

# Tolerance or avoidance: drought frequency determines the response of an N<sub>2</sub>-fixing tree

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## Summary

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- Climate change is increasing drought frequency, which may affect symbiotic N<sub>2</sub> fixation (SNF), a process that facilitates ecosystem recovery from disturbance. Here, we assessed the effect of drought frequency on the ecophysiology and SNF rate of a common N<sub>2</sub>-fixing tree in eastern US forests.
- We grew *Robinia pseudoacacia* seedlings under the same mean soil moisture, but with different drought frequency caused by wet–dry cycles of varying periodicity.
- We found no effect of drought frequency on final biomass or mean SNF rate. However, seedlings responded differently to wet and dry phases depending on drought frequency. Under low-frequency droughts, plants fixed carbon (C) and nitrogen (N) at similar rates during wet and dry phases. Conversely, under high-frequency droughts, plants fixed C and N at low rates during dry phases and at high rates during wet phases.
- Our findings suggest that *R. pseudoacacia* growth is resistant to increased drought frequency because it employs two strategies – drought tolerance or drought avoidance, followed by compensation. SNF may play a role in both by supplying N to leaf tissues for acclimation and by facilitating compensatory growth following drought. Our findings point to SNF as a mechanism for plants and ecosystems to cope with drought.

## Introduction

Global climate change is increasing the temporal variability of precipitation to terrestrial ecosystems (IPCC, 2013), resulting in greater frequency of drought events (Sheffield & Wood, 2008), and posing a threat to the services these systems provide (Vose *et al.*, 2016). Increased drought frequency may have direct negative effects on ecosystem primary productivity as plants are exposed to more frequent fluctuations in soil moisture and periods of low water availability (Knapp *et al.*, 2002). However, increased drought frequency may indirectly reduce productivity via symbiotic N<sub>2</sub> fixation (SNF), a key ecosystem process facilitated by plants. SNF supplies the majority of new nitrogen (N) to terrestrial ecosystems (Vitousek & Howarth, 1991), particularly those recovering from disturbances (Boring & Swank, 1984; Batterman *et al.*, 2013), but is sensitive to changes in soil N supply and plant N demand that may be driven by fluctuations in soil moisture. Thus, if increased drought frequency negatively affects SNF, it could reduce terrestrial ecosystem productivity and resilience to disturbance.

The response of SNF to increased drought frequency depends on how N<sub>2</sub>-fixing plants (hereafter N<sub>2</sub>-fixers) respond to repeated drought. Although N<sub>2</sub>-fixers appear to increase SNF during individual drought events (Tobita *et al.*, 2010; Wurzburger & Miniatt, 2014; Mantovani *et al.*, 2015; but see Serraj *et al.*, 1999),

field studies have presented conflicting evidence on whether SNF increases or decreases across ecosystems with declining annual precipitation (Schulze *et al.*, 1991; Aranibar *et al.*, 2004; Soper *et al.*, 2015). In addition, it remains unclear how repeated drought and recovery cycles affect growth and SNF activity. A critical consideration is how drought frequency influences the soil N cycle. As SNF is more costly than soil N uptake (Gutschick, 1981; Vitousek & Field, 1999), facultative N<sub>2</sub>-fixers regulate SNF rate in response to soil N availability by constructing or excising root nodules, the organs in which SNF takes place. Thus, how SNF responds to increased drought frequency depends on how wet and dry phases affect the balance between soil N supply and plant N demand, time lags in SNF regulation and the costs associated with the regulation of fixation.

Variation in soil moisture may shift the balance between soil N supply and plant N demand, altering the extent to which N<sub>2</sub>-fixers carry out SNF. During periods of low soil moisture, reductions in N mineralization (Stanford & Epstein, 1974) and mass diffusion (Chapin, 1991) can decrease the supply of N to plant roots. Concurrently, plant N demand can decrease, remain stable or increase, depending on the physiological response to drought. Plant N demand may decrease if stomatal limitation on photosynthesis leads to decreased growth, but may recover if plants acclimate to drought and return to an ambient growth rate. Plant N demand may even increase during drought if plants

acclimate by investing N in photosynthetic enzymes that increase the maximum rate of carboxylation ( $V_{\text{cmax}}$ ) and thus increase water-use efficiency (WUE) (Wright *et al.*, 2001, 2005; Kitao *et al.*, 2007). However, as soils rewet, N supply may increase as moisture limitation of mineralization and diffusion are alleviated. Indeed, rewetting events are often accompanied by large, transient pulses of mineral N that can exceed predrought levels (Tie-mann & Billings, 2011).  $\text{N}_2$ -fixers may respond to these changes in N availability by adjusting investment in SNF (Barron *et al.*, 2011), because SNF is substantially more costly than soil N uptake (Vitousek & Field, 1999). As such,  $\text{N}_2$ -fixers may increase carbon (C) investment to symbionts (either through construction of root nodules or greater flow of C to existing nodules) when soil N supply is limited (Tobita *et al.*, 2010; Wurzbürger & Miniat, 2014), and decrease C supply to symbionts when soil N is more abundant. Thus, the effect of increased drought frequency on SNF depends on moisture-induced changes in both N supply and demand.

Frequent fluctuations in soil moisture may create time lags in the regulation of SNF. If soil moisture changes more rapidly than  $\text{N}_2$ -fixers can respond, allocation to SNF becomes suboptimal (Menge *et al.*, 2009). Such a mismatch between N acquisition strategy and the environment could result in reductions in  $\text{N}_2$ -fixer growth. Time lags are difficult to predict because we lack an understanding of the time scales of SNF regulation. Most  $\text{N}_2$ -fixers adjust the SNF rate by constructing or excising root nodules (Pearson & Vitousek, 2001; Barron *et al.*, 2011). However, some species may rapidly fine-tune SNF by adjusting photosynthate supply to existing nodules, thereby altering the nodule mass-specific SNF rate on a time scale of minutes to hours (Johnsen & Bongarten, 1991). Thus, the significance of time lags may depend on how  $\text{N}_2$ -fixers track changes in N availability.

Increased drought frequency could reduce the growth and biomass of  $\text{N}_2$ -fixers because of the C costs associated with the regulation of SNF (Menge *et al.*, 2009, 2011). The up- or down-regulation of SNF may result in energetic costs relating to cell growth, metabolism or protein synthesis. Nodule construction, in particular, represents a substantial 'start-up' cost to SNF, which can be balanced by the benefits of increased N acquisition (Gutschick, 1981). If  $\text{N}_2$ -fixers construct and excise nodules more frequently, the 'return on nodule investment' may decline because of the shorter nodule lifespan. These costs may increase with increasing drought frequency (Menge *et al.*, 2011), and could result in reduced  $\text{N}_2$ -fixer growth and thus reduced SNF.

Here, we examined the effect of drought frequency on SNF with a manipulative experiment on a model  $\text{N}_2$ -fixing tree, *Robinia pseudoacacia*, a widespread and ecologically important  $\text{N}_2$ -fixer in eastern US forests (Boring, 1984; Boring & Swank, 1984). We hypothesized that seedling biomass and growth would be lower under high vs low drought frequency, even though both treatments had the same mean soil water content over the duration of the study, because of lag times in regulation and the costs of frequent nodule construction and excision. We also hypothesized that the SNF rate and WUE would be lower for seedlings under high vs low drought frequency, as a result of lag times in physiological response.

## Materials and Methods

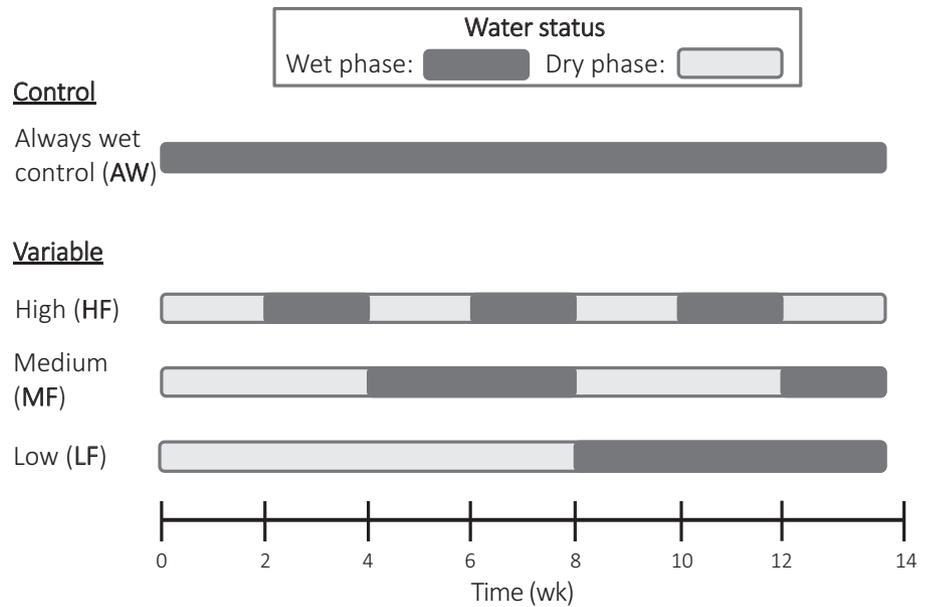
### Experimental design

To assess the effect of drought frequency on SNF rate by *Robinia pseudoacacia* L., we conducted a glasshouse experiment in Athens, Georgia, USA. We germinated 140 *R. pseudoacacia* individuals from seed (Sheffield's Seed Co., Locke, NY, USA) in 5-l pots. Each pot contained a nutrient-poor potting mix consisting of a 1 : 1 ratio of local forest soil (Cecil series; Typic Kanhapludult), passed through a 4-mm sieve to remove roots and coarse organic matter, and coarse granite sand. To ensure seedlings had access to  $\text{N}_2$ -fixing symbionts, we inoculated each pot with 10 ml of a slurry consisting of field-collected *R. pseudoacacia* root nodules homogenized with deionized water. After 4 months of growth, we fertilized all pots with one-quarter strength Hoagland solution minus N.

### Drought frequency treatments

After 5 months of growth, we randomly assigned each seedling to one of four 14-wk drought frequency treatments: always wet control (AW), and three variable soil moisture treatments that cycled through 'wet' and 'dry' phases at low (LF), medium (MF) and high (HF) frequency (treatment  $n=33$ ; see Fig. 1). The three variable soil moisture treatments had identical mean volumetric water content (VWC), averaged over the 14-wk experiment, and the same total number of weeks in the dry phase, but differed in the frequency of their wet–dry cycles. We maintained AW treatment pots and variable soil moisture pots during wet phases at 15% VWC, which was 70% of the field capacity for our soil. Variable soil moisture pots during the dry phases were held at 5% VWC, a level which caused early stomatal closure of *R. pseudoacacia* seedlings in preliminary tests, but was above the wilting point for our soil (3% VWC). HF, MF and LF treatments cycled between wet and dry phases every 2, 4 and 8 wk, respectively. These treatments mimic the conditions observed in *R. pseudoacacia* stands in Cowee, NC, USA, where 2-wk droughts occurred multiple times per growing season and 8-wk droughts occurred once in 3 yr (J. M. Minucci *et al.*, unpublished data). Although we exposed seedlings to similar levels of water stress during all 'dry' periods, it is possible that the 8-wk drought of the LF treatment posed an additional stress on seedlings as a result of C depletion, and the short but frequent droughts of the HF treatment may have stressed plants because of increased temporal variability. We randomly arranged all seedlings on a single row of tables in the glasshouse, and table was used as a blocking variable.

We randomly selected two seedlings from each treatment and outfitted their pots with 12-cm-long time domain reflectometer probes to estimate soil moisture (CS655; Campbell Scientific, Logan, UT, USA). As seedling roots reached the bottom of the pots by harvest one, these probes integrated moisture for the entire soil volume that the seedlings accessed. We used the average soil VWC of these pairs to infer the water status of the treatment group and did not harvest or otherwise measure these seedlings at any point during the experiment. When a pair of



**Fig. 1** Schedule of the water status for each drought frequency treatment. Wet phases and dry phases corresponded to 15% and 5% volumetric water content, respectively. All 'variable' treatments consisted of a total of 8 wk in the dry phase and 6 wk in the wet phase.

moisture probe-equipped pots dropped 3% below the target VWC, we supplied every seedling in the treatment group with 0.25 l of water to increase VWC by *c.* 6%. Drought frequency treatments began on 10 July 2014 and ran for 14 wk under ambient light conditions.

#### Ecophysiological measurements and biomass determination

Every 2 wk, we randomly selected five individuals from each treatment group for ecophysiological measurements and destructive harvesting. To quantify plant-available soil moisture at the time of harvest, we measured pre-dawn leaf water potential ( $\Psi_{pd}$ , MPa; PMS Instruments, Albany, OR, USA) on one fully extended leaf per individual. We measured leaf net C assimilation rate ( $A_{net}$ ,  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ leaf area s}^{-1}$ ) at saturating light ( $1800 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ), stomatal conductance ( $g_s$ ,  $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ) and transpiration ( $E$ ,  $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ) between 11:00 and 13:00 h, on the day of harvest (LI-6400XT; Li-Cor, Lincoln, NE, USA). Measurements were taken on the terminal leaflet of the fully extended leaf closest to the apical meristem. When the leaflet did not fill the chamber, we estimated the leaflet area by measuring the width and length with calipers and using an allometric relationship previously established with glasshouse-grown *R. pseudoacacia* seedlings (Wurzburger & Miniat, 2014). Intrinsic WUE or C gain per unit water lost ( $\text{WUE}_i$ ,  $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$ ) was estimated as  $A_{net}$  divided by  $g_s$ .

We then destructively harvested the five seedlings to estimate leaf, stem, root and nodule biomass pools. Biomass pools were separated and oven dried at 65°C to constant weight and then weighed to the nearest 0.001 g. Because our results indicated strong relationships in nodule mass fraction and  $A_{net}$  in some treatment groups, but not others, we estimated leaf N content (% DW) of HF and LF seedlings, and a subset of AW seedlings (50% randomly selected across all harvest dates) *post hoc*. For

these samples, we ground the dried leaves to a fine powder in a ball-mill grinder. We then packed 15 mg of ground tissue into tin capsules and combusted samples according to the Dumas method on an elemental analyzer (Flash EA 1112 NC analyzer; Elantech, Lakewood, NJ, USA).

Immediately on harvest, we removed a subset of live root nodules from each seedling with forceps to estimate the  $\text{N}_2$  fixation rate via acetylene reduction assay (ARA,  $\mu\text{mol N}_2 \text{ fixed g}^{-1} \text{ h}^{-1}$ ; Barron *et al.*, 2011). We immediately placed each set of excised nodules into a tightly sealed 250-ml glass jar fitted with a rubber septum. We then replaced 10% of the jar headspace with  $\text{C}_2\text{H}_2$ , and incubated the nodules for 20 min, taking 10-ml gas samples at 10 and 20 min with a syringe. We also ran control incubations without nodules to account for background  $\text{C}_2\text{H}_4$  concentration. All samples were measured for ethylene production using a gas chromatograph equipped with a flame ionization detector (SRI Instruments, Torrance, CA, USA). The ratio of nodule ethylene production to  $\text{N}_2$  fixation was determined with a one-time set of ten 98%-atom  $^{15}\text{N}$  (Sigma-Aldrich) incubations run in parallel with ARAs. The mean (and SE) conversion ratio was 0.06 ( $\pm 0.01$ )  $\mu\text{mol C}_2\text{H}_4 : \mu\text{mol N}_2$ , which is at the low end of the range of values observed for woody  $\text{N}_2$ -fixers (Anderson *et al.*, 2004).

#### Soil moisture and nutrient analysis

To determine pot-level variability in soil moisture, we estimated soil moisture at the time of harvest by sampling *c.* 20 g of homogenized soil from the pot of each harvested seedling. Samples were weighed and oven dried at 100°C to a constant weight to determine the percentage gravimetric soil moisture. To test whether soil dissolved inorganic nitrogen (DIN) pools changed in response to treatments, we quantified ammonium and nitrate pools with a 2 M KCl extraction on the first and last harvest. For each seedling harvested, we sampled 25 g of soil, agitated it in

50 ml of 2 M KCl for 4 h, and filtered it through 1- $\mu$ m glass fiber filters. Ammonium and nitrate concentrations in the filtrate were quantified using continuous flow analysis (RFA300 Auto-analyzer; Alpkem Corp., Clackamas, OR, USA).

### Statistical analysis

To confirm that seedlings in wet and dry phases had different VWC and  $\Psi_{pd}$  at the time of harvest, we used one-way analysis of variance (ANOVA). To test whether drought frequency treatment and water status (i.e. wet/dry phase) affected our response variables, we used a two-way ANCOVA with type III sums of squares (R; CAR package). Explanatory variables in our models were drought frequency treatment, water status (i.e. wet or dry phase) and their interaction. The AW treatment was not included when assessing the interaction term, as AW seedlings experienced only the wet phase. We included total seedling biomass as a covariate to account for scaling relationships between plant size and response variables. Glasshouse table position was initially included as a blocking variable to account for spatial heterogeneity in glasshouse conditions, but it was not a significant predictor and was subsequently removed from the analyses. When we detected a significant interaction between drought frequency treatment and water status, suggesting that seedlings responded differently to drought depending on their treatment, we used a set of linear contrasts to compare mean response values of wet and dry phases within each drought frequency treatment. We also compared means for each water status and treatment combination with the AW control. The family-wise type I error rate for contrasts was controlled at  $\alpha = 0.05$  using a Bonferroni correction. If there was no interaction, *post-hoc* separation of means was carried out with Tukey's honestly significant difference (HSD) test. All statistical analyses were performed with R v.3.1.1 (R Development Core Team, 2016).

## Results

### Soil resources

Soils in wet phases had greater soil moisture than those in dry phases ( $F_{1,129} = 366.5$ ,  $P < 0.001$ ), with 14.96% soil moisture compared with 5.79% (Table 1). Mean pre-dawn water potential

**Table 1** Effect of water status on gravimetric soil moisture (% SM), pre-dawn leaf water potential ( $\Psi_{pd}$ ) and KCl-extractable ammonium and nitrate across treatments

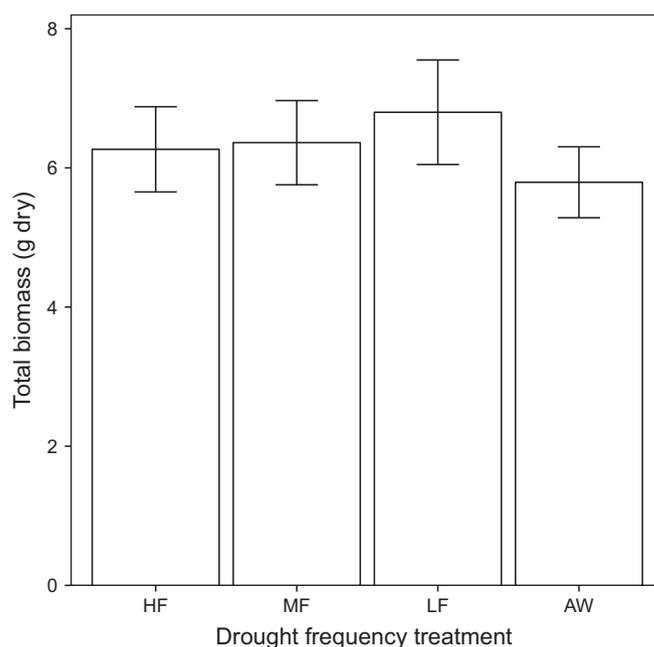
	Wet phase	Dry phase	<i>P</i> value
% SM	14.96% ( $\pm 0.36$ )	5.79% ( $\pm 0.24$ )	<b>&lt; 0.001</b>
$\Psi_{pd}$	-0.13 MPa ( $\pm 0.01$ )	-0.46 MPa ( $\pm 0.05$ )	<b>&lt; 0.001</b>
NH <sub>4</sub> <sup>+</sup>	0.17 ppm ( $\pm 0.03$ )	0.13 ppm ( $\pm 0.03$ )	0.41
NO <sub>3</sub> <sup>-</sup>	0.08 ppm ( $\pm 0.06$ )	0.07 ppm ( $\pm 0.07$ )	0.91

Values represent means ( $\pm$  SE). *P* values are given for the water status effect in ANOVA. Bold *P* values indicate a statistically significant difference ( $P < 0.05$ ).

was also higher for the wet phase soils compared with the dry phase soils ( $F_{1,130} = 66.4$ ,  $P < 0.001$ ). Mean pre-dawn water potential for dry phases fell within the range of pre-dawn water potentials observed on summer days in natural *R. pseudoacacia* stands in Cowee, NC, USA (J. M. Minucci *et al.*, unpublished data). DIN soil pools did not differ from the first harvest to the final harvest (NH<sub>4</sub><sup>+</sup>:  $F_{1,31} = 0.003$ ,  $P = 0.42$ ; NO<sub>3</sub><sup>-</sup>:  $F_{1,31} = 0.007$ ,  $P = 0.93$ ), nor did they vary among drought frequency treatments (NH<sub>4</sub><sup>+</sup>:  $F_{3,31} = 0.3$ ,  $P = 0.83$ ; NO<sub>3</sub><sup>-</sup>:  $F_{3,31} = 1.3$ ,  $P = 0.30$ ) or between wet and dry phases (NH<sub>4</sub><sup>+</sup>:  $F_{1,31} = 0.7$ ,  $P = 0.41$ ; NO<sub>3</sub><sup>-</sup>:  $F_{1,31} = 0.01$ ,  $P = 0.91$ ).

### Biomass partitioning and final biomass

At the end of the experiment, there were no differences in total biomass among drought frequency treatments ( $F_{3,16} = 0.44$ ,  $P = 0.73$ ; Fig. 2). However, biomass partitioning among plant organs, specifically roots, differed throughout the experiment depending on treatment (drought frequency by water status interaction:  $F_{2,90} = 3.43$ ,  $P = 0.04$ ; Supporting Information Table S1). The proportion of root biomass to total biomass (i.e. root mass fraction) increased for seedlings in the LF drought treatment during the wet phase. For this treatment, all weeks of the wet phase occurred consecutively following an 8-wk drought. There was no effect of drought frequency treatment or water status on the fraction of biomass partitioned to leaves (drought frequency:  $F_{3,124} = 2.33$ ,  $P = 0.08$ ; water status:  $F_{1,124} = 0.60$ ,  $P = 0.44$ ; Table S1), and we did not observe a significant amount of leaf shedding during the dry phases.



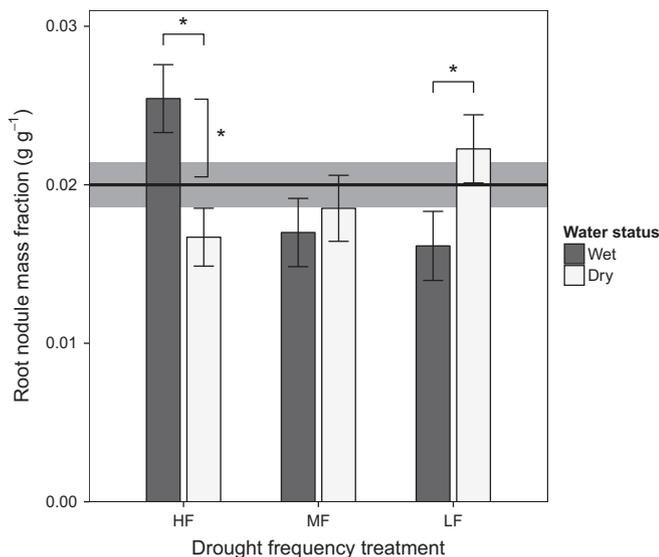
**Fig. 2** Final biomass for *Robinia pseudoacacia* plants in each drought frequency treatment. Bar heights represent untransformed means and error bars denote  $\pm$  SE. Treatments: HF, high frequency; MF, medium frequency; LF, low frequency; AW, always wet control.

### Symbiotic N<sub>2</sub> fixation

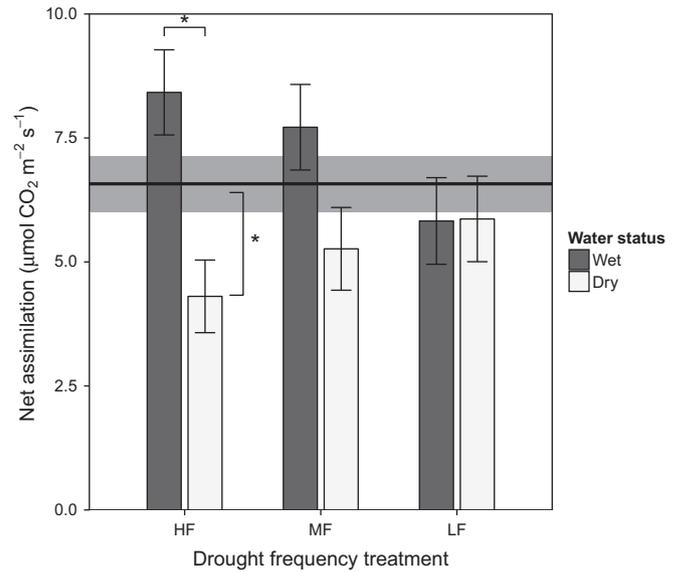
Seedlings responded to wet and dry phases by the up- or down-regulation of nodule biomass rather than by a change in the nodule mass-specific SNF rate, and the direction of this response was dependent on drought frequency (drought frequency by water status interaction:  $F_{2,90} = 12.6$ ,  $P < 0.001$ , Fig. 3). For seedlings in the HF treatment, nodule mass fraction increased by 49% during the wet phases compared with the dry phases. Conversely, LF treatment seedlings increased nodule mass fraction by 43% during the dry phase. MF treatment seedlings exhibited an intermediate response, with no differences in nodule mass fraction between wet and dry phases. When averaged across wet and dry phases, drought frequency did not affect nodule mass fraction ( $F_{2,90} = 1.53$ ,  $P = 0.22$ ). The nodule mass-specific SNF rate was consistent across drought frequency treatments and water statuses, with a mean rate of  $16.8 (\pm 1.3) \mu\text{mol C}_2\text{H}_4 \text{ g}^{-1} \text{ h}^{-1}$ , or  $264.9 (\pm 20.6) \mu\text{mol N}_2 \text{ fixed g}^{-1} \text{ h}^{-1}$  based on our empirically derived  $\text{C}_2\text{H}_4 : \text{N}_2$  conversion factor.

### Leaf gas exchange

Drought frequency altered how net C assimilation responded to variation in soil moisture (drought frequency by water status interaction:  $F_{2,90} = 3.1$ ,  $P = 0.048$ ; Fig. 4). Seedlings in the HF treatment assimilated 92% more C in wet vs dry phases, whereas MF and LF seedlings assimilated a similar amount of C in both phases. When averaged across wet and dry phases, drought frequency did not alter net C assimilation ( $F_{2,90} = 0.28$ ,  $P = 0.76$ ).



**Fig. 3** Effect of drought frequency and water status on *Robinia pseudoacacia* root nodule mass fraction. Bar heights represent untransformed least-square means standardized to mean total biomass (5.01 g DW). Error bars represent  $\pm$  SE. Horizontal line and shaded region represent mean and SE values for the always wet control. Horizontal brackets and asterisks denote significant differences between wet and dry phases for a given treatment. Vertical brackets and asterisks denote significant differences from the always wet control. Treatments: HF, high frequency; MF, medium frequency; LF, low frequency.



**Fig. 4** Effect of drought frequency and water status on *Robinia pseudoacacia* net carbon assimilation (net assimilation). Bar heights represent untransformed least-square means standardized to mean total biomass (5.01 g DW). Error bars represent  $\pm$  SE. Horizontal line and shaded region represent mean and SE values for the always wet control. Horizontal brackets and asterisks denote significant differences between wet and dry phases for a given treatment. Vertical brackets and asterisks denote significant differences from the always wet control. Treatments: HF, high frequency; MF, medium frequency; LF, low frequency.

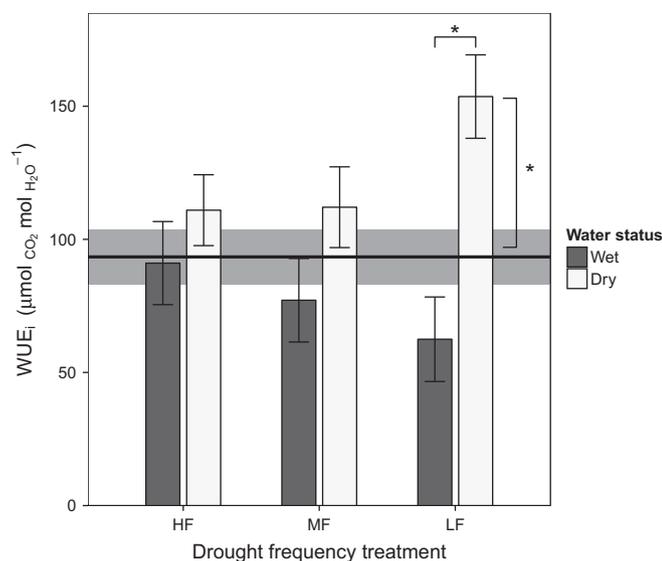
Stomatal conductance and transpiration were dependent on water status ( $g_s$ :  $F_{1,90} = 33.7$ ,  $P < 0.001$ ;  $E$ :  $F_{1,90} = 36.1$ ,  $P < 0.001$ ) and were not affected by drought frequency. Seedlings in wet phases had 113% and 105% higher  $g_s$  and  $E$ , respectively, than those in dry phases. However, drought frequency altered how  $\text{WUE}_i$  responded to variation in soil moisture (drought frequency by water status interaction:  $F_{2,90} = 4.77$ ,  $P = 0.01$ ; Fig. 5). Seedlings in the LF treatment increased  $\text{WUE}_i$  by 68% during the dry phase, whereas MF and HF seedlings maintained consistent  $\text{WUE}_i$  across wet and dry phases.

### Leaf N content

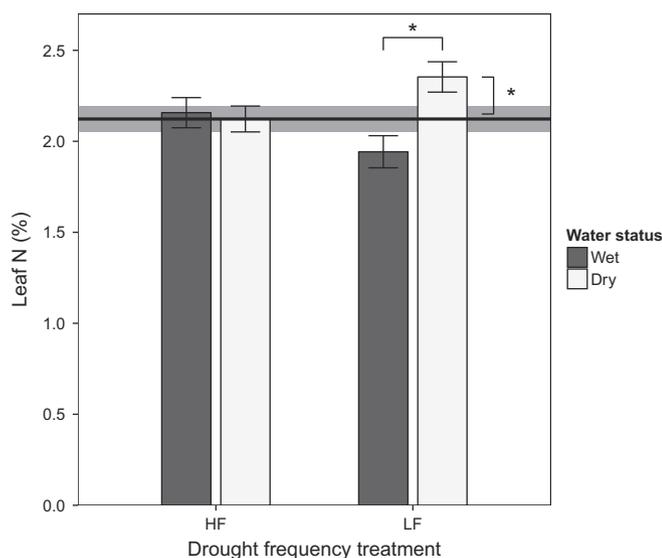
Drought frequency altered how the leaf N content responded to dry vs wet phases (drought frequency by water status interaction:  $F_{1,80} = 8.446$ ,  $P = 0.005$ ; Fig. 6). Seedlings in the LF treatment increased leaf N content by 21% during dry phases compared with wet phases, and by 10% compared with AW control seedlings. Leaf N content for HF seedlings was not significantly different from the AW control for either wet or dry phases.

### Discussion

*Robinia pseudoacacia* seedlings did not differ in their final biomass across treatments, and thus we did not find support for our hypothesis that increased drought frequency would reduce N<sub>2</sub>-fixer growth through costs associated with lag times or SNF regulation. Interestingly, SNF and other physiological traits responded differently to dry and wet phases depending on



**Fig. 5** Effect of drought frequency and water status on intrinsic water-use efficiency ( $WUE_i$ ) of *Robinia pseudoacacia*. Bar heights represent untransformed least-square means standardized to mean total biomass (5.01 g DW). Error bars represent  $\pm$  SE. Horizontal line and shaded region represent mean and SE values for the always wet control. Horizontal brackets and asterisks denote significant differences between wet and dry phases for a given treatment. Vertical brackets and asterisks denote significant differences from the always wet control. Treatments: HF, high frequency; MF, medium frequency; LF, low frequency.



**Fig. 6** Effect of drought frequency and water status on *Robinia pseudoacacia* leaf nitrogen (N) concentration (%). Bar heights represent untransformed least-square means standardized to mean total biomass (5.01 g DW). Error bars represent  $\pm$  SE. Horizontal line and shaded region represent mean and SE values for the always wet control. Horizontal brackets and asterisks denote significant differences between wet and dry phases for a given treatment. Vertical brackets and asterisks denote significant differences from the always wet control. Treatments: HF, high frequency; LF, low frequency.

whether drought occurred at LF or HF. Plant response strategies to drought include those that promote tolerance of water stress (e.g. acclimation) and those that promote avoidance of water

stress (McDowell *et al.*, 2008). In our study, seedlings exposed to LF drought cycles exhibited traits associated with drought tolerance, whereas those exposed to HF drought cycles exhibited traits associated with drought avoidance, followed by compensatory growth on soil rewetting. *Robinia pseudoacacia* may employ these contrasting strategies depending on how drought affects plant physiology and the availability of soil N.

### Drought tolerance response

Seedlings under LF responded to a single 8-wk drought by increasing  $WUE_i$ , root mass fraction, leaf N content and SNF rate (via greater nodule mass fraction). These responses reached maximum values relative to the AW control after six or more weeks of drought (Fig. S1), suggesting that drought tolerance was achieved through acclimation. Seedlings attained greater  $WUE_i$  by reducing  $g_s$  whilst maintaining a similar net assimilation to seedlings in constant well-watered conditions. Photosynthetic acclimation can result from increased N investment in Rubisco (Wright *et al.*, 2001), allowing for a greater maximum rate of carboxylation ( $V_{cmax}$ ) (Pankovic *et al.*, 1999; Kitao *et al.*, 2007) and  $WUE_i$ , and SNF may facilitate this response (Tobita *et al.*, 2010; Adams *et al.*, 2016). In support of this mechanism, we observed higher leaf N content in LF seedlings under dry vs wet phases. In addition, we observed a significant positive relationship between leaf N content and nodule mass fraction (Pearson's  $r = 0.43$ ,  $P < 0.001$ ), which suggests that investment in SNF may provide *R. pseudoacacia* with the N required to acclimate to drought.

Both soil N supply and plant N demand can help to explain plant investment in SNF under drought. Prolonged drought can reduce the N available to plants (Rennenberg *et al.*, 2009; He & Dijkstra, 2014) by suppressing microbial activity (Stanford & Epstein, 1974) and mass diffusion (Chapin, 1991), thus triggering an increase in SNF investment (Wurzburger & Miniat, 2014). In our study, although we did not detect differences in soil  $NH_4^+$  or  $NO_3^-$  supply in response to drought frequency or water status, we observed an increase in nodule mass fraction in the dry phases of LF treatment, suggesting that differences in plant N demand were driving the response of SNF. Plant N demand is largely determined by the net C assimilation rate, as plants must acquire sufficient N to maintain C:N stoichiometry during growth (Elser *et al.*, 2010). Thus, N demand may decrease during drought, as a result of stomatal limitation on C assimilation, and hence growth. However, if plants acclimate to drought, as observed in our LF treatment,  $A_{net}$  may recover to rates observed in well-watered plants (Flexas *et al.*, 2006), and thereby maintain N demand. If, however, plants acclimate to drought specifically by increasing N investment in Rubisco, as earlier, N demand may become even greater than under wet conditions, and result in the upregulation of SNF (Wright *et al.*, 2001, 2005; Tobita *et al.*, 2010).

### Drought avoidance and compensatory growth response

In contrast with LF seedlings, individuals grown under high drought frequency (HF) did not acclimate during repeated 2-wk

droughts, and they fixed C and N at low rates during these periods. This finding supports our hypothesis that seedlings would show reduced drought acclimation under high drought frequency. The lack of acclimation in HF seedlings is consistent with the idea that acclimation may require longer time periods of drought (i.e. weeks to months) (Flexas *et al.*, 2006; Zhou *et al.*, 2016), and thus may be inhibited when droughts are interspersed with brief wet periods, as in our HF treatment. However, the lack of acclimation did not reduce final biomass, as HF seedlings exhibited a strong compensatory growth response, with rapid net photosynthesis and SNF rates during wet, inter-drought periods.

Seedlings grown under HF drought fixed N more rapidly during wet, inter-drought periods than during drought periods, a pattern which may be related to the effect of drought cycles on plant N demand. Short (2-wk) drought periods in our HF treatment may have induced stomatal limitation of photosynthesis, and hence reduced plant N demand as a result of a diminished growth rate. On rewetting, however, SNF increased to a rate even greater than that in plants under constant moisture, and this response may be explained by greater plant N demand associated with rapid growth. Greater SNF following rewetting was not accompanied by increased leaf N content, which suggests that fixed N was not stored in leaf tissues, but instead fueled new growth. Interestingly, this increase in growth compensated for reductions during dry phases, resulting in no differences in final biomass relative to the control. Our findings of rewetting-induced compensatory growth may be vital for plant resistance to increased drought frequency. Such a response may be triggered by root production of cytokinins, which are translocated to shoots to promote rapid cell division (Wang *et al.*, 2016). Interestingly, cytokinin production is dependent on sufficient N supply to roots (Tamaki & Mercier, 2007), which suggests the possibility that N<sub>2</sub>-fixing plants may be particularly adapted for drought-induced compensatory growth.

### Ecosystem-scale implications

Our findings suggest that *R. pseudoacacia* seedlings are resistant to both prolonged drought and increased drought frequency, and that SNF facilitates this resistance. As ecosystem-level SNF is determined by the resource allocation strategies of individual plants, our findings may reveal the physiological basis of the ecosystem SNF response to drought. If ecosystem-level SNF is unaffected by drought frequency, it lends natural resilience to terrestrial ecosystems recovering from disturbance (Boring & Swank, 1984; Menge *et al.*, 2012; Batterman *et al.*, 2013). However, the application of our findings on tree seedlings to natural systems depends on how drought mediates the effects of competition between N<sub>2</sub>-fixers and nonfixers (Wurzburger & Miniati, 2014), which may depend on access to deep water sources (Giordano *et al.*, 2011), susceptibility to herbivory (Gaylord *et al.*, 2013) and the successional stage of the ecosystem (Menge & Hedin, 2009; Batterman *et al.*, 2013). In addition, as we fertilized our seedlings with all nutrients except N, it is possible that other nutrients, such as phosphorus, may limit SNF rates in natural settings. Finally, drought frequency may

affect the timing of SNF (i.e. whether it occurs during dry or wet phases), as it did in our experiment, and it is unclear whether this difference affects how fixed N enters and cycles within the plant–soil system.

### Conclusions

In our study, the growth and mean SNF rate of *R. pseudoacacia* seedlings were unaffected by increased drought frequency, and we found no evidence for costs associated with time lags and SNF regulation. However, we found that drought frequency determined how seedlings responded to individual drought events, where seedlings employed a tolerance strategy under LF drought and an avoidance strategy followed by rapid growth under HF drought. Interestingly, N fixation may supply the N needed in both strategies, by facilitating rapid growth following drought, and by accumulating N in leaf tissues for acclimation. Although the response of natural forests to drought will depend on how N<sub>2</sub>-fixing and nonfixing trees compete for, and acquire, water and soil N, our findings point to the potential role of SNF in ecosystem resistance to increased drought frequency.

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### Author contributions

J.M.M., N.W., C.F.M. and R.O.T. designed the research. J.M.M. performed the research. J.M.M., N.W. and C.F.M. analyzed the data and wrote the manuscript. R.O.T. edited final drafts of the manuscript.

### References

- Adams MA, Turnbull TL, Sprent JI, Buchmann N. 2016. Legumes are different: leaf nitrogen, photosynthesis, and water use efficiency. *Proceedings of the National Academy of Sciences, USA* 113: 4098–4103.
- Anderson MD, Ruess RW, Uliassi DD, Mitchell JS. 2004. Estimating N<sub>2</sub> fixation in two species of *Alnus* in interior Alaska using acetylene reduction and <sup>15</sup>N<sub>2</sub> uptake. *Ecoscience* 11: 102–112.
- Aranibar JN, Otter L, Macko SA, Feral CJW, Epstein HE, Dowty PR, Eckardt F, Shugart HH, Swap RJ. 2004. Nitrogen cycling in the soil–plant system along a precipitation gradient in the Kalahari sands. *Global Change Biology* 10: 359–373.

- Barron AR, Purves DW, Hedin LO. 2011. Facultative nitrogen fixation by canopy legumes in a lowland tropical forest. *Oecologia* 165: 511–520.
- Batterman SA, Hedin LO, van Breugel M, Ransijn J, Craven DJ, Hall JS. 2013. Key role of symbiotic dinitrogen fixation in tropical forest secondary succession. *Nature* 502: 224–227.
- Boring LR. 1984. Symbiotic nitrogen fixation in regenerating black locust (*Robinia pseudoacacia* L.) stands. *Forest Science* 30: 528–537.
- Boring LR, Swank WT. 1984. The role of black locust (*Robinia pseudoacacia*) in forest succession. *Journal of Ecology* 72: 749–766.
- Chapin FS. 1991. Effects of multiple environmental stresses on nutrient availability and use. In: Mooney HA, Winner WE, Pell EJ, eds. *Response of plants to multiple stresses*. San Diego, CA, USA: Academic Press, 67–88.
- Elser JJ, Fagan WF, Kerkhoff AJ, Swenson NG, Enquist BJ. 2010. Biological stoichiometry of plant production: metabolism, scaling and ecological response to global change. *New Phytologist* 186: 593–608.
- Flexas J, Bota J, Galmés J, Medrano H, Ribas-Carbó M. 2006. Keeping a positive carbon balance under adverse conditions: responses of photosynthesis and respiration to water stress. *Physiologia Plantarum* 127: 343–352.
- Gaylord ML, Kolb TE, Pockman WT, Plaut JA, Yopez EA, Macalady AK, Pangle RE, McDowell NG. 2013. Drought predisposes piñon–juniper woodlands to insect attacks and mortality. *New Phytologist* 198: 567–578.
- Giordano CV, Guevara A, Boccalandro HE, Sartor C, Villagra PE. 2011. Water status, drought responses, and growth of *Prosopis flexuosa* trees with different access to the water table in a warm South American desert. *Plant Ecology* 212: 1123–1134.
- Gutschick VP. 1981. Evolved strategies in nitrogen acquisition by plants. *American Naturalist* 118: 607–637.
- He M, Dijkstra FA. 2014. Drought effect on plant nitrogen and phosphorus: a meta-analysis. *New Phytologist* 204: 924–931.
- IPCC. 2013. Stocker TF, Qin D, Plattner G-K, Tignor M, Allen SK, Boschung J, Nauels A, Xia Y, Bex V, Midgley PM, eds. *Climate change 2013: the physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge, UK & New York, NY, USA: Cambridge University Press.
- Johnsen KH, Bongarten BC. 1991. Allometry of acetylene reduction and nodule growth of *Robinia pseudoacacia* families subjected to varied root zone nitrate concentrations. *Tree Physiology* 9: 507–522.
- Kitao M, Lei TT, Koike T, Kayama M, Tobita H, Maruyama Y. 2007. Interaction of drought and elevated CO<sub>2</sub> concentration on photosynthetic down-regulation and susceptibility to photoinhibition in Japanese white birch seedlings grown with limited N availability. *Tree Physiology* 27: 727–735.
- Knapp AK, Fay PA, Blair JM, Collins SL, Smith MD, Carlisle JD, Harper CW, Danner BT, Lett MS, McCarron JK. 2002. Rainfall variability, carbon cycling, and plant species diversity in a mesic grassland. *Science* 298: 2202–2205.
- Mantovani D, Veste M, Boldt-Burisch K, Fritsch S, Koning LA, Freese D. 2015. Carbon allocation, nodulation, and biological nitrogen fixation of black locust (*Robinia pseudoacacia* L.) under soil water limitation. *Annals of Forest Research* 58: 259–274.
- McDowell N, Pockman WT, Allen CD, Breshears DD, Cobb N, Kolb T, Plaut J, Sperry J, West A, Williams DG *et al.* 2008. Mechanisms of plant survival and mortality during drought: why do some plants survive while others succumb to drought? *New Phytologist* 178: 719–739.
- Menge DNL, Ballantyne F IV, Weitz JS. 2011. Dynamics of nutrient uptake strategies: lessons from the tortoise and the hare. *Theoretical Ecology* 4: 163–177.
- Menge DNL, Hedin LO. 2009. Nitrogen fixation in different biogeochemical niches along a 120 000-year chronosequence in New Zealand. *Ecology* 90: 2190–2201.
- Menge DNL, Hedin LO, Pacala SW. 2012. Nitrogen and phosphorus limitation over long-term ecosystem development in terrestrial ecosystems. *PLoS One* 7: e42045.
- Menge DNL, Levin SA, Hedin LO. 2009. Facultative versus obligate nitrogen fixation strategies and their ecosystem consequences. *American Naturalist* 174: 465–477.
- Pankovic D, Sakac Z, Kevres S, Plesnicar M. 1999. Acclimation to long-term water deficit in the leaves of two sunflower hybrids: photosynthesis, electron transport and carbon metabolism. *Journal of Experimental Botany* 50: 127–138.
- Pearson HL, Vitousek PM. 2001. Stand dynamics, nitrogen accumulation, and symbiotic nitrogen fixation in regenerating stands of *Acacia koa*. *Ecological Applications* 11: 1381–1394.
- R Development Core Team. 2016. *R: a language and environment for statistical computing, version 3.3.1*. [WWW document] URL <https://www.R-project.org/>. Vienna, Austria: R Foundation for Statistical Computing.
- Rennenberg H, Dannenmann M, Gessler A, Kreuzwieser J, Simon J, Papen H. 2009. Nitrogen balance in forest soils: nutritional limitation of plants under climate change stresses. *Plant Biology* 11: 4–23.
- Schulze ED, Gebauer G, Ziegler H, Lange OL. 1991. Estimates of nitrogen fixation by trees on an aridity gradient in Namibia. *Oecologia* 88: 451–455.
- Serraj R, Sinclair TR, Purcell LC. 1999. Symbiotic N<sub>2</sub> fixation response to drought. *Journal of Experimental Botany* 50: 143–155.
- Sheffield J, Wood EF. 2008. Projected changes in drought occurrence under future global warming from multi-model, multi-scenario, IPCC AR4 simulations. *Climate Dynamics* 31: 79–105.
- Soper F, Richards A, Siddique I, Aidar M, Cook G, Hutley L, Robinson N, Schmidt S. 2015. Natural abundance (δN) indicates shifts in nitrogen relations of woody taxa along a savanna–woodland continental rainfall gradient. *Oecologia* 178: 297–308.
- Stanford G, Epstein E. 1974. Nitrogen mineralization–water relations in soils. *Soil Science Society of America Journal* 38: 103.
- Tamaki V, Mercier H. 2007. Cytokinins and auxin communicate nitrogen availability as long-distance signal molecules in pineapple (*Ananas comosus*). *Journal of Plant Physiology* 164: 1543–1547.
- Tiemann LK, Billings SA. 2011. Changes in variability of soil moisture alter microbial community C and N resource use. *Soil Biology and Biochemistry* 43: 1837–1847.
- Tobita H, Uemura A, Kitao M, Kitaoka S, Utsugi H. 2010. Interactive effects of elevated CO<sub>2</sub>, phosphorus deficiency, and soil drought on nodulation and nitrogenase activity in *Alnus hirsuta* and *Alnus maximowiczii*. *Symbiosis* 50: 59–69.
- Vitousek PM, Field CB. 1999. Ecosystem constraints to symbiotic nitrogen fixers: a simple model and its implications. *Biogeochemistry* 46: 179–202.
- Vitousek P, Howarth R. 1991. Nitrogen limitation on land and in the sea: how can it occur? *Biogeochemistry* 13: 87–115.
- Vose JM, Miniati CF, Luce CH, Asbjornsen H, Caldwell PV, Campbell JL, Grant GE, Isaak DJ, Loheide SP, Sun G. 2016. Ecohydrological implications of drought for forests in the United States. *Forest Ecology and Management* 380: 335–345.
- Wang X-L, Wang J-J, Sun R-H, Hou X-G, Zhao W, Shi J, Zhang Y-F, Qi L, Li X-L, Dong P-H *et al.* 2016. Correlation of the corn compensatory growth mechanism after post-drought rewatering with cytokinin induced by root nitrate absorption. *Agricultural Water Management* 166: 77–85.
- Wright IJ, Reich PB, Cornelissen JHC, Falster DS, Groom PK, Hikosaka K, Lee W, Lusk CH, Niinemets Ü, Oleksyn J *et al.* 2005. Modulation of leaf economic traits and trait relationships by climate. *Global Ecology and Biogeography* 14: 411–421.
- Wright IJ, Reich PB, Westoby M. 2001. Strategy-shifts in leaf physiology, structure and nutrient content between species of high and low rainfall, and high and low nutrient habitats. *Functional Ecology* 15: 423–434.
- Wurzburger N, Miniati CF. 2014. Drought enhances symbiotic dinitrogen fixation and competitive ability of a temperate forest tree. *Oecologia* 174: 1117–1126.
- Zhou S-X, Medlyn BE, Prentice IC. 2016. Long-term water stress leads to acclimation of drought sensitivity of photosynthetic capacity in xeric but not riparian *Eucalyptus* species. *Annals of Botany* 117: 133–144.

## Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

**Fig. S1** Physiological differences of *Robinia pseudoacacia* seedlings exposed to low-frequency (LF) drought treatment vs always wet control (AW) through time.

**Table S1** Effect of drought frequency treatment and water status on root mass fraction, leaf mass fraction and nodule mass-specific symbiotic N<sub>2</sub> fixation (SNF) rate of *Robinia pseudoacacia*

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