Title: Water, nutrients, or both? Effects of multiple belowground resources on early seedling growth and physiology in eight species of tropical dry forest trees

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*Introduction*

Plants require multiple belowground resources including water and a variety of nutrients. Differences in both how plants acquire these resources and how they respond to variability in the availability of soil-borne resources contribute to niche differentiation and the maintenance of diversity (Levine and HilleRisLambers 2009, Holste et al. 2011).  Because the seedling stage is a vulnerable time in trees’ life histories during which populations suffer elevated mortality (Ribbens et al. 1994), many studies have evaluated how water and nutrients affect seedling growth rates (Yavitt 199?,Baraloto et al. 2006), allocation to roots versus shoots (Burslem et al. 1996, Canham et al. 1996), physiological process rates (Graciano et al. 2005), and relationships with symbionts such as mycorrhizae and nitrogen fixing bacteria in Fabaceae (Birhane et al. 2015).  These studies typically find that responses to multiple belowground resources are often species-specific and also may depend upon whether above- or belowground organs are considered (Freschet et al. 2013).  Thus, we still lack a general framework for understanding the joint effects of belowground resources on seedling performance, particularly in environments with large seasonal or temporal variability in soil moisture and nutrient availability (Lodge et al. 1994), such as seasonally dry tropical forests.

Paragraph 2 introduce TDF..

Seedling growth in TDF is thought to be strongly constrained by water availability and is typically restricted to the wet season (Gerhardt 1993, Maza-Villalobos et al. 2013).  However, studies have also shown that TDF seedlings respond positively to increased nutrient availability (Huante et al. 1995a, Huante et al. 1995b, Ceccon et al. 2003).  Surprisingly, few studies have investigated how nutrients and soil water jointly affect tropical dry forest tree seedlings.  There are a number of possible responses to increased water and nutrients, from additive, synergistic, antagonistic, or no response.

How does drought/nutrients affect relationships with symbionts, i.e. N fixing bacteria or mycorrhizae?Paragraph 3 Goals and Specific Objectives

Here we examine the effects of water and nutrient addition on early growth and above- and belowground traits  of eight species from the tropical dry forest of Costa Rica. We used three species cable of fixing nitrogens and five non-ixers species from different plant families. We hypothesized that water and/or nutrient addition will cause plants to shift their traits in order to uptake the new resources available, which will lead to a biomass gain. (ref)

We used a diverse sample of tropical dry forest tree species from different functional groups in a seedling pot experiment to answer the following questions:

*(Q1) What is the relative influence of water vs. nutrient availability on tropical dry forest seedling growth and biomass allocation.*

*(Q2) How does increased nutrient and/or water availability influence seedling water- and nutrient-use traits and relationships with symbionts?*

*(Q3) Are traits coordinated along the “fast to slow” continuum and to what extent does this correlate with growth rates among species?*

Based on previous studies (Smith et al, 2017), we expected nitrogen fixing legumes to have higher growth rates and respond differently to treatments compared to non-fixing species.

***Methods***

*Study site and species*

Our experiment took place between June to November, 2015, in a shade house located at the Estacion Experimental Forestal Horizontes, Área de Conservación Guanacaste, Costa Rica (10°420 46″ N, 85°350 44″). Rainfall is highly seasonal, with a dry period of 5-6 months. Mean annual precipitation at nearby Sector Santa Rosa is 1729 mm and mean temperature is 25 C (Becknell and Powers 2014), although Horizontes likely has lower rainfall than Santa Rosa (Gutiérrez-Leitón 2013). Our study occurred during one of the driest years on record (Cooley et al. 2019), wherein rainy season rainfall was less than X of average due to the very strong El Niño Southern Oscillation (ENSO) event of 2015; thus we considered ambient rainfall as a drought.  We used 8 species that belong to 5 different families. The Fabaceae included *Enterolobium cyclocarpum, Gliricidia sepium,, Dalbergia retusa,* all of which fix nitrogen, as well as the non-fixer *Hymenea courbaril.* The other families and species we studied included Meliaceea-*Swietenia macrophylla,* Simaroubaceae *-Simarouba glauca,* Bignoniaceae-*Tabebuia ochraceae* and Bombaceae-*Pachira quinata* (Table 1). Collectively, these species represent the full range of functional trait variation that defines different tropical dry forest plant functional types in ecosystem simulation models (Xu et al 2016), including nitrogen (N) fixing and non-N fixing legume species, and evergreen and deciduous leaf phenologies.

*Seed germination, growing conditions*

The shade house was covered with a 50% shade cloth. All  seeds were sown directly into pots containing a soil mixture of 2 parts of locally collected soil and 1 part sand. To improve germination rates, we applied two different treatments to the seeds. *Enterolobium* seeds were subject to water baths alternating from boiling to cold water for 30 seconds for breaking the seed coat. All other species were soaked in water overnight.

Seeds were sown directly into pots in early June, 2015.  Following germination and establishment, pots were thinned to one seedling per pot.  All plants received supplemental water when needed during this period.  After ~12 weeks, an initial harvest of four individuals per species was made (see Methods below). Watering and/or fertilizer treatments on the remaining seedlings were started two days later, and were imposed for 12 weeks (N = 4 plants per species per treatment) prior to harvest.

*Watering and fertilization treatments*

At 12 weeks, plants were randomly assigned to one of four treatments: control (no additions), nutrient addition, water addition, water + nutrient addition.  Water additions consisted of 500 mL added every two weeks.  The nutrient addition treatment consisted of a commercial fertilizer containing NPK and complete micronutrients.  Nutrient addition rates were corresponded to 150 kg N ha-1 yr-1, prorated to the duration of the experiment (3 months). Fertilizer was dissolved in water and added to the surface soil in the pots every two weeks in 20 mL doses with a syringe. Soil moisture readings were taken in all pots (0-5 cm soil depth) immediately prior to watering treatments and then ~1 day following watering, and these measurements were repeated six equally spaced times during the growing season after the treatments began. This allowed us to quantify the magnitude of the watering effect on soil moisture, and also allowed us to know whether elevated soil moisture in the watered pots persisted beyond two weeks. These measurements were made with a SM150 Soil Moisture Sensor (Delta-T Devices, Ltd, Cambridge, England).

*Functional trait  measurements*

We measured morphological and physiological traits related to carbon, nutrient, water acquisition and use for each individual seedling (N = ZZ at the end of the experiment). Prior harvesting the plants at the end of the experiment, we measured maximum photosynthetic capacity at 1200 PAR (Amax; μmol CO2 m−2 s−), stomatal conductance (gs; mmol m−2 s−1) and instantaneous water use efficiency (WUE; μmol CO2 mmol H2O−1) with a LCi portable photosynthesis system (ADC Bioscientific Ltd. Hoddesdon, UK).

For specific leaf area (SLA), leaf area-based nitrogen concentration (Narea) and leaf mass-based nitrogen concentration (Nmass) one leaf per plant was scanned and dried at ~60 °C for 48 h and weighed. SLA was calculated by dividing leaf area by leaf weight. After measuring SLA, this same leaf was ground to fine powder and wrapped into tin capsules to quantify leaf nitrogen concentration and the stable isotope of δ13C. δ13C was measured on a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon, Cheshire, UK) at the Stable Isotope Facility at the University of California, Davis. We then calculated Nmass and Narea by dividing leaf nitrogen concentration by its dry weight and leaf area respectively.

All these leaf traits were measured on the untreated individuals harvested at the beginning of the experiment and all individuals in the final harvest, while the physiological traits were only measured in the individuals at the end of the experiment prior to the harvest.

*Plant performance: Biomass and Growth.*

Every two weeks height was measured on each individual seedling to calculate absolute growth rate in terms of height (AGRh). We calculated AGRh as (Hf) - Hi)/(t2-t1) where Hi is the initial height of the plant and Hf is the final height of the plant.

After the gas exchange measurements at the end of the experiment, we harvested each individual seedling for biomass quantification. First, we divided the plants into leaves and stems, then the roots were carefully extracted and washed from the pots and for *Enterolobium cyclocarpum, Gliricidia sepium and Dalbergia retusa* every single nodule was counted. After this, all roots, stems, and leaves were dried at ~ 60° C for 48h then weighed.

*Symbiotes*

For quantifying mycorrhizal colonization we stainined the roots with alanine blue (Koske & Gemma 1989). Three washed 2-3 cm long roots of each seedling were cleared in 20% KOH for 3-5 d at room temperature (highly pigmented roots received the KOH treatment, then spent 3 hr at 90 C, and finally were submerged in H2O2), rinsed with DI water and acidified in 1% HCl overnight. Roots were stained in 0.05% alanine blue for 3 days and destained in 14:1:1 mixture of lactic acid, glycerol and water for 3 days. Stained root pieces were mounted on slides and root colonization was quantified microscopically using the magnified intersection method (McGonigle et al. 1990) on ~100 intersections per root fragment. We calculated colonization as the percentage of root length colonized by AM fungi.

Data analysis

We hypothesized that N fixing legumes and non-legumes would respond differently to treatments, so we considered N-fixing status as a main effect in our analyses (Powers and Tiffin, 2010; Pineda-Garcia et al 2016, Adams et al 2016).

To address how the addition of water and/or nutrients affected plant biomass and allocation (Q1*)*, we built linear mixed models  (gaussian error distribution) with treatment (ambient rain, ambient rain plus water, ambient rain plus nutrients, ambient rain plus water and nutrients) and fixer category (fixer- vs. non-fixer) and their interaction as the main effects, initial height as a covariate to control for the differences in height between plants, and species as a random effect. We constructed separate models as described above for the following response variables: total biomass, AGRh, root mass fraction (RMF), stem mass fraction (SMF), and leaf mass fractions (LMF).  The mass fractions were calculated as the proportion of a given tissue mass relative to total seedling biomass and express the relative allocation of biomass to different functions.

For all models we performed likelihood ratio F-tests on type-III ANOVAs to detect significant model terms (to account for the unbalanced seedlings within treatments; *car* package), then used Tukey’s HSD post-hoc tests to detect differences between groups using the *emmeans* R package (ref). For each linear model we also determined marginal (R2m; fixed effects) and conditional (R2c; fixed and random effects) R2 values (performance R package; Nakagawa and Schielzeth 2013).

Then, to investigate *how water and/or nutrient addition influenced seedling functional traits (Q2)* we used the same mixed modeling approach as above, instead using Amax, gs, and instantaneous WUE, SLA, Nmass, Narea, foliar δ13C (as an integrated metric of water use efficiency), as response variables. This allowed us to interpret any changes in seedling traits after we imposed the watering and fertilization treatments within the context of developmental changes over the growing season.

Finally, to determine *if seedling traits across nutrient and/or water addition treatments were related with differences in seedling growth rates among treatments (Q3)* we used the same mixed model framework as above, this time adding principal components axes to integrate across all seedling-level functional traits. First, we performed a principal components analysis to reduce data dimensionality and selected the principal component axes that explained most of the variation in the data using scaled and centered functional trait data for each individual seedling (Amax, Nmass, Narea, WUE, gs, SLA, and foliar δ13C). The PCA provides information on trait coordination and integration.

Then, we built linear mixed models with two and three-way interactions (gaussian error distribution) and selected the model most appropriate to determine how seedling-level functional traits (PCs), treatment, fixer-category influenced seedling-level absolute growth rates.

 We used the model:

*AGRh ~ treatment + PCn +  nfixer +*

*initial seedling height + random effect(species)*

Last, we assessed the strength of relationships between functional traits (PCs) and seedling absolute growth rate in each treatment in the presence of significant interaction terms (i.e., Treatment \* PC1). If the slope of the relationship between PC and a treatment was steeper we interpreted this as evidence that overall shifts in seedling-level traits within a treatment were correlated with higher seedling growth rates. We visualized these relationships by plotting interaction plots for the full model (*emmeans* R package). Mixed models were carried using the lme4 R package.

Finally for checking that model assumptions were not violated we used the R package performance to assess the normality of the residuals and the homogeneity of variances. All analyses were done using R.

**Results**

***(Q1) What is the relative influence of water vs. nutrient availability on tropical dry forest seedling performance?***

***This needs to be improved, I am listing every single result, sounds boring***

Overall, the effects of the treatments over total plant biomass, aboveground, belowground and AGRh depended on the capacity of the species to fix nitrogen FIGURE XXX

N-Fixer species increased by

responded significantly to all treatments when compared to the ambient rain treatment. When water was added, total biomass increased 32%, while the addition of nutrients increased 64% and the addition of nutrients and water increased it 67% . In the case of non-Nfixer species, total biomass only increased significantly in the plus nutrients treatment by 39% and 41% in the plus nutrients and water treatment.

Following the same trend as total biomass, the effect of the treatment on the aboveground biomass depended on whether  the species were N-fixers or non-Nfixers (F = 5.94, p-values = 0.0008). In the case of N-fixer species,  aboveground biomass increased significantly by 47% in the plus nutrient treatment and 82% in the plus nutrient and water addition. While for non-fixers, aboveground biomass increased significantly 31% when nutrients were added and 44% when nutrients and water were added (FIGURE XXX).

For belowground biomass (only roots), all species responded to the treatments no matter if they were Nfixers or non-Fixers (F = 9.76, p-values < 0.001).  The addition of water increased belowground biomass in all plants by 28% while the addition of nutrients increased it 64% and the addition of both water and nutrients increased it 49% (FIGURE XXX). No significant treatment effects were found on the mass fractions (root,stem and leaf).

Finally for AGRh, the treatment effects depended on whether the species were N-fixers or non-Nfixers (F = 9.816, p-values < 0.0001). For N-fixers, AGRh increased 62% in the nutrient addition treatment and 77% in nutrient and water treatment (FIGURE XXX). The AGR did not change significantly in any of the treatments when compared to the ambient rain for non-fixers.

***(Q2) How does increased nutrient or water availability influence water- and nutrient-use traits?***

For the three physiology traits measured, only for Amax, the response to the treatments depended on being N-fixers or non-fixers (F = 7.383 , pvalue < 0.0001).  In the plus nutrients treatment,  Amax for N-Fixer species decreased significantly 15% while, in the plus water treatment increased 14% on the (FIGURE XXX). For non-Nfixers, Amax did not change significantly in any of the treatments applied (FIGURE XXX). While gs and iWUE responded to the treatments (F = 5.534, pvalue = 0.0014549, F = 6.3961, pvalue = 0.0208896, respectively) and varied significantly depending on being N-fixer or non fixer (F = 4.7960, pvalue = 0.003619, F = 5.9854, pvalue = 0.041038, respectively). No significant interaction between NFixer and treatment were found.

For these two traits, an addition of water (plus water and plus water and nutrients treatments) led to a higher gs and lower iWUE in all plants irrespectively of the capacity of the species to fix nitrogen (FIGURE XXX), being NFixers species 50% more water efficient when compared to the Non-Nfixer species (FIGURE XXX).

Leaf traits

For the other five leaf traits measured, Gincreased for fixers 45% when nutrients were added and 60% when nutrients and water were added  while for non fixers this same trait increased 81% and 71% respectively. In the case of Nmass, this trait decreased 18% when water was added for the fixer species.

The addition of nutrients and/or water did not have an effect over the Mycorrhizal colonization and this did not change significantly between fixer and non-Fixer species.

***(Q3) Are shifts in seedling traits in response to increased water and/or nutrient addition associated with differences in seedling-level growth rates?***

Overall the first three principal axes explained 83% in the data. The first PC axis accounted for 48% of total variation and showed strong loadings on Amax, Narea, Nmass and WUE (in descending order). The second PC accounted for 19.4% of total variation with gs and SLA having the strongest loadings. Finally the third PC axes accounted for 15.8%  (Table #).

The most appropriate model for  determining how seedling-level functional traits (PCs), treatment and fixer-category influenced seedling absolute growth rates was the one that only included the two-way interactions (df = 12, Pr(>Chisq) = 0.2505). We found a significant interaction between treatment and PC1 (F= 9.50, df = 3, P = 0.0000158) and treatment and PC2 (F = 2.782, df = 3, P = 0.045) , indicating that shifts in seedling-level trait values treatments may have differentially influenced seedling growth rates among treatments.

Compared to the ambient treatment, PC1 had a much stronger relationship with seedling growth rates (steeper slope) in both the nutrient (~15-fold stronger effect; t = -3.62; P = 0.003) and water + nutrient addition treatments (~16-fold stronger effect; t = -4.13; P < 0.001The relationships between PC1 and growth rates in the ambient and water treatments (t = -0.22; P =  0.99) and the nutrient and nutrient + water treatments (t =  -0.37; P = 0.98) were equivalent. In the case of PC2, although we found a significant interaction between treatments and the PC2, the Post-Hoc test showed only significant differences in the seedling growth rates of the treatments plus water and plus water and nutrients  (FIGURE XXX)

Discussion

Total biomass

Overall, the addition of nutrients and not the addition water increased our performance metrics

Message that I want to c: The addition of nutrients increased our metrics of plants performance (Total biomass, above,below and AGR) but NFixers species responded stronger to the addition on nutrients

*Leaf traits*

*No traits responded independently to the treatments*

Supplement information

**Soil moisture**

*Biomass partition figures: root, stem and leaf mass fractions Supplementary*