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Research paper

CO₂ refixation is higher in leaves of woody species with high mesophyll and stomatal resistances to CO₂ diffusion

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The percentage of respiratory and photorespiratory CO₂ refixed in leaves (P_r) represents part of the CO₂ used in photosynthesis. The importance of P_r as well as differences between species and functional types are still not well investigated. In this study, we examine how P_r differs between six temperate and boreal woody species: *Betula pendula*, *Quercus robur*, *Larix decidua*, *Pinus sylvestris*, *Picea abies* and *Vaccinium vitis-idaea*. The study covers early and late successional species, deciduous broadleaves, deciduous conifers, evergreen conifers and evergreen broadleaves. We investigated whether some species or functional types had higher refixation percentages than others, whether leaf traits could predict higher P_r and whether these traits and their impact on P_r changed during growing seasons. Photosynthesis CO₂ response (A/C_i)-curves, measured early, mid and late season, were used to estimate and compare P_r , mesophyll resistance (r_m) and stomatal resistance (r_s) to CO₂ diffusion. Additionally, light images and transmission electron microscope images were used to approximate the fraction of intercellular airspace and cell wall thickness. We found that evergreens, especially late successional species, refixed a significantly higher amount of CO₂ than the other species throughout the entire growing season. In addition, r_m , r_s and leaf mass per area, traits that typically are higher in evergreen species, were also significantly, positively correlated with P_r . We suggest that this is due to higher r_m decreasing diffusion of (photo) respiratory CO₂ out of the leaf. Cell wall thickness had a positive effect on P_r and r_m , while the fraction of intercellular airspace had no effect. Both were significantly different between evergreen conifers and other types. Our findings suggest that species with a higher r_m use a greater fraction of mitochondria-derived CO₂, especially when stomatal conductance is low. This should be taken into account when modeling the overall CO₂ fertilization effect for terrestrial ecosystems dominated by high r_m species.

Keywords: boreal trees, CO₂ refixation, ecophysiology, leaf mass per area, mesophyll resistance/conductance, photosynthesis, stomatal resistance/conductance.

Introduction

When illuminated, the CO₂ concentration inside plant leaves, around Rubisco, determines photosynthetic activity (Campbell et al. 1988, Terashima et al. 2011). Although the majority of CO₂ fixed may be derived from the atmosphere (Parkhurst 1994), there is a substantial amount of CO₂ produced and released inside mesophyll cells through respiration and photorespiration (TL Sage and RF Sage 2009, Tholen et al. 2012, Sage and Khoshravesh 2016, Walker et al. 2016). The degree

to which plants utilize this intercellular source of CO₂, the significance thereof compared with atmospheric CO₂, and whether any conditions promote the refixation of (photo)respiratory CO₂, are all questions to consider when modeling the overall photosynthetic capacity of plants. For example, not incorporating mesophyll resistance to CO₂ diffusion (r_m) results in up to a 75% underestimation of the maximum carboxylation rate of Rubisco (V_{cmax}) (Sun et al. 2014a). Knowing more about the percentage of (photo)respiratory CO₂ being refixed, P_r

might be similarly important for accurate modeling of terrestrial CO₂ uptake, especially when considering future increased CO₂ conditions (Sun et al. 2014b, Sage and Khoshravesh 2016).

CO₂ produced through (photo)respiration either diffuses directly from the mitochondria into the chloroplasts due to their close proximity (Sage and Khoshravesh 2016) or leaks to the intercellular air spaces (von Caemmerer 2013, Walker et al. 2016). Little is known about either of these pathways. If respiratory CO₂ joins the CO₂ in the intercellular airspaces, the probability of it diffusing into another cell, and being refixed by Rubisco, should be affected by the same conditions that influence CO₂ concentrations in the intercellular airspaces overall. One major factor could therefore be r_m , which directly influences the movement of CO₂ inside leaves (Warren 2008). It is generally observed that lower r_m facilitates CO₂ uptake and correlates with larger concentrations of CO₂ around Rubisco, increasing photosynthetic rates (Warren 2008). However, as respiratory CO₂ is not diffusing all the way from the atmosphere, this does not necessarily hold true for refixation percentages. Rather, higher resistance to CO₂ diffusion should lower the fraction of respiratory CO₂ that escapes out of the leaf once it has left the cell it derived from. We therefore predict a positive correlation between r_m and the amount of refixation that occurs.

Several studies show that cell wall thickness and the surface area of chloroplasts exposed to intercellular airspaces are among the most important factors influencing r_m (Tomás et al. 2013, Jimei et al. 2017, Veromann-Jürgenson et al. 2017). However, the level of restriction of CO₂ diffusion due to different anatomical traits varies among species and foliage structure (Tomás et al. 2013). It is likely that plant species with similar phenotypical characteristics, plant functional types (PFTs), will also have similar factors influencing r_m (Chapin III et al. 1996). Plant functional types are often used in vegetation models for climate and land use monitoring (Lavorel et al. 2007), and while photosynthetic biochemical parameters and species-specific capacities for CO₂ assimilation are described across PFTs for tree species (Wullschlegel 1993, Wright et al. 2004, Niinemets et al. 2015), information on P_r and how it is influenced by r_m is still incomplete. It would therefore be useful to provide more information about the fate of respiratory-derived CO₂ for different PFTs to increase the accuracy of such models. Some major PFTs that have been shown to have very different physiological and morphological traits in the boreal zone are broadleaved, coniferous, deciduous and evergreen trees (Chapin et al. 1996). Significant differences in the leaf investment strategies of these types may be found. For example, boreal evergreen conifer leaves typically have thick cell walls, sunken stomata and resin channels inside the leaves (Grassi and Bagnaresi 2001, Ghimire et al. 2015, Fan et al. 2019). Morphological differences could be one explanation for significant differences in average P_r between the PFTs. Another way of separating plant species into types is sorting them

according to pioneer and climax species. We know, for example, that pioneer species tend to have thinner leaves than climax species (Sobrado 2008, Han et al. 2010), as well as a tendency for higher photosynthetic rates per unit of leaf area (Bazzaz 1979).

In addition to anatomical differences between species and PFTs, leaf structure and organelle position may vary during a growing season: some evergreen broad-leaved tree species, such as for example *Quercus glauca*, have higher leaf mass per area (LMA) and net photosynthetic rates late in the growing season (Miyazawa et al. 1998). Furthermore, Miyazawa and Terashima (2001) found that the surface area of chloroplasts facing the intercellular air spaces on a leaf area basis increased within a period of up to 40 days after full leaf expansion. This merits exploring if P_r is influenced by seasonality and foliar maturation. Our hypothesis is that differences in leaf anatomy, whether due to functional type or leaf development stage, will correlate with differences in P_r .

Stomatal resistance to CO₂ diffusion (r_s) substantially affects CO₂ concentrations at the photosynthetic sites (Wong et al. 1979). It is the most direct means for plants to prevent cellular water loss; stomatal closure (high r_s) simultaneously slows diffusion of CO₂ and preserves water by slowing transpiration (Wong et al. 1979, Schulze 1986, Franks and Farquhar 1999). However, as discussed in connection with r_m , mitochondria-derived CO₂ does not need to diffuse from the leaf exterior. High r_s might therefore trap CO₂ produced in the mitochondria and thus correlates with higher P_r . Furthermore, r_s has a tendency to increase with increasing LMA when data are pooled from several PFTs and species (Onoda et al. 2017). Comparing r_s and P_r across species and functional types should therefore give sufficient variation in r_s for a correlation with P_r to potentially be revealed.

In this paper, we investigated anatomical and physiological traits that affected leaf-level CO₂ diffusion. We considered if these traits also influenced P_r , and compared several plant functional types. In addition, we evaluated if and how these traits changed as the foliage developed throughout the growing season. Specifically, we tested whether:

- (i) Evergreen and conifer species will have greater P_r compared with deciduous species. We suspect that this might in part be due to greater values of r_m and r_s , therefore;
- (ii) The refixation percentage is positively affected by greater rates of r_m and r_s . As these physiological factors might be affected by leaf maturation, and thus during the growing season when measuring takes place, we therefore finally test whether;
- (iii) Leaves show greater P_r , r_m and r_s later in the growing season, and whether differences in LMA, cell wall thickness and average area fraction of intercellular airspace have an effect on P_r , r_m and r_s .

Materials and methods

Study site and plant material

The plant material was collected in an open, mixed deciduous forest in Växjö, southern Sweden (56°50′26.6″N 14°49′20.6″E). The 30-year means, monthly air temperature and precipitation from May to September, averages around 12.2 °C and 618 mm. The climate is coastal temperate with mild winters. Data were collected in 2017. The average monthly air temperatures and precipitation in May, July and September that year were 11.9 °C and 16.8 mm, 15.3 °C and 39.7 mm and 12.2 °C and 63.1 mm, respectively (Swedish Meteorological and Hydrological Institute 2018).

Current-year folia samples from mature Roth. *Betula pendula*, *L. Quercus robur*, Mill. *Larix decidua*, *L. Pinus sylvestris*, (L.) Karst. *Picea abies* and *L. Vaccinium vitis-idaea* were collected from a south-facing site at 1.3 m (with the exception of *V. vitis-idaea*, where the whole plant was collected). Individuals were sampled at three different time points during the growing season: early (late May), mid (early July) and late season (late August and early September). *Betula pendula* and *Q. robur* are deciduous broadleaved trees, *L. decidua* is a deciduous conifer tree and *P. sylvestris* and *P. abies* are evergreen conifer trees, whereas *V. vitis-idaea* is an evergreen broadleaved shrub. In southern Sweden, *B. pendula*, *Q. robur*, *L. decidua* and *P. sylvestris* are grouped as early to mid-successional species (primary species), and *P. abies* and *V. vitis-idaea* are late successional species (climax species).

Eight to 10 branches per species were cut, brought back to the laboratory and placed in water, after which the stems were recut while submerged, avoiding cavitation. Other studies using detached branches have shown gas exchange measurements to be stable at least 14 h after sampling (Dang et al. 1997, Mitchell et al. 1999). We measured gas exchange no more than 8 h after branch collection. Leaves adjacent to the ones used for the gas exchange measurements were removed and prepared for microscopy (see Microscopy and leaf trait analysis section). This procedure was repeated at each time point of the growing season with the same species and the number of samples collected at the same site.

Gas exchange measurements

Gas exchange measurements were done using a 6-cm² chamber of the LI-6400 and LI-6800 with red-blue light emitting diodes light sources (LICOR Inc., Lincoln, NE, USA). The reference CO₂ concentration was set at 400 p.p.m., block temperature at 25 °C, flow rate at 500 μmol s⁻¹, relative humidity at 50 ± 10% and irradiance at 1000 μmol quanta m⁻² s⁻¹. These conditions were kept constant while the leaves acclimated to the chamber. The leaves were considered acclimated when the photosynthetic rate and stomatal conductance had reached steady state (usually achieved after 20–40 min). Photosynthesis

CO₂ response (*A/C_i*) curves were generated by sequential adjustment of the reference CO₂ concentration between 50 and 1200 p.p.m. (400, 300, 200, 100, 50, 400, 600, 800, 1000 and 1200 p.p.m.). A total of 156 *A/C_i* curves were generated. Values for stomatal resistance *r_s* included in the results were taken at the first 400-p.p.m. CO₂ concentration.

A/C_i curves were analyzed with the LeafWeb online tool and database (www.leafweb.org), which estimated the maximum rate of CO₂ fixation (*A_{max}*), *V_{cmax}*, the maximum rate of electron transport for a given light intensity (*J_{max}*), maximum rate of triose phosphate use (TPU), *r_m* and *P_r* by fitting a modified version of the Farquhar–von Caemmerer–Berry model (Gu et al. 2010). LeafWeb calculates refixation percentage according to the equation presented by Tholen et al. (2012). The equation uses the fraction of respiratory and photorespiratory CO₂ that has not escaped to the atmosphere; it includes the cytosolic partial pressure of mitochondrial CO₂, the diffusion resistance to these molecules imposed by the chloroplasts, the resistance represented by carboxylation reaction itself, and the resistance derived by the cell wall and plasma membrane. The estimated amount of (photo)respiratory CO₂ that escapes to the atmosphere is the leakage flux divided by the sum of the two fluxes originating in the mitochondria (Tholen et al. 2012). Hence, the relative fraction of respiratory and photorespiratory CO₂ presumed to be refixed and used in photosynthesis, *P_r*, is estimated as 1 minus the relative amount of CO₂ that escapes to the atmosphere, calculated according to Tholen et al. (2012)

$$P_r = 1 - \left(\frac{x_{py}}{r_{wp} + r_{sc}} / \left(\frac{x_{py}}{r_{ch} + k^{-1}} + \frac{x_{py}}{r_{wp} + r_{sc}} \right) \right) = \left(\frac{r_{wp} + r_{sc}}{r_{ch} + k^{-1}} + 1 \right)^{-1}.$$

Here, *r_{ch}* is the resistance represented by the chloroplast, *x_{py}* is the cytosolic partial pressure of (photo)respiratory CO₂ molecules, and resistance from the cell wall, plasma membrane and stomata is indicated as *r_{wp}* and *r_{sc}*, respectively. In addition, the resistance derived from the carboxylation reaction itself was included ($k^{-1} \equiv (p_c - O)/V_c$). For the reported values, the resistances are expressed in Pa s⁻¹ m⁻² μmol⁻¹ because diffusion inside the leaf is a process driven by the gradient in partial pressure (e.g., Pa) rather than concentration. The conversion factor between *r_m* (m² s Pa μmol⁻¹) and *r_m* (m² s mol⁻¹) is given by

$$r_m \text{ in m}^2 \text{ s Pa } \mu\text{mol}^{-1} = P \times 10^{-6} r_m \text{ in m}^2 \text{ s mol}^{-1}.$$

Here, *P* is the total atmospheric pressure in Pa.

After gas exchange measurements, projected leaf area for *L. decidua*, *P. sylvestris*, *P. abies* and *V. vitis-idaea* was calculated using ImageJ (Schneider et al. 2012) and adjusted in the gas exchange output accordingly. All samples (either a 2.27-cm²

leaf disc from *B. pendula* and *Q. robur*, or the total leaf area used for the gas exchange measurement from *L. decidua*, *P. sylvestris*, *P. abies* and *V. vitis-idaea*) were subsequently placed into separate envelopes and dried in an oven at 70 °C around 48 h until dry. Fresh weight, dry weight and leaf area were then used to calculate water content and LMA as described by Cornelissen et al. (2003).

Microscopy and leaf trait analysis

Leaf tissues (mid leaf, avoiding the major veins) from early, mid and late season were prepared for light microscopy (LM) and transmission electron microscopy (TEM). Samples of $\sim 2 \times 3$ mm were fixed in Karnovsky's fixative (5% glutaraldehyde, 4% paraformaldehyde and 0.1 M sodium cacodylate buffer) including a vacuum treatment, washed in the buffer and post-fixed in buffered 1% osmium tetroxide. Samples were step-wise dehydrated in a graded acetone series, infiltrated with three different ratios of Spurr resin to acetone and embedded in Spurr resin within flat molds. The resin was polymerized in an oven at 60 °C for 8 h. The samples were sectioned into semi-thin (3 μ m) and ultrathin (50 nm) sections for LM and TEM, respectively, using a SuperNova ultra-microtome (Reichert-Jung/LKB) with a diamond knife. Light microscopy sections were left overnight on a hot plate to ensure proper attachment of the section to the glass slide and then stained with either Toluidine Blue, Safranin or periodic acid–Schiff and imaged in a Leica DM5000B microscope. The ultrathin sections were collected on carbon-coated copper grids and contrasted with 1% uranyl acetate and lead citrate (2.7% in 3.5% sodium citrate) and examined in a Philips CM 100 TEM at 80 kV (Philips, Amsterdam, The Netherlands). The LM sections were made for all three time points of the growing season. Only samples from early and mid-season were used for TEM and subsequent cell wall thickness measurements. Hence, the cell wall data were pooled, and TEM data variation was not investigated for seasonal effects.

Images were analyzed with ImageJ (Schneider et al. 2012) with the 'Trainable Weka Segmentation' plugin to measure the area fraction of intercellular airspaces (F_{ias}) in the mesophyll tissue from LM sections. The plugin allows a manual choice of colors for analysis of the pixel composition of the LM sections (see Figure 1). We used the average of two LM pictures per sample analyzed this way, resulting in a total of three samples per season per species ($n = 54$).

Quantification of mesophyll cell wall thickness (T_{cw}) was performed from TEM images taken at 7900 $\times$ magnification (see Figure 1), as done in Nafisi et al. (2015). For each species, 100 measurements were done distributed over three samples (three individual plants) for the mid-season samples, and two to three for the early season samples. As we pooled the data, the T_{cw} measurements are from a total of five to six different individuals per species.

Statistical analysis

Linear regression (ordinary least squares) was used to analyze the effects of r_m , r_s and LMA on P_r as well as the effects of LMA, T_{cw} , water content and F_{ias} on r_m . Analysis of variance was used to detect differences in P_r , r_m , r_s , A_{max} , V_{cmax} , J_{max} , TPU, r_d , leaf water content, LMA, F_{ias} and T_{cw} between the species and growing seasons. Where necessary, the data were transformed to meet the assumptions of normality and equal variance, which were tested using Shapiro–Wilk tests and qq-plots. When significant differences between means were detected, Tukey's honest significant difference test was used to determine which species or growing seasons differed ($P < 0.05$). All calculations were performed using R Studio Version 1.1.456 (RStudioTeam 2015).

Results

CO₂ refixation is highest in evergreens and in climax species

The percentage of (photo)respiratory CO₂ that was refixed inside the mesophyll (P_r) ranged from about 20 to over 90% (Figure 2). Functional type mattered (Table S1 available as Supplementary data at Tree Physiology Online, Figure 2), with the climax species *P. abies* and *V. vitis-idaea* showing significantly greater values of mean P_r (55.5 and 59.6%, respectively) than the early/mid successional species *B. pendula*, *Q. robur*, *L. decidua* and *P. sylvestris*, which displayed mean values of 41.8, 35.7, 42.0 and 40.6% (Figure 2, Table S1 available as Supplementary data at Tree Physiology Online). Deciduous species refixed on average significantly less CO₂, only 40% of the respired CO₂, while evergreen species on average refixed significantly more CO₂, about 52% of their respired CO₂. Deciduous broadleaved species had significantly lower P_r than evergreen conifers, with mean ($\pm$ SD) values of 38.7 ($\pm$ 8.5)% compared with 50.1 ($\pm$ 15.9)%, (Figure 2). Deciduous conifer species (*L. decidua*) had mean P_r values of 42 ($\pm$ 9.5)%, which was significantly lower than the evergreen broadleaved (*V. vitis-idaea*) values of 55%.

We found a highly significant ($P < 0.01$) difference between species for the variables: P_r , r_m , r_s , A_{max} , V_{cmax} , J_{max} , TPU, r_d , leaf water content, LMA, intercellular airspace and cell wall thickness. In addition, r_s , A_{max} , V_{cmax} , J_{max} , TPU, r_d , leaf water content and LMA were also affected to some degree by seasonality with differences being specifically pronounced between early and late in the season (Table 1, Table S1 available as Supplementary data at Tree Physiology Online). The photosynthetic parameters and LMA were lowest early in the growing season, while at the same time, leaf water percentage was highest. Mean values of P_r , however, only changed significantly between seasons for two species: *Q. robur* and *L. decidua* (Figure 3, Table S1 available as Supplementary data at Tree Physiology Online). *Quercus robur* was not refixating as much CO₂ during early season as

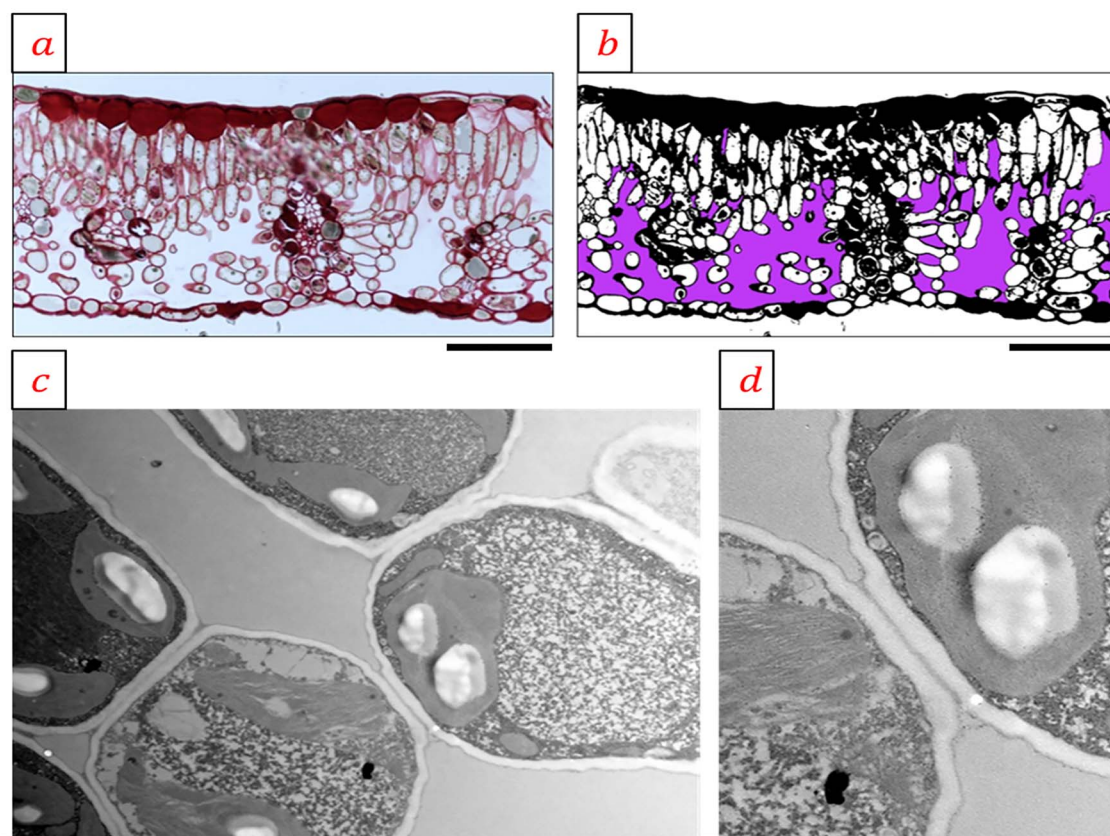


Figure 1. Leaf anatomy and examples of anatomical trait measurements in *B. pendula*. (a) Semi-thin section stained with periodic acid–Schiff and imaged in a brightfield microscope shows the stacked layers of small palisade cells, air-filled spongy mesophyll, the stomata on the lower leaf side and phenol-rich epidermis cells, and (b) after image segmentation for area fraction of intercellular air spaces. (c) Ultra thin section of mesophyll cells imaged in transmission electron microscope visualizing the diffusion path defined by the cells and the intercellular airspaces. (d) Details from an area selected for wall thickness measurements. Bar = 100 μm (a and b), 2 μm (c and d).

compared with mid and late season, while *L. decidua* showed the highest percentages in the middle of the season. Although we expected r_m to change throughout the season as the leaves matured, only *B. pendula* had a significantly ($P < 0.02$) lower r_m early in the growing season (Figure 4, Table S1 available as Supplementary data at *Tree Physiology* Online) compared with late season.

Cell wall thickness significantly affected P_r and r_m

Cell wall thickness (T_{cw}) was significantly greater in the evergreen *P. abies*, with an average of 1.2 (± 0.08) μm , compared with the deciduous species *B. pendula*, *Q. robur* and *L. decidua*, which had a T_{cw} of 0.2 (± 0.009), 0.22 (± 0.02) and 0.39 (± 0.08) μm , respectively (Figure 5). Although we had few TEM samples, we observed a significant effect between T_{cw} and P_r ($P = 0.025$, $r^2 = 0.18$) and between T_{cw} and r_m ($P = 0.05$, $r^2 = 0.15$) using an ordinary least squares model. We found no support for a relationship between intercellular airspace percentage in the mesophyll and P_r , or r_m (results not shown), but we observed a species variation that could be explained by functional type (Figure 5, Table 1, Table S1 available as

Supplementary data at *Tree Physiology* Online). The coniferous evergreen species had significantly ($P < 0.01$) less air in their mesophyll than broadleaved species and the deciduous conifer (Figure 5). Light microscope pictures showed that average ($\pm$ SD) intercellular airspace percentage in the mesophyll was about 38.2 (± 9.7), 28.8 (± 6.9), 35.5 (± 6.0), 39.7 (± 6.4), 15.8 (± 5.5) and 21.7 (± 5.1)% for *B. pendula*, *Q. robur*, *L. decidua*, *V. vitis-idaea*, *P. sylvestris* and *P. abies*, respectively (Figures 1 and 5, Table S1 available as Supplementary data at *Tree Physiology* Online).

r_m , r_s and LMA explain differences in P_r

We found a significant effect on P_r for r_m , r_s and LMA; all had positive correlations (Table 2). Although the relationship between r_m and P_r differed among species, with some species displaying a negative relationship, a highly significant relation was observed across species (Figure 5). This species variation is not surprising considering that multiple morphological and physiological traits affected P_r (Table 2) and that these traits also varied between species and season (Table 1, Table S1 available as Supplementary data at *Tree Physiology* Online).

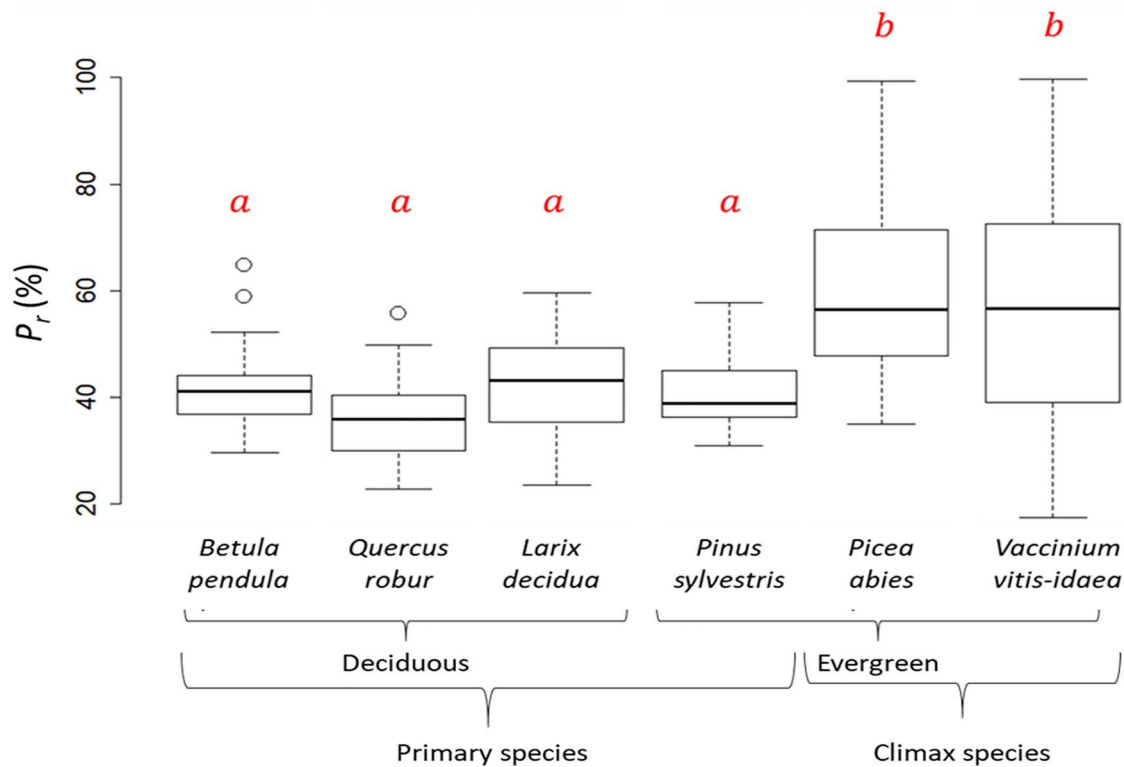


Figure 2. Percentage of CO₂ refixed (P_r) for six different woody species, separated into functional group (deciduous, evergreen, and primary and climax species). The center thick line of each boxplot represents the median. Different letters (a and b) indicate significant statistical difference at the $P < 0.01$ level using Tukey HSD. $N = 156$.

Table 1. ANOVA results: The effect of species and growing season on leaf traits: P_r (percentage of respiratory CO₂ refixed), r_m (mesophyll resistance to CO₂ diffusion), r_s (stomatal resistance to CO₂ diffusion), $A_{\max}$ (photosynthetic capacity), $V_{\max}$ (maximum carboxylation rate of Rubisco), $J_{\max}$ (maximum electron transport rate), TPU (triose phosphate use), r_d (respiration in the light), leaf water content, LMA (leaf mass per area), F_{ias} (average fraction of intercellular airspaces in the mesophyll tissue as estimated from light microscope sections), and T_{cw} (average thickness of mesophyll cell wall as measured from transmission electron microscope). F-values and p-values with species and light environment as main effects. Bold numbers represent p-values less than 0.05 ($p > 0.05$).

Variables	Species		Growing season (early, mid and late)	
	F-value	P-value	F-value	P-value
P_r (%)	14.4	1.6e-11	0.4	0.7
r_m ($\text{p}_a \text{ s}^{-1} \text{ m}^{-2} \mu\text{mol}^{-1}$)	3.6	0.0044	0.9	0.4
r_s ($\text{p}_a \text{ s}^{-1} \text{ m}^{-2} \mu\text{mol}^{-1}$)	15.0	6.49e-12	11.9	1.57e-05
$A_{\max}$ ($\mu\text{mol m}^{-2} \text{ s}^{-1}$)	12.56	3.5e-10	5.0	0.008
$V_{\max}$ ($\mu\text{mol m}^{-2} \text{ s}^{-1}$)	20.2	5.16e-15	6.0	0.00324
$J_{\max}$ ($\mu\text{mol m}^{-2} \text{ s}^{-1}$)	13.6	8.04e-11	6.5	0.002
TPU ($\mu\text{mol m}^{-2} \text{ s}^{-1}$)	12.5	4.24e-10	4.1	0.018
r_d ($\mu\text{mol m}^{-2} \text{ s}^{-1}$)	11.4	53e-09	3.8	0.024
Leaf water content (%)	7.3	3.97e-06	61.2	<2e-16
LMA (g m^{-2})	182.1	<2e-16	7.8	6.07e-4
T_{cw} (μm)	28.27	3.25e-07	—*	—*
F_{ias} (%)	915.7	4.42e-11	0.3	0.8

*Cell wall thickness data is from one point of the growing season (mid-season)

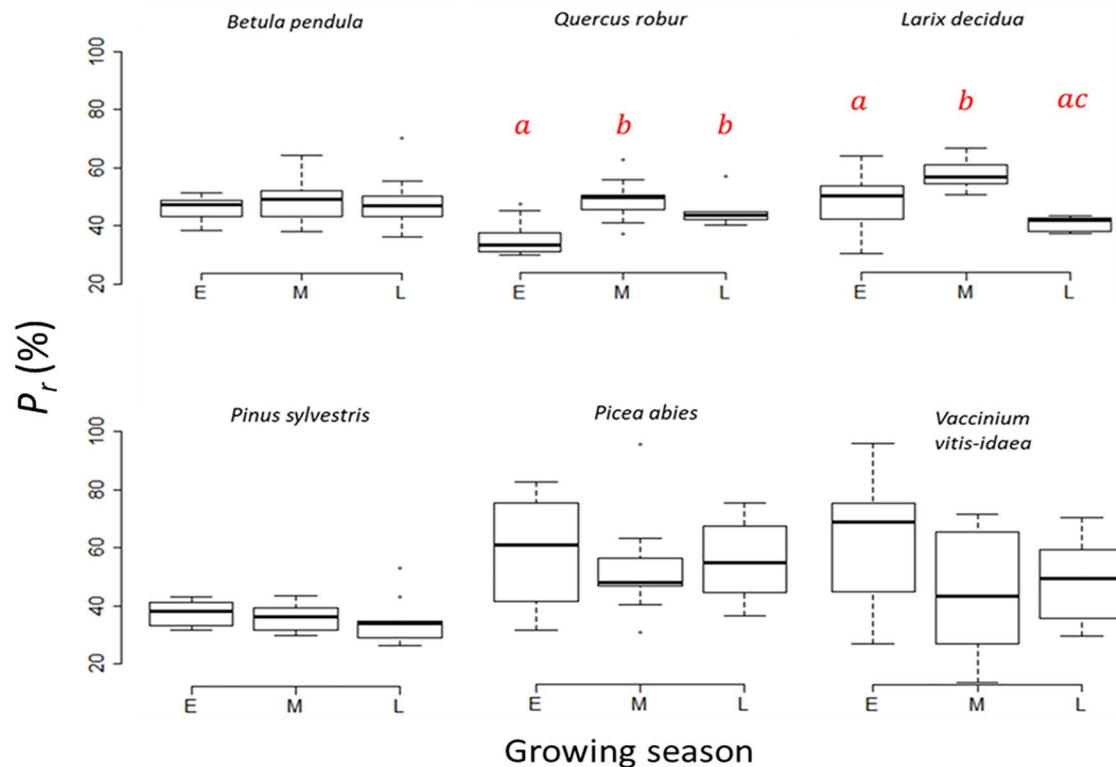


Figure 3. Percentage of CO₂ refixed (P_r) for six different woody species early (E), mid (M) and late (L) in the growing season. The center thick line of each boxplot represents the median. Different letters (a and b) indicate significant statistical difference at the $P < 0.01$ level using Tukey HSD. Differences were tested with Tukey HSD. $N = 156$.

Discussion

Evergreen and climax species are efficient in using their respiratory CO₂

We showed that the two evergreen species *P. abies* and *V. vitis-idaea* had the highest P_r of all species measured (Figure 2). Efficient refixation of (photo)respiratory CO₂ will be an advantage when stomata are closed, such as during drought, high temperature stress or during winter-hibernating periods. Conifers growing in mild winter climates can have significant carbon fixation all year round (Fry and Phillips 1977, Harrington et al. 1994), while conifers in colder, northern environments seem to limit carbon fixation to irregular periods with temperatures above freezing (Jurik et al. 1988, Schaberg et al. 1995). Being able to utilize even a small amount of CO₂ without having to open stomata might be an advantage for evergreens, especially since there are examples of water stress (Kincaid and Lyons 1981, Teskey et al. 1984) and stomatal closure (Delucia 1987) being limiting factors of winter photosynthesis.

Although also an evergreen, *P. sylvestris* had refixation percentages that were more similar to the deciduous species. *Picea abies* and *V. vitis-idaea* can be defined as climax species, or 'late' successional species, while *P. sylvestris* and the deciduous species are all pioneer, or 'early' successional species in Sweden (Hannon et al. 2018). It is therefore possible, and a potential

subject for further investigation, that climax species overall have higher P_r compared with the early successional species. It has been shown that early and late successional species can have different physiological capabilities; for example, early successional species seem to have higher net photosynthetic rates, both on an area and mass basis (Kloeppel et al. 1993, Matsuki and Koike 2006, Bussotti 2008). P_r could on the other hand be a trait that is more prominent in late succession.

Refixation percentage is positively correlated with r_m , r_s and LMA

A central point of our results was that higher r_m , r_s and LMA correlated with higher P_r (Figure 5). There were large variations in the data when all species were pooled, with some species even displaying a negative relationship to one or more of the above traits. However, all species that had significant relationships between P_r and r_m , r_s and LMA had positive correlations similar to the average trend (Figure 6). It is possible that some of the same adaptations or physiological states that increase r_m and LMA also increase P_r . Alternatively, r_m and LMA directly affect P_r . Both high r_m and high r_s can decrease photosynthetic activity due to a slowdown of CO₂ diffusion to the photosynthetic sites from the exterior (Parkhurst 1994, Warren 2008, Jimei et al. 2017). This is the basis for our

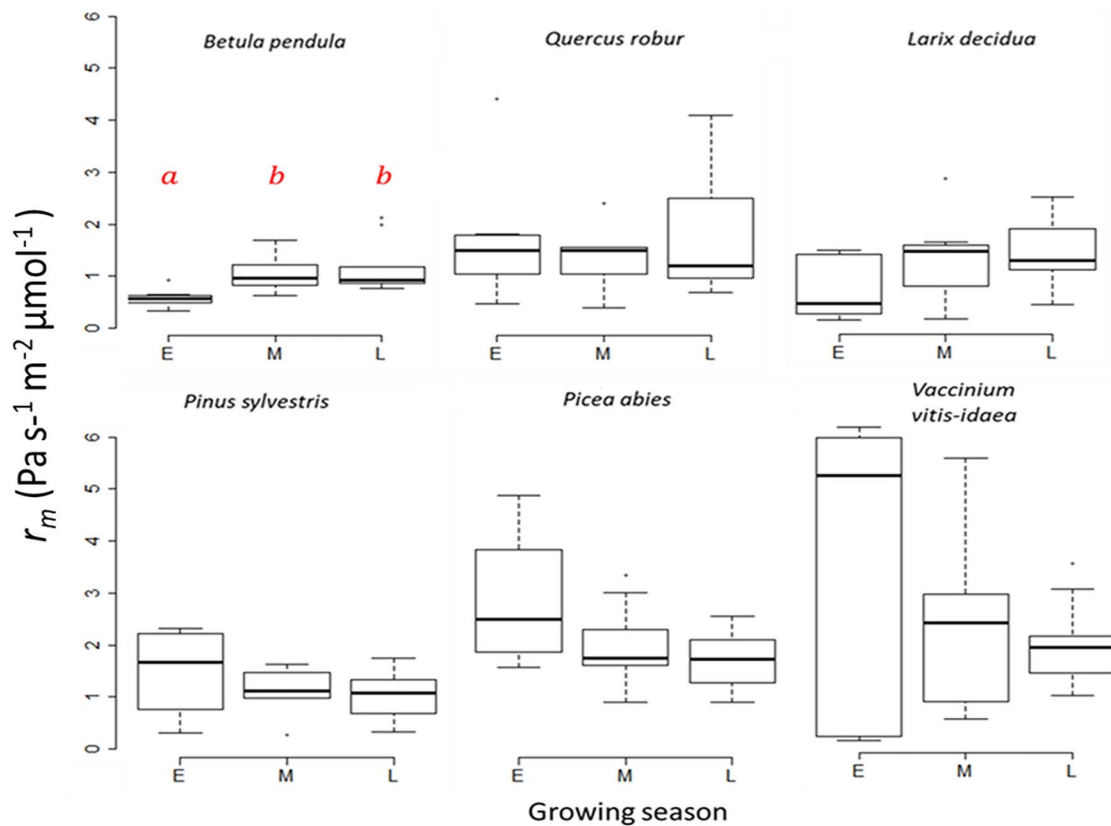


Figure 4. Mesophyll resistance to CO₂ diffusion (r_m) of six different woody species early (E), mid (M) and late (L) in the growing season. The center thick line of each boxplot represents the median. Different letters (a and b) indicate significant statistical difference at the $P < 0.01$ level using Tukey HSD. Differences were tested with Tukey HSD. $N = 136$.

Table 2. Linear regression results: The effect of physiological and morphological traits on the dependent variable, P_r (percentage of refixed respiratory CO₂). Model estimate from three (1–3) ordinary least squares linear models, with T-values and p-values in parentheses. Physiological traits: r_m (mesophyll resistance to CO₂ diffusion), r_s (stomatal resistance to CO₂ diffusion), and morphological trait: LMA (leaf mass per area). Bold numbers represent p-values less than 0.05.

	1	2	3
Independent variables			
r_m (Pa s ⁻¹ m ⁻² μmol ⁻¹)	5.77 (6.25, 5.13e-09)	5.04 (5.51, 1.8e-07)	4.90 (5.61, 1.24e-07)
r_s (Pa s ⁻¹ m ⁻² μmol ⁻¹)		0.59 (3.51, 6.2e-04)	0.63 (3.88, 1.7e-04)
LMA (g m ⁻²)			0.05 (4.2, 4.74e-05)
Intercept	36.7 (21.0, <2e-16)	32.33 (15.7, <2e-16)	26.35 (10.5, <2e-16)
Degrees of freedom	134	132	126
Adjusted r^2	0.22	0.28	0.36
F-statistic	39.04	27.55	25.59
P-value	5.135e-9	1.005e-10	5.375e-13

underlying hypothesis: a long, low-conductive pathway for CO₂ diffusion (high r_m) from the cell to the atmosphere, and closed stomata (high r_s), would help capture (photo)respiratory CO₂ produced in the mesophyll, increasing P_r . Our results are in line with this hypothesis. However, they also suggest that more factors influence P_r , which should be further expanded on in later studies. The same methods have previously been applied on some of the same species studied here (Eckert et al. 2020). There was no significant correlation between P_r and r_m in the

findings of Eckert et al. (2020); however, there was a positive trend. The previous article did find a correlation between V_{cmax} that was not obvious in the current study. It is possible that more data are needed in order to make larger-scale correlations obvious.

It should be mentioned that the LeafWeb model (see Materials and methods) is using both stomatal and leaf conductance to calculate P_r . These factors are thus not independent from each other. The model assumes that changes in r_m and r_s

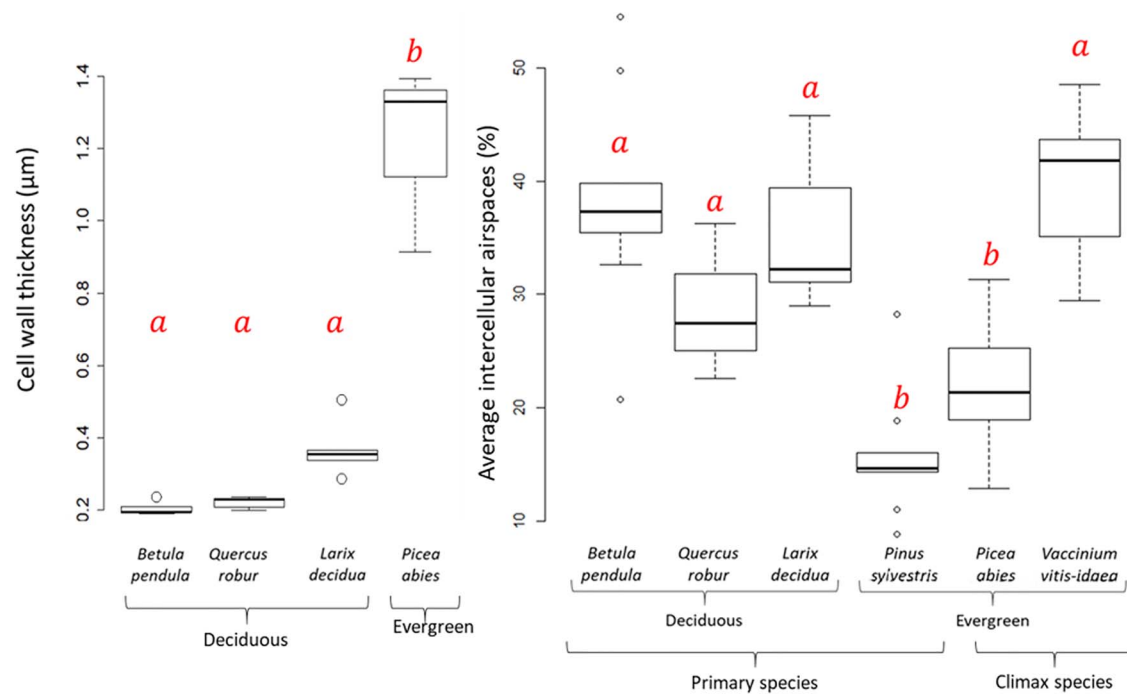


Figure 5. Cell wall thickness (T_{cw}) of mesophyll cells, and average fraction of intercellular airspace (F_{ias}) of leaves from different woody species. Which functional group (deciduous, evergreen, and primary and climax species) each species belongs to is indicated. The center thick line of each boxplot represents the median. Different letters (a and b) indicate significant statistical difference at the $P < 0.01$ level using Tukey HSD. $N = 12$ (cell wall thickness), $n = 54$ (average intercellular airspace).

lead to changes in P_r . While experimental validation is needed, the assumptions are justifiable because carboxylation does not differentiate between CO_2 sources, whether from mitochondria or intercellular airspace. What should matter to the fate of mitochondria-derived CO_2 is the relative diffusion-resistance to the carboxylation sites versus relative resistance to the outside of the leaf. In this regard, the fact that LMA (and cell wall thickness) had a positive correlation with P_r gives some evidence that this assumption is correct. Furthermore, the study by Busch et al. (2013) measured refixation directly and found that a continuous layer of chloroplasts covered the cell periphery. The chloroplasts captured the (photo)respired CO_2 and boosted photosynthesis. Similarly, high efflux resistance due to chloroplast positioning is one of the assumptions made by the LeafWeb model. Busch et al. (2013) also found that refixating (photo)respiratory CO_2 boosted photosynthesis more when ambient atmospheric CO_2 concentrations were low ($200 \mu mol^{-1}$). Efficiently recycling mitochondria-derived CO_2 has implications for water-use efficiency. During drought, high P_r would allow species to close stomata while keeping up a limited amount of photosynthesis. The meta-study by Ainsworth and Long (2005) showed that plants overall increase r_s when exposed to elevated CO_2 . This might make (photo)respiratory-derived CO_2 a larger fraction of the CO_2 used in photosynthesis, especially in high r_m species, such as the evergreens in this study.

We expected LMA to correlate with P_r because it can be a predictor of thick cell walls and tightly packed mesophyll (Niinemets et al. 2009, Onoda et al. 2017), which simultaneously enhances r_m (Flexas et al. 2008). Although LMA also increases with more mesophyll layers, this does not necessarily increase r_m if r_m is measured per unit of leaf area (supplementary material in Sun et al. 2014b). In our data, r_m and LMA were not correlated, which might be due to r_m being measured on a leaf-area basis. A new paper by Veromann-Jürgenson et al. (2020) found that variation in LMA did not correlate to structural traits known to control r_m across species. Veromann-Jürgenson et al. (2020) concluded that more detailed knowledge of the underlying traits affecting r_m are needed for accurate prediction, and further showed evidence that chloroplast area exposed to intercellular airspaces and cell wall thickness are important drivers of r_m . Likely, these two traits are therefore also important drivers of P_r .

Environmental factors should also be considered, as they have been shown to rapidly induce changes in r_m (but not in LMA) for different species: increases in temperature generally lower r_m until a certain threshold (Bernacchi et al. 2002), and soil water availability, salinity and growth irradiance may all affect r_m (Flexas et al. 2008, Flexas et al. 2009). It would therefore be interesting to further investigate these elements in relation to P_r to further clarify if r_m is directly affecting P_r , or another factor affecting both P_r and r_m simultaneously.

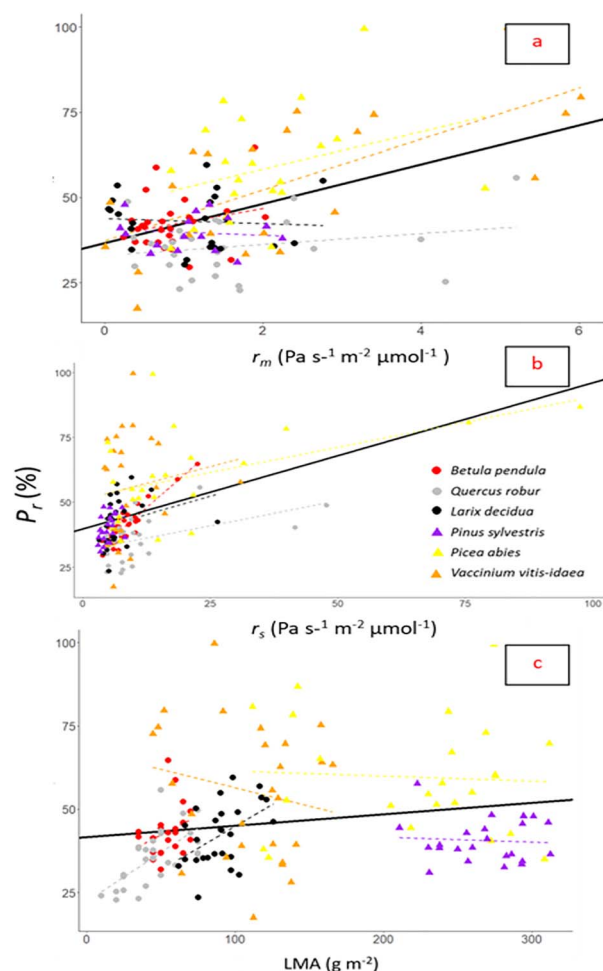


Figure 6. Relationship between the percentages of CO₂ refixed (P_r) and (a) mesophyll resistance to CO₂ diffusion (r_m), (b) stomatal resistance to CO₂ diffusion (r_s) and (c) LMA in six different woody species. Deciduous species are marked with circles, and evergreens are marked with triangles. Regression lines: (a) all species (black solid line, $y = 36.7 + 5.8x$, $P < 0.01$, adj. $r^2 = 0.22$), *B. pendula* (dashed red line, $y = 38.7 + 4.0x$, $P = 0.22$, adj. $r^2 = 0.02$), *Q. robur* (dashed gray line, $y = 33.0 + 1.6x$, $P = 0.25$, adj. $r^2 = 0.02$), *L. decidua* (dashed black line, $y = 43.4 + 0.8x$, $P = 0.75$, adj. $r^2 = -0.04$), *P. sylvestris* (dashed purple line, $y = 40.6 \pm 0.9x$, $P = 0.71$, adj. $r^2 = -0.06$), *P. abies* (dashed yellow line, $y = 47.3 + 5.5x$, $P = 0.16$, adj. $r^2 = 0.05$) and *V. vitis-idaea* (dashed orange line, $y = 37.3 + 7.5x$, $P < 0.01$, adj. $r^2 = 0.43$); (b) all species (black solid line, $y = 39.8 + 0.56x$, $P < 0.01$, adj. $r^2 = 0.17$), *B. pendula* (dashed red line, $y = 26.5 + 1.7x$, $P < 0.01$, adj. $r^2 = 0.784$), *Q. robur* (dashed gray line, $y = 31.5 + 0.38x$, $P = 0.011$, adj. $r^2 = 0.19$), *L. decidua* (dashed black line, $y = 37.6 + 0.62x$, $P = 0.14$, adj. $r^2 = 0.05$), *P. sylvestris* (dashed purple line, $y = 32.6 + 1.5x$, $P = 0.06$, adj. $r^2 = 0.12$), *P. abies* (dashed yellow line, $y = 51.7 + 0.4x$, $P = 0.01$, adj. $r^2 = 0.23$) and *V. vitis-idaea* (dashed orange line, $y = 50.9 + 0.51x$, $P = 0.5$, adj. $r^2 = -0.02$) and (c) all species (black solid line, $y = 41.6 + 0.034x$, $P = 0.012$, adj. $r^2 = 0.035$), *B. pendula* (dashed red line, $y = 30.7 + 0.23x$, $P = 0.13$, adj. $r^2 = 0.06$), *Q. robur* (dashed gray line, $y = 22.28 + 0.29x$, $P < 0.01$, adj. $r^2 = 0.40$), *L. decidua* (dashed black line, $y = 19.51 + 0.25x$, $P = 0.025$, adj. $r^2 = 0.15$), *P. sylvestris* (dashed purple line, $y = 44.6 - 0.01x$, $P = 0.76$, adj. $r^2 = -0.04$), *P. abies* (dashed yellow line, $y = 62.9 - 0.02x$, $P = 0.793$, adj. $r^2 = -0.04$), *V. vitis-idaea* (dashed orange line, $y = 67.5 - 0.11x$, $P = 0.344$, adj. $r^2 = -0.003$). $N = 136$.

Season, cell wall thickness and fraction of intercellular airspace

The point in the growing season (early, mid or late) at which the measurements were taken did not seem to matter for r_m and was only significant for P_r in *L. decidua* and *Q. robur*, both of which showed highest refixation mid-season (Figure 3). However, most of the other physiological and morphological traits investigated did change throughout the growing seasons (r_s , A_{max} , V_{cmax} , J_{max} , TPU, r_d , leaf water content and LMA, Table 1, Table S1 available as Supplementary data at Tree Physiology Online). This is in line with previous findings that mature leaves from both mid and late successional species had higher photosynthetic rates than young leaves (Yu et al. 2020). The fact that so many other traits did change throughout the growing season shows that the leaves underwent some maturation, but that it did not have a great effect on P_r and r_m . On the other hand, both *L. decidua* and *Q. robur* refixed more of their (photo)respiratory CO₂ in the middle of the growing season, which means that for these two species, leaf maturation might have had an effect. In addition to anatomical and physiological traits, it is likely that the environmental conditions around leaves influence P_r . Particularly temperature and irradiance, as they are known to affect both respiration and photosynthesis, and as they have been shown to strongly correlate with refixation in photosynthetic bark of *Pinus monticola* (Cernusak and Marshall 2001). Another explanation for a seasonal effect on P_r for *L. decidua* and *Q. robur* might therefore be that these two species had a different response to the mid-season climate than the others.

Even though we do not have as many TEM pictures as P_r measurements, we did find a correlation between cell wall thickness and r_m . Of the anatomical traits investigated in our study, cell wall thickness is known to have a strong positive correlation with r_m (Tomás et al. 2013, Onoda et al. 2017, Veromann-Jürgenson et al. 2017). Species differences were found where *P. abies* had the thickest cell walls (Figure 5). It is possible that a stronger correlation would have emerged if species and growing season samples had been represented. This is also true for cell wall thickness and P_r , where we also found a significant, positive effect. *Picea abies* and *V. vitis-idaea* were the two species with the highest P_r . While we have no data on cell wall thickness for *V. vitis-idaea*, *P. abies* clearly also has thicker cell walls than the other species tested (Figure 5). In the literature, a close relative of *V. vitis-idaea*, *Vaccinium oxycoccus*, shows a cell wall thickness of 0.61–1.06 μm . If similar numbers can be found for *V. vitis-idaea*, it would be close to *P. abies* and significantly thicker than the deciduous species (Figure 5). Thick cell walls can thus be a predictor of high P_r . More data on cell wall thickness for different species would help investigate this further.

Comparing intercellular airspace between species shows that the two evergreen conifers had much more tightly packed

mesophyll than the others, which is in line with expectations given their sturdy leaf anatomy (Niinemets et al. 2009). Intercellular airspace percentage has in some cases been shown to have an influence on r_m . However, it seems largely to be determined as only affecting r_m to a small degree, as CO₂ diffusion in air does not meet much resistance compared with diffusion across cell walls (Tholen et al. 2012). This might then be the same reason it did not affect P_r .

Conclusions

When studying the percentage of respiratory CO₂ refixation (P_r) between different species and functional types, we found that evergreen, late successional species, especially *V. vitis-idaea* and *P. abies*, utilized significantly more of their mitochondria-derived CO₂ than deciduous and early successional species. Measuring P_r at various points of the growing season showed that P_r was relatively constant and that the two evergreen species had the highest percentages of P_r throughout the entire season. Among the anatomical and physiological traits we investigated, mesophyll resistance to CO₂ diffusion (r_m), stomatal resistance to CO₂ diffusion (r_s) and leaf dry matter content (LMA) were significantly, positively correlated with P_r . We suggest that this is due to higher r_m and r_s decreasing diffusion of (photo)respiratory CO₂ out of the leaf. Cell wall thickness was significantly different between conifers and broadleaves, and cell wall thickness positively affected both P_r and r_m in our study.

Our findings suggest that species with higher r_m and thicker cell walls might be more efficient with their mitochondria-derived CO₂. P_r should be considered when modeling the overall CO₂ fertilization effect for terrestrial ecosystems dominated by high- r_m species. This is because some species develop a lower number of stomata under prolonged elevated atmospheric CO₂, or close more of the stomata (increasing r_s). They might in that way be able to keep photosynthetic rates high enough while also preserving water. There are thus implications for water-use efficiency and overall CO₂ drawdown.

Supplementary data

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

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