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Can legume inclusion in pasture systems improve forage carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) nutrition? Findings from Tsolo, Eastern Cape, South Africa.

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Abstract

The supply of nitrogen (N) to plants limits primary productivity in numerous ecosystems and these limitations in N concentrations in plants also limit herbivores, by limiting their productivity in relation to both plant nutritional quantity and quality. Carbon isotope ratio can provide insight about the photosynthetic pathways utilized by different plant species. Plant C and N isotope ratios were studied for different species growing in old arable lands in Kubedlana communal area, located at 32°11'53 S and 28°14'1 E and at 1020.8 m altitude in the Eastern Cape, South Africa. The vegetation type of the study area is the Foothill Moist Grassland. The area receives an annual rainfall between 600 mm and 750 mm. The study was aimed at testing whether there was seasonal shift amongst different plant species in relation to $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopes. Leaf samples of *L. cuneata* (legume), forbs (weeds) and native grasses from the area were sampled to determine carbon and nitrogen isotope ratios. Plant samples were dried for 48 h at 70 °C and ground with a ball mill. All sampling was done once in spring (November) 2022, summer (February) 2023 and autumn (March) 2023 and winter (May 2023) seasons, respectively. Permutational multivariate analysis of variance (PERMANOVA) was used to test the effect of plant type (forbs, grass and legume) on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopes. There was a significant difference ($P = 0.001$) between the three plant species and seasonal change regarding $\delta^{15}\text{N}$ ratios. $\delta^{13}\text{C}$ ratios, plant type also significantly ($P = 0.001$) affected plant carbon ratios. These findings emphasize the importance of

legume inclusion into old lands which is crucial in promoting nutrient cycling in pastures and ultimately nutritive value which is critical for improved animal performance.

Introduction

Nitrogen (N) is a significant limiting resource in numerous terrestrial ecosystems and N cycling influences most aspects of ecosystem function (Thomas *et al.*, 2013). The supply of N to plants limits primary productivity in a myriad of ecosystems (Oberson *et al.*, 2013). The limitations in N concentrations in plants also result in limitations to herbivores, by constraining the productivity of herbivores in relation to both nutritional quantity and quality of plants (Craine *et al.* 2015b; Zavala *et al.*, 2013). It is therefore crucial to understand how patterns in terrestrial N cycling occur within and across ecosystems in order to predict patterns of plant productivity, ecosystem carbon sequestration, nutrient fluxes and trace gas losses to the atmosphere (Goll *et al.*, 2012; Hudman *et al.*, 2012). Photosynthesis is also strongly affected by nitrogen availability because the photosynthetic machinery accounts for more than half of the N in the leaves (Pinder *et al.*, 2012). Typically, plant leaves are used as an index of $\delta^{15}\text{N}$ of the whole plant. Although there may be differences that exist among leaves, roots, and stems (Unkovich, 2013), the N isotope ratios generally correlate among plant fractions and any average differences are generally minor. For example, a study that was done, across 90 grass species collected from 67 sites in four grassland regions worldwide for the determination of $\delta^{15}\text{N}$, the $\delta^{15}\text{N}$ of leaves averaged just 0.3‰ less than those of roots compared to a range of 18‰ for leaves and 14‰ for roots (Craine *et al.*, 2015b). Although the N cycle is consisting of numerous processes that are difficult to measure, the ratios of $\delta^{15}\text{N}$ in plants or soils provide an indication on patterns of crucial aspects of the N cycle. These aspects comprise of the following: N supply rates to ecosystems and plants, N availability to plants, the N pathways by which N is lost from ecosystems, and the quantity of N lost. There is evidence that plant species differ in their capacity to assimilate inorganic and organic N however, there is limited known information and how interactions with companion plants affect the use of different N sources (Unkovich, 2012).

Therefore field (farm) research was carried with the aim to conduct a full investigation into the plant carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) nutrition of *L. cuneata*, a leguminous plant species, and its companion plants growing in old arable lands at Kubedlana communal area near Tsolo, Eastern Cape, South Africa. The study of *L. cuneata* and its companion plants' carbon and nitrogen nutrition is particularly relevant for understanding the long-term effects of land-use changes and the potential for ecological restoration. By examining the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures of *L. cuneata* and its companion plants, researchers can clarify the plant-soil-microbe interactions, nutrient cycling, and the potential for these abandoned lands to support diverse and resilient plant communities. Additionally, this analysis can contribute to an in-depth understanding of the nitrogen cycling dynamics within the old arable lands' ecosystem. Furthermore, the insights gained from this research can inform sustainable land management practices, such as the integration of *L. cuneata* and other leguminous species into agroecosystems, to enhance soil fertility, improve nutrient cycling, and promote the overall ecological health of these agricultural landscapes. This research was also aimed at elucidating the complex interplay between the carbon and nitrogen dynamics within this plant community, with a particular focus on understanding the adaptive strategies and resource partitioning mechanisms employed by *L. cuneata* and its associated plant species in the context of the old arable land ecosystem as influenced by seasonal change.

Study Area

The research was conducted in old arable lands at Kubedlana communal area near Tsolo under OR Tambo district municipality in the Eastern Cape Province, South Africa. Kubedlana is located at 32°11'53 S and 28°14'1 E and at 1020.8 m altitude. The vegetation type in the study area is classified as Foothill Moist

Grassland (Mucina and Rutherford, 2006). The annual rainfall of the area is estimated to range between 600 mm and 750 mm, while temperatures range between 3 °C in winter and 28 °C in summer. The soil chemical properties are as follows: P (5.99 mg/kg), K (0.20 mg/kg), Ca (4.26 mg/kg), Mg (1.16 mg/kg), and Zn (6.28 mg/kg).

Plant sampling and preparation

Samples of the uppermost fully expanded leaves of legumes, forbs (weeds) and native grasses from the legume and control (grass only) plots were sampled to determine dry matter production and other biochemical contents. Leaf samples were dried for 48 h at 70 °C and ground with a ball mill. Leaf nitrogen and carbon isotope ratios (i.e. foliar $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$) and N were determined from approximately 3 mg with an isotope-ratio mass spectrometer (IRMS; Deltaplus XP and Delta C prototype Finnigan MAT, respectively, Finnigan MAT, Bremen, Germany; 0.1‰ precision). The $\delta^{15}\text{N}$ values represent nitrogen isotopic composition of the sample relative to that of atmospheric dinitrogen in ‰: $\delta^{15}\text{N} = (\text{R}_{\text{sample}} / \text{R}_{\text{standard}} - 1) \times 1000$ (‰), where R = sample is the sample isotope ratio ($^{15}\text{N}/^{14}\text{N}$) and R_{standard} is the $\delta^{15}\text{N}$ ratio for atmospheric N_2 .

Data analysis

R vegan package statistical package was used to analyse all data, utilising a permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001; McArdle & Anderson, 2001). PERMANOVA was used to test the effect of the season (summer, winter, spring, and autumn) and plant type (forbs, grass and legume /*L. cuneata*) on foliar nutrient content. To graphically ordinate variation in plant isotope composition among the plant types and seasons, nonmetric multidimensional scaling (NMDS) was applied. Then a Non-Metric Multidimensional Scaling (NMDS) using Manhattan distance to assess the compositional dissimilarity among samples based on plant nutrient and isotope data was also used. The NMDS analysis was performed in R using the vegan package, with the dimensionality reduced to two axes to facilitate visualization. In addition, the generalised linear mixed model (GLMM) for each variable, with season as a random effect was also used to analyse data.

Results

There is a difference in the $\delta^{13}\text{C}$ isotope composition, and the shift is clear and inclined to plant type. Grasses had high ^{13}C content in comparison to forbs and legumes (Figure 3). In contrast, forbs and legumes usually utilising the C3 photosynthetic pathway, have a different carbon-concentrating mechanism as C4 plants, and they tend to have a higher discrimination against the ^{13}C isotope during carbon fixation. Consequently, C3 plants, including forbs and legumes, typically have lower ^{13}C content (more negative $\delta^{13}\text{C}$ values) compared to C4 plants. There was also a distinct observed differences in the ^{15}N isotope composition among the plant types in the old arable lands and this can be related to the fact that legumes, such as *L. cuneata*, can fix atmospheric nitrogen (N_2) through a symbiotic relationship with nitrogen-fixing bacteria called *Rhizobium*. The biological nitrogen fixation process preferentially incorporates the lighter ^{14}N isotope into the plant biomass, resulting in the lower (more negative) $\delta^{15}\text{N}$ values observed in legumes in current study. Grasses and forbs primarily obtain their nitrogen from the soil, and soils often have higher $\delta^{15}\text{N}$ signature due to various soil nitrogen transformation processes such as mineralization, nitrification, denitrification. Therefore, the non-leguminous plants tend to have higher $\delta^{15}\text{N}$ values as they assimilate the heavier ^{15}N isotope from the soil. When the data were classified seasonally according to drier (Winter and Autumn) and rainy seasons (Spring and Summer), there was a notable response ($p = 0.001$) in $\delta^{15}\text{N}$ values to variations in precipitation. Higher ($p = 0.001$) $\delta^{15}\text{N}$ values were observed during the dry and lower during rainy seasons, respectively. This seasonal tendency was observed throughout sampling seasons, but a

variation in the scale on $\delta^{15}\text{N}$ values was noted, both for legume and non-legume species. (Table 1; Figure 2).

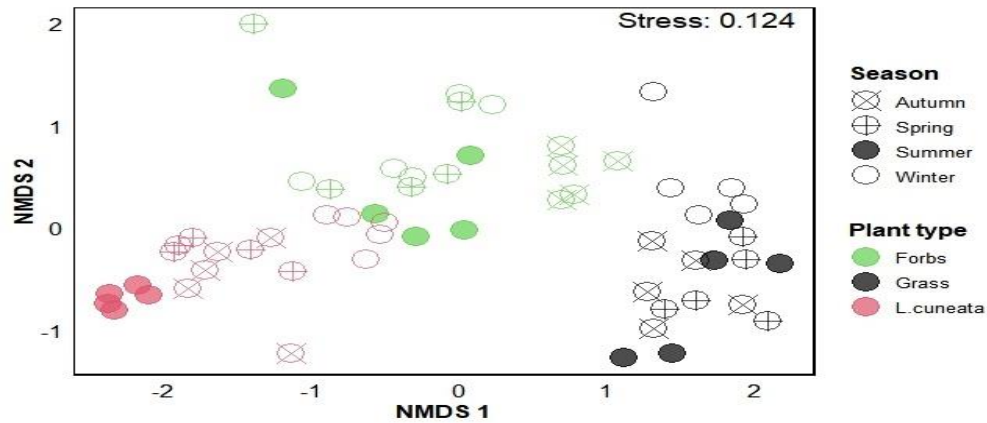


Figure 1: NMDS ordination of samples based on species plant nutrition and isotope data using Manhattan distance. Sites that are closer together in the plot have more similar plant nutrition and isotope compositions, as determined by Manhattan distance. Plant nutrients and isotope variables showed distinct spatial separation between the three plant types.

Table 1: Seasonal and plant type effect and their interaction on $\delta^{15}\text{N}$ content

Group variable	df	R ²	F	P
Season	3	0.072	10.399	0.001
Plant type	2	0.691	148.398	0.001
Season*Plant type	6	0.124	8.918	0.001
Residual	48	0.111		
Total	59	1.000		

There are significant differences in season ($r^2 = 0.072$ and $P = 0.001$), plant type ($r^2 = 0.691$ and $P = 0.001$), and in their interaction ($r^2 = 0.124$ and $P = 0.001$).

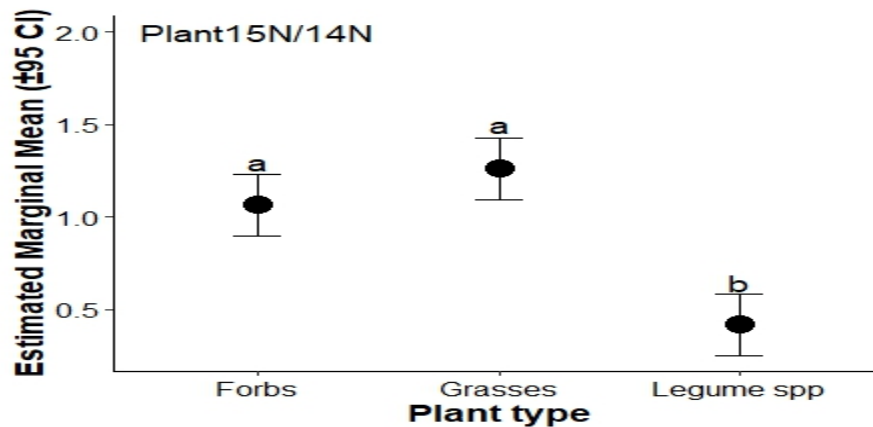


Figure 2: Nitrogen isotope ($\delta^{15}\text{N}$) nutrition of legume (*L. cuneata*) and different companion plant species growing in Kubedlana communal area.

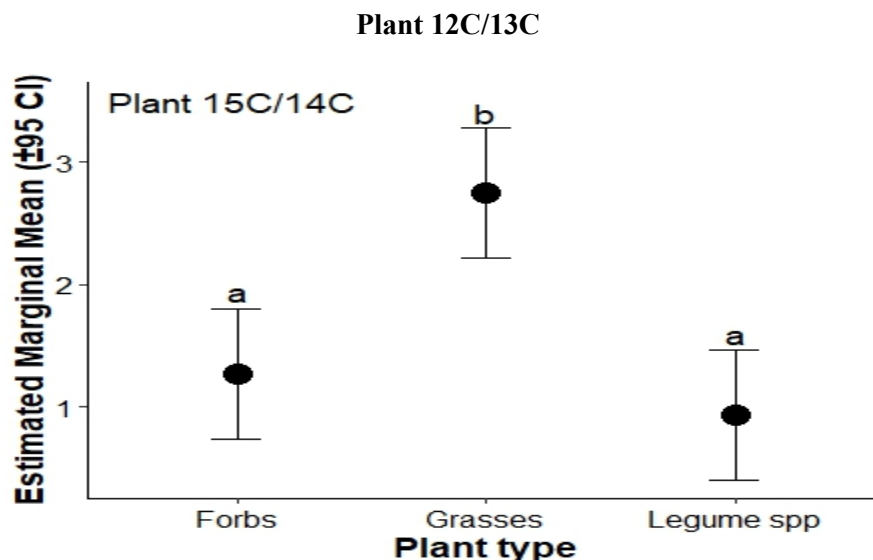


Figure 3: Carbon isotope ($\delta^{13}\text{C}$) nutrition of legume (*L. cuneata*) and different companion plant species growing in Kubedlana communal area.

Discussion

The $\delta^{13}\text{C}$ signature of grasses was significantly higher (less negative) than that of forbs and legumes. The type of pasture had a significant effect on $\delta^{13}\text{C}$. Grasses typically have higher $\delta^{13}\text{C}$, whereas legumes typically lower $\delta^{13}\text{C}$ than both forbs and grasses due to differences in C isotopic fractionation during CO_2 assimilation. The basis of the use of $\delta^{13}\text{C}$ procedure is that legumes (C3) species discriminate against ^{13}C during photosynthesis to a greater extent than do grasses (C4) species. Due to this fractionation during photosynthesis, C3 plants typically contain approximately 14 parts per thousand less carbon-13 than C4 plants. There was a notably clustering of plant functional groups where legumes and grasses separated along NMDS 1. The observed range of foliar $\delta^{15}\text{N}$ values for the legumes was notably smaller in comparison to grasses and forbs; respectively. This finding is consistent with the findings made by (Gerschlauer *et al.*,2019); who argued that $\delta^{15}\text{N}$ values of plants that mainly rely on N_2 fixation are usually - 0 ‰, reflecting

atmospheric isotopic N values (Enriquez-Hidalgo *et al.*, 2015). Craine *et al.*, 2015b), also alluded that most N₂-fixing plants show significant departures from 0 ‰ due to differences in reliance on fixed N. The strong depletion of $\delta^{15}\text{N}$ in legumes compared to the forbs and grasses is an indication that legumes largely relied on atmospheric N. Foliar N concentration ranging at (-3.14 ‰ and -7.02 ‰) for legumes, which was lower than (4.65 ‰ and 7.91 ‰) for grasses and (-0.88 ‰ and 4.35 ‰) for forbs, respectively were found in the current study. Unkovich (2013); also argued that variations in $\delta^{15}\text{N}$ of symbiotic N₂ fixation were not necessarily the product of N₂-fixation, but rather a combination of measurement errors, intra-plant fractionation events resulting in tissue differences and possible preferential losses of ¹⁵N-depleted NH₃. Higher values of $\delta^{15}\text{N}$ depicted by grasses are an indication that grasses depend, to some extent, on N from non-symbiotic N₂ fixation or from the soil. The $\delta^{15}\text{N}$ value, is a widely used indicator of nitrogen cycling and sources within ecosystems. The observed pattern in the current study, where grasses and forbs exhibit higher $\delta^{15}\text{N}$ values compared to legumes, can be ascribed to numerous underlying mechanisms such as nitrogen fixation, nitrogen cycling and fractionation, nitrogen acquisition strategies and environmental conditions. Legumes have the unique ability to fix atmospheric nitrogen (N₂) through a symbiotic relationship with nitrogen-fixing bacteria (e.g., *Rhizobium*). Consequently, legumes typically exhibit lower $\delta^{15}\text{N}$ values compared to plants that rely on soil-derived nitrogen sources, such as grasses and forbs.

Conclusion

In conclusion, the observed differences in the ¹³C isotope composition among the plant types (grasses, forbs, and legumes) growing in the old arable lands of the Eastern Cape, South Africa, are primarily driven by their distinct photosynthetic pathways and carbon acquisition strategies. The observed shift in ¹³C content, where grasses exhibited higher ¹³C content compared to forbs and legumes, can be attributed to the distinct photosynthetic pathways employed by these plant types. Grasses, which primarily utilize the C4 photosynthetic pathway, usually possess higher ¹³C content due to the efficient carbon-concentrating mechanisms involved in this pathway. In contrast, forbs and legumes, which predominantly utilize the C3 photosynthetic pathway, exhibit lower ¹³C content. This difference in ¹³C content reflects the distinct carbon isotope discrimination patterns associated with the C3 and C4 photosynthetic pathways. Therefore, the observed differences in the ¹⁵N isotope composition among the plant types (grasses, forbs, and legumes) in the old arable lands of the Eastern Cape, South Africa, can be attributed to the distinct nitrogen acquisition and cycling processes associated with each plant functional group. The higher $\delta^{15}\text{N}$ values in grasses and forbs reflect their reliance on soil-derived nitrogen, while the lower $\delta^{15}\text{N}$ values in legumes are a result of their ability to fix atmospheric nitrogen. The observed higher $\delta^{15}\text{N}$ values during the dry season and lower values during the rainy season can be attributed to the variations in water availability and its impact on nitrogen cycling processes. Several studies have shown that water availability is a key driver of $\delta^{15}\text{N}$ values in ecosystems, regardless of other factors such as soil nitrogen stock. During drier periods, reduced water availability can lead to increased soil nitrogen mineralization, nitrification, and volatilization, which preferentially remove the lighter ¹⁴N isotope and enrich the remaining soil nitrogen with the heavier ¹⁵N isotope.

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