Tolerance or avoidance: drought frequency determines the response of an N2-fixing tree

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Summary

- Climate change is increasing drought frequency, which may affect symbiotic N2 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 N2-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 N2 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 N2-fixing plants (hereafter N2-fixers) respond to repeated drought. Although N2-fixers appear to increase SNF during individual drought events (Tobita et al., 2010; Wurzburger & Miniat, 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 N2-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 N2-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 \((V_{\text{max}})\) and thus increase water-use efficiency (WUE) \((\text{Wright et al., 2001, 2005; Kitao et al., 2007})\). 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 \((\text{Tiemann & Billings, 2011})\). N\(_2\)-fixers may respond to these changes in N availability by adjusting investment in SNF \((\text{Barron et al., 2011})\), because SNF is substantially more costly than soil N uptake \((\text{Vitousek & Field, 1999})\). As such, N\(_2\)-fixers may increase carbon \((C)\) investment to symbionts \((\text{either through construction of root nodules or greater flow of C to existing nodules})\) when soil N supply is limited \((\text{Tobita et al., 2010; Wurzburger & Miniat, 2014})\), 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 N\(_2\)-fixers can respond, allocation to SNF becomes suboptimal \((\text{Menge et al., 2009})\). Such a mismatch between N acquisition strategy and the environment could result in reductions in N\(_2\)-fixer growth. Time lags are difficult to predict because we lack an understanding of the time scales of SNF regulation. Most N\(_2\)-fixers adjust the SNF rate by constructing or excising root nodules \((\text{Pearson & Vitousek, 2001; Barron et al., 2011})\). 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 \((\text{Johnsen & Bongarten, 1991})\). Thus, the significance of time lags may depend on how N\(_2\)-fixers track changes in N availability.

Increased drought frequency could reduce the growth and biomass of N\(_2\)-fixers because of the C costs associated with the regulation of SNF \((\text{Menge et al., 2009, 2011})\). The up- or down-regulation of SNF may result in energetic relations 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 \((\text{Gutschick, 1981})\). If 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 \((\text{Menge et al., 2011})\), and could result in reduced 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 N\(_2\)-fixing tree, Robinia pseudoacacia, a widespread and ecologically important N\(_2\)-fixer in eastern US forests \((\text{Boring, 1984; Boring & Swank, 1984})\). 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 \((\text{Sheffield’s Seed Co., Locke, NY, USA})\) in 5-l pots. Each pot contained a nutrient-poor potting mix consisting of a 1:1 ratio of local forest soil \((\text{Cecil series; Typic Kanhapludult})\), passed through a 4-mm sieve to remove roots and coarse organic matter, and coarse granite sand. To ensure seedlings had access to 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 \((\text{treatment n = 33; see Fig. 1})\). 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 \((\text{J. M. Minucci et al., unpublished data})\). 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 \((\text{CS655; Campbell Scientific, Logan, UT, USA})\). 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 pots and variable soil moisture pots during wet phases at 15% VWC, which was 70% of the field capacity for our soil.

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 \((\text{Sheffield’s Seed Co., Locke, NY, USA})\) in 5-l pots. Each pot contained a nutrient-poor potting mix consisting of a 1:1 ratio of local forest soil \((\text{Cecil series; Typic Kanhapludult})\), passed through a 4-mm sieve to remove roots and coarse organic matter, and coarse granite sand. To ensure seedlings had access to 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.
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 (Ψpd, MPa; PMS Instruments, Albany, OR, USA) on one fully extended leaf per individual. We measured leaf net C assimilation rate (Anet, μmol CO2 m⁻² leaf area s⁻¹) at saturating light (1800 μmol photons m⁻² s⁻¹), stomatal conductance (gs, μmol H2O m⁻² s⁻¹) and transpiration (E, μmol H2O m⁻² 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 (WUEi, μmol CO2 mol⁻¹ H2O) was estimated as Anet divided by gs.

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 Anet 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 five root nodules from each seedling with forceps to estimate the N2 fixation rate via acetylene reduction assay (ARA, μmol N2 fixed g⁻¹ 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 C2H2, 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 C2H4 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 N2 fixation was determined with a one-time set of ten 98%-atom 15N (Sigma-Aldrich) incubations run in parallel with ARAs. The mean (and SE) conversion ratio was 0.06 (± 0.01) μmol C2H4 : μmol N2, which is at the low end of the range of values observed for woody N2-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

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

Statistical analysis
To confirm that seedlings in wet and dry phases had different VWC and Ψpd 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 α = 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 (F1,129 = 366.5, P < 0.001), with 14.96% soil moisture compared with 5.79% (Table 1). Mean pre-dawn water potential was also higher for the wet phase soils compared with the dry phase soils (F1,130 = 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 (NH4+: F1,31 = 0.003, P = 0.42; NO3−: F1,31 = 0.007, P = 0.93), nor did they vary among drought frequency treatments (NH4+: F3,31 = 0.3, P = 0.83; NO3−: F3,31 = 1.3, P = 0.30) or between wet and dry phases (NH4+: F1,31 = 0.7, P = 0.41; NO3−: F1,31 = 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 (F3,16 = 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: F2,99 = 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: F3,124 = 2.33, P = 0.08; water status: F1,124 = 0.60, P = 0.44; Table S1), and we did not observe a significant amount of leaf shedding during the dry phases.

Table 1 Effect of water status on gravimetric soil moisture (% SM), pre-dawn leaf water potential (Ψpd) and KCl-extractable ammonium and nitrate across treatments

<table>
<thead>
<tr>
<th></th>
<th>Wet phase</th>
<th>Dry phase</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>% SM</td>
<td>14.96% (+0.36)</td>
<td>5.79% (+0.24)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ψpd</td>
<td>−0.13 MPa (+0.01)</td>
<td>−0.46 MPa (+0.05)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NH4+</td>
<td>0.17 ppm (+0.03)</td>
<td>0.13 ppm (+0.03)</td>
<td>0.41</td>
</tr>
<tr>
<td>NO3−</td>
<td>0.08 ppm (+0.06)</td>
<td>0.07 ppm (+0.07)</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Values represent means (+ SE). P values are given for the water status effect in ANOVA. Bold P values indicate a statistically significant difference (P < 0.05).
Symbiotic N\textsubscript{2} 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 $16.8 (\pm 1.3) \mu mol \text{C}_2\text{H}_4 \text{g}^{-1} \text{h}^{-1}$, or $264.9 (\pm 20.6) \mu mol \text{N}_2 \text{fixed g}^{-1} \text{h}^{-1}$ 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 ± 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.](image)

Stomatal conductance and transpiration were dependent on water status ($g$: $F_{1,90} = 33.7, P < 0.001$; $E$: $F_{1,90} = 36.1, P < 0.001$) and were not affected by drought frequency. Seedlings in wet phases had 113\% and 105\% higher $g$ and $E$, respectively, than those in dry phases. However, drought frequency altered how WUE\textsubscript{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 WUE\textsubscript{i} by 68\% during the dry phase, whereas MF and HF seedlings maintained consistent WUE\textsubscript{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\textsubscript{2}-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...
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, 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, by reducing g, 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 carbon assimilation (V_{cmax}) (Pankovic et al., 1999; Kitao et al., 2007) and WUE, 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 N2-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 N2-fixers and nonfixers (Wurzburger & Miniat, 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 N2-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


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$_2$ fixation (SNF) rate of *Robinia pseudoacacia*