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 could not explain observed growth responses. Thus, as photosynthesis and growth were not coordinated a pool of excess 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.

Key Words

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

Introduction

Understanding plant growth requires knowledge of the mass balance that must be achieved between C uptake and subsequent allocation to growth, storage, and respiration. It is commonly assumed plant growth is limited by C availability, yet it has long been demonstrated that correlations between A and growth are weak or seldom present. Körner (2013) argues that growth instead controls A, as it is the norm for sink activity to feedback on source activity. This is supported by evidence that growth of plants under environmental stress is not limited by the supply of photoassimilates (Palacio et al., 2014). As woody plants have highly integrated systems of competing carbohydrate sinks (Kozlowski, 1992), growth should principally depend on the transport of these photoassimilates between different tissue and organ sinks. Despite a wealth of studies, however, large uncertainties still remain regarding the coordination of C supply, via A, and growth of woody species.

In woody species, how A controls growth has been studied with manipulations of C source activity. Examples included elevated CO_2 experiments, for example FACE (reviewed in Ainsworth and Long (2005), and partial defoliation experiments. Elevated CO_2 has been shown to increase A rates (Drake et al., 1997; Ainsworth and Rogers, 2007) and across four FACE experiments this resulted in a conserved increase in forest production (Norby et al., 2005). Evidence from elevated CO_2 experiments, however, also reveals that the growth response tends to be much smaller than the photosynthetic enhancement (Kirschbaum, 2011). In defoliation experiments, compensatory increases in A are commonly shown yet are attributed to variable mechanisms, including reduction in end product inhibition (Iglesias et al., 2002; Zhou and Quebedeaux, 2003; Handa et al., 2005), enhanced biochemical activity (Ovaska et al., 1993a; Layne and Flore, 1995), increased stomatal conductance (Layne and Flore, 1995), leaf nutrient status (Turnbull et al., 2007), and regulatory sugar signaling (Eyles et al., 2013). However, increases in A did not always produce increased growth due to reductions in meristem sink strength (Palacio et al. 2012), C limitation to mycorrhizal colonization (Markkola et al. 2004), or an overall decrease in whole plant C gain (Ovaska et al., 1993b). These manipulations of C source activity expose unresolved issues with how changes in A do not always infer similar responses in growth.

Alternatively, tissue sink activity can restrict biomass production when limited by environmental or developmental constraints (Körner, 2003). This is because metabolic signaling networks, relaying information on C and nitrogen (N) status of different tissues, can down regulate photosynthetic activity (Paul and Foyer, 2001). Whether this down regulation, via sink inhibition, exists in woody species has been tested through fruit removal, girdling, and low temperatures. In these studies, down regulation of A was frequently correlated to carbohydrate accumulation resulting from reduced tissue sink strength (Iglesias et al., 2002; Hoch et al., 2002; Urban and Alphonsout, 2007; Haouari et al., 2013). However, reductions in A were also attributed to biochemical limitations prior to carbohydrate accumulation (Nebauer et al., 2011), irreversible photo-oxidative damage (Duan et al., 2008), and stomatal limitation (Li et al., 2005). These mixed results are not surprising as we still know little about the pathways in which plants achieve balance between assimilation, storage, and growth across temporal scales (Smith and Stitt, 2007). As these manipulations likely impact source as well as sink activity simultaneously, affect water transport, are very extreme, or are specific to large annual fruiting sinks, they tell us little about source-sink coordination in normal field-grown woody species. This coordination is further confounded by the fact that under normal growing conditions, A is not always correlated to photosynthate accumulation, as with fir needles (Little and Loach, 1973). Unfortunately, there is still limited understating of the physiological roles of carbohydrates in photosynthetic regulation and the elements triggering the down-regulation process (Nebauer et al., 2011).

An alternative approach is to lower belowground C sink strength in tree seedlings by manipulating rooting volume, by varying the container size. The advantage of this approach is it allows a large range of manipulations, can be easily compared to naturally planted seedlings and mimics natural conditions as seedlings compete for space or reach bedrock. Seedlings undergo many physiological and morphological changes in response to rooting volume, including biomass partitioning, A, water relations, nutrient uptake and respiration (NeSmith and Duval, 1998, and refer-

ences therein). The rooting volume available to plants can decrease C sink strength by reducing root growth. Container size studies frequently have photosynthetic down-regulation, likely as a result of sink limitation (Arp, 1991; McConnaughay and Bazzaz, 1991; Gunderson and Wullschleger, 1994; Sage, 1994; Maina et al., 2002; Ronchi et al., 2006). A meta-analysis by Poorter et al. (2012a) concluded that A is the process likely to be the strongest affected by pot size and may best explain the observed effect on biomass seen in the large number of studies where containers are used. This conclusion arises because plants grown in small containers are shown to accumulate leaf starch while having lower C exchange and assimilate export rates (Robbins and Pharr, 1988). However, evidence in support for a trade-off between C storage and growth in trees is, to date, inconclusive (Palacio et al., 2014). Based on these previous studies, using container size as a sink-strength manipulation can be used to empirically test how growth and A are coordinated.

Another barrier in understanding the coordination between A and growth is a lack of knowledge regarding the allocation of C in woody species. Understanding the fate of assimilated C is crucial when source or sink manipulations do not directly lead to equivalent changes in growth. As programmed plasticity in C allocation naturally occur as plants growth and develop, this must be accounted for when evaluating induced variation in growth (Wright and Mcconnaughay, 2002). Thus, when C allocation is altered by a treatment, it should be shown whether these allocation patterns differ from common sized untreated plants (Reich et al., 2002; Poorter et al., 2012b). Only then can we test if functional balance preservation or optimal foraging occurs from changes in C uptake. For example, seedlings in containers may shift allocation to leaves if root restriction occurs or alter fine root morphology to increase nutrient uptake. In woody species, shifts in allometric relationships of leaves, stems and roots can reveal if growth is ontogenetically constrained or actively adjusted when A is affected. This then allows additional pools of C, such as root exudation or changes in tissue respiration, to be accounted for and evaluated.

This study utilizes a novel field design to investigate the coordination between growth and A in *Eucalyptus tereticornis* Sm. seedlings, by manipulating container size and thus rooting volume. Seedlings were maintained under well watered conditions in order to evaluate only the effect of restricted soil volume and the limited nutrient resource pool. We used freely-rooted seedlings as a control for the container size treatments. Empirical results were combined with a simple plant growth model to simulate seedling growth with a C mass balance approach, which was then compared to observed harvested seedling mass. The model used whole-plant C gain, scaled from instantaneous rates of leaf A, to quantify seedling production over 120 days.

1). The manipulations of container size were hypothesized to induce a belowground sink limitation in these seedlings which was expected to be largest in the smallest containers, resulting in physical constraints to growth independently of A.

2). Reducing soil volume was expected to induce rapid negative effects on growth and physiology compared to free seedlings (container effect), with accumulation of leaf nonstructural carbohydrates triggering photosynthetic down regulation through time as a function of available soil volume.

3). Last, the growth model was expected to find agreement between simulated and observed seedling mass, through direct correlation of the effects of soil volume on rates of leaf A.

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 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,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 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 (A_{sat}) and saturating light and [CO_2] (A_{max}) 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). 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 (g_s) were logged 5 times for both A_{sat} and A_{max}. Photosynthetic CO_2 response (AC$_i$) 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 AC$_i$ curve began at the reference [CO_2] of 400 µl l^{-1} and then consisted of 12 additional steps from [CO_2] of 50 to 1800 µl l^{-1}at 25°C at saturating light (above). From these curves the photosynthetic parameters, J_{max} and Vc_{max}, were quantified using the biochemical model of Farquhar et al. (1980). 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. Reported values of R_d reported are predicted rates at 25°C using a Q10 value (1.86) developed on 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 (Ψ_{pd}) and midday (Ψ_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 AC$_i$ and R_d. Leaves were detached and immediately stored inside foil covered bags before water potential measurements were performed. Ψ_{pd} was measured before sunrise and Ψ_l at 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 and subsamples of harvested roots were dried to a constant mass and milled for analysis of N content, δ^{13}C, 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. N concentrations of leaf and soil 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 δ^{13}C 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 CO_2, 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 α-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, m^2 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 ($leaf_i$, $stem_i$ and $root_i$) and a starting leaf area (LA_i) measured prior to planting. The initial biomass of roots was divided evenly between fine and coarse roots. The daily net biomass production of seedlings (P_i) is then given by

$$P_i = L \left(C_{day,i} \, M \, \epsilon_c^{-1} \right) - R \tag{1}$$

where L is standing cumulative leaf area, $C_{day,i}$ is the predicted daily carbon assimilation, M is a self shading parameter, ϵ_c is a biomass conversion efficiency parameter and R is the total respiration of all tissue components. Total respiration was calculated as

$$R = \Sigma(R_c \, M_c) \tag{2}$$

where R_c is tissue respiration of fine roots, coarse roots or stems on a mass basis and M_c is the standing biomass of each component. R_{leaf} is represented in the calculation of C_{day} (described below). The change in individual component biomass, here solved on a daily time step, is given by

$$\frac{dM_c}{dt} = A_c \, P_i - (\Lambda_c \, M_c) \tag{3}$$

where A_c is the component specific biomass partitioning to whole plant biomass (%) and Λ_c is component specific turnover rate. Due to the duration of the experiment Λ_{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.

C_{day} 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 (R_d, J_{max}, V_{cmax}, and g_1) 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 g_1 parameter was generated by fitting observed g_s 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 (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 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 C_{day} 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 C_{day} 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 (M) 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 M for each of the 61 digitized seedling, independently for each treatment. Next, the linear relationship between M and total leaf area was for determined across digitized seedlings, within each treatment. For the growth model, M was then predicted for

each daily time step using the previous days cumulative leaf area and this value was then applied to $C_{day,i}$. All default parameters used in model simulations are reported in Table. 3.

We then utilized this model to test the hypothesis that the effects of belowground sink limitation on rates of leaf A where sufficient to accurately predict overall seedling production after 120 days. Each model run utilized changes in A and leaf mass fraction by treatment with published or local vaules of stem and root respiration rates to generate total seedling mass and leaf area after 120 days. Culutaive total carbon gain for each treatment was equal to the sum of each value of $C_{day,i}$ over 120 days and final seedling carbon was assumed to equal half of the final mass for both modelled and observed seedlings. First, a default model was optimized to produce a final leaf mass fraction that correctly predicted both the final leaf mass and total biomass of the harvested free seedling controls (M_0). This optimized leaf mass fraction was then applied to model runs with treatment specific C_{day} to determine if changes in leaf A alone could accurately predict biomass (M_1). Next, model sensitivity to different C allocation scenarios, including non-optmized treatment specific leaf mass fraction and up or down regulation or non-leaf tissue respiration by ± 50 % of default values ($M_{2,3,4, respectively}$), was tested to investigate any possible sources of missing C from initial model simulations and observed harvest biomass. For all cases, seedling production was compared between model output and harvested seedlings with treatment specific mean daily carbon assimilation by first scaling values to the free seedling control.

Data Analysis

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 volume treatments were different. Mixed model ANOVAs of A_{max} and leaf chemistry were performed using the 'nlme' package (Pinheiro et al., 2014) in R and r^2 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 $P \leq 0.05$.

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 container volumes the experiment was chosen to be harvested after 120 days. Over this 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 5th week of the experiment. Following this period both height (8th week) and then diameter (9th 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 soil volumes across the final two months of the experiment. Seedlings maintained diameter growth throughout the experiment, although marginal with smaller soil volumes 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.

Harvested total seedling biomass at harvest was significantly different across 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, consistent with the meta-analysis of Poorter et al. (2012a). 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 (Fig. 3a,b). Across all treatments, the final harvested root:shoot was conserved in these seedlings, with a slightly higher shoot than root mass on average ($\overline{x}$=0.904, Fig. 3c).

SRL of harvested fine roots was not different across soil volumes (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 ($P<0.001$).

Leaf chemistry

Leaf N % was significantly higher in free seedlings and the largest container volume at the onset of gas exchange measurements (6th week, $P<0.001$). Over the remaining duration of the experiment the smallest container volume had a significant reduction in leaf N % compared to other soil volumes, while free seedlings maintained a greater leaf N % (Table. 1, $P<0.001$). Leaf starch content in the smallest container was double that of free seedlings ($P=0.039$), while leaf soluble sugars did not differ across treatments throughout the experiment (Table. 1). Differences in leaf 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. 4) and A_{max} (Table. 2) were both significantly higher in the largest container volume and the free seedling at the first measurement campaign (both $P<0.001$). Across all measurement campaigns A_{sat} was consistently higher in free seedlings than in containers (Figure 3, $P<0.001$). The interaction between photosynthetic capacity, leaf starch, and leaf N on a mass basis was marginally significant ($P=0.0584$) but A_{max} was highly correlated to both leaf N content and 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 leaf starch (Fig. 3b)

The photosynthetic parameters J_{max} and Vc_{max} were not different within treatments at the beginning and end of gas exchange campaigns, therefore the parameter means per treatment are reported here (Table. 2). Overall, both J_{max} and Vc_{max} were significantly higher in free seedlings with little variation between soil volume treatments ($P=0.0012$ & 0.0021, respectively). Leaf dark respiration rates were not significantly different across soil volumes (Table. 2). The g_1 parameter, generated for each seedling from the Medlyn et al. (2012) optimal stomatal conductance model, was lowest in the free seedling and was marginally different across soil volume treatments (Table. 2). Predicted values of g_s, using the g_1 parameter, where highly correlated with observed values (r^2= .74, $P<0.001$, data not shown).

Neither Ψ_{pd} nor Ψ_l were different across treatments, with mean values of -0.27 and -1.2 mPa across all seedlings, respectively. Although g_s in free seedlings was generally higher than those in containers (Table. 2, $P=0.001$), the mean rates for all seedlings were high at 0.37 mol H_2O m$_{-2}$ s$_{-1}$ and did not decline significantly across the experiment duration. Additionally, leaf $\delta^{13}C$ at final harvest was not different across treatments (Table. 1). Combined these indices provide strong evidence that water stress was not apparent on these well-watered seedlings throughout the experiment. Soil N % at harvest was not different across soil volumes ($\overline{x}$=04.5 %) 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

Model M_0, was able to converge on an optimum leaf mass fraction (21.6 %) which accurately predicted the mean harvest total biomass of free seedlings within 1.2 %. Using this leaf mass fraction to drive the interaction betwen leaf A rates, via C_{day}, and seedling biomass production M_1 overestimated seedling mass carbon by an average of 63.7$\pm$0.05 % across the entire range of predicted total carbon gain in seedlings with soil volume restriction (Fig. 6a). When modelled and oberserved total carbon mass were compared with the mean reduction in daily assimilated carbon (Fig. 6a) it is apparent that the reductions in leaf A were not sufficiently high enough to explain the reduction in harvested seedling biomass with soil volume restriction. Thus, a large pool of excess carbon remained unexplained, generated from A, that did not end up in biomass.

The remaining model simulations tested carbon allocation scenarios in an attempt to account for this remaining pool of missing carbon. Neither increase or decreases of 50 % in non-leaf tissue respiration ($M_{3,4}$) suffiently improved biomass estimates in seedlings with soil volume restriction (Fig. S1b,c). It was further determined that in order to account for this pool of carbon the non-tissue respiration rates in modelled seedlings would have to have increased by 250 %. Constraining the model with the observed treatment specific leaf mass fraction from the final harvest (M_2) still overestimated seedling total carbon, but vastly improved model predictions (Fig. S1b,c). This indicates that the belowground sink limitation in this experiment likely severely altered carbon allocation in leaves in addition to reductions in photosynthesis. This is evident from the 7-61 % reduction in harvested leaf mass fraction from large to small soil volumes compared to the optimized free seedling control.

Discussion

were then utilized to investigate how different C allocation scenarios, not explicitly tested within the framework of this experiment, might account for these pools of unexplained carbon.

Tables

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 $\delta^{13}C$ 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.

Volume (L)	Seedling mass (g)	SLA$_{TNC-free}$ (m² kg⁻¹)	Leaf Starch (%)	Leaf Sugars (%)	Leaf Nitrogen (%)	Root Nitrogen (%)	SRL (cm m⁻¹)	Leaf $\delta^{13}C$ (‰)
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 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 A_{max} and R_{dark} are $\mu mol\ m^{-2}\ s^{-1}$ and g_s are mol $m^{-1}\ s^{-1}$, each at at 25°C. Values of A_{max}, g_s and g_1 represent overall means across measurement campaigns (n=6). R_{dark}, J_{max} and Vc_{max} 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.

| Volume (L)	A_{max}	R_{dark}	J_{max}	Vc_{max}	g_s	g_1
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 (0.00) 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
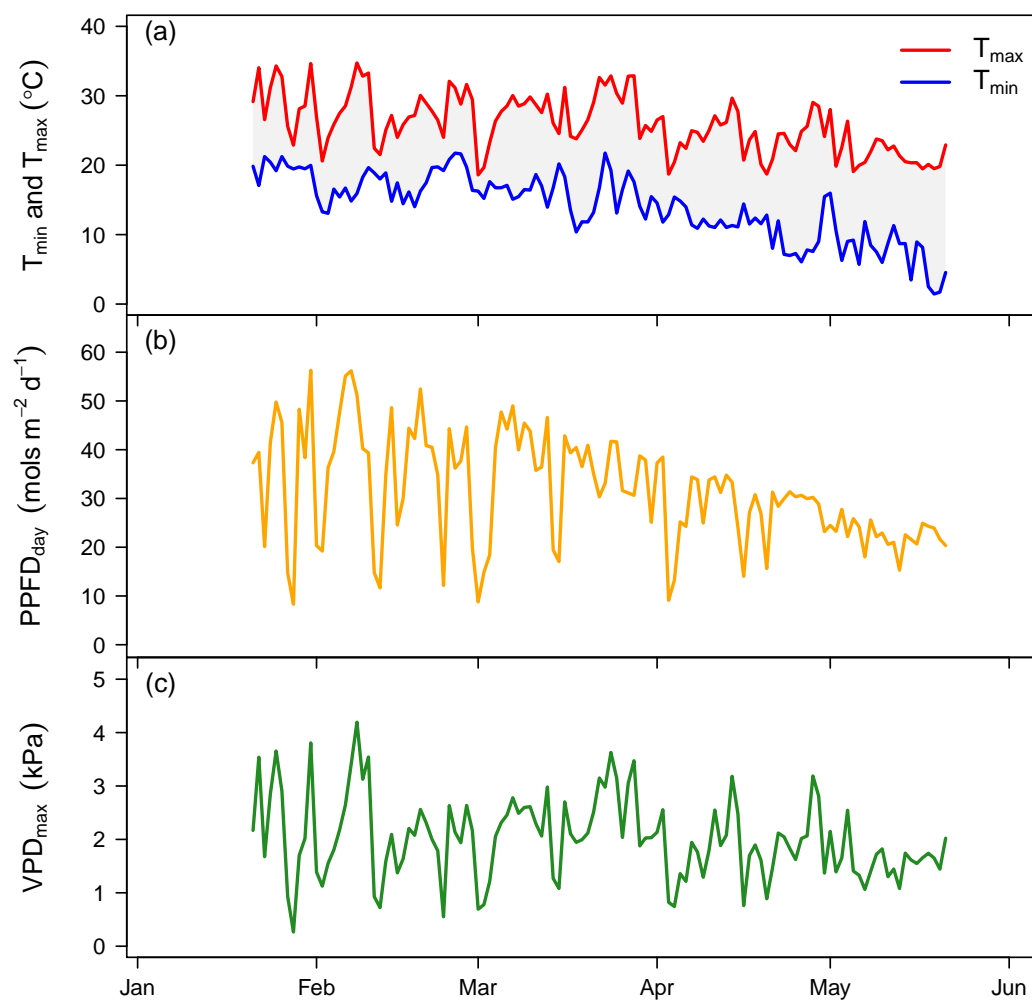
Figures

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.
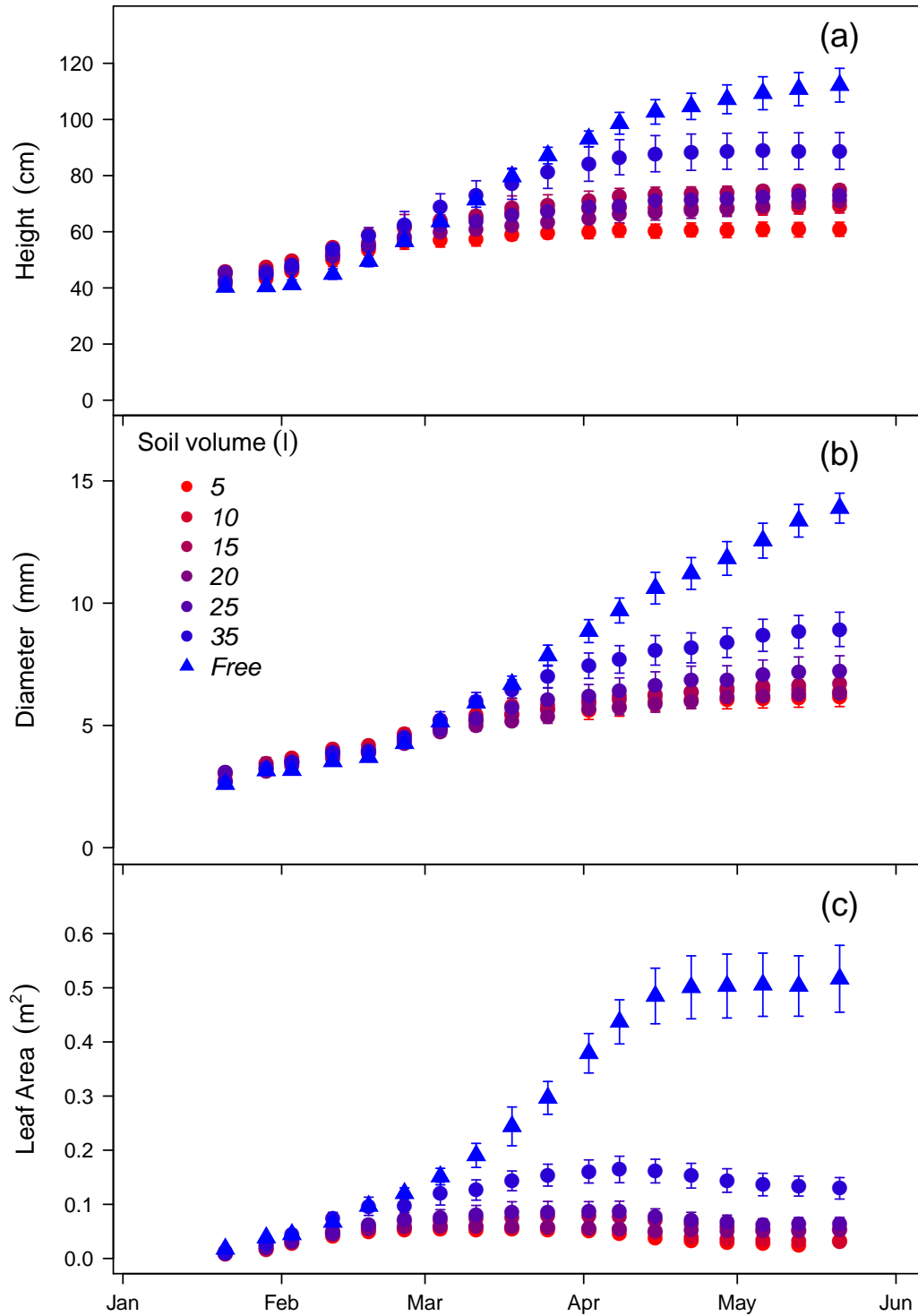
Figure 2: Soil volume treatment means ± standard error of height growth (a), diameter growth (b), and interpolated seedling leaf area (c) measured weekly of *Eucalyptus tereticornis* seedlings across the experiment duration in 2013.
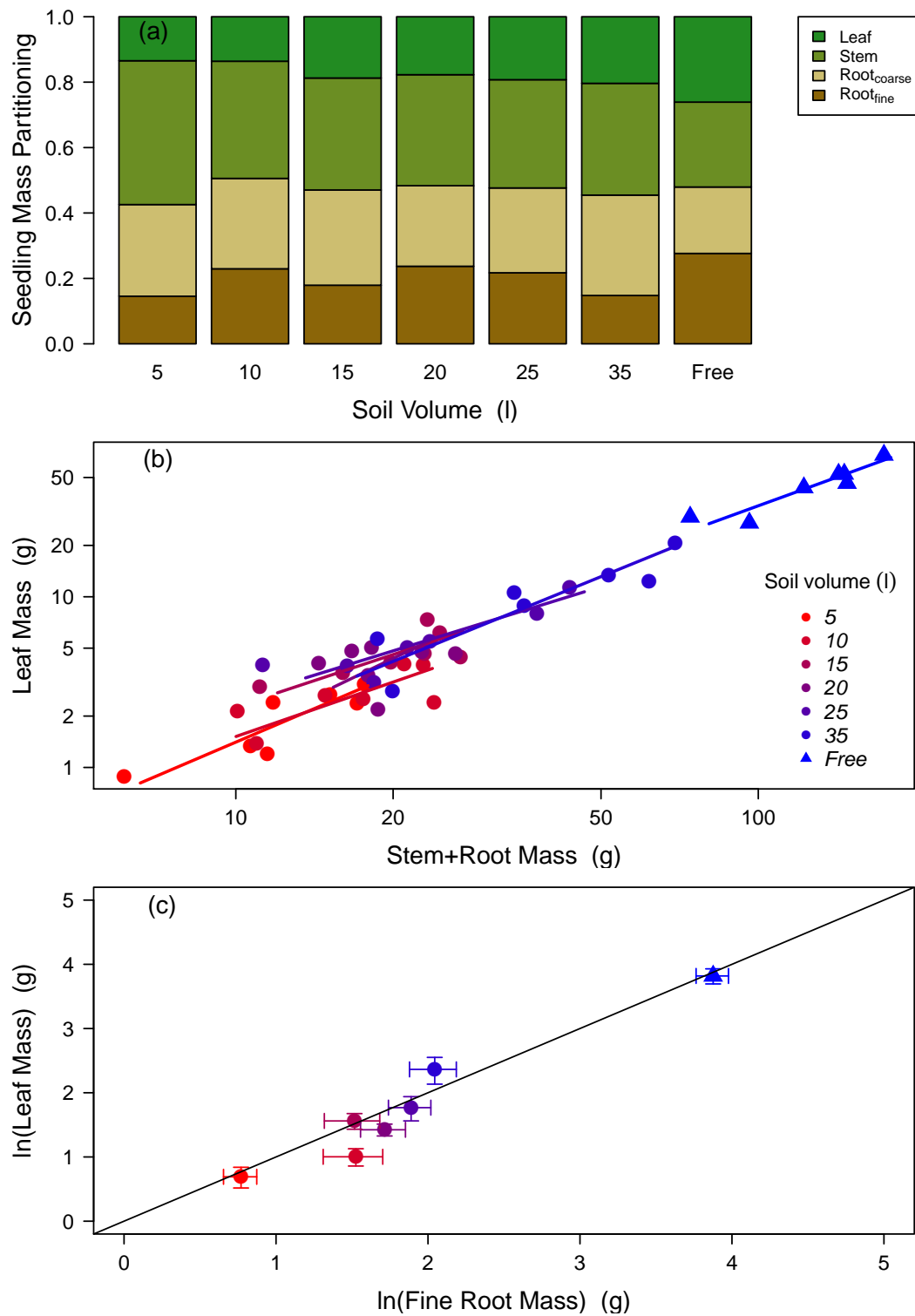
Figure 3: Soil volume treatment means of mass partitioning to leaves, stems, and roots (a), bivariate relationships between mass allocation to leaves and stems + roots (b) and leaf mass as a function of fine root biomass ± standard error (c). For (b) lines represent standardized major axis fitting of the log transformed allometric relationships of leaf mass fraction by treatment.
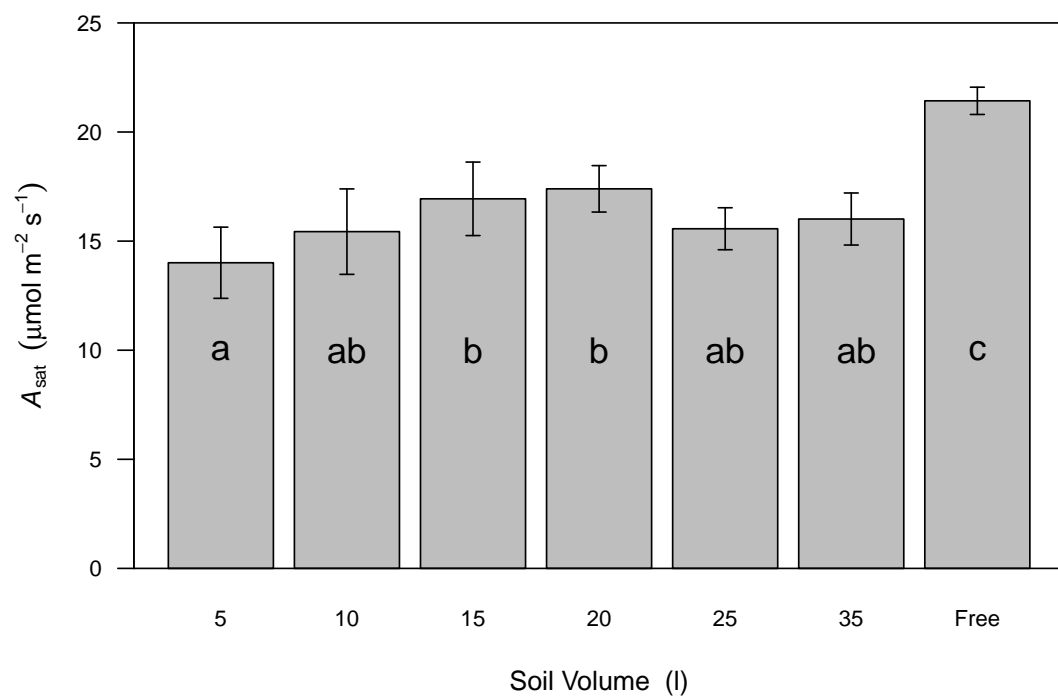
Figure 4: Soil volume treatment means $\pm$ standard error, across all measurement campaigns (n=6), of light saturated rates of photosynthesis at 25°C. Different letters represent significant differences between treatments.
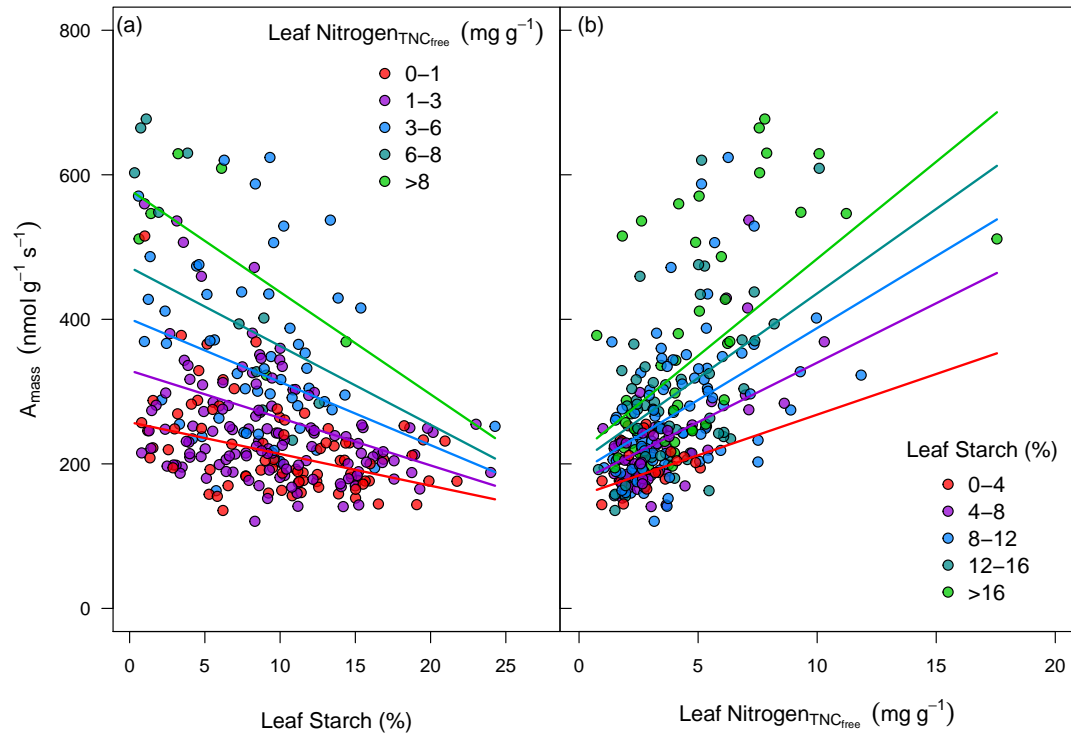
Figure 5: Photosynthetic capacity, on a leaf mass basis, as a function of accumulation of leaf starch (a) and leaf nitrogen content without TNC (b). Colors represent bins levels (n=5) of both leaf starch and nitrogen grouped from low to high . Lines represents predictions, for each bin level, from the linear mixed effects model equation of 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.
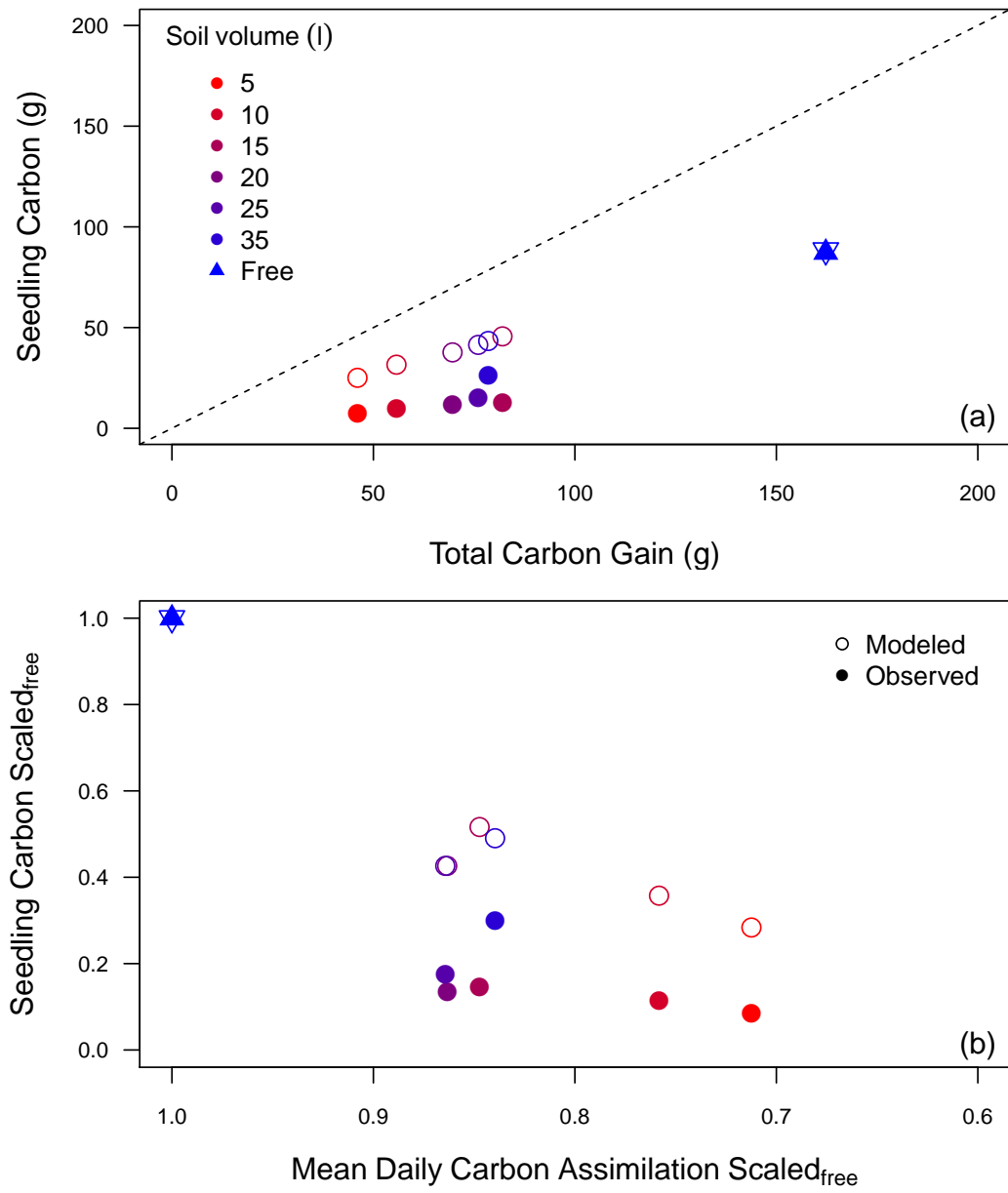
Figure 6: Total carbon mass for harvested and modeled seedlings versus predicted total carbon gain after 120 days (a) and reductions in final seedling carbon mass, both modeled and observed, as a function of the reduction in leaf photosynthesis across treatments (b). For (b) both seedling carbon mass and daily carbon assimilation were first scaled to the free seedling control.

1 Supporting Information Figures

Table 3: Seedling Growth Model Default Parameters

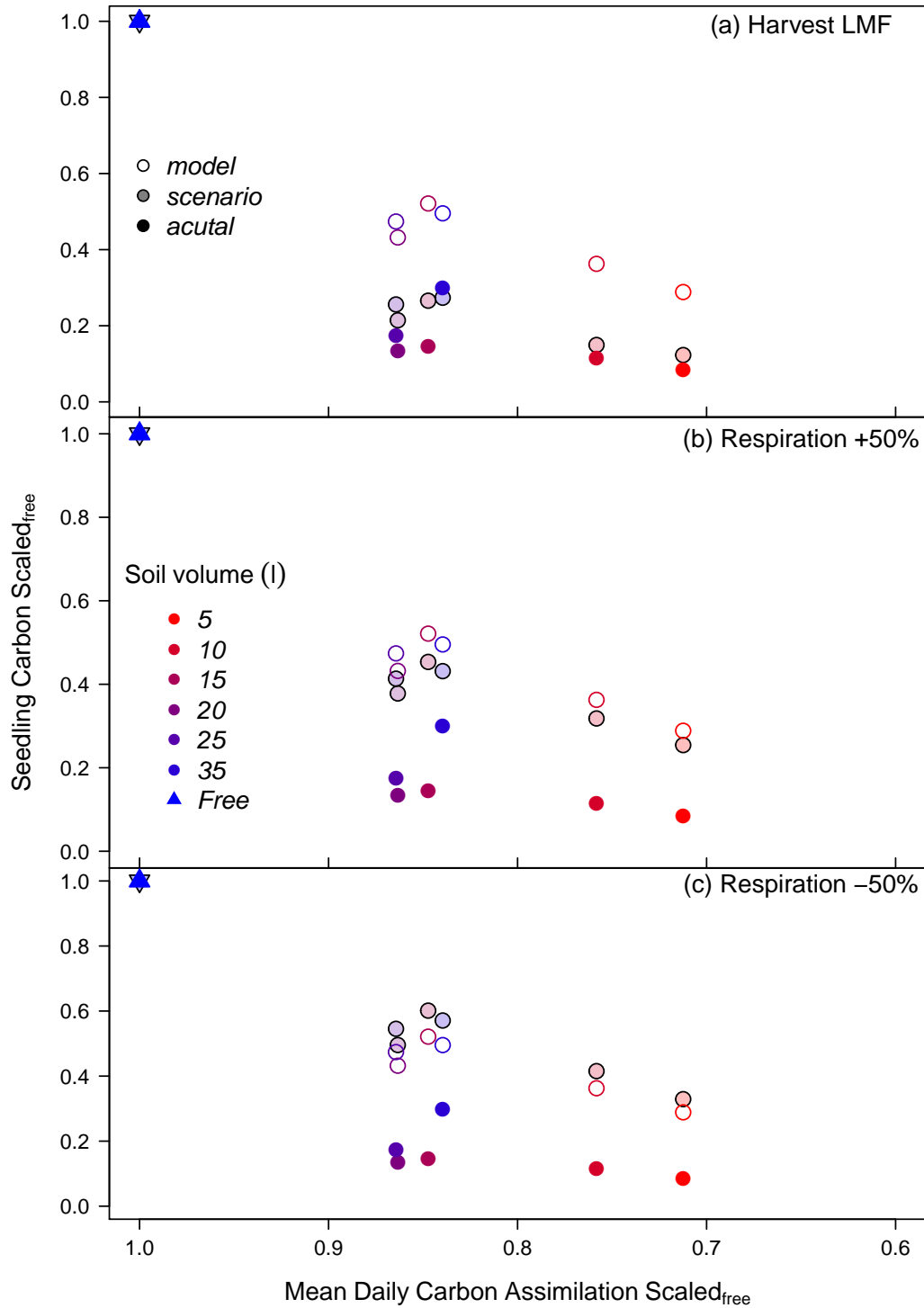| Variable	Default Value	Units	Source
Leaf area$_i$	0.035	m^2	this study
Leaf mass$_i$	3.45	g	this study
Stem mass$_i$	1.51	g	this study
Root mass$_i$	0.99	g	this study
ε_c	.65	g C g mass^{-1}	Mäkelä (1997)
$R_{coarse\ root}$	0.00124	g C g root^{-1}	Marsden et al. (2008)
$R_{fine\ root}$	0.010368	g C g root^{-1}	Ryan et al. (2010)
R_{stem}	0.00187	g C g stem^{-1}	Drake 2014 (unpublished)
C_{day}	5.4-7.6	g C m^{-2} d^{-1}	this study
Λ	1/365	yr^{-1}	theoretical

Figure S1: Sensitivity testing of seedling growth model to different carbon allocation strategies in-cluding; constraints of leaf mass fraction to treatment specific final harvest values (a) and changes in respiration of non-leaf tissue components by ± 50 % (b,c respectively). 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.

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