.01$, Fig. 5) and the non-legumes ($r = 0.51$, $P < 0.01$, Fig. 5), but there was no difference in slopes for legumes and non-legumes ($P > 0.05$, homogeneity of slopes test). The leaf P concentration was not significantly different between legumes and non-legumes (Table 2). Overall, however, leaf P was positively correlated with leaf N concentrations ($r = 0.74$, $P < 0.01$, Fig. 5).

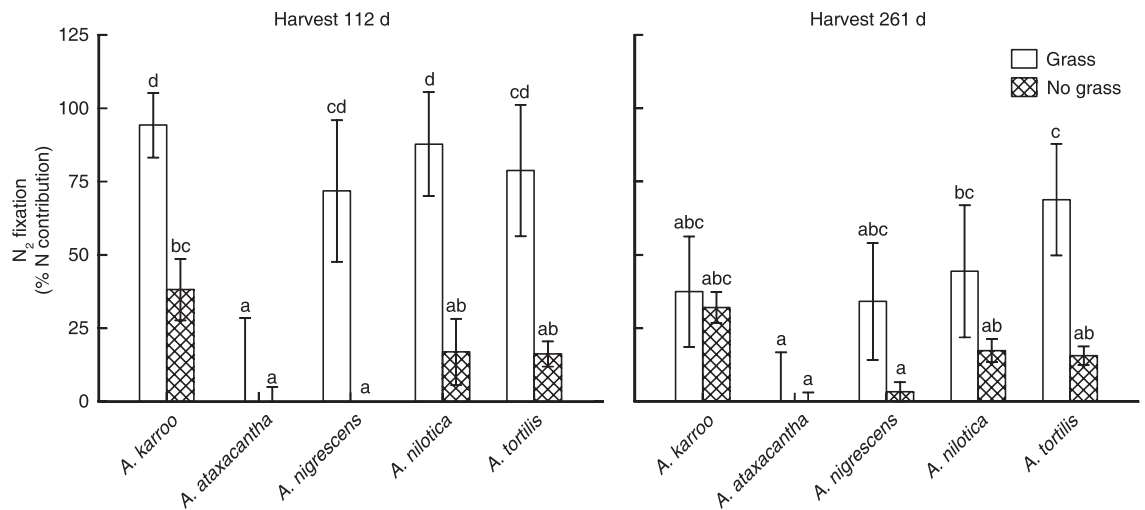


Fig. 4. The percentage contributions of N_2 fixation to leaf N calculated from the $\delta^{15}N$ values of *A. karroo*, *A. nigrescens*, *A. tortilis* and *A. nilotica* using *A. ataxacantha* as a reference plant. Statistics on graph as for Fig. 3. Three-way ANOVA (factors: species, presence/absence grass, harvest time) revealed that there was a significantly ($F_{1,240} = 5.68$, $P = 0.02$) smaller contribution of N_2 fixation to the N budget after 261 days compared with 112 days. Species ($F_{4,240} = 9.1$, $P < 0.01$) and the presence/absence of grass ($F_{4,240} = 37.1$, $P < 0.01$) had significant effects on the contribution of N_2 fixation. There was significant interaction between the presence/absence of grass and species ($F_{4,240} = 2.98$, $P = 0.02$). There were no significant three-way interactions.

Table 1. The proportion (%) of excavated leguminous saplings (0.7–1.3 m height) that had nodules on their roots. Plant height is the mean for the number of plants excavated

Species	Plant height (m)	Number nodulated/ number excavated	Nodule frequency (%)
<i>A. ataxacantha</i>	1.1	0/6	0
<i>A. burkei</i>	1.0	0/6	0
<i>A. caffra</i>	0.9	5/7	71
<i>A. gerrardi</i>	0.7	6/7	86
<i>A. grandicornuta</i>	1.8	1/6	17
<i>A. karroo</i>	1.3	4/7	57
<i>A. nigrescens</i>	1.3	0/6	0
<i>A. nilotica</i>	1.3	1/6	17
<i>A. robusta</i>	0.9	0/7	0
<i>A. tortilis</i>	1.3	0/6	0
<i>Dichrostachys cinerea</i> ssp. <i>africana</i>	0.7	0/6	0
<i>Dichrostachys cinerea</i> ssp. <i>nyassana</i>	1.1	3/7	43

Table 2. The $\delta^{15}N$ (‰), N and P concentrations (% of leaf DW) and the contribution of N_2 fixation to the N budget of the legumes calculated from the $\delta^{15}N$ values of the legume and non-legume species. The values are the mean \pm SE. There were 16 legume and 24 non-legume species (see Appendix S1 for species list). The P -values were determined from two-tailed Student's t -tests

	Legume	Non-legume	P
N (%)	2.28 \pm 0.08	1.85 \pm 0.11	0.01
P (%)	0.098 \pm 0.005	0.098 \pm 0.008	0.94
$\delta^{15}N$ (‰)	0.98 \pm 0.32	2.15 \pm 0.32	0.02
%N from N_2 fixation	54.6 \pm 15.1		

Discussion

The predominance of leguminous trees (77% of trees) in the savanna in the Hluhluwe-iMfolozi system

investigated (Appendix S4) that are capable of fixing N_2 (Fig. 1) deserves an explanation. As only 17.5% of the savanna tree species in Hluhluwe-iMfolozi were legumes, it is unlikely that the mere availability of legume species in the flora (Crews 1999) drives the predominance of leguminous trees in this system. Midgley & Bond (2001) listed multiple constraints (including seed survival/dispersal, seedling establishment, fire, herbivory and competition) on the survival of African *Acacias*, including competition with grass, but did not consider nutrition as a possible constraint. The growth of legume tree seedlings was indeed strongly reduced by the presence of grass (Fig. 2). This reduction in growth could result from competition for nutrients, water and light, or a combination of these. The literature on tree–grass interactions includes examples of both facilitation and competition. In some cases, trees facilitate grass growth through increased nutrient availability under

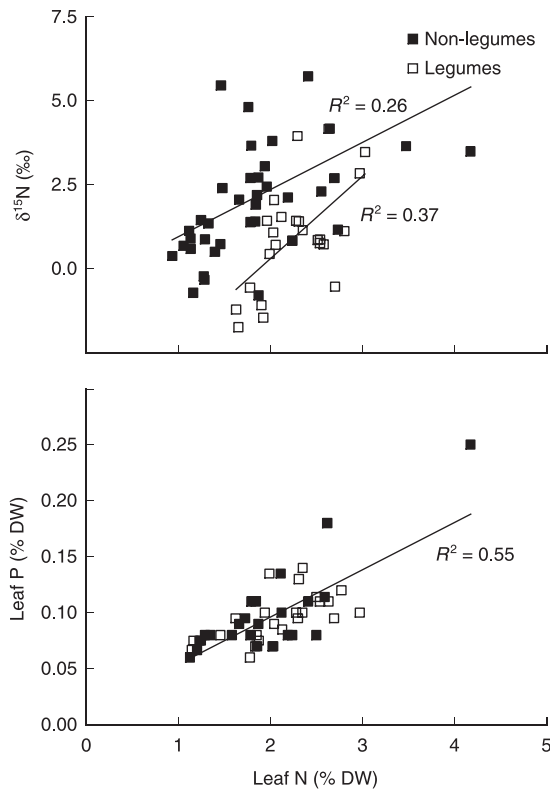


Fig. 5. Variation in $\delta^{15}\text{N}$ and leaf P concentration with leaf N concentration for leaves of 24 legume and 38 non-legume species (see Appendix S1 for species list). The leaves were collected from species growing in the Hluhluwe-iMfolozi area in April to May 2005 and March to April 2006.

the tree canopy and through hydraulic lift of water into the root zone occupied by the grass (Ludwig *et al.* 2004). In others, grasses have been shown to facilitate tree seedling establishment (O'Connor 1995). However, trees do compete with grass for both nutrients and water (Scholes & Archer 1997) as detailed for both adult trees (Knoop & Walker 1985; Belsky *et al.* 1993) and seedlings (Kraaij & Ward 2006). In the field experiment, 'grass' and 'no grass' plots were irrigated to reduce water limitations on tree-seedling growth. The *Acacia* spp. grown without grass in the field experiments had less negative $\delta^{13}\text{C}$ values than those grown with grass, especially in the resprout growth (Fig. 3). If trees and grass were competing for water in this experiment, we would have expected plants growing with grass to have restricted stomatal conductances, and thus more positive $\delta^{13}\text{C}$ values. We conclude that competition for water by grass was probably not a major limiting factor for the growth of the *Acacia* spp. in this experiment.

Competition for light is likely to have been a major factor in the reduced growth of the *Acacia* spp. in the 'grass' plots. This competition developed as the grasses grew from being cut back until they over-topped the tree seedlings. The analysis of young and mature leaf N concentrations showed reduced N concentrations in the mature leaves, possibly due to remobilization of N from mature to younger tissues (Appendix S3). Remobilization of N could be responsible for the lack

of differences in the $\delta^{15}\text{N}$ values between young and mature leaves (Appendix S3). Remobilization of N is likely under N-deficient conditions or when older leaves become shaded. Thus competition for light could have limited the N_2 fixation capacity of the tree seedlings, especially during the latter stages of the experiment. Generally, legumes cope poorly with low light, because of the nodule demand for energy, and shade-tolerant legumes are largely restricted to the tropics (Sprent 1999). Grass competition for light is likely to be a constraint on the survival of most *Acacia* spp. The death of several tree seedlings in the presence of grass and the lack of growth of the remaining seedlings could be attributable to shading. The fact that the period over which growth was measured included winter resulted in retarded growth, although the plants in the 'no grass' treatment did continue to grow slowly over this period. The lack of growth and reduced survival in the grass sward could limit seedling survival in the savanna system, especially when combined with fire and herbivory damage to the young plants. However, survival of the seedlings in the grass sward and eventual escape above the grass sward height may be contingent on periodic reduction of grass cover through grass consumption by herbivory or fire and subsequent resprouting by the trees.

Higgins *et al.* (2000) argued that tree cover in savannas is limited by demographic bottlenecks to tree recruitment, particularly in arid savannas with low and variable rainfall. In mesic savannas the establishment bottleneck would be less important, but sapling escape from the grass and grass-fuelled fires could be a major limitation on tree survival. The capacity of some *Acacia* spp. to resprout enables the plants to grow rapidly enough to escape the kill-zone of the frequent savanna fires, which are fuelled by the combustible grass layer (Bond & van Wilgen 1996). Resprout growth was associated with low $\delta^{15}\text{N}$ values, probably indicating high N_2 -fixation dependence, in the presence of grass, but not in the absence of grass (Fig. 3). The resprouting plants grown with grass may have had a greater pre-existing capacity for N_2 fixation or might have been more dependent on this capacity due to the presence of grass roots. An earlier limitation on *Acacia* spp. survival than fire may be escape from the light-limitation imposed by the grass. However, our experiments only tracked mortality over a limited time period and do not allow us to clearly differentiate between above- and below-ground competition.

The rate of growth of *A. ataxacantha* with grass was significantly slower than that of the other *Acacia* spp. In the Hluhluwe-iMfolozi area *A. ataxacantha* is restricted to the forest margins and thickets and does not occur in the open savanna areas. This might indicate that this species is not adapted to the presence of grass in the savanna. *A. ataxacantha* has been reported not to nodulate (Hernández-Lucas *et al.* 1995; Harrier *et al.* 1997; Pueppke & Broughton 1999), associated with slow root hair production (Harrier *et al.* 2000). This species failed to produce nodules in both the field

experiment (data not shown) and the pot experiment, and nodules were not observed on the roots of saplings excavated in natural populations. In the field experiment with grass and in the pot experiment (all treatments), *A. ataxacantha* had the highest $\delta^{15}\text{N}$ values, indicating a lack of N_2 fixation. The slow growth of *A. ataxacantha* seedlings with grass is consistent with the lack of nodulation of this plant.

The soils in the Hluhluwe-iMfolozi area are generally replete with total N, as was the case in our field experiment site (Appendix S2). The pH, N and P-values for the field experiment site were similar to those from a wider sampling of the park in 2000 in which the average soil pH (1 M KCl) was 5.73 ± 0.03 , the total N concentration was $2.09 \pm 0.02 \text{ g kg}^{-1}$ and the total P concentration was $333 \pm 4 \text{ g kg}^{-1}$ ($n = 712$; W. J. Bond & W. Stock, unpublished data). Total N does not, however, reflect the availability of inorganic N for plant uptake, which depends on the rate of N mineralization and the rate of loss from the soil as a consequence of consumption by the biota and leaching/volatilization. Competition with the *Acacia* spp. for inorganic N by grass is likely to be intense. This is the probable cause of extensive N_2 fixing activity in the plants grown with grass, resulting in low $\delta^{15}\text{N}$ values for the *Acacia* spp. White clover (*Trifolium repens*), red clover (*Trifolium pratense*) and alfalfa (*Medicago sativa*) have also been shown to fix a greater proportion of plant N when grown together with grasses (Carlsson & Huss-Danell 2003). This was attributed to grasses being strong competitors for N and the induction of N_2 -fixation to N-deprivation. Similarly, after 112 days of growth with grass, the *Acacia* seedlings had significantly lower leaf N concentrations than those grown without grass, possibly indicating an N constraint in the plants grown with grass, although light limitation also reduces leaf Rubisco concentration and thus leaf N content (e.g. Seeman 1989). As in *A. auriculiformis* (Goi *et al.* 1993), the *Acacia* spp. engaged in N_2 fixation are likely to have simultaneously acquired inorganic N from the soil, depending on the availability of combined N in the soil. Uptake of N from high concentrations of soil N results in increased leaf $\delta^{15}\text{N}$ values due to discrimination against ^{14}N as a result of preferential efflux of ^{14}N from the roots (Evans 2001). This partially accounts for the positive correlation between leaf N concentration and $\delta^{15}\text{N}$ for both the legumes and the non-legumes (Fig. 5). Furthermore, a positive correlation between leaf N concentration and $\delta^{15}\text{N}$ has been previously reported and related to greater fractionation in more N-replete systems (Martinelli *et al.* 1999). Leaf $\delta^{15}\text{N}$ values can also be reduced by the utilization of N from deeper soils with more negative $\delta^{15}\text{N}$ values than those of the surface soils (Högberg 1997). In contrast, in the 'no-grass' plots in the field experiment the *Acacia* spp. that had deeper roots actually had more positive $\delta^{15}\text{N}$ values than those with shallower roots in the grass plot. Thus the major influence on *Acacia* spp. leaf $\delta^{15}\text{N}$ was N_2 fixation, rather than rooting depth. This

dependence on N_2 fixation was reduced as the *Acacia* spp. got older, possibly due to increased shading by the grass sward.

Högberg (1997) suggested that the difference in nitrogen isotope values between N_2 -fixers and reference plants should be at a minimum of at least 5‰. This requirement for such large differences between $\delta^{15}\text{N}$ values of putative N_2 fixers and non-fixers is a product of the complex nature of N isotope discrimination. One reason that N_2 fixation in the field is difficult to demonstrate using $\delta^{15}\text{N}$ is that both legume and non-legume $\delta^{15}\text{N}$ values are variable and are correlated with leaf tissue N concentrations and N cycling (Fig. 5). This means that extensive data need to be collected and that the leaf N concentrations of legumes and non-legumes need to be considered, as we have done. Comparison of legume and non-legume $\delta^{15}\text{N}$ values without consideration of the leaf tissue N concentrations results in insensitivity of the technique and the requirement for large differences in $\delta^{15}\text{N}$ between N_2 -fixers and reference plants to demonstrate N_2 -fixation. The $\delta^{15}\text{N}$ values of the legumes in this study (0.98, Table 2) were smaller than those reported previously for Kruger National Park (2.14, Aranibar *et al.* 2003). However, the values for the non-legumes were also smaller in our study (2.15, Table 2) than in Kruger (4.74), based on re-analysis of the data of Aranibar *et al.* (2003) by grouping legumes and non-legumes. The higher $\delta^{15}\text{N}$ at the Kruger site (*c.* 350 km north of Hluhluwe-iMfolozi) may be related to differences in rainfall; Hluhluwe-iMfolozi receives higher rainfall (780 mm per annum) than Kruger (550 mm per annum) and $\delta^{15}\text{N}$ generally decreases with rainfall (Aranibar *et al.* 2004). This difference in $\delta^{15}\text{N}$ values does not translate to differences in N_2 fixation because re-analysis of the data of Aranibar *et al.* (2003) with the same formula for percentage N derived from N_2 fixation that we used also yields a result of 55% N derived from N_2 fixation. Although the estimate of the contribution of N_2 fixation to the N-budget of the plant may not be entirely quantitative (Högberg 1997), it does indicate that significant amounts of N are likely to be derived from N_2 fixation in both Hluhluwe-iMfolozi and Kruger National Park.

Leaf P concentrations did not differ between legumes and non-legumes, and the correlations between leaf P and leaf N had similar slopes for legumes and non-legumes. This indicates that the legumes did not have a particularly high requirement for P, as suggested by Hartwig (1998). Although soil total P concentrations in both the field experiment site (Appendix S2) and in the survey of Hluhluwe-iMfolozi were high, the Bray II available P at the field site was surprisingly low. The low available P concentration and the correlation (Fig. 5) of leaf P with leaf N concentration indicate that the trees may be limited by P to some extent, as has been described previously for savanna trees in tropical Africa (Högberg 1986a,b) and suggested by the results of Ludwig *et al.* (2004). The Bray II soil P levels in the

grass plots were half of those in the 'no grass' plots after only 112 days. Thus the ability of legumes to fix N₂ may liberate them from N constraints, but other nutrients, such as P, may well be a constraint.

Conclusion

Vitousek *et al.* (2002) suggested that relatively high N availability in lowland tropical forests permits legumes to maintain an N-demanding lifestyle without always being required to pay the costs of fixing N. We believe that this also applies to the Hluhluwe-iMfolozi savanna where legumes are abundant. In this savanna, leguminous tree seedlings utilize N₂ fixation to cope with intense competition from nitrogen-use efficient C₄ grasses for N while niche segregation (Walter 1971) for nutrient acquisition is impossible. In frequently burnt and herbivore-impacted savannas, loss of soil N and competition with grass may provide a niche for N₂ fixing leguminous trees.

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Supplementary material

The following supplementary material is available for this article:

Appendix S1. List of legume and non-legume species sampled from the field.

Table S2. Soil characteristics of field plots on which *Acacia* spp. were grown.

Figure S3. Comparison of the leaf N concentrations and the leaf $\delta^{15}\text{N}$ values of the young and mature leaves of five *Acacia* spp. grown in field plots.

Table S4. The number of legumes and non-legume trees occurring in savanna areas within Hluhluwe-iMfolozi.

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