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

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# Abstract

Interpreting limitations to plant growth requires understanding of the balance between carbon (C) source and sink activity in order to assess C allocation and biomass partitioning. 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 naturally sown 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 (A). 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. We developed a seedling growth model that utilized leaf A rates to allocate daily C uptake towards mass growth of stems, leaves and roots. We then asked whether the observed reductions in A explained the observed differences in seedling biomass. We found that although belowground sink limitation resulted in the down regulation of A, these reductions were not significant enough to explain observed growth responses. Thus, as photosynthesis and growth were not coordinated an excess pool of non-biomass C resulted in seedlings with soil volume restriction. This research highlights the need to further utilize mass balance approaches when evaluating plant C allocation and confirms that A and growth are not always directly related.

# Keywords

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

# Introduction

# Materials and Methods

## Experimental design

This experiment was located at 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 phosphorus respectively) with low organic matter (0.7%) and low water holding capacity. At this site a soil hard layer exists at ~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, 1980b). 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 35 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. A Imidacloprid (BAYER CropScience) insecticide tablet was planted 5 cm below the roots of each seedling. Containers were planted flush with the soil surface inside metal sleeves, designed to minimize excess air space between the container and outside soil while also allowing for container removal. This allowed for soil temperatures in containers to reflect conditions of naturally sown (free) seedlings. Each experimental block (n=7) contained a complete replicate set of container volumes as well as one free seedling, with 1 m2 spacing. For each free seedling, used as the control, a 1 m2 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 around each subplot to exclude local vegetation.

Plants were watered weekly or when needed, accounting for natural precipitation, to maintain soil moisture at field capacity (13-15%). 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 to collect excess 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 excess 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 21st 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 m2 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 (Asat) and saturating light and [CO2] (Amax) on new fully expanded leaves. Measurements were initiated only after sufficient 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). Asat measurements were made at PPFD of 1800 mol m-1 s-1 and [CO2] of 400 l l-1 and Amax with [CO2] of 1600 l l-1 and PPFD of 1800 mol~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 CO2 assimilation rate and stomatal conductance (gs) were logged 5 times for both Asat and Amax.

Photosynthetic CO2 response (ACi) curves were also developed at 25°C 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). Each ACi curve began at the reference [CO2] of 400 l l-1 and then consisted of 12 additional steps from [CO2] of 50 to 1800 l-1 at 25°C at saturating light (above). From these curves the photosynthetic parameters, Jmax and Vcmax, were quantified using the biochemical model of (Farquhar et al. 1980).

Leaf dark respiration rates (Rd) was measured on each seedling during the same dates as ACi 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 [CO2] of 400 l l-1 while leaf temperature was maintained at current ambient conditions. Reported values of Rd are standardized rates at 25°C using a Q10 value (1.86) developed for these seedlings in a separate experiment (Drake et al. unpublished). Leaf area and dry mass were recorded for each leaf during gas exchange campaigns.

## Leaf water potential

Predawn (pd) and midday (l) leaf 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 ACi and Rd. Leaves were detached and immediately stored inside foil covered bags before water potential measurements were performed. pd was measured before sunrise and l at midday 13:00-14:30 h. These measurements were used as a measure of static water stress on the seedlings , 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 and subsamples of harvested roots were dried to a constant mass and milled for analysis of N content, 13C, and total non-structural carbohydrates (TNC). 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. Nitrogen concentrations of leaf and soil samples were determined using a Carlo Erba CE1110 elemental analyzer with thermal conductivity and mass spectromic detection (of N2 and CO2). The percentage of N in the sample was calculated by comparison with known standards. Leaf 13C was analyzed with an Delta V Advantage coupled to a Flash HT and Conflo IV isotope ratio mass spectrometer. Leaf samples were flash combusted at 1000°C to convert to to CO2, feed to the mass spectrometer and isotopic signatures are reported relative to the VPDP scale. Leaf TNC concentration was analyzed using a total starch assay kit (Megazyme International 303 Ireland Ltd., Wicklow, Ireland) and includes the starch (mg g-1) and soluble sugar (mg g-1) concentrations. Starch was quantified using a thermostable -amylase and amyloglucosidase assay (McCleary et al. 1997) and soluble sugars were determined following the anthrone method (Ebell 1969). Complete methods of the TNC assay are described in (Mitchell et al. 2013). Specific leaf area (SLA, m2 kg-1), for leaves sampled during gas exchange campaigns, was then calculated by first subtracting the TNC content from individual dry leaf mass before dividing leaf area by leaf mass.

## Seedling growth model

We developed a simple seedling growth model that utilized leaf A rates to allocate daily C assimilate towards biomass production of stems, leaves, fine roots and coarse roots. The model begins with mean initial tissue component biomass (leafi, stemi and rooti) and a starting leaf area (LAi measured prior to planting. The initial biomass of roots was divided evenly between fine and coarse roots. The daily net biomass production of seedlings (Pi) is then given by

(1)

where L is total plant leaf area, Cday,i is the predicted daily carbon assimilation, s is a self shading parameter, $\textepsilon$c is a biomass conversion efficiency parameter and R is the total respiration of all tissue components. Total respiration was calculated as

(2)

where Rc is tissue respiration of fine roots, coarse roots or stems on a mass basis and Mc is the standing biomass of each component. Rleaf is represented in the calculation of Cday (described below). The change in individual component biomass (Mc), here solved on a daily time step, is given by

(3)

where Ac is the component specific biomass partitioning to whole plant biomass (%) and c is component specific turnover rate. Due to the duration of the experiment $stem was assumed to equal 0. Total seedling biomass, per time step, was then equal to the sum of all biomass components; leaves, stems, fine roots and coarse roots.

Cday was predicted by fitting a coupled photosynthesis - stomatal conductance model (Farquhar et al. 1980, Medlyn et al. 2011) in the 'plantecophys' package in R (Duursma 2014) to the mean photosynthetic parameters (Rd, Jmax, Vcmax, and g1) for each treatment and meteorological data from an onsite weather station. Examples of the photosynthesis model are described in Medlyn et al. (2002) and the approach of the coupled leaf gas exchange model are described in Duursma et al (2014). The g1 parameter was generated by fitting observed gs 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, leaf A rates (molCO2~ 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. Cday was calculated by converting predicted rates to mass C gain over 15~min (g~m-2) and then summed for 24 h. This resulted in 120 unique values of Cday for each soil volume treatment, one value for each day of the experiment. Thus, each daily time step for model runs included a value of Cday that represented both treatment specific photosynthetic parameters and meteorological constraints across the duration of the experiment.

It was further necessary to calculate a self-shading parameter (s) when scaling leaf A with cumulative seedling leaf area. This was accomplished by utilizing 61 previously digitized Eucalyptus seedlings, covering 5 total species which include *E. tereticornis*, from Duursma et al. (2012) to run in 'YplantQMC' package in R (fix cite YplantQMC) to build a 3d plant structure based on digitized metrics of plant allometry and crown structure. Inputting the same treatment specific physiological parameters listed above, 'YplantQMC' outputs total A, using total leaf area, for seedlings assuming self-shading as well as for a full sun large horizontal leaf. The ratio of total A with self-shading to horizontal leaf was then used to calculate s for each of the 61 digitized seedling, independently for each treatment. Next, the linear relationship between s and total leaf area was for determined across digitized seedlings, within each treatment. For the growth model, s was then predicted for each daily time step using the previous days cumulative leaf area and this value was then applied to Cday,i. All default parameters used in model simulations are reported in Table.~.

## Data analysis

Differences in experimental parameters with soil volume were analysed by one-way analysis of variance (ANOVA) in R (R Development Core Team 2011) with individual containers as random effects and soil volume as a categorical fixed effect. Tukey’s post-hoc tests were performed in conjunction with ANOVA to determine which specific paired comparisons among soil volume treatments were different. Mixed model ANOVAs of Amax and leaf chemistry were performed using the 'nlme' package (Pinheiro et al. 2014) in R and R2 values of mixed models were computed as in (Nakagawa and Schielzeth 2013). Tests of allometric relationships between biomass components were implemented using major axis regression in the 'smatr' package in R (Warton et al. 2012). Results were considered significant at P≤0.05..

# Results

## Growth and morphology metrics

## Leaf chemistry

## Gas exchange and photosynthetic parameters

## Modelling seedling biomass

# Discussion

# Tables

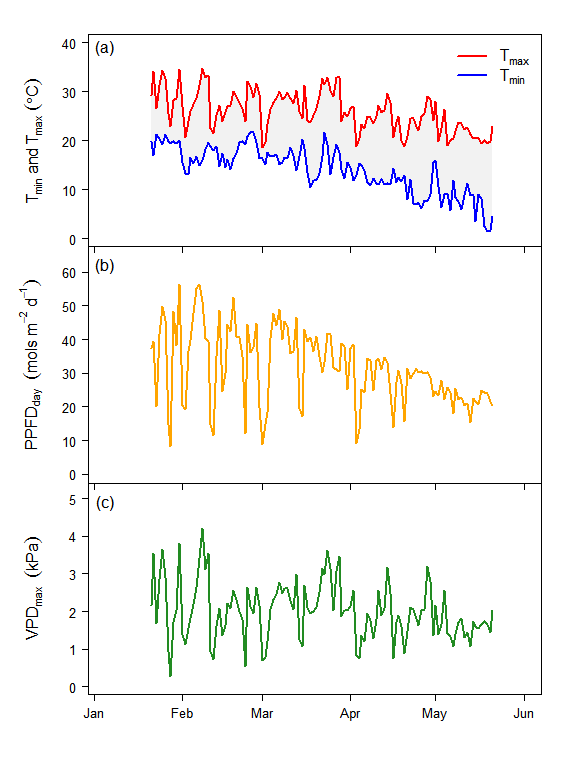
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| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Volume (L)** | **Seedlingmass(g)** | **SLA(mkg)** \*\*L | eafStarch(%)\*\* \*\*Le | afSugars(%)\*\* \*\*Lea | fNitrogen(%)\*\* \*\*Root | Nitrogen(%)\*\* \*\*SRL~( | cm~m)\*\* \*\*{Leaf~ | }C~()\*\* |
| 5 | 14.8 (1.82) a | 11.8 (0.32) a | 12.7 (0.97) b | 6.4 (0.28) a | 1.1 (0.02) a | 0.78 (0.04) ab | 39.1 (5.47) a | -30.1 (0.26) a |
| 10 | 20.0 (2.38) ab | 11.7 (0.31) a | 9.4 (0.75) ab | 6.7 (0.25) a | 1.3 (0.04) ab | 0.75 (0.02) a | 34.2 (5.83) a | -30.2 (0.25) a |
| 15 | 25.4 (2.49) ab | 12.7 (0.48) a | 7.3 (0.73) a | 7.2 (0.28) a | 1.4 (0.06) ab | 0.71 (0.02) a | 37.6 (4.63) a | -30.3 (0.36) a |
| 20 | 23.4 (1.63) ab | 11.8 (0.37) a | 9.5 (0.88) ab | 6.6 (0.26) a | 1.4 (0.05) ab | 0.76 (0.04) a | 45.3 (5.50) a | -29.7 (0.28) a |
| 25 | 30.4 (5.49) ab | 12.4 (0.40) a | 9.8 (0.71) ab | 6.9 (0.24) a | 1.3 (0.06) ab | 0.74 (0.02) a | 47.0 (7.10) a | -29.7 (0.25) a |
| 35 | 52.2 (9.55) b | 13.5 (0.46) ab | 9.8 (0.65) ab | 6.8 (0.22) a | 1.5 (0.08) b | 0.77 (0.03) ab | 50.6 (11.61) a | -30.6 (0.38) a |
| Free | 174.5 (18.02) c | 15.1 (0.47) b | 6.8 (0.65) a | 7.4 (0.25) a | 2.4 (0.09) c | 0.9 (0.03) b | 43.7 (6.24) a | -30.0 (0.34) a |
| Container Effect (P) | 0.001 | 0.001 | 0.039 | 0.128 | 0.001 | 0.015 | 0.662 | 0.458 |

**Table 1.** Responses of plant and leaf characterisitics of *Eucalyptus tereticornis* seedlings to soil volume treatments. Each value reflects the mean(standard error) for each treatment. Seedling mass, SRL, root nitrogen and leaf ^13C values are from final harvest. Values of leaf starch, sugars, nitrogen and SLA represent overall means across measurement campaigns (n=6). Different letters represent significant differences between treatments. The container effect P value represents the overall difference between seedlings with soil volume restriction and the control seedlings.

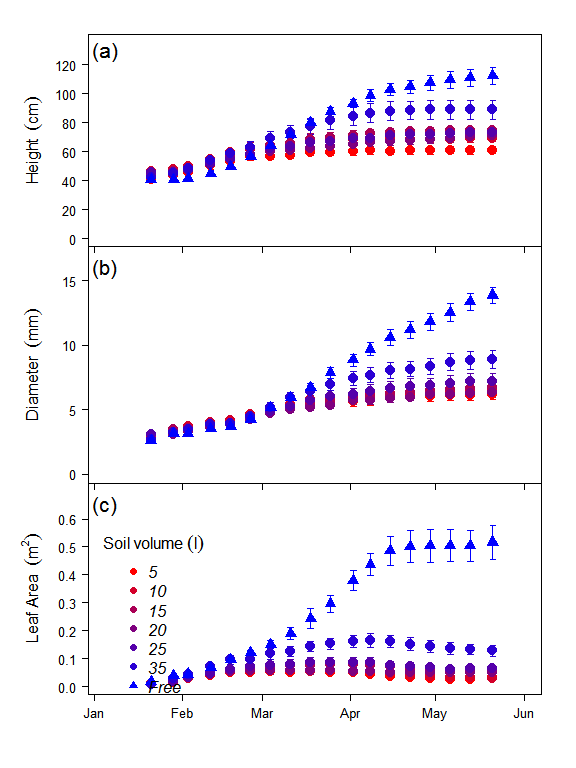
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| --- | --- | --- | --- | --- | --- | --- |
| **Volume~(L)** | \*\* | \*\* \*\* | extit{J}\*\* \*\* | tit{Vc}\*\* \*\* | t{g}\*\* \*\*\textit{ | g}\*\* |
| 5 | 21.2 (0.9) a | 0.61 (0.04) a | 104.5 (3.3) a | 63.3 (2.5) a | 0.30 (0.01) a | 5.1 (0.1) bc |
| 10 | 22.3 (1.4) ab | 0.79 (0.06) a | 116.5 (7.5) a | 69.4 (4.7) a | 0.36 (0.01) ab | 5.4 (0.1) cd |
| 15 | 23.3 (1.2) ab | 0.70 (0.05) a | 125.4 (7.8) a | 80.8 (5.1) ab | 0.42 (0.01) ab | 5.8 (0.1) d |
| 20 | 26.1 (0.7) b | 0.73 (0.11) a | 131.5 (8.6) a | 82.1 (4.7) ab | 0.37 (0.01) ab | 4.9 (0.1) ac |
| 25 | 23.9 (0.9) ab | 0.53 (0.13) a | 132.8 (13.1) a | 79.0 (8.7) a | 0.30 (0.01) a | 4.5 (0.1) a |
| 35 | 25.0 (1.0) ab | 0.61 (0.04) a | 127.2 (6.1) a | 82.4 (3.6) a | 0.31 (0.01) a | 4.4 (0.2) a |
| Free | 33.1 (0.7) c | 0.64 (0.07) a | 169.0 (8.2) b | 100.4 (3.3) b | 0.44 (0.01) b | 4.5 (0.1) ab |
| Container Effect (P) | 0.001 | 0.039 | 0.001 | 0.002 | 0.001 | 0.079 |

**Table 2.** Responses of leaf level gas exchange parameters of *Eucalyptus tereticornis* seedlings to soil volume treatments. Each value reflects the mean(standard error) for each treatment. Units for Amax and Rdark are mol m-2 s-1 and gs are mol m-2 s-1, each at at 25°C. Values of Amax, gs and g1 represent overall means across measurement campaigns (n=6). Rdark, Jmax and Vcmax values are means of two measurement campaigns at beginning and end of gas exchange measurements. Different letters represent significant differences between treatments. The container effect P value represents the overall difference between seedlings with soil volume restriction and the control seedlings.

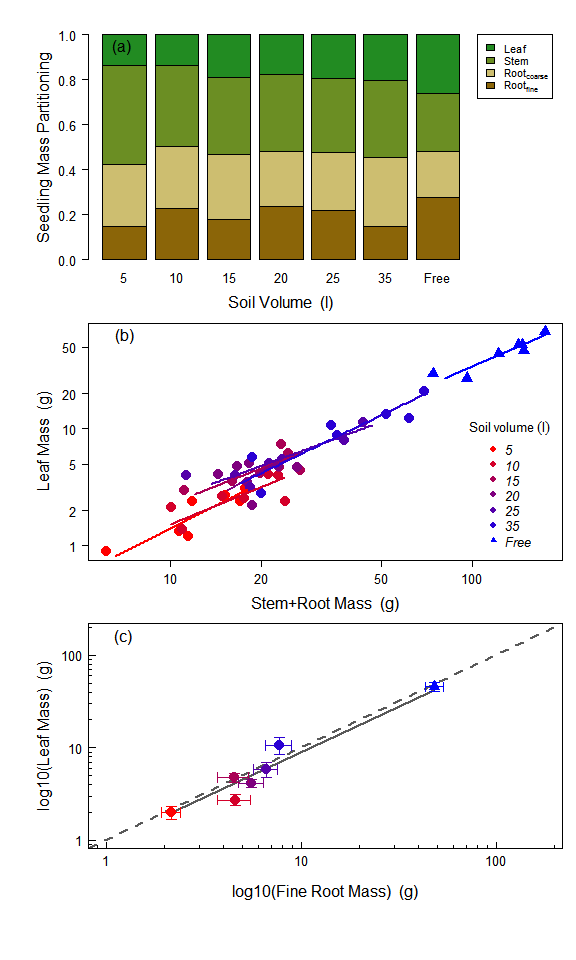
# Figures



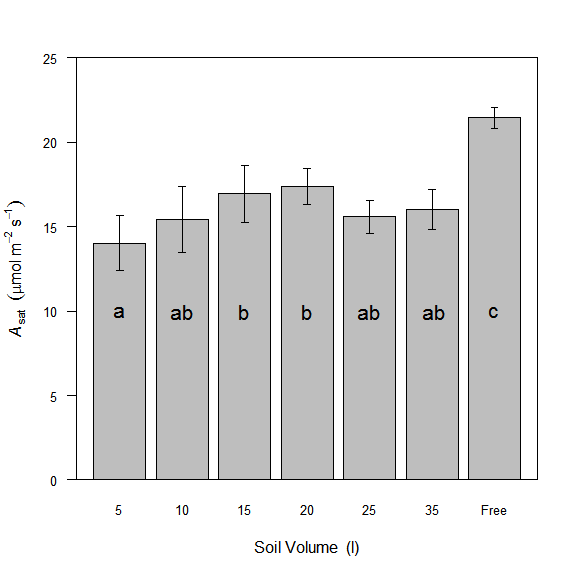
**Figure 1**. Daily maximum and minimum temperature (a), cumulative daily PPFD (b), and daily maximum vapour pressure deficit (c) across the experiment duration in 2013.



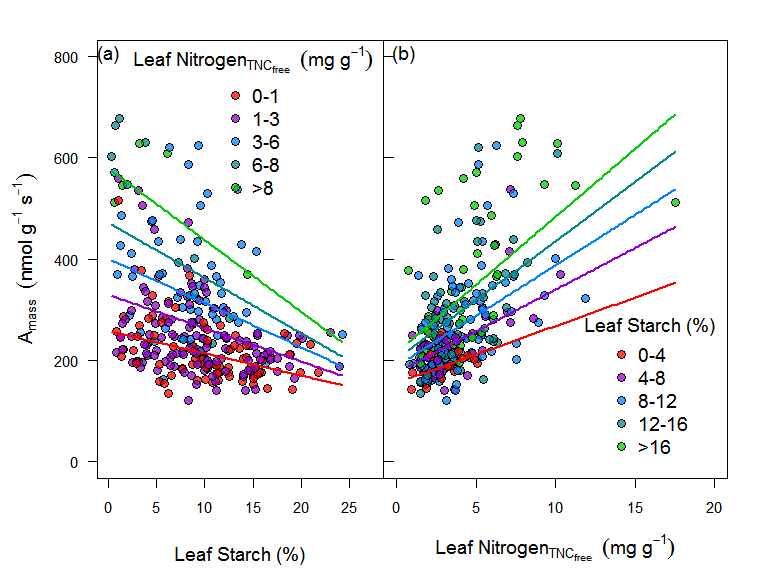
**Figure 2**. Soil volume treatment means ± standard error of height growth (a), diameter growth (b), and interpolated seedling leaf area (c) measured weekly of *Eucalyptus tereticornis* seedlings across the experiment duration in 2013.



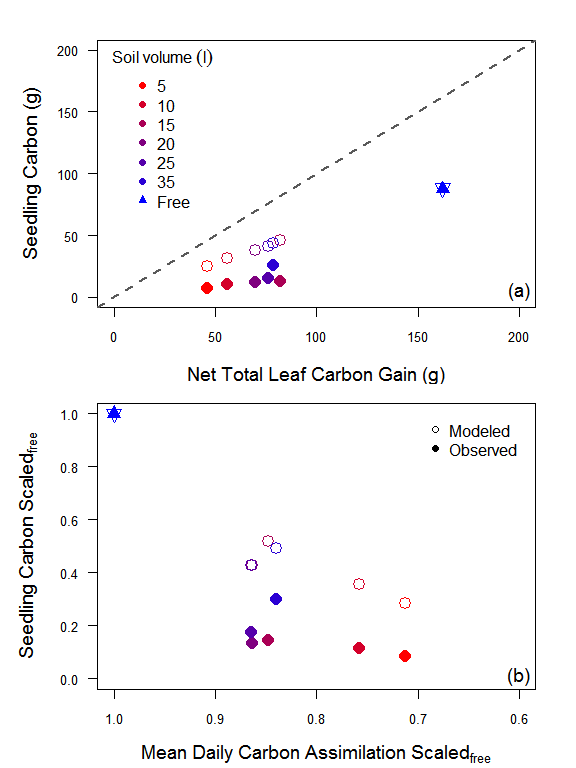
**Figure 3**. Soil volume treatment means of mass partitioning to leaves, stems, and roots at harvest (a), bi-variate relationships between mass allocation to leaves and stems + roots (b) and leaf mass as a function of fine root biomass with ± standard error (c). For (b) lines represent standardized major axis fitting of the log transformed allometric relationships of leaf mass fraction by treatment. For (c) the dashed line is the 1:1 relationship and the solid line represents the significant linear model fit (R2=0.81).



**Figure 4**. Soil volume treatment means ± standard error, across all measurement campaigns (n=6), of light saturated rates of photosynthesis at 25°C. Different letters represent significant differences between treatments.

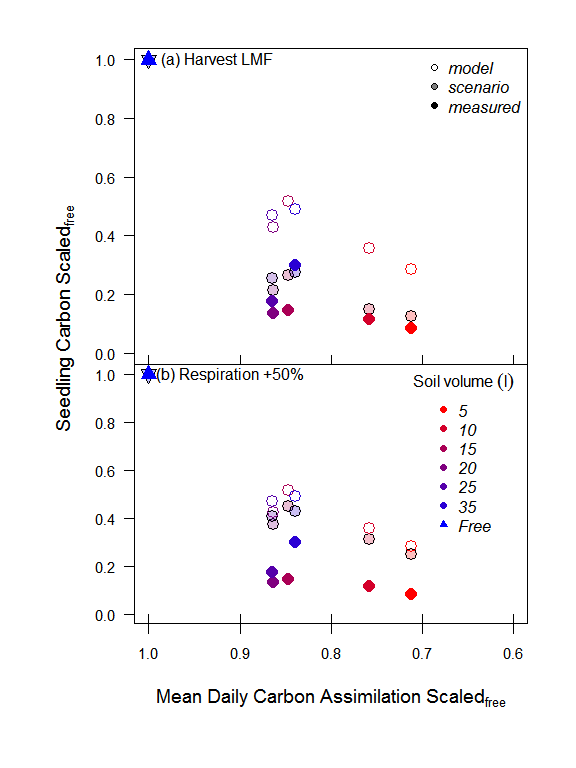


**Figure 5**. Photosynthetic capacity, on a leaf mass basis, as a function of accumulation of leaf starch (a) and leaf nitrogen content without TNC (b). Colors represent bins levels (n=5) of both leaf starch and nitrogen grouped from low to high . Lines represents predictions, for each bin level, from the linear mixed effects model equation of Amax as a function of starch and nitrogen. The marginal R2 (fixed effects only) was 0.37 and the conditional R2 (fixed and random effects) was 0.48 for the complete model.



**Figure 6**. Total carbon mass for harvested and modeled seedlings versus predicted total carbon gain after 120 days (a) and reductions in final seedling carbon mass, both modeled and observed, as a function of the reduction in leaf photosynthesis across treatments (b). For (a) the dashed 1:1 identifies the difference between net total leaf carbon gain and gross seedling production. For (b) both seedling carbon mass and daily carbon assimilation were first scaled to the free seedling control.

# Supporting Information



**Figure S1**. Sensitivity testing of seedling growth model to different carbon allocation strategies including; constraints of leaf mass fraction to treatment specific final harvest values (a) and increases in respiration of non-leaf tissue components by 50% (b). Open and filled symbols represent default model and harvest values, while shaded symbols represent model sensitivity to each scenario by soil volume treatment. Both seedling carbon mass and daily carbon assimilation were first scaled to the free seedling control.

**Table S1** Seedling Growth Model Default Parameters

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