Effects of below-ground space limitation on performance of Eucalyptus seedlings: Does photosynthesis really control growth?

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Abstract

Negative feedbacks to photosynthesis are commonly interpreted as the primary factor responsible for growth limitation in plants under stress. This study used manipulations of soil volume to test how growth is coupled to physiology, allocation, and sink activity in Eucalyptus tereticornis seedlings. We grew seedlings in a large range of container sizes and planted containers flush to the soil alongside freely rooted seedlings (free). Reduced soil volume was expected to induce rapid negative effects on growth and physiology compared to free seedlings. It was hypothesized that soil volume effect would be largest in the smallest containers, resulting in physical constraints to growth independently of photosynthesis. Photosynthesis would then become sink-limited, resulting in the build-up of leaf nonstructural carbohydrates eventually leading to photosynthetic down regulation. We observed a negative container effect on aboveground growth soon after the experiment started. Although growth was consistently different across soil volumes mass, partitioning to leaves, stems, roots was conserved after 120 days. Photosynthetic capacity was also significantly reduced in containers, and was related to both leaf nitrogen content and starch accumulation, however starch effects were weaker. We developed a simple seedling growth model that utilized leaf photosynthesis rates to allocate daily carbon (c) uptake towards mass growth of stems, leaves and roots. We then asked whether the observed reductions in photosynthesis explained the observed differences in seedling biomass. We found that photosynthesis reductions alone was not sufficient and the inclusion of additional carbon and mass allocation strategies were necessary to better predict the observed growth responses to decreasing soil volume. Overall, we found limited evidence for sink-limitation of photosynthesis by constraining seedling growth in containers, and argue that shifts in C allocation are necessary to explain the experimental results. This research highlights the need to further understand adaptive strategies of plant C allocation, and confirms that photosynthesis and growth are not always directly related.

Key Words

photosynthesis, sink regulation, growth, carbon allocation, soil volume

Introduction

Methods

Experimental Design

This experiment was located on the Hawkesbury Forest Experiment site in Richmond, NSW, Australia. Plots were located in open cover with a site history that consists of a paddock that was converted from native pasture grasses. Top soils at this site, used for the study, are an alluvial formation of low-fertility sandy loam soils (380 and 108 mg kg^{-1} total N and P respectively) with low organic matter (0.7 %) and low water holding capacity. At this site a soil hard layer exists at $\sim$1.0 m with a transition to heavy clay soils. The climate for the region is classified as sub-humid temperate.

Eucalyptus tereticornis seedlings, 20 weeks old and approximately 40 cm tall in tube stock, were chosen from a single local Cumberland plain cohort. Previous experiments have confirmed that species with tap roots (similar to *E. tereticornis*) use the center of the container as the medium for thick roots leaving the periphery of the soil as the most active sites for fine root proliferation (Biran and Eliassaf, 1980a,b). This is generally hypothesized to be a different response than seedlings with no taproot. By using a species with tap root growth and manipulations of container length rather than width, it is believed that a more realistic test of inhibition of growth through constrained soil volume would be achieved. Six seedlings were harvested before planting to measure initial leaf area and dry mass of leaves, stems and roots.

Six container volumes were used ranging from 5 l to 3 l, with a 22.5 cm diameter, and lengths ranging from 15 to 100 cm. Containers were constructed of PVC pipe and were filled with local top soil (described above). Soil in each container was packed to achieve a target soil bulk density of 1.7 g m^{-3}. Each seedling was given an Imidacloprid (BAYER CropScience) insecticide tablet planted 5 cm below the roots. Containers were planted flush with the soil surface inside metal sleeves, designed to minimize excess air space between the container and outside soil while allowing container removal. This allowed for soil temperatures in containers to reflect conditions of naturally sown seedlings. Each experimental block (n=7) contained a complete replicate set of container volumes as well as one free seedling, with 1 m^2 spacing. For each free seedling, a 1 m^2 subplot was excavated to 0.5 m and replaced with the same soil used in each container. A border of root exclusion material was buried 0.25 m deep and extended 0.25 m above the ground surface to exclude local vegetation.

Plants were watered bi-weekly or when needed, accounting for natural precipitation, to maintain soil moisture at field capacity (13-15 %). To achieve field capacity all soil filled containers were weighed and soil moisture was measured (Time Domain Reflectometer at 10 cm depth) before watering. Derived equations based on container weight at planting (when soil moisture was 3 %) and at field capacity was used to calculate watering requirements for each individual plant. Drain systems were built into each pot to prevent pooling of water in containers before root expansion, from reduced root uptake, or from large rainfall events. These conditions could lead to an anaerobic environment around the root that could hinder the uptake of water through reduced root conductance (Poorter et al., 2009), an undesired experimental artifact. A collection compartment in the bottom of containers, containing gravel covered by root exclusion mesh, was used collect water for 20, 25, and 35 l containers. Plastic tubing (6 mm diameter) was inset into the gravel layer and extended through the top of the container. A lysimeter pump was then used to suction excess water, through the tubing, as needed. As small containers (5, 10, and 15 l) have a larger irradiation effect a simple bottom plug was used to drain water from the gravel compartment.

Growth and morphology metrics

Seedlings were planted on January 21st 2013 and stem height, diameter at 15 cm and leaf count were measured weekly thereafter. Once the growth rate of individual plants had significantly declined a full biomass harvest was completed (May 5th 2013). Dry mass of leaves, stems, roots and cumulative leaf area (LI-3100C Area Meter; LI-COR, Lincoln, NE, USA) was measured for each seedling. Mean individual leaf area for each harvested seedling was calculated by dividing cumulative leaf area by total leaf count of only fully expanded leaves. This value was then used to interpolate cumulative leaf area through time with weekly leaf counts. Root mass was collected by passing soil from each container through a 1 mm sieve, washing, separating into fine and coarse roots ($<$2 mm and $>$2 mm diameter, respectively) and then drying to a constant mass. Roots from the free seedlings were collected by excavating each 1 m^2 subplot to 0.5 m depth. 25 g fresh weight subsamples of washed fine roots were analyzed, using WhinoRhizo software (Regent Instruments Inc.), for specific root length (SRL, cm m^{-1}).

Photosynthetic parameters

Leaf gas exchange measurements were performed bi-weekly at saturating light (A_{sat}) and saturating light and [CO_2] (A_{max}) on new fully expanded leaves. Measurements were initiated only after new leaf growth occurred (March 17th, 2013), approximately 6 weeks following planting, and continued until the biomass harvest. Leaf level gas exchange was measured with a standard leaf chamber equipped with blue-red light emitting diodes using a portable gas exchange system (LI-6400, LI-COR, Lincoln, NE, USA). A_{sat} measurements were made at PPFD of 1800 μmol m^{-1} s^{-1} and [CO_2] of 400 μl l^{-1} and A_{max} with [CO_2] of 1600 μl l^{-1} and PPFD of 1800 μmol photons m^{-1} s^{-1}. This choice of light level to achieve light saturation is consistent with other studies on *Eucalyptus* species (Kallarackal and Somen, 1997; Pinkard et al., 1998; Crous et al., 2013; Drake et al., 2014). These measurements were conducted during midday (10:00-14:00 h) with leaf temperature maintained at 25°C. After leaves acclimated to the chamber environment, net CO_2 assimilation rate and stomatal conductance were logged 5 times for both A_{sat} and A_{max}. Photosynthetic CO_2 response (AC_i) curves were also constructed on a random subset of each container size (n=3) after new leaves were first produced and immediately prior to the final harvest (May 23rd 2013). For each AC_i curve the reference [CO_2] was set as follows: 400, 50, 100, 150, 230, 330, 400, 650, 900, 1200, 1500, 1600, and 1800 μl l^{-1}. From these curves the photosynthetic parameters, J_{max} and Vc_{max}, were quantified using the leaf photosynthesis model by farquhar1980biochemical and a Ball-Berry type model of stomatal conductance ()within the 'plantecophys' package in R v. 3.0.1 (**?**R Development Core Team, 2011). Leaf dark respiration rates (R_d) was measured on each seedling during the same dates as AC_i curves using detached leaves inside a conifer chamber attached to the Licor 6400 at least 1 hour after sundown. Measurements were taken at a reference [CO_2] of 400 μl l^{-1} while leaf temperature was maintained at current ambient conditions. Specific leaf area (SLA, m^2 g^{-1}) was calculated by measuring leaf area and dry mass for all leaves sampled during gas exchange campaigns.

Leaf water potential

Predawn (Ψ_{pd}) and midday (Ψ_l) water potentials were measured for each seedling using a PMS 1505D pressure chamber (PMS Instruments, Albany, OR, USA) on fully expanded leaves during the same time period as AC_i and (R_d). Ψ_{pd} and Ψ_l leaves were sampled and immediately stored inside foil covered bags and then transported to the laboratory for measurements of water potential. Ψ collections were completed before sunrise and midday 13:00-14:30 h. These measurements were used as a measure of static water stress on the seedlings (Sellin, 1999), and to ensure that the bulk soil water availability was high enough for plants as they became larger and roots filled the soil volume.

Leaf, root and soil chemistry

Leaves used in each gas exchange measurements were dried to a constant mass and milled for analysis of N content. Pre-planting soil samples (n=6) and subsamples of soil from each container following harvest were sieved to remove organic material, air dried and milled for analysis. N concentrations of milled samples were determined using a Carlo Erba CE1110 elemental analyzer with thermal conductivity and mass spectromic detection (of N_2 and CO_2). The percentage of N in the sample was calculated by comparison with known standards. Leaf total non-structural carbohydrates concentration was analyzed using a total starch assay kit (Megazyme International 303 Ireland Ltd., Wicklow, Ireland) on gas exchange leaves (above) and represents the sum of starch (mg g^{-1}) and soluble sugar (mg g^{-1}) concentrations. Starch was quantified using a thermostable α-amylase and amyloglucosidase assay (McCleary et al.) and soluble sugars were determined following the anthrone method (Ebell, 1969). Full methods of the TNC assay are described in (Mitchell et al., 2013).

Seedling growth model

We developed a simple seedling growth model that utilized leaf photosynthesis to allocate daily carbon assimilate towards mass production of stems, leaves and roots. The model starts with mean initial seedling mass ($leaf_i$, $stem_i$ and $root_i$) and a starting leaf area (LA_i) measured prior to planting. Biomass production for each day of the experiment was estimated by multiplying the whole tree seedling carbon uptake gain per day (C_{day}) by a biomass conversion efficiency parameter (ξ_c, Zhu et al. (2008)), allocating new carbon to the previous days biomass and then accounting for carbon loss via tissue respiration. C_{day} was calculated for each seedling by fitting a coupled photosynthesis - stomatal conductance model (Farquhar et al., 1980; Farquhar and Sharkey, 1982; Medlyn et al., 2002) in the 'plantecophys' package in R (**?**) to the mean photosynthetic parameters (R_d, J_{max}, V_{cmax}, and g_1) generated for each soil volume and meteorological data from an onsite weather station. The g_1 parameter was generated by fitting observed stomatal conductance values into the optimal stomatal conductance model from (Medlyn et al., 2012). Combined with the meteorological parameters PPFD, air temperature, and relative humidity, at 15 m intervals, photosynthesis rates (μmol CO_2 m^{-2} s^{-1}) were then predicted for each soil volume treatment. Rates were assumed to be representative of the entire 15 min meteorological interval. C_{day} was then calculated by converting predicted rates to mass carbon gain over 15 min (g m^{-2}) and then summed for 24 h. Additionally, it was necessary to calculate a self-shading parameter (M) when scaling flat leaf photosynthesis to seedling cumulative leaf area. This was accomplished by utilizing 61 previously digitized Eucalyptus seedlings (*E. tereticornis, E. haemastoma, E. pilularis, E. saligna*, (Duursma et al., 2012)) from run in 'YplantQMC' package in R (Cieslak, Mik. Uses code by Robert Pearcy and Medlyn.) to build a 3d plant structure based on digitized metrics of plant allometry and crown structure. Inputting the same physiological parameters listed above, 'YplantQMC' outputs total photosynthesis, using total leaf area, for seedlings assuming self-shading as well as for a full sun large horizontal leaf. The ratio of self-shading to flat leaf total photosynthesis was then used to calculate M, which was then applied to modeled rates of photosynthesis when scaling to seedling cumulative leaf area. All parameters used in model simulations are reported in Table 3.

We then utilized this model to to investigate whether the effects of soil volume on photosynthesis were enough to accurately predict overall seedling production after 120 days or whether changes in carbon allocation or mass partitioning where also necessary to predict biomass. First, the model was simulated using only changes in photosynthesis rates across treatments combined with mean values of mass partitioning and either published or local data of stem and root respiration rates. Next, different mass partitioning and carbon allocation scenarios were simulated to investigate sources of missing carbon from the initial model simulation. This included testing model sensitivity by altering leaf carbon allocation, root respiration rates, and fine root carbon allocation. For all cases, mass production was compared between model output and actual harvested seedling mass to investigate what carbon allocation strategies beyond reduction in photosynthesis may better predict the seedling growth responses induced by limiting soil volume.

Statistics

Differences in experimental parameters with soil volume were analysed by one-way analysis of variance (ANOVA) in R with individual containers as random effects and soil volume as a categorical fixed effect. Tukeys post-hoc tests were performed in conjunction with ANOVA to determine which specific paired comparisons among soil volumes were different. Mixed model ANOVAs of A_{max} and plant chemistry were performed using the nlme package (Pinheiro et al., 2014) in R and r^2 values of mixed models were computed as in 'citenakagawa2013general. Tests of allometric relationships between biomass components were implemented using major axis regression in the smatr package (Warton et al., 2012).

Results

Growth and morphology metrics

In this open field study, colder temperatures and reductions in cumulative PPFD per day (Fig. 1) most likely lead to the reduced growth in the free seedlings in the final weeks of the experiment (Fig. 2). Combined with severe growth reductions in the smallest containers the experiment was chosen to be harvested after 120 days. Across the experiment duration height, diameter, and leaf area diverged between container volumes (Fig. 2). First, seedling leaf area significantly diverged between soil volumes ($p<0.026$) during the 5^{th} week of the experiment. Following this period both height (8^{th} week) and then diameter (9^{th} week) significantly deviated across soil volumes ($p<0.002$ & 0.001, respectively). Negative growth effects then manifested as severely reduced height gain and declining leaf area through time with small container volumes across the final two months of the experiment. Seedlings maintained diameter growth throughout the experiment, although marginal with smaller containers in the final month. Final seedling height significantly increased with increasing soil volume ($p<0.001$). Increases in both final stem diameter ($p<0.001$) and cumulative leaf area (both $p<0.001$) were found with increasing soil volume and these differences were driven mainly by the largest container and the free seedling.

Total plant biomass at harvest was significantly different between all container volumes ($p<0.001$) and with free seedlings ($p<0.001$, Table. 1). We analyzed the relationship between biomass growth with each fold increase in soil volume and found an increase of 34 % with a doubling of pot size, in line with the meta-analysis of Poorter et al. (2012). Additionally, plant biomass was highly correlated with total leaf area across all treatments (r^2 = .97, $p<0.001$). Differences in biomass partitioning to leaves, stems, and roots were not different across soil volumes when variation in seedling biomass within treatments was factored in the analysis (p=0.937, 0.823 & 0.843, respectively). Across all treatments, the final harvested root:shoot appears to be conserved with these seedlings, with a slightly higher shoot than root mass on average (p=0.2271, $\overline{x}$=0.904).

SRL of harvested fine roots was not different across soil volumes (p=0.662, Table. 1). Over the duration of the experiment SLA was higher in free seedlings but was not different across containers sizes (Table. 1, $p<0.001$) and this pattern was evident in the first gas exchange measurement campaign (6th week, $p<0.001$).

Leaf and root chemistry

Foliar leaf N was significantly higher in free seedlings and the largest container at the onset of gas exchange measurements (6th week, $p<0.001$). Over the remaining duration of the experiment the smallest container had a significant reduction in leaf N compared to all other containers, while the free seedling maintained greater leaf N (Table. 1, $p<0.001$). Additionally, mean starch content in the smallest container was double that of free seedlings (p=0.042), while leaf soluble sugars did not differ across treatments (p=0.1368) throughout the experiment (Table. 1). Differences in

starch between the free seedling and the smallest container were also evident during the first gas exchange campaign (p=0.0013).

Gas exchange and photosynthetic parameters

A_{sat} (Fig. 3) and A_{max} (Table. 2) were both significantly higher in the largest container and the free seedling at the first measurement campaign on new fully expanded leaves (both $p<0.001$). Across measurement campaigns A_{sat} was consistently higher rates in free seedlings than in containers (Figure 3, $p<0.001$). The interaction between photosynthetic capacity on a mass basis, leaf starch, and foliar N was marginally significant (p=0.0584) but A_{max} was highly correlated to both leaf N content leaf starch (both $p<0.001$). Across all measurement campaign A_{max} was higher when foliar N was also higher, usually associated with low levels of leaf starch (Fig. 3a). A_{max} was also lower when leaf starch was high as higher leaf N often did not coincide with high starch (Fig. 3b)

The photosynthetic parameters J_{max} and Vc_{max} were not different across the two measurement campaigns (p=0.1251 & 0.0754, respectively), therefore the parameter means per volume are reported here (Table. 2). Overall, both J_{max} and V_{cmax} were significantly higher in free seedlings with little variation between container sizes (p=0.0012 & 0.0021, respectively). Leaf dark respiration rates were not significantly different across soil volumes (Table. 2, p=0.5581). From the Medlyn et al. (2012) optimal stomatal conductance model the g_1 parameter, generated for each seedling, was not different across soil volumes (Table. 2, p=0.3043). Predicted values of stomatal conductance, using g_1 parameter, where highly correlated with observed values (r^2= .74, $p<0.001$, data not shown).

Neither predawn nor midday water potentials were different across treatments (p=0.8161 & 0.442, respectively) across the treatments, with mean water potentials of -0.27 and -1.2 mPa across seedlings, respectively. Although stomatal conductance in free seedlings was generally higher than those in containers (Table. 2, p=0.0023), the mean rates for all seedlings were high at 0.37 mol H_20 m_{-2} s_{-1} and did not decline significantly across the experiment duration. Combined these indices provide strong evidence that water stress was probably not apparent on these well-watered seedlings throughout the experiment. Soil N (%) at harvest was not different across soil volumes ($\bar{x}$=04.5 %, p=0.5718) and decreased approximately 3 % across all containers over the experiment duration. This indicates that nutrient leaching from free seedlings or from draining of containers following natural rainfall events did not differ between treatments.

Modelling seedling biomass

The initial model simulation, testing only treatment specific photosynthesis rates, was unable to accurately predict the responses of actual harvested seedling biomass to reduced soil volume. When both C_{day} and biomass of modeled and harvested seedlings were scaled to to the 'free' seedling the initial model design overestimated biomass for all treatments (Fig. 6a). This suggests that reductions in photosynthesis alone could not realistically capture the reduced growth response of these seedlings. Next, the model was re-run using the mean mass fractions of leaves, stems and roots from the final harvest, per treatment, to drive daily mass partitioning and seedling production. Combined with treatment specific photosynthesis rates this model still overestimated seedling production for all treatments (Fig. 6b).

To test whether potential sources of missing carbon could improve the modeled response of seedling mass production to the model was then run with 3 different carbon allocation scenarios that could not be tested within the framework of this experiment. This was accomplished by testing the sensitivity of the model to these scenarios by adjusting allocation to leaves or rates and root respiration rates by $\pm$50 %. The model output of these scenarios was then compared to both the original model output and with harvested seedling biomass. Increasing C allocation to either leaves or fine roots,in coalescence with photosynthesis rates, did improve the model but

neither were sufficient to accurately predict the actual biomass response (Fig. S1a,c). Altering respiration rates of fine and coarse roots did not have a noticeable deviation from the original model (Fig. S1b).

Discussion

Tables

Table 1: Responses of plant and leaf characterisitcs of Eucalyptus tereticornis to soil volume treatments. Each value reflects the mean(standard error) of each treatment.

Volume (L)	Seedling mass (g)	SLA (m^2 g^{-1})	Leaf Nitrogen (mg g^{-1})	Leaf Sugars (%)	Leaf Starch (%)	SRL (cm m^{-1})	Root Nitrogen (%)
5	14.8 (1.8)	0.009 (0.0002)	1.9 (0.1)	6.4 (0.3)	12.7 (1.0)	39.1 (5.5)	0.82 (0.05)
10	20.0 (2.4)	0.010 (0.0002)	2.4 (0.1)	6.7 (0.2)	9.4 (0.7)	34.2 (5.8)	0.75 (0.02)
15	25.4 (2.5)	0.011 (0.0005)	2.7 (0.2)	7.2 (0.3)	7.2 (0.7)	37.6 (4.6)	0.71 (0.02)
20	23.4 (1.6)	0.010 (0.0003)	2.8 (0.2)	6.6 (0.3)	9.5 (0.9)	45.3 (5.5)	0.76 (0.04)
25	30.4 (5.5)	0.010 (0.0004)	2.8 (0.2)	6.9 (0.2)	9.8 (0.7)	47.0 (7.1)	0.74 (0.02)
35	52.2 (9.6)	0.011 (0.0004)	3.5 (0.2)	6.8 (0.2)	9.8 (0.6)	50.6 (11.6)	0.77 (0.03)
Free	174.5 (18)	0.013 (0.0004)	6.4 (0.3)	7.4 (0.2)	6.8 (0.6)	43.7 (6.2)	0.87 (0.04)

Table 2: Responses of leaf level gas exchange parameters of *Eucalyptus tereticornis* to soil volume treatments. Each value reflects the mean(standard error) of each treatment. Units for A_{max} and R_{dark} are μmol m^{-1} s^{-1} and g_s are mol m^{-1} s^{-1}.

Volume (L)	A_{max}	R_{dark}	J_{max}	Vc_{max}	g_s	g_1
5	21.2 (0.9)	2.8 (0.3)	103.7 (5.8)	62.9 (2.8)	0.30 (0.01)	5.1 (0.1)
10	22.3 (1.4)	2.7 (0.4)	116.5 (5.9)	69.4 (2.9)	0.36 (0.01)	5.4 (0.1)
15	23.3 (1.2)	1.4 (0.1)	123.1 (15.4)	79.4 (10.0)	0.45 (0.01)	6.2 (0.2)
20	26.1 (0.7)	1.7 (0.1)	130.0 (14.1)	81.4 (6.0)	0.38 (0.01)	5.2 (0.2)
25	23.9 (0.9)	1.2 (0.1)	131.9 (5.9)	78.6 (2.6)	0.32 (0.01)	4.8 (0.2)
35	25.0 (1.0)	1.5 (0.2)	121.2 (3.7)	78.0 (3.5)	0.33 (0.02)	4.7 (0.2)
Free	33.1 (0.7)	1.3 (0.1)	171.7 (18.6)	101.2 (5.7)	0.49 (0.02)	5.0 (0.2)

Figures

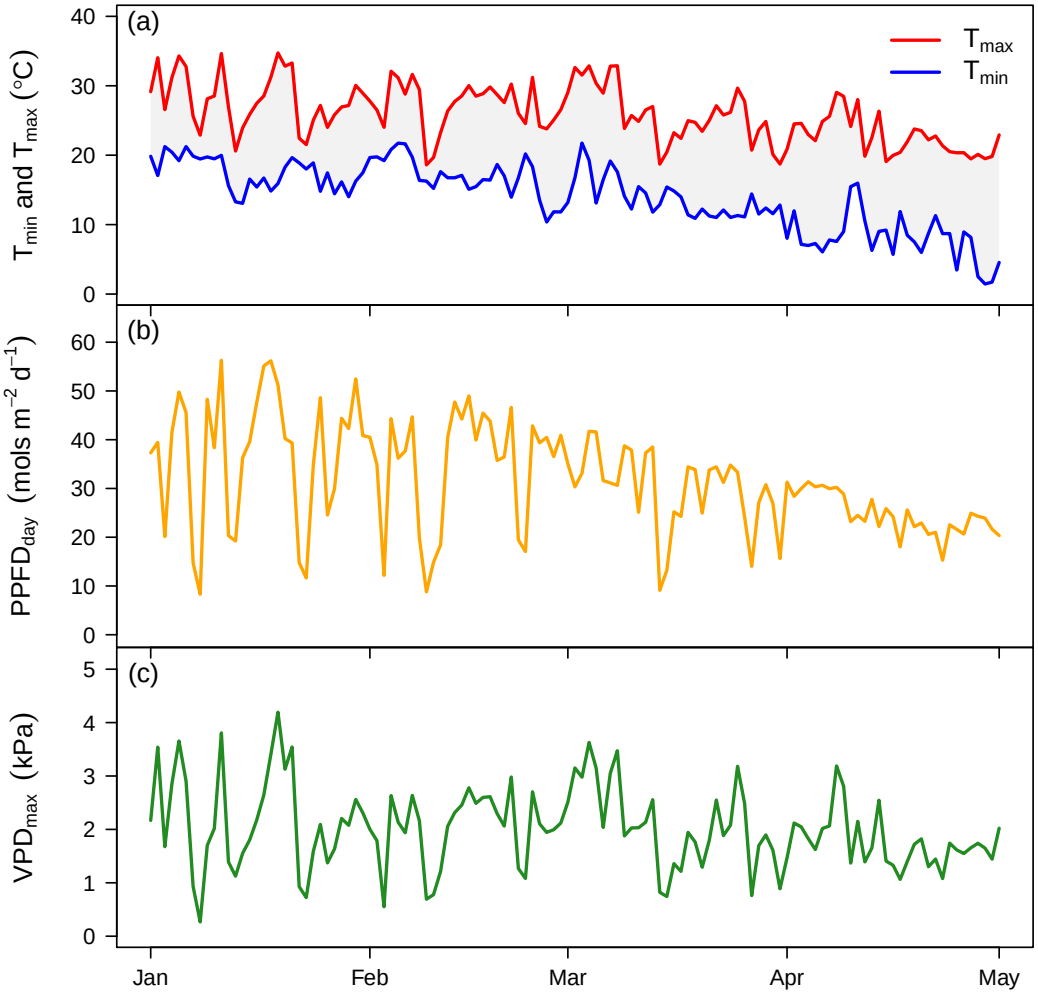

Figure 1: Daily maximum and minimum temperature (a), cumulative daily PPFD (b), and daily maximum vapour pressure deficit (c) across the experiment duration.

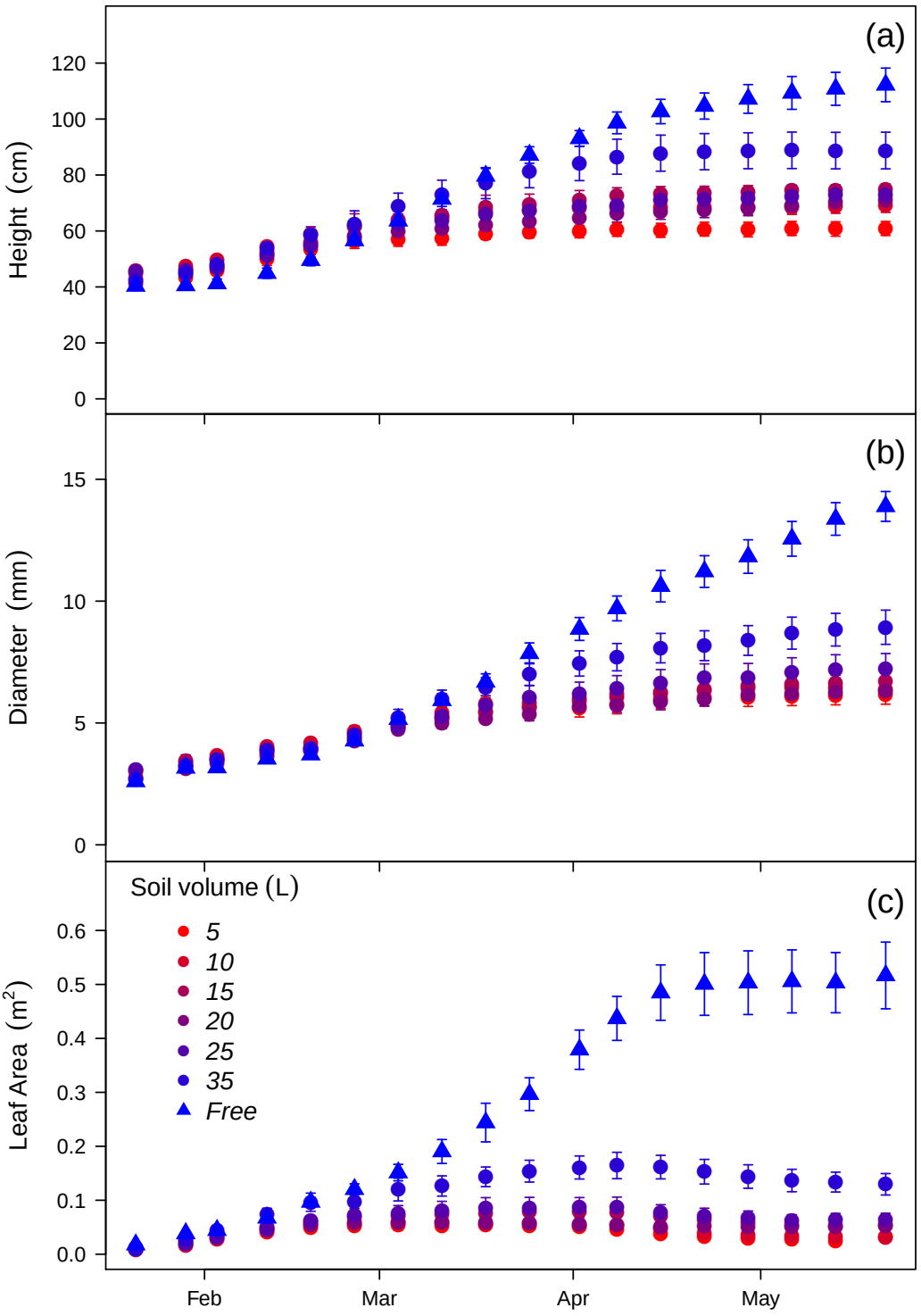

Figure 2: Soil volume treatment means ± SE (n=7) of height growth (a), diameter growth (b), and interpolated seedling leaf area (c) measured weekly of *Eucalyptus tereticornis* seedlings over 120 days.

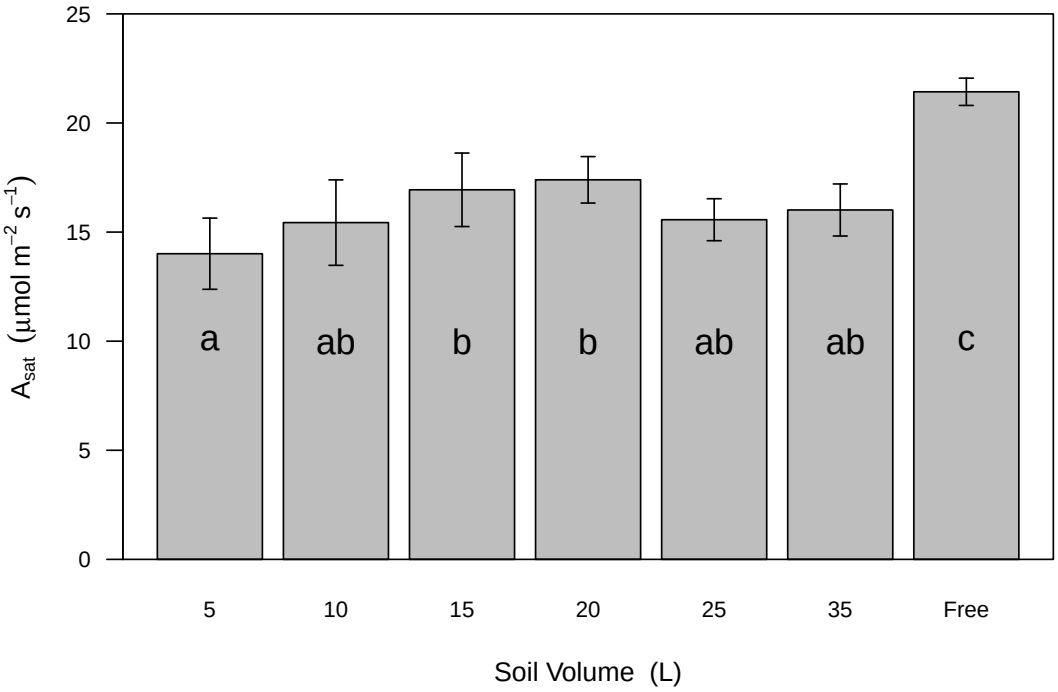

Figure 3: Soil volume treatment means ± SE (n=7), across all measurement dates (n=6), of light saturated rates of photosynthesis at 25°C

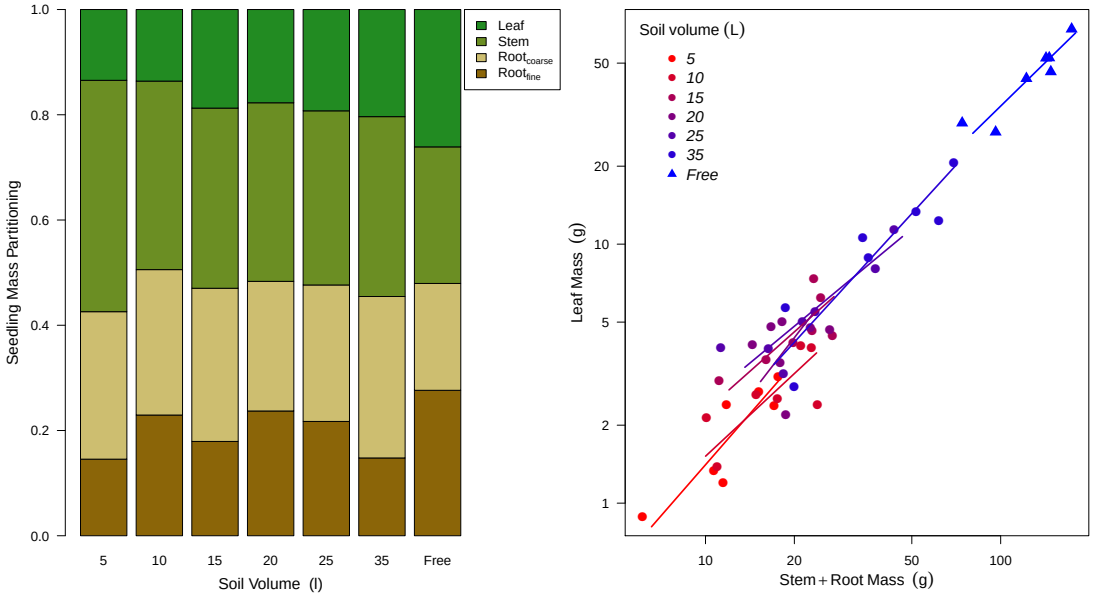

Figure 4: Soil volume treatment means (n=7) of mass partitioning to leaves, stems, and roots (a) and bi-variate relationships between mass allocation to leaves and stems + roots (b). Lines represent standardized major axis fitting of the log transformed allometric relationships of leaf mass fraction by treatment.

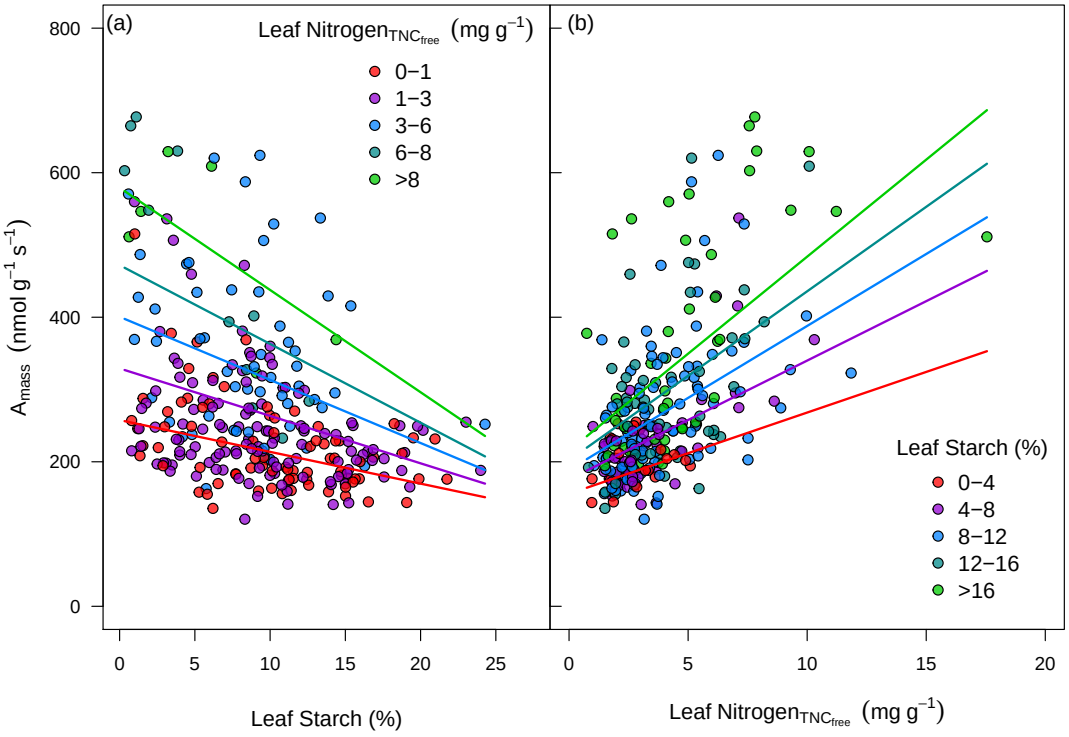

Figure 5: Photosynthetic capacity, on a leaf mass basis, as a function of accumulation of leaf starch (a) and leaf nitrogen (b) content. Colors represent bins levels (n=5) of leaf starch and nitrogen grouped from low to high . Lines represents predictions, for each bin level, from the linear mixed effects model equation of A_{max} as a function of starch and nitrogen. The marginal (r^2 (fixed effects only) was 0.37 and the conditional (r^2 (fixed and random effects) was 0.48 for the complete model.

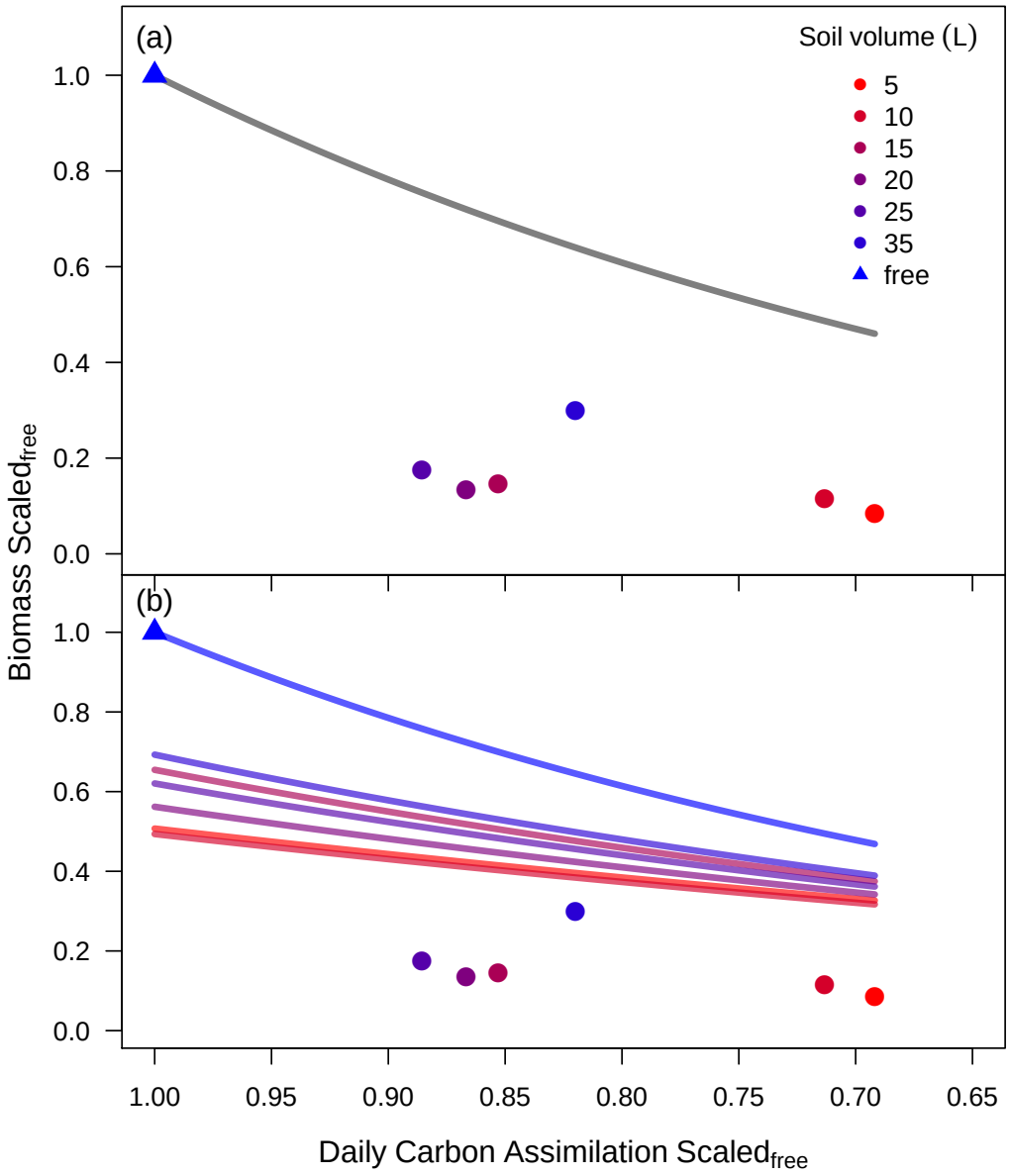

Figure 6: Modeled and harvested seedling biomass (g) versus daily assimilated carbon gain (g m^2). Values of biomass and carbon gain are scaled the free seedling with unlimited soil volume.

1 Supporting Information Figures

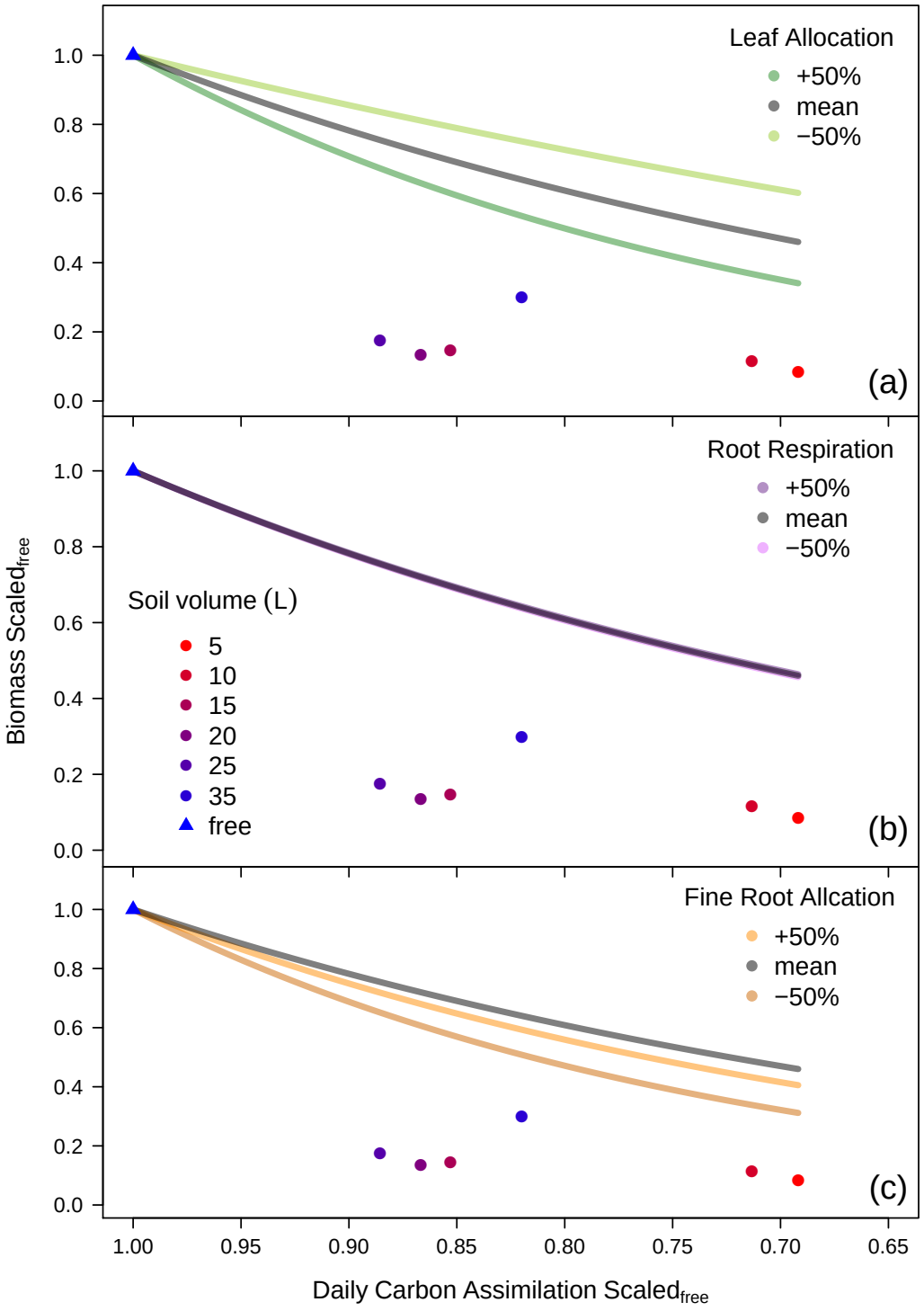

Figure S1: Modeled and harvested seedling biomass (g) versus daily assimilated carbon gain (g m^2) including sensitivity testing for unmeasured carbon allocation scenarios. Model parameters of leaf allocation (a), root respiration (b), and fine root allocation (c) were increased or decreased by 50%.

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