Tree Physiology 00, 1–17
https://doi.org/10.1093/treephys/tpab136

Research paper

The effect of elevated CO_2 on aboveground and belowground carbon allocation and eco-physiology of four species of angiosperm and gymnosperm forest trees

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Received April 13, 2021; accepted October 7, 2021; handling Editor David Tissue

Although atmospheric CO_2 concentration ($[CO_2]$) continues to rise, the question of how tree carbon (C) allocation is affected by this change remains. Studies show that C assimilation increases under elevated CO_2 (eCO_2). Yet, no detailed study has determined the fate of the surplus C, i.e., its compartment and physiological process allocation, nor in multiple species together. In this project, we grew 2-year-old saplings of four key Mediterranean tree species (the conifers *Cupressus sempervirens* L. and *Pinus halepensis* Mill., and the broadleaf *Quercus calliprinos* Webb. and *Ceratonia siliqua* L.) to $[CO_2]$ levels of 400 or 700 p.p.m. for 6 months. We measured the allocation of C to below and aboveground growth, respiration, root exudation, storage and leaf litter. In addition, we monitored intrinsic water-use efficiency (WUE), soil moisture, soil chemistry and nutrient uptake. Net assimilation, WUE and soil nitrogen uptake significantly increased at eCO_2 across the four species. Broadleaf species showed soil water savings, which were absent in conifers. All other effects were species-specific: *Cupressus* had higher leaf respiration, *Pinus* had lower starch in branches and transiently higher exudation rate and *Quercus* had higher root respiration. Elevated CO_2 did not affect growth or litter production. Our results are pivotal to understanding the sensitivity of tree C allocation to the change in $[CO_2]$ when water is abundant. Species-specific responses should be regarded cautiously when predicting future changes in forest function in a higher CO_2 world.

Keywords: carbon sinks, carbon uptake, root exudation, soil nutrients, water saving, water-use efficiency.

Introduction

Due to human activity, atmospheric $[CO_2]$ has been on a continuous rise over the past two centuries (IPCC et al. 2014). In contrast, until the industrial revolution it was stable at ~280 p.p.m. for millennia. The current $[CO_2]$ level, of ~410 p.p.m., is the highest since 3–3.3 Mya. According to some models, this increment is projected to exceed 900 p.p.m. in the late 21st century (Cech et al. 2003, IPCC et al. 2014, NOAA ESRL). Forest trees play an important role in the global (C) carbon cycle, and therefore they are potentially capable of serving to buffer against the ongoing $[CO_2]$ elevation (Brinck et al. 2017, Smallman et al. 2017, Moomaw et al. 2020, Walker et al. 2021). Trees assimilate CO_2 and fix it into sugar, which can move on to several C sink processes. A major part of the C is emitted back to the atmosphere via respiration, whereas the rest goes to structural growth or storage, and some also reaches the soil through root exudation and litter (Klein and Hoch 2015). To simplify, there is a single source of C uptake by the plants (A, assimilation), whereas its allocation is dynamically divided between different sinks (R, respiration; G, growth; S, storage; E, export to soil and L, litter production):

$$A = G + S + R + E + L \quad (1)$$

Notably, plant C sinks also include volatile organic compounds, osmolytes and defense compounds (Chapin et al. 1990) as well as reproductive sinks like flowers and fruits. We argue, however, that these sinks are smaller in magnitude,

particularly in forest trees (e.g., in a pine species, C allocation to cones was calculated at <5%; Klein and Hoch 2015).

Many studies have shown plant responses to elevated atmospheric [CO_2]. Most research focused on aboveground responses, as belowground measurements are harder to achieve, especially for trees. Klein et al. (2016) showed, in mature *Picea abies* under elevated CO_2 (eCO_2), significantly higher assimilation than those grown under ambient conditions. This surplus C did not translate into higher growth, or extra litter, but rather there was a trend towards more root biomass. Yet the question of 'where is all this surplus C allocated?' has persisted. An increase in available nitrate concentration in the rhizosphere, at similar moisture levels and without any sign of reduced tree nitrogen (N) uptake, suggested higher microbial N mineralization, presumably due to enhanced root exudation (priming effect; Schleppi et al. 2019). Root exudation, the release of a variety of compounds into the rhizosphere by plant roots, includes compounds secreted by both passive and active processes from intact root tissues (Strehmel et al. 2014). The estimated amount of C being released by root exudates is 21% of all C allocated into fine roots (Sun et al. 2017), which may comprise ~10% of net primary productivity in some forests (Kannenberg and Phillips 2017). This flux to soil is influenced by many biotic and abiotic factors such as drought, nutrient deficiency and soil microbiota (Vranova et al. 2013). Previous studies have shown that exudation rates might increase under stress, despite a lower assimilation rate (Karst et al. 2017, Jakoby et al. 2020). Because root exudates occur in a narrow zone of soil around roots and are rapidly taken up by soil microbes, they represent one of the most poorly quantified components of the C cycle. Phillips et al. (2009) demonstrated an increase in root exudation rate in *Pinus taeda* seedlings when exposed to eCO_2. Other studies also showed changes in the quality and quantity of root exudates (Jones and Darrah 1996, Hodge and Millard 1998) due to enhanced plant photosynthesis and growth (Leakey et al. 2009).

Respiration and the other C sinks were also studied in earlier experiments. Janssens et al. (1998) reported an increase in root respiration in *Pinus sylvestris* seedlings as a response to eCO_2 conditions. Drake et al. (2008) have also shown an increase in root respiration in mature *P. taeda* exposed to eCO_2, however when combining eCO_2 with N fertilization treatment a reduction in root respiration rate was observed. Leaf respiration rate response to eCO_2 seems to be species-specific and also dependent on experimental conditions and duration of exposure to eCO_2 (Wang and Curtis 2002, Aspinwall et al. 2017). Few studies have shown that N uptake by the plant has increased under eCO_2 (Nie and Pendall 2016, Schleppi et al. 2019). Cotrufo et al. (2005) demonstrated no significant increase in annual leaf litter production in three *Populus* species exposed to eCO_2. Tjoelker et al. (1998) showed a species-specific response to eCO_2 in terms of starch accumulation. A meta-analysis done by Ainsworth and Long (2005) found a positive response of photosynthetic rate and showed that tree growth generally increased under eCO_2, but this response varied among species and experimental conditions. Cohen et al. (2018) suggest that under eCO_2 plants allocate more C to belowground organs. Changes in growth and root/shoot ratio can affect other fluxes when considering the whole-tree C flux changes.

Surveying previous studies on stomatal sensitivity to [CO_2] among species, we showed that the responses of trees to eCO_2 diverge among functional groups (Klein and Ramon 2019). Unlike gymnosperm (conifers) species, angiosperms (broadleaf) reduced stomatal conductance (g_s) as a response to eCO_2, their water-use efficiency (WUE) has improved and soil moisture around the trees was higher (Cech et al. 2003, Bader et al. 2009). Studies made on young saplings in controlled conditions found stronger and more significant responses to eCO_2 than in the field (Oberbauer et al. 1985, Körner 2003, Pokorný et al. 2013). The decrease in g_s meant that eCO_2 could partially relieve tree drought stress, as shown in young lemon trees (*Citrus limon*; Paudel et al. 2018). Under eCO_2, g_s was reduced, and, consequently, soil water content (SWC) remained higher than under ambient CO_2 (aCO_2). Exposed to drought conditions, those saplings showed a lesser drop in leaf water potential and a slower decrease in photosynthesis than trees growing in aCO_2. Killi et al. (2018) conducted an experiment on two *Quercus* species exposed to eCO_2 and showed in *Quercus ilex* the same response chain of g_s reduction, which leads to a higher WUE, inducing a water-saving potential. *Quercus cerris*, a deciduous species, did not show the same trend. Water savings and increased soil moisture have been also shown in other eCO_2 experiments (Pendall et al. 2004) and eCO_2 models (Hovenden et al. 2019) but not in others (Gimeno et al. 2018).

Overall, past studies demonstrated different, and, sometimes, contradictory results. Plant response to eCO_2 is affected by tree species, age, soil chemistry and physical structure. In addition, different experimental or environmental conditions such as light, water availability, relative humidity, temperature, nutrient availability, soil microbiome, etc., have an effect on the response. Although limited in their ability to simulate mature tree growth in the forest, studies on young trees in controlled conditions are still important data sources (Hartmann et al. 2018). Here, we aimed to cover the whole-tree C balance, and specifically, to test the hypothesis of belowground allocation and root exudation as an extra C 'disposal channel'. For our purpose, young trees were the practical choice, also allowing the comparison between conifer and broadleaf tree species in a single experiment.

The main goal of our project was to elucidate the sensitivity of C allocation of Mediterranean angiosperm and gymnosperm forest trees to atmospheric [CO_2] levels. Specifically, we aimed to: (i) examine the whole-tree C balance under different atmospheric [CO_2] levels, including C uptake, respiration, growth,

exudation, litter production and storage; (ii) pinpoint the sink of surplus C under elevated atmospheric [CO_2], either species-specific or general, focusing on belowground allocation and root exudates; and (iii) test for the existence of major eco-physiological effects, namely on (a) intrinsic water-use efficiency (WUEi), (b) soil water saving and (c) soil N uptake.

Materials and methods

Climate controlled growth rooms

This project took place in two new, custom-made and climate controlled growth rooms. Each room was 3 × 5 m by area, and 2.5 m by height. The first room retained an aCO_2 concentration of ∼410 p.p.m., whereas the second room held an eCO_2 concentration of 650–750 p.p.m.. Ideally, young trees would swap between the rooms, to cancel any potential room effect. However, this was not possible without damaging the trees, because of the measurement systems, and, in particular, root exudation systems (see below). Nevertheless, we made sure that all conditions but [CO_2] were highly similar between the rooms. Measurements carried out during the acclimation period, i.e., prior to the eCO_2 application, showed a zero room effect. In both rooms, eCO_2 was maintained by a regulatory system consisting of a compressed CO_2 cylinder and an infrared [CO_2] sensor (0–2000 p.p.m. CO_2, PolyGard Transmitter, Pocking, Germany). When the cylinder valve opens, the [CO_2] in the room increases until the sensor shows a concentration of 650 p.p.m. (or 410 p.p.m. in the other room). Subsequently, the valve automatically closes. We found that the [CO_2] in the room continues to rise up to 750 p.p.m., but it starts to decrease because of diffusion and plant consumption until the concentration is below 650 p.p.m.. Then the valve opens automatically again, and the cycle is repeated. Large fans installed in each room ensured homogeneous distribution of the CO_2. All other conditions were kept the same across the two rooms: day temperature ∼24 °C, night temperature ∼21 °C and 12/12 h day/night cycle. Relative humidity was not directly controlled, however it was maintained at 60–70%. The light level was controlled by an array of high-intensity LED lamps (Regiolux, Königsberg, Germany), producing a photosynthetically active radiation that ranged from ∼150 (μmol photons m^{-2} s^{-1}) at pot height to ∼750 (μmol photons m^{-2} s^{-1}) at treetop.

Plant and soil material

In both rooms, eight saplings from each of four Mediterranean forest tree species were grown for 6 months, with 64 plants in total. In addition, three saplings from each species were sacrificed for compartment biomass assessment at the outset of the experiment. The two broadleaved species, *Quercus calliprinos* Webb. and *Ceratonia siliqua* L., and the two conifer species, *Pinus halepensis* Mill. and *Cupressus sempervirens* L., were selected from a JNF (Jewish National Fund) Forest service nursery, on the basis of the same phenotype, phenology and origin (see Figure S1 available as Supplementary data at *Tree Physiology* Online). They were 1.5 years old and 0.5- to 1-m tall. The tree seeds or seedlings were collected by the JNF from February to April 2017. All originated in the Jerusalem Mountains, Israel, except for the *Cupressus*, which was collected from Beit She'arim in the Galilee, Israel. All were transferred to plastic 'quick-pots 585' (200 ml plugs, 5 × 5 cm) in the Eshtaol nursery. There, the plants were grown with 2% starter fertilization and irrigated with fertilization until September 2018. At the Weizmann Institute, Israel, all trees were transplanted to the 10-l pots. To avoid shock and enable the root system to develop, they were grown under a sustained irrigation regime (60 ml three times a day) without fertilization and they received a natural light regime in a glasshouse until the transfer to the rooms. After a 3-month acclimation process, the saplings were transferred to the two controlled growth rooms and were exposed to the same conditions for further acclimation of 2 months, before [CO_2] treatment began to be applied in the eCO_2 room. Pots were adapted to facilitate root system access and enable root sample collection, root exudation and root respiration measurement with minimal soil and sapling interference. Three windows were cut in each pot in such a way that they can be opened for sample collection or monitoring, and closed during the rest of the time. All pots contained natural forest soil mixed with washed sand and tuff (5/10/1 v/v). During the experiment, saplings were drip-irrigated with 30 ml three times a day (90 ml day^{-1}). Soil water content was measured point-wise during the experiment. The planting soil was not fertilized, and the saplings were not sprayed. Soil structure and nutrient composition were monitored at the beginning and end of the experiment (see below).

Measurement campaigns

Compartment biomass assessment (tap root/lateral roots/stem/branches/leaves) was determined on 12 saplings, which were destructively harvested before the experiment started. Stem diameter, at 3-cm aboveground, was monitored for all saplings at the beginning and at the end of the experiment. During the experiment, leaf gas exchange (net assimilation, transpiration, g_s and respiration) was measured on all saplings and leaf litter was collected. Destructive measurements such as root respiration and root exudation collection were performed on five saplings per species per treatment ($4 \times 5 \times 2 = 40$). The other three saplings per species per treatment ($4 \times 3 \times 2 = 24$) were kept for compartment biomass assessment (tap root/lateral roots/stem/branches/leaves) at the end of the experiment. Therefore, during the experiment they were measured only for leaf gas exchange. Lastly, root and branch samples were measured at the end of the experiment for starch content analysis.

Growth measurements

Three saplings of each species were taken at the beginning of the experiment (March 2019), for compartment biomass assessment. All leaves were cut from each sapling, the naked stem was cut from the base, above the first lateral root split. Roots were separated from the soil and then washed to remove attached particles. Soil was sieved through a 2-mm sieve and detached roots were added to the rest. Saplings were divided into five compartments: tap root, lateral roots, stem, branches and leaves. Each compartment was kept in a paper bag and dried at 60 °C for 3 days in order to measure biomass. A sample of fresh leaves and roots of each sapling was taken to the lab for leaf and root area calculation using a flatbed scanner HP color jet pro MFP m477fdn. The leaves were scanned over a background of A4 (210 × 297 mm) white paper and their area was calculated using Fiji package for image processing (Schindelin et al. 2012). The measured area was separated from the background with the color threshold tool and then calculated using the particle analyzer tool. After scanning, leaves and roots were also transferred into a 60 °C oven in order to measure biomass. The same process repeated for three saplings of each species from each room at the end of the experiment (August 2019). Stem diameters were taken for all saplings at stem base at the beginning and end of the experiment (March and July 2019).

Gas exchange measurements

Rates of net leaf assimilation (A, μmol CO_2 m^{-2} s^{-1}), transpiration (T, mmol H_2O m^{-2} s^{-1}), stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}) and leaf and root respiration (R, μmol CO_2 m^{-2} s^{-1}) were measured using the portable gas exchange system GFS-3000 (Walz, Effeltrich, Germany). The sensor head was equipped with a red–blue LED light source inside a standard chamber (8 cm^2). For each measurement cycle of A, T and g_s, light intensity inside the cuvette was adjusted to the light intensity in the room at the average height of the leaves and kept at 300 μmol m^{-2} s^{-1}. The temperature and relative humidity inside the chamber were maintained as in the rooms, i.e., 24 ± 1 °C and 60–70%, respectively. Due to these temperature and humidity ranges, vapor pressure deficit within the chamber fluctuated between 0.84 and 1.27 kPa. For leaf and root respiration measurements, the cuvette was kept closed and dark. The CO_2 mixer was set to provide a stable concentration of 400 p.p.m. for all measurements in the aCO_2 room, and of 700 p.p.m. in the eCO_2 room. Flow rate was set to 750 μmol s^{-1} and impeller speed was set to step 7 (2.2 m s^{-1}) inside the sample cuvette. In each measurement, a single mature leaf of the broadleaved and the *Cupressus* saplings, or eight fascicles of needles of the *Pinus* saplings, or a fine root, was randomly sampled. Leaves were either cut or remained intact and were measured immediately (<30 s) inside the cuvette for 3–4 min. Preliminary measurements on cut leaves showed that gas exchange rates were identical to those of intact leaves for at least 12 min following cutting. Roots for respiration measurements were gently dug up from soil through the pre-installed windows in the pots, then washed and dried out on paper for a minute before measuring inside the cuvette. Subsequently, the leaves and the roots were cut and transferred to the lab for leaf and root area calculation using a flatbed scanner. All gas exchange parameters were calculated per projected area. In addition, gas exchange measurements were used to calculate the intrinsic WUE at the leaf level (WUEi = A/gs, μmol CO_2 mol^{-1} H_2O). For leaf respiration measurements, the light in the rooms was turned off and a green light was used instead to enable vision without inducing photosynthesis. Gas exchange measurement cycles occurred one time before [CO_2] treatment was applied, in February 2019, and then on three additional campaigns in April, May and June 2019, following the application of eCO_2 at the end of February 2019.

Root exudation collection

Root exudates were collected from intact lateral fine roots using a non-soil syringe system modified from Phillips et al. (2009). Root tips from the middle of the pot were dug up gently without harming them and then sampled. They remained attached to the target trees during the entire procedure until harvest. The intact fine roots were gently washed with a spray bottle, using autoclaved C-free nutrient solution (0.5 mM NH_4NO_3, 0.1 mM KH_2PO_4, 0.2 mM K_2SO_4, 0.2 mM $MgSO_4$ and 0.3 mM $CaCl_2$) and fine forceps to remove soil particles and other possible contaminants. Roots were placed into a 20-ml sterile plastic syringe and filled with 0.5- to 1.3-mm acid-washed glass beads and 10-ml autoclaved C-free nutrient solution. Then, the syringes were covered with parafilm to avoid soil particles or organic matter from entering the syringe. Syringe were placed in a stable position and covered with aluminum foil to block light. After 48 h, the nutrient solution was collected from each syringe system. An additional 10 ml of double-distilled water was flushed through the syringe system to obtain a representative C recovery. Each campaign included between 34 and 45 samples, accompanied by 14–17 control replicates. Control syringes were installed according to the same procedure, but with no root placed inside. All the solutions were filtered immediately through a 0.22-μm sterile syringe filter (Millex PVDF, Millipore Co., Billerica, MA, USA) in the growth rooms and stored in the lab at −80 °C until analysis. The solutions were analyzed for dissolved organic C on a total organic carbon analyzer (Shimadzu VCPH—Carbon and Nitrogen analyzer, Kyoto, Japan). Root exudation rates were calculated as the total amount of C flushed from each root system over the incubation period divided by root surface area of the investigated root strand, and hereafter referred to as specific exudation rate (μg C cm^{-2} root day^{-1}). After root exudate collection, roots were cutoff the tree and stored

at 4 °C for no longer than 1 week until processing. Root surface area was measured by flatbed scanner and with ImageJ analysis software (https://imagej.net/ImageJ). Subsequently, root biomass was determined by oven drying (48 h, 60 °C) and weighing. Root exudation collection occurred one time before [CO_2] treatment was applied (February 2019), and then three additional times in March, May and June 2019, when introduction to an eCO_2 began at the end of February 2019.

Starch content analysis

Small branches and roots (second–third order, 1- to 3-mm diameter) were sampled from five saplings per species of each room at the end of the experiment (July 2019). Roots were dug up from the middle of the pot. Samples were immediately cooked in a microwave three times for 40 s, to stop any enzymatic or metabolic process (Landhäusser et al. 2018). Samples were transferred to a 60 °C oven for 3 days. All samples were ground using a ball mill (Retsch, Haan, Germany) at a frequency of 20 s^{-1} until tissues had turned into fine powder (~5 min). Dried wood powder (29–31 mg) was extracted with 2-ml deionized water at 90 °C for 30 min. Starch was quantified according to Landhäusser et al. (2018) by an enzymatic reaction. Unfortunately, samples for soluble sugars were lost due to human error, and could not be replicated. Starch was degraded to glucose with alpha amylase (from *Bacillus licheniformis*, cat no. A4551-100 mg, Sigma Aldrich) and amyloglucosidase (from *Aspergillus niger*, Sigma Aldrich, St. Louis, Missouri, USA). Enzyme blend of glucose assay reagent (cat no. G3293-50 ML, Sigma Aldrich, St. Louis, Missouri, USA) was added to the final solution that was quantitatively analyzed using spectrophotometry by evaluating the reaction product. The total amount of formed gluconate-6-phosphate was determined as the increase in NADH+ H+ using a photometer (HR 700; Hamilton, Reno, NE, USA). Starch concentrations were calculated as percentages (w/w) from dry matter.

Soil structure, moisture and nutrient content

Soil samples were collected from three different locations of the mixture pile, before pots were filled. Soil was sampled again, this time from the pots, at the end of the experiment (August 2019). Soil samples were collected from three pots per group. However, due to the minimum amount of soil required for the analyses, replicates were later lumped together. After plant organic materials were removed, soil from three pots per species from each room were mixed and sent to Gilat Field Services Laboratory, Israel, for soil analysis. The following examinations were performed: soil porosity (SP), electrical conductivity (EC), pH, sodium absorption ratio (SAR), calcite content, physical structure (sand, silt and clay content) and mineral content (Cl, Na, Ca, Mg, ammonium-nitrogen ($N\text{-}NH_4$), nitrate-nitrogen ($N\text{-}NO_3$), Olsen-P and K concentrations). Soil moisture was monitored during two consecutive days (48 h) at 3 months following the start of the experiment, for five saplings for each species and tratment (n = 5). In each pot, a calibrated EC-5 dielectric constant soil moisture sensor was installed at mid-depth (Meter, Pullman, WA, USA). Volumetric SWC was measured constantly and recorded each 10 min on a EM50 datalogger (Meter). The mean soil moisture for each individual sapling was claculated as the avergae of these readings over 48 h.

Scaling of carbon allocation fluxes and a sapling-scale carbon balance

Carbon allocation fluxes were up-scaled to the whole-sapling scale using measured values of leaf and root biomass. All fluxes were transformed into g C day^{-1}, using the mass balance approach described in Klein and Hoch (2015). Briefly, total leaf and root areas per sapling were calculated from direct measurements of leaf and lateral root biomass, supplemented by biomass/area ratios measured for each species. Assimilation and leaf respiration rates were summed across sapling crowns and along 12-h periods (the gas exchange system measured net assimilation, and thus accounted for leaf respiration during the day). Correction factors were applied for two of the four species: for *Ceratonia*, values were adjusted to account for the ~50% lower assimilation rates in immature leaves, and for *Cupressus*, values were adjusted to account for the 3D structure of the scales. Root respiration and exudation rates were summed along 24-h periods and across sapling lateral roots and fine roots (50% of lateral root area), respectively. Respiration rates were measured at ~24 °C, the temperature of the growth rooms in which saplings were growing. Carbon allocation into leaf, root and stem growth were calculated as 40% of the biomass change, temporally down-scaled into diurnal resolution. Leaf litter was also temporally down-scaled from monthly into diurnal resolution. Overall, values were averaged across replicates per species and treatment (n = 3–8, depending on the flux). Finally, diurnal C sink fluxes were summed and compared with the diurnal C source flux (assimilation) per species and treatment.

Synthesis and statistical analysis

Raw data analysis, statistical computing and graphics were done using R statistical software (R Development Core Team). Data were checked for normality of the residuals using a Shapiro–Wilk test, and homogeneity of the variances were performed using a Levene's test before applying statistical analyses. We confirmed the normality of the residuals, with P-values ranging from e.g., 0.281 for assimilation to 0.858 for leaf growth in *Pinus* and the homogeneity of the variances with P-values ranging from e.g., 0.184 for root starch to 0.959 for root growth in *Quercus*. Two major statistical analyses were applied: (i) a linear model testing the effects of CO_2, species, CO_2 × species and time, on C allocation fluxes, eco-physiological parameters and biomass allocation across the four studied tree species (Table 1); and (ii)

Table 1. *P*-values for the effects of CO_2, species, CO_2 × species and time, on carbon allocation fluxes, eco-physiological parameters and biomass allocation in the studied tree species. R an, respiration; G, growth; St, starch content; g_s, stomatal conductance; WUE, water-use efficiency; B, biomass. Significant differences are in boldface.

Effect	Assimilation	R_{leaf}	R_{root}	G_{stem}	Exudation	Litter	St_{branch}	St_{root}
CO_2	<**0.0001**	0.1246	0.2624	**0.0051**	0.5823	0.9649	0.1654	0.5761
Species	<**0.0001**	<**0.0001**	**0.0027**	<**0.0001**	0.0955	**0.0002**	<**0.0001**	0.3401
CO_2 ×species	0.4077	0.0851	0.4120	0.9875	0.1512	0.5322	0.5252	**0.0153**
Time	0.2496	**0.0277**	0.1173	N.A.	0.0819	**0.0013**	N.A.	N.A.

Effect	Transpiration	g_s	WUE	B_{leaf}	B_{stem}	B_{root}
CO_2	0.1801	0.4542	<**0.0001**	0.1017	0.2125	0.6219
Species	**0.0422**	<**0.0001**	**0.0003**	**0.0047**	**0.0532**	**0.0004**
CO_2 ×species	0.3052	0.6371	0.9130	0.1393	0.3094	0.2439
Time	**0.0006**	**0.0007**	<**0.0001**	N.A.	N.A.	N.A.

N.A., not available.

split plot analysis of variance (ANOVA) tests for the sensitivity of measured responses to eCO_2, separately for each of the four species at each of four time-points (see Tables S1–S3 available as Supplementary data at *Tree Physiology* Online), which were followed by a post hoc Tukey honest significance test, where needed. For statistical test of dynamic responses (see Supplementary data at *Tree Physiology* Online) a repeated measures ANOVA was used. In the case of root exudations and root and leaf respiration, the change from the baseline mean was calculated before statistical analyses were applied.

Results

The effect of eCO_2 on aboveground and belowground carbon allocation

Carbon allocation sinks were calculated at the whole-sapling scale and their partitioning was presented in relation to Carbon source of each species and treatment (Figure 1). Overall, C sinks were well balanced with the C source of 0.5–1.3 g C day^{-1}, and differences could relate to missing sinks (e.g., stem respiration not measured here), or allocation to storage. Across species and treatments, root respiration was the largest sink, accounting for 25–32% and 41–59% of sinks in conifers and broadleaved species, respectively. Leaf respiration was larger in conifers, 16–24% of sinks, compared with 7–10% in broadleaf species. Growth processes consumed 29–47% of the assimilated C, mostly allocated to woody tissues (stem and roots), whereas root exudation was a smaller sink of 4–16% of all sinks, and litter production merely 1–2%. Elevated CO_2 shifted C allocation fluxes in species-specific manner: in *Quercus*, root respiration increased on expense of root growth and exudation. In *Ceratonia*, stem growth increased whereas the share of leaf respiration and root exudation decreased. In *Pinus*, root exudation and stem growth increased on expense of root growth and respiration. In *Cupressus*, leaf growth and respiration increased whereas stem growth decreased. The

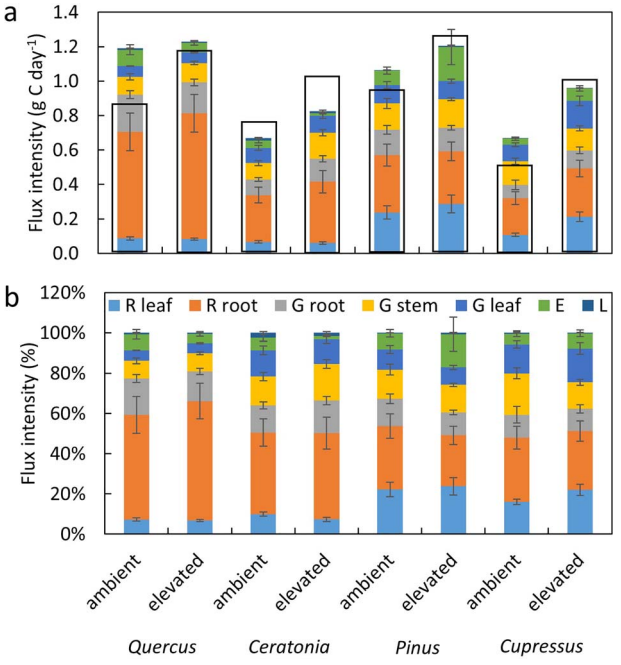

Figure 1. C source (black frames in a) and partitioning of C sinks (bars indicating g C day^{-1} in a, and % of total sinks in b) in *Quercus*, *Ceratonia*, *Pinus* and *Cupressus* under ambient and eCO_2 conditions. Values are means ± SE (n = 3–8, depending on C flux). R, respiration; G, growth; E, exudation; L, litter production.

following sections present the eCO_2 effect on each specific C flux, and its statistical significance.

eCO_2 effect on carbon uptake (net assimilation)

There was a highly significant increase in photosynthesis under eCO_2 compared with ambient conditions (Figure 2; 8.0 and 5.9 μmol m^{-2} s^{-1}, respectively, $P < 0.001$, n = 8; see Table S1 available as Supplementary data at *Tree Physiology* Online). Photosynthesis was also significantly different among the species (Table 1). The highest assimilation rate occurred in *Quercus* saplings under eCO_2 during the last campaign, with a mean

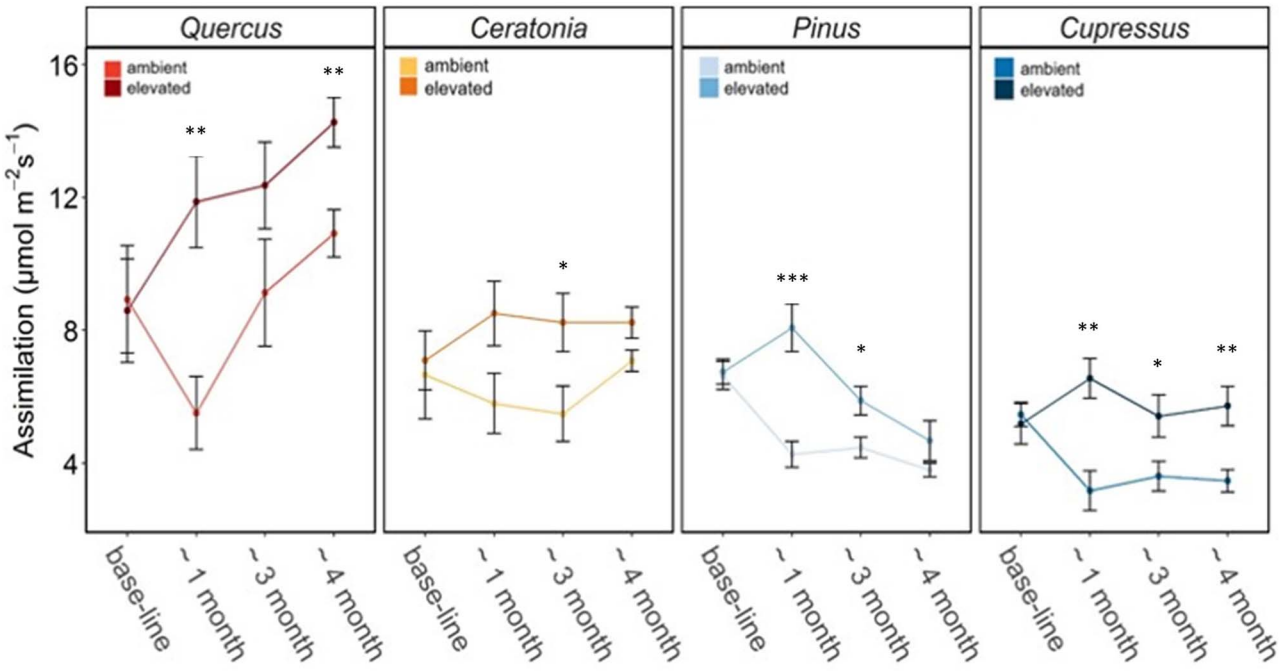

Figure 2. Leaf net assimilation dynamics in saplings of four tree species in two climate controlled rooms, with aCO_2 vs eCO_2, in four time points during the experiment. Dots represent the mean assimilation, for each group, with the standard error (n = 8). Significant differences between aCO_2 and eCO_2 within species are noted by asterisks (***P-value <0.001, **P-value <0.01 and *P-value <0.05).

of 14.3 µmol m^{-2} s^{-1}. The lowest assimilation rate occurred in *Cupressus* saplings under ambient conditions, at the second campaign, with an assimilation rate mean of 3.2 µmol m^{-2} s^{-1}. Conifer assimilation rates might have practically been even lower, since our calculation using projected leaf area neglected needle and scale thickness. This rate remained steady during the next campaigns (with a mean of ~3.5 µmol m^{-2} s^{-1}; Figure 2). Differences were most significant at the second campaign, 1 month after treatment began. In the next campaigns, differences progressively decreased (e.g., for *Pinus*) or decreased with fluctuations (as the others).

The fate of surplus carbon

Across the species, both above and belowground biomass growth showed no significant effect of the treatment (Table 1). Nevertheless, *Cupressus* and *Ceratonia* showed a slight, positive, response to the [CO_2] increase (Figure 3), whereas *Pinus* did not demonstrate any change. *Quercus* did not show a response in aboveground biomass either, but it demonstrated a small decrease in belowground biomass. At the compartment level, a characteristic biomass distribution within plant tissues was typical for each species (see Figure S2 available as Supplementary data at *Tree Physiology* Online). *Quercus* saplings allocated more C to roots than to leaves and stems, whereas all other species allocated more C in aboveground tissues than in root systems (see Figure S2 available as Supplementary data at *Tree Physiology* Online). Again, no statistically significant effect was shown. Under eCO_2, *Quercus* root system had less biomass increment than under aCO_2 (mean of 54.1 and 64.5 g). This decrease appeared in both tap and lateral roots. *Ceratonia* saplings under eCO_2 had larger stem and root system, than those growing under aCO2. Stem and lateral root increment under eCO_2 with P-values of 0.071 and 0.087, respectively (see Table S2 available as Supplementary data at *Tree Physiology* Online). Shoot diameters were measured for all saplings in both rooms (n = 32), pre- and post-experiment, as another approach to follow growth (see Figure S3 available as Supplementary data at *Tree Physiology* Online). A positive eCO_2 effect was shown in shoot diamenter increment (see Table S2 available as Supplementary data at *Tree Physiology* Online; $P < 0.001$). Differences were significant for gymnosperm species, but not for angiosperms (Table 2).

Leaf and root respiration rates were measured by IRGA for three to five samples in each group, divided into four campaigns. The aggregated data indicated species differences (Table 1), where only *Cupressus* showed a significant increase in leaf respiration rate as a response to the [CO_2] elevation (P = 0.008). Regarding root respiration rates, only *Quercus* demonstrated a significant effect (P = 0.003). Under ambient conditions, *Quercus root* respiration rates decreased, whereas for eCO_2 they increased (Figure 4). Leaf respiration rate of *Cupressus* under eCO_2 conditions increased significantly in comparison with the baseline, whereas under ambient conditions it remained stable. *Ceratonia* and *Pinus* under eCO_2

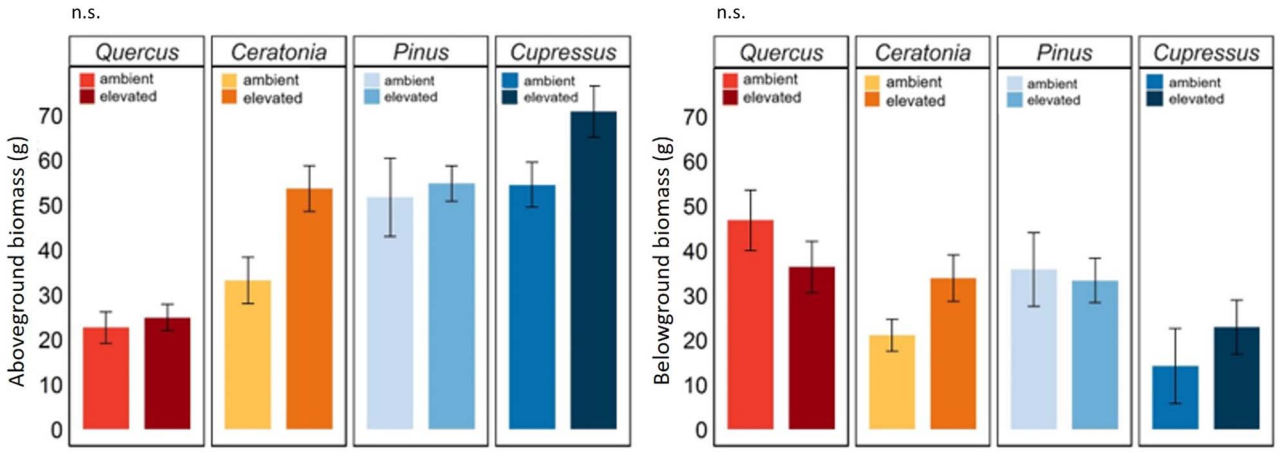

Figure 3. Above- and belowground biomass growth in saplings of four species in two climate controlled rooms, with aCO_2 vs eCO_2. Bars represent the means of post-experiment biomass minus the means of pre-experiment biomass for each species, with standard errors (n = 3). Differences between aCO_2 and eCO_2 within species were not significant (n.s.).

Table 2. Shoot diameter increment of four tree species after 6 months growing under aCO_2 vs eCO_2 conditions.

Species	Treatment	Shoot diameter increment (mm)	SE	*P*-value	
Quercus	Ambient	1.91	0.34	0.446	
	Elevated	2.39	0.50		
Ceratonia	Ambient	4.11	0.52	0.312	
	Elevated	4.89	0.52		
Pinus	Ambient	2.17	0.11	<0.001	***
	Elevated	3.47	0.27		
Cupressus	Ambient	1.95	0.46	0.005	**
	Elevated	3.55	0.15		

***, P-value < 0.001; **, P-value < 0.01.

showed a higher decrease from the baseline, in comparison with saplings under aCO_2. Those changes were significant for *Ceratonia* but not for *Pinus* (P = 0.015 and 0.207). Nonetheless, at the third campaign, the leaf respiration rate of *Pinus* under aCO_2 showed a highly significant decrease (P < 0.001; Figure 4).

Leaf litter from five saplings for each species was collected in both rooms, three times during the experiment. The aggregated data showed no significant difference between ambient and eCO_2 leaf litter mass (see Table S1 available as Supplementary data at *Tree Physiology* Online). However, *Pinus* under eCO_2 showed a trend of increasing leaf litter production (Figure 5), with a significant effect of time across the four species (Table 1). Root exudates were collected at four campaigns and were analyzed for dissolved organic C. Root exudation transiently increased in *Quercus* and *Pinus*, and decreased in *Ceratonia* and *Cupressus* in comparison with the baseline values. There were no significant differences between the treatments (Figure 6). The opposite trend occurred with *Pinus* exudates, which remained stable under ambient conditions, but increased under eCO_2 (P = 0.187). *Cupressus* demonstrated a significant decrease under eCO_2 conditions at the last campaign (P = 0.007).

The effect of eCO_2 on eco-physiological traits

In general, angiosperms maintained ~10 times more starch in branches than gymnosperms (~5% starch in angiosperms vs ~0.5% in gymnosperms; Figure 7; a significant species effect in Table 1). Among the species, only *Pinus* branches demonstrated a significant decrease in starch content (P = 0.005; see Table S2 available as Supplementary data at *Tree Physiology* Online), from ~0.7% to ~0.1%. Root starch was not significantly different either, but *Quercus* saplings under eCO_2 held a trend of more starch in roots than under aCO_2, still not significant (P = 0.059). In contrast, roots of gymnosperm species, *Pinus* and *Cupressus*, contained less starch when grown under eCO_2 conditions (P = 0.084 and 0.101, respectively). *Ceratonia* roots contained about the same level of starch under both conditions (~0.67%). As a result, a significant interaction between species and CO_2 level emerged (Table 1).

Leaf WUEi was calculated based on the ratio between net assimilation and stomatal conductance (see Figure S4 available

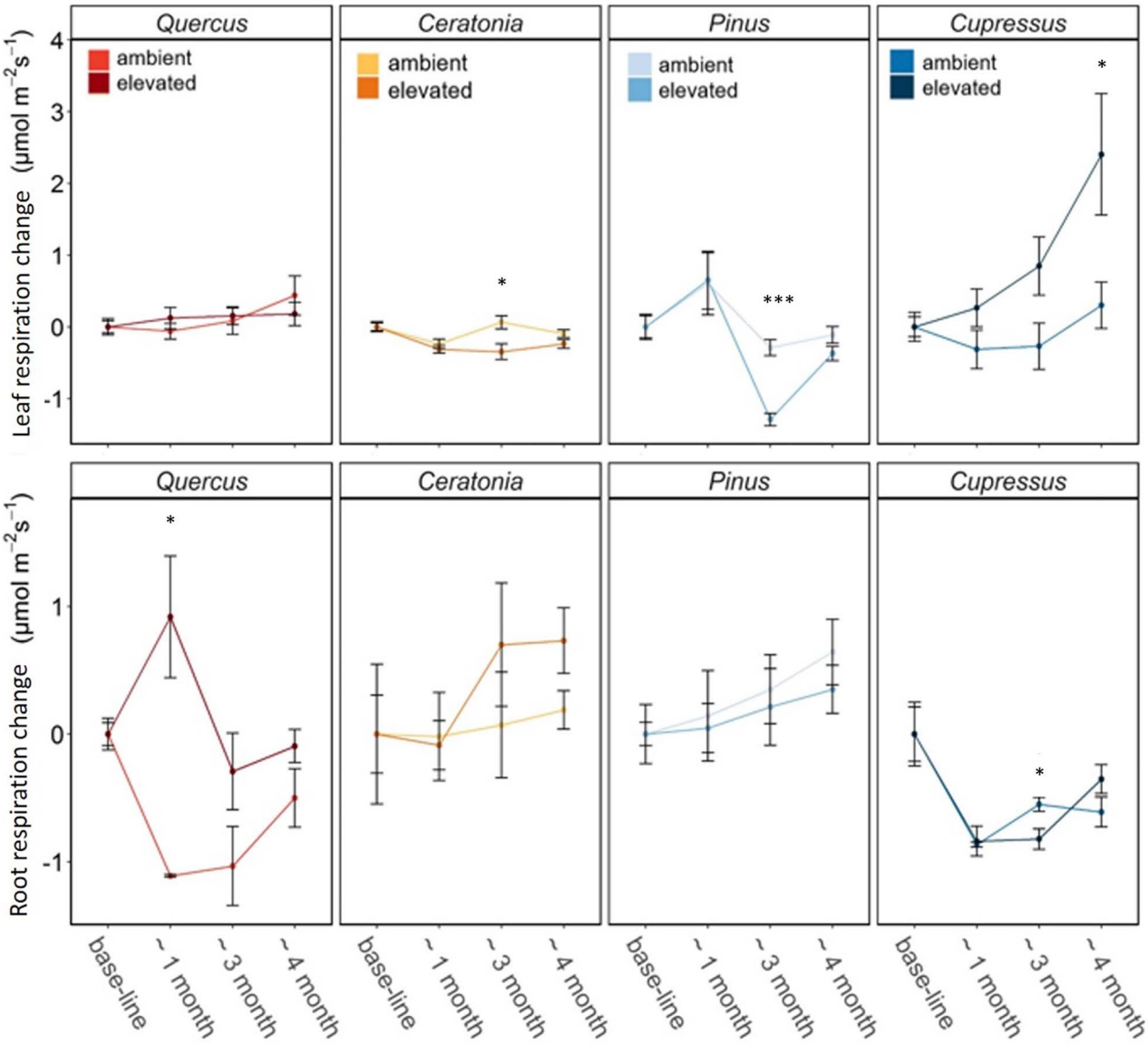

Figure 4. Leaf and root respiration dynamics in saplings of four species in two climate controlled rooms, with aCO_2 vs eCO_2, in four time points during the experiment. Dots represent the mean of differences from the baseline rates for each group with the standard error (n = 5 for leaf respiration rate, and 3–5 for root respiration rate). Significant differences between aCO_2 and eCO_2 within species are noted by asterisks (***P-value <0.001, **P-value <0.01 and *P-value <0.05).

as Supplementary data at *Tree Physiology* Online) in four campaigns during the experiment. Aggregated data of all saplings combined for each room (n = 32) showed a highly significant increase in WUEi under eCO_2 conditions (Table 1 and see Table S3 available as Supplementary data at *Tree Physiology* Online; P < 0.001). Significant differences were also observed within species, more so for gymnosperms than for angiosperms (Figure 8).

Soil moisture was monitored during 2 days for five saplings for each species and tratment (n = 5). Except for soil of *Pinus* saplings, soil moisture was higher under eCO_2 than under aCO_2 (Figure 9). Differences between pots under eCO_2 and aCO_2 were significant for angiosperm species, *Quercus* and *Ceratonia* (P = 0.01 and 0.003, respectively), but not for the gymnosperm species. All saplings of both rooms received precisely the same amount of water throughout the experiment and the pre-experiment processes. During the experiment, water did not drip from the pots. Therefore, soil moisture can represent the tree water-use, along with the constant, and equal, evaporation from the pot surface. Hence, it may be assumed that angiosperm species used less water when growing under eCO_2. Considering the transpiration measurements of the last campaign in June 2019 (the closest to the time that SWC were monitored), higher transpiration rates were measured for saplings under aCO_2 than

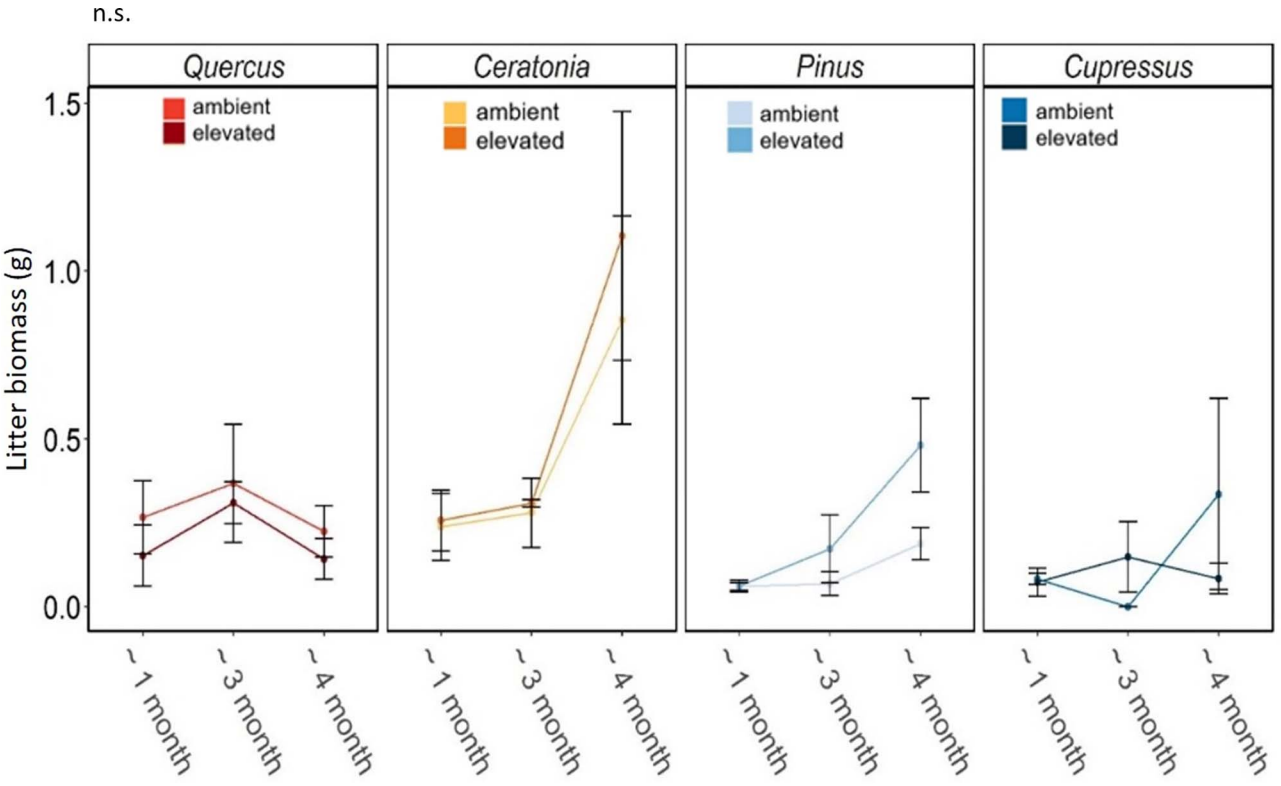

Figure 5. Leaf litter biomass dynamics in saplings of four species in two climate controlled rooms, with aCO_2 vs eCO_2, in three time points during the experiment (litter at baseline was either zero or negligible). Dots represent the mean assimilation, for each group, with the standard error (n = 5). Differences between aCO_2 and eCO_2 within species were not significant (n.s.).

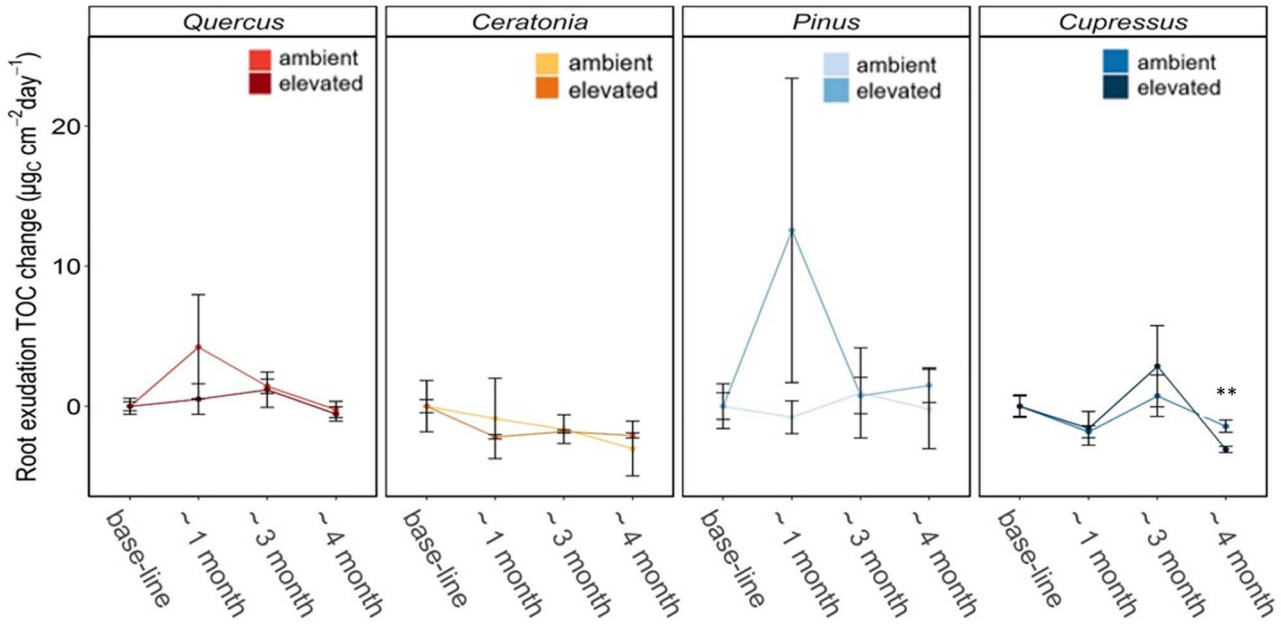

Figure 6. Root exudation dynamics in saplings of four species in two climate controlled rooms, with aCO_2 vs eCO_2, in four time points during the experiment. Dots represent the mean of differences from the baseline rates for each group, with standard error (n = 4). Significant differences between aCO_2 and eCO_2 within species are noted by asterisks (**P-value < 0.01).

under eCO_2 (see Figure S5 available as Supplementary data at *Tree Physiology* Online). Nevertheless, the difference was significant only for *Ceratonia* ($P < 0.001$).

In most soil parameters, there were differences between aCO_2 and eCO_2. In the case of pH, except for *Quercus* soil, a lower pH value was shown in soil from the eCO_2 room (highest

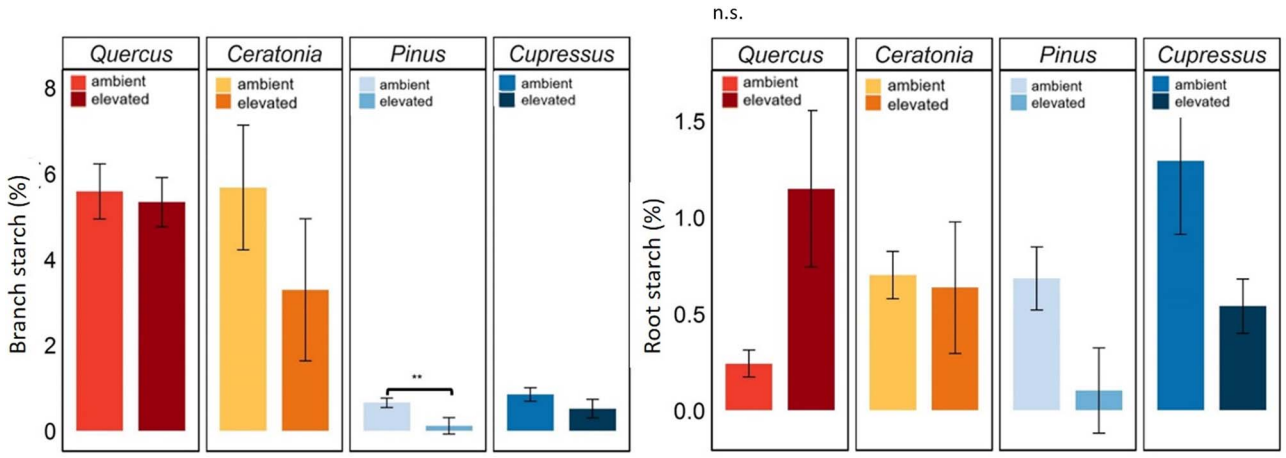

Figure 7. Post-experiment starch content, in fine roots and branches, in four sapling species in two climate controlled rooms, with aCO_2 vs eCO_2. Bars represent the means of post-experiment starch content of both tissues for each group, with standard errors (n = 5). Significant differences between aCO_2 and eCO_2 within species are noted by asterisks (**P-value <0.01). Differences in root starch between aCO_2 and eCO_2 within species were not significant (n.s.).

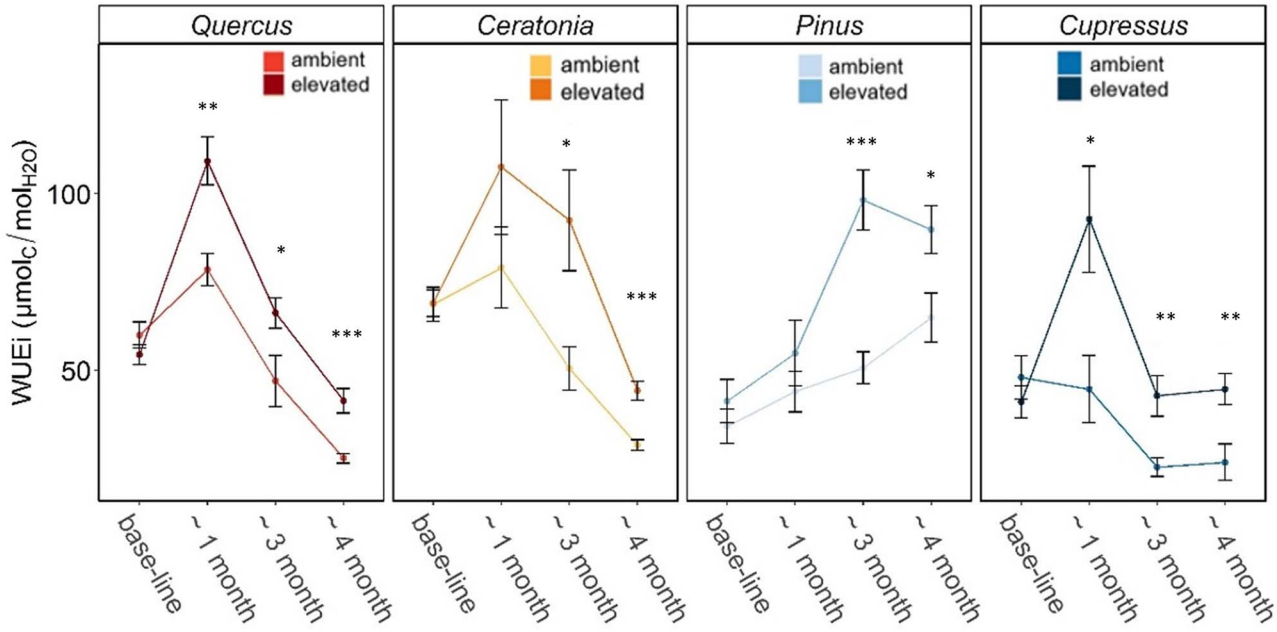

Figure 8. Leaf WUEi dynamics in saplings of four species in two climate controlled rooms, with aCO_2 vs eCO_2. Dots represent the mean leaf WUE, for each group, with standard errors (n = 8). Significant differences between aCO_2 and eCO_2 within species are noted by asterisks (***P-value <0.001, **P-value <0.01 and *P-value <0.05).

value for the *Ceratonia* and *Cupressus* soil of the aCO_2 room, lowest value for the *Pinus* soil of the eCO_2 room; Table 3). The same happened with chlorine (Cl) content, which increased in all samples, probably due to high level of Cl in the irrigation water, but here *Quercus* soil showed much lower value in the aCO_2 room compared with *Quercus* soil in the eCO_2 room. Electrical conductivity highly increased in all samples, more so under eCO_2. Sodium (Na), calcium (Ca) and magnesium (Mg) were higher in all samples compared with pre-experiment samples. Sodium and Ca were always higher in samples from the aCO_2 room. Magnesium had the same trend of lower value in soil from the eCO_2 room, but only for angiosperm species. Soil from gymnosperm species pots showed the opposite trend. Post-experiment values of potassium (K) remained under standard error values range of pre-experiment samples. Lower values of K content were shown in soil from gymnosperms under eCO_2. Phosphorus (P) decreased in all samples. Except for samples from *Pinus* saplings (for both treatments), in all other samples the values of P were lower than detection threshold (below 3 mg kg^{-1}). *Pinus* samples

Table 3. Soil properties from pots where saplings of four tree species were grown under treatments of aCO_2 and eCO_2 conditions. 'Before' row show mean values of soil that were taken from three different locations of the pile, before pots were filled up, with standard errors (n = 3). Other rows represent the values of soil that were mixed together, post-experiment, from three pots per group.

Soil chemistry: pH, EC, SAR, and minerals content (Cl, Na, Ca, Mg, N-NH_4, N-NO_3 and Olsen-P concentrations). Samples were taken from three pots per group (for technical reasons, replicates were lumped together; see Materials and methods).

Group	Treatment	pH	EC ds m^{-1}	Cl mg L^{-1}	Na mEq L^{-1}	Ca mg L^{-1}	Mg mg L^{-1}	SAR	N-NO3 mg kg^{-1}	N-NH4 mg kg^{-1}	K of CaCl2	P mg kg^{-1}
Before	Pre	7.5 ± 0.06	1.9 ± 0.07	224.8 ± 7.56	6.1 ± 0.4	270.1 ± 10.67	30.4 ± 2.24	2.1 ± 0.12	18.9 ± 3.04	25.6 ± 3.41	49.4 ± 3.93	11.7 ± 2.96
Quercus	Ambient	7.3	8.00	759.2	34.2	733.3	162.7	6.83	13.1	4.55	46.6	3.3
Quercus	Elevated	7.3	6.78	1027.3	28.3	677.3	146.3	5.92	7.2	4.97	44.3	<3
Ceratonia	Ambient	7.4	8.21	1528.5	35.0	473.9	160.1	8.16	14.1	3.81	44.3	<3
Ceratonia	Elevated	7.2	6.48	1080.1	28.3	364.4	137.4	7.38	2.5	4.1	46.6	<3
Pinus	Ambient	7.2	8.33	1598.1	35.8	550	175.6	7.83	14.5	7.56	46.6	3.8
Pinus	Elevated	6.9	7.84	1483.5	35.0	442.7	182.0	8.13	1.5	7.07	39.6	3.9
Cupressus	Ambient	7.4	8.37	1347.3	35.0	855.7	165.8	6.59	15.1	2.96	42.0	<3
Cupressus	Elevated	7.3	7.81	601.7	34.2	268.2	176.3	9.15	7.4	2.87	39.6	<3

Physical structure: sand, silt and clay content, calcite content ($CaCO_3$) and SP.

Group	Treatment	Sand %	Silt %	Clay %	$CaCO_3$%	Saturation %
Before	Pre	9.00 ± 3.46	19.67 ± 2.08	71.33 ± 5.51	3.67 ± 1.15	37.33 ± 2.08

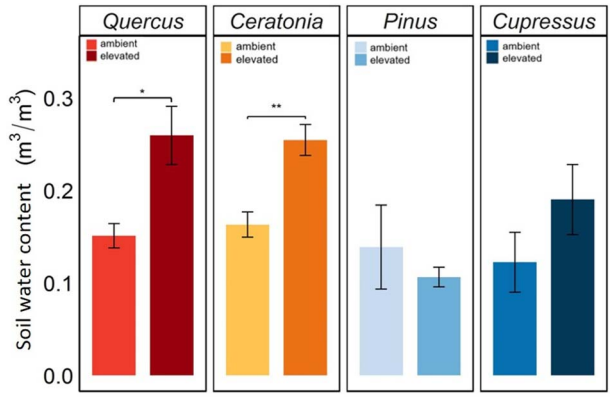

Figure 9. Soil water content in pots of saplings of four species in two climate controlled rooms, with aCO_2 vs eCO_2. Bars represent the mean for average of 48 h continuous measurements from five pots for each group, with standard errors (n = 5). Significant differences between aCO_2 and eCO_2 within species are noted by asterisks (**P-value <0.01 and *P-value <0.05).

had a detectable, still low, values of 3.8–3.9 mg kg^{-1}. Nitrogen decreased in all samples (Figure 10). Ammonium-nitrogen (N-NH_4) similarly decreased for each species without a clear effect of the treatment. *Cupressus* soil had the lowest content of N-NH_4 at the end of the experiment, 2.86–2.96 mg kg^{-1}, whereas *Pinus* soil contained 7.07–7.56 mg kg^{-1}, compared with 25.6 ± 3.41 mg kg^{-1} in pre-experiment soil. Nitrate-nitrogen (N-NO_3) decreased under eCO_2, when N–NO_3 in soil from pots of the aCO_2 room was quite conserved among the different species (13.1–15.1 mg kg^{-1}, compared with 18.9 ± 3.04 mg kg^{-1} in pre-experiment soil). Soil samples of *Quercus* and *Cupressus* under eCO_2 contained 45% and 51% less N–NO_3, respectively, than soil of the same species under aCO_2. In soil of *Ceratonia* and *Pinus*, N–NO_3 contents were ~82% and 90%, respectively, lower in soil from the eCO_2 room than from the aCO_2 room.

Discussion

The fate of carbon in saplings of four species under eCO_2

We showed that assimilation, WUE and soil N uptake significantly increased at eCO_2 across four species grown under controlled conditions. Broadleaf species showed soil water savings, which were absent in conifers. These results are well in line with those of previous studies (e.g., Curtis and Wang 1998, Cernusak et al. 2013), which tested some, but not all, of the parameters tested here. All other effects were species-specific, e.g., an increased root respiration in *Quercus* and leaf respiration *Cupressus*, a decreased leaf respiration in *Ceratonia* and decreased wood starch in *Pinus*. Elevated CO_2 did not affect growth, nor litter production, and the effects on root exudation rates were inconsistent, with transient increases in *Pinus* and *Quercus*, but not in *Ceratonia*. Still, the answer to the question of the fate of this surplus C is more complex. This study tracks, for the first time, almost all major fluxes that are potential sinks of the C. The first conclusion that appeared from the results of this experiment is that the response to [CO_2] elevation is mostly species-specific and not consistent across angiosperms or gymnosperms. This result was repeated in both the C balance approach (Figure 1) and the individual flux responses (Figure 11). In the case of *Quercus*, saplings under eCO_2 demonstrated more root activities such as root starch storage and root respiration, whereas aboveground activity did not significantly change. Young *Quercus* trees are known to have a slow shoot growth and higher investment in soil acclimation and root system development (Collet et al. 2006), and they indeed invested more in belowthan aboveground growth. *Ceratonia* saplings accumulated more biomass when growing under eCO_2, both above- and belowground. Trends were recorded for higher stem and lateral root biomass (P = 0.071 and 0.087). At the same time, leaf respiration rate in those saplings were decreased compared with saplings under ambient conditions. Root respiration

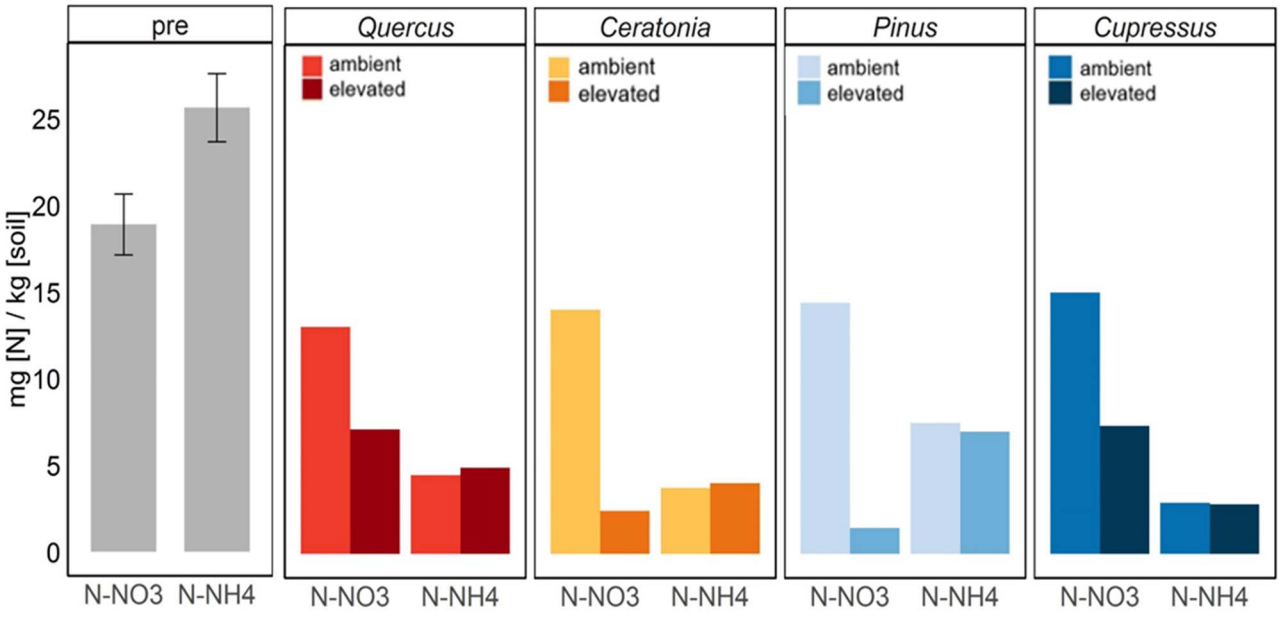

Figure 10. Post-experiment ammonium-nitrogen($N\text{-}NO_3$) and nitrate-nitrogen($N\text{-}NO_4$) content in soil from pots where saplings of four tree species were grown. 'Pre' bars (gray) represent mean value of N content in soil that were taken from three different locations of the pile, before filled in pots with standard errors (n = 3). Other bars represent N content in soil post-experiment, from three pots per group (for technical reasons, replicates were lumped together; see Materials and methods).

rates of *Ceratonia*, measured in the two treatments, did not show statistically significant difference. *Pinus* saplings under eCO_2 contained almost no starch storage in fine roots and small branches (~0.1%), whereas under aCO_2 they contained ~0.65%. This finding was unexpected considering earlier eCO_2 experiments (Li et al. 2018), and that the use of starch is usually stress-related (Paudel et al. 2018), and specifically in *Pinus halepensis* (Klein et al. 2014). Soluble sugars (not quantified here) might have buffered this change in starch, as they tend to increase under eCO_2 (Li et al. 2018). In our case, not only were the saplings not limited in their assimilation capacity, the C uptake through assimilation even increased. One explanation could be that because of the exposure to eCO_2 conditions, the need to store C decreased. Although no other significant responses were recorded, a trend of an increase in leaf litter and root exudation was shown in *Pinus* (P = 0.07 and 0.187). Taken together, these observations may indicate the multiple ways by which the surplus C escapes the tree, either in a form of dissolved C through root exudation, or otherwise as a solid matter in leaf litter. It should be noted that although no significant biomass growth effect was detected in *Pinus*, shoot diameter increment during the experiment was significantly higher under eCO_2 ($P < 0.001$). *Cupressus* showed a higher aboveground activity under eCO_2. Its higher leaf growth at eCO_2 was accompanied by an increase in leaf respiration, whereas under ambient conditions, leaf respiration remained approximately stable during the experiment. Among the gymnosperm species, two parameters seemed to have a common response: similar to *Pinus*, root starch content in *Cupressus* also decreased under eCO_2, although not significantly (P = 0.101). However, this limitation may explain the moderation that appeared in some parameters at the third and the fourth campaign (Figures 2, 4 and 6 and Figure S5 available as Supplementary data at *Tree Physiology* Online).

Root–soil interaction and water relations in saplings of four species under eCO_2

In addition to the direct effect that eCO_2 has on the tree C balance, a few indirect effects were recorded in terms of root–soil interactions and water relations. Previous studies have shown that enriched atmospheric [CO_2] is associated with plant water status (e.g., Leuzinger et al. 2005, Paudel et al. 2018). The closest association is through stomatal activity. Higher [CO_2] enables the plant to reduce stomatal aperture without reducing C uptake, in turn improving its WUE. This leads to lower water consumption and higher soil moisture (Cech et al. 2003, Bader et al. 2009, Paudel et al. 2018). However, we do not know to what extent the WUEi increase was related to properties of new leaves grown under the eCO_2 (potentially having, e.g., lower stomatal density; Steinthorsdottir et al. 2013), rather than older leaves. By the end of the experiment, the foliage of most saplings had equal amounts of old and new leaves, except for *Cupressus* under eCO_2, with 63% new leaves. It was also found that gymnosperm stomata are less [CO_2] sensitive than angiosperm stomata (Klein and Ramon 2019). In this experiment, the increase in WUE was overarching. This might be due to comprehensive increase in

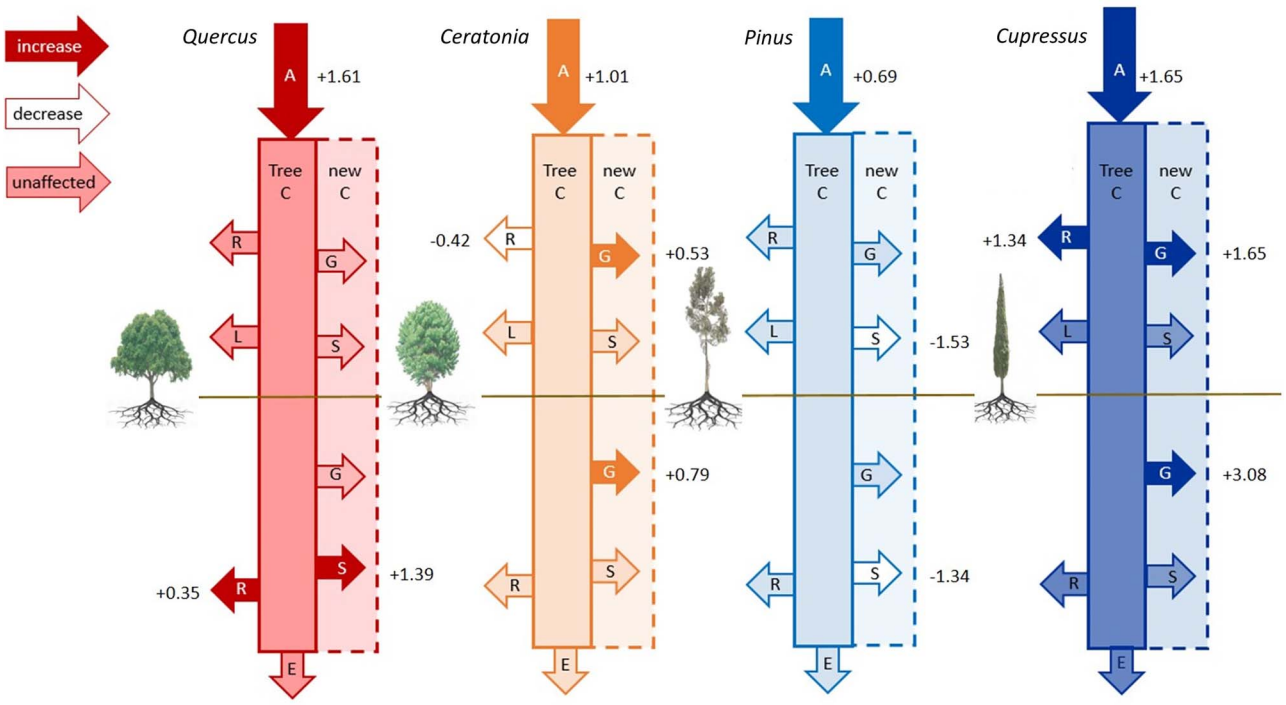

Figure 11. The fate of surplus C in *Quercus*, *Ceratonia*, *Pinus* and *Cupressus* (left to right) under eCO_2 conditions. Sapling response to eCO_2 was assessed in nine major parameters. A represents the surplus C uptake by the plant through photosynthesis. G, R and S represent the effect on above and belowground C sink for growth, respiration and starch content, respectively, whereas L and E represent the effect on leaf litter and root exudation, respectively. Filled arrows represent an increase in the parameter under eCO_2 compared with saplings under ambient conditions; empty (white) arrows represent a decrease in the parameter and transparent arrow represent unaffected parameter (legend). Numbers are effect sizes. Effects were determined for $P < 0.1$.

C uptake, while, overall, transpiration rate was not significantly changed. Nevertheless, SWC significantly increased in pots of the angiosperm, but not gymnosperm, species, indicating lower water consumption in the former. *Cupressus* had higher leaf biomass, therefore it could be expected to transpire more and therefore to have lower soil moisture. The same species with higher soil moisture, correspondingly showed a decrease in g_s at the last campaign (the closest to the SWC measurement time; Figure S4 available as Supplementary data at *Tree Physiology* Online), although a statistical significance was noted only for *Ceratonia* ($P = 0.011$). Higher soil moisture under angiosperms should not be related to higher leaf turnover, as leaf litter biomass results show same values for the *Pinus* and the *Quercus* saplings (Figure 5). Root biomass was also unaffected by CO_2 (see Figure S2 and Table S2 available as Supplementary data at *Tree Physiology* Online). A potential explanation for the water savings is an eCO_2-induced decrease in specific leaf area (Ainsworth and Long 2005), however we did not measure it here. These divergent stomatal conductivity responses to the eCO_2 treatment, might be related to the longer evolutionary lineages of gymnosperms, which originated in a higher $[CO_2]$ world, and that the decrease in atmospheric $[CO_2]$ at the time that angiosperm species originated, is an evolutionary driver of high stomatal sensitivity (Klein and Ramon 2019).

Soil chemistry differences between treatments were shown among all species. Since all pots were filled from the same mixture pile of unified soil, and received the same irrigation regime and the same water source, we may assume that all pots had the same soil chemistry and physical properties. Therefore, differences in post-experiment mineral contents were attributed to $[CO_2]$ effects and species, inherent properties. At the end of the experiment, all species were under phosphorus limitation. This may have an impact and may mediate the effect of the treatment, e.g., to limit a potential growth increase response (Schleppi et al. 2019). Nitrogen uptake responses to treatments were also comprehensive among species. In the case of $N\text{-}NH_4$ in soil, it decreased with no relation to eCO_2 in all species. In contrast, $N\text{-}NO_3$ was consumed more under eCO_2, and almost depleted from soil in *Pinus* and *Ceratonia*. Previous studies showed that plant N uptake increased under eCO_2 (Norby et al. 2010, Nie and Pendall 2016). It is also known that the positive effect of eCO_2 on plant production and C allocation to rhizosphere may decline because soil N becomes a limiting factor (Luo et al. 2004, Usyskin-Tonne et al. 2020). Phillips et al. (2009) studied the increase in growth and root exudation in *Pinus taeda* seedlings, and found that it increased the mass-specific root exudation under eCO_2, but only under N limitation in soil. This may be the case in our study, where *Pinus* soil

contained the lowest N-NO_3, and showed a small increase in root exudates. Another factor that can affect and be affected by the soil nutrient and plant response to eCO_2 is the soil microbial community. It was demonstrated that N cycle-related activities, e.g., denitrification, are effected by eCO_2 via the change in quality and quantity of plant exudates (Smart et al. 1997, Drigo et al. 2010, Yu et al. 2018). In this experiment, we used a native forest soil containing a variety of microbial species, including *Suillus granulatus*, which is a well-known arbuscular mycorrhiza of pine (Prieto et al. 2016, García-Rodríguez et al. 2017). Indeed, in *Pinus* roots, mycorrhizal hyphae appeared in both treatments (see Figure S6 available as Supplementary data at *Tree Physiology* Online). Soil samples from the rhizosphere were collected and future identification and quantification of fungi and bacteria should be done.

Concluding remarks

This study's results are important to understanding the sensitivity of tree C allocation to the ongoing [CO_2] increment. Here we broadly confirm the claim that eCO_2 results in increased C uptake, WUE and soil N uptake, as recently reviewed by Walker et al. (2021). However, higher C uptake will appear only until other elements are depleted. In our case, these elements are N and P, as shown in other forest tree species (Norby et al. 2010, Crous et al. 2019), but there can be other limiting nutrients. Other field studies have already shown that at the current CO_2, C is not the limiting factor for trees (Körner 2003). In some forests, a deficit in available nutrients such as N, P and Mg was already demonstrated to limit tree production and growth in spite of atmospheric [CO_2] elevation (Luo et al. 2004, Bader et al. 2010, Schleppi et al. 2019). Nutrient limitation then probably moderates the increase in WUE too. Water savings shown by broadleaf and not conifers confirm the results from the Swiss FACE near Basel (Körner et al. 2005, compared with Leuzinger and Bader 2012) and also in our global, multi-species analysis (Klein and Ramon 2019). Considering the above, it can be assumed that nutrients or at least macro-elements will be a limiting factor in future forests. Combining this work with the results of our recent meta-analysis (Klein and Ramon 2019) indicates a potentially inherent difference between gymnosperms and angiosperms in terms of stomatal sensitivity to the change in [CO_2] and hence in water saving in the favor of the latter, although other evidence have also been shown, including in a water-limited forest (Gimeno et al. 2018). This difference may have an important role in a high [CO_2] future, and has to be taken into consideration when making predictions of global water cycle. Since gymnosperm and angiosperm species distribution diverges across forest types, the effect of eCO_2 in the future will be diverse among ecosystems. While gymnosperms are dominant in the upper latitudes, tropical, subtropical and temperate angiosperm ecosystems might demonstrate higher soil moisture in the future then in temperate and boreal gymnosperm ecosystems. Other global change factors that which we did not consider in this study are global warming and longer periods of drought (IPCC et al. 2014). Their interactions with eCO_2 in affecting tree functions are not sufficiently understood, and it is possible, e.g., that warming and drying could cancel out water savings related with eCO_2 effects on broadleaf species (Picon et al. 1996, Paudel et al. 2018). However, all other effects in our studies were species-specific, hence we conclude that the sinks for surplus C depend on the species, and that physiological responses to eCO_2 should not be generalized. Further research is required to determine which eCO_2 effects are conserved at the phylogenetic/functional type, and which are not.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

Authors' contributions

D.D. performed the study designed by T.K. The authors wrote the paper jointly.

Acknowledgments

We would like to thank: first, the Jewish National Fund (KKL) for providing the saplings for the experiment (through the nursery in Eshtaol); second, M. Poraz Ltd for installation of the CO_2 monitoring system; third, the Weizmann institute greenhouse facility crew for their help with the establishment of the controlled growth room; and finally, the Editor and reviewers who helped us to further develop the messages of the paper.

Funding

This work was supported by the Merle S. Cahn Foundation and the Monroe and Marjorie Burk Fund for Alternative Energy Studies; Mr and Mrs Norman Reiser, together with the Weizmann Center for New Scientists; the Yeda-Sela Center for Basic Research and the Edith & Nathan Goldberg Career Development Chair. The authors declare no conflict of interest.

Data availability statement

All data used in this study is reported in the paper.

Conflict of Interest

The authors declare no conflict of interest in the preparation of this research paper.

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