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RESEARCH ARTICLE

N_2 fixation is linked to the ability to encroach in African savanna trees

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Abstract

1. Encroachment is a globally ubiquitous phenomenon, characterised by increasing indigenous tree densities in savanna and grassland. Encroachment has been attributed to rising atmospheric CO_2 concentrations fertilising tree growth and shifting the competitive balance between trees and grasses. However, only a subset of savanna tree species are currently described as encroachers, raising the hypothesis that CO_2 responsiveness differs among species. Within southern African savannas, encroachment is driven primarily by nitrogen (N_2)-fixing species, implying the CO_2 response may be mediated via traits that enhance plant-available N.
2. Using an open-topped chamber system, we experimentally manipulated atmospheric CO_2 concentrations and soil moisture for 12 savanna tree species (six encroachers and six non-encroachers) under ambient (a)CO_2 (~397.9 ppm) or elevated (e)CO_2 (~545.1 ppm) treatments and water limited or well-watered soil moisture treatments. We measured N-dynamics traits including nodule mass fraction (NMF), leaf $\delta^{15}N$, stem $\delta^{15}N$, and percentage of N derived from fixation (%Ndfa).
3. We found that encroachers and non-encroachers differ in short-term N-dynamics but share similar long-term N allocation strategies. Encroachers exhibited lower leaf $\delta^{15}N$, indicating greater utilisation of N_2 fixation products to meet immediate short-term protein synthesis. Long-term N allocation strategies (NMF, stem $\delta^{15}N$, and %Ndfa) were similar between encroachers and non-encroachers, with plants fixing more N_2 under the eCO_2 and well-watered treatment. In encroachers, leaf and stem $\delta^{15}N$ were unrelated, in contrast to the positive relationship in

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non-encroachers, pointing to distinct tissue-level N allocation, possibly reflecting differential N utilisation and retention.

4. We demonstrate the significance of N_2 fixation in mediating the CO_2 responsiveness of encroaching savanna trees. N_2 fixation increases plant-available N, likely enabling encroachers to meet immediate N demands even under water limitation and increasing CO_2. The potential feedback loop, where eCO_2 enhances photosynthesis, facilitating greater C allocation for N_2 fixation, helps to explain the ecological success of the subset of species driving encroachment under increased atmospheric CO_2.

KEYWORDS
CO_2, encroachment, N_2 fixation, nodulation, savanna, *Senegalia*. Legumes, *Vachellia*

1 | INTRODUCTION

Global change over the last century has driven rapid increases in the abundance of indigenous woody plants in savannas, a phenomenon termed encroachment (Archer et al., 2017; Eldridge et al., 2011; Stevens et al., 2016). Savanna ecosystems cover over half of the African continental landmass (Scholes & Archer, 1997). African savannas have a unique disturbance-adapted flora and fauna that significantly contributes to global biodiversity and ecosystem functioning (Murphy et al., 2016; Osborne et al., 2018; Van Vooren et al., 2018). Savanna vegetation structure is comprised of a discontinuous cover of C_3 woody plants and a continuous ground layer of shade-intolerant C_4 grasses (Ratnam et al., 2011). However, encroachment poses a major threat to savanna biodiversity, functioning and services (Archer et al., 2017; Luvuno et al., 2022; Wieczorkowski & Lehmann, 2022).

The environmental drivers of savanna encroachment are multi-scalar and interlinked. Recruitment of savanna woody plants has historically been constrained by bottom-up controls in resource and water availability, along with top-down controls, such as herbivory and fire (Higgins et al., 2000; Sankaran et al., 2005). Rising atmospheric CO_2 and changing rainfall patterns (Nackley et al., 2018; Stevens et al., 2017; Venter et al., 2018), alongside changes in fire regimes, land management practices and megafaunal loss (Stevens et al., 2022), are hypothesised to be significant factors creating a release from this demographic bottleneck. Globally, rising atmospheric CO_2 is a key driver of vegetation change, since increasing levels can enhance C_3 plant water use efficiency (WUE) to alleviate bottom-up constraints on water availability (Nackley et al., 2018; Polley, 1997; Polley et al., 2003). Plants absorb CO_2 through their stomata, a process that inherently involves water vapour loss via transpiration. However, in *Vachellia farnesiana* and other mimosoid species such as *Prosopis glandulosa* elevated atmospheric (e)CO_2 levels can induce partial stomatal closure, reducing water loss, while maintaining photosynthetic efficiency, resulting in rapid plant growth rates (Polley, 1997; Polley et al., 2003). When bottom-up constraints on growth are reduced, plants have an increased potential to escape top-down disturbance-based traps, such as fire and herbivory (Bond & Midgley, 2000).

In savanna woody plants, CO_2 fertilisation of growth allows individuals to rapidly escape size-related fire and herbivory traps and reach a state where disturbances have a minimal impact on survival (LaMalfa et al., 2019; Skowno et al., 1999; Staver & Bond, 2014). However, CO_2 fertilisation is limited by the availability of other nutrients, such as nitrogen (N), phosphorus (P) and potassium (K) (Cech et al., 2010; Rance et al., 2023; Ripley et al., 2022). The role of plant nutrient economies in mediating CO_2 responses in savanna woody plants remains unknown. In savannas, resource limitation is exacerbated by competition between woody plants and the grass layer. C_4 grasses dominate the savanna ground layer, and their ability to maintain high internal CO_2 concentrations during carbon (C) fixation, limits their sensitivity to eCO_2 (Hager et al., 2016). Overall, eCO_2 likely disrupts the balance between woody plants and C_4 grasses in savannas by altering competitive interactions (Bond & Midgley, 2000, 2003; Eamus & Palmer, 2007). However, some limitations in trees can be overcome through resources gained from symbiotic partners. In forest ecosystems, CO_2 fertilisation of tree growth is limited by soil N and P and modulated by mycorrhizal association (Terrer et al., 2019). It is unknown whether similar nutrient limitations on CO_2-responsiveness exist in savanna woody plants.

In African savannas, woody vegetation across southern regions is dominated by *Vachellia* and *Senegalia* species (Bouchenak-Khelladi et al., 2010; Osborne et al., 2018). All *Vachellia* species and 95% of *Senegalia* species, as well as the broader Mimosoideae subfamily, fix gaseous N_2 to supplement acquisition of N from sources in the (Sprent, 2009; Telford et al., n.d.). Plant N_2 fixation is underpinned by mutualistic symbiosis with rhizobial bacteria which are hosted within root nodules that form in response to colonisation by the bacteria (Schultze & Kondorosi, 1998). Within nodules, rhizobia catalyse the fixation of atmospheric N_2 into plant-usable NH_3, in exchange for fixed C (Kambatuku et al., 2013). Rhizobial symbioses consume up to 16% of the host's photosynthetically fixed C to maintain growth, activity and reserves (Kaschuk et al., 2010). Supplementation of plant N intake via N_2 fixation can confer a competitive growth advantage in certain environments, given the crucial role of N in plant growth and performance (Fathi, 2022; Ye et al., 2022). In savanna ecosystems, where disturbances like fire and herbivory are frequent and N availability is often low, N_2 fixation provides a key advantage, enabling plants to regrow following these events (Batterman et al., 2013; Vitousek et al., 2013). The individual plant N_2 fixation rate varies considerably among *Vachellia* and *Senegalia* species (Cramer et al., 2010; Pellegrini et al., 2016). Species-level differences are further reflected in plant encroacher status, with only a few species driving encroachment (Telford et al., n.d.). The relationship between the ability to encroach and N_2 fixation ability has not been addressed, but could be significant given that many of the species driving encroachment nodulate (Telford et al., n.d.).

The understanding of how atmospheric CO_2 concentrations and water availability impacts plant growth and N-dynamics has been developed separately in various contexts. Elevated CO_2 conditions favour N_2-fixing C_3 dicots, providing a competitive advantage over non-fixing C_3 and C_4 plants via increased growth rates (Poorter & Navas, 2003). This advantage arises because N_2-fixing C_3 plants maintain higher internal N levels and effectively use increased C availability under eCO_2 to enhance photosynthesis and reduce respiration (Chen & Markham, 2021; Dusenge et al., 2019; Li et al., 2022). For instance, *V. farnesiana* fixed four times the amount of N_2 when grown under eCO_2 (980 ppm) compared to ambient $(a)CO_2$ (385 ppm) (Polley, Johnson, & Mayeux, 1997a). Possibly, the stimulation of N_2 fixation in woody plants could amplify CO_2-driven vegetation change and dominance of certain species (Polley et al., 2003; Polley, Mayeux, et al., 1997b). However, plant responsiveness to eCO_2 is modulated by limiting water availability, which constrain eCO_2 effects on plant growth and N_2 fixation (Reich et al., 2014; Wullschleger et al., 2002). When water is not limited, N_2-fixing plants create positive feedback between photosynthesis, N_2 fixation and growth (Schortemeyer et al., 1999, 2002). Conversely, low soil moisture hinders rhizobia mobility, limiting symbiosis and N_2 fixation (Deans et al., 1993). The effect of low soil moisture is not uniform; *V. sieberiana* has been observed to increase nodule biomass under water limitation (Telford et al., 2023). The interplay between N_2 fixation, eCO_2 and water limitation, and their role in driving woody encroachment remains insufficiently understood.

With the aim of understanding how N_2 fixation interacts with CO_2 and water availability in encroaching and non-encroaching woody savanna plants, we compared N-dynamic traits of 12 species of *Vachellia* and *Senegalia* (six encroachers and six non-encroachers). We measured nodule mass fraction (NMF) as an indicator of plant resource allocation strategy and potential N_2 fixation capacity, along with the natural abundance of stable N isotopes ($\delta^{15}N$; the ratio of ^{15}N to ^{14}N relative to atmospheric N_2) in stem and leaf tissues and the % nitrogen derived from fixation (%Ndfa) as proxies for plant N-dynamics (Table 1). We tested the hypothesis that N_2 fixation in encroachers increases in response to eCO_2 and increased water availability. We expected that under eCO_2 encroachers would allocate more C to rhizobial bacteria, thereby increasing the catalysation of N_2 into a plant usable form, ultimately enhancing N_2 fixation. The positive feedback between host plant and rhizobia increases plant N availability, facilitating growth and photosynthetic capacity (Figure 1; Schortemeyer et al., 1999, 2002).

2 | METHODS

2.1 | Study species and their ecological traits

Vachellia (187 species) and *Senegalia* (227 species) are genera in the Fabaceae family, formally known as *Acacia*. *Vachellia* and *Senegalia* are found predominantly in savanna ecosystems throughout the tropics (Ringelberg et al., 2023; Telford et al., n.d.), across wide rainfall and temperature gradients (Bouchenak-Khelladi et al., 2010; Ringelberg et al., 2023).

We examined nine *Vachellia* and three *Senegalia* species indigenous to African savannas (Table 2). These 12 species are facultative N_2-fixers (Pillay et al., 2023); utilising N_2 fixation and soil-derived N sources depending on environmental conditions and N availability (Menge et al., 2009). Six species currently show field evidence of encroachment across large geographical areas in the African savannas, while the other six show no evidence of encroaching behaviour to date (Table 2).

We initially planned to sample pairs of closely related encroaching and non-encroaching species across the phylogenetic tree for a balanced study species selection. However, several species failed to germinate, requiring substitutions. This resulted in an uneven sampling structure, with encroaching species clustering in the same phylogenetic area (Figure S3).

2.2 | Seed germination and seedling establishment

Seeds for the 12 species were procured from SilverHill Seeds (http://www.silverhillseeds.co.za; Cape Town, South Africa). In November 2021, seed dormancy was broken to initiate germination by heat-shocking the seeds in 80 °C water for 10 min (Bodede et al., 2018; Cramer et al., 2007). Germinated seedlings were transplanted into individual cylindrical pots (diameter 20 cm × length 75 cm) to limit the risk of plants becoming pot-bound (Arp, 1991; Raubenheimer & Ripley, 2022). Pots had holes at the base to enable water drainage and contained soil from a nearby savanna where *Vachellia* species naturally occur (Fort Beaufort, Eastern Cape, South Africa). The soil was sieved to remove large rock fragments. The soil texture was classified as loam sand (10% clay, 5% silt, 85% sand). Soil P and ammonium (NH_4-N) levels were very low

TABLE 1 An overview of the four N-dynamic traits measured in this experiment. The four traits include nodule mass fraction (NMF), leaf $\delta^{15}N$, stem $\delta^{15}N$ and % of N derived from fixation (%Ndfa).

N-dynamic trait	Theoretical basis	Method	Strengths	Limitations	References
Nodule mass fraction (NMF)	• Nodule biomass correlates with plant N_2 fixation rate at the time of collection • Structural trait that indicates plant nodule allocation	• Destructive harvesting • Biomass collection	• Useful for result interpretation when combined with other measurements	• Requires destructive sampling • Does not capture seasonal variability	Alon et al. (2021), Batterman et al. (2013), Hardarson and Danso (1993) and Telford et al. (2023)
Leaf $\delta^{15}N$	• Reflects the ratio of ^{15}N:^{14}N in leaf tissue • During N_2 fixation, biological processes discriminate against heavier ^{15}N (fractionation) • N_2-fixing plants have lower leaf $\delta^{15}N$ due to preferential uptake of ^{14}N • Indicates short-term N use for the synthesis of photosynthetic protein	• Mass spectrometry	• Proxy for N_2 fixation • Widely used	• Requires destructive harvesting • Does not capture seasonal variability	Ariz et al. (2015), Chalk et al. (2016), Delwiche and Steyn (1970), Hardarson and Danso (1993), Peoples et al. (2002); Shearer and Kohl (1986) and Unkovich et al. (2008)
Stem $\delta^{15}N$	• Reflects the ratio of ^{15}N:^{14}N in stem tissue • During N_2 fixation, biological processes discriminate against heavier ^{15}N (fractionation) • N_2-fixing plants have lower stem $\delta^{15}N$ due to preferential uptake of ^{14}N • Indicates long-term N storage and transport of fixed N	• Mass spectrometry	• Proxy for N_2 fixation • Widely used	• Requires destructive harvesting • Does not capture seasonal variability	Ariz et al. (2015), Chalk et al. (2016), Delwiche and Steyn (1970), Hardarson and Danso (1993), Peoples et al. (2002), Shearer and Kohl (1986) and Unkovich et al. (2008)
% of N derived from fixation (%Ndfa)	• %Ndfa estimated using shoot $\delta^{15}N$ values • Higher %Ndfa indicates greater reliance on atmospheric N_2	• Shoot $\delta^{15}N$ is averaged from leaf and stem values • A non-fixing reference plant used for comparison	• Allows N_2 fixation assessment in field and glasshouse experiments	• Subject to soil $\delta^{15}N$ variability • Seasonal and annual variation in N assimilation • %Ndfa values may fall outside 0–100 • Scientists debate which plant components (leaves, stems, roots, nodules) to include in calculation	Boddey et al. (2000), Cramer et al. (2007, 2010), Cramer and Bond (2013), Evans, 2001, Kambatuku et al. (2013), Peoples et al. (1995, 2002), Schulze et al. (1991), Shearer et al. (1983) and Unkovich et al. (1994)

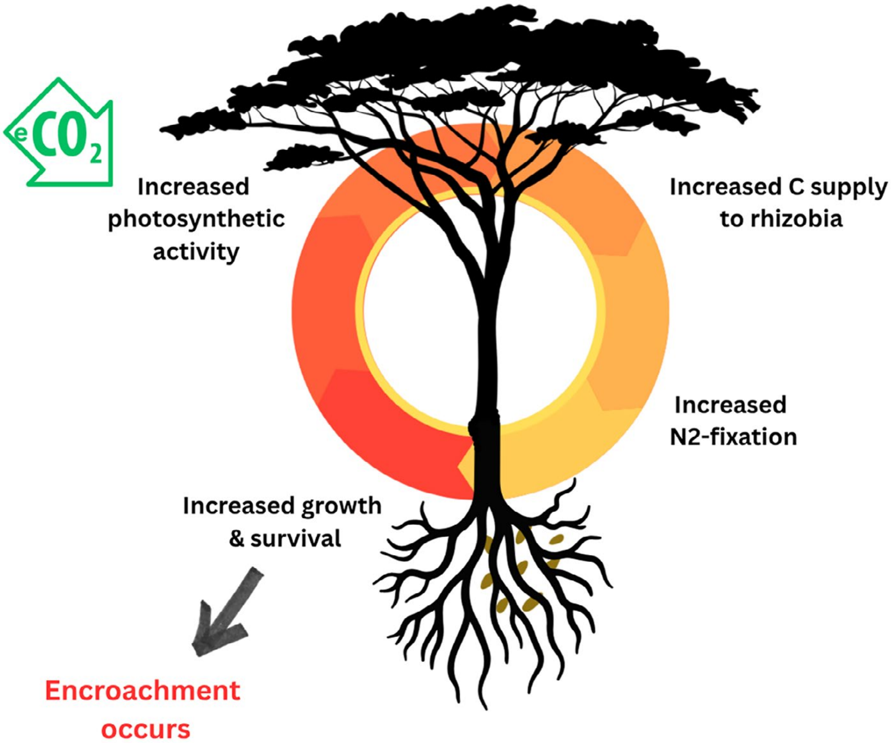

FIGURE 1 A schematic diagram illustrating our hypothesis. A potential feedback loop between host plant and rhizobia bacteria under eCO_2, whereby increased CO_2 availability stimulates N_2 fixation, enhances plant growth and survival and ultimately drives encroachment in the savanna.

at 0.2 and 0.45 mg/L, while nitrogen (NO_3-N) levels were moderate at 14.12 mg/L (Table S1).

2.3 | Experimental design and environmental treatments

Study plants were subjected to experimental treatments at the Rhodes University Elevated CO_2 facility (RUECF; Makhanda, South Africa). The RUECF location has a mean annual temperature of 17.9 °C and a mean annual precipitation of 600 mm (www.worldclim.com). The RUECF consists of 16 × 3 m diameter open-top chambers (OTCs) (Figure S1; for a complete description of OTC construction, see Baso et al., 2021; for instrument details, see Raubenheimer & Ripley, 2022).

Eight OTCs were randomly selected for the eCO_2 treatment, while the remaining eight OTCs were the control (aCO_2) treatment. In the eCO_2 treatment, the median daytime CO_2 concentration was 545.1 ppm with 90% of values falling between 496–588 ppm. For the aCO_2 treatment, the median daytime CO_2 concentration was 397.9 ppm with 90% of values falling 383–411 ppm. The eCO_2 treatment concentration reflects the Representative Concentration Pathway (RCP) 8.5 predictions for the turn of the 21st century (IPCC, 2021, 2022). CO_2 treatments began in December 2021.

To assess the effect of water limitation, each OTC was divided in half, and a soil moisture treatment was applied to one subset of pots. Two soil moisture levels were contrasted to represent water-watered versus well-limited conditions. Well-watered (WW) pots were irrigated daily for 10 min using an automated drip system, which delivered on average 176.1 mL per 5 min (≈352.2 mL per 10 min), maintaining soil moisture near field capacity (mean volumetric soil moisture content, SMC = 0.30). Water-limited (WL) pots received ambient rainfall through the open chamber roof, except for two manual watering events during an extended dry spell to prevent mortality (SMC = 0.24; Figure S2). During the experiment, WL pots received a cumulative 68.4 L of water per pot, equivalent to 2444 mm of rainfall, approximately 0.30 mm/day based on pot surface area.

The average SMC difference between WW and WL treatments was 0.07, with the difference being more pronounced during the dry season (e.g. 0.116 difference in the lowest-rainfall quarter of the year; May, June and July) relative to the wet season (e.g. 0.054 difference in the wettest quarter; December, January and February; Figure S2). These water treatments were designed to simulate natural variation in dry season water availability typical of Makhanda, where soil moisture can fall below 25% during drought. CO_2 treatment did not influence soil moisture differences (mean SMC difference; aCO_2 = 0.07; eCO_2 = 0.07).

The study involved 222 pots with approximately five replicates per species for each of the four treatments (aCO_2; eCO_2 and WW; WL; see Tables S2 and S3 for replicate numbers). Due to germination issues replication was uneven in some species (e.g. *V. swazica*, n = 5).

TABLE 2 Distribution data of the 12 study species in this experiment. Encroacher (Encr.) and non-encroacher (Non-encr.) status, range size (km^2), mean annual temperature (MAT) (°C) and mean annual precipitation (MAP) (mm) range of each species were taken from Telford et al. (n.d.).

Species	Encr. status	Encr. status reference	Distribution	Range size (km^2)	MAT (°C)	MAP (mm)
Vachellia exuvialis (I. Verd.) Kyal. & Boatwr.	Encr.	Bredenkamp (1986) and Turpie et al. (2019)	East and southern Africa	38,000	20–25	500–1000
Vachellia grandicornuta (Gerstner) Seigler & Ebinger	Encr.	Combrink (2017) and Turpie et al. (2019)	East southern Africa	120,000	15–25	500–1000
Vachellia karroo (Hayne) Banfi & Galasso.	Encr.	Muvengwi et al. (2017), Skhosana (2023), Tiawoun et al. (2022) and Turpie et al. (2019)	Africa	1,200,000	10–20	>1000
Vachellia nilotica (L.) P.J.H. Hurter & Mabb.	Encr.	Liao et al. (2018), Mupangwa et al. (2023), Muvengwi et al. (2017), Shikangalah and Mapani (2020), Skhosana (2023), Turpie et al. (2019) and Yusuf et al. (2011)	Sub-Sahel & Southern Africa	1,600,000	20–30	>1000
Vachellia sieberiana (DC.) Kyal. & Boatwr.	Encr.	Skhosana (2023) and Turpie et al. (2019)	Africa	840,000	10	800–1100
Vachellia tortilis (Forssk.) Galasso & Banfi.	Encr.	Haile et al. (2021); Skhosana (2023), Tefera et al. (2008), Tiawoun et al. (2022), Turpie et al. (2019) and Yusuf et al. (2011)	Sub-Saharan Africa	1,300,000	20–30	>1000
Senegalia burkei (Benth.) Kyal. & Boatwr.	Non-encr.	NA	Southern Africa	150,000	15–20	>1000
Senegalia caffra (Thunb.) P.J.H. Hurter & Mabb.	Non-encr.	NA	Southern Africa	220,000	15–30	>1000
Senegalia nigrescens (Oliv.) P.J.H. Hurter.	Non-encr.	NA	Southern Africa	570,000	18–23	500–1000
Vachellia erioloba (E. Mey.) P.J.H. Hurter.	Non-encr.	NA	Southern Africa	340,000	18–23	>500
Vachellia robusta (Burch.) Kyal. & Boatwr.	Non-encr.	NA	East southern Africa	290,000	10–20	500
Vachellia swazica (Burtt Davy) Kyal. & Boatwr.	Non-encr.	NA	East southern Africa	33,000	16–23	250–1000

2.4 | Plant harvest for N-dynamic traits

The plants in the 222 pots were destructively harvested in a random order at 11 months old from October to November 2022.

2.4.1 | Plant traits

Aboveground tissues were separated by cutting the stem at the soil surface, oven-dried at 60°C for 72h and weighed using a three-point balance.

Belowground tissues were extracted from pots and washed in fine mesh bags to minimise fine root loss. The belowground tissues were oven-dried in two steps: 40 °C for 48h, followed by 70 °C for 24h, and weighed using a three-point balance.

Nodules were separated from washed roots (Telford et al., 2023). Dead and decaying nodules were discarded to ensure that only fixing nodules were collected. Nodules were dried in silica gel-filled Eppendorf tubes, refrigerated and weighed using a four-point balance (Howieson & Dilworth, 2016).

2.4.2 | Plant N-dynamic traits

Dry belowground biomass and nodule biomass data were used to calculate nodule mass fraction using the following equation (Table 1):

$$\text{NMF}\ (\%\,\text{biomass}) = \left(\frac{\text{nodule biomass (g)}}{\text{belowground biomass (g)}}\right) \times 100$$

Dried leaf and stem tissues were separately ground to a powder using a tissue lyser (Qiagen, Hilden, Germany), and a subsample of 3mg of milled material was weighed into tin capsules (Electrical Microanalysis Ltd., Okehampton, UK) for δ^{15}N analysis at the University of Sheffield, UK (Table 1). Mass spectrometry was performed using an Elementar vario PYRO cube Elemental Analyser (Elementar-Straße 1, 63,505 Langenselbold, Germany) and an Elementar PRECISION stable isotope ratio mass spectrometer (Elementar-Straße 1, 63,505 Langenselbold, Germany). Internal standards with known isotope ratios were used to calibrate the mass spectrometer. The $\delta^{15}N$ values for each sample were calculated using the formula:

$$\delta^{15}\mathrm{N}\,(‰) = \left(\frac{R\text{ sample}}{R\text{ standard}} - 1\right) \times 1000$$

Where R_{sample} is the ratio of ^{15}N:^{14}N in the sample, and R_{standard} is the ^{15}N:^{14}N ratio in the standard (atmospheric $N=0$). Shoot $\delta^{15}N$ was calculated as a weighted average of leaf $\delta^{15}N$ and stem $\delta^{15}N$.

$$\text{shoot }\delta^{15}\mathrm{N}\,(‰) = \left(\frac{(\text{leaf }\delta^{15}\mathrm{N}\,(‰) \times \text{leaf biomass (g)} + \text{stem }\delta^{15}\mathrm{N}\,(‰) \times \text{stem biomass (g)})}{\text{leaf biomass (g)} + \text{stem biomass (g)}}\right)$$

Estimates of per cent N derived from fixation (%Ndfa) were calculated using shoot $\delta^{15}N$ values using the following equation (Cramer et al., 2010; Cramer & Bond, 2013; Kambatuku et al., 2013):

$$\%\,\mathrm{Ndfa} = \left(\frac{\text{reference shoot }\delta^{15}\mathrm{N}\,(‰) - \text{shoot }\delta^{15}\mathrm{N}\,(‰)}{\text{reference shoot }\delta^{15}\mathrm{N}\,(‰)}\right) \times 100$$

Reference shoot $\delta^{15}N$ values were derived from a small number ($n=6$) of non-nodulating *V. erioloba* individuals, grown under identical conditions to the study plants. These were suitable reference values as their $\delta^{15}N$ values (8.75–11.07) were comparable to pot soil $\delta^{15}N$ (9.66). Bulk soil $\delta^{15}N$ was determined by collecting 2.00 g of soil from the top 30 cm of 30 randomly selected pots. The soil samples were homogenised and freeze-dried. About 30.00 mg of ball-milled soil was analysed using mass spectrometry techniques (Electrical Microanalysis Ltd., Okehampton, UK).

2.5 | Statistical analyses of N-dynamic traits

Data is available from the Dryad Digital repository: https://doi.org/10.1111/1365-2656.12537 (Telford et al., 2025). The 12 study species were not entirely independent, as nine belonged to *Vachellia* and three to *Senegalia*, necessitating a phylogenetic component for distinguishing encroachers and non-encroachers. A phylogenetic comparative analysis helps differentiation between the two pathways, explaining how species can exhibit similar ecological traits. (1) Independent evolution via convergent adaptation to similar environments. (2) Phylogenetic niche conservatism (PNC), where traits have been inherited from a common ancestor (Crisp & Cook, 2012; Westoby et al., 2023).

We constructed a phylogeny for *Vachellia* and *Senegalia* species using plastid regions trnL-F intergenic spacer, matK and trnH-psbA via sequences downloaded from GenBank (Table S4). The three plastid markers were aligned with Muscle 5.1 (Edgar, 2004) and concatenated. A time-calibrated phylogenetic tree was constructed using Bayesian inference with BEAST v.2.7.5 (Bayesian evolutionary analysis by sampling trees; Drummond & Rambaut, 2007). A secondary calibration fixed the divergence date at 40.6 million years ago (Mya) with a standard deviation of 0.0001 between the outgroup (*Desmanthus bicornutus*) and one ingroup sample (*V. karroo*) (Bouchenak-Khelladi et al., 2010). Three different analyses were run with 100,000,000 generations, sampling a tree every 1000 generations, using a Yule speciation process, a random local clock and the GTR+G model. Convergence was verified using Tracer v.1.7.2 (Hadfield, 2010). All trees were concatenated, and median ages were mapped onto the nodes of the maximum credibility tree.

The phylogenetic tree showed that *Vachellia* and *Senegalia* were monophyletic groups (Figure S3). Within the *Vachellia* group, encroacher *V. karroo* is a sister to non-encroacher *V. swazica*, and encroacher *V. exuvialis* is sister to these two species (Figure S3). Further, encroacher *V. grandicornuta* is a sister to non-encroacher *V. robusta* in a wider clade also related to encroachers *V. sieberiana* and *V. tortillis* (Figure S3). Non-encroacher *V. erioloba* and encroacher *V. nilotica* are successively sister species to all remaining *Vachellia* species (Figure S3). Within the *Senegalia* group, non-encroachers *S. nigrescens* are a sister to *S. burkei*, with *S. caffra* their sister species (Figure S3).

To account for non-independence and correlated random effects from phylogenetic relationships, we used MCMCglmm for multivariate analyses in R (R Core Team, 2024), implementing Markov Chain Monte Carlo (MCMC) routines for fitting generalised linear mixed models (MCMCglmm function; 'MCMCglmm' package; Hadfield, 2010). This approach accounts for non-independence and correlated random effects arising from phylogenetic relationships (Hadfield, 2010). N-dynamic traits (NMF, leaf $\delta^{15}N$, stem $\delta^{15}N$ and %Ndfa) were analysed relative to encroacher status, CO_2 treatment, soil moisture treatment and their interaction. Data were log-transformed where necessary to fulfil assumptions of the statistical models. Models ran for 100,000 iterations with a thinning interval of 10 and weakly informative priors (V = diag(1), nu = 0.002):

$$\begin{aligned}&\text{N dynamic trait} \sim \text{encroacher status} \times \mathrm{CO_2}\text{ treatment}\\&\times \text{soil moisture treatment} + (1|\mathrm{OTC}) + (1|\text{phylogeny})\end{aligned}$$

where 'N-dynamic trait' was the dependent variable. 'Encroacher status' was a categorical variable (encroacher; non-encroacher). 'CO_2 treatment' was a categorical variable (aCO_2 (~397.9 ppm); eCO_2 (~545.1 ppm)). 'Soil moisture treatment' was a categorical variable (WL; WW). 'OTC' was a random effect accounting for OTC number (1–16). 'Phylogeny' was a random effect accounting for the evolutionary relationships among species.

Phylogenetic signal was measured using Pagel's lambda (Pagel, 1999), in MCMCglmm (Hadfield, 2010). Accounting for phylogenetic signal captures the variation in species trait values arising from shared evolutionary history (i.e. PNC), ensuring accurate interpretations of ecological and evolutionary trait patterns

(Münkemüller et al., 2012). Lambda quantifies the strength of the phylogenetic signal (range: 0–1); $\lambda = 0$ indicates trait evolution is independent of phylogeny (no phylogenetic signal); $\lambda = 1$ indicates trait evolution follows the phylogeny (strong phylogenetic signal) (Pearse et al., 2023).

We tested the significance of phylogenetic signal further using the model:

$$\text{N dynamic trait} \sim \text{encroacher status} \times \text{CO}_2 \text{ treatment}$$
$$\times \text{ soil moisture treatment} + (1|\text{OTC})$$

The significance of each model fit was evaluated using parameter estimates and confidence intervals, with effects deemed significant if intervals did not intersect zero.

We used standardised major axis regression (SMA) (smatr function; 'smatr' package; Warton et al., 2012) to assess bivariate N-dynamic trait relationships across experimental treatments.

3 | RESULTS

CO_2 and soil moisture treatments had distinct effects on the N-dynamic traits of encroacher and non-encroacher species. Across all treatments, encroachers exhibited higher NMF and %Ndfa than non-encroachers (Figures 2a and 2d). Leaf and stem $\delta^{15}N$ were consistently lower in encroachers (Figures 2b and 2c), particularly for *V. exuvialis*, *V. grandicornuta*, *V. karroo*, *V. tortilis* and *V. sieberiana* grown under eCO_2 and WW treatments (Figures S6 and S7).

3.1 | Nodule mass fraction (NMF)

NMF did not differ significantly between encroachers and non-encroachers (Figure 2; with phylogeny and without phylogeny). However, NMF increased significantly under eCO_2 (Figure 3; with phylogeny; posterior mean (pm) = 0.43 [95% confidence interval (CI) = 0.09, 0.78]; $p < 0.01$; without phylogeny; pm = 0.46 [CI = 0.10, 0.83]; $p < 0.01$) and under the WW soil moisture treatment (Figure 3; with phylogeny; pm = 0.57 [CI = 0.22, 0.89]; $p < 0.01$; without phylogeny; pm = 0.57 [CI = 0.23, 0.93]; $p < 0.01$). The interaction of these factors had a negative effect, indicating that the eCO_2 effect is different under the WL and WW soil moisture treatments (Figure 3; with phylogeny; pm = −0.55 [CI = −1.02, −0.06]; $p < 0.05$). Variation in NMF was not attributable to species' evolutionary relatedness (Pagel's $\lambda = 0.24$; likelihood ratio test against $\lambda = 1$: $p = 0.54$; $\lambda = 0$: $p = 0.01$).

3.2 | Leaf δ^{15}N values

Leaf $\delta^{15}N$ values did not differ significantly between encroachers and non-encroachers (Figure 3; with phylogeny). In models excluding

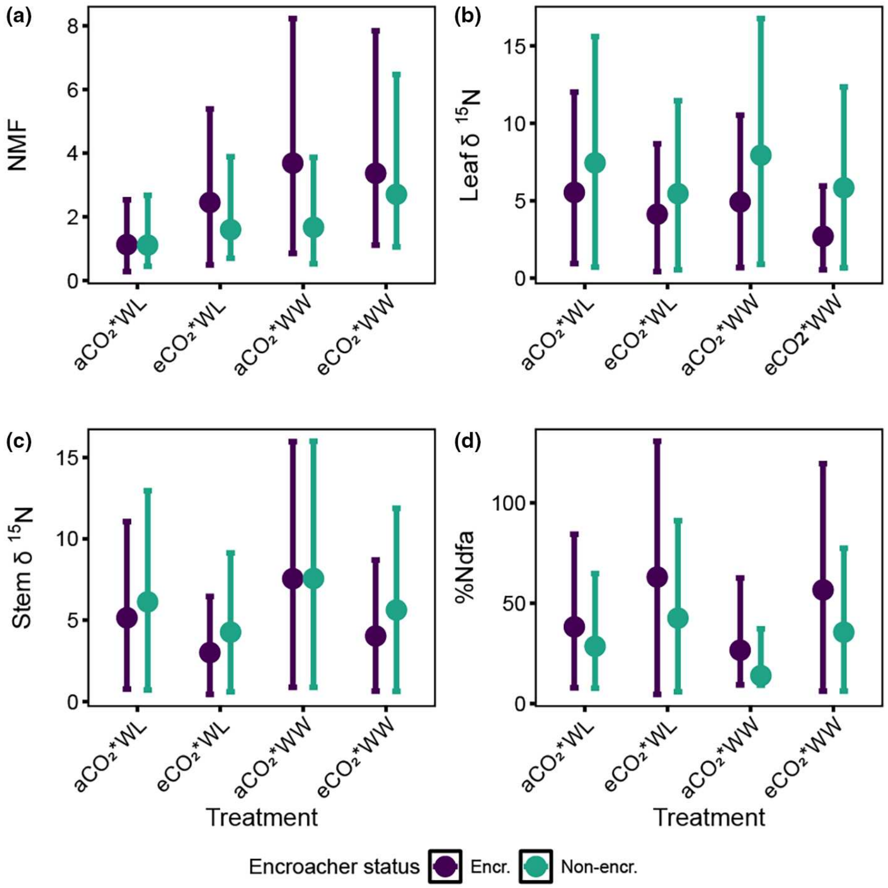

FIGURE 2 Plant N-dynamics: CO_2 and soil moisture effects on encroacher (Encr.) and non-encroacher (Non-encr.) species. A summary of the mean (a) nodule mass fraction (NMF) (% biomass), (b) leaf $\delta^{15}N$ (‰), (c) stem $\delta^{15}N$ (‰) and (d) the percentage of N_2 derived from the atmosphere (%Ndfa) across CO_2 (aCO_2 (~397.9 ppm); eCO_2 (~545.1 ppm)) and soil moisture (water-limited [WL]; well-watered [WW]) treatments. The data presented include the mean and upper and lower standard error calculated directly from the data ($n = 222$; Tables S2 and S3).

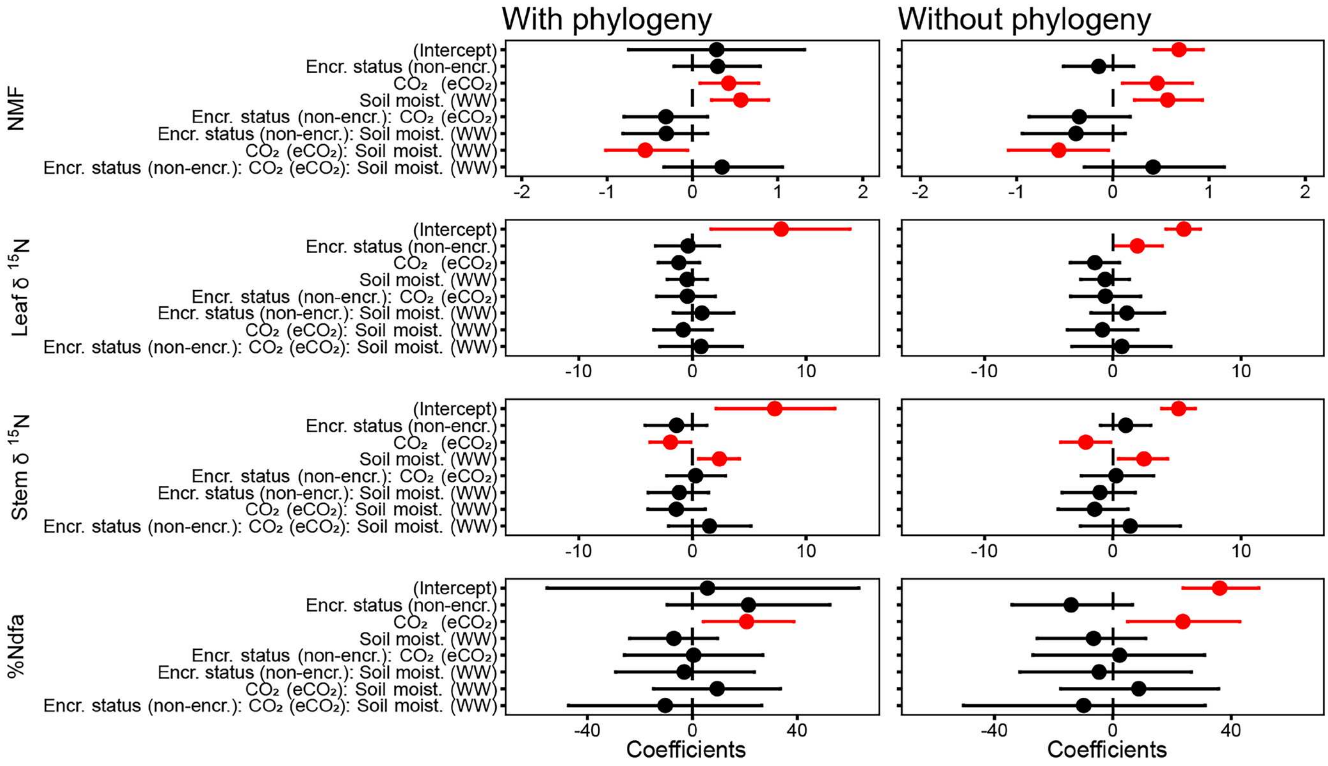

FIGURE 3 Coefficients estimated from MCMC models examining the response of four N-dynamic traits (nodule mass fraction [NMF], leaf δ^{15}N, stem δ^{15}N and % nitrogen derived from fixation [%Ndfa]) of encroachers (encr.) and non-encroachers (non-encr.) across CO_2 (aCO_2 [~397.9 ppm]; eCO_2 [~545.1 ppm]) and soil moisture (water limited [WL]; well-watered [WW]) treatments. The model output where phylogeny (with phylogeny) is a random effect and (without phylogeny) not included. Each point represents the estimated coefficient value. The whiskers represent the 95% confidence intervals (CI) around these coefficients. Significant factors (CI does not intercept 0) are highlighted in red, and non-significant factors (CI intercepts 0) are black ($n = 222$; Tables S2 and S3).

phylogeny, leaf δ^{15}N was significantly lower in encroachers (Figure 3; without phylogeny; pm = 1.91 [CI = 0.03, 3.86]; $p < 0.05$). However, leaf δ^{15}N was not affected by environmental treatment, and there was no interaction effect (Figure 3; with phylogeny and without phylogeny). Variation in leaf δ^{15}N reflected a shared evolutionary history among species (Pagel's $\lambda = 0.63$; likelihood ratio test against $\lambda = 1$: $p = 0.93$; $\lambda = 0$: $p = 0.04$).

3.3 | Stem δ^{15}N values

Stem δ^{15}N did not significantly differ between encroachers and non-encroachers (Figure 3; with phylogeny and without phylogeny). Stem δ^{15}N significantly decreased in plants grown under eCO_2 (Figure 3; with phylogeny; pm = −1.93 [CI = −3.73, −1.15]; $p < 0.05$; without phylogeny; pm = −2.01 [CI = −4.07, −0.18]; $p < 0.05$) and increased in WW treatment (Figure 3; with phylogeny; pm = 2.37 [CI = 0.55, 4.12]; $p < 0.01$; without phylogeny; pm = 2.43 [CI = 0.45, 4.26]; $p < 0.01$). We observed no interaction between encroacher status and environmental treatments on stem δ^{15}N (Figure 3; with phylogeny and without phylogeny). Variation in stem δ^{15}N is explained by shared evolutionary history among species (Pagel's $\lambda = 0.53$; likelihood ratio test against $\lambda = 1$: $p = 0.84$; $\lambda = 0$: $p = 0.22$).

3.4 | % N derived from fixation (%Ndfa)

Percentage of Ndfa did not significantly differ between encroachers and non-encroachers (Figure 2; with phylogeny and without phylogeny). However, %Ndfa increased under eCO_2 (Figure 3; with phylogeny; pm = 20.65 [CI = 4.12, 38.71]; $p < 0.05$; without phylogeny; pm = 23.64 [CI = 4.98, 42.89]; $p < 0.05$). Soil moisture did not affect %Ndfa (Figure 3; with phylogeny and without phylogeny). Further, we found no interaction effect between encroacher status and environmental treatments on %Ndfa (Figure 3; with phylogeny and without phylogeny). The %Ndfa was correlated with the evolutionary relatedness of species (Pagel's $\lambda = 0.59$; likelihood ratio test against $\lambda = 1$: $p = 0.85$; $\lambda = 0$: $p = 0.32$).

3.5 | Bivariate N-dynamic trait relationships

We found no significant relationships between leaf and stem δ^{15}N for encroachers under any environmental treatments (Figure 4). In contrast, non-encroachers exhibited a positive relationship between leaf and stem δ^{15}N across all environmental treatments (Figure 4; $p < 0.05$). Intercepts and slopes differed significantly between encroachers and non-encroachers in $aCO_2 \times WW$ and $eCO_2 \times WL$ treatments (Figure 4; $p < 0.05$).

Patterns between leaf δ^{15}N and NMF varied with encroacher status and environmental treatments. For encroachers, a negative correlation between leaf δ^{15}N and NMF was observed under the $aCO_2 \times WW$ treatment (Figure 5a; $p < 0.05$). In non-encroachers, negative relationships were reflected in the $aCO_2 \times WL$ ($p < 0.05$), $aCO_2 \times WW$ ($p < 0.05$) and $eCO_2 \times WW$ ($p < 0.01$) treatments (Figure 5a). There were no significant differences in intercepts and slopes between encroachers and non-encroachers.

Associations between stem δ^{15}N and NMF differed across encroacher status and environmental treatments. A negative relationship between stem δ^{15}N and NMF was observed in encroachers grown under $aCO_2 \times WW$ ($p < 0.01$) and in non-encroachers grown under $eCO_2 \times WW$ (Figure 5b; $p < 0.05$). The intercepts and slopes of encroachers and non-encroachers significantly differed in the $aCO_2 \times WL$ treatment (Figure 5b; $p < 0.01$).

Covariation between %Ndfa and NMF differed among encroacher status and environmental treatments. The %Ndfa and NMF were positively associated in encroachers grown under $aCO_2 \times WW$ ($p < 0.01$) and for non-encroachers grown under $eCO_2 \times WW$ (Figure 5c; $p < 0.05$). Significant differences in intercepts and slopes between encroacher and non-encroacher were observed in the $aCO_2 \times WW$ ($p < 0.05$) and $eCO_2 \times WL$ treatments (Figure 5c; $p < 0.05$).

4 | DISCUSSION

Encroacher status in African savanna tree species appears linked to N_2 fixation dynamics. Our hypothesis that N_2 fixation in encroachers increases in response to eCO_2 and increased water availability compared to non-encroachers is partially supported by our data. Lower leaf δ^{15}N values in encroachers suggest a greater reliance on N_2 fixation, pointing towards a short-term N allocation strategy prioritising immediate uptake for growth and metabolic demands. In contrast, stem δ^{15}N, indicative of long-term N storage and transport, was more responsive to environmental factors such as eCO_2 and soil moisture rather than encroacher status. Under eCO_2 and well-watered treatments, encroachers and non-encroachers increased the storage and transport of N_2 fixation products (indicated by decreased stem δ^{15}N). Strong phylogenetic signals in leaf and stem δ^{15}N, as well as %Ndfa suggest that N allocation and storage strategies are conserved through PNC, with these traits being inherited from a common ancestor. In contrast, NMF did not differ between encroachers and non-encroachers, and there was no support for a phylogenetic signal, suggesting that nodule biomass allocation is primarily influenced by environmental factors.

Our findings suggest that encroachers possess N traits that are responsive to short-term environmental change, indicated by consistently lower leaf δ^{15}N values regardless of CO_2 levels and soil moisture treatment. Plant δ^{15}N is strongly influenced by internal N demand, which regulates uptake, assimilation and allocation processes. These physiological mechanisms drive isotope fractionation within the plant, impacting the leaf δ^{15}N values (Ariz et al., 2015; Tcherkez, 2011). Mobilising N_2 fixation products to leaves provides several ecological advantages, as leaves are primary sites for energy-intensive processes, such as photosynthesis and protein synthesis (Ariz et al., 2015; Canadell et al., 2007; Tsukaya, 2018). Encroacher success could be driven by their ability to prioritise the allocation of N_2 fixation products to leaves for access to N resources immediately required for metabolic demands.

Facultative N-fixers adjust N_2 fixation to meet their needs, while obligate N_2-fixers fix N at the same rate regardless of environmental conditions (Menge & Hedin, 2009). Encroachers could be efficient facultative N_2-fixers that increase fixation and transport products to leaves as required. Flexible short-term N-dynamics can increase

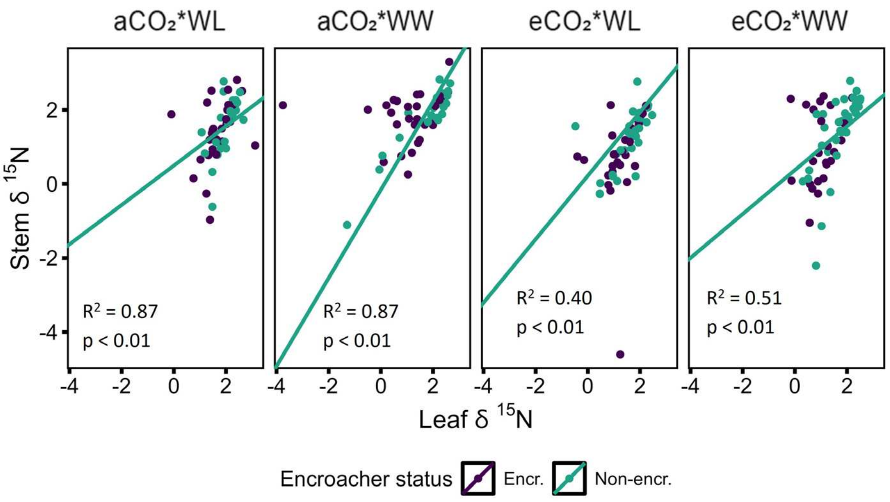

FIGURE 4 Bivariate relationship between plant N-dynamics. Standardised major axis regressions (SMAs) for leaf δ^{15}N (‰) and stem δ^{15}N (‰) in encroacher species and non-encroacher species across CO_2 (aCO_2 [~397.9 ppm]; eCO_2 [~545.1 ppm]) and soil moisture (water-limited [WL]; well-watered [WW]) treatments. The trend lines for encroachers (purple) and non-encroachers (turquoise) have been calculated separately. Only significant trendlines, R^2 and p-values are shown ($n = 222$; Tables S2 and S3).

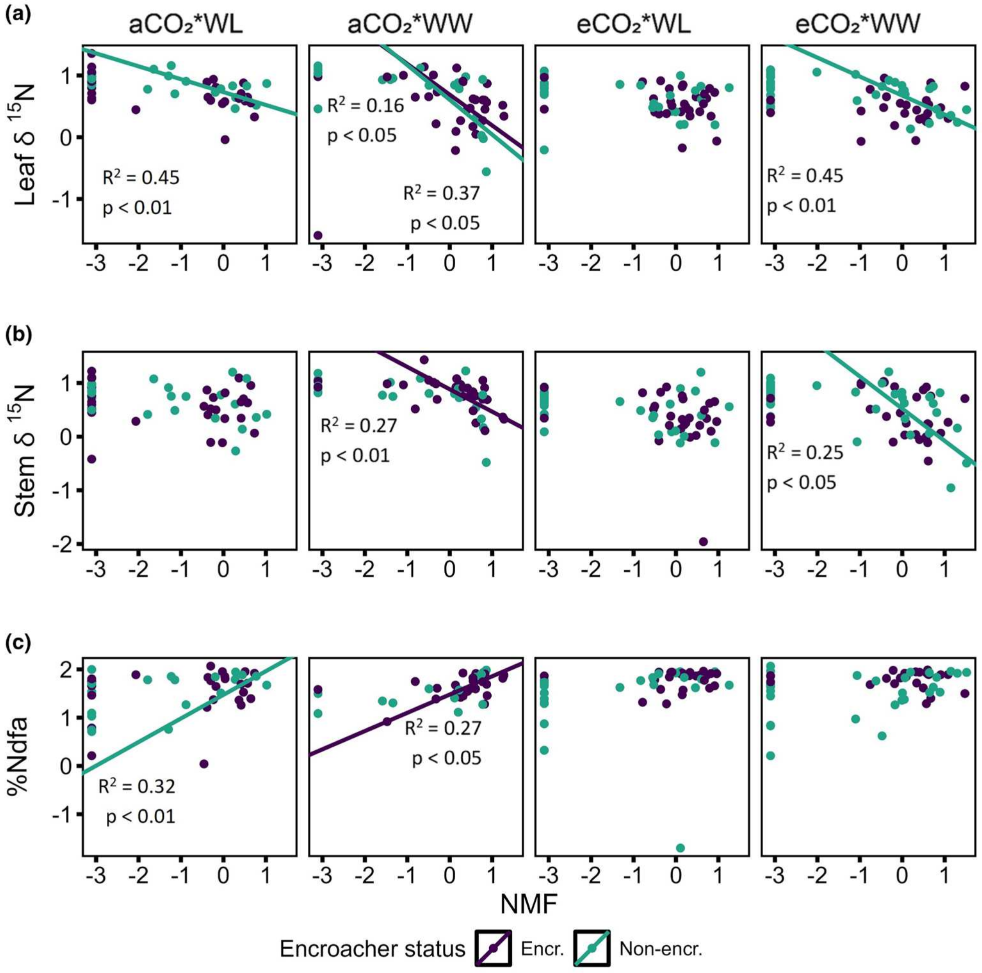

FIGURE 5 Bivariate relationships among N-dynamics traits. Standardised major axis regressions (SMAs) for nodule mass fraction (NMF) (% biomass) of encroacher and non-encroacher groups of species for (a) leaf δ^{15}N (‰), (b) stem δ^{15}N (‰) and (c) % nitrogen derived from the atmosphere (%Ndfa). Treatment refers to the CO_2 (aCO_2 [~397.9 ppm]; eCO_2 [~545.1 ppm]) and soil moisture (water-limited [WL]; well-watered [WW]) treatment. The trend lines for encroachers (purple) and non-encroachers (turquoise) have been calculated separately. Only significant trendlines, R^2 and p-values are shown ($n = 222$; Tables S2 and S3).

plant N use efficiency, which entails a proportional allocation of N use and resources to tissue storage (Berendse & Aerts, 1986). Increased allocation of N_2 fixation products to the leaves ensures plants can adequately support critical metabolic activities (Baral et al., 2016; Unkovich et al., 2008). Flexible N_2 fixation and allocation could provide encroachers with a short-term competitive advantage that supports higher rates of photosynthesis, promoting growth and survival in the disturbance-prone savanna ecosystem.

In this experiment, stem δ^{15}N values, NMF and %Ndfa did not differ between encroachers and non-encroachers. However, under eCO_2 encroachers and non-encroachers invested more biomass in nodules, stem δ^{15}N values decreased, and %Ndfa increased. Similar patterns of increased nodule biomass allocation and decreased stem δ^{15}N were observed under the well-watered treatment across the 12 species. These findings suggest all species exhibited enhanced nodulation and increases in fixed N_2 under eCO_2 and well-watered treatments. It is widely acknowledged that increased CO_2 concentrations can enhance N assimilation and uptake in plants, resulting in reduced tissue δ^{15}N values (Ariz et al., 2015; Polley, Johnson, & Mayeux, 1997a). Similarly, well-watered conditions can facilitate nodule formation and N uptake via fixation, as low soil moisture restricts the mobility of free-living rhizobia (Deans et al., 1993). These results suggest that strategies involving nodule development and the storage and transport of N_2 fixation products are species-specific responses to environmental factors rather than traits associated with encroacher status.

The absence of a correlation between leaf and stem δ^{15}N values in encroachers, contrasting with the observed patterns in non-encroachers, suggests distinct N dynamics and allocation strategies between these groups. In non-encroachers, the correlation indicates a consistent pattern of N movement and allocation from roots to leaves (Ariz et al., 2015; Peuke et al., 2013). Conversely, the lack of correlation in encroachers suggests a more flexible N allocation strategy, potentially involving differential N utilisation and retention across various plant parts. More research into the δ^{15}N values of other plant components is required to explore this further.

Our analysis suggests that evolutionary history influences N-dynamic traits in encroachers. Strong phylogenetic signals for leaf δ^{15}N, stem δ^{15}N and %Ndfa indicate that patterns in these traits have likely arisen due to PNC. However, the limited size of our phylogeny must be considered when interpreting these results. *Vachellia* and *Senegalia* collectively contain over 400 species (Ringelberg et al., 2023), whereas our phylogeny includes only 12 species, which may not accurately capture broader evolutionary patterns (Hillis, 1996). Therefore, conclusions on phylogenetic signals across N-dynamic traits in this study should be interpreted with caution, as broader patterns remain unexamined (Boettiger et al., 2012).

5 | CONCLUSIONS

Our study provides some of the first insights into the physiological mechanisms governing encroachment of woody plant species in savannas. Our findings suggest that N_2 fixation enhances N use efficiency, enabling encroacher species to meet immediate N demands and thrive in savannas under changing environmental conditions. The potential feedback loop between N_2 fixation and photosynthesis allows plants to capitalise on additional resources, enhancing their growth. This mechanism, where eCO_2 enhances photosynthesis, which in turn supports greater allocation of C to rhizobia to catalyse more N_2, to increase N_2 fixation, promoting plant growth, may explain the ecological success of a select few species in driving encroachment (Figure 6).

Our findings indicate that the woody species which have previously demonstrated an ability to encroach under past CO_2 increases enhance their N_2 fixation to a greater extent compared to woody species that have not previously shown encroacher behaviour. The rate of woody encroachment will likely increase in concert with predicted CO_2 rises. However, since N_2 fixation in all species increased under eCO_2, future encroachment may be driven by a wider selection of species than those currently observed. Alternatively, increased N_2 fixation in response to CO_2 may not be a key functional trait defining encroacher status. Encroachers likely possess suites of traits that enable them to capitalise on changing environmental conditions. Looking ahead, we advocate for a deeper understanding of the biotic mechanisms driving encroachment, particularly how shifts in the N acquisition strategies of woody plants interact with competition, fire regimes and soil feedbacks, to better anticipate long-term impacts on savanna ecosystem structure and function.

AUTHOR CONTRIBUTIONS

Elizabeth M. Telford and Caroline E. R. Lehmann conceived the ideas and designed the methodology; Elizabeth M. Telford, Katie J. Field, Richard Carkeek, Russell B. Dixon, Emma Jones, Sarah L. Raubenheimer and Edith Singini collected the data; Elizabeth M. Telford and Caroline E. R. Lehmann analysed the data with input from Brad Ripley, Colin P. Osborne, Kimberley Simpson and Lorna Street; Elizabeth M. Telford led the writing of the manuscript with comments from Caroline E. R. Lehmann, Kimberley Simpson, Lorna Street and Colin P. Osborne. Emily Fletcher prepared plant tissues

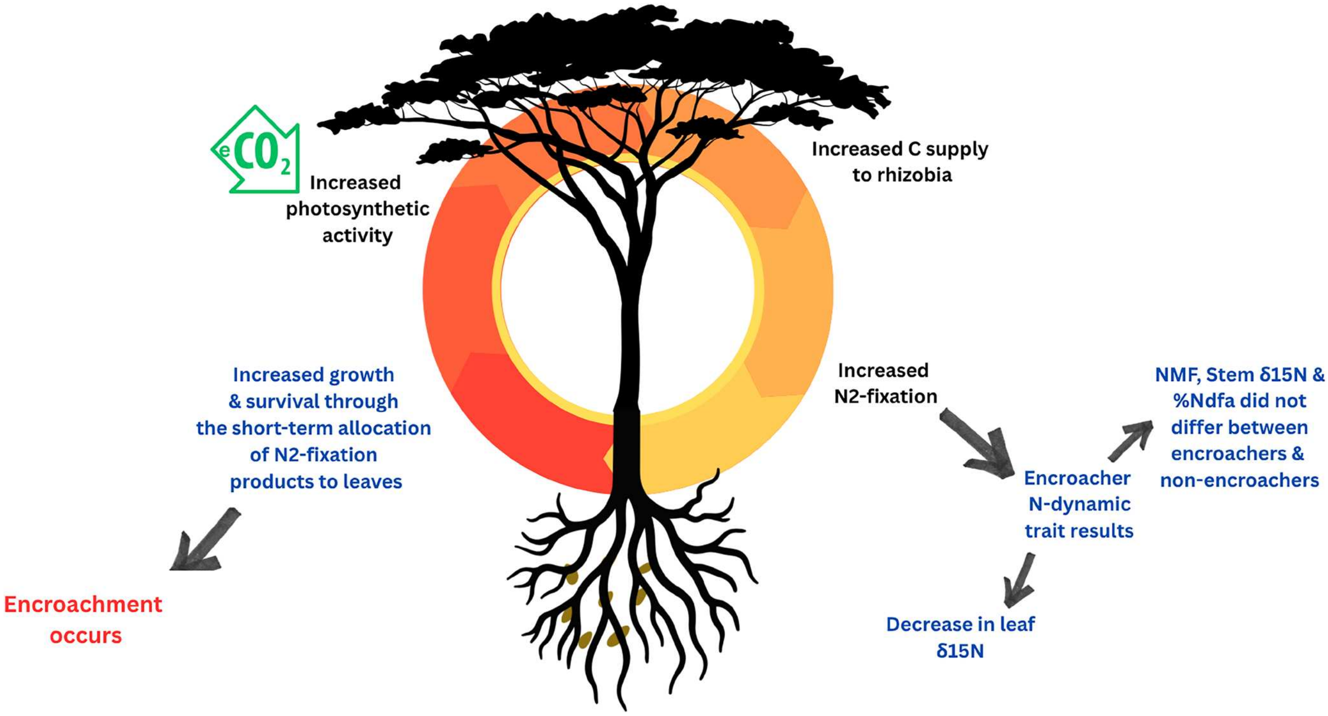

FIGURE 6 A schematic diagram depicting our results (blue text) in the context of our original hypothesis (black text). A proposed feedback loop between host plant and rhizobia bacteria under eCO_2, whereby increased CO_2 availability stimulates N_2 fixation and the transportation of N products to leaves to meet immediate metabolic demands, enhancing plant growth and survival and ultimately drives encroachment in the savanna.

for isotope analysis; Kimberley Simpson prepared the phylogeny and advised on statistical analysis; and Lorna Street advised on calculations. All authors contributed critically to the drafts and gave final approval for publication.

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CONFLICT OF INTEREST STATEMENT

We declare that there is no conflict of interest between authors. Katie J. Field. is a senior editor for *Functional Ecology* but has played no role in the editorial or peer review process for this paper.

DATA AVAILABILITY STATEMENT

Data are available at https://doi.org/10.5061/dryad.gqnk98t2f (Telford et al., 2025). Code is available at https://doi.org/10.5281/zenodo.17580879 (Telford, 2025).

STATEMENT ON INCLUSION

Our study brings together authors from multiple countries, including scientists based in South Africa where the research was conducted. All authors were actively involved from the early stages of study design and research planning.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1. Soil characteristics of potting soil used in this experiment. (SD) standard deviation.

Table S2. The number of replicates used in this study to compare the N-dynamics of encroachers and non-encroachers. Number of replicates (n) for each treatment combination: CO_2 treatment (ambient, aCO_2: ~398 ppm; elevated, eCO_2: ~545 ppm) × soil moisture treatment (water-limited; well-watered) ($n = 222$).

Table S3. The number of replicates used in this study to compare the N-dynamics of encroachers and non-encroachers. Number of replicates (n) for each treatment combination: CO_2 treatment (ambient, (a)CO_2: ~398 ppm; elevated, (e)CO_2: ~545 ppm) × soil moisture treatment (water-limited; well-watered) ($n = 222$).

Table S4. GenBank accession details for the sequences used to create a phylogenetic tree of the study species.

Figure S1. Layout of 16 open-top chambers (OTC) at the Rhodes University CO_2 facility. CO_2 (ambient (a)CO_2: ~397.9 ppm; elevated (e)CO_2:~545.1 ppm) × soil moisture treatment (water limited; well-watered). There were 222 pots, each with an individual plant, approximately 20 pots per chamber and 10 pots per treatment within each OTC.

Figure S2. Mean daily soil water volumetric content (SMC) for 222 pots across the experiment. Blue line represents the well-watered treatment. The red line represents the water limited treatment. The solid line represents ambient (a)CO_2 (~397.9 ppm). The dashed line represents elevated (e)CO_2 (~545.1 ppm).

Figure S3. Phylogenetic tree of the 12 study species with Desmanthus birocornutus as an outgroup. Refer to Table S4 for the accession numbers of the strains used to make the tree.

Figure S4. Bivariate relationships between plant components. Standardised major axis regressions (SMAs) for nodule biomass (grams) and belowground biomass (grams) in encroacher species and non-encroacher species across CO_2 (ambient (a)CO_2: ~397.9 ppm; elevated (e)CO_2: ~545.1 ppm) × soil moisture (water limited (WL); well-watered (WW) treatments). Only significant correlations are shown with a trend line. Note that data and axes have been natural log transformed (*n* = 222; Table S2).

Figure S5. Boxplots comparing the nodule mass fraction (NMF) (%) of 12 species. (a) CO_2 ambient (a)CO_2: ~397.9 ppm; elevated (e)CO_2: ~545.1 ppm) and (b) soil moisture (water limited (WL); well-watered (WW) treatment for nine Vachellia species and three Senegalia species (*n* = 222; Table S2).

Figure S6. Boxplots comparing the leaf $\delta^{15}N$ of 12 species. (a) CO_2 ambient (a)CO_2: ~397.9 ppm; elevated (e)CO_2: ~545.1 ppm) and (b) soil moisture (water limited (WL); well-watered (WW) treatment for nine Vachellia species and three Senegalia species (*n* = 222; Table S2).

Figure S7. Boxplots comparing the stem $\delta^{15}N$ of 12 species. (a) CO_2 ambient (a)CO_2: ~397.9 ppm; elevated (e)CO_2: ~545.1 ppm) and (b) soil moisture (water limited (WL); well-watered (WW) treatment for nine Vachellia species and three Senegalia species (*n* = 222; Table S2).

Figure S8. Boxplots comparing the % nitrogen derived from the atmosphere (%Ndfa) of 12 species. (a) CO_2 ambient (a)CO_2: ~397.9 ppm; elevated (e)CO_2: ~545.1 ppm) and (b) soil moisture (water limited (WL); well-watered (WW) treatment for nine Vachellia species and three *Senegalia* species (*n* = 222; Table S2).

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