

The native distribution of a common legume shrub is limited by the range of its nitrogen-fixing mutualist

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Summary

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- Plant–microbe mutualisms, such as the legume–rhizobium symbiosis, are influenced by the geographical distributions of both partners. However, limitations on the native range of legumes, resulting from the absence of a compatible mutualist, have rarely been explored.
- We used a combination of a large-scale field survey and controlled experiments to determine the realized niche of *Calicotome villosa*, an abundant and widespread legume shrub.
- Soil type was a major factor affecting the distribution and abundance of *C. villosa*. In addition, we found a large region within its range in which neither *C. villosa* nor *Bradyrhizobium*, the bacterial genus that associates with it, were present. Seedlings grown in soil from this region failed to nodulate and were deficient in nitrogen. Inoculation of this soil with *Bradyrhizobium* isolated from root nodules of *C. villosa* resulted in the formation of nodules and higher growth rate, leaf N and shoot biomass compared with un-inoculated plants.
- We present evidence for the exclusion of a legume from parts of its native range by the absence of a compatible mutualist. This result highlights the importance of the co-distribution of both the host plant and its mutualist when attempting to understand present and future geographical distributions of legumes.

Introduction

The fundamental niche is defined as the set of environmental factors required by a species to sustain a viable population (Hutchinson, 1957). The environmental factors defining the fundamental niche are typically abiotic factors such as the climatic and edaphic conditions that allow the species to successfully grow and reproduce. Species distributions are also affected by biotic factors including the presence of competitors, parasites and/or pathogens, limiting the presence of host species to a ‘realized’ niche, as exemplified by the success of invasive species when released from parasite or pathogen pressure in their new habitat (Torchin & Mitchell, 2004). By contrast, the presence of mutualists such as pollinators and/or dispersal vectors can increase the realized niche of a species (Guisan & Thuiller, 2005).

In plants, another important biotic factor to consider is the presence and abundance of microbial mutualists in the soil, such as bacteria or mycorrhizal fungi. One of the most studied mutualistic relationships in plants is the symbiosis between legumes and N₂-fixing bacteria (rhizobia), as this symbiosis is a main natural source of nitrogen (N) for terrestrial ecosystems (Vitousek *et al.*, 2013; Davies-Barnard & Friedlingstein, 2020). Legumes and their rhizobial partners are naturally found across a wide range of habitats in nearly all terrestrial biomes and climates

(Sprent *et al.*, 2017). Yet, symbiotic N₂ fixation is often limited by environmental conditions affecting the growth and function of either the host plant, the bacterial mutualist, or both (Taylor & Menge, 2018; Tamme *et al.*, 2021; Sepp *et al.*, 2023). For example, insufficient availabilities of soil micronutrients such as iron or molybdenum limit the process of bacterial N₂ fixation (O’Hara, 2001; Brear *et al.*, 2013). Additionally, highly acidic or alkaline soils limit the growth of some rhizobial species, as well as the development of root nodules, the structures in which legume plants host the bacteria (Rupela & Saxena, 1987; Graham, 1992; Shen *et al.*, 2013; Ramoneda *et al.*, 2023). Environmental limitations resulting in the complete absence of a compatible rhizobial partner from an otherwise permissive environment have been reported in several cases, primarily in the context of their effect on productivity in agricultural systems (Graham, 2003; Lugtenberg, 2015).

In natural ecosystems, the human-facilitated introduction of many legume species into non-native habitats often resulted in successful and even aggressive invasions by these species (Richardson *et al.*, 2000; Graham, 2003; Rodríguez-Echeverría *et al.*, 2011). Yet, studies show that a successful invasion by a legume occurs either if its symbiosis is not specific (i.e. the host plant promiscuously associates with diverse mutualists) or when the specific bacterial mutualist is present or co-introduced

(Rodríguez-Echeverría *et al.*, 2011; Gehlot *et al.*, 2013; Klock *et al.*, 2015; Simonsen *et al.*, 2017). Thus, the absence or scarcity of a compatible mutualist is expected to limit the ability of a host legume to invade new habitats far from its natural range (Parker, 2001; Parker & Parker., 2006; Delavaux *et al.*, 2022). However, studies of the effects of mutualist presence–absence are generally concerned with the colonization of novel habitats, and rarely present examples of said absence within the native range (but see Stanton-Geddes & Anderson, 2011; Afkhami *et al.*, 2014).

In this study, we evaluated the factors that constitute the niche breadth of *Calicotome villosa* (Poir.) Link, a common legume shrub abundant throughout the Mediterranean region of Israel, and tested whether the absence of a compatible mutualist explains its absence from specific regions inside its native range. That is, we asked the following: Can a legume be excluded from a geographic region within its native range (i.e. with suitable abiotic conditions), but where the lack of compatible mutualists makes the conditions unsuitable?

To determine the most important environmental factors influencing the distribution of *C. villosa*, we conducted a large-scale field survey of its presence and abundance across a precipitation gradient and in two distinct soil types. During the survey, we found several large regions within its abiotic niche in which *C. villosa* was completely absent. We then conducted a series of controlled experiments to determine the cause of the absence of *C. villosa* from these regions. We compared the growth and nodulation of seedlings grown in soils in which *C. villosa* is abundant to soils in which it is absent, with and without inoculation with its symbiont, and with and without fertilization with all required nutrients other than nitrogen. We hypothesized that: (1) Seedlings grown in soil from a habitat in which *C. villosa* is abundant will outperform those grown in soil from a habitat in which *C. villosa* is absent because the latter will be limited in nitrogen derived from N₂ fixation; (2) seedlings grown in soil from a habitat in which *C. villosa* is abundant will outperform those grown in soil from a habitat in which *C. villosa* is absent because the later will be limited by other nutrients (e.g. phosphorus, iron); and (3) inoculation with either a symbiont isolated from wild *C. villosa* populations or soil collected from a site where *C. villosa* is abundant will restore seedling performance in soils from a habitat in which it is absent.

Materials and Methods

Field survey

We conducted a large-scale field survey of the presence and abundance of *Calicotome villosa*. The purpose of the survey was to evaluate the niche breadth of *C. villosa* based on a large dataset of populations sampled in diverse sites. A total of 39 sites were surveyed, with no recent (at least in the last decade) history of disturbance or human intervention. Sites were stratified to include a sufficient sampling effort for each environmental factor. We evaluated the effect of the following environmental factors: two types of soil (Terra-Rossa derived from limestone (Chromic Luvisols,

FAO) and Rendzina derived from chalk) and mean annual precipitation (MAP).

Within each site, we randomly selected five circular sampling plots (6 m radius, *c.* 113 m²), located at least 50 m apart. In each sampling plot, we recorded GPS coordinates at the center of the plot, tallied all *C. villosa* individuals within the plot and measured the height and canopy perimeter of each individual. We then estimated the biomass for each shrub using an allometric equation developed by Dovrat & Sheffer (2018). Data on rock and soil types and mean annual precipitation at each site were obtained by crossing GPS locations with geological and precipitation maps, freely available on the websites of the Israel Meteorological Service (<https://ims.gov.il/>) and Israel Geological Society (<https://www.igs.org.il/>).

To gain a more quantitative description of the age structure of *C. villosa* populations, we used the Jenks natural breaks algorithm on the estimated biomass of all measured individuals, to divide the data into classes that most likely represent the different size groups. We then defined Class 1 (the lowest biomass) as the seedlings class (Jenks, 1967). Size class divisions were subsequently applied to each sampled plot, to calculate the proportion of seedlings within the plot. The mean proportion of seedlings within each site was used as an indicator of recruitment rates. Specifically, a right-skewed distribution of individuals was indicative of a higher proportion of the small size groups, that is seedlings. We compared models composed of the effects of different linear combinations of mean annual precipitation and soil type on the density of *C. villosa* (individuals per hectare) in the surveyed plots and selected the most parsimonious model based on the Akaike Information Criterion corrected for a small sample size using the AICcmodavg R package (AICc; Burnham & Anderson, 2004).

Isolation and identification of the bacterial mutualist

We chose to isolate N₂-fixing bacteria from the root nodules of *C. villosa* shrubs growing in Ramat-Hanadiv (32°33'08.0"N, 34°56'27.9"E), which is the survey site where we found the highest abundance of *C. villosa* shrubs (Fig. 1). We uprooted five medium-sized shrubs during late winter, which is the season when nodule biomass and bacterial activity within the nodules reach a yearly maximum (Dovrat & Sheffer, 2018), and collected a portion of the root system that contained nodules from each shrub. These samples of root systems were placed in sealed plastic bags inside a cooler and transferred to the laboratory. In the laboratory, we separated the nodules from the root systems and surface-sterilized them in 70% ethanol for 30 s followed by commercial bleach (3% sodium hypochlorite) for 2 min. After sterilization, we washed the nodules five times with sterile water and crushed them using a Mini-BeadBeater™ by Biospec Products.

We spread 50 µl of the crushed nodule slurry on Petri dishes containing yeast extract mannitol agar with Congo Red dye (Mannitol 10.0 g, K₂HPO₄ 0.5 g, MgSO₄·7H₂O 0.2 g, NaCl 0.1 g, yeast extract 0.5 g, 25 mg Congo Red and sterile distilled water to 1.0 L), either with no dilution or diluted 10-fold. We differentiated between the bacteria by colony morphology and color. We isolated a subset of the colonies and confirmed their identities

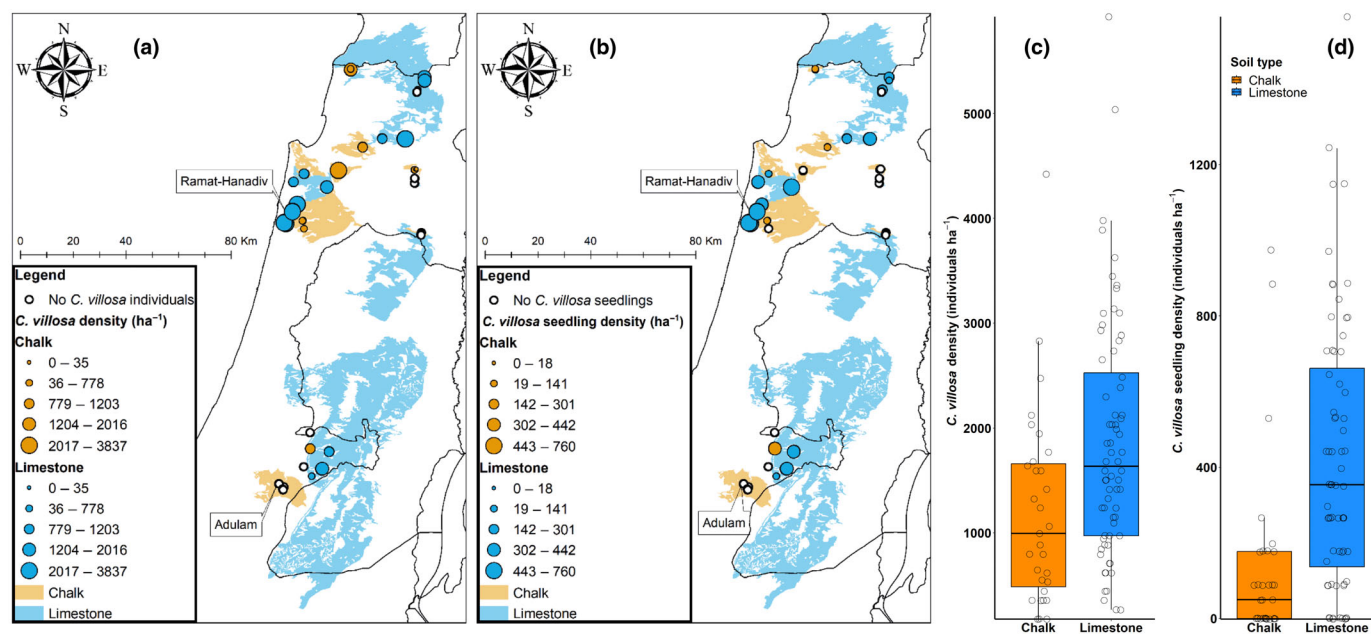


Fig. 1 Density maps of *Calicotome villosa* individuals (all sizes) (a) and seedlings (b) growing on soil derived from chalk (orange) and limestone (blue) bedrock in the surveyed field sites throughout the Mediterranean region of Israel ($n = 39$). The size of the circle in each site is correlated with the density of individuals or seedlings found in the site; open circles represent surveyed sites where *C. villosa* was absent. Two locations are denoted in the maps: the site of highest abundance – Ramat-Hanadiv, and the region where *C. villosa* is absent – Adulam. Panels (c, d) show the density of individuals (all sizes; c) and seedlings (d) in all sites as a function of rock type.

with PCR and Sanger sequencing using primers for both the 16S rRNA gene (338f:5'-ACTCCTACGGAGGCWGCAG-3'; 806r:5'-CGTCAATTCCTTTTTRAGTTT-3') and the *nifH* gene (25f:5'-TAYGGNAARGGNGGNATYGGNAARTC-3'; 457r:5'-GCCATCATYTCNCCNGA-3'). PCR Conditions were as follows: 98°C for 5 min followed by 95°C for 30 s, 50°C for 15 s, 72°C for 15 s repeated 35 times, and ending with 72°C for 5 min.

We compared the sequences to the NCBI database using the BLAST algorithm and used the top 10 results that had the highest percent identity. We selected an isolate that produced *nifH* bands in gel electrophoresis and was found to match *Bradyrhizobium* reference sequences according to Sanger sequencing of both the *nifH* and 16S PCR products. *Bradyrhizobia* are the most reported symbionts of the legumes in the Genistee tribe and therefore the chosen isolate was the most likely to successfully nodulate *C. villosa* roots. To further confirm the identity of the isolate, we grew it in yeast extract mannitol, extracted culture DNA and sent it to SeqCenter LLC (Pittsburgh, PA, USA) for whole-genome sequencing. We annotated the genome using Prodigal (Hyatt *et al.*, 2010).

To accurately place the isolate within the *Bradyrhizobium* phylogeny, we downloaded all available *Bradyrhizobium* genomes from the NCBI database, and extracted protein sequences of 31 universal single-copy genes from each of these genomes using the AMPHORA2 package (Wu & Scott, 2012). We concatenated these into one pseudo-protein per genome, aligned them together with the protein sequences from our isolate and used FASTTREE (Price *et al.*, 2009) to construct a phylogenetic tree. We then performed a comparative pan-genome analysis of the phylogenetic cluster our isolate belongs to using Anvi'o (Eren *et al.*, 2021). In

addition, we constructed phylogenetic trees for nodulation factor genes *nodA*, *nodB* and *nodC*. We used the HMMER software (v.3.3.2), and publicly available HMM profiles for each gene, to find these genes within each genome, then aligned them using MUSCLE (Edgar, 2004) and constructed the trees with FastTree as above with *nod* genes from a *Sinorhizobium* isolated from soil collected in the region of Adulam (Fig. 1, collected for a different study), as an outgroup.

Controlled inoculation experiments

We performed a set of controlled experiments aimed at evaluating the causes for the patterns of *C. villosa* presence and abundance that we found in the survey. First, to explain the lower densities of *C. villosa* seedlings in chalk-derived soils compared with limestone-derived soils (see the survey results section below), we hypothesized that chalk-derived soil is deficient in necessary nutrients other than nitrogen. We tested this by growing *C. villosa* seedlings in two different soils (mixed with perlite 1:1 volume): limestone-derived Terra-Rossa soil from the site in which the abundance of *C. villosa* was highest (Ramat-Hanadiv), and chalk-derived Rendzina soil from a site where *C. villosa* was absent (31°39'03.1"N, 34°57'58.5"E Adulam; Fig. 1, Supporting Information Fig. S1).

We tested for nutrient limitation by fertilizing half of the plants grown in each soil with a sufficient supply of N-free Hoagland nutrient solution, which contains also all the necessary micronutrients, while the other half was irrigated with water only. Second, to explain the absence of *C. villosa* from a large area within its abiotic range (Adulam, Judean lowlands, Israel), we

hypothesized that a compatible rhizobial partner is absent from the soil in this region. We tested this hypothesis by inoculating half of the seedlings in each combination of soil type and fertilization treatment with the *Bradyrhizobium* strain we isolated from the nodules of plants growing in the field. Third, to test for a potential influence of non-rhizobial microbes critical for the survival and growth of *C. villosa* seedlings, we compared inoculation of seedlings grown in Adulam soil with the isolated *Bradyrhizobium* to inoculation with a small amount (a tablespoon ~15 ml) of soil from Ramat-Hanadiv on the surface of the soil from Adulam, to ensure access to a suite of local bacterial mutualists. To ensure reproducibility, we conducted the controlled experiment twice, once with five replicate plants per treatment and once with seven replicate plants per treatment.

Experimental setup – soil collection and analyses

We hypothesized that the soils in sites in which the density of *C. villosa* adults and seedlings was the highest (compared to the entire survey), contained large enough populations of suitable mutualists. We therefore collected soils from Ramat-Hanadiv (Limestone-derived Terra-Rossa soil, 562 mm MAP, mean *C. villosa* density $c. 2500$ individuals ha^{-1}) and Adulam (Chalk-derived Rendzina soil, 424 mm MAP, no *C. villosa* individuals; Figs 1, S1). Within each site, we collected the soil from 10 randomly placed locations, both under the canopies of diverse woody vegetation and in open patches where only ephemeral herbaceous vegetation grows (soil collected in early summer 2022). We collected soil by first removing the plant litter and organic matter and collecting the top 10 cm of soil. We then thoroughly mixed the soils from each site before using them in the experimental pots and analyzing the content of nutrients and bacteria in each of these soils.

From each soil pool, we took two to three technical subsamples for extraction in a 2 M KCl solution to estimate the concentration of inorganic nitrogen (Maynard *et al.*, 1993). Nitrate and ammonium concentrations in extracted soil solutions were measured colorimetrically using an EpochTM Microplate Reader (Norman & Stucki, 1981). We extracted from each soil pool three technical subsamples with 0.5 M sodium bicarbonate at pH 8.5, to estimate the concentration of available phosphorus using the P-Olsen colorimetric method (Olsen & Sommers, 1982). Soil solution pH was measured by mixing with a magnetic stirrer three samples of 25 g of soil from each soil type with 50 ml of 0.01 M CaCl_2 solution. Soils were stirred for 30 min, allowed to settle for 1 h and then the supernatant was measured with Orion StarTM A211 Benchtop pH Meter (Thermo Fisher Scientific, Waltham, MA, USA).

To test for the presence and relative abundance of *Bradyrhizobium* in the soils collected from Ramat-Hanadiv and Adulam, we extracted total microbial DNA from each of the soil pools using the DNeasy PowerSoil Pro Kit (Qiagen). We amplified the V3–V4 regions of the bacterial 16S rRNA gene using the following primers: 357f:5'-ACTCCTACGGGAGGCWGCAG-3' and 806r:5'-CGTCAATTCCTTTTRAGTTT-3'. PCR conditions were as follows: First PCR (16S adapters): 98°C for 30 s followed

by 98°C for 10 s, 78°C for 15 s, 55°C for 15 s and 72°C for 20 s repeated 30 times, and ending with 72°C for 5 min. Second PCR (Nextera dual indexes): 95°C for 5 min followed by 98°C for 20 s, 55°C for 15 s and 72°C for 1 min, repeated 10 times, and ending with 72°C for 1 min. We cleaned the PCR amplicons using magnetic beads. Samples were multiplexed using Nextera dual indexes, and sequencing was performed on an Illumina MiSeq instrument using a 600-cycle V3 chemistry kit. Results were analyzed using the DADA2 and PHYLOSEQ packages in R (McMurdie & Holmes, 2013; Callahan *et al.*, 2016; R Core Team, 2022).

Seed germination and setup in the glasshouse

We collected seeds of *C. villosa* from individuals growing on different soil types and under a wide range of MAP regimes. Seeds were then mixed to eliminate any effect of genetic differences between populations. We sterilized the seeds in commercial bleach (3% sodium hypochlorite) for 2 min, washed them in distilled water, and chipped them with a scalpel to break the hard seedcoat and ensure germination. We then germinated the treated seeds on 1% water agar in Petri dishes. After a week in the Petri dishes, we transferred the seedlings to the experimental pots. We filled each pot with $c. 450$ ml of a 1 : 1 mixture of inert perlite medium and soil from its corresponding soil type treatment (i.e. Adulam and Ramat-Hanadiv soils). We grew the plants in a controlled glasshouse under short-day conditions (9 h : 15 h, light : dark with 22°C : 16°C ambient temperature, respectively) to simulate the winter conditions in which *C. villosa* seedlings grow in the field.

We grew our *Bradyrhizobium* isolate in yeast extract mannitol medium for 3 d at 28°C and in darkness. We allowed the experimental seedlings to establish in the pots for 4 d and then inoculated half of the replicates in each of the inoculation treatments (with and without fertilization, $n = 10$ and 14 in the first and second experiment, respectively). We removed the bacteria from the spent medium by centrifugation and resuspension in 10 mM MgCl_2 solution and inoculated each plant with 1 ml of the resuspended culture (OD~0.25). At the same time, we added to the rest of the plants 1 ml of sterile 10 mM MgCl_2 solution as a control.

Measurements of seedling performance

Beginning 1 month after transplantation into pots, we measured the height of each seedling weekly for 7 wk. After 105 d since germination, we harvested the plants by first washing off the soil from the root system then separating the plant stem, leaves and roots into separate paper bags. The biomass of stems, leaves, roots and nodules (after first separating and counting the nodules) was oven-dried at 60°C for 72 h and weighed. We milled the dry biomass of the leaves, stems and roots to a powder using a mini bead beater and measured their concentrations of carbon (C) and nitrogen using an elemental analyzer (FLASH 2000; Thermo Scientific).

The plant growth rate was calculated from the slope of the fitted linear regression (change in height (cm) vs week). Nodule

mass fraction (NMF) was calculated as the dry biomass of all root nodules in each plant divided by its total dry plant biomass. Total plant nitrogen content was calculated by multiplying the nitrogen concentrations of each tissue by its respective dry biomass. We used ANOVA to evaluate how the type of inoculation and fertilization treatment and their interaction influenced biomass, nitrogen concentrations and content, and growth rates (in cm per week) in each of the two soil types. In the figures, we show *P*-values of pairwise comparisons between all pairs of treatments within the same soil type. First, we used a linear mixed effect model with experimental repeat as a random effect. Since this random effect was statistically insignificant, we removed it as a term in the model, and the final analyses were done on pooled data from both repeats of the experiment. The statistical analysis shown in Table 2 (see later) is for each soil type separately due to the asymmetrical design of the experiment (inoculation of Adulam soil with Ramat-Hanadiv soil). For an analysis that includes soil type as an independent variable, but does not include the plants grown in Adulam soil and inoculated with Ramat-Hanadiv soil, see Table S1.

Data analysis software

All statistical analyses were performed in R and figures were created using GGPLOT2 (Bates *et al.*, 2015; Wickham, 2016; R Core Team, 2022). Maps were created using ARCGIS® software by Esri.

Results

Bedrock type is the main determinant of *C. villosa* abundance

We conducted a large-scale field survey to evaluate niche breadth of *C. villosa* based on a large dataset of populations sampled in diverse sites. A total of 39 sites were surveyed, with no recent (at least in the last decade) history of disturbance or human intervention. The survey revealed that the abundance of *C. villosa* was affected mainly by bedrock-derived soil type. The densities of *C. villosa* plants (adults and seedlings combined) were 39% lower in chalk than in limestone-derived soils (1166 ± 167 and 1872 ± 138 individuals ha^{-1} mean $\pm$ SE, respectively; $F=8.103$ $P=0.005$; Fig. 1a). The density of seedlings was especially low in chalk-derived soils with 75% lower densities than limestone-derived soils (132 ± 41 and 419 ± 42 seedlings ha^{-1} , respectively; $F=16.29$, $P<0.001$; Fig. 1b).

Moreover, we did not find populations of *C. villosa* in one region, Adulam Park, and found very low abundances in eight additional sites with a combination of Rendzina soil on chalk bedrock and relatively low MAP (<550 mm). Surprisingly, the density (and cover) of *C. villosa* individuals was not related to mean annual precipitation (Table S2). The fit of the model for the density of seedlings was slightly improved by combining soil type and precipitation but was not statistically significant. Therefore, it is unlikely that low precipitation alone excluded *C. villosa* from these regions, especially since populations of *C. villosa* are

found also in sites with lower MAP (to the east and southwest of Adulam; Fig. S1), but not on chalk-derived soil. Full statistical results for the most parsimonious model are summarized in Table S2.

N and P availabilities do not limit *C. villosa* in chalk-derived soil

To test whether soil nutrient levels contributed to the distribution of *C. villosa*, we measured the concentrations of available nitrogen and phosphorus in the soils collected from the site of highest abundance and the site with zero abundance (Ramat-Hanadiv and Adulam, respectively). We found that Adulam soil contained twice as much available nitrogen (nitrate + ammonium) as Ramat-Hanadiv soil (4.14 ± 0.14 vs 1.97 ± 0.02 $\mu\text{g g}^{-1}$ soil, respectively) and *c.* 50% more phosphorus (3.48 ± 0.1 vs 2.38 ± 0.1 $\mu\text{g g}^{-1}$ soil, respectively) (Table 1). Soil pH was higher in Adulam compared to Ramat-Hanadiv soil, with 7.21 ± 0.01 and 6.8 ± 0.03 , respectively (Table 1). Additionally, a comparison of the biomass of seedlings grown with and without fertilization with all nutrients other than nitrogen showed a positive effect on plants grown in Ramat-Hanadiv soil, or on inoculated plants grown in Adulam soil, but did not increase the growth of uninoculated seedlings in Adulam soil (Table 2). We therefore concluded that *C. villosa* is not absent from Adulam due to nutrient deficiency in this soil.

The symbiont of *C. villosa* belongs to the *Bradyrhizobium* genus

We hypothesized that the absence of *C. villosa* from Adulam is due to the absence of a suitable symbiont. Testing this hypothesis required the isolation and characterization of a N_2 -fixing symbiont from within the plant's native range. We isolated and identified a N_2 -fixing symbiont of *C. villosa* growing in Ramat-Hanadiv. The strain is a member of the *Bradyrhizobium* genus and is closely related to *B. cosmicum* and *B. japonicum* ('RH 3a 1' in Figs 2, S2). The strain's draft genome contains 8155 711 base pairs with 63% GC content and 7908 protein-coding open reading frames, and is available online in the NCBI database under TaxID: 1404864.

Using a phylogenomic analysis based on single copy house-keeping genes, we identified a clade of 19 closely related *Bradyrhizobia* (Fig. 2). These strains were isolated from various plant species growing in different climates across the world from Australia to Canada with no clear geographical or host

Table 1 Nitrogen and phosphorus availabilities and pH $\pm$ SE of the two examined soils.

	Adulam soil	Ramat-Hanadiv soil
Nitrate (NO_3^-) ($\mu\text{g g}^{-1}$ soil)	1.50 ± 0.1	Not detected
Ammonium (NH_4^+) ($\mu\text{g g}^{-1}$ soil)	2.82 ± 0.01	1.97 ± 0.02
Phosphate (PO_3^-) ($\mu\text{g g}^{-1}$ soil)	3.48 ± 0.1	2.38 ± 0.1
Soil pH	7.21 ± 0.01	6.8 ± 0.03

Table 2 Statistical results of ANOVAs of the effects of fertilization, inoculation with *Bradyrhizobium* or inoculation with soil from Ramat-Hanadiv, and their interaction on biomass production and allocation, and nitrogen economy in plants grown in Adulam or Ramat-Hanadiv soil.

Soil source	Dependent variable	Independent variable	df	F value	P-value	Significance
Adulam	Shoot biomass	Fertilizer	1	0.002	0.964	ns
		Inoculation	2	23.99	4.5e−08	***
		Fertilizer × Inoculation	1	4.945	0.031	*
	Root biomass	Fertilizer	1	2.998	0.089	ns
		Inoculation	2	1.473	0.239	ns
		Fertilizer × Inoculation	1	7.544	0.008	*
	Root mass fraction	Fertilizer	1	2.673	0.1	ns
		Inoculation	2	37.81	8.5e−11	***
		Fertilizer × Inoculation	1	1.972	0.16	ns
	Nodule number	Fertilizer	1	27.46	3.1e−06	***
		Inoculation	2	75.5	5.7e−16	***
		Fertilizer × Inoculation	1	28.91	1.9e−06	***
	Nodule mass fraction	Fertilizer	1	0.37	0.5	ns
		Inoculation	2	67.6	4.55e−15	***
		Fertilizer × Inoculation	1	2.21	0.1	ns
	Total plant N	Fertilizer	1	1.271	0.265	ns
		Inoculation	2	49.24	1.2e−12	***
		Fertilizer × Inoculation	1	11.34	0.001	**
	Leaf N conc. (%)	Fertilizer	1	0.017	0.896	ns
		Inoculation	2	1.473	1.3e−10	***
		Fertilizer × Inoculation	1	0.178	0.675	ns
Ramat-Hanadiv	Shoot biomass	Fertilizer	1	35.84	5.4e−07	***
		Inoculation	1	0.395	0.533	ns
		Fertilizer × Inoculation	1	1.141	0.29	ns
	Root biomass	Fertilizer	1	9.007	0.005	**
		Inoculation	1	0.002	0.963	ns
		Fertilizer × Inoculation	1	0.001	0.974	ns
	Root mass fraction	Fertilizer	1	16.08	0.0003	***
		Inoculation	1	3.181	0.082	ns
		Fertilizer × Inoculation	1	2.754	0.105	ns
	Nodule number	Fertilizer	1	20.85	4.9e−05	***
		Inoculation	1	11.01	0.002	**
		Fertilizer × Inoculation	1	8.65	0.005	**
	Nodule mass fraction	Fertilizer	1	31.7	1.8e−06	***
		Inoculation	1	11	0.002	**
		Fertilizer × Inoculation	1	3.8	0.05	ns
	Total plant N	Fertilizer	1	17.02	1.9e−5	***
		Inoculation	2	1.291	0.263	ns
		Fertilizer × Inoculation	1	0.276	0.602	ns
	Leaf N conc. (%)	Fertilizer	1	1.166	0.287	ns
		Inoculation	2	2.737	0.107	ns
		Fertilizer × Inoculation	1	3.597	0.066	ns

Significance codes: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, $P > 0.05$.

association pattern, and most but not all were collected from root nodules. Interestingly, five of these genomes did not contain nodABC genes, which are required for successful nodulation. In terms of orthologous gene content, our isolate contained no orthologous genes unique to it within this clade (Fig. S3). Due to the importance of nodulation genes for determining host–mutualist interactions, we constructed a new phylogenetic tree consisting only of genomes containing the nodABC genes (Fig. S2). A phylogeny based only on nodABC genes identified the most closely related strain to RH 3a 1 as *Bradyrhizobium rifense* strain CTAW71, isolated from the root nodules of *Cytisus villosus* grown in Morocco (Chahboune *et al.*, 2012). This was true for both the individual nod gene trees and for a concatenated nodABC tree (Figs S4, S5).

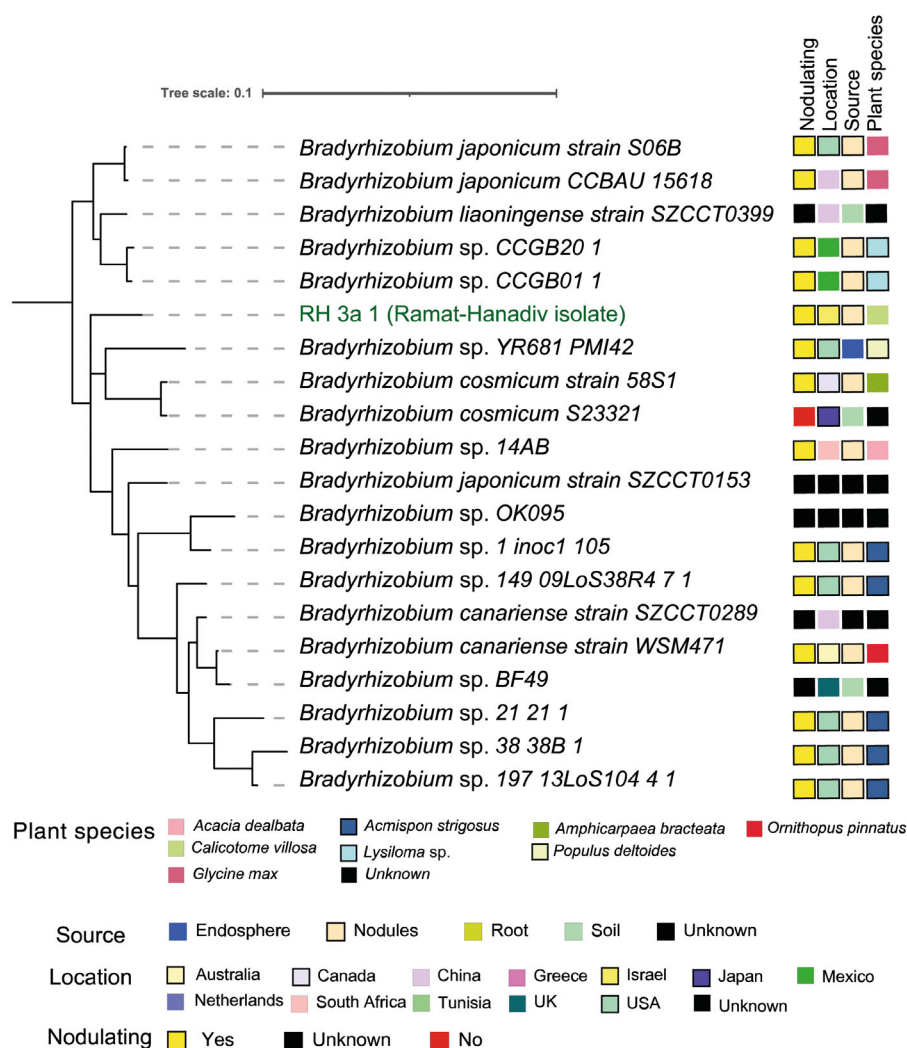
Furthermore, the majority of the strains in the most closely related clades based on nod genes, originate from nodules of plant species that grow in Mediterranean climates from various countries and continents (Fig. 3).

Taken together, these results suggest that the association of this symbiont with *C. villosa* is a relatively recent evolutionary occurrence, and that in the process of adaptation, it acquired nod genes typical of arid regions in the southern Mediterranean coast.

Inoculation with the isolated *Bradyrhizobium* induced nodulation

To test whether the low abundance of *C. villosa* in chalk-derived soils is due to the absence of its symbiont or the lack of a limiting

Fig. 2 Phylogenetic tree of *Bradyrhizobia* closely related to the strain isolated in the current study from root nodules of *Calicotome villosa* in Ramat-Hanadiv (RH 3a 1, marked in green), constructed with a concatenated alignment of 31 core proteins using the AMPHORA2 package (Wu & Scott, 2012). This tree was pruned to only show *Bradyrhizobia* which are closely related to our isolate out of 788 available *Bradyrhizobium* genomes included in the analyses (see Supporting Information Fig. S2). The bars to the right of the tree provide information on the plant species, source tissue and location of isolation of the respective strain. The nodulating category shows whether the strain was actively nodulating the plant species, was unable to nodulate, or its nodulation capacity was unknown.



nutrient, we performed an inoculation experiment in fertilized and unfertilized Adulam soil and compared it to similar treatments in soil from Ramat-Hanadiv. Uninoculated plants did not nodulate in Adulam soil (see comment below), compared with substantial nodulation observed in Ramat-Hanadiv soil, providing a probable reason for the absence of *C. villosa* from Adulam (Fig. 4a). Inoculation of Adulam soil with either the isolated symbiont or a small amount of Ramat-Hanadiv soil strongly induced nodulation. The number of root nodules on each plant was highest in the fertilized and inoculated treatments in both Adulam and Ramat-Hanadiv soils, with 88.33 ± 10.74 and 36.83 ± 6.24 nodules, respectively (Fig. 4a). In unfertilized Adulam soils, plants inoculated with *Bradyrhizobium* had more than twice as many nodules as those inoculated with Ramat-Hanadiv soil (35.83 ± 4.82 vs 14.50 ± 3.41 , respectively, $F = 27.55$ and $P < 0.0001$). By contrast, plants grown in unfertilized Ramat-Hanadiv soil showed no effect of the inoculation with *Bradyrhizobium* on the number of nodules per plant ($P = 0.551$, Fig. 4a).

Seedlings that were inoculated with *Bradyrhizobium* and fertilized had a similar and high nodule mass fraction (the proportion

of nodule biomass out of total plant biomass) in both Adulam and Ramat-Hanadiv soils, with a mean of $6 \pm 0.5\%$. In comparison, in unfertilized soils the result was quite different, with twice as high nodule mass fraction in Adulam soil in plants inoculated with either *Bradyrhizobium* or soil from Ramat-Hanadiv ($5 \pm 0.8\%$ and $3\% \pm 0.3\%$, respectively; Fig. 4b) compared to inoculated and uninoculated plants in Ramat-Hanadiv soil ($2 \pm 0.3\%$, and $1 \pm 0.2\%$, respectively; Fig. 4b). In the first repeat of the experiment, we found a small number of nodules also in six (out of 10 uninoculated plants in Adulam soil) replicate plants grown in uninoculated Adulam soil, with four of them producing only one very small nodule, and two producing three and four nodules. Since this was not replicated in the second repeat of the experiment, nor previous preliminary experiments (Alon, 2021), we suspected that this was due to minor contamination from the inoculation of other plants, but did not exclude these data from the analysis. We conclude that the native microbiota of Adulam soil lacks the capacity to nodulate *C. villosa* while our *Bradyrhizobium* isolate and the microbiota in Ramat-Hanadiv successfully nodulated the seedlings of *C. villosa*.

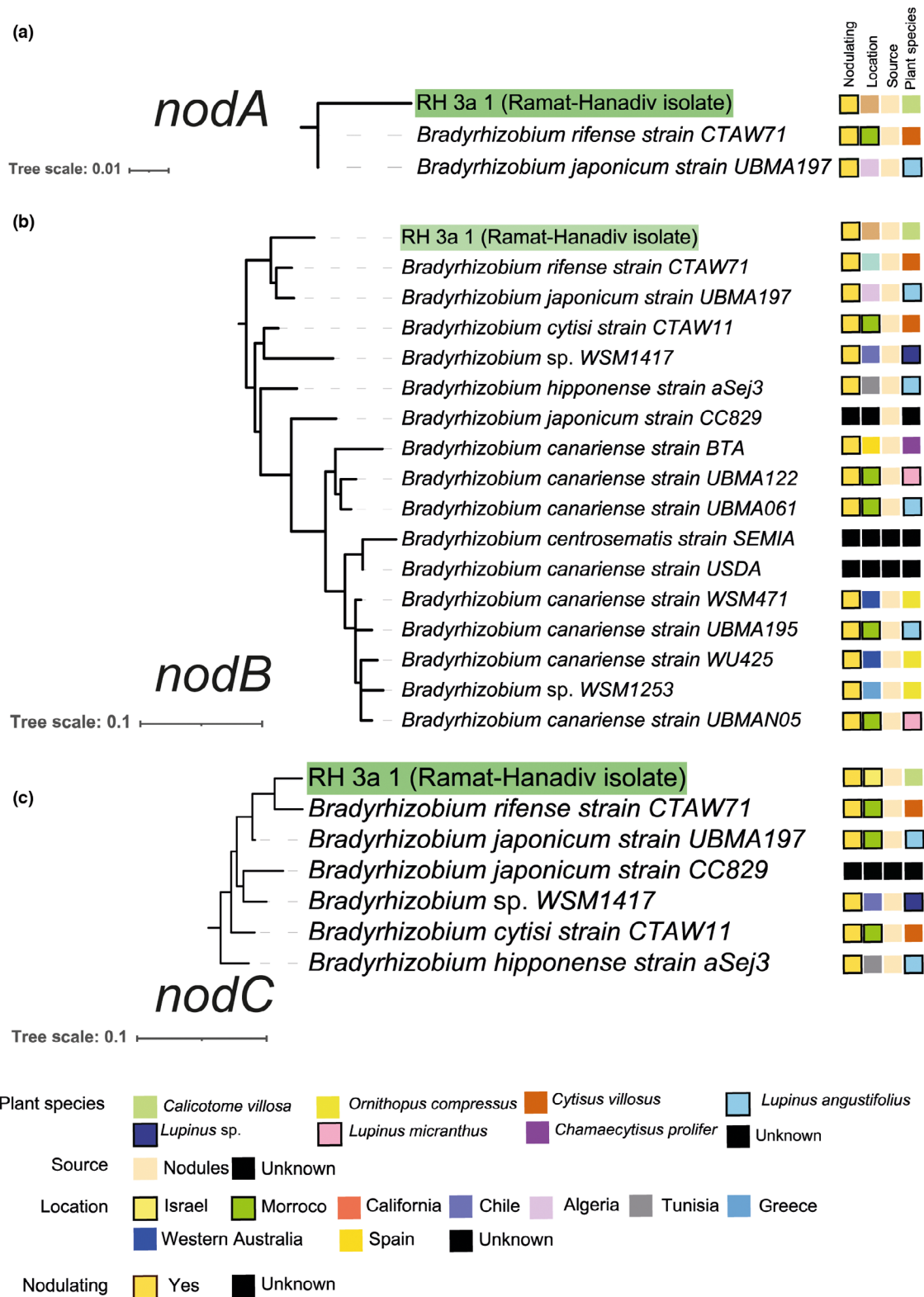


Fig. 3 Phylogenetic trees of *Bradyrhizobia* closely related to the strain isolated in the current study from root nodules of *Calicotome villosa* in Ramat-Hanadiv (RH 3a 1, marked in green), constructed with (a) *nodA*, (b) *nodB* and (c) *nodC* protein sequences. This tree was pruned to only show *Bradyrhizobia* which are closely related to our isolate out of 788 available *Bradyrhizobium* genomes included in the analyses (see Supporting Information Fig. S2). The bars to the right of the tree provide information on the plant species, source tissue and location of isolation of the respective strain. The nodulating category shows whether the strain was actively nodulating the plant species, was unable to nodulate, or its nodulation capacity was unknown.

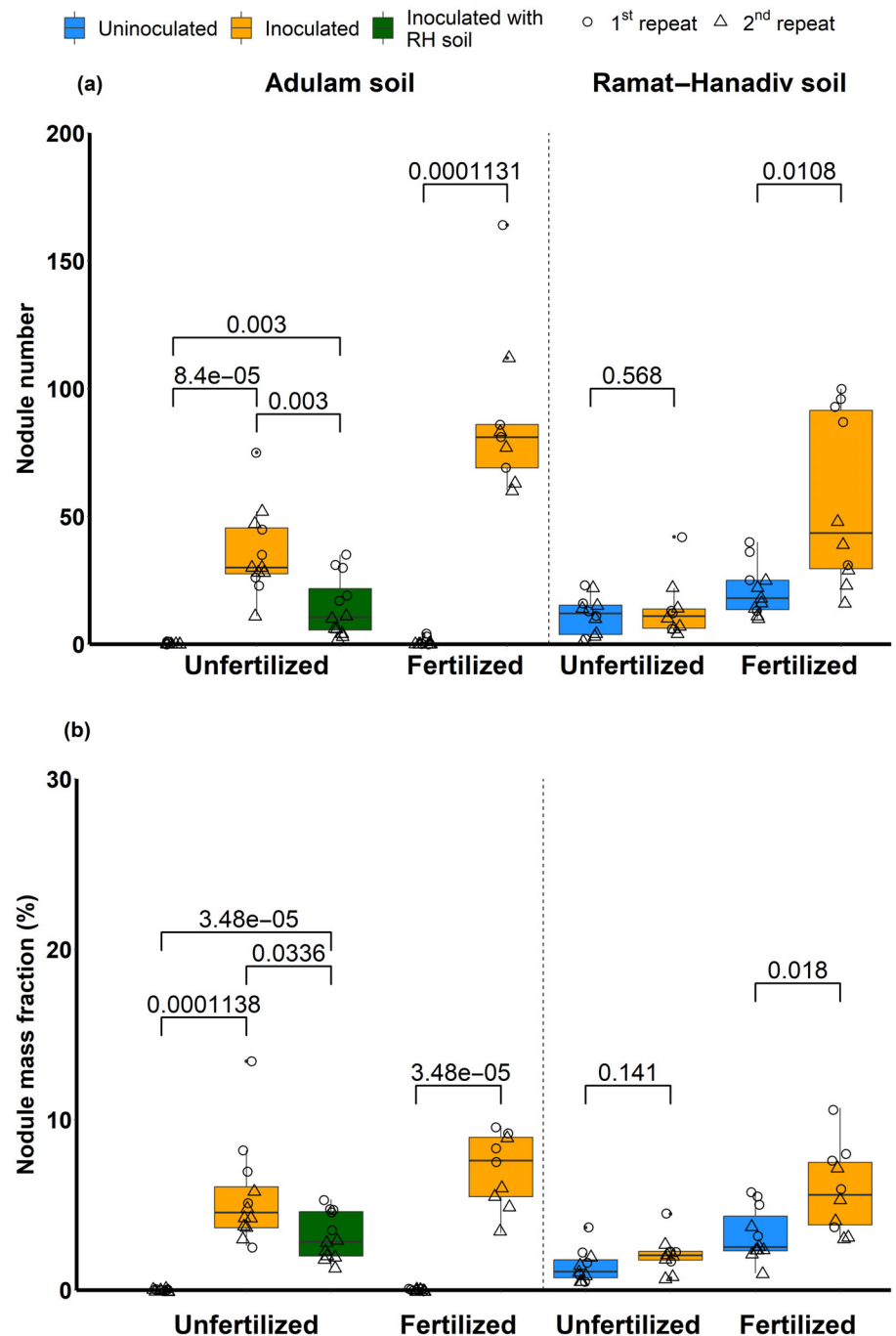


Fig. 4 Response of nodulation of seedlings of *Calicotome villosa* to soil source, inoculation and fertilization treatments. The number of root nodules (a) and nodule mass fraction (NMF) (b) as a function of seedlings grown in soil from the site in which *C. villosa* is absent (Adulam; left side) and from the site in which it is abundant (Ramat-Hanadiv; right side) as a function of inoculation with a *Bradyrhizobium* isolate vs inoculation with Ramat-Hanadiv (RH) soil and fertilization treatment, for two repeats of the experiment pooled together ($n = 5$, and $n = 7$). Bars in boxplots are the interquartile range (IQR) between the 25th and the 75th percentiles, and lines inside bars are the median. Values above each pair are the P -values for that specific comparison.

Inoculation, not fertilization, restores the growth of *C. villosa* in Adulam soil

We measured the response of *C. villosa* seedlings grown in unfertilized Adulam soil, in terms of plant biomass and nitrogen content, to inoculation with either *Bradyrhizobium* or a tablespoon of soil from Ramat-Hanadiv and found that inoculation resulted in significantly higher shoot biomass compared to uninoculated plants (0.28 ± 0.02 , 0.41 ± 0.02 and 0.17 ± 0.01 g, respectively; $F = 27.89$, $P < 0.0001$; Fig. 5a,d; Table 2). We measured the same pattern in fertilized Adulam soil with 0.44 ± 0.06 g and

0.16 ± 0.04 g total shoot biomass in inoculated vs uninoculated plants, respectively ($F = 13.63$, $P = 0.001$). Confirming our first hypothesis, that nutrients in the soil are not the limiting factor for *C. villosa* growth in Adulam, fertilization in this experiment did not affect the shoot biomass of uninoculated seedlings but significantly increased shoot biomass in inoculated seedlings (0.44 ± 0.06 and 0.28 ± 0.02 , respectively; $F = 5.94$, $P = 0.02$; Fig. 5a; Table 2). By contrast, fertilization doubled shoot biomass in both inoculated and uninoculated seedlings grown in Ramat-Hanadiv soil ($F = 40.53$, $P < 0.0001$ and $F = 7.643$, $P = 0.01$, respectively; Fig. 5a). Thus, we concluded that Ramat-Hanadiv

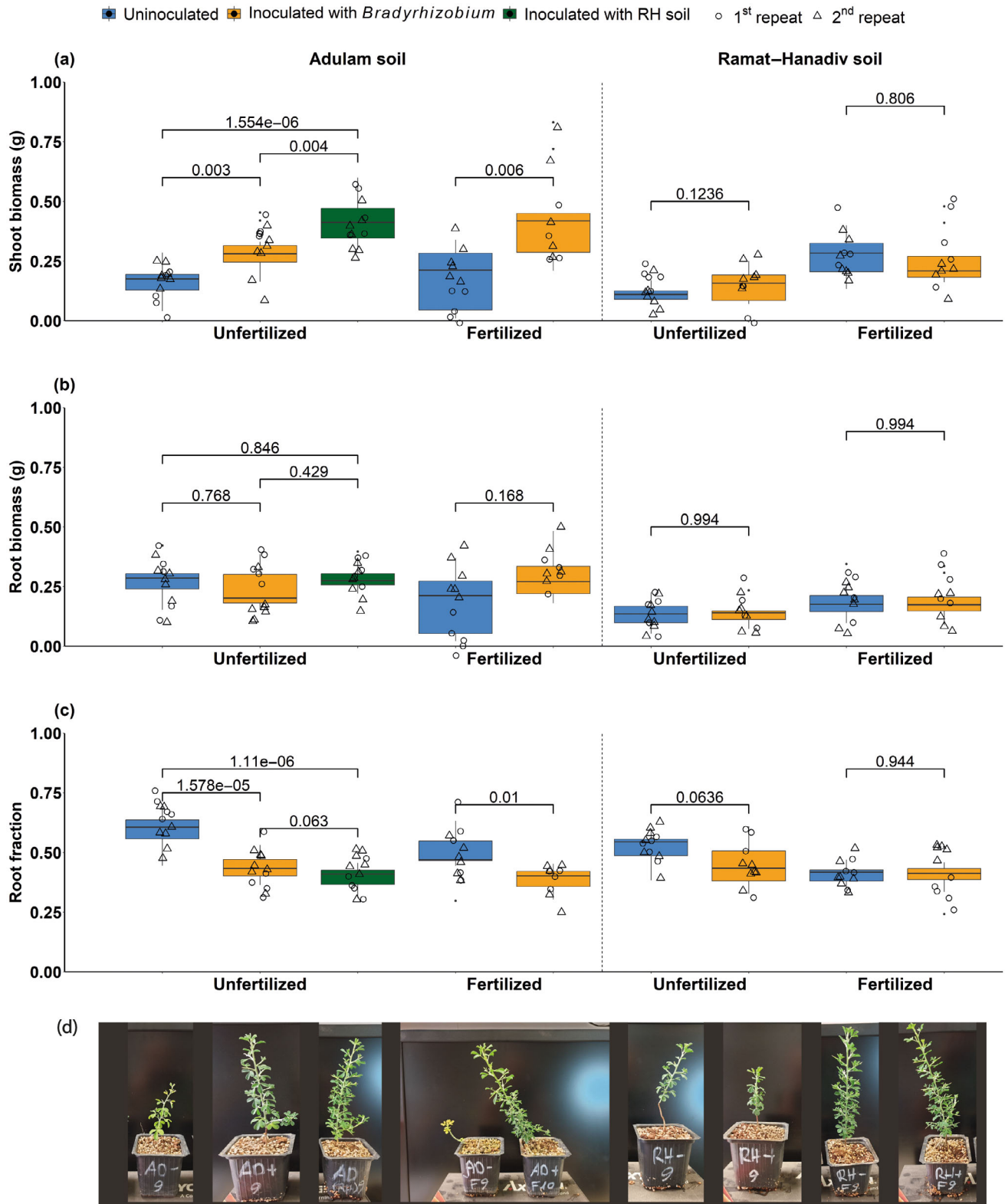


Fig. 5 Response of biomass and biomass allocation of seedlings of *Calicotome villosa* to soil source, inoculation and fertilization treatments. (a) Shoot biomass (g), (b) root biomass (g), and (c) root mass fraction (root biomass/total biomass) of seedlings grown in soil from the site in which *C. villosa* is absent (Adulam; left side) and from the site in which it is abundant (Ramat-Hanadiv; right side) as a function of inoculation with a *Bradyrhizobium* isolate vs inoculation with Ramat-Hanadiv soil and fertilization treatment, for two repeats of the experiment pooled together ($n = 5$, and $n = 7$). Bars in boxplots are the interquartile range (IQR) between the 25th and the 75th percentiles, and lines inside bars are the median. Values above each pair are the P -values for that specific comparison. (d) Representative photos of plants from each treatment are arranged below their respective boxplot.

soil is deficient in nutrients other than nitrogen needed for either plant growth or symbiotic N₂-fixation, while Adulam soil is relatively rich in nutrients.

Root biomass was not affected by inoculation, except for fertilized Adulam soil, where inoculation increased root biomass only in one of the experimental repeats ($F=5.394$, $P=0.0321$, Fig. 5b). In accordance, the root mass fraction roughly mirrors shoot biomass: significantly higher in Adulam soil in uninoculated plants compared to inoculated plants in both unfertilized ($F=40.95$, $P<<0.0001$; Fig. 5c; Table 2) and fertilized treatment ($F=9.277$, $P=0.006$). Importantly, in Ramat-Hanadiv soil, where the native microbiota consistently nodulated *C. villosa* (Fig. 2a,b), inoculation did not have a significant effect on shoot and root biomass (Fig. 4a,b,d; Table 2).

In unfertilized Adulam soil, inoculated plants were also taller and grew aboveground faster than uninoculated plants, with no significant difference between inoculation with *Bradyrhizobium* culture or with Ramat-Hanadiv soil (1.15 ± 0.05 and 0.99 ± 0.02 cm wk⁻¹ for plants inoculated with Ramat-Hanadiv soil and with *Bradyrhizobium*, and 0.69 ± 0.02 cm wk⁻¹ for

uninoculated plants, respectively; $F=33.95$, $P<<0.0001$; Figs 5d, S6). We measured a similar effect in unfertilized Ramat-Hanadiv soil with plant shoots growing at a rate of 0.67 ± 0.04 and 0.38 ± 0.01 cm wk⁻¹ in inoculated vs uninoculated treatments, respectively ($F=54.74$, $P<<0.0001$). In fertilized Adulam soil, inoculation had no significant effect on the shoot growth rate, but in fertilized Ramat-Hanadiv soil the shoots of inoculated plants grew significantly slower than uninoculated plants, potentially due to increased allocation to the growth of roots and root nodules (0.47 ± 0.03 vs 0.82 ± 0.07 cm wk⁻¹, respectively; Fig. S6).

In fertilized Adulam soil, inoculated plants had twice the leaf N concentration compared to uninoculated plants, and similar to the concentration of nitrogen in the leaves of seedlings grown in both unfertilized and fertilized Ramat-Hanadiv soil (Fig. 6a; Table 2). Plants grown in unfertilized Adulam soil and inoculated with *Bradyrhizobium* or Ramat-Hanadiv soil had significantly higher leaf nitrogen concentrations compared to uninoculated plants ($4.02 \pm 0.17\%$ and $4.08 \pm 0.16\%$ vs $2.02 \pm 0.12\%$, respectively; $F=28.83$, $P<<0.0001$; Fig. 6a).

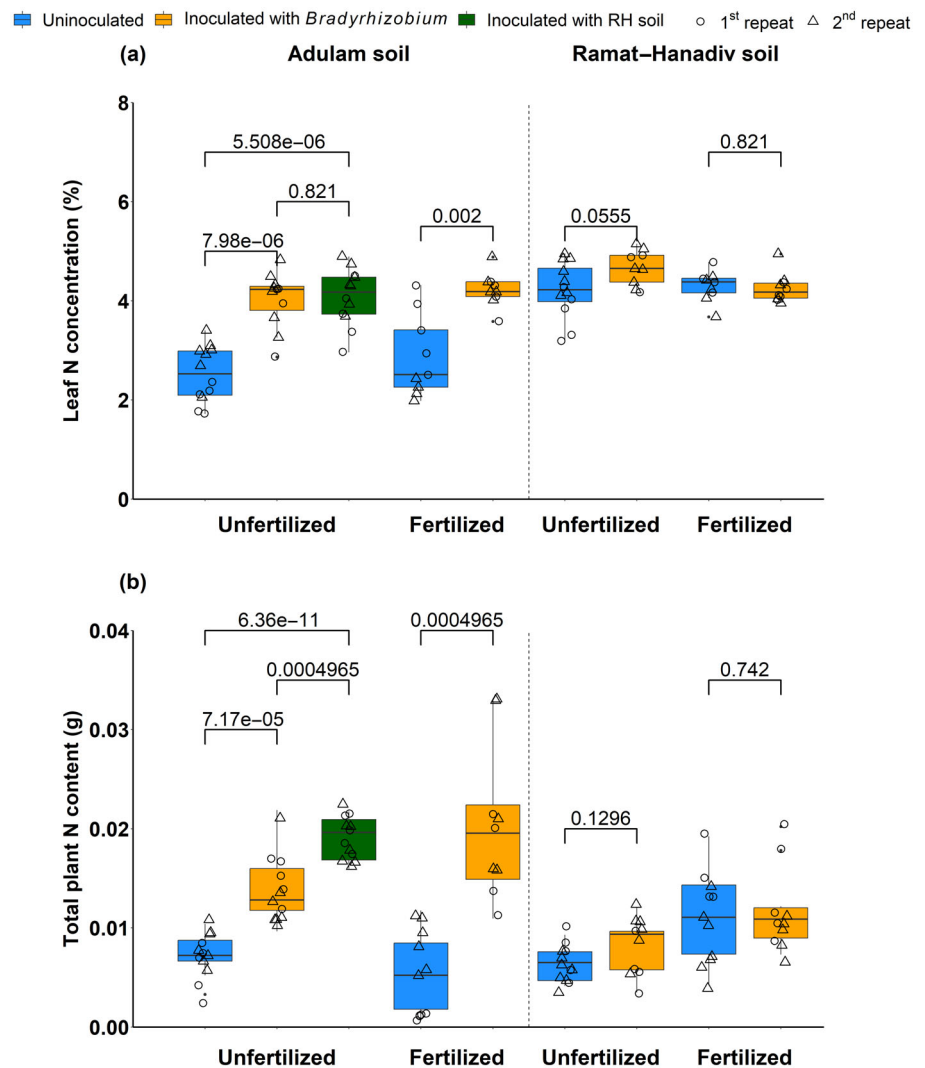


Fig. 6 Response of leaf and plant nitrogen of seedlings of *Calicotome villosa* to soil source, inoculation and fertilization treatments. Leaf N concentration (%) (a) and total plant N content (g) (b) of seedlings grown in soil from the site in which *C. villosa* is absent (Adulam; left side) and from the site in which it is abundant (Ramat-Hanadiv; right side) as a function of inoculation with a *Bradyrhizobium* isolate vs inoculation with Ramat-Hanadiv soil and fertilization treatment, for two repeats of the experiment pooled together ($n=5$, and $n=7$). Bars in boxplots are the interquartile range (IQR) between the 25th and the 75th percentiles, and lines inside bars are the median. Values above each pair are the P -values for that specific comparison.

Inoculation had a similar yet smaller effect in plants grown in unfertilized Ramat-Hanadiv soil, with $4.67 \pm 0.11\%$ vs $4.14 \pm 0.16\%$ in inoculated and uninoculated plants, respectively ($F = 4.423$, $P = 0.037$; Fig. 6b). Total plant nitrogen content showed similar patterns to shoot biomass in all treatments. Therefore, we concluded that inoculation of Adulam soil with the *Bradyrhizobium* symbiont recovered the plant's ability to symbiotically fix N_2 and thus restored its growth and nitrogen economy.

The bacterial genus *Bradyrhizobium* is absent from Adulam soil

Given these results, we hypothesized that *C. villosa* is absent from Adulam because its N_2 -fixing symbiont is absent. To test this hypothesis, we compared the composition of the bacterial community in the soils collected from Adulam vs Ramat-Hanadiv using 16S rRNA gene amplicon sequencing. The bacterial community composition of both soil types at the genus level is shown in Fig. S7. Adulam soil samples were found to be highly heterogeneous in bacterial community composition compared to Ramat-Hanadiv soils (Fig. S8). Despite this heterogeneity, we found that the *Bradyrhizobium* genus was completely absent from all Adulam soil samples, whereas in Ramat-Hanadiv, it was highly abundant, comprising an average of $3.27 \pm 0.22\%$ of the bacterial community's 16S reads. Moreover, we found that the relative abundance of the entire *Alphaproteobacteria* class, which contains most symbiotic N_2 -fixing bacteria, was lower in Adulam soil compared to Ramat-Hanadiv ($7.74 \pm 3.5\%$ vs $29.73 \pm 1.17\%$, respectively; Kruskal–Wallis test due to low sample size: chi-squared = 3.8571 $P = 0.049$; Fig. S9).

Discussion

The main objectives of this study were to characterize the niche breadth of a common dryland perennial legume, and to evaluate whether the absence of a suitable N_2 -fixing mutualist can lead to a reduction of its realized niche. Indeed, soils sampled from the region in which *C. villosa* was absent contained no 16S rRNA reads that mapped to the *Bradyrhizobium* genus, which are the only known N_2 -fixing bacteria that form viable nodules in *C. villosa*. Although this is based on a small sample of the soils, it was surprising given that many species of herbaceous legumes are present in this region (Adulam, with at least 30 different species; R. Dagan, pers. comm.), and are known to actively nodulate and associate with N_2 -fixing bacteria under field conditions (A. B. Pokhrel, unpublished). A field survey of a larger scale will be needed to further confirm the absence of *Bradyrhizobium* from these soils and to understand the reasons behind it. Nevertheless, we found that seedlings of *C. villosa* failed to form nodules in Adulam soil unless inoculated with an external source of *Bradyrhizobium* bacteria. Inoculation of soil from Adulam with *Bradyrhizobium* induced nodulation and improved the growth and nitrogen accumulation of seedlings, supporting our hypothesis that the absence of the N_2 -fixing symbiont from Adulam soil is a major factor explaining the absence of *C. villosa* from this region.

Calicotome villosa is limited in its association with N_2 -fixing symbionts

Co-dependence between the distributions of host plants and their relevant mutualists plays a significant role in shaping plant distributions, in addition to facilitative effects induced by the presence of the mutualist (Parker, 2001; Afkhami *et al.*, 2014). The failure of a legume to establish itself in a new habitat, due to a lack of a suitable bacterial mutualist, was shown to be the reason why some legume species fail to colonize far from their native habitat (Parker & Parker, 2006; Simonsen *et al.*, 2017). Our comprehensive survey showed that viable populations of *C. villosa* exist within a radius of 5 km from Adulam. Additionally, Adulam and the entire Mediterranean region have seen significant human activity (e.g. livestock grazing and human migration) for millennia, making a dispersal limitation of either plant seeds or soil bacteria seem unlikely (Arroyo *et al.*, 2008).

Annual legumes are common and diverse in the region of Adulam, and therefore, we assumed that many species of N_2 -fixing bacteria inhabit the soil, but found that these are primarily *Microvirga*, *Sinorhizobia* and *Neorhizobia* (R. Dagan *et al.*, unpublished). As an example, the *Sinorhizobium* we used as an outgroup in constructing the nod genes' phylogenetic trees, which was isolated from plants grown in Adulam soil, was genetically distant to RH 3a 1, and therefore unlikely to associate with *C. villosa* roots. Furthermore, it has been shown that some legumes can associate with different mutualists in their native habitat when edaphic conditions change (de Castro *et al.*, 2018; Rath *et al.*, 2018). Thus, the lack of nodules in seedlings grown in Adulam soil suggests that *C. villosa* can form a mutualism only with a specific bacterial genus, that is *Bradyrhizobium*, as is known for other legumes of the Genista tribe (Gonzalez-Andres & Ortiz, 1999; Stepkowski *et al.*, 2018).

The legume-rhizobia symbiosis can be characterized by a spectrum between host-mutualist specificity and promiscuity and varies significantly between species belonging to the same family or tribe (Andrews & Andrews, 2017). Mutualist specificity has been previously reported for the Genista tribe, to which *C. villosa* belongs (Gonzalez-Andres & Ortiz, 1999; Cardinale *et al.*, 2008), but whether *C. villosa* exhibits specificity to a particular clade within the *Bradyrhizobium* genus is as of yet unclear. However, the fact that strains closely related to our isolate were found to associate with a wide range of plant species suggests that this strain is not an exclusive symbiont of this particular plant species. The wide diversity of genome-sequenced *Bradyrhizobium* isolates (Fig. S2) can provide an excellent system for studying the boundaries of specificity in legume-rhizobia mutualisms. Interestingly, the phylogenetic trees derived from the AMPHORA2 genes and the nod genes were markedly different. While the 31 housekeeping genes used by AMPHORA2 indicated a wide variety of possible origins of the RH 3a 1 strain, the nod-based trees point to a more recent acquisition of these genes upon association to local plant hosts, adapted to a Mediterranean and semi-arid climate. Thus, these nod genes were likely acquired by RH 3a 1 via horizontal gene transfer from local nodulating bacteria, a phenomenon known

to occur in *Bradyrhizobium* (Minamisawa *et al.*, 2002; Moulin *et al.*, 2004).

Association with a symbiont alters the N economy of *C. villosa*

The results of our controlled experiments suggest that *C. villosa* is critically dependent on symbiotic N₂ fixation as its main source of N, even when nitrogen is available in the soil. Adulam soils had twice as much available N as Ramat-Hanadiv soils in our experiment, and yet leaf N concentration was half in plants growing in uninoculated plants in the former vs the latter (Fig. 5a). Furthermore, biomass allocation to roots was also significantly higher in uninoculated plants grown in Adulam soil, suggesting higher investment by these plants in acquiring soil nutrients. These results suggest that *C. villosa* may be limited in its ability to acquire available soil N exclusively through fine root uptake. In addition, inoculated plants in unfertilized Adulam soil grew more and eventually contained more nitrogen compared to plants in unfertilized Ramat-Hanadiv soil, probably due to the combination of symbiotic N₂ fixation with the higher availability of phosphorus in Adulam soil compared to Ramat-Hanadiv soil.

Previous studies, including both controlled and field experiments, found that *C. villosa* regulated its investment in nodule construction and within-nodule bacterial activity in response to the availability of N in the soil (i.e. facultative regulation of fixation *sensu* Hedin *et al.*, 2009; Dovrat *et al.*, 2018; Alon *et al.*, 2021), therefore suggesting that *C. villosa* plants can grow without symbiotic N fixation. However, our findings show that without its symbiont, *C. villosa* acquires very little N through root uptake even though N is available in the soil, and with significantly higher root biomass. Another possibility is that in the absence of the symbiont, *C. villosa* alters its N economy by lowering the concentration of N throughout the plant, primarily in the leaves, to adapt to unfavorable conditions (Wolf *et al.*, 2017; Dovrat *et al.*, 2020).

Interestingly, plants grown without fertilization in Adulam soils had higher shoot biomass when inoculated with Ramat-Hanadiv soil compared to only *Bradyrhizobium*. This suggests that either there is some micronutrient that is deficient in Adulam soil and was supplemented by Ramat-Hanadiv soil, or that there is some microbe present in Ramat-Hanadiv soil that improves the growth of *C. villosa* beyond our *Bradyrhizobium* isolate. It is also conceivable that a higher diversity of symbiotic N-fixers would be more productive. However, based on the available results we cannot currently confirm or reject any of these hypotheses.

Abiotic limitations on the *Bradyrhizobium* symbiont in chalk-derived soils

It is now established that soil pH is one of the main drivers of soil microbial community composition and, in particular, has a strong influence on the abundance of N₂-fixing bacteria (Lemaire *et al.*, 2015; Fierer, 2017; de Castro *et al.*, 2018; Ramoneda *et al.*, 2023; Sepp *et al.*, 2023). Alkaline soils limit the growth

and survival of some species of Bradyrhizobia and may limit their geographical range (Tang *et al.*, 1991; Graham, 1992; Rathi *et al.*, 2018; Bouhnik *et al.*, 2022). Furthermore, it is well known that in alkaline soils, such as Adulam soil, the availability of iron is greatly reduced (Colombo *et al.*, 2014) which may inhibit both nodulation and the N₂ fixation process itself (Rupela & Saxena, 1987; Tang *et al.*, 1991).

Our results show that the soil in Adulam is slightly more alkaline compared to the soil in Ramat-Hanadiv, therefore suggesting that this difference is a possible factor in the absence of *Bradyrhizobium* from Adulam. We also showed that fertilization, which included iron and all other micronutrients needed for nitrogen fixation, increased growth and fixation in Adulam soil. A study on a closely related strain of *Bradyrhizobium* identified in *C. villosa*, showed a significant decrease in the colony-forming unit (CFU) count with increasing pH of the bacterial growth medium, even at small pH differences of 0.5 (Meghvansi *et al.*, 2005). Although most soils in Israel are alkaline due to their high content of calcium carbonate, the pH of chalk-derived soils in regions with low rainfall is significantly higher than the pH of limestone-derived soils, therefore imposing stronger limitations on *Bradyrhizobium* growth (Singer, 2007; Gehlot *et al.*, 2012; Guerrouj *et al.*, 2013). Indeed, as we found in the survey, *C. villosa* was mainly absent from sites with chalk bedrock within the lower range of mean annual precipitation. We note, however, that although inoculation in Adulam soil did induce the formation of viable nodules in *C. villosa*, the inoculation solution contained a very large number of bacteria inoculated directly on the base of the plant, potentially reducing the influence of the soil conditions on microbial performance. *Bradyrhizobium* could therefore be limited in its *persistence* in the soil (e.g. during rainless summers; Dovrat & Sheffer, 2018) rather than in its immediate survival during inoculation.

Conclusions

Although *C. villosa* is adapted to a wide set of environmental conditions, we show here that the realized niche of this common shrub is likely constrained by the local absence of a compatible N₂-fixing symbiont. Furthermore, the high specificity of this legume in associating with soil N₂-fixing bacteria is necessary to maintain its N economy. Thus, the apparent low capacity of *C. villosa* to efficiently acquire available soil N hints at a trade-off between symbiotic N₂ fixation and uptake of soil nitrogen by fine roots.

The absence of the *Bradyrhizobium* genus as well as the lower relative abundance of other N₂-fixing bacteria in Adulam soil is striking, as this is likely the first reported case of a limitation of the realized niche of a legume within its native range, by the niche limitations of its symbiont. In other words, we show that the fundamental niche of the host legume must overlap with the range of its compatible mutualist. This leads to the conclusion that the distribution patterns of legumes, and therefore inputs of new fixed N into terrestrial ecosystems, cannot be fully understood without knowledge of the co-distribution of their respective mutualists.

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Competing interests

None declared.

Author contributions

MA, YW, ES and OMF designed the study and experimental methodology. MA performed all experimental work and data analyses. MA and YW conducted all fieldwork. MA, OMF and ES wrote the manuscript, and all authors contributed to the final manuscript and gave final approval for publication.

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Data availability

The data that supports the findings of this study are available in the supporting information of this article. Sequencing data are available online at NCBI with the following accessions: whole genome of RH 3 1 isolate TaxID: 1404864, Biosample accessions for 16S sequencing data: SAMN35741709, SAMN35741710, SAMN35741711, SAMN35741712, SAMN35741713, SAMN35741714.

References

- Afkhami ME, McIntyre PJ, Strauss SY. 2014. Mutualist-mediated effects on species' range limits across large geographic scales. *Ecology Letters* 17: 1265–1273.
- Alon M. 2021. *Linking strategies of symbiotic nitrogen fixation with the nitrogen cycle in drylands*. PhD Thesis. Rehovot, Israel: The Hebrew University of Jerusalem.
- Alon M, Dovrat G, Masci T, Sheffer E. 2021. Soil nitrogen regulates symbiotic nitrogen fixation in a legume shrub but does not accumulate under it. *Ecosphere* 12: 1045–1061.
- Andrews M, Andrews ME. 2017. Specificity in legume-rhizobia symbioses. *International Journal of Molecular Sciences* 18: 705.
- Arroyo J, Aparicio A, Albaladejo RG, Muñoz J, Braza R. 2008. Genetic structure and population differentiation of the Mediterranean pioneer spiny broom *Calicotome villosa* across the strait of gibraltar. *Biological Journal of the Linnean Society* 93: 39–51.
- Bates D, Mächler M, Bolker B, Walker S. 2015. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 67: 1–48.
- Bouhnik O, Alami S, Lamin H, Lamrabet M, Bennis M, Ouajdi M, Bellaka M, Antri SE, Abbas Y, Abdelmoumen H *et al.* 2022. The fodder legume *Chamaecytisus albidus* establishes functional symbiosis with different bradyrhizobial symbiovars in morocco. *Microbial Ecology* 84: 794–807.
- Brear EM, Day DA, Smith PMC. 2013. Iron: an essential micronutrient for the legume-rhizobium symbiosis. *Frontiers in Plant Science* 4: 1–15.
- Burnham KP, Anderson DR. 2004. Multimodel inference: understanding AIC and BIC in model selection. *Sociological Methods & Research* 33: 261–304.
- Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. DADA2: high-resolution sample inference from illumina amplicon data. *Nature Methods* 13: 581–583.
- Cardinale M, Lanza A, Bonni ML, Marsala S, Puglia AM, Quatrini P. 2008. Diversity of rhizobia nodulating wild shrubs of Sicily and some neighbouring islands. *Archives of Microbiology* 190: 461–470.
- de Castro PR, dos Reis Junior FB, Zilli JE, Fischer D, Hofmann A, James EK, Simon MF. 2018. Soil characteristics determine the rhizobia in association with different species of Mimosa in central Brazil. *Plant and Soil* 423: 411–428.
- Chahboune R, Carro L, Peix A, Ramírez-Bahena M-H, Barrijal S, Velázquez E, Bedmar EJ. 2012. *Bradyrhizobium rifense* sp. nov. isolated from effective nodules of *Cytisus villosus* grown in the moroccan rif. *Systematic and Applied Microbiology* 35: 302–305.
- Colombo C, Palumbo G, He JZ, Pinton R, Cesco S. 2014. Review on iron availability in soil: interaction of Fe minerals, plants, and microbes. *Journal of Soils and Sediments* 14: 538–548.
- Davies-Barnard T, Friedlingstein P. 2020. The global distribution of biological nitrogen fixation in terrestrial natural ecosystems. *Global Biogeochemical Cycles* 34: 1–17.
- Delavaux CS, Weigelt P, Magnoli SM, Kreft H, Crowther TW, Bever JD. 2022. Nitrogen-fixing symbiotic bacteria act as a global filter for plant establishment on islands. *Communications Biology* 5: 1–6.
- Dovrat G, Bakhshian H, Masci T, Sheffer E. 2020. The nitrogen economic spectrum of legume stoichiometry and fixation strategy. *New Phytologist* 227: 365–375.
- Dovrat G, Masci T, Bakhshian H, Mayzlish Gati E, Golan S, Sheffer E. 2018. Drought-adapted plants dramatically downregulate dinitrogen fixation: evidences from Mediterranean legume shrubs. *Journal of Ecology* 106: 1534–1544.
- Dovrat G, Sheffer E. 2018. Symbiotic dinitrogen fixation is seasonal and strongly regulated in water-limited environments. *New Phytologist* 221: 1866–1877.
- Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32: 1792–1797.
- Eren AM, Kiehl E, Shaiber A, Veseli I, Miller SE, Schechter MS, Fink I, Pan JN, Yousef M, Fogarty EC *et al.* 2021. Community-led, integrated, reproducible multi-omics with anvi'o. *Nature Microbiology* 6: 3–6.
- Fierer N. 2017. Embracing the unknown: disentangling the complexities of the soil microbiome. *Nature Reviews Microbiology* 15: 579–590.
- Gehlot HS, Panwar D, Tak N, Tak A, Sankhla IS, Poonar N, Parihar R, Shekhawat NS, Kumar M, Tiwari R *et al.* 2012. Nodulation of legumes from the Thar desert of India and molecular characterization of their rhizobia. *Plant and Soil* 357: 227–243.
- Gehlot HS, Tak N, Kaushik M, Mitra S, Chen W-M, Poweleit N, Panwar D, Poonar N, Parihar R, Tak A *et al.* 2013. An invasive mimosa in India does not adopt the symbionts of its native relatives. *Annals of Botany* 112: 179–196.
- Gonzalez-Andres F, Ortiz JM. 1999. Specificity of rhizobia nodulating genista monspessulana and genista linifolia *in vitro* and in field situations. *Arid Soil Research and Rehabilitation* 13: 223–237.
- Graham PH. 1992. Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditions. *Canadian Journal of Microbiology* 38: 475–484.
- Graham V. 2003. Legumes: importance and constraints to greater use. *Plant Physiology* 131: 872–877.
- Guerrouj K, Ruiz-Díez B, Chahboune R, Ramírez-Bahena M-H, Abdelmoumen H, Quinones MA, El Idrissi MM, Velázquez E, Fernández-Pascual M, Bedmar EJ *et al.* 2013. Definition of a novel symbiovar (sv. retamae) within *Bradyrhizobium retamae* sp. nov., nodulating *Retama sphaerocarpa* and *Retama monosperma*. *Systematic and Applied Microbiology* 36: 218–223.
- Guisan A, Thuiller W. 2005. Predicting species distribution: offering more than simple habitat models. *Ecology Letters* 8: 993–1009.

- Hedin LO, Brookshire ENJ, Menge DNL, Barron AR. 2009. The nitrogen paradox in tropical forest ecosystems. *Annual Review of Ecology, Evolution, and Systematics* 40: 613–635.
- Hutchinson GE. 1957. Concluding remarks. *Cold Spring Harbor Symposia on Quantitative Biology* 22: 415–427.
- Hyatt D, Chen GL, LoCasio PF, Land ML, Larimer FW, Hauser LJ. 2010. Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* 11: 119.
- Jenks GF. 1967. The data model concept in statistical mapping. *International Yearbook of Cartography* 7: 186–190.
- Klock MM, Barrett LG, Thrall PH, Harms KE. 2015. Host promiscuity in symbiont associations can influence exotic legume establishment and colonization of novel ranges. *Diversity and Distributions* 21: 1193–1203.
- Lemaire B, Dlodlo O, Chimphango S, Stirton C, Schrire B, Boatwright JS, Honnay O, Smets E, Sprent J, James EK *et al.* 2015. Symbiotic diversity, specificity and distribution of rhizobia in native legumes of the Core Cape Subregion (South Africa). *FEMS Microbiology Ecology* 91: 1–17.
- Lugtenberg B. 2015. *Principles of plant-microbe interactions: Microbes for sustainable agriculture*. Leiden, The Netherlands: Springer.
- Maynard DG, Kalra YP, Crumbaugh JA. 1993. Nitrate and exchangeable ammonium nitrogen. *Soil Sampling and Methods of Analysis* 1: 25–38.
- McMurdie PJ, Holmes S. 2013. PHYLOSEQ: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* 8: e61217.
- Meghvansi MK, Prasad K, Mahna SK. 2005. Identification of pH tolerant *Bradyrhizobium japonicum* strains and their symbiotic effectiveness in soybean [*Glycine max* (L.) Merr.] in low nutrient soil. *African Journal of Biotechnology* 4: 663–666.
- Minamisawa K, Itakura M, Suzuki M, Ichige K, Isawa T, Yuhashi K, Mitsui H. 2002. Horizontal transfer of nodulation genes in soils and microcosms from *Bradyrhizobium japonicum* to *B. elkanii*. *Microbes and Environments* 17: 82–90.
- Moulin L, Béna G, Boivin-Masson C, Stępkowski T. 2004. Phylogenetic analyses of symbiotic nodulation genes support vertical and lateral gene co-transfer within the *Bradyrhizobium* genus. *Molecular Phylogenetics and Evolution* 30: 720–732.
- Norman RJ, Stucki JW. 1981. The determination of nitrate and nitrite in soil extracts by ultraviolet spectrophotometry. *Soil Science Society of America Journal* 45: 347–353.
- O'Hara GW. 2001. Nutritional constraints on root nodule bacteria affecting symbiotic nitrogen fixation: a review. *Australian Journal of Experimental Agriculture* 41: 417–433.
- Olsen SR, Sommers LE. 1982. Determination of available phosphorus. *Method of Soil Analysis* 2: 2018.
- Parker MA. 2001. Mutualism as a constraint on invasion success for legumes and rhizobia. *Diversity and Distributions* 7: 125–136.
- Parker M, Malek W, Parker IM. 2006. Growth of an invasive legume is symbiont limited in newly occupied habitats. *Diversity and Distributions* 12: 563–571.
- Price MN, Dehal PS, Arkin AP. 2009. FASTTREE: computing large minimum evolution trees with profiles instead of a distance matrix. *Molecular Biology and Evolution* 26: 1641–1650.
- R Core Team. 2022. *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.
- Ramonedá J, Stallard-Olivera E, Hoffert M, Winfrey CC, Stadler M, Niño-García JP, Fierer N. 2023. Building a genome-based understanding of bacterial pH preferences. *Science Advances* 9: ead8998.
- Rathi S, Tak N, Bissa G, Chouhan B, Ojha A, Adhikari D, Barik SK, Satyawada RR, Sprent JI, James EK *et al.* 2018. Selection of *Bradyrhizobium* or *Ensifer* symbionts by the native Indian caesalpinoid legume *Chamaecrista pumila* depends on soil pH and other edaphic and climatic factors. *FEMS Microbiology Ecology* 94: fuy180.
- Richardson DM, Allsopp N, D'antonio CM, Milton SJ, Rejmánek M. 2000. Plant invasions — the role of mutualisms. *Biological Reviews* 75: 65–93.
- Rodríguez-Echeverría S, Le Roux JJ, Crisóstomo JA, Ndlovu J. 2011. Jack-of-all-trades and master of many? How does associated rhizobial diversity influence the colonization success of Australian Acacia species? *Diversity and Distributions* 17: 946–957.
- Rupela OP, Saxena MC. 1987. Nodulation and nitrogen fixation in chickpea. *The Chickpea* 191: 115–125.
- Sepp S-K, Vasar M, Davison J, Oja J, Anslan S, Al-Quraishy S, Bahram M, Bueno CG, Cantero JJ, Fabiano EC *et al.* 2023. Global diversity and distribution of nitrogen-fixing bacteria in the soil. *Frontiers in Plant Science* 14: 1100235.
- Shen C, Xiong J, Zhang H, Feng Y, Lin X, Li X, Liang W, Chu H. 2013. Soil pH drives the spatial distribution of bacterial communities along elevation on Changbai Mountain. *Soil Biology and Biochemistry* 57: 204–211.
- Simonsen AK, Dinnage R, Barrett LG, Prober SM, Thrall PH. 2017. Symbiosis limits establishment of legumes outside their native range at a global scale. *Nature Communications* 8: 1–9.
- Singer A. 2007. *The soils of Israel*. Berlin, Heidelberg, Germany: Springer.
- Sprent JI, Ardley J, James EK. 2017. Biogeography of nodulated legumes and their nitrogen-fixing symbionts. *New Phytologist* 215: 40–56.
- Stanton-Geddes J, Anderson CG. 2011. Does a facultative mutualism limit species range expansion? *Oecologia* 167: 149–155.
- Stępkowski T, Banasiewicz J, Granada CE, Andrews M, Passaglia LMP. 2018. Phylogeny and phylogeography of rhizobial symbionts nodulating legumes of the tribe genisteae. *Genes* 9: 163.
- Tamme R, Pärtel M, Kõljalg U, Laanisto L, Liira J, Mander Ü, Moora M, Niinemets Ü, Öpik M, Ostonen I *et al.* 2021. Global macroecology of nitrogen-fixing plants. *Global Ecology and Biogeography* 30: 514–526.
- Tang C, Robson AD, Dilworth MJ. 1991. Inadequate iron supply and high bicarbonate impair the symbiosis of peanuts (*Arachis hypogaea* L.) with different *Bradyrhizobium* strains. *Plant and Soil* 138: 159–168.
- Taylor BN, Menge DNL. 2018. Light regulates tropical symbiotic nitrogen fixation more strongly than soil nitrogen. *Nature Plants* 4: 655–661.
- Torchin ME, Mitchell CE. 2004. Parasites, pathogens, and invasions by plants and animals. *Frontiers in Ecology and the Environment* 2: 183–190.
- Vitousek PM, Menge DNL, Reed SC, Cleveland CC. 2013. Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. *Philosophical Transactions of the Royal Society, B: Biological Sciences* 368: 20130119.
- Wickham H. 2016. *GGPLOT2: elegant graphics for data analysis*. New York, NY, USA: Springer-Verlag.
- Wolf AA, Funk JL, Menge DNL. 2017. The symbionts made me do it: legumes are not hardwired for high nitrogen concentrations but incorporate more nitrogen when inoculated. *New Phytologist* 213: 690–699.
- Wu M, Scott AJ. 2012. Phylogenomic analysis of bacterial and archaeal sequences with AMPHORA2. *Bioinformatics* 28: 1033–1034.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Observations of *Calicotome villosa* as documented in the BioGIS database (BioGIS, 2020).

Fig. S2 Phylogenetic tree of the *Bradyrhizobium* genus with this study's isolate closely related clade in green.

Fig. S3 Pan-genomic analysis of RH 3a 1 (Ramat-Hanadiv isolate) and its closely related strains.

Fig. S4 Phylogenetic trees of the *Bradyrhizobium* genus with this study's isolate closely related clade in green, based on alignments of the nodA, nodB and nodC genes.

Fig. S5 Phylogenetic trees of the *Bradyrhizobium* genus with this study's isolate closely related clade in green, based on the concatenated alignment of the nodA, nodB and nodC genes.

Fig. S6 Plant height change over the time of the experiment as a function of each inoculation and fertilization treatment in soil collected from Adulam and from Ramat-Hanadiv.

Fig. S7 Relative abundance of bacterial rRNA 16S reads of the different bacterial genera in Adulam and Ramat-Hanadiv soil samples, based on three replicate samples from each soil pool.

Fig. S8 Composition of the bacterial community in the soil samples collected from Adulam and Ramat-Hanadiv using PCoA ordination.

Fig. S9 Relative proportion of rRNA 16S reads of the *Bradyrhizobium* genus and the *Alphaproteobacteria* class in the soils used in the controlled experiment.

Table S1 Statistical results of ANOVAs of the effects of fertilization, inoculation with *Bradyrhizobium* and soil type and their interaction on biomass production and allocation, and nitrogen economy in plants grown in Adulam or Ramat-Hanadiv soil.

Table S2 Results of ANOVAs and multiple regression of the density of all *Calicotome villosa* individuals (adults + seedlings) and seedlings as influenced by: soil type and mean annual precipitation, and their combinations.

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