Title: Water, nutrients, or both? Effects of multiple belowground resources on early seedling growth, physiology, and symbionts 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.

 DESCRIBE THE TYPES OF RESPONSES… (Table 1).

Paragraph 2 introduce TDF..

Seedling growth in TDF is 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.

\* SYNERGISTIC if plant didn't respond to each resource individually, but does respond to them added together, OR if LRR for both resources together is outside the 95% CI for an individual resource

\*\*\* what does it mean if the resources are not balanced?  Can a plant take advantage of added water or nutrients, if it does not have the other resource?

Paragraph 3 Goals and Specific Objectives

Here we examine the effects of water and nutrient addition on biomass gain and leaf trait of eight species from the tropical dry forest of Costa Rica. We used three fixers and five non fixers species. We hypothesized that water and/or nutrient addition will cause seedlings to shift their leaf 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 biomass allocation of tropical dry forest legumes and non-legume?*

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

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

***Methods***

*Study site and species*

Our experiment took place between June to November, 2015, in a shade house located at the Estación Experimental Forestal Horizontes, Area 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: *Enterolobium cyclocarpum, Gliricidia sepium, Hymenea courbaril, Dalbergia retusa, Swietenia macrophylla, Simarouba glauca, Tabebuia ochraceae* and *Pachira quinata* (Table 2). 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.

*Seed germination, growing conditions*

The shade house was covered with a 50% shade cloth. All the seeds 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.  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. At the end of the experiment, we experience the mortality of 4 plants, which reduce our sample size from 128 to 124.

*Watering and fertilization treatments*

At 12 weeks, seedlings were randomly assigned to one of four treatments: control (no additions), nutrient addition, water addition, water and 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 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 resource-acquisition and hydraulic traits to understand how seedlings respond to uptake the resources available (Q2) and how these influence their performance (Q3) (ref). Prior harvesting the seedlings, 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 (WUEi; μmol CO2 mmol H2O−1) in 117 seedlings with a LCi portable photosynthesis system (ADC Bioscientific Ltd. Hoddesdon, UK) (ref).

For specific leaf area (SLA, cm2/g ), leaf area-based nitrogen concentration (Narea, g/m2 ) and leaf mass-based nitrogen concentration (Nmass, mg/g) one leaf per seedling 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 concentrations by dividing leaf nitrogen concentration by its dry weight and leaf area respectively. We also calculated total seedling nitrogen content for each individual seedling by multiplying leaf mass-based nitrogen concentration by the total dry mass of all leaves for a seedling at the end of the experiment.

*Biomass and Growth*

To evaluate how nutrients and water influence seedling’s performance (Q1) every two weeks height was measured on each individual seedling to calculate absolute growth rate (AGR) in terms of height of each seedling. We calculated AGR as (Hf - Hi)/ (t2-t1) where Hi is the initial height of the seedling and Hf is the final height of the seedling.

After the gas exchange measurements, we harvested each individual seedling for biomass quantification. First, we divided the seedlings into leaves and stems, then the roots were carefully extracted and washed from the pots. All roots, stems, and leaves were dried at ~60° C for 48 h then weighed.

Data analysis

We calculated these for legumes and non-legume taxa separately, as numerous studies have suggested that the direction and magnitude of trait correlations differ between legumes and non-legumes (Powers and Tiffin, 2010; Pineda-Garcia et al 2016, Adams et al 2016).

To address determine how the addition of water and/or nutrients affected seedling biomass allocation (Q1), we built linear mixed models (Gaussian error distribution) with treatment (control, ambient rain + water, ambient rain + nutrients, ambient rain + water & 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 seedlings, and species as a random effect. To determine how treatments affected individual seedling biomass allocation and growth we used total seedling biomass (leaves + stems + roots), belowground biomass (roots), aboveground biomass (stems and leaves), leaves, stems, or roots separately, and AGR as response variables. For all models we performed likelihood ratio chi-squared (χ2) 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 (*emmeans* package). For each linear model we also determined marginal (R2m; fixed effects) and conditional (R2c; fixed and random effects) R2 values (performance 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 WUE, SLA, Nmass, Narea, canopy nitrogen content, 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 shifts in 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 to two principal components axes (hereafter, PC1 and PC2) using scaled and centered functional trait data for each individual seedling (Amax, WUE, gs, SLA, canopy nitrogen content, and foliar δ13C). Using Nmass or Narea in place of canopy nitrogen content in this analysis produced the same overall results. Then, we built a linear mixed model (gaussian error distribution) to determine how seedling-level functional traits (PC1 and PC2), treatment, fixer-category, and the two-way interactions between treatment, fixer category, and PCs (predictors) influenced seedling-level absolute growth rates (response). *Full model:* absolute height growth rate ~ treatment \* PC1 +  fixer category \* PC1 + treatment \* PC2 +  nfixer \* PC2 + initial seedling height + species [random effect]). 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 X PC1). If the slope of the relationship between PC1 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* package).