

Soil nitrogen regulates symbiotic nitrogen fixation in a legume shrub but does not accumulate under it

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Abstract. Legumes in dryland ecosystems face the challenge of maintaining energetically costly symbiosis with N₂-fixing rhizobia, in a water- and nutrient-limited environment. Controlled experiments showed a strong reduction in symbiotic N₂ fixation in response to elevated levels of nitrogen availability, but this regulation of N₂ fixation was not found in dryland field settings. Here, we ask whether regulation of N₂ fixation occurs in the field and what are the possible consequences for dryland soil nitrogen. We measured plant investment in root nodules and rates of bacterial activity, in seedlings and adults of a common N₂-fixing shrub, *Calicotome villosa*, in five field sites naturally varying in soil nitrogen and phosphorus availabilities. Additionally, we measured nitrogen concentrations and availabilities in the soil under *C. villosa* and a reference non-fixing shrub. Biomass investment in root nodules was significantly reduced in response to elevated levels of soil nitrate in adult shrubs, but no such effect was found for seedlings. Soil nitrogen concentration under *C. villosa* shrubs was low compared to the soil under a non-fixing reference shrub. We provide evidence that symbiotic N₂-fixing plants in drylands tightly regulate their investment in symbiotic N₂ fixation, therefore controlling nitrogen inputs into the soil. These findings challenge the long-held notion that the growth of N₂-fixing plants necessarily leads to the accumulation of soil nitrogen in their surroundings over time.

Key words: *Calicotome villosa*; dryland; ecosystem; Fabaceae; facultative strategy; Mediterranean; nodulation; soil.

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INTRODUCTION

Symbiotic dinitrogen (N₂) fixation in plants (hereafter N₂ fixers) is the main natural mechanism by which bio-available nitrogen enters terrestrial ecosystems (Cleveland et al. 1999, Vitousek et al. 2013). The presence of actively N₂-fixing plants in an ecosystem, in theory, should diminish soil nitrogen deficiency over time, by continuous recycling of the nitrogen-rich litter that is characteristic of N₂-fixing plants (McKey 1994). As a consequence, N₂ fixers may become competitively disadvantaged in soils with sufficient nitrogen availability due to the

high cost of maintaining symbiotic N₂ fixation (Gutschick 1981). Yet, in many tropical forests, N₂ fixers are present and sometimes abundant even when soil nitrogen is not limiting, which invoked a paradox in the understanding of symbiotic N₂ fixation (ter Steege et al. 2006, Hedin et al. 2009, Batterman et al. 2013a).

In the pursuit of resolving this paradox, studies using a combination of models and field observations have provided evidence of a suite of strategies by which N₂-fixing plants regulate fixation, in response to naturally occurring availability of nitrogen in the soil (Hedin et al. 2009, Menge et al. 2009). A strategy of investment in

fixation regardless of soil nitrogen availability (i.e., no regulation) was defined as “obligate,” as observed in actinorhizal plants in temperate and boreal forests (Menge and Crews 2016). Alternatively, a strategy of regulation of the investment in fixation in response to naturally occurring availability of soil nitrogen was defined as “facultative” (i.e., fixing when nitrogen availability is low and downregulating fixation when soil nitrogen is sufficient; Menge et al. 2009). The facultative strategy has been proposed as an explanation for the persistence of N₂-fixing plants in the nitrogen-rich soils of tropical forests (Menge et al. 2009). Recent studies show a spectrum of the degree of regulation of symbiotic fixation in response to soil nitrogen rather than a binary strategy (Menge et al. 2015, Taylor and Menge 2018, Dovrat et al. 2020). However, so far, most of the studies that attempted to detect regulation of symbiotic N₂ fixation used controlled experiments (Batterman et al. 2013b, Menge et al. 2015, Dovrat et al. 2018), with only one such study performed in a field setting of a Neotropical forest (Barron et al. 2011).

In dryland ecosystems, water limitation influences both the performance of plants and bacterial processes such as nitrogen fixation, litter decomposition, and mineralization, thus constraining the supply of available soil nitrogen. Under water-limited conditions, symbiotic N₂ fixation potentially is constrained also by the limited availability of other soil nutrients such as phosphorus and iron (Bremer et al. 1988, Henkin et al. 2007, Austin 2011, Vitousek et al. 2013, McCulloch et al. 2021), which may consequently limit the abundance of N₂ fixers. Despite these limitations, both annual and perennial legumes are highly diverse and abundant in many drylands, in climates varying from arid desert to mesic Mediterranean, and most of these legumes are potential N₂ fixers (Hely and Ofer 1972, Agnew and Zohary 1974, Sprent and Gehlot 2010, Dovrat et al. 2018, 2020). Furthermore, water-limited conditions might benefit the survival of N₂-fixing legumes over non-fixing plants throughout succession, compared to the decreasing abundance of N₂-fixing legumes in late successional wet tropical forests (see also Adams et al. 2016, Minucci et al. 2017, Gei et al. 2018). Therefore, symbiotic N₂ fixation by legumes in dryland ecosystems potentially plays an

important role in the nitrogen cycle. However, the effects of dryland N₂ fixers on the content of nitrogen in the soil at the local and ecosystem scales remain unresolved.

Originally, models describing the various strategies of regulation of N₂ fixation were mainly concerned with N₂ fixers from temperate and tropical forests (Menge et al. 2009, Sheffer et al. 2015). Recently, controlled experiments showed that most of the common perennial legumes in dryland habitats employ a facultative strategy of regulation of fixation. The most common of these species, *Calicotome villosa* Poir., also showed a strong seasonal pattern of fixation, corresponding with the availability of water in the soil (Dovrat and Sheffer 2018, Dovrat et al. 2018). Dovrat and Sheffer (2018), further found significant differences in the regulation of symbiotic fixation between *C. villosa* seedlings and adults, which they attributed to the greater nitrogen demand and energetic constraints of young plants resulting in strict regulation of fixation in the first year. However, field measurements of fixation in *C. villosa* provided no conclusive evidence for facultative regulation of fixation under naturally varying availabilities of soil nitrogen.

To address the above-mentioned gaps—the influence of dryland N₂ fixers on soil nitrogen availability and occurrence of facultative regulation of fixation in the field—we studied symbiotic N₂ fixation in a range of field sites, using *C. villosa* as a model perennial N₂ fixer. The N₂-fixing shrub *C. villosa* is widespread around the Mediterranean Basin and is abundant in habitats with a wide range of soil types and disturbance histories and across a large range of rainfall conditions (Agnew and Zohary 1974, Henkin et al. 2007). Shrubs are summer deciduous, allowing them to endure the long dry summer typical of the Mediterranean climate while maintaining photosynthesis in their green stems (Yiotis et al. 2008).

Our goals in this study were as follows: (1) to evaluate whether facultative regulation of fixation occurs within the natural range of soil nitrogen availability, (2) to examine the patterns of regulation of N₂ fixation both in seedlings and adult shrubs, and (3) to evaluate whether a dominant nitrogen-fixing shrub influences soil nitrogen availability in dryland ecosystems. To test the effects of naturally varying levels of soil

nitrogen and phosphorus availabilities on plant investment in symbiotic N_2 fixation by *C. villosa*, we evaluated fixation in five adjacent field sites, chosen to represent different conditions in terms of soil nitrogen and phosphorus availabilities. In all sites, we assessed plant nodulation (biomass allocation to root nodules) and within-nodule rate of bacterial activity in naturally growing *C. villosa* shrubs, as well as the availability of soil nitrogen and phosphorus. We hypothesized that (1) plant investment in symbiotic N_2 fixation will decrease with increasing availability of soil nitrogen (Dovrat and Sheffer 2018), (2) regulation will be stronger in seedlings compared to adults shrubs, (3) plant investment in symbiotic N_2 fixation will increase with increasing availability of soil phosphorus (Wurzbarger et al. 2012, Batterman et al. 2013b), and (4) the concentration of nitrogen in the soil under *C. villosa* shrubs will be high compared to the soil under non-legume plants, due to the high foliar nitrogen concentration and low nitrogen use efficiency of the nitrogen-fixing shrub (Grünzweig et al. 2010).

MATERIALS AND METHODS

Study sites and shrub selection

We selected five adjacent study sites located in the Judean Mountains, Israel, that differ in land use and fire history (Appendix S1: Table S1). The variety of site histories was likely to result in a range of soil nitrogen and phosphorus availabilities as well as shrubs at different developmental stages. All the sites are on north-facing slopes with Terra-Rossa soil (Chromic Luvisols, FAO) on dolomite bedrock.

The area of each site is about 0.01 km², and all five sites are confined within a 5 km² area, minimizing spatial heterogeneity and reducing the possibility of weather events affecting one site and not the other (Appendix S1: Fig. S1). Given the time since the last fire, two sites included seedlings of *C. villosa* only (one- to two-year-old plants) and no adult shrubs and the three remaining sites included mostly adult shrubs (up to 15-year-old plants). Seedling recruitment in these ecosystems is very slow (except following fire); thus, it was impractical to locate seedlings in the latter three sites.

At each site, we randomly selected either ten seedlings or twenty adult shrubs of similar sizes

and measured their height and perimeter, which we later used to calculate total shoot biomass using an allometric equation (Dovrat and Sheffer 2018).

Measurements of N_2 fixation in the field

We estimated plant investment in root nodules for symbiotic fixation (nodulation) and the momentary rate of bacterial activity within nodules in all selected shrubs in the peak season of fixation in *C. villosa* (February 2019, according to Dovrat and Sheffer 2018). We carefully excavated the central root system of each plant, searching for nodules attached to the roots or detached in the soil. Since excavating the entire root system is impractical in these rocky field sites, we used a standard sampling effort confined to the root-zone area under the canopy of each shrub. Based on personal experience and observations, both in the field and in pot experiments, the vast majority of nodules are located at or close to the taproot and close to the surface.

The nodules that were found first (1–2 nodules per plant) were immediately placed in a sealed jar for analysis of the momentary rate of bacterial activity. We collected an additional volume of six liters of shallow soil (0–10 cm) from around the base of each shrub. When no nodules were found in the field after a standardized search effort (up to 20 min), we moved on to the next plant. In the laboratory, we meticulously searched for all nodules in the soils collected under each shrub. In total, we excavated 19–21 adult shrubs per site in the three adult sites and 12–13 seedlings per site in the two seedling sites.

We used the first nodules that we found from each shrub to evaluate the momentary rate of bacterial activity within nodules, using acetylene reduction assay (ARA, Hardy et al. 1968). While the validity of this method has been criticized for providing inaccurate values of nitrogen fixation (due to factors such as nodule O₂ limitation and plant disturbance; Minchin et al. 1986, Witty and Minchin 1988), we use ARA only as a comparative measure of the activity of the bacteria hosted in the root nodules at the different sites (Vessey 1994). We incubated the nodules that were placed in the jars with 10% of the air replaced by acetylene (from a tank) in a dark cooler and analyzed ethylene (C₂H₄) production after 30 and 60 min using a gas chromatograph (Focus GC;

Thermo Fisher Scientific, Waltham, Massachusetts, USA). For each set of ARA incubations, control incubations with acetylene and without nodules were used to measure background rates of spontaneous conversion of acetylene to ethylene.

After ARA incubation, all nodules were cleaned, cut open to check viability (indicated by a pink color inside), oven-dried at 60°C for 48 h, and weighed. We used the biomass of incubated nodules to calculate the rate of bacterial activity within nodules as the rate of ethylene produced per gram of incubated nodule biomass ($\mu\text{mol C}_2\text{H}_4 \cdot \text{h}^{-1} \cdot \text{g}^{-1}$ nodule), and the biomass of all nodules found per plant to calculate nodule mass fraction as the proportion of biomass allocated to nodule construction out of total shoot biomass.

Soil nitrogen and phosphorus sampling

We sampled the soil in all study sites at the peak of the growing season (February 2019) and sites with adult shrubs also at the end of the dry season (October 2019), to evaluate the concentrations of nitrogen and phosphorus. Only samples taken at the end of the dry season were considered when estimating the long-term effects of *C. villosa* shrubs on soil nitrogen because at this time the cycling and transformations of soil nitrogen are minimal and thus soil nitrogen is relatively stable and representative of long-term conditions (Robertson et al. 1999). Within each site, we defined three types of soil patches: inter-shrub soil typically covered by herbaceous vegetation during the growing season (herbaceous patch), soil under an N_2 -fixing shrub (*C. villosa* patch), and soil under a reference non-fixing shrub (*Rhamnus lycioides* patch). The soil under the non-fixing reference plant was used to test a general enrichment of the soil caused solely by the presence of a perennial shrub, that is, to exclude an “island of fertility” effect (Schlesinger et al. 1996).

On each sampling campaign, two replicate pooled samples were collected from each patch type per site to characterize the mean soil conditions instead of the local spatial heterogeneity. Each pooled sample was composed of five soil samples collected by first removing surface plant litter and organic matter and collecting the top 10 cm of soil. For shrub samples, this included soils from five different shrubs, and for herbaceous samples, five different inter-shrub patches. Soils from all five samples were then thoroughly mixed. From each

soil pool, we took two-three technical subsamples for extraction in a 2 mol/L KCl solution to estimate the concentration of inorganic nitrogen (Maynard et al. 1993). Nitrate and ammonium concentrations in extracted soil solutions were measured colorimetrically (Norman and Stucki 1981). This amounted to a total of six representative samples per patch type (i.e., two per site) used for the calculation of soil nutrient availabilities.

In the laboratory, we extracted from each soil sample two technical subsamples with 0.5 mol/L sodium bicarbonate at pH 8.5, to estimate the concentration of available phosphorus using the P-Olsen colorimetric method (Olsen and Sommers 1982). The remaining soils were oven-dried at 60°C for 72 h and milled to powder using a mixer mill. We measured the concentration of organic carbon (C) and total nitrogen (including all organic and inorganic forms) using an elemental analyzer (FLASH 2000; Thermo Scientific), after removing calcareous carbonates from soil samples with HCl (Harris et al. 2001).

We used Western Ag PRS[®] root simulating probes to estimate the fluxes of available soil anions and cations from the beginning of the rainy season to the peak of the season (100 d) when fixation was measured. In each site, we inserted four-probe couples into the shallow soil (0–10 cm depth) in five locations (both under *C. villosa* shrubs and in inter-shrub open areas) to capture local variability.

Statistical analyses

We defined soil nitrogen and phosphorus availabilities as ordinal predictors since they were measured at the site level and therefore could not be used for standard regression analysis. We then analyzed the effects of three ordinal levels of soil nitrogen or phosphorus in the three sites with adult shrubs on the rate of bacterial activity within nodules and nodule mass fraction using the Jonckheere-Terpstra test for ordinal predictors (Jonckheere 1954). The Jonckheere-Terpstra is a non-parametric analysis of variance test, designed specifically to account for the effects of ordered variables. We conducted separate analyses for the effects of soil nitrogen and soil phosphorus since these are congruent measurements for each site. Plants for which no nodules were found were excluded from these statistical analyses while maintaining a

minimum of ten replicates per treatment (in one site $n = 9$). All statistical analyses were performed using R (R Core Team 2018), and figures were created using ggplot2 (Wickham 2016).

RESULTS

*Symbiotic N_2 fixation in *C. villosa* adult shrubs*

We found a decrease in plant investment in nodulation with increasing availability of soil

nitrate and increasing nitrate flux (Fig. 1a, b; Table 1). A 2.5-fold difference in soil nitrate availability (1.23 and $3.16 \mu\text{g NO}_3^- \text{g}^{-1}$ soil) resulted in a twofold difference in the nodule mass fraction of adult shrubs which was higher in the site with lower soil nitrate ($0.0027 \pm 0.0013\%$ and $0.0055 \pm 0.002\%$, respectively). A 1.5-fold higher availability of soil phosphorus resulted in a twofold difference in the estimated rates of bacterial activity within nodules (15.17 ± 2.46 and

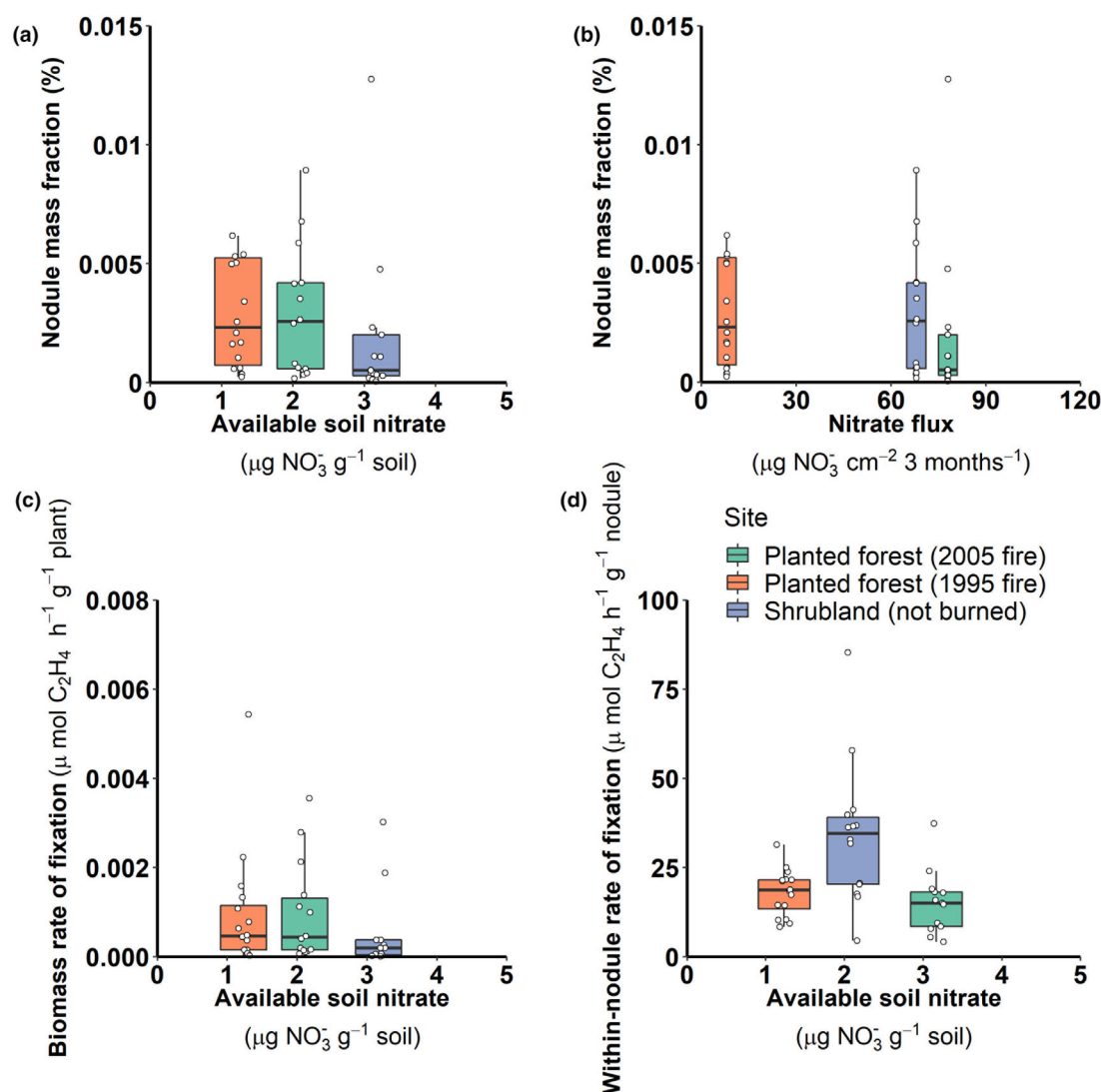


Fig. 1. Effects of soil nitrate availability and flux on adult *C. villosa* shrub nodulation (nodule mass fraction; (a, b), biomass rate of fixation (c), and within-nodule rate of fixation (d). Different colors of the bars indicate the different sites. Bars in boxplots are the interquartile range (IQR) (25th(Q1) to the 75th percentile(Q3)), lines inside bars are the median.

Table 1. Results of Jonckheere-Terpstra tests for the effects of soil inorganic nitrogen and phosphorus availabilities on fixation measures in *C. villosa* adult shrubs.

Dependent variable	Independent variable	<i>P</i>	Alternative hypothesis
Nodule mass fraction (%)	Available NO ₃ ⁻	0.015	Decreasing
	Nitrate flux	0.015	Decreasing
	Available NH ₄ ⁺	n.s.	Decreasing
	Available PO ₃ ⁻	0.034	Increasing
	Phosphorus flux	0.034	Increasing
Within-nodule rate of fixation (μmol C ₂ H ₄ ·h ⁻¹ ·g ⁻¹ nodule)	Available NO ₃ ⁻	n.s.	Decreasing
	Nitrate flux	n.s.	Decreasing
	Available NH ₄ ⁺	n.s.	Decreasing
	Available PO ₃ ⁻	0.0002	Increasing
	Phosphorus flux	0.0002	Increasing
Biomass rate of fixation (μmol C ₂ H ₄ ·h ⁻¹ ·g ⁻¹ plant)	Available NO ₃ ⁻	n.s.	Decreasing
	Nitrate flux	n.s.	Decreasing
	Available NH ₄ ⁺	n.s.	Decreasing
	Available PO ₃ ⁻	0.036	Increasing
	Phosphorus flux	0.036	Increasing

Notes: The null hypothesis is that the population medians are equal (i.e., $H_0 : \theta_1 = \theta_2 = \dots = \theta_k$), and the alternative hypothesis can be that the medians are either decreasing or increasing with increasing order of the predictors (i.e., $H_1 : \theta_1 \geq \theta_2 \geq \dots \geq \theta_k$) with at least one strict inequality.

$34.14 \pm 5.3 \mu\text{mol C}_2\text{H}_4\cdot\text{h}^{-1}\cdot\text{g}^{-1}$ nodule), and almost twofold difference in biomass-specific fixation rates ($5 \times 10^{-5} \pm 2 \times 10^{-5}$ and $9 \times 10^{-5} \pm 3 \times 10^{-5} \mu\text{mol C}_2\text{H}_4\cdot\text{h}^{-1}\cdot\text{g}^{-1}$ plant; Fig. 2; Table 1). However, we found no effect of soil nitrate on the estimated rates of bacterial activity within the nodules. No significant response was detected for any of these variables to the availability of ammonium in the soil (Appendix S1: Fig. S2).

Symbiotic N₂ fixation in *C. villosa* seedlings

Within-nodule rates of bacterial activity in seedlings were more than twofold higher in the site with higher phosphorus availability in the soil (22.02 ± 7.18 compared to $46.03 \pm 6.52 \mu\text{mol C}_2\text{H}_4\cdot\text{h}^{-1}\cdot\text{g}^{-1}$ nodule), similar to the pattern found in adults (Fig. 3i; Table 2). However, in contrast to adult shrubs, we found that the rate of bacterial activity in the nodules of seedlings was also influenced by the availability of soil ammonium and nitrate. The rate of bacterial activity within seedling nodules was twofold lower in the site with higher availability of soil ammonium and nitrate (Fig. 3c, f; Table 2). There was no significant difference in either nodule mass fraction or biomass rate of bacterial activity of seedlings between the two sites sampled (Fig. 3; Table 2).

Soil nitrogen distribution

The availability of inorganic nitrogen in the soil (ammonium and nitrate) was not significantly different between *C. villosa* and herbaceous patches, both in the mid-rainy season and at the end of the dry season. At the end of the dry season, total nitrogen (organic + inorganic) concentration in soil patches under *R. lycioides* shrubs was 35% greater than in either herbaceous or *C. villosa* patches ($F = 20.72$, $P < 0.0001$). We found no significant differences between the patch types in the availabilities of either soil ammonium or nitrate (Fig. 4). Site-specific results of soil nutrient measurements can be found in Appendix S1: Table S1.

DISCUSSION

The main aim of this study was to determine whether the strategy of facultative regulation of fixation, previously shown mainly in controlled experiments, occurs also in natural field settings. We found that plant nodulation was low in adult plants growing in sites with higher availability of soil nitrate compared to sites with lower soil nitrate, while estimates of the within-nodule rate of fixation showed a parallel negative response to increased availability of soil nitrogen only in seedlings. The rate of bacterial activity within

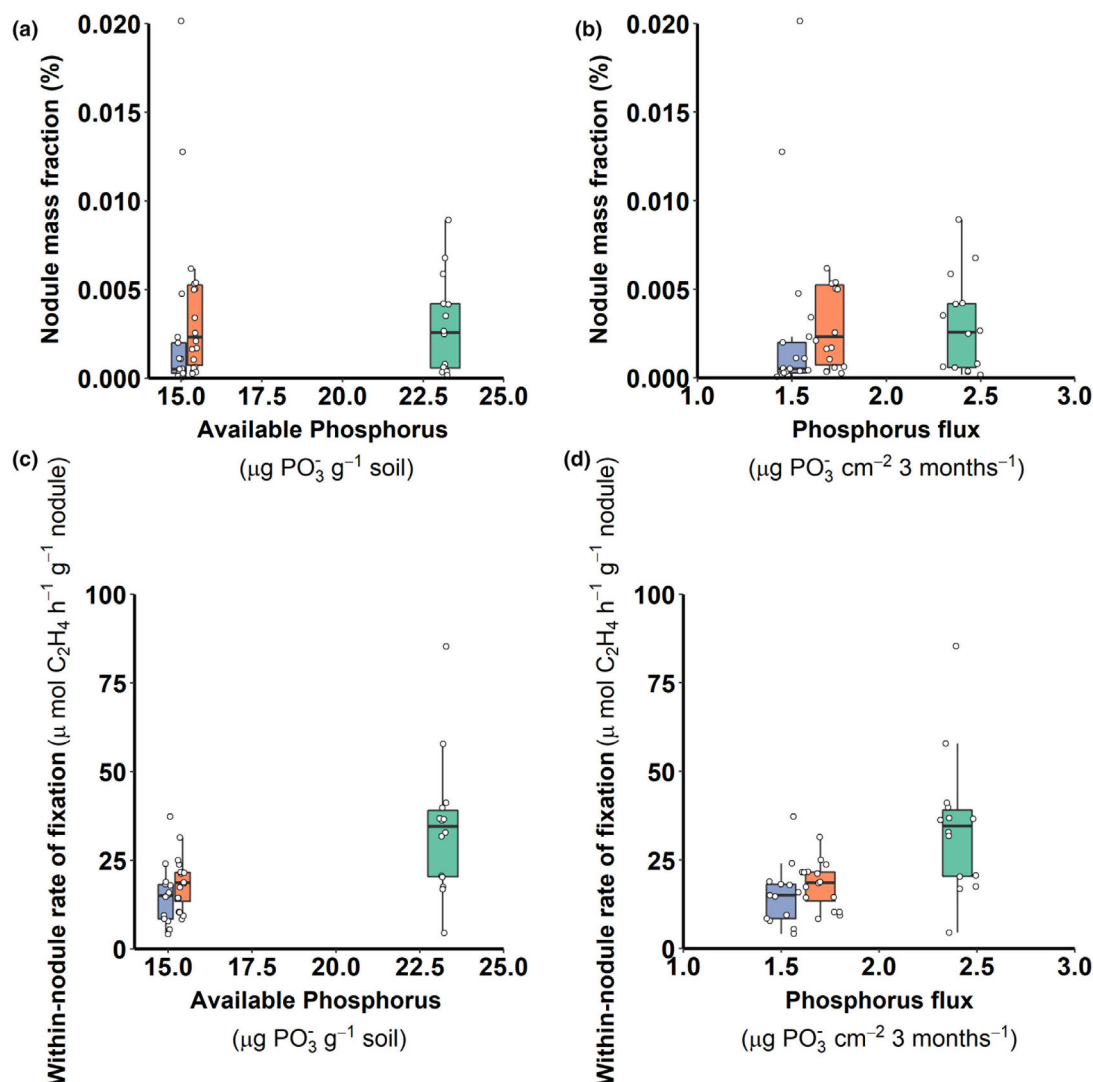


Fig. 2. Effects of soil phosphorus availability and flux on adult *C. villosa* shrub nodulation (nodule mass fraction; (a, b), biomass rate of fixation (c), and within-nodule rate of fixation (d). Different colors of the bars follow coloring for sites in Fig. 1. Bars in boxplots are the interquartile range (IQR) (25th(Q1) to the 75th percentile(Q3)), lines inside bars are the median.

nodules was positively associated with soil phosphorus availability, with a more pronounced response in seedlings.

The second aim of the current study was to evaluate the possible long-term effects of the presence of a perennial N_2 fixer on the concentration of nitrogen in the soil. We found an opposite pattern, whereby the concentration of total nitrogen in the soil was low and similar under *C. villosa* and neighboring soil patches that are not

covered by a perennial plant, compared to the soil under a local non-fixing shrub.

Nodulation is regulated by soil nitrate availability

We found a significant negative correlation between nodule mass fraction of adult shrubs and soil nitrate availability only and no clear response of nodulation to soil ammonium or total nitrogen availability. This result is in agreement with one of the few and first studies

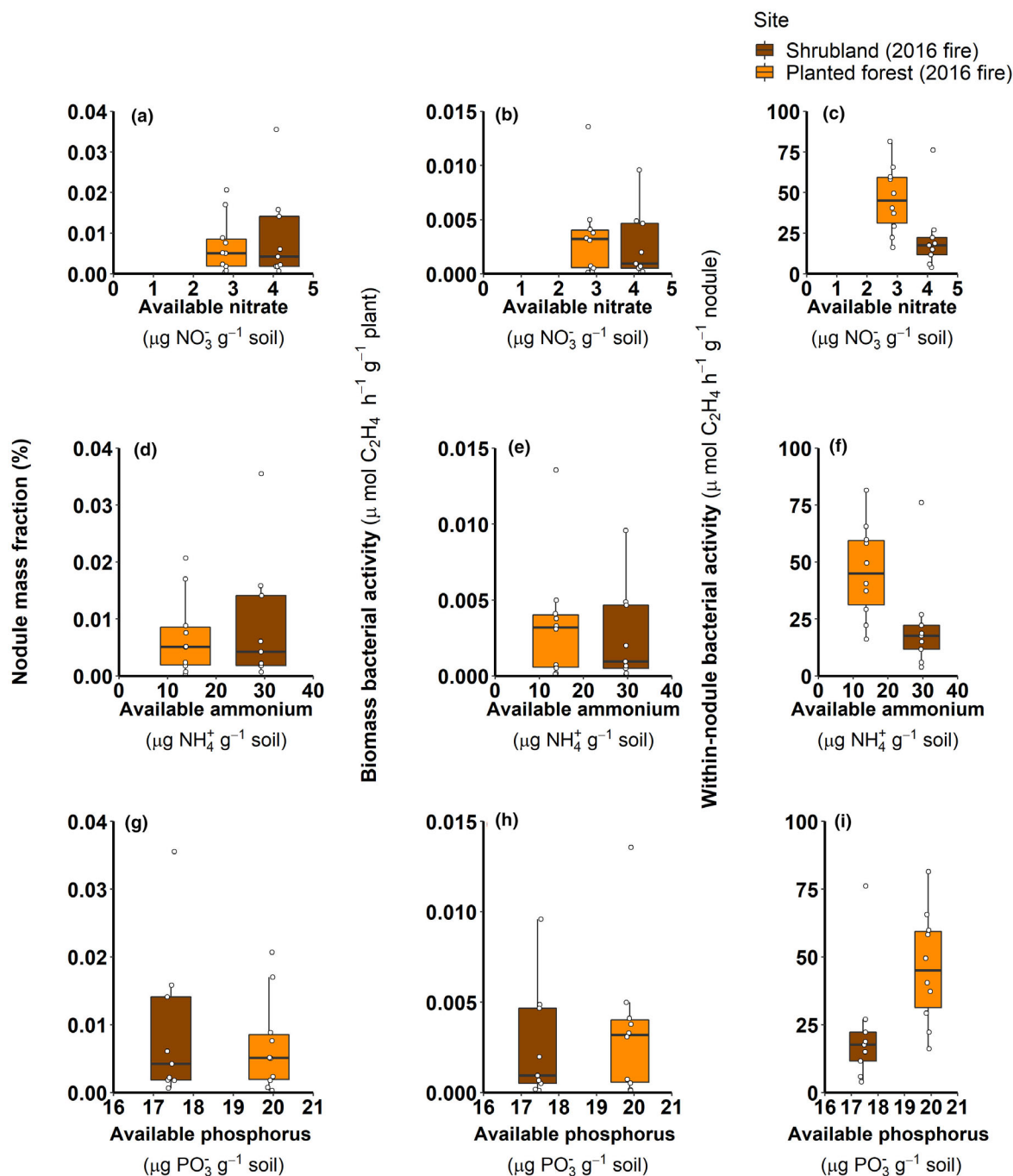


Fig. 3. Effects of soil nitrate, ammonium, and phosphorus availability on *C. villosa* seedling nodulation (nodule mass fraction; a, d, g, respectively), biomass bacterial activity (b, e, h, respectively), and within-nodule bacterial activity (c, f, i, respectively). Different colors of the bars indicate the different sites. Bars in boxplots are the interquartile range (IQR) (25th(Q1) to the 75th percentile(Q3)), lines inside bars are the median.

Table 2. Results of Jonckheere-Terpstra test for the effects of soil inorganic nitrogen and phosphorus availabilities on fixation measures in *C. villosa* seedlings.

Dependent variable	Independent variable	<i>P</i>	Alternative hypothesis
Nodule mass fraction (%)	Available NO_3^-	n.s.	Decreasing
	Available NH_4^+	n.s.	Decreasing
	Available PO_3^-	n.s.	Increasing
Within-nodule rate of fixation ($\mu\text{mol C}_2\text{H}_4\cdot\text{h}^{-1}\cdot\text{g}^{-1}$ nodule)	Available NO_3^-	0.005	Decreasing
	Available NH_4^+	0.005	Decreasing
	Available PO_3^-	0.005	Increasing
Biomass rate of fixation ($\mu\text{mol C}_2\text{H}_4\cdot\text{h}^{-1}\cdot\text{g}^{-1}$ plant)	Available NO_3^-	n.s.	Decreasing
	Available NH_4^+	n.s.	Decreasing
	Available PO_3^-	n.s.	Increasing

Notes: The null hypothesis is that the population medians are equal (i.e., $H_0: \theta_1 = \theta_2 = \dots = \theta_k$), and the alternative hypothesis can be that the medians are either decreasing or increasing with increasing order of the predictors (i.e., $H_1: \theta_1 \geq \theta_2 \geq \dots \geq \theta_k$) with at least one strict inequality.

showing facultative downregulation of fixation in the field, where nodulation was low in sites with increasing concentrations of soil nitrate in legume trees (*Inga* sp.) in a tropical forest (Barron et al. 2011). Furthermore, molecular studies show the role of soil nitrate in the pathways of nodule formation and regulation of nodulation (Streeter and Wong 1988, Ruffe et al. 2008, Ferguson et al. 2019).

In contrast to adult shrubs, we did not detect any differences in nodulation between the seedlings in the two study sites. Nodule mass fraction was generally higher for seedlings compared to adults, in agreement with controlled studies that compared plant investments in fixation at different life stages (Jacobs et al. 2007, Dovrat and Sheffer 2018). The nodule mass fraction values in the studied seedlings in the field resembled those of seedlings grown with high availability of nitrogen (ammonium-nitrate) in controlled conditions ($0.7 \pm 0.2\%$ and $0.3 \pm 0.1\%$, respectively; Dovrat et al. 2018). Seedlings grown without added nitrogen supply in controlled experiments had much higher nodule mass fractions ($4.4 \pm 0.5\%$ in Dovrat and Sheffer 2018, and $3.44 \pm 0.43\%$ in Alon et al. unpublished results).

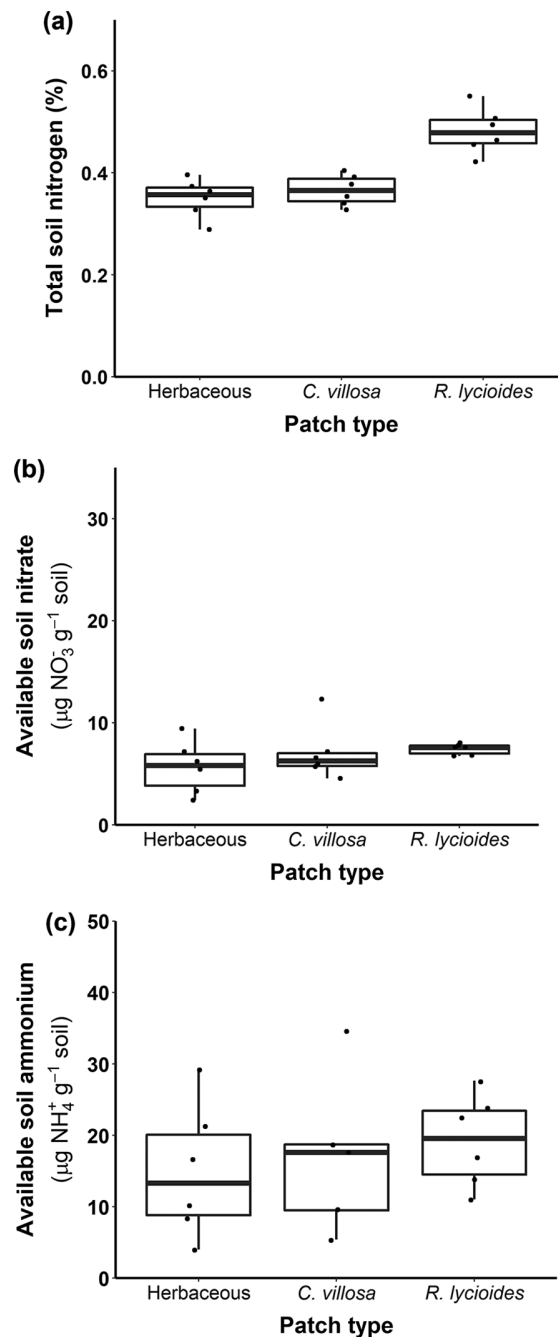


Fig. 4. Total soil nitrogen (inorganic + organic) (a), nitrate (b), and ammonium (c) availabilities in soils of the three sampled patch types (herbaceous, *C. villosa* (fixer), and *R. lycioides* (non-fixer)) at the end of the dry season. Bars in boxplots are the interquartile range (IQR) (25th (Q1) to the 75th percentile (Q3)), lines inside bars are the median.

The low values of nodule mass fraction that we found in the field, therefore, suggest that *C. villosa* seedlings were not strongly limited by soil nitrogen in these field sites, or that fixation was limited by other environmental factors. Consistently, the availability of soil nitrate was higher in the two early-successional sites with seedlings compared to the lower availability of soil nitrate in the three sites with adult shrubs.

Within-nodule bacterial activity rates

We found lower rates of acetylene reduction within nodules in sites with higher soil nitrogen availability (for both nitrate and ammonium) only in the seedlings. This result reinforces the findings of the two previous studies in *C. villosa*, showing strong downregulation of within-nodule bacterial activity rates with increasing availability of soil nitrogen (Dovrat and Sheffer 2018, Dovrat et al. 2018). *Calicotome villosa* seedlings must maintain a strict nitrogen budget while conserving other resources (e.g., photosynthesis products and water), as this is critical especially at the early stages of establishment in nitrogen-deficient dryland ecosystems (Schlesinger et al. 2006, LeBauer and Treseder 2008). However, since soil nitrogen and phosphorus availabilities were correlated, we could not separate the effects of either nutrient on within-nodule bacterial activity rates.

In contrast to the availability of soil nitrogen, we found a strong and positive correlation between the rate of bacterial activity within nodules and the availability of phosphorus in the soil for both age groups, as known for many different legume species, and in a variety of ecosystems (Uliassi and Ruess 2002, Power et al. 2010, Wurzbacher et al. 2012, Vitousek et al. 2013, Ament et al. 2018). Phosphorus limitation on bacterial activity rates may be exacerbated by the ongoing increase in atmospheric CO₂ concentrations (Trerweiler et al. 2018). We, therefore, suggest that in these sites, within-nodule bacterial activity rates are affected primarily by soil phosphorus availability, rather than soil nitrogen. Differences in within-nodule rates of bacterial activity may also be related to a heterogeneous composition of rhizobial partners in the different soil communities of our study sites (Rodríguez-Echeverría et al. 2003).

Effect of C. villosa on soil nitrogen

The presence of symbiotic N₂-fixing plants in an ecosystem is often associated with a gradual increase in soil nitrogen concentration (Vitousek and Walker 1989, Bellingham et al. 2001, Hughes and Denslow 2005, Goergen and Chambers 2009, Mitchell and Ruess 2009). Yet, we found that the concentration of nitrogen in the soil was low under adult N₂-fixing shrubs compared to soils under an abundant, similarly structured yet non-fixing shrub. Furthermore, both in the middle of the rainy season and at the end of the dry season, the availability of inorganic nitrogen in the soil under *C. villosa* shrubs was similar to neighboring herbaceous patches. Finding no significant addition of nitrogen to the soil by *C. villosa* was shown before (Grünzweig et al. 2010, Dovrat and Sheffer 2018), but here we also exclude a possible “island of fertility” effect by comparing to the reference non-fixer (Schlesinger et al. 1996, Moro et al. 1997).

The high concentration of soil nitrogen under the reference shrub, *R. lycioides*, was likely enhanced by a relatively high foliar nitrogen concentration in this shrub compared to other local non-fixers ($2.16 \pm 0.05\%$, mean $\pm$ SE; Loshkovoy, unpublished data). Yet, nitrogen concentrations in *C. villosa* leaves ranging 3–4.7% in our sites were higher than most non-legume shrubs and, on average, twofold higher than *R. lycioides*. One potential explanation for the limited effect of *C. villosa* on the nitrogen cycle in its vicinity is the small amount of leaf litter shed by *C. villosa* annually at the onset of summer (as much as 2.5 times lower compared to neighboring Mediterranean shrubs and trees; Grünzweig et al. 2010), in addition to the resorption of nitrogen from the leaves before shedding them in late spring (Grünzweig et al. 2010, Dovrat and Sheffer 2018). Moreover, the diversity and biomass of herbaceous plants are relatively high under *C. villosa* shrubs, compared to neighboring shrubs, due to its open canopy and deciduousness (Grünzweig et al. 2010), although herbaceous biomass under *C. villosa* did not harbor more nitrogen compared to similar vegetation that did not grow under *C. villosa* (Dovrat and Sheffer 2018).

Unexpectedly low levels of total nitrogen concentration in the soil under *C. villosa* probably are also related to the facultative N₂-fixation strategy of these shrubs. In theory, facultative

fixers avoid competitive exclusion by non-fixers by adjusting their investment in symbiotic N₂ fixation as soil nitrogen availability changes over time, keeping soil nitrogen at limiting levels (Menge et al. 2009, Sheffer et al. 2015). Thus, the sustained nitrogen limitation in soils under adult *C. villosa* shrubs is probably related to a combination of (i) efficient regulation of plant investment in fixation, (ii) nitrogen use by herbaceous plants throughout the growing season, (iii) low quantities of litter, and (iv) allocation of fixed nitrogen to protein-rich seeds (McKey 1994).

CONCLUSIONS

In this study, we provide evidence that facultative N₂ fixers in a dryland ecosystem maintain a tight nitrogen economy, effectively avoiding increases in nitrogen availability in the surrounding soil and therefore conserving their competitive advantage. The fact that a nitrogen surplus does not seem to accumulate in the soil around active N₂ fixers hints at the efficient use of soil nitrogen when available and minimal loss of newly fixed nitrogen through the litter.

The detection of facultative regulation of fixation in the field is challenging, due to the complexity of natural ecosystems, the difficulty in locating all root nodules in the field, and the notoriously high variance of fixation measurements. This may be the reason that a previous field study of *C. villosa* did not detect a relationship between soil nitrogen availability and investments in symbiotic nitrogen fixation. To address these issues in future studies, we suggest that large sample size, simultaneous use of different methods, and concomitant data from controlled studies must be used to provide as complete a picture as possible of fixation strategies in the field.

Furthermore, we call for an update of the large-scale assessments and models of the inputs of symbiotically fixed nitrogen into terrestrial ecosystems, which so far have not taken fixation strategies into account.

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LITERATURE CITED

- Adams, M. A., T. L. Turnbull, J. I. Sprent, and N. Buchmann. 2016. Legumes are different: leaf nitrogen, photosynthesis, and water use efficiency. *Proceedings of the National Academy of Sciences of USA* 113:4098–4103.
- Agnew, A. D. Q., and M. Zohary. 1974. Geobotanical foundations of the middle east. *Journal of Ecology* 62:349.
- Ament, M. R., J. A. Tierney, L. O. Hedin, E. A. Hobbie, and N. Wurzbarger. 2018. Phosphorus and species regulate N₂ fixation by herbaceous legumes in longleaf pine savannas. *Oecologia* 187:281–290.
- Austin, A. T. 2011. Has water limited our imagination for aridland biogeochemistry. *Trends in Ecology & Evolution* 26:229–235.
- Barron, A. R., D. W. Purves, and L. O. Hedin. 2011. Facultative nitrogen fixation by canopy legumes in a lowland tropical forest. *Oecologia* 165:511–520.
- Batterman, S. A., L. O. Hedin, M. Van Breugel, J. Ransijn, D. J. Craven, and J. S. Hall. 2013a. Key role of symbiotic dinitrogen fixation in tropical forest secondary succession. *Nature* 502:224–227.
- Batterman, S. A., N. Wurzbarger, and L. O. Hedin. 2013b. Nitrogen and phosphorus interact to control tropical symbiotic N₂ fixation: a test in *Inga punctata*. *Journal of Ecology* 101:1400–1408.
- Bellingham, P. J., L. R. Walker, and D. A. Wardle. 2001. Differential facilitation by a nitrogen-fixing shrub during primary succession influences relative performance of canopy tree species. *Journal of Ecology* 89:861–875.
- Bremer, E., D. A. Rennie, and R. J. Rennie. 1988. Dinitrogen fixation of lentil, field pea and fababean under dryland conditions. *Canadian Journal of Soil Science* 68:553–562.
- Cleveland, C. C., et al. 1999. Global patterns of terrestrial biological nitrogen (N₂) fixation in natural ecosystems. *Global Biogeochemical Cycles* 13:623–645.

- Dovrat, G., H. Bakhshian, T. Masci, and E. Sheffer. 2020. The nitrogen economic spectrum of legume stoichiometry and fixation strategy. *New Phytologist* 227:365–375.
- Dovrat, G., T. Masci, H. Bakhshian, E. Mayzlish Gati, S. Golan, and E. Sheffer. 2018. Drought-adapted plants dramatically downregulate dinitrogen fixation: evidences from Mediterranean legume shrubs. *Journal of Ecology* 106:1534–1544.
- Dovrat, G., and E. Sheffer. 2018. Symbiotic dinitrogen fixation is seasonal and strongly regulated in water-limited environments. *New Phytologist* 221: 1866–1877.
- Ferguson, B. J., C. Mens, A. H. Hastwell, M. Zhang, H. Su, C. H. Jones, X. Chu, and P. M. Gresshoff. 2019. Legume nodulation: the host controls the party. *Plant Cell and Environment* 42:41–51.
- Gei, M., et al. 2018. Legume abundance along successional and rainfall gradients in Neotropical forests. *Nature Ecology and Evolution* 2:1104–1111.
- Goergen, E. M., and J. C. Chambers. 2009. Influence of a native legume on soil N and plant response following prescribed fire in sagebrush steppe. *International Journal of Wildland Fire* 18:665–675.
- Grünzweig, J., Y. Navon, D. Kanas, I. Dirks, and R. Dumbur. 2010. Study of major processes in the biogeochemical cycles of the *Phillyrea latifolia* shrubland in Ramat Hanadiv. Hebrew University of Jerusalem, Ramat Hanadiv, Israel.
- Gutschick, V. P. 1981. Evolved strategies in nitrogen acquisition by plants. *American Naturalist* 118: 607–637.
- Hardy, R. W. F., R. D. Holsten, E. K. Jackson, and R. C. Burns. 1968. The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. *Plant Physiology* 43:1185–1207.
- Harris, D., W. R. Horwath, and C. Van Kessel. 2001. Acid fumigation of soils to remove carbonates prior to total organic carbon or carbon-13 isotopic analysis. *Soil Science Society of America Journal* 65:1853–1856.
- Hedin, L. O., E. N. J. Brookshire, D. N. L. Menge, and A. R. Barron. 2009. The nitrogen paradox in tropical forest ecosystems. *Annual Review of Ecology, Evolution, and Systematics* 40:613–635.
- Hely, F. W., and I. Ofer. 1972. Nodulation and frequencies of wild leguminous species in the northern Negev region of Israel. *Australian Journal of Agricultural Research* 23:267–284.
- Henkin, Z., N. G. Seligman, and I. Noy-Meir. 2007. Successional transitions and management of a phosphorus-limited shrubland ecosystem. *Rangeland Ecology and Management* 60:453–463.
- Hughes, R. F., and J. S. Denslow. 2005. Invasion by a N_2 -fixing tree alters function and structure in wet lowland forests of Hawaii. *Ecological Applications* 15:1615–1628.
- Jacobs, S. M., N. E. Pettit, and R. J. Naiman. 2007. Nitrogen fixation by the savanna tree *Philenoptera violacea* (Klotzsch) Schrire (Apple leaf) of different ages in a semi-arid riparian landscape. *South African Journal of Botany* 73:163–167.
- Jonckheere, A. R. 1954. A distribution-free k-sample test against ordered alternatives. *Biometrika* 41:133.
- LeBauer, D. S., and K. K. Treseder. 2008. Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecology* 89:371–379.
- Maynard, D. G., Y. P. Kalra, and J. A. Crumbaugh. 1993. Nitrate and exchangeable ammonium nitrogen. *Soil Sampling and Methods of Analysis* 1:25–38.
- McCulloch, L. A., D. Piotto, and S. Porder. 2021. Drought and soil nutrients effects on symbiotic nitrogen fixation in seedlings from eight Neotropical legume species. *Biotropica* 53:703–713.
- McKey, D. 1994. Legumes and nitrogen: the evolutionary ecology of a nitrogen-demanding lifestyle. *Advances in Legume Systematics* 5: the Nitrogen Factor 5:211–228.
- Menge, D. N. L., and T. E. Crews. 2016. Can evolutionary constraints explain the rarity of nitrogen-fixing trees in high-latitude forests? *New Phytologist* 211:1195–1201.
- Menge, D. N. L., S. A. Levin, and L. O. Hedin. 2009. Facultative versus obligate nitrogen fixation strategies and their ecosystem consequences. *American Naturalist* 174:465–477.
- Menge, D. N. L., A. A. Wolf, and J. L. Funk. 2015. Diversity of nitrogen fixation strategies in Mediterranean legumes. *Nature Plants* 1:15064.
- Minchin, F. R., J. E. Sheehy, and J. F. Witty. 1986. Further errors in the acetylene reduction assay: effects of plant disturbance. *Journal of Experimental Botany* 37:1581–1591.
- Minucci, J. M., C. F. Miniati, R. O. Teskey, and N. Wurzbarger. 2017. Tolerance or avoidance: drought frequency determines the response of an N_2 -fixing tree. *New Phytologist* 215:434–442.
- Mitchell, J. S., and R. W. Ruess. 2009. N_2 fixing alder (*Alnus viridis* spp. *fruticosa*) effects on soil properties across a secondary successional chronosequence in interior Alaska. *Biogeochemistry* 95:215–229.
- Moro, M. J., F. I. Pugnaire, P. Haase, and J. Puigdefábregas. 1997. Mechanisms of interaction between a leguminous shrub and its understorey in a semi-arid environment. *Ecography* 20:175–184.
- Norman, R. J., and J. W. Stucki. 1981. The determination of nitrate and nitrite in soil extracts by

- ultraviolet spectrophotometry. *Soil Science Society of America Journal* 45:347–353.
- Olsen, S. R., and L. E. Sommers. 1982. Determination of available phosphorus. *Method of Soil Analysis* 2:2018.
- Power, S. C., M. D. Cramer, G. A. Verboom, and S. B. M. Chimphango. 2010. Does phosphate acquisition constrain legume persistence in the fynbos of the Cape Floristic Region? *Plant and Soil* 334:33–46.
- R Core Team. 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
- Robertson, G. P., D. C. Coleman, P. Sollins, and C. S. Bledsoe. 1999. *Standard soil methods for long-term ecological research*. Oxford University Press on Demand.
- Rodríguez-Echeverría, S., M. A. Pérez-Fernández, S. Vlaar, and T. Finnan. 2003. Analysis of the legume-rhizobia symbiosis in shrubs from central western Spain. *Journal of Applied Microbiology* 95:1367–1374.
- Ruffe, S., et al. 2008. Systemic signaling of the plant nitrogen status triggers specific transcriptome responses depending on the nitrogen source in *Medicago truncatula*. *Plant Physiology* 146:2020–2035.
- Schlesinger, W. H., J. A. Raikks, A. E. Hartley, and A. F. Cross. 1996. On the spatial pattern of soil nutrients in desert ecosystems. *Ecology* 77:364–374.
- Schlesinger, W. H., S. L. Tartowski, and S. M. Schmidt. 2006. *Nutrient cycling within an arid ecosystem. Structure and function of a Chihuahuan Desert ecosystem*. Oxford University Press, New York, New York, USA. Pages 133–149.
- Sheffer, E., S. A. Batterman, S. A. Levin, and L. O. Hedin. 2015. Biome-scale nitrogen fixation strategies selected by climatic constraints on nitrogen cycle. *Nature Plants* 1:1–25.
- Sprent, J. I., and H. S. Gehlot. 2010. Nodulated legumes in arid and semi-arid environments: Are they important? *Plant Ecology and Diversity* 3:211–219.
- Streeter, J., and P. P. Wong. 1988. Inhibition of legume nodule formation and N_2 fixation by nitrate. *Critical Reviews in Plant Sciences* 7:1–23.
- Taylor, B. N., and D. N. L. Menge. 2018. Light regulates tropical symbiotic nitrogen fixation more strongly than soil nitrogen. *Nature Plants* 4:655–661.
- ter Steege, H., et al. 2006. Continental-scale patterns of canopy tree composition and function across Amazonia. *Nature* 443:444–447.
- Trierweiler, A. M., K. Winter, and L. O. Hedin. 2018. Rising CO_2 accelerates phosphorus and molybdenum limitation of N_2 fixation in young tropical trees. *Plant and Soil* 429:363–373.
- Uliassi, D. D., and R. W. Ruess. 2002. Limitations to symbiotic nitrogen fixation in primary succession on the tanana river floodplain. *Ecology* 83:88–103.
- Vessey, J. K. 1994. Measurement of nitrogenase activity in legume root nodules: in defense of the acetylene reduction assay. *Plant and Soil* 158:151–162.
- Vitousek, P. M., D. N. L. Menge, S. C. Reed, and C. C. Cleveland. 2013. Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. *Philosophical Transactions of the Royal Society B: Biological Sciences* 368:20130119.
- Vitousek, P. M., and L. R. Walker. 1989. Biological invasion by *Myrica faya* in Hawai'i: plant demography, nitrogen fixation, ecosystem effects. *Ecological Monographs* 59:247–265.
- Wickham, H. 2016. *ggplot2: Elegant graphics for data analysis*. Springer-Verlag, New York, New York, USA.
- Witty, J. F., and F. R. Minchin. 1988. Measurement of nitrogen fixation by the acetylene reduction assay; myths and mysteries. Pages 331–344 in *Nitrogen fixation by legumes in Mediterranean agriculture*. Springer, Berlin, Germany.
- Wurzburger, N., J. P. Bellenger, A. M. L. Kraepiel, and L. O. Hedin. 2012. Molybdenum and phosphorus interact to constrain asymbiotic nitrogen fixation in tropical forests. *PLOS ONE* 7:2–8.
- Yiotis, C., G. K. Psaras, and Y. Manetas. 2008. Seasonal photosynthetic changes in the green-stemmed Mediterranean shrub *Calicotome villosa*: a comparison with leaves. *Photosynthetica* 46:262–267.

DATA AVAILABILITY STATEMENT

Data are available from Figshare: <https://figshare.com/s/b5224cbf0c718d2dafc8>

SUPPORTING INFORMATION

Additional Supporting Information may be found online at: <http://onlinelibrary.wiley.com/doi/10.1002/ecs2.3843/full>