Drought-adapted plants dramatically downregulate dinitrogen fixation: Evidences from Mediterranean legume shrubs

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Abstract
1. The importance of symbiotic dinitrogen (N\textsubscript{2}) fixation in shaping the coupled nitrogen–carbon cycle is now known for most humid terrestrial ecosystems. However, whether N\textsubscript{2} fixation can play a key role in the nitrogen and carbon budget of water-limited and seasonally dry ecosystems remains a mystery.

2. The maintenance of metabolically and physiologically costly symbiotic fixation in water-limited environments is highly complex. These costs are particularly high during the first developmental season, when allocation to deep rooting and drought resistance mechanisms is essential for seedling survival of prolonged seasonal drought.

3. We, therefore, evaluated how drought-adapted legume species change their allocation to symbiotic nitrogen fixation as a function of soil nitrogen availability. We tested this on seedlings of a suite of four common Mediterranean legume shrubs with a strong seasonal behaviour, which we grew under controlled nitrogen and phosphorus availabilities. We asked: (1) Do species differ in their investment and regulation of nitrogen fixation? (2) Is fixation regulated via plant allocation to nodules, fixation rate or both? and (3) Does phosphorus availability limit symbiotic nitrogen fixation?

4. All Mediterranean perennial legumes in the experiment established and grew, nodulated, and fixed nitrogen, even under severe nitrogen limitation. The four species reacted similarly to nitrogen supply, by strongly downregulating fixation through both decreased nodulation and lower rate of fixation. However, we found a significant interspecific difference in fixation (both nodulation and rate), biomass production and growth rate. Our experimental species presented a range of fixation investment strategies corresponding to life-history and resource partitioning patterns. Phosphorus limitation had a minor influence on both fixation and plant growth.

5. Synthesis. The high physiological cost of symbiotic fixation imposes the need to tightly regulate fixation in perennial legumes coping with severe water stress. Control of fixation allows legume species to colonize recently disturbed nitrogen-deficient habitats, cope with grazing, survive long seasonal droughts and recover nitrogen fixation later in the wet season, and survive over time by reducing nitrogen inputs to the ecosystem.

Keywords
BNF, Fabacea, fixation rate, leaf nitrogen content, Mediterranean ecosystems, resource partitioning, symbiosis, water limitation
1 | INTRODUCTION

Dinitrogen (N\textsubscript{2}) fixation is the principal source of non-anthropogenic nitrogen in terrestrial ecosystems (Cleveland et al., 1999; Vitousek, Menge, Reed, & Cleveland, 2013). N\textsubscript{2} fixation in plants (hereafter “fixers”), by symbiotic root bacteria, has been shown to play a key role in the coupled carbon–nitrogen cycle in tropical (Batterman, Hedin, et al., 2013), temperate and boreal (Binkley, Senock, & Cromack, 2003; Binkley, Sollins, Bell, Sachs, & Myrold, 1992; Crocker & Major, 1955) forests, and potentially, is also functionally important in Mediterranean, water-limited, seasonal and nitrogen-deficient dryland ecosystems (Sprent & Gehlot, 2010).

Seasonal droughts and water limitation influence processes from plant-level physiology to vegetation structure and ecosystem functioning (Minucci, Miniat, Teskey, & Wurzburger, 2017; Tenhunen, Serra, Harley, Dougherty, & Reynolds, 1990). Water limitation can constrain the uptake of nutrients by plants, and therefore exerts a direct influence on plant nutrient availability, or indirectly constrain the supply of soil nutrients, by limiting most nutrient cycling processes (Evans & Belnap, 1999; Schlesinger, Abrahams, Parsons, & Wainwright, 1999; West, 1991). Therefore, nitrogen cycling in seasonally dry environments is influenced by rainfall patterns (Aranibar et al., 2004), and the availability of nitrogen is usually limited and pulsed (Grünzweig, Navon, Kanas, Dirks, & Dumbur, 2010). Likewise, nitrogen fixation must be influenced by water availability.

Strategies of symbiotic N\textsubscript{2} fixation in plants (Barron, Purves, & Hedin, 2011; Menge, Wolf, & Funk, 2015), and their ecosystem consequences (Hedin, Brookshire, Menge, & Barron, 2009; Houlton, Wang, Vitousek, & Field, 2008; Vitousek et al., 2013), have been the focus of major recent breakthroughs in the understanding of the ecology of tropical and extra-tropical forests (Barron et al., 2011; Batterman, Hedin, et al., 2013; Batterman, Wurzburger, & Hedin, 2013; Menge & Hedin, 2009; Wurzburger & Hedin, 2016). Although N\textsubscript{2} fixation and N\textsubscript{2}-fixing plants (mostly legumes, Fabacea) have been described in many drylands globally (see Adams, Turnbull, Sprent, & Buchmann, 2016; Sprent & Gehlot, 2010), their fixation strategies and functional influences in such ecosystems are largely unresolved.

Symbiotic fixation is especially complex for plants in dry seasonal environments. Dry conditions limit nutrient availability and dramatically constrain plant metabolism for sustained parts of the year. Therefore, the competitive advantage of having an endogenous source of nitrogen can easily be offset by the high metabolic and physiological costs of hosting N\textsubscript{2}-fixing bacteria (Gutschick, 1981; McKey, 1994). Fixation costs also are associated with higher energetic, phosphorus (P) and molybdenum demands (Barron et al., 2009; Binkley et al., 2003; Wurzburger, Bellenger, Kraepiel, & Hedin, 2012), and plant investment in building a moist, protected and controlled environment for the bacterial symbionts in their root nodules.

Furthermore, dry environments present an acute trade-off between plant allocation towards drought tolerance and survival (e.g. a deep root system, root:shoot partitioning, leaf traits; Dovrat, 2015; Valladares, Martínez-Ferri, Balaguer, Perez-Corona, & Manrique, 2000), and plant allocation towards the maintenance of fixation. Mediterranean fixers, for example, must be adapted to drought and at the same time committed to: (1) maintenance of vital root nodules in the dry upper soil layers; (2) allocation towards a root system for coping with limitation by water and other nutrients (Barron et al., 2009); and (3) high foliar nitrogen, which potentially increases water-use efficiency (Adams et al., 2016), but makes them more sensitive to the effects of herbivory (McKey, 1994). The latter is especially important in the Mediterranean Basin, where plants have adapted to a long history of intensive grazing (Perevolotsky & Seligman, 1998). We, therefore, hypothesize that Mediterranean N\textsubscript{2} fixers will use fixation sparingly, and strongly downregulate fixation when soil nitrogen is available.

Conversely, water stress, seasonality and pulsed precipitation all result in high fluctuations in the availability of soil nitrogen, as well as a highly confined growing season. An obligate fixation pattern (although perhaps seasonal), without regulation, could be more adaptive under such fluctuating and unpredictable conditions (Menge, Ballantyne, & Weitz, 2011). In fact, obligate fixers (such as actinorhizal Alders and Coriaria) typically dominate in mesic ecosystems with sustained nitrogen deficit, usually following large disturbances (Menge, Levin, & Hedin, 2009; Sheffer, Batterman, Levin, & Hedin, 2015). However, since fixers contribute to gradual soil nitrogen recovery, over time obligate fixers are excluded, while facultative fixers coexist with competing non-fixing plants even in late successional stages.

We, therefore, ask how N\textsubscript{2} fixers in water-limited seasonal ecosystems react to soil nitrogen availability. To test this we evaluated strategies of N\textsubscript{2} fixation in a selection of common perennial legumes from Mediterranean ecosystems. The flora of the Mediterranean Basin contains an incredible diversity of legume species. Mediterranean flora in Israel alone contains ca.
100 perennial, and an additional 200 annual legume species, most of which are potential N\textsubscript{2} fixers (GRIN database, USDA, 2015; http://www.ars-grin.gov). These perennial fixers range from fast-growing herbaceous plants to slow-growing large woody shrubs, and often dominate after disturbance (Broza, Lahav, & Izhaki, 1992; Galié et al., 2015). Moreover, evidence now suggests that legumes first evolved 60 million years ago in the area that today is part of the Mediterranean Basin (Schrire, Lewis, & Lavin, 2005), and acquired the ability to nodulate around the same time (Lavin, Herendeen, & Wojciechowski, 2005; Sprent, 2007). However, the fact that seasonally dry conditions evolved in these areas only 40–50 million years later calls for an explanation of the diversification and survival of N\textsubscript{2}-fixing species and strategies in the region since.

Perennial woody plants in seasonally dry Mediterranean ecosystems are adapted to cope with and survive through prolonged summer droughts, and, as a consequence, influence their environment by forming patches of organic matter and water accumulation (islands of fertility; Moro, Pugnaire, Haase, & Puigdefábregas, 1997). The specific strategies of adaptation and fixation in perennial legumes have not been studied in this context. Few studies have focused on patterns of nitrogen fixation in Mediterranean herbaceous annual plants (Menge et al., 2015; Wolf, Funk, & Menge, 2016), or on the
presence of fixation in perennial Mediterranean plants (Atallah et al., 2008; Valladares et al., 2002). We, therefore, examined how plant investment in nodulation and symbiotic fixation is influenced by nitrogen and phosphorus availability in perennial drought-adapted plants, using controlled experiments with a suite of legume species.

Our experiment was designed to test whether perennial Mediterranean fixers downregulate fixation in response to soil nitrogen supply, a facultative strategy (sensu Barron et al., 2011), or maintain constant fixation regardless of soil nitrogen availability, an obligate strategy. We grew saplings, the life stage in which plant nitrogen demand is maximized, of four perennial legume species. Calicotome villosa Poir. and Ononis spinosa L. represented fast-growing plants (woody and herbaceous respectively) dominant in recently disturbed habitats, which are spiny and highly protected from grazing. Spartium junceum L. and Anagyris foetida L. represented woody shrubs, slower at colonizing new habitats and contain secondary metabolites for grazing protection. Specifically, we asked: (1) Do Mediterranean species (and populations) differ in their regulation of N$_2$ fixation in response to nitrogen supply? (2) Is fixation regulated via plant allocation to nodules, fixation rates or both? and (3) Does phosphorus availability limit N$_2$ fixation and/or plant growth?

2 | MATERIALS AND METHODS

2.1 | Experimental design

We conducted a controlled pot experiment to study how the availability of soil resources influences the nutrient acquisition strategy of legume saplings: allocation to roots and root nodules, and rate of symbiotic N$_2$ fixation. We tested this on a suite of four perennial legume species that are widespread in the Mediterranean region of Israel: O. spinosa L. (dwarf-shrub), C. villosa Poir., S. junceum L. and A. foetida L. (shrubs). We also included two distinct populations of C. villosa: a northern population near Tiberias, Gallilee, growing on Terra-Rosa soil 250 m a.s.l., and a central population near Rehovot, coastal lowlands, growing on red-sandy Hamra soil 80 m a.s.l.

Specifically, our experiment was designed to evaluate how nitrogen and phosphorus limitation influence plant growth, within-plant biomass and nitrogen allocation, and fixation. We used three nutrient addition treatments (Table 1). NPK treatment with complete nutrition (at N:P:K ratio of 70:20:60, equal to an addition of 80 mg N m$^{-2}$ day$^{-1}$ as NH$_4$NO$_3$, and 22 mg P m$^{-2}$ day$^{-1}$ as KH$_2$PO$_4$; ICI Ltd.) with concentrations that meet plants nutrient demands (Haifa 2017; http://www.haifa-group.com/knowledge_center). A PK treatment without nitrogen (N:P:K ratio 0:20:60). And K treatment without nitrogen and with low phosphorus (N:P:K ratio 0:2:60), with phosphorus concentration similar to phosphorus availability in Mediterranean shrublands (Grünzweig et al., 2010; Henkin, Seligman, & Noy-Meir, 2010).

Overall, the experiment included 15 treatments (four species, with 2 C. villosa populations × 3 treatments) with 8–10 replicates per treatment (depending on sapling survival in the first stages of growth). All treatments were supplemented with an addition of nitrogen and phosphorus-free micro-nutrient solution to ensure that all other macro- and micronutrients (including potassium and molybdenum) were balanced and non-limiting.

The experiment was carried out during the natural growing season (autumn to spring), in a net house with transparent rain-shed polycarbonate roof and net walls. The net house allowed us to grow the plants under natural climate conditions (temperature and RH), but without rain. All plants received non-limiting water inputs regularly, using controlled drip irrigation (PJC Netafim®; with two drips per pot at 1 L/hr capacity).

2.2 | Setup of experimental saplings

We used seeds of the four species of legumes and two C. villosa populations collected by the Israeli gene bank (IGB, 2017). Gene bank seeds are collected from wild population, cleaned and separated from dispersal tissues, and stored initially in dry conditions (RH 15 ± 5%, Temp. 15 ± 1°C) and eventually sealed and frozen at −18°C until use. Before the experiment, we acclimated the seeds for 24 hr in dry conditions at room temperature.

Seeds were germinated in agar in an incubator (12–12 light–dark, 20°C), according to protocols developed for each species with IGB based on available databases (IGB, 2017) and preliminary tests. We developed specific germination protocols to ensure simultaneous germination of all seeds, to diminish variance in plant age.

We transplanted the seedlings into 8-L pots, filled with perlite inert medium (Agrekal Perlite. 0.2 mm granules, density 2.2 g/cm$^3$) that does not react with water or chemicals. We also chose to grow the plants in the perlite medium because it is easy to wash off the

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**TABLE 1** Three resource availability treatments in the experiment. In all treatments water and microelements, including molybdenum and iron, were provided equally and were not limited. Values in irrigation represent the concentration of nutrients that were available for the plant in the inert medium. Total addition values represent the amounts added per area throughout the experiment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NPK Concentration in irrigation (ppm)</th>
<th>Total addition (g/m$^2$)</th>
<th>PK Concentration in irrigation (ppm)</th>
<th>Total addition (g/m$^2$)</th>
<th>K Concentration in irrigation (ppm)</th>
<th>Total addition (g/m$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen</td>
<td>70</td>
<td>13.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>20</td>
<td>3.8</td>
<td>20</td>
<td>3.8</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>Potassium</td>
<td>60</td>
<td>11.5</td>
<td>60</td>
<td>11.5</td>
<td>60</td>
<td>11.5</td>
</tr>
</tbody>
</table>
roots, and allows an even distribution of the water and nutrients in the pot. Fixers received a homogenized inoculum of soils (30 ml of soil, containing c. 0.35 mg of available nitrogen) collected from the rhizosphere of legume species (experimental species and other species) in native Mediterranean ecosystems to ensure access to a full suite of bacterial symbionts.

2.3 Plant response measures

We monitored the height of plants along plant development for the duration of the experiment. We harvested plants after 24 weeks. We quantified above-ground plant biomass, below-ground (root) biomass, root nodule biomass, fixation activity, root : shoot allocation, and tissue carbon and nitrogen contents. We measured fixation using acetylene reduction assay (Hardy, Holsten, Jackson, & Burns, 1968) with a sample of fresh nodules collected from the roots during plant harvest. We incubated nodules for 30 and 60 min, and analysed ethylene (C₂H₄) production on a gas chromatograph (Focus GC, Thermo-Finnigan). Following incubation we opened the nodules to verify their activity, and measured their dry biomass. We calculated the rate of fixation per g of incubated nodule biomass, and the rate of fixation per whole plant (by multiplication of fixation rate by total plant nodule biomass).

We weighed the biomass of incubated nodules, all nodules, root and shoot after drying at 60°C for 72 hr. Stems and leaves were then separately milled to a fine powder and analysed for tissue carbon and nitrogen by a FLASH2000 CN Elemental Analyser (Thermo Scientific). We calculated the total plant nitrogen content, as a proxy of plant nitrogen demand and evaluated the relative contributions of N-fertilizer, seed nitrogen and fixed nitrogen to the N demand, by extrapolating from the accumulation of fixed nitrogen in the no-N treatments and the measured rates of fixation. Investment in nodulation and roots were calculated as the ratio of plant nodule biomass (nodule mass fraction, NMF) or root biomass (root mass fraction, RMF) to whole-plant biomass.

2.4 Statistical analysis

We used multifactorial ANOVA to test the effects of the species and experimental treatment on the different measured variables: plant biomass, nodule biomass, nodule mass fraction, RMF, foliar nitrogen, rate of fixation per nodule mass and whole-plant fixation. Basic assumptions for ANOVA, that is, normally distributed residuals and homogeneity of variances, were examined using the Kolmogorov-Smirnov and Shapiro–Wilk tests and Levene's test respectively. Data not meeting these assumptions were either mathematically transformed or analysed using nonparametric analyses following Conover and Iman (1981). We used linear and power regressions to analyse the influence of fixation rate on biomass growth, foliar nitrogen and RMF respectively.

We used maximum likelihood to analyse if and how factors such as species, treatment and time affected plant height growth along plant development. We used a power model in the form $H(t) = a_j x_i^b_j$, where the height of the plant at time $t$ $H(t)$ is a function of day from the start on the experiment ($x$), and $a_j$ and $b_j$ are parameters that quantify the magnitude of the effects for a combination of the $j^{th}$ species and $j^{th}$ treatment. We compared specific models for each species vs. a pooled model for all species together, to find that species-specific models are more parsimonious. We then compared for each species a model that includes all three experimental treatments, to a model that compares NPK and no-N (PK and K) treatments. For C. villosa we also compared a model that compares the two populations to a model that pools individuals from all populations together. We used the Akaike information criterion corrected for a small sample size (AICc) to compare the strength of evidence for competing alternative models.

We solved for the maximum likelihood parameter values for each model in our set of models, using simulated annealing in the “likelihood 1.3” package in R (Murphy, 2012). Residuals ($r$) for the different variables were normally distributed, so the error terms were modelled accordingly. We used asymptotic two-unit support intervals to assess the strength of evidence for individual maximum likelihood parameter estimates (Edwards, 1992). To evaluate each alternate model we calculated the slope and the $R^2$ of the regression of observed vs. predicted values as measures of model bias and goodness-of-fit respectively.

Statistical analyses were performed in SPSS (SPSS 14 for Windows SPSS Inc, Chicago, IL, USA, for frequentist analysis), and in the r programming environment version 2.8.0 (R Development Core Team, 2008, for the maximum likelihood analysis).

3 RESULTS

3.1 Symbiotic nitrogen fixation

All four species in our experiment established after germination and grew, even when growth relied on fixed nitrogen only (except for minor initial inputs of nitrogen from the seed and inoculation soil, Figure 1a). After 24 weeks, we found active root nodules in all plants in the experiment, but strong downregulation of fixation when nitrogen was supplied regularly (i.e. in the NPK treatment) in all species (Figure 1). Fixation patterns differed in both plant allocation to nodulation and rates of fixation within nodules, with differences related to both treatment and species.

Plant nodulation in the no-N treatments was 1.6 and up to 35-fold higher compared to nodulation in the NPK treatment (with no significant difference between PK and K treatments; Figure 1b, Table 2), with both treatments and species each explaining 20% of total variability. Each of the species in the experiment showed a different proportion of allocation to nodulation, as well as different patterns of downregulation, as indicated by a significant interaction between the effects of species and treatment. The perennial herb O. spinosa presented the highest nodulation of all species in the no-N treatments, while its nodulation decreased 3.6-fold in the NPK treatment. In no-N treatments, the woody species, C. villosa (both populations) and S. junceum, presented moderate nodulation (three
times smaller) compared to O. spinosa, and downregulated nodulation by about half in the NPK treatment. Anagyris foetida presented an intermediate level of nodulation compared to all other species in the no-N treatments, but had the strongest downregulation, with almost no fixation in the NPK treatment.

Like nodulation, the rate of fixation (measured as rate of acetylene reduction per g nodule, and per plant) was both species-specific (explaining 40% of the variability) and downregulated when nitrogen was available (with treatment explaining 15% of the variability, Figure 1c, Table 2). The rate of fixation within nodules was 2–10 times lower in plants supplied with nitrogen compared to no-N treatments. This downregulation of fixation rates within nodules was similar in all four species (i.e. no species × treatment interaction). However, we found that the overall rate of fixation per plant was both influenced

**TABLE 2** Results of multifactorial ANOVA of the effects of species (random factor) and treatment (fixed factor) on: total plant biomass, nodule biomass, nodule mass fraction, root mass fraction, within-nodule rate of fixation and whole-plant rate of fixation

<table>
<thead>
<tr>
<th>Model variable</th>
<th>Factor</th>
<th>df</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant biomass</td>
<td>Species</td>
<td>3</td>
<td>12.96</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>2</td>
<td>30.81</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>Species × treatment</td>
<td>6</td>
<td>7.37</td>
<td>***</td>
</tr>
<tr>
<td>Nodule biomass</td>
<td>Species</td>
<td>3</td>
<td>6.09</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>2</td>
<td>9.03</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Species × treatment</td>
<td>6</td>
<td>4.97</td>
<td>***</td>
</tr>
<tr>
<td>Leaf N content</td>
<td>Species</td>
<td>3</td>
<td>2.25</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>2</td>
<td>24.6</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Species × treatment</td>
<td>6</td>
<td>5.75</td>
<td>***</td>
</tr>
<tr>
<td>Nodule mass fraction</td>
<td>Species</td>
<td>3</td>
<td>1.91</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>2</td>
<td>60.65</td>
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</tr>
<tr>
<td></td>
<td>Species × treatment</td>
<td>6</td>
<td>11.82</td>
<td>***</td>
</tr>
<tr>
<td>Root mass fraction</td>
<td>Species</td>
<td>3</td>
<td>27.36</td>
<td>**</td>
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<td></td>
<td>Treatment</td>
<td>2</td>
<td>13.79</td>
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<tr>
<td></td>
<td>Species × treatment</td>
<td>6</td>
<td>8.37</td>
<td>***</td>
</tr>
<tr>
<td>Fixation per g nodule</td>
<td>Species</td>
<td>3</td>
<td>42.6</td>
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<tr>
<td></td>
<td>Treatment</td>
<td>2</td>
<td>19.25</td>
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</tr>
<tr>
<td></td>
<td>Species × treatment</td>
<td>6</td>
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<td>ns</td>
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<tr>
<td>Fixation per plant</td>
<td>Species</td>
<td>3</td>
<td>44.8</td>
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<tr>
<td></td>
<td>Treatment</td>
<td>2</td>
<td>20.71</td>
<td>**</td>
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<td></td>
<td>Species × treatment</td>
<td>6</td>
<td>1.32</td>
<td>ns</td>
</tr>
</tbody>
</table>

*p < .05, **p < .005, ***p < .0001.
by the experimental treatment, and significantly different between species (Tables 2 and 3). The fast-growing herb *O. spinosa* had the highest rate and *A. foetida* the lowest rate of fixation per plant (Figure 1d). The rate of fixation per plant was always significantly higher in the no-N treatments. We further found a positive correlation between overall fixation rate and sapling growth (Pearson correlation coefficient with total biomass $r = .987$, $p < .0001$; with height growth rate $r = .855$, $p < .01$, Figure 3a).

Table 3 summarizes indices that reflect plant allocation to fixation, and how allocation to nodules and fixation changed with and without nitrogen supply. These measures indicate that: (1) nodule mass fraction varied between species, and (2) nodulation decreased dramatically in all species with nitrogen supply. The ratio between NPK and PK treatments in both nodulation and rate of fixation show strong downregulation in the species with the lowest rate of fixation (*A. foetida*), mild downregulation in intermediate fixers (*C. villosa* and *S. junceum*) and only moderate downregulation in the species with the highest rate of fixation (*O. spinosa*). Nonetheless, these results highlight the importance of comparing relative indices with absolute measures of fixation. For example, a small relative change in fixation (1:2 or 1:3, for NPK: PK treatments) in the strong fixer *O. spinosa* is a much larger absolute difference in fixation compared to the change in all other examined species. By comparing both absolute and size-corrected relative measures we show that most of the variability that we find in fixation is related to differences among species and their response to fertilization treatments.

### 3.2 | Growth and resource allocation

Biomass was 2–26 times higher in the NPK treatment compared to the no-N treatments (which did not differ significantly, Figure 1a, Table 1), with treatment explaining 50% of the variability in biomass, and species explaining an additional 30%. The most productive fast-growing species, *O. spinosa*, is the only species in which the biomass of no-N plants approximated that of NPK plants. In each species we found a different biomass response between NPK and no-N treatments (i.e. a significant species × treatment interaction), which coincided with the pattern of change in the total rate of fixation between the treatments. Furthermore, we found similar differences in the contribution of fixed nitrogen to total plant nitrogen, among species (Figure S1).

Much like biomass production, we also found higher contents of nitrogen in the foliage of NPK treatment plants (similarly high for all species) compared to no-N plants (Figure 2a). Here, we did find significantly more foliar nitrogen in the PK treatment with sufficient phosphorus availability, compared to the low phosphorus availability K treatment, only when all species were pooled, which suggests that phosphorus limitation does influence plant foliar nitrogen and potentially also plant photosynthetic efficiency and growth. In the no-N plants, foliation nitrogen content was highest in the species with higher fixation, *O. spinosa*, and slightly lower in species that did not fix as much. We found an increase in foliar nitrogen content as a function of the rate of fixation per plant (Figure 3b).

Our developmental analysis indicated that plant height differed between species, and that for each species height growth was faster and plants were taller in NPK treatment compared to slower growth and shorter plants in no-N treatments (Table S1). Although differences between the no-N treatments were small, we did find that in the two species with intermediate rates of fixation, *C. villosa* and *S. junceum*, plants in the PK treatment grew significantly taller compared to those in the K treatment. The plants from the two different populations of *C. villosa* also differed in their height growth. However, both the strong fixer *O. spinosa*, and the slowest fixer *A. foetida*, showed the opposite trend, with K plants growing taller than plants in the PK treatment.

Finally, biomass allocation to the root system (as indicated by the RMF) increased in the nutrient-deficient treatments (PK and K; Figure 2b). In no-N treatments, RMF was also inversely related to biomass allocation towards nodulation and fixation (Figure 3c). This allocation trade-off varied between species with high investment in fixation and low allocation to roots (*O. spinosa*), and species with low nodulation and high allocation to roots (*A. foetida*). We further found that allocation to roots is a trait that is mostly species-specific (species explaining 69% of RMF variability), while nodulation is mostly nutrient sensitive (with treatment explaining 67% of NMF variability).

### 4 | DISCUSSION

All the saplings of common Mediterranean perennial drought-adapted legumes in our experiment established and grew, nodulated, and fixed atmospheric dinitrogen, even in severe nitrogen limitation. The four species reacted similarly to nitrogen supply, by dramatically downregulating N$_2$ fixation through both decreased nodulation and lower rate of fixation. Phosphorus limitation had...
a minor influence on both fixation and plant growth. However, species did differ in fixation (both nodulation and rate), and in resource partitioning. Our findings show that N₂ fixation is tightly regulated in plants that have been adapted to cope with severe water stress.

4.1 Mediterranean perennial fixers are facultative

Controlled experiments are the best method to determine strategies of fixation (e.g. Menge et al., 2015). Although we found that all species in the experiment used a facultative fixation strategy, there are interesting differences in the strength of the control of

![Figure 2](image-url)  
**Figure 2** Response of nitrogen and biomass allocation of Mediterranean shrub seedlings (four species + two populations) to three nitrogen and phosphorus addition treatments (ample N and P supply = NPK, in black bars, no N = PK in grey and no N low P = K in white bars), M ± SE of leaf nitrogen content (a), nodule mass to total plant biomass ratio (NMF) (b) and root mass to total plant biomass ratio (RMF) (c). Different letters above columns indicate significant differences (Post hoc Games–Howell in a, and Bonferroni in b, c, p < .05).

![Figure 3](image-url)  
**Figure 3** Plant fixation rate and: total biomass (a), leaf nitrogen content (b) and root mass to total biomass, RMF (c). Means (M) and standard errors (SE) of four Mediterranean shrub seedlings: *Ononis spinosa* (OS), *Calicotome villosa* (CV), *Spartium junceum* (SJ) and *Anagyris foetida* (AF), in two nitrogen and phosphorus addition treatments (no N, and ample P supply = PK, in white symbols, and no N low P = K, in grey symbols)
nodule formation and within-nodule rate of fixation among species. While the control of nodule formation varied widely among species, the control of within-nodule rate of fixation was similar in all species, despite having initially different rates of fixation. This could be related to the heterogeneous composition of rhizobia partners, given a similar mixture of different inoculation sources that we provided to all plants in the experiment (i.e. promiscuous symbiosis; Rodríguez Echeverría, Pérez-Fernández, Vlaar, & Finnan, 2003). Further research is needed to understand how rhizobia composition within plant nodules influence plant fixation rate, fixation strategy and growth (but see Denison & Kiers, 2004).

Finding a combined control of fixation via both nodulation and within-nodule rate of fixation seems to be a robust strategy in Mediterranean perennial legume species, as shown by the group of species in our experiment. Similar strategies have been found in perennial plants in both Mediterranean (Valladares et al., 2002), and other ecosystems (Wurzburger & Hedin, 2016).

The two mechanisms of regulation of fixation allow plants to control their investment in the symbiotic partners (Sprent, 2001), which is especially important in seasonally dry conditions and in habitats with strong spatial and temporal heterogeneity in soil resources (Menge & Levin, 2017). Under these conditions, tight regulation of plant investment in fixation is necessary to improve the chance of saplings to establish during the short rainy season, and survive during the long dry season. By allocating less resources to fixation when nitrogen is not limiting, saplings can invest in other resource acquisition strategies and tissues (Poorter et al., 2012) and grow deep roots, which are essential for plant establishment and survival in drylands (Dovrat, 2015; Schenk & Jackson, 2002). We further hypothesize that in drought-adapted plants, allocation to roots (for water) is primary to fixation, and therefore enforce facultative fixation, as supported by our results.

The instantaneous rates of N₂ fixation measured in our Mediterranean saplings resembled fixation rates by facultative tropical legume saplings (e.g. Battersman, Wurzburger, et al., 2013), and by a facultative Mediterranean shrub (Retama sphaerocarpa (L.) Boiss; Valladares et al., 2002). However, symbiotically fixed nitrogen did not supply the potential demand for nitrogen by the growing saplings (as compared to growth with nitrogen supply). The species in our experiment demonstrate a range of fixation patterns, which are related to differences in life history and drought adaptation of each species. The herb O. spinosa is an extreme example of a fast-growing and early flowering species, with high fixation rates, which allowed similar biomass in individuals growing with and without nitrogen supply. The three other slow-growing species had moderate to low rates of fixation, with different strategies of downregulation. The species that fixed the least, A. foetida, allocated mostly to root growth. This interspecific variability is especially pronounced in the absolute nodulation, and total fixation values, and is a manifestation of interspecific differences in plant growth rates, and accordingly their nitrogen demand. Phyllogenetic differences in fixation rates and strategies have been recently shown for tropical fixers (Wurzburger & Hedin, 2016), but here we show that the trade-off with drought adaptation mechanisms enforces a strong species-specific influence on fixation strategy.

4.2 | Influence of P limitation

N₂ fixers are often associated with phosphorus-rich niches (Chimphango, Potgieter, & Cramer, 2015), and evidence exists for P limitation on fixer growth (Binkley et al., 2003) and fixation (Battersman, Wurzburger, et al., 2013; Wurzburger et al., 2012). In the conditions of our experiment, over 6 months in the absence of nitrogen addition, phosphorus did not limit seedling growth or nitrogen fixation. P limitation partially affected nitrogen accumulation in the leaves in some of our experimental species. Furthermore, in most species the rate of fixation was even higher in the low phosphorus treatment.

However, phosphorus limitation could develop in these fixers at later growth stages, when P demands increase. We believe that phosphorus limitation could influence the growth of plants, as indicated by increasing cover and height of C. villosa in a phosphorus fertilization experiment in a P-limited field site (Henkin et al., 2010). However, we think that for Mediterranean species growing in these conditions phosphorus does not limit the rates of fixation directly, but probably will limit fixation indirectly along plant growth. More research is needed to understand P demands in Mediterranean fixers, and whether P-mining strategies (e.g. excretion of extracellular phosphatases; Baribault, Kobe, & Finley, 2012; Houlton et al., 2008; and symbiosis with mycorrhiza fungi) are used by fixers to overcome P limitation.

Interestingly, the fast-growing O. spinosa was the only species in the experiment that demonstrated P limitation, by allocating more biomass to roots in the low phosphorus treatments compared to nitrogen-deficient, phosphorus-sufficient treatment. We hypothesize that the vigorous growth and high rates of fixation in these plants suggest that their growth was not limited by nitrogen, and therefore were more susceptible to phosphorus limitation.

4.3 | Comparison of two C. villosa populations

Most research on N₂ fixation rates and strategies focused on among-species comparisons (e.g., Atallah et al., 2008; Menge et al., 2015; Rodríguez-Echeverría et al., 2003; Wurzburger & Hedin, 2016). Here, we evaluated within-species differences in fixation strategies in a dominant N₂-fixing species, with distinct independent populations that developed on contrasting soil types (no cross-pollination between populations; Arroyo, Aparicio, Albaladejo, Munoz, & Braza, 2008).

We found primarily morphological difference between plants from the two C. villosa populations. But, the populations did not differ in plant biomass, fixation rates and fixation strategies, suggesting that fixation strategy is a robust trait associated with plant phylogeny and biome conditions. However, facultative fixation can also be evaluated as a phenotypic adaptation that is highly flexible and influenced by habitat (or experimental treatment) conditions. Similarity
in fixation among populations, but differences in morphology, was also found for the dryland legume shrub *R. sphaerocarpa* (Valladares et al., 2002).

### 4.4 | Resource partitioning trade-off in Mediterranean fixers

Biomass partitioning theories suggest that plants change their biomass allocation between organs according to resource availability, and therefore are better adapted to changing habitat conditions (Coleman, McConnaughay, & Ackerly, 1994; Grime, 1979; Tilman, 1988). Optimal partitioning theory predicts that plants will allocate more biomass to the part responsible for assimilating the resource that is limiting plant growth (e.g. Coleman et al., 1994; Wright & McConnaughay, 2002). For example, Poorter and Nagel (2000) showed in a review that plant root: shoot ratio increases when nutrients are limiting.

Our results also show higher allocation to roots in nitrogen-, and nitrogen- and phosphorus-deficient plants. This pattern was strong in the slow-growing shrub saplings, but we did not find more allocation to roots in the fast-growing herb, which also was the strongest fixer (perhaps partly due to ontogenetic differences in development; McConnaughay & Coleman, 1999). Together, these findings support the hypothesis that downregulation of fixation, via reduced allocation to both nodule maintenance and bacteria maintenance, allows Mediterranean fixers to allocate more resources to competitive plant organs: root, shoot and leaves.

### 4.5 | Individual strategies influence long-term species survival

Facultative vs. obligate fixation strategies influence both the survival of the individual fixers (by affecting plant economy), and the survival of the species in the community (by affecting plant–soil ecosystem feedbacks; Sheffer et al., 2015). Paradoxically, obligate N₂ fixers influence soil nitrogen recovery over time, and by that create ecosystem conditions that allow their competitive exclusion by non-fixing plant (Hedin et al., 2009). A facultative strategy allows fixers to coexist with non-fixers in the community over time, and to diminish excessive nitrogen inputs to the ecosystem (Menge & Hedin, 2009).

Furthermore, symbiotic N₂ fixers in the Mediterranean Basin have adapted to a long history of intensive grazing pressure (Perevolotsky & Seligman, 1998) and man-made disturbance (fire and clear cutting; Naveh & Carmel, 2004). The present assembly of Mediterranean legumes, therefore, contains only highly protected grazing-adapted species, which produce spines (e.g. *O. spinosa* and *C. villosa*) and/or secondary-metabolite toxins (e.g. *A. foetida* and *S. juncum*). In the latter case, fixed nitrogen not only increases plant susceptibility to grazing but also allows plants to invest this nitrogen to deter herbivores (Wink, 2013).

We, therefore, suggest that strong up- and downregulation of fixation allows Mediterranean legumes species to: (1) colonize recently disturbed and typically nitrogen-deficient habitats, (2) cope with grazing, (3) survive over time in the community and reduce nitrogen inputs to the ecosystem, and (4) survive prolonged seasonal droughts.

In conclusion, symbiotic fixation is an efficient and evolutionary-preserved strategy, which has been adapted to the seasonal dry conditions that characterize Mediterranean ecosystems. A facultative strategy seems to be the rule for Mediterranean perennial fixers, presenting a range of different levels of investment in and regulation of fixation, corresponding to life history and resource partitioning.

We propose that in addition to the direct effect on plant economy, N₂ fixation strategies influence succession of the plant community and ecosystem state following wildfire and grazing disturbances, and ecosystem functions such as soil nitrogen cycling and seasonal productivity in Mediterranean ecosystems. This research highlights the need to further study the ecology of fixers and patterns of fixation in drylands. Future research will be necessary to elucidate what controls regulate fixation during the dry season, and how dry conditions influence the potential environmental feedback of those fixers, at the plant and ecosystem levels.

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**AUTHORS’ CONTRIBUTIONS**

G.D. and E.S. designed the study and experimental methodology, performed all data analyses and wrote the manuscript; T.M. conducted the gas chromatography and Elemental analysis; G.D. and H.B. performed the experimental work; E.M.G. and S.G. designed the seed storage and germination methodologies. All authors contributed critically to the drafts and gave final approval for publication.

**DATA ACCESSIBILITY**

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.cm922 (Sheffer, 2018).

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article.