



# Nodulation alleviates the stress of lower water availability in *Vachellia sieberiana*

Elizabeth M. Telford<sup>1,2</sup> · Nicola Stevens<sup>3,4</sup> · Guy F. Midgley<sup>5</sup> · Caroline E. R. Lehmann<sup>1,2</sup>

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## Abstract

The genus *Vachellia* (Fabaceae) has a pan-tropical distribution and numerous *Vachellia* species are currently observed to be expanding their indigenous ranges and/or are invasive. Most *Vachellia* species have the capacity to enhance nitrogen uptake via an N<sub>2</sub>-fixing rhizobial mutualism that manifests in specialized root nodule structures enabling the catalysis of atmospheric N<sub>2</sub> into a plant useable form. Improved understanding of nodulation may provide new insight to the changing patterns of ecological success of *Vachellia* species. Here, we investigated how the seedling growth, allometry and nodulation of two common *Vachellia* species, the arid *Vachellia erioloba* and the mesic *Vachellia sieberiana*, responded to varied levels of water availability. Seedlings were grown at 4%, 8% and 16% soil moisture content (SMC) for four months. The seedling growth and allometry of *V. erioloba* was unresponsive to changing water availability, and no nodulation was observed. The allometry of *V. sieberiana* was responsive to changing water availability and nodulation was observed; with the highest nodule biomass and growth rate recorded at 4% SMC. These patterns suggest that *V. erioloba* does not require the rhizobial mutualism, possibly due to lower competitive interactions between woody plants and grass in the arid savanna. Whereas, due to the competitive vegetation interactions typical in the mesic savanna, N<sub>2</sub>-fixation via nodule development could provide *V. sieberiana* a competitive advantage over grass not only in limited N conditions, but also during periods of lower water availability.

**Keywords** Nodules · *Vachellia* · Water-availability · Soil moisture · Savanna · N<sub>2</sub>-fixation

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✉ Elizabeth M. Telford  
S1014831@ed.ac.uk

<sup>1</sup> School of Geosciences, University of Edinburgh, Drummond Building, 1 Drummond Street, Edinburgh EH8 9XP, UK

<sup>2</sup> Royal Botanic Gardens Edinburgh, 20a Inverleith Row, Edinburgh EH3 5LT, UK

<sup>3</sup> Environmental Change Institute, School of Geography and the Environment, University of Oxford, Oxford OX13QY, UK

<sup>4</sup> Centre for African Ecology, School of Animal, Plant and Environmental Sciences, University of the Witwatersrand, Wits, Johannesburg 2050, South Africa

<sup>5</sup> School for Climate Studies and Department of Zoology and Botany, Natural Sciences, Building, 20 Merriman Ave, Stellenbosch 7600, South Africa

## Introduction

Savannas are open ecosystems characterised by discontinuous woody cover and a continuous herbaceous ground layer that is shade intolerant (Scholes and Archer 1997; House et al. 2003). They account for a fifth of the earth's land surface across four continents, including half of Africa (Scholes and Archer 1997; Sankaran et al. 2005). A major threat to savanna ecosystems is woody encroachment, defined as the increase in dominance and cover of woody species (Stevens et al. 2017; Devine et al. 2017). In Africa, woody cover is increasing at an average rate of ~2.4% per decade (Venter et al. 2018); threatening biodiversity, grazing provision, hydrology, and nutrient cycling (Parr et al. 2012; Honda and Durigan 2016; Lehmann and Parr 2016; Leitner et al. 2018). However, it remains unclear why only a small number of species are responsible for encroachment (Liu et al. 2013). Across Africa, the majority of encroaching woody species are species of *Vachellia*, *Senegalia* and *Dichrostachys* (Stevens et al. 2017). One hypothesis is that the ability to fix

atmospheric nitrogen ( $N_2$ ) can contribute to this pattern (Stevens 2017). Indeed, many of these encroaching species have the capacity to fix  $N_2$  via a rhizobial mutualism (Sprent 1995; Scholes and Archer 1997). The rhizobial mutualism manifests via the formation of root nodules; where  $N_2$  is transformed into plant useable  $NH_4$  (Kambatuku et al. 2013).

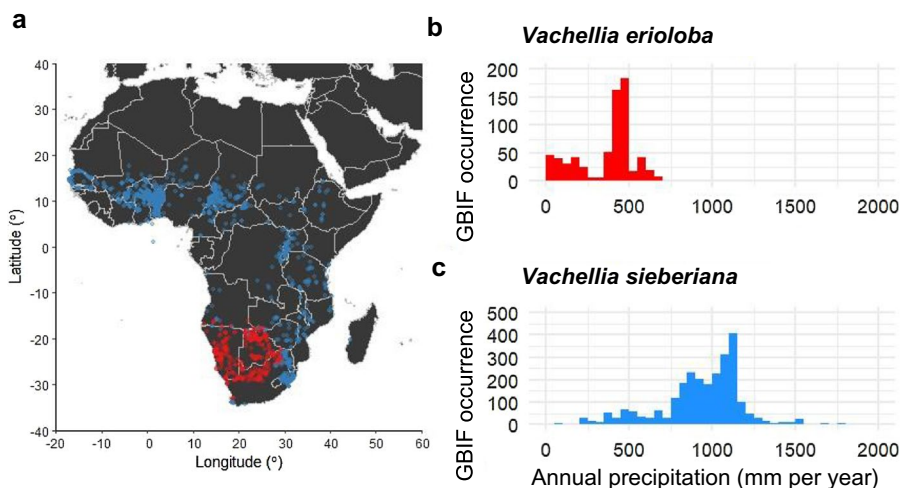
Previous experiments have shown that the legume-rhizobia symbiosis is sensitive to climatic extremes. Globally, terrestrial  $N_2$ -fixation has been found to be inhibited by increased drought (Dovrat and Sheffer 2019; Zheng et al. 2020). This is possibly because the mobility of rhizobia is impeded by low soil moisture (Deans et al. 1993), reducing symbiosis establishment (Swaine et al. 2007; Fall et al. 2011). However, drought has been found to trigger an 80% increase in nodule biomass in temperate forest tree *Robinia pseudoacacia* L. (Wurzburger and Miniati 2014). If the sensitivity of nodulation to drought is widespread it may explain why woody legumes that seldom nodulate, such as *Vachellia erioloba* (E.Mey.) P.J.H. Hurter, are more common within the arid savanna (< 450 mm mean annual precipitation (MAP); Fig. 1b). It has also been suggested that if N is not a limit on plant growth, the ability to nodulate is of limited advantage, as substantial energy must be invested in traits that facilitate survival in water limited conditions (Sprent and Gehlot 2010). Nodulating legumes, such as *Vachellia sieberiana*; (DC.) Kyal. & Boatwr., are more common in mesic savannas (~ 800–1000 mm MAP; Fig. 1c). Mesic savannas are typically N limited; due to high rates of soil nutrient leaching (Zahran 1999), competition with grasses (due to increased productivity associated with higher annual rainfall) (Cramer et al. 2007, 2010) and frequent fire events leading to N loss (Archibald and Hempson 2016; Pellegrini et al. 2016). Further, nodulation enables woody legume

seedlings to compensate for limited soil N in a competitive environment at a critical, but vulnerable establishment phase (Kambatuku et al. 2013).

Savanna ecosystems are typified by bottlenecks in recruitment (Bond and Midgley, 2003). Particularly within semi-arid and arid savannas, water availability is the primary cause of a bottleneck at the seedling stage (Higgins et al. 2007; Stevens et al. 2014). Additionally, climate change predictions suggest that Africa will become drier and hotter, with changing patterns of seasonal rainfall and experience an increase in drought events (IPCC 2022). Encroachment is widespread but the drivers between mesic and arid landscapes are likely to differ. In arid savannas, maximum woody cover is limited by water availability so the potential for woody encroachment could be constrained (Sankaran et al. 2005). Whereas, within mesic savannas, there is sufficient water availability for canopy closure but disturbances from fire, herbivory and humans prevent canopy closure (Kraaij and Ward 2006). Therefore woody encroachment (and canopy closure) potential is high in mesic savannas (Osborne et al. 2018). However, it remains unclear whether drier conditions under climate change could slow or accelerate woody encroachment by either limiting or stimulating seedling growth.

In Africa, the woody component of savanna vegetation is often dominated by mimosoids *Vachellia* and *Senegalia* (Bouchenak-Khelladi et al. 2010). Previous research has identified that *Vachellia* contains the most aggressive encroachers (Lewis et al. 2021). Therefore, understanding how seedling growth and recruitment of *Vachellia* species responds to drier conditions at this critical demographic stage is useful. In particular, a seldom considered aspect of savanna seedling success is how lower water availability

**Fig. 1** a A map showing the distribution of *Vachellia erioloba* (red) and *Vachellia sieberiana* (blue) across Africa (Bivand and Nicholas 2019; GBIF.org 2020a; GBIF.org 2020b). Histograms of the number of recorded species occurrence based on GBIF records of **b** *V. erioloba* and **c** *V. sieberiana* in relation to mean annual precipitation (MAP) (mm) (GBIF.org 2020a; GBIF.org 2020b; Hijmans 2020). *V. erioloba* GBIF occurrence records are highest at ~ MAP 500 mm and *V. sieberiana* occurrence is highest at ~ MAP 1000 mm



alters the functionality of the legume rhizobium symbiosis (Serraj 2003; Sankaran 2019). Previously it has been proposed that lower water availability can reduce  $N_2$ -fixation by reducing carbon nodule metabolism, introducing oxygen limitation, thus causing reduction of  $N_2$ -fixation product transport (Serraj 2003). Understanding the interactions between lower water availability and nodule production related to plant growth could help determine the functional role of  $N_2$ -fixation in seedling success.

Here we investigated how soil moisture relates to the growth and nodulation response of two woody *Vachellia* species, one arid and one mesic, both considered encroachers in a controlled glasshouse experiment (Hauwanga et al. 2018; Russell et al. 2019). We asked (1) does water availability affect seedling growth rates? We expected that the growth of arid-adapted *V. erioloba* would be unaffected by extremes in water availability in comparison with mesic adapted *V. sieberiana* (Seymour 2003). We predicted that the aboveground growth *V. sieberiana* would be positively correlated with water availability (Kraaij and Ward 2006). (2) Does water availability affect nodulation? We predicted *V. sieberiana* would exhibit lower levels of nodulation (nodule biomass and nodule mass fraction) when grown in the lowest soil moisture treatment, as rhizobia movement would be limited (Swaine et al. 2007; Fall et al. 2011). The literature notes that nodulation in *V. erioloba* is rare with previous research suggesting this occurs as *V. erioloba* obtains N from ground water rather than  $N_2$ -fixation (Barnes et al. 1997). Therefore, we assumed that it would be unlikely that *V. erioloba* would develop nodules across any of the soil moisture treatments.

## Materials and methods

### Description of study species

*Vachellia erioloba* is herbivore dispersed and occurs in arid savannas where rainfall is less than ~450 mm MAP (Fig. 1a; Fig. 1b) (Seymour 2008). Nodulation is infrequent for the this species, this is postulated to be due to its long roots that are able to access mineral N in ground water (Barnes et al. 1997; Sprent 2009). *V. erioloba* is a drought resistant, slow growing species characteristic of acidic sandy soils (Seymour 2008), and is recognised as a mild encroacher (Stevens et al. 2017; Hauwanga et al. 2018). Recruitment is generally episodic, and wet season dependent (Seymour 2008). Within its native range herbivore densities are low and fire is infrequent (Barnes 2001; Seymour 2008).

*Vachellia sieberiana* is a herbivore-dispersed species found across southern Africa where rainfall ranges from

800–1100 mm MAP (Fig. 1a; Fig. 1c) (Bunney 2014) and is known to nodulate (Sprent 2009). *V. sieberiana* grows on deep, well-drained, light sandy and medium loamy acid soils (Tadesse et al. 2007). This species is fast growing (Sunmonu and Van Staden 2014), and considered to be a woody encroacher (Stevens et al. 2017; Russell et al. 2019). Browse pressure is primarily on adult trees by *Giraffe camelopardalis* (Zinn et al. 2007). Within *V. sieberiana*'s range, fire is frequent and fuelled by highly productive grasses (February et al. 2013; Bunney 2014).

### Experimental design

Seeds were purchased from Silverhill Seeds (<http://www.silverhillseeds.co.za>) (Cape Town, South Africa). The seeds of both species had coat-imposed dormancy, and pre-germination treatments were required. Therefore, the seeds were soaked in sodium hypochlorite (NaClO) for two minutes, to reduce fungal and mould growth and then soaked in boiling water for 10 minutes (Cramer et al. 2007). Treated seeds were germinated in petri dishes containing 10% Agar gel placed in a growth chamber (Conviron A1000, Conviron Europe Ltd, Isleham, Cambridgeshire, B7 5RJ, UK) at 30 °C for three-four days until germination occurred. There was approximately ~90% germination success in both species.

Seedlings were grown from May to September 2018 (winter) at Stellenbosch University's glasshouses heated using two standard garden infra-red heaters mounted three metres apart across the glasshouse ceiling. The average glasshouse air temperature was 25 °C and soil temperatures were between 17 °C and 35 °C. Temperature was measured using Thermochron iButtons (Thermochron, Baulkham Hills, Australia).

The germinated *V. erioloba* and *V. sieberiana* seedlings were transplanted into individual two litre pots. The soil was a mixture of a native alluvial sand aggregate taken from the Kalahari Desert and vermiculate (two parts sand: one part vermiculate). Seedlings were randomly distributed in the glasshouse and were moved every three days to ensure a homogenised growing environment. In total, the experiment consisted of 180 plants of two species.

All seedlings were provided with 5 ml of Strake Ayres: Nutrifeed; a water soluble fertiliser during week one (Strake Ayres, South Africa) (Macronutrient quantities available in Appendix Table 3). The soil was not inoculated with rhizobia. As the origin of the sand was the Kalahari desert where *V. erioloba* is a keystone species we assumed that a compatible rhizobia would be present (van der Merwe et al. 2019). Given the limited understanding of in-situ growth traits of *Vachellia* species we chose to use present free-living rhizobia in the sand (Winters et al. 2018). We believed that

this would provide an accurate representation of the natural ability of rhizobia to survive water limited conditions (Shetta 2015).

### Soil moisture treatments

Three watering treatments were imposed (measured in volumetric soil moisture content), where for each species 30 individuals were watered at an average of 4% soil moisture content (SMC) ( $0.100 \text{ m}^3/\text{m}^3$ ); average 8% SMC ( $0.180 \text{ m}^3/\text{m}^3$ ); and average 16% SMC ( $0.280 \text{ m}^3/\text{m}^3$ ). During the first two weeks 200 ml of water was given to each pot every two days to ensure establishment (Kraaij and Ward 2006). During weeks three and four, seedlings received 200 ml water every three days. At the beginning of week five (approximately one month after being transplanted into individual pots) soil moisture treatments were applied.

The appropriate SMC for this experiment was determined through a pilot study involving already germinated seedlings *Vachellia exuvialis* (I. Verd.) Kyal. & Boatwr. Here, 10 *V. exuvialis* seedlings per water treatment were subjected to the following water treatments: 1% SMC, 2% SMC, 4% SMC, 6% SMC, 8% SMC, 14% SMC, 16% SMC, 18% SMC, and 20% SMC. On this basis, the soil moisture treatments applied in the experiment reported here were determined by recording the health and survival of *V. exuvialis* to a range of watering regimes for 30 days. In the pilot, we observed for signs of root rot and leaf longevity in the higher SMCs. We used *V. exuvialis* in the pilot study due to seed availability, and it is fast growing species with a high germination success rate. Supplementary information on metrics of performance of *V. erioloba* and *V. sieberiana* in relationship to water availability were gathered from the literature (Cramer et al. 2007; Shetta 2015; Azad and Sumon 2016, 2017).

Soil moisture was recorded throughout the main experiment and pilot study using an HS2 HydroSense II Display (Campbell Scientific Ltd, Loughborough, UK). Soil moisture readings were collected every three days and the pots were watered accordingly.

### Plant and leaf measurements at harvest

Plants were harvested at three ages, providing ten replicates per species x water treatment x harvest. Ten seedlings of each species of each water treatment were harvested at three points. Harvests were at one (Harvest 1), two (Harvest 2) and three months (Harvest 3) post water treatment. Height (mm) of each seedling was measured weekly.

Seedlings were separated into above and belowground biomass, and roots were carefully washed to maintain fine root mass. Nodules were removed from the roots using forceps

and cleaned using a paint brush. For each harvested plant, the final plant height, dry aboveground and belowground biomass (grams), and nodule dry biomass (grams) were recorded. We chose to focus upon nodule biomass rather than nodule count as this is keeping with the dominant approach in the literature (Voisin et al. 2003; Cramer et al. 2012; Menge et al. 2015). We also calculated nodule mass fraction using the following equation:

$$\text{Nodule mass fraction} = \frac{\text{dry nodule biomass (grams)}}{\text{dry whole plant biomass (grams)}}$$

All plant material was oven dried at 65 °C for 36 hours (Kambatuku et al. 2013).

### Statistical analyses

Linear mixed effect models were fitted to test the effect of water availability on height (mm) and absolute growth (mm) (lme4 package; Bates et al. 2015). Height and absolute growth measurements were log transformed prior to analysis. Week and water treatment were fitted as fixed effects, and individual tag number (Species-Treatment-Pot) was fitted as a random effect. Due to the large difference in the niches occupied by these two species they were separated when creating the model assessing height related to water treatment. Only height and absolute growth for individuals that were harvested during Harvest 3 for *V. sieberiana* and *V. erioloba* was used in this analysis. This was because we were not interested in the temporal response of plant growth.

The seedling biomass and allocation data [below ground biomass (grams), above: belowground ratio and aboveground biomass (grams)] were analysed using a two-way ANOVA (Car: Companion to Applied Regression package; Fox and Weisberg 2019). The data was normal in distribution. Post-hoc Tukey significant difference (HSD) tests were carried out to separate the effects of water availability on the biomass for each species (MulticompView: Visualizations of Paired Comparisons package; Graves et al. 2019). To check homogeneity of variance a Levene's test was used as a robust test of deviations from normality (Car: Companion to Applied Regression package; Fox and Weisberg 2019). Only the biomass data that was collected during Harvest 3 for *V. sieberiana* and *V. erioloba* was used in this analysis. This was because we were not interested in the temporal response of plant growth.

A two-way ANOVA was used to explore whether there was a correlation between soil moisture content, harvest and nodulation (nodule biomass (grams), nodule mass fraction (grams) and nodule count), using the Car: Companion to Applied Regression package (Fox and Weisberg 2019). Nodule biomass (grams) and nodule mass fraction data was log

transformed prior to analysis. A post-hoc Tukey significant difference (HSD) and a Levene’s test were also applied. Only nodulation data collected from *V. sieberiana* across all three harvesting efforts was used in this analysis. This decision was informed by the lack of nodulation displayed by *V. erioloba*. All figures were created using the ggplot2; elegant graphics for data package (Wickham 2016) All analyses was conducted in the R environment 3.5.1 (R Core Team 2020).

## Results

### Does water availability affect seedling growth rates?

The height and aboveground biomass of the four month-old *V. sieberiana* seedlings (collected in harvest 3) was affected by water treatment (Table 1; Table 2; Appendix Table 4). *V. sieberiana* seedlings grown in the driest conditions (4% SMC) were significantly taller (Fig. 2a; Table 1) and had the largest aboveground biomass (Fig. 3b; Appendix Table 5) than seedlings grown at 8% SMC and 16% SMC. In contrast, the belowground biomass of *V. sieberiana* was not affected by water availability ( $df=2, F=1.45, p>0.050$ ; Fig. 3b; Appendix Table 4).

**Table 1** The output of a mixed model for the effect of water treatment on the log transformed growth (height) of *Vachellia erioloba* (VE) and *Vachellia sieberiana* (VS) seedlings

		Effect of water availability on the logged height of <i>Vachellia erioloba</i>		
		Estimates	CI	<i>p</i>
Intercept		1.08	0.99–1.16	< 0.001**
Week		0.09	0.08–0.09	< 0.001**
Treatment 8%		0.00	– 0.10–0.10	0.959
Treatment 16%		– 0.04	– 0.14–0.05	0.350
Random Effects $\sigma^2$	0.08			
<sup>T</sup> 00 IndividualID	0.00			
ICC	0.06			
N IndividualID	26			
Observations	390			
Marginal R <sup>2</sup>	0.636			
Conditional R <sup>2</sup>	0.658			
		Effect of water availability of the logged height of <i>Vachellia sieberiana</i>		
		Estimates	CI	<i>p</i>
Intercept		1.06	0.96–1.15	< 0.001**
Week		0.11	0.10–0.11	< 0.001**
Treatment 8%		– 0.14	– 0.26–– 0.03	<b>0.012*</b>
Treatment 16%		– 0.16	– 0.27–– 0.05	<b>0.005*</b>
Random Effects $\sigma^2$	0.09			
<sup>T</sup> 00 IndividualID	0.01			
ICC	0.09			
N IndividualID	28			
Observations	420			
Marginal R <sup>2</sup>	0.696			
Conditional R <sup>2</sup>	0.724			

4%, 8% and 16% correspond the soil moisture content the seedlings were grown in. Height measurements were taken three weeks following germination for 15 weeks. Soil moisture treatments were applied at week two. The individual seedling was tested as a random effect. Only seedlings that were harvested during Harvest 3 were used in this analysis

P- value significance codes are indicated by: 0 ‘\*\*\*\*’ 0.001 ‘\*\*\*’ 0.01 ‘\*\*’ 0.05 ‘.’ Significance is indicated in bold

**Table 2** The output of a mixed model for the effect of water treatment on the log transformed absolute growth of *Vachellia erioloba* (VE) and *Vachellia sieberiana* (VS) seedlings

		Effect of water availability on the logged absolute growth of <i>Vachellia erioloba</i>		
		Estimates	CI	<i>p</i>
Intercept		0.97	0.81–1.13	<b>&lt; 0.001**</b>
Week		0.01	– 0.15–0.17	0.870
Treatment 8%		0.10	– 0.05–0.25	0.180
Treatment 16%		– 0.01	– 0.02–0.00	0.116
Random Effects $\sigma^2$	0.36			
<sup>T</sup> 00 IndividualID	0.00			
ICC	0.01			
N IndividualID	26			
Observations	390			
Marginal R <sup>2</sup>	0.012			
Conditional R <sup>2</sup>	0.019			
		Effect of water availability of the logged absolute growth of <i>Vachellia sieberiana</i>		
		Estimates	CI	<i>p</i>
Intercept		0.99	0.84–1.14	<b>&lt; 0.001**</b>
Week		– 0.16	– 0.31–0.02	0.029
Treatment 8%		– 0.01	– 0.16–0.13	0.849
Treatment 16%		0.00	– 0.01–0.02	0.583
Random Effects $\sigma^2$	0.36			
<sup>T</sup> 00 IndividualID	0.00			
ICC	NA			
N IndividualID	28			
Observations	420			
Marginal R <sup>2</sup>	0.016			
Conditional R <sup>2</sup>	NA			

4%, 8% and 16% correspond the soil moisture content the seedlings were grown in. Height measurements were taken three weeks following germination for 15 weeks. Soil moisture treatments were applied at week two. The individual seedling was tested as a random effect. Only seedlings that were harvested during Harvest 3 were used in this analysis

*P*-value significance codes are indicated by: 0 ‘\*\*\*\*’ 0.001 ‘\*\*\*’ 0.01 ‘\*\*’ 0.05 ‘.’ Significance is indicated in bold.

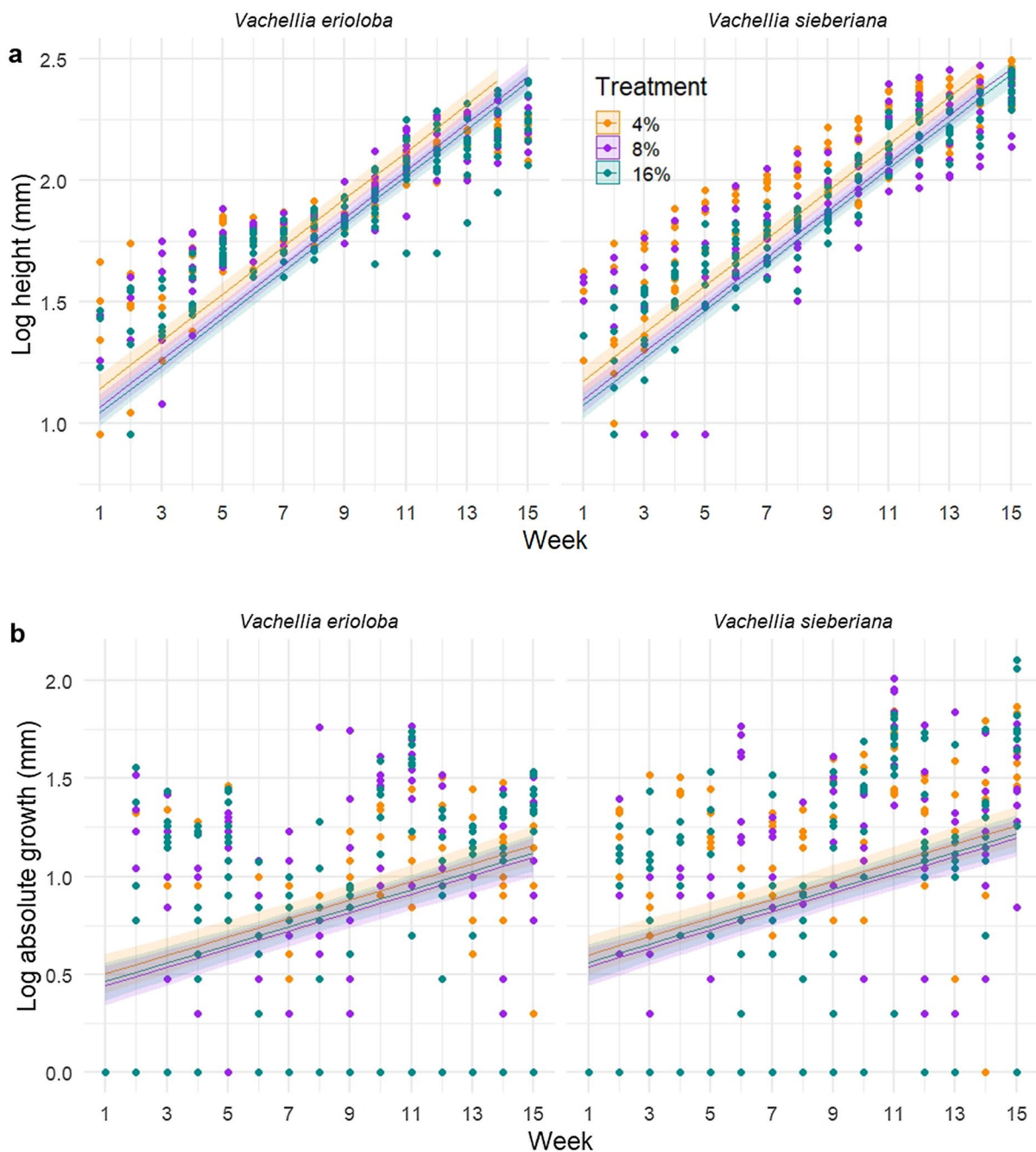
The growth and allocation patterns of the arid *V. erioloba* did not vary across water treatment ( $df=2$ ,  $F=1.45$ ,  $p>0.050$ ; Fig. 3a; Fig. 3b; Appendix Table 5). Relative to *V. sieberiana*, *V. erioloba* seedlings were shorter ( $df=2$ ,  $p>0.050$ ; Fig. 2a; Table 1), but proportionally had a larger belowground biomass ( $df=1$ ,  $F=57.42$ ,  $p<0.010$ ; Fig. 3c; Appendix Table 4).

### Does water availability affect nodulation?

As expected, *V. erioloba* did not develop nodules across any the water treatments. Nodule count and nodule biomass

in *V. sieberiana* increased with age ( $df=2$ ,  $F=129.194$ ,  $p<0.001$ ; Fig. 4a; Appendix Table 5; Appendix Table 6). There was an 800% increase in the number of individuals that nodulated between Harvest 1 (two months old) and Harvest 2 (three months old) (Appendix Table 5). There was an 11% increase in the number of individuals that nodulated between Harvest 2 (three months old) and Harvest 3 (four months old) (Appendix Table 5).

Nodule mass fraction was affected by water treatment ( $df=2$ ,  $F=3.098$ ,  $p<0.05$ ; Appendix Table 5). Proportional to total plant biomass, *V. sieberiana* seedlings grown in 4% SMC and 8% SMC produced nodules with the largest biomass



**Fig. 2** A comparison of how water treatment effected **a** The log transformed height (mm) and **b** The log transformed absolute growth (mm) of *Vachellia erioloba* and *Vachellia sieberiana* measured at weekly intervals over a four-month period. The seedlings shown in

this graph were harvested during Harvest 3. Treatment 4% (orange), 8% (purple) and 16% (blue) correspond with the soil moisture content. Height measurements began three weeks following germination. Soil moisture treatment was applied during week 2

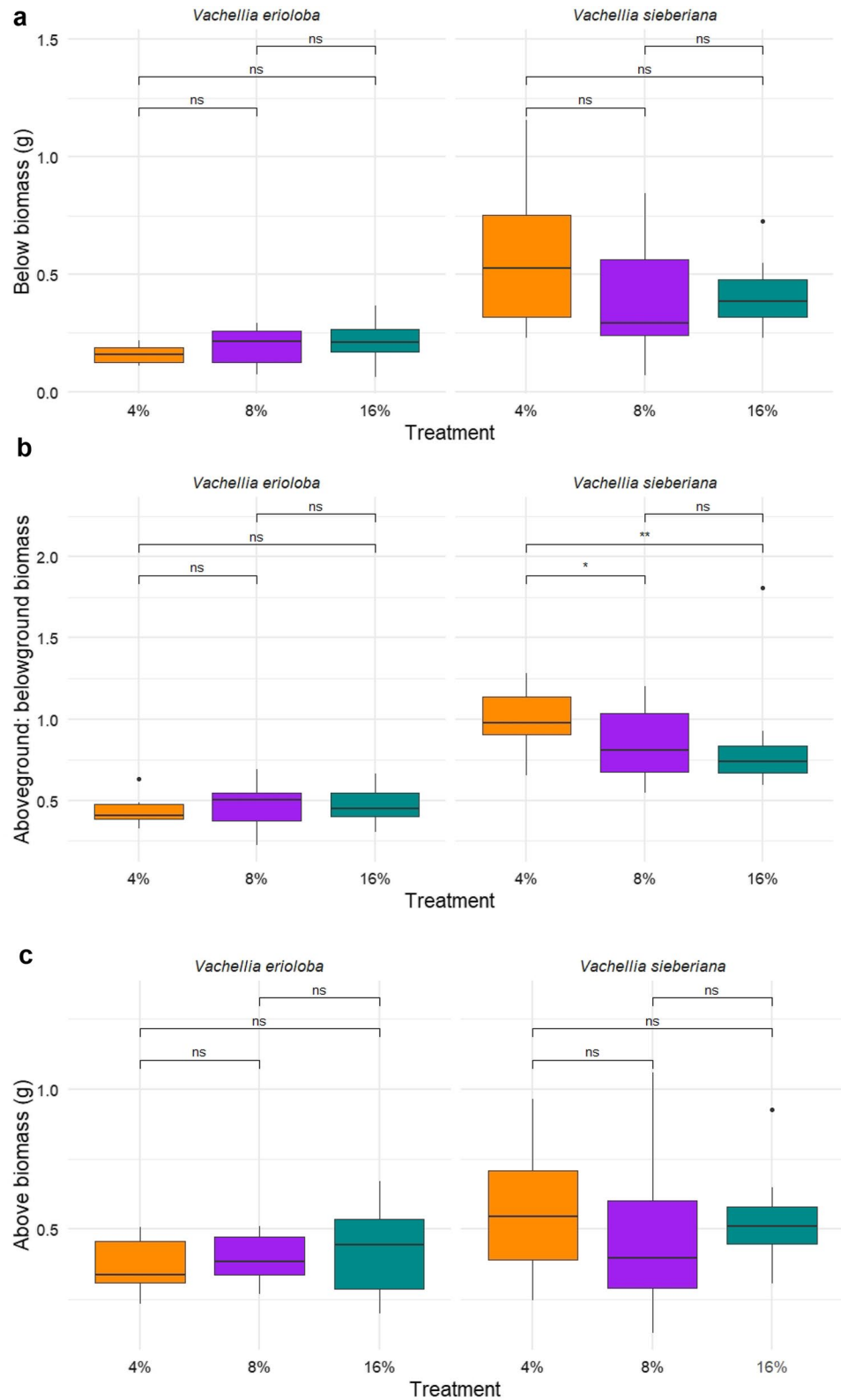
(Fig. 4c). Further, *V. sieberiana* seedlings grown at 4% SMC produced fewer nodules overall, but with the highest total nodule biomass. Whereas *V. sieberiana* seedlings grown at 16% SMC produced a higher number of nodules, with a lower nodule biomass than *V. sieberiana* seedlings grown at 4% SMC (Appendix Table 6).

## Discussion

### Does water availability affect seedling growth rates?

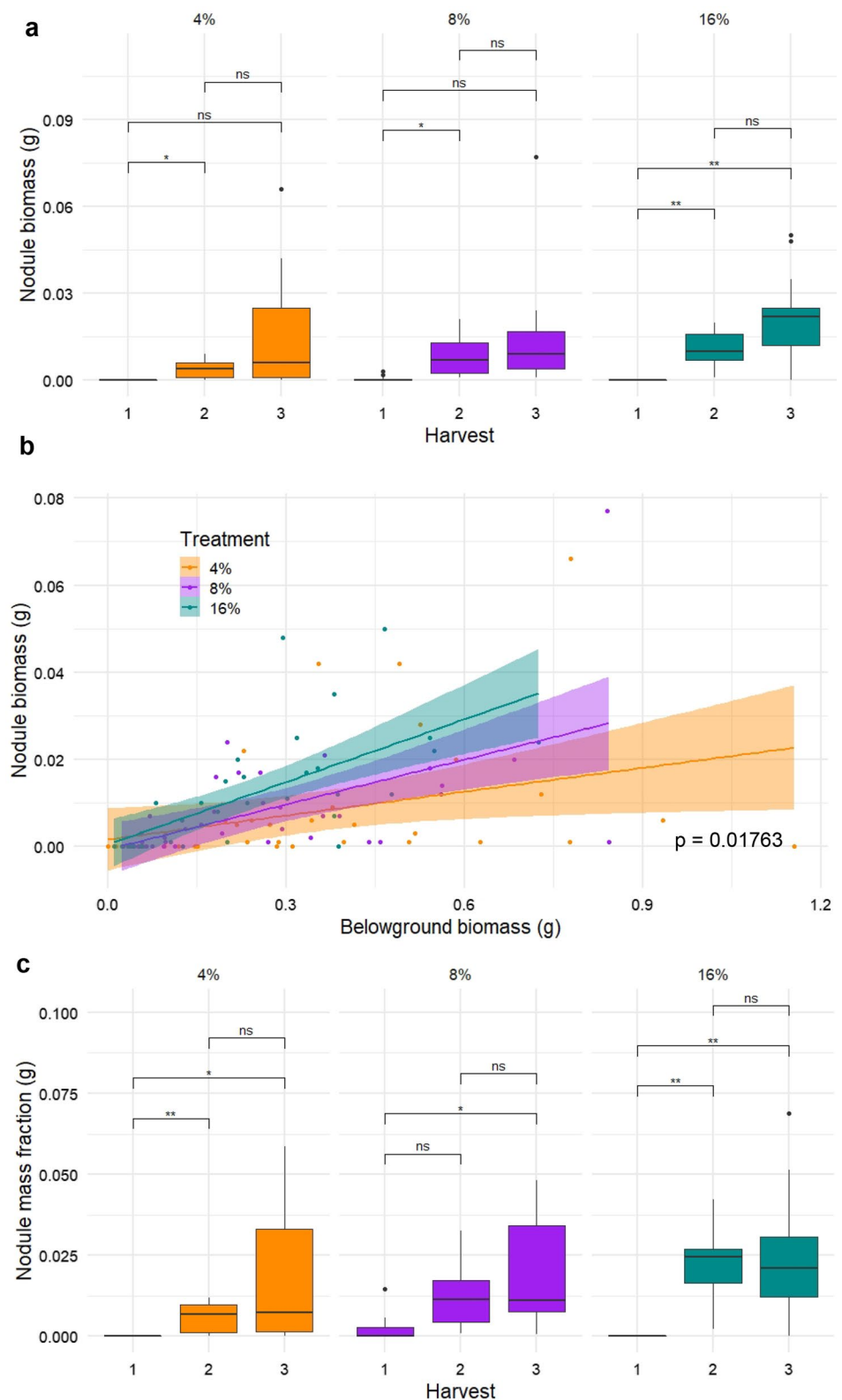
The growth of *V. sieberiana* seedlings increased with a decline in water availability, whereas the growth of *V.*

**Fig. 3** Comparison of how water treatment effected **a** The belowground biomass (grams), **b** The above: belowground biomass ratio, **c** The aboveground biomass (grams) of *Vachellia erioloba* and *Vachellia sieberiana*. All individuals were harvested during Harvest 3 at four months old. The water treatment corresponds to 4%, 8% and 16% soil moisture content. The brackets and numbers represent the p-values for pairwise comparisons across water treatments





**Fig. 4** A comparison of how water treatment effected **a** The nodule biomass (grams) of *Vachellia sieberiana* seedlings at three sequential harvests over a four month period. Harvest 1 occurred when the plants were two month old, Harvest 2 occurred when the plants were three months old, and Harvest 3 occurred when the plants were four months old. Nodules were not present during Harvest 1 for *V. sieberiana* seedlings grown in 4% SMC (orange) and 16% SMC (blue). The brackets and numbers represent the p-values for pairwise comparisons between harvests. **b** The belowground biomass (grams) plotted against the nodule biomass (grams). The p-value was calculated using a linear model to test the effect of water treatment on the relationship between nodule biomass and belowground biomass. **c** The nodule mass fraction of *V. sieberiana* over a four month period. The nodule mass fraction was calculated by dividing the nodule biomass (grams) by whole plant biomass (grams). The data points included are from the nodules produced by *V. sieberiana* across all three harvests. The brackets and numbers represent the p-values for pairwise comparisons between harvests. The zeros pertain to plants from Harvest 1 that did not develop nodules



*erioloba* was unresponsive. Correlations between reduced water availability and increased growth/success has been seen in other *Vachellia* species; rapid above and below-ground growth in *Vachellia tortilis* and *Vachellia raddiana* has been reported during the dry season in Southern Israel (Winters et al. 2018). These two species are known to nodulate (Sprent 2009), and are native to the semi-arid (500–1000 MAP) savanna (Ludwig et al. 2001). In some *Vachellia* species, soil water deficits have been found to lower nutrient absorption due to decreased mobility of nutrients to the root surface (Moura and Vieira 2020). Under drought conditions, slow nutrient diffusion from the soil to the root surface reduces nutrient translocation to leaves (Vieira et al. 2019). Therefore, unless the plant responds with accessing additional sources of N photosynthetic rates and enzymatic activity could decline, resulting in a reduction in growth rates (Moura and Vieira 2020). In addition, it is possible that lower soil water availability may also signal a competitive environment, triggering investment in nodulation to enhance plant growth (Sprent et al. 2010; Foxx and Fort 2019). *V. sieberiana* is native to the mesic savanna, where vegetation structure is driven by competition between grass and trees (Sankaran et al., 2004). In this experiment, the increased growth in low water availability displayed by *V. sieberiana* could be explained by the lack of competition.

As predicted the growth and allometry of *V. erioloba* was unaffected by changing soil moisture. Plant growth rate traits are only one of many elements of species life history strategy. These traits must be considered alongside the ability to survive and reproduce under a range of environmental conditions (Adams et al. 2016). Arguably, flexible growth patterns that increase water uptake under water-stressful conditions would not benefit *V. erioloba* survival in arid environments. Therefore traits favouring slow growth that require limited water are possibly advantageous for persistence (Seymour 2003). In *V. erioloba* a lack of phenotypic plasticity in growth and allocation likely facilitates the growth of deep roots that in arid conditions enables deep water access, and thereby resilience to drought in environments where competition is unlikely to limit growth. The lack of phenotypic plasticity is further demonstrated as tap root construction is favoured even when water availability is not limiting (Seymour 2008; February et al. 2013) and it is a rigid pattern of belowground investment that allows mature individuals of *V. erioloba* to survive in water limited environments (Barnes 2001).

### Does water availability affect nodulation?

Increases in *V. sieberiana* plant height and total biomass in the driest soil conditions (4% SMC) were correlated with an increase in nodulation expressed nodule biomass and

nodule mass fraction. Similar patterns of increasing nodulation with plant biomass have been found in *Albizia saman* and *Leucaena leucocephala* (Azad et al. 2013). An increase in nodule biomass is indicative of increased N<sub>2</sub>-fixing bacteria concentration within the nodules (Gwata et al. 2004). Hence, increased activity likely to correlate with increased available N for plant growth. We propose this pattern of increased height in *V. sieberiana* under lower water availability may be mediated through nodulation. In this species, low water availability possibly signals a belowground competitive environment and further where water stress can result in plant tissue damage that triggers jasmonic acid responses that in turn triggers increased nodulation (Sun et al. 2006; Hause and Schaarschmidt 2009). In some cases, under such conditions, if a plant has sufficient C reserves to maintain the rhizobia mutualism there will be an increase in the N available for growth. Our findings were contrary to our original prediction as movement of rhizobia can be inhibited by a lack of water in the soil (Swaine et al. 2007; Fall et al. 2011) and highlights remaining uncertainties in understanding N<sub>2</sub>-fixation.

*Vachellia sieberiana* develops larger but fewer nodules within a drier environment. We assume the relationship between nodule count and biomass can be explained through the interaction of rhizobia mobility (Swaine et al. 2007; Fall et al. 2011) and root cell wall thickness associated with root plasticity in response to low soil moisture (Mantovani et al. 2015; Chaulagain and Frugoli 2021). *Vachellia sieberiana* has been found to develop a tap root when soil water availability is low (Mugunga and Sahinkuye 2020). Tap roots tend to have a thicker cell wall which can hinder rhizobia infection (Gavrin et al. 2017). This can reduce the number of nodules developed, as rhizobia will opt to infect the root via the already developed nodules (Shetta 2015). Therefore, it is possible that the flexible development of nodules, in response to root plasticity, could mean that drought is less of a limiting factor to seedling recruitment than previously hypothesized for *V. sieberiana* (Case et al. 2020).

### Future research

Future projections suggest southern Africa will experience an increase in extreme weather events, such as drought (IPCC 2022). Our research gives some indication of how the seedling establishment phase of these encroaching species is impacted by water stress. Our results indicate that for an arid species such as *V. erioloba*, whose allometry remains unaffected by lower water availability, a hotter, drier climate may permit an extension of geographical range. However, the ability of *V. sieberiana* plants to nodulate and fix N<sub>2</sub> could provide a competitive advantage in conditions where water availability is low. Some studies have reported *V. sieberiana* is extending to arid areas as a result of vegetation

change (Mugunga and Sahinkuye 2020). However, the relationship between savanna tree cover and water availability is not straight-forward (Sankaran et al. 2005), as water availability interacts with fire and herbivory which can strengthen or weaken recruitment bottlenecks (Bond 2008). Further, the understanding of seedling, sapling and adult survival under prolonged drought is not well understood. We suggest future experiments examining water availability consider the interactive impacts of fire and herbivory on plant growth and nodulation in *Vachellia*. As this will give a more complete picture of the factors that characterise recruitment bottlenecks in savanna woody plants.

Within arid environments *Vachellia* species are thought to establish only in years of above-average rainfall (Seymour 2008), and it is likely changing rainfall will alter establishment and survival (Van Der Merwe et al. 2020). In this experiment, to allow seedlings the opportunity to establish they were exposed to four weeks of continuous watering before treatments were imposed. We suggest that future experiments should identify the minimum watering period for seedling establishment to understand how seedlings will adapt to these climatic changes.

Further, experiments testing the effects of water availability are difficult to execute in-situ as many factors influence how ecosystems experience change in precipitation (Vicca et al. 2012). The most important factor is not the amount of precipitation, but the amount of water that plants can access. This ‘plant available water’ is dependent on soil texture, and rooting depth (Tolk 2003) and estimations of the magnitude is often complicated by runoff water and stem flow (Fall et al. 2011). With this in mind, we suggest that future experiments take plant size into consideration when controlling soil moisture content. Further this experiment would have benefitted from the inclusion of measurements such as the  $^{15}\text{N}$  natural abundance method that has been successfully applied to quantify  $\text{N}_2$ -fixation by legumes (Senthilkumar et al. 2021). We suggest that future experiments incorporate these measurements to provide a more in depth understanding of nodulation.

## Conclusion

Water availability has a wide range of effects on closely related *Vachellia* species relative to their environmental niche and associated growth traits. For *V. sieberiana* to be successful in the mesic environment it requires extended periods of growth to escape fire and compete with grass.

Nodulation could enable a flexible N supply to enhance growth over such time periods. In this experiment the increased nodulation of *V. sieberiana* associated with low water availability suggests nodulation potentially assists the withstanding of low water availability within its environmental niche. This trait could be useful during periods of drought. The growth traits of *V. erioloba*, remained unaffected by lower water availability, potentially attributable to its high tolerance to aridity (Barnes et al. 1997). Perhaps, the measure of success for *V. erioloba* is not rapid growth but being able survive in a water limited environment via methods of belowground investment. These patterns suggest that effective life history strategies for the arid-adapted species can preclude the requirement for a rhizobial mutualism, as typically soil N is relatively high and there are lower competitive interactions (Aranibar et al. 2004). Whereas in the mesic savanna, where soil N is scarce there is higher competitive stress, woody plants may favour nodulation, especially under low water supply that limits root access to soil N (Aranibar et al. 2004; Veldhuis et al. 2016).

## Appendix

See Tables 3, 4, 5, 6, and Fig. 5.

**Table 3** A breakdown of the quantitative values of macro nutrients provided to all seedlings during week one of the experiment. The fertilizer brand name was strake Ayres: Nutrifeed. The plants were provided 5 ml per pot with a concentration of 5 g of feed dissolved in 5 L of water (Starke Ayres 2023)

Element	Analysis
Nitrogen (N)	6.5%
Phosphorous (P)	2.7%
Potassium (K)	13.0%
Calcium (Ca)	7.0%
Magnesium (Mg)	2.2%
Sulphur (S)	7.5%
Iron (Fe)	Trace
Manganese (Mn)	Trace
Boron (B)	Trace
Zinc (Zn)	Trace
Copper (Cu)	Trace
Molybdenum (Mo)	Trace

**Table 4** The output from 2-way ANOVA for testing the effect of water availability treatment and differences on belowground biomass (grams), above: below ground ratio and aboveground biomass (grams) of *Vachellia erioloba* and *Vachellia sieberiana* seedlings that were harvested at four months old (during Harvest 3). Treatment refers to the water availability treatment

Plant trait	Factor	Df	Mean Sq	F value	P value
Belowground biomass (grams)	Treatment	2	0.195	0.786	0.460
	Species	1	14.514	58.620	<b>&lt;0.0001***</b>
	Treatment: Species	2	0.519	2.096	0.130
	Residuals	59	0.248		
Aboveground: Belowground ratio	Treatment	2	0.087	1.473	0.236
	Species	1	8.543	145.038	<b>&lt;0.0001***</b>
	Treatment: Species	2	0.173	2.934	0.060
	Residuals	59	0.059		
Aboveground biomass (grams)	Treatment	2	0.017	0.563	0.572
	Species	1	0.238	7.681	<b>0.001**</b>
	Treatment: Species	2	0.023	0.756	0.473
	Residuals	59	0.031		

P- value significance codes are indicated by: 0 '\*\*\*\*' 0.001 '\*\*\*' 0.01 '\*\*' 0.05 '.' Significance is indicated in bold

**Table 5** The output from 2-way ANOVA for testing the effect of water availability treatment on nodule biomass harvested at two months (Harvest 1), three months (Harvest 2) and four months old (Harvest 3) *Vachellia sieberiana* seedlings. Treatment refers to the water availability treatment

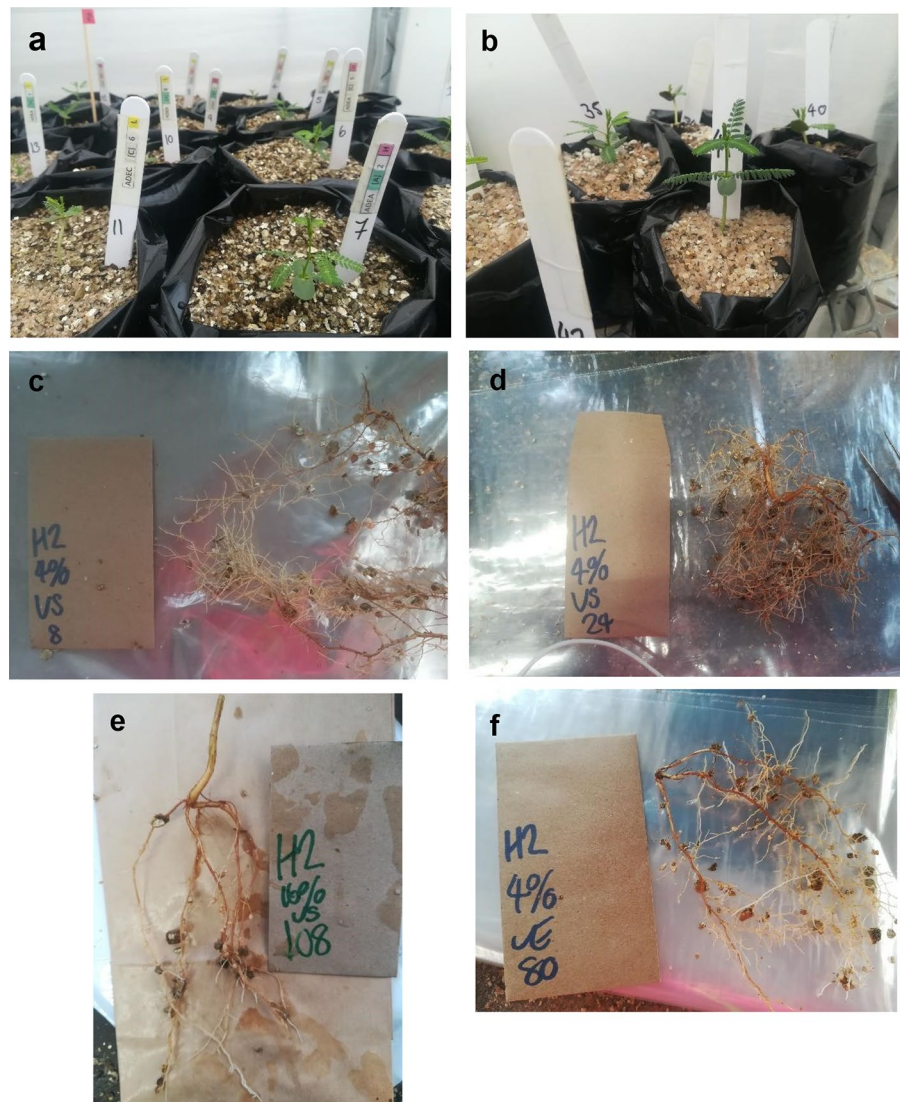
Plant trait	Factor	Df	Mean Sq	F value	P value
Nodule biomass (grams)	Treatment	2	0.000146	1.087	0.341
	Harvest	2	0.00280	20.903	<b>&lt;0.0001***</b>
	Treatment: Harvest	4	0.0000881	0.658	0.623
	Residuals	101	0.000134		
Nodule count	Treatment	2	5.54	7.015	<b>0.0014**</b>
	Harvest	2	97.78	123.914	<b>&lt;0.0001***</b>
	Treatment: Harvest	4	1.71	2.172	0.0774
	Residuals	101	0.79		
Nodule mass fraction (grams)	Treatment	2	0.000488	3.098	<b>0.049*</b>
	Harvest	2	0.00343	21.675	<b>&lt;0.0001***</b>
	Treatment: Harvest	4	0.000203	1.288	0.280
	Residuals	101	0.000158		

P- value significance codes are indicated by: 0 '\*\*\*\*' 0.001 '\*\*\*' 0.01 '\*\*' 0.05 '.' Significance is indicated in bold.

**Table 6** The proportion (%) of *Vachellia sieberiana* seedlings that developed nodules. Treatment refers to the water availability treatment (SMC = soil moisture content). The seedlings were harvested at two months (Harvest 1), three months (Harvest 2) and four months old (Harvest 3). There were 10 replicates per Harvest\*Treatment

	Treatment	Number of plants nodulated	Total nodule biomass (grams)	Total nodule count	Percentage of plants nodulated	Percentage increase between harvests
Harvest 1	4% SMC	0	0.000	0	0%	–
Harvest 1	8% SMC	3	0.006	18	30%	–
Harvest 1	16% SMC	0	0.000	0	0%	–
Harvest 2	4% SMC	8	0.040	120	80%	800
Harvest 2	8% SMC	10	0.090	230	100%	233
Harvest 2	16% SMC	9	0.142	417	80%	900
Harvest 3	4% SMC	10	0.249	379	100%	25
Harvest 3	8% SMC	10	0.215	571	100%	0
Harvest 3	16% SMC	10	0.239	658	100%	11

**Fig. 5** A selection of photos taken throughout the experiment **a** is a *Vachellia sieberiana* seedling at 3 weeks old **b** is a *V. sieberiana* seedling at 6 weeks old grown in 16% soil moisture content (SMC) water treatment. **(c)** Is the root network of a *V. sieberiana* seedling grown in 4% SMC. **d** The root network of a *V. sieberiana* seedling grown in 4% SMC. **e** The root network of a *V. sieberiana* seedling grown in 16% SMC **(f)** is the root network of *Vachellia erioloba* grown in 4% SMC



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**Data Availability** See online resources and Github folder: [https://github.com/Elizabeth261191/Telford\\_et\\_al\\_2022](https://github.com/Elizabeth261191/Telford_et_al_2022)

## Declarations

**Conflict of interest** The authors declare that there is no conflict of interest.

**Ethical approval** This study was approved by University of Edinburgh School of GeoScience Research Ethics & Integrity Committee.

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