Effects of leaf age and tree size on stomatal and mesophyll limitations to photosynthesis in mountain beech (*Nothofagus solandrii* var. *cliffortioides*)

David Whitehead1,7, Margaret M. Barbour1,2, Kevin L. Griffin3, Matthew H. Turnbull4 and David T. Tissue5,6

Mesophyll conductance, $g_m$, was estimated from measurements of stomatal conductance to carbon dioxide transfer, $g_s$, photosynthesis, $A$, and chlorophyll fluorescence for Year 0 (current-year) and Year 1 (1-year-old) fully sunlit leaves from short (2 m tall, 10-year-old) and tall (15 m tall, 120-year-old) *Nothofagus solandrii* var. *cliffortioides* trees growing in adjacent stands. Rates of photosynthesis at saturating irradiance and ambient CO₂ partial pressure, $A_{satQ}$, were 25% lower and maximum rates of carboxylation, $V_{cmax}$, were 44% lower in Year 1 leaves compared with Year 0 leaves across both tree sizes. Although $g_s$ and $g_m$ were not significantly different between Year 0 and Year 1 leaves and $g_s$ was not significantly different between tree heights, $g_m$ was significantly (19%) lower for leaves on tall trees compared with leaves on short trees. Overall, $V_{cmax}$ was 60% higher when expressed on the basis of CO₂ partial pressure at the chloroplasts, $C_r$, compared with $V_{cmax}$ on the basis of intercellular CO₂ partial pressure, $C_i$, but this varied with leaf age and tree size. To interpret the relative stomatal and mesophyll limitations to photosynthesis, we used a model of carbon isotopic composition for whole leaves incorporating $g_m$ effects to generate a surface of ‘operating values’ of $A$ over the growing season for all leaf classes. Our analysis showed that $A$ was slightly higher for leaves on short compared with tall trees, but lower $g_m$ apparently reduced actual $A$ substantially compared with $A_{satQ}$. Our findings showed that lower rates of photosynthesis in Year 1 leaves compared with Year 0 leaves were attributable more to increased biochemical limitation to photosynthesis in Year 1 leaves than differences in $g_m$. However, lower $A$ in leaves on tall trees compared with those on short trees could be attributed in part to lower $g_m$ and higher stomatal, $L_s$, and mesophyll, $L_m$, limitations to photosynthesis, consistent with steeper hydraulic gradients in tall trees.

**Keywords**: carbon isotope discrimination, carboxylation, mesophyll conductance, mesophyll limitation, stomatal conductance

Introduction

The regulation of photosynthesis rate in leaves is closely related to leaf nitrogen concentration (*Field and Mooney 1986, Evans 1989*), with lower rates of photosynthesis in older leaves generally attributable to lower nitrogen concentrations and decreasing ratio of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) to chlorophyll content (*Warren and Adams 2001*). However, the biochemical limitation of photosynthesis does not account fully for the observed decreases in photosynthesis with leaf ageing (*Warren 2006*). The inclusion of stomatal limitation to photosynthesis, $A$, in models at leaf scales is widely accepted (*Farquhar et al. 1980, Farquhar and Sharkey 1982*), but there is increasing recognition that limitation to photosynthesis by mesophyll conductance is as
important as stomatal limitation (Harley et al. 1992, Loreto et al. 1992, Flexas et al. 2008, Warren 2008). Inclusion of mesophyll conductance to CO\(_2\) transfer, \(g_m\), in models of photosynthesis reduces estimates of CO\(_2\) partial pressure at the chloroplasts, \(C_s\), relative to the partial pressure in the intercellular air spaces, \(C_i\). Using \(C_s\) rather than \(C_i\) corrects underestimation of the maximum rate of Rubisco carboxylation, \(V_{\text{max}}\), derived from the analysis of \(A/C\) curves, which assumes that \(g_m\) is infinitely large (Flexas et al. 2008, Warren 2008).

Recent reviews have summarized current understanding of the variability in \(g_m\) between species and its dynamic regulation by environmental variables, but the mechanisms of the responses associated with anatomical, morphological and biochemical characteristics remain largely unknown (Flexas et al. 2008, Warren 2008). Early work (Evans and Loreto 2000) supported relationships between \(g_m\) and structural properties of leaves, particularly mesophyll cell thickness and intercellular air space. However, measurements with a wide range of species show that while \(g_m\) may be limited by leaf structure, it may vary widely within leaves of different species with a similar area to mass ratio (Flexas et al. 2008). It has also been suggested that \(g_m\) may be related to the surface area of chloroplasts bordering sub-stomatal cavities (Evans 1999, Hanba et al. 2001). Parallel increases in \(g_m\) and \(A\) have been observed during early leaf development in broadleaved trees (Miyazawa and Terashima 2001, Eichelmann et al. 2004), but \(g_m\) tends to decrease with increasing leaf age following full expansion within weeks for herbaceous plants (Loreto et al. 1997) and deciduous trees (Grassi and Magnani 2005) and within years for evergreen broadleaved trees (Niinemets et al. 2005, 2006) and conifers (Ethier et al. 2006, Warren 2006).

In canopies, regulation of photosynthesis by leaf scale morphological and biochemical characteristics is further complicated by variability associated with light environment and nitrogen allocation to Rubisco activity (Niinemets et al. 2006). Further, hydraulic limitations may be responsible for age-related decline in photosynthesis and tree growth (Ryan and Yoder 1997, McDowell et al. 2002, 2005), although the mechanisms remain uncertain (Ryan et al. 2006). Photosynthesis is dependent on the diffusive pathways in leaves, and stomatal conductance to CO\(_2\) transfer, \(g_s\), and \(g_m\) may be influenced at the tree scale by water potential gradients associated with differences in tree size during leaf development (Koch et al. 2004, Woodruff et al. 2008, 2009). Given that it is likely that hydraulic regulation of developmental changes in leaf properties and their associated effects on photosynthesis are attributable to path length effects on hydraulic conductance, it is more appropriate to interpret differences in \(g_s\) and \(g_m\) in relation to tree size rather than tree age (Mencuccini et al. 2005).

Warren et al. (2003) were the first to measure changes in \(g_m\) with height through the crown of a 50-year-old, 30-m-tall coniferous Pseudotsuga menziesii tree and showed that \(g_s\) and \(g_m\) limited photosynthesis by 30 and 20%, respectively, but changes in \(g_m\) with height were small. In contrast, Woodruff et al. (2009) measured reductions of 47 and 42% in \(g_m\) and \(A\), respectively, in P. menziesii trees ranging in height from 5 to 55 m and attributed these differences to increased needle thickness and mesophyll cell thickness with increased tree height associated with the effects of water potential gradients on needle development during expansion.

We used an existing successional sequence of Nothofagus solandrii var. clifortioides (mountain beech) trees growing at Craigieburn Forest, central South Island, New Zealand, to investigate the effects of leaf age (Year 0 and Year 1 leaves) and hydraulic constraints associated with tree size (2- and 15-m-tall trees) on stomatal and mesophyll limitations to photosynthesis. Our first hypothesis was that \(g_s\) and \(g_m\) would be lower in Year 1 leaves compared with Year 0 leaves because of increased path length for CO\(_2\) diffusion within older leaves. Our second hypothesis was that \(g_s\) and \(g_m\) would be lower for leaves of the same age on tall trees compared with short trees because of increased hydraulic constraints on leaf development in tall trees.

We estimated \(g_m\) from measurements of photosynthesis and chlorophyll fluorescence using the ’constant J’ method (see below; Loreto et al. 1992). We also used carbon isotopes to interpret the relationship between the long-term effects of leaf age and hydraulic path length on \(g_s\) and \(g_m\). The stable carbon isotope composition of leaf tissue provides an integrated record of the balance of CO\(_2\) supply (stomatal conductance) and demand (photosynthesis) (Farquhar et al. 1982), and it is used commonly to interpret changes in \(C_i/C_s\) (Farquhar et al. 1989). McDowell et al. (2002) attributed linear relationships between isotopic composition and tree size to hydraulic constraints. Similarly, Woodruff et al. (2009) used measurements of carbon isotopic composition to derive relationships between \(A\), \(g_s\) and \(g_m\) integrated across the lifetime of needles. In addition to interpretation of measurements of carbon isotopic composition, we extend the model of isotope discrimination to include a component attributable to \(g_m\) following Barbour et al. (2010). Using this approach, we determined the potential combinations of \(g_s\), \(g_m\) and \(A\) that give the measured isotopic values and we relate this to leaves of the same age from trees of different sizes.

**Materials and methods**

**Site and plant material**

Measurements were made in an age sequence of N. solandrii var. clifortioides trees growing at Craigieburn Forest, central South Island, New Zealand (latitude 43.3°S, longitude 171.0°E, elevation ~1100 m), consisting of plots of short trees in sapling (10 years) and tall trees in pole (120 years) stages of succession. Trees at the sapling stage had resulted from re-growth following disturbance from wind-thrown of canopy trees and...
consisted of a dense canopy of trees with small diameter at 1.3 m above ground level (<30 mm) and a height of ~2 m. The pole stand consisted of a large number of small-diameter (<200 mm) trees undergoing self-thinning, with heights of ~15 m (Davis et al. 2003). The soils in both stands was a Katrine silt loam, predominantly high-country yellow-brown earths (Soil Survey Staff 1998), equivalent to Acidic Allophanic Brown Soil in the New Zealand classification (Hewitt 1998) and to Andic Dystrochrept in the USDA classification, derived from greywacke, loess and colluvium. Mean annual temperature and precipitation at the site are 8 °C and 1447 mm, respectively (McCracken 1980).

**Sampling protocol and leaf characteristics**

Gas exchange measurements were conducted in mid-summer (February) on Year 0 (current-year) and Year 1 (1-year-old) leaves collected from 12 replicate trees in the short and tall stands. Leaves of both ages were fully expanded and Year 0 and Year 1 leaves had emerged ~3 and ~15 months, respectively, before the measurements were made. Fully sunlit shoots were removed from trees using a pruning saw (short trees) and shotgun (tall trees), stems were re-cut under water immediately and samples were taken to the laboratory. The shoots were kept in water and exposed to ambient conditions until gas exchange measurements were made.

Following the measurements of photosynthesis, leaf samples were placed flat and photographed using a digital camera, and leaf area was estimated using digitizing software with the scanned images. Subsequently, the samples were dried at 70 °C and weighed to allow calculation of the ratio of leaf area to leaf dry mass, S. Dried samples were ground and measured for nitrogen concentration and carbon isotope composition with an isotope ratio mass spectrometer (Europa Scientific 20/20) interfaced to a Dumas elemental analyser (Europa Scientific ANCA-SL, Europa Scientific Ltd, Crewe, UK). Nitrogen concentrations are expressed on a mass, N_m, and area, N_s, basis and isotope ratios are presented (as %) in the familiar delta notation as δ^13C = (σ_sample/σ_standard) − 1, where σ is the isotope ratio (13C/12C) and the standard used is CO_2 from Vee Dee Pee Belemnite (VDPB).

**Measurements of photosynthesis**

Measurements of photosynthesis were made using four portable photosynthesis systems (Model 6400; LiCor, Inc., Lincoln, NE, USA) equipped with CO_2 control modules. Three photosynthesis systems were equipped with standard 20 x 30 mm chambers with light sources consisting of blue–red light-emitting diodes (Model 6400-02B) to provide irradiance (400–700 nm), Q, of varying intensity. One photosynthesis system was equipped with an integrated fluorescence detector (Model LI-6400-40 leaf chamber; LiCor, Inc.) for measurements of chlorophyll fluorescence (see below). The four photosynthesis systems were calibrated and matched for CO_2 and water vapour concentrations before use. Measurements of gas exchange on excised shoots were started after stomatal conductance, g_s, and photosynthesis, A, had reached maximum values and g_s remained high during the entire measurement period. The length and width of the leaves were ~5 mm, so several adjacent leaves were placed in the chamber. There was no contribution to gas exchange from leaf area covered by the gaskets around the edge of the chamber because all leaves were fully contained within the chamber and we assumed that the effects of stomatal heterogeneity on measurements of photosynthesis were insignificant. Values of stomatal conductance to CO_2 transfer, g_s, were calculated by dividing values for water vapour transfer by 1.6 (Jones 2002), accounting for differences in the rates of diffusion of water vapour and CO_2 in air. All measurements were made at a constant leaf temperature of 20 °C, determined using an energy balance approach and maintained using thermoelectric coolers. Leaf-to-air vapour pressure deficit was generally between 0.5 and 1.1 kPa. All data are expressed on the basis of one-sided leaf surface area.

The response of photosynthesis, A, to varying intercellular CO_2 partial pressure, C_i, was measured by varying the CO_2 partial pressures in the leaf chamber, C_a, in 13 steps, decreasing from 150 to 0 Pa, using a flow rate of 500 µmol s⁻¹ at a saturating irradiance, Q (400–700 nm), of 1000 µmol m⁻² s⁻¹. Measurements were recorded automatically at each C_a set point when photosynthesis had equilibrated, which was typically <2 min. We analysed the A/C_i curves using the Farquhar et al. (1980) model to estimate the maximum carbon fixation rate of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), V_{c,max}, the maximum rate of photosynthesis at saturating Q and ambient CO_2 partial pressure (C_a = 38 Pa), A_{sat}, the maximum rate of photosynthesis at saturating Q and saturating CO_2 partial pressure (80 Pa), A_{max}, and g_s at saturating Q and ambient CO_2 partial pressure. Following Farquhar et al. (1980), calculation of these variables assumed that g_m was infinitely large. We used the Michaelis–Menten constants of Rubisco described in Bernacchi et al. (2001), adjusted to 20 °C, such that K_c (rate constant for Rubisco carboxylation) = 23.12 Pa, K_m (rate constant for oxygenation) = 21.39 Pa and τ (specificity factor for Rubisco) = 2823 (Jordan and Ogren 1984). Subsequently, when fitting the response of A to the CO_2 partial pressure at the chloroplasts, C_i, we used values of K_m = 15.34 Pa and K_c = 14.02 Pa adjusted to 20 °C following Bernacchi et al. (2002).

Measurements of chlorophyll fluorescence on light-adapted leaves were made simultaneously with measurements of photosynthesis at each value of C_i to determine the photochemical efficiency of photosystem II (Φ_{PSII}) from (F_{m'} - F)/F_{m'}, where F and F_{m'} are the steady and maximal fluorescence, respectively (Schreiber et al. 1994). Φ_{PSII} is related directly to the rate of electron transport, J (Genty et al. 1989), and is used to determine the portion of the A/C_i curve where J is constant.
We estimated values of the rate of mitochondrial respiration in the light, \( R_m \), and the CO\(_2\) compensation point at the chloroplasts, \( \Gamma^* \), using the photo-compensation point method of Laisk and Oja (1998). Additional \( A/C \) curves were measured at three low levels of \( Q \) (300, 150 and 50 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)) at seven values of \( C_c \) (decreasing from 20 to 0 Pa) to generate relationships on the linear part of the \( A/C \) curve. Extrapolation of the common point of intersection of the three \( A/C \) curves to the \( A \) axis generated an estimate of \( R_m \), while extrapolation to the \( C_c \) axis generated an estimate of the intercellular CO\(_2\) compensation point in the absence of day respiration, \( C_c^* \), and this was assumed to be equal to \( \Gamma^* \) (DeLucia et al. 2003). Mean values of \( R_m \) and \( \Gamma^* \) were calculated for each leaf class and used in the estimation of \( g_m \).

**Estimation of \( g_m \) using the constant \( J \) method**

Mesophyll conductance, \( g_m \), was estimated using the ‘constant \( J \)’ method (Loreto et al. 1992), which assumes that when the rate of photosynthetic electron transport, \( J \), becomes constant, further increases in \( A \) with increasing \( C_c \) are due to suppression of photorespiration because the rate of carboxylation progressively substitutes the rate of oxygenation (Harley et al. 1992, Long and Bernacchi 2003, Singsaas et al. 2003). In these conditions, photosynthesis is related to the CO\(_2\) partial pressure at the site of fixation in the chloroplasts, \( C_c \), and the relative specificity of Rubisco to CO\(_2\) and O\(_2\), which is normally described by the chloroplastic CO\(_2\) compensation point, \( \Gamma^* \). Estimates of \( g_m \) were obtained from values of \( \Gamma^* \) and \( R_m \) and data from the linear part of the \( A/C \) response following Harley et al. (1992). Values of \( g_m \) were obtained from three or more measurements of photosynthesis from the \( A/C \) response curves at high \( C_c \) partial pressure when the rate of electron transport, \( J \), was constant (Singsaas et al. 2003, Warren 2006). The value for \( g_m \) was resolved iteratively as the value that provided the lowest variance of \( J \) associated with \( A \).

**Data analysis**

All analyses were undertaken at the leaf level using SPSS software (SPSS for Windows, version 11.0.1, 2001; SPSS, Inc., Chicago, IL, USA). Variables were tested for normality and homogeneity of variance, and logarithmic transformations were made as necessary to meet the underlying statistical assumptions of the models. The main and interactive effects of leaf age and tree size were tested using analysis of variance.

**Stomatal and mesophyll limitations to photosynthesis**

The responses of photosynthesis to ambient, \( C_a \), intercellular, \( C_c \), and chloroplastic, \( C_i \), CO\(_2\) partial pressures were derived from estimates of average values of \( g_s \) and \( g_m \) for each of the four leaf classes using the relationships (Farquhar and Sharkey 1982)

\[
A = g_s (C_a - C_i) = g_m (C_i - C_c).
\]  # (1)

This assumes that boundary layer effects were negligible (Lanigan et al. 2008), which is reasonable, considering the small size of *Nothofagus* leaves and the good mixing within the leaf chamber. The limitations to photosynthesis imposed by stomatal, \( L_s \), and mesophyll, \( L_m \), conductance were estimated from calculations of \( A \) using measured values of \( g_s \) and \( g_m \) or assuming that they were infinite, following the procedure adopted by Warren et al. (2003). Rates of photosynthesis, \( A_m \), at saturating irradiance (1000 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)) and ambient CO\(_2\) partial pressure (38 Pa) were estimated using average measurements of stomatal, \( g_s \), and mesophyll \( g_m \), conductance. Rates of photosynthesis at saturating irradiance when \( C_s = C_i \) were estimated using measured \( g_s \) and assuming \( g_m \) was infinite, \( A_m \), and rates of photosynthesis at saturating irradiance when \( C_i = C_s \) were estimated using measured \( g_m \) and assuming \( g_s \) was infinite, \( A_s \). Stomatal and mesophyll limitations to photosynthesis were then calculated from

\[
L_s = \frac{(A_n - A_s)}{A_s} \quad \text{and} \quad L_m = \frac{(A_n - A_m)}{A_m}.
\]  # (2)

**Estimation of the effects of \( g_m \) on operating values of \( A \) and \( g_s \)**

Fractionation of carbon isotope composition during photosynthesis, \( \Delta \), is described by Farquhar et al. (1989) as

\[
\Delta = b + (b - a) \frac{C_i}{C_a}
\]  # (3)

where \( a \) (4.4\%) and \( b \) (assumed to be 27\%) are diffusional and biochemical isotope fractionation factors, respectively. However, it is the chloroplastic CO\(_2\) partial pressure, \( C_i \), that is important for discrimination, as is evident when the full derivation of Eq. (3) is considered (Brugnoli and Farquhar 2000, Barbour et al. 2010):

\[
\Delta = a_0 \frac{C_a - C_i}{C_a} + a \frac{C_i - C_c}{C_a} + (b_s + a) \frac{C_i - C_e}{C_a} + b \frac{C_i}{C_a} - f \frac{\Gamma^*}{C_a} - e' \frac{R_d}{A} \frac{C_a - \Gamma^*}{C_a}
\]  # (4)

where \( a_0 \) (2.9\%, noting that we have assumed very high boundary layer conductance for *Nothofagus* leaves given their small size) and \( a_1 \) (0.7\%) are fractionations associated with diffusion through the boundary layer and leaf water, respectively; \( b_s \) is fractionation as CO\(_2\) moves into solution (1.1\% at 25 °C); \( C_s \) is the CO\(_2\) partial pressure at the leaf surface, and \( e \) (−3\%, Bickford et al. 2009) and \( f \) (11.6\%, Lanigan et al. 2008) are fractionations associated with mitochondrial respiration in the light and photorespiration, respectively. \( \Gamma^* \) is the CO\(_2\) compensation point in the absence of mitochondrial
respiration, and \( R_d \) is measured as described above. Note that the value of \( b \) in Eq. (4) is \( \sim 30\% \), the true discrimination by Rubisco and other carboxylating enzymes, rather than the approximate value to account for other fractionations within the mesophyll as in Eq. (3) above.

Measured values of leaf tissue isotopic composition, \( \delta^{13}C_p \), were converted to discrimination, \( \Delta \), using

\[
\Delta = \frac{\delta^{13}C_a - \delta^{13}C_p}{1 + \delta^{13}C_p}
\]

where \( \delta^{13}C_a \) is the isotopic composition of CO₂ in air and was assumed to be \( \sim 8\% \). Equations (3) and (4) may be solved for \( A \) at a range of values for stomatal conductance to CO₂, \( g_s \), incorporating Eq. (1) using values for the parameters listed above. The model was implemented using the Solver function in Excel (version 2007) where a value of \( g_s \) was input and Eqs (1), (3) and (4) were solved iteratively for \( A \) until \( \Delta \) matched the measured value for each leaf class. We assumed that Eqs (3) and (4) relate directly to bulk leaf material, i.e., that post-photosynthetic fractionation within the leaves is negligible, although we recognize that this is not strictly true. For example, lignin is known to be \( \sim 4\% \) more depleted in \( \delta^{13}C \) than sucrose (Schmidt and Gleixner 1998) and lignin content likely varies with leaf age (Miyazawa et al. 2003) and covaries with the ratio of leaf area to dry mass in trees of different heights (Ninemets et al. 1999). For this reason, we restrict our interpretation of fitted values to a comparison between the simple model (Eq. 3) that assumes infinite \( g_m \) and the more comprehensive model (Eq. 4) that includes \( g_m \) for each leaf class.

Results

Leaf properties and rates of photosynthesis

Leaf area to mass ratio, \( S \), was significantly lower for Year 1 leaves compared with Year 0 leaves in stands of both ages (average difference 5%), and \( S \) was also significantly lower for both ages in tall trees (120-year-old) compared with short trees (10-year-old, average difference was 9%, Table 1). This was associated with a significantly lower value for nitrogen concentration per unit mass, \( N_m \), in Year 1 leaves compared with Year 0 leaves (average difference 18%) but, on a leaf area basis, nitrogen concentration, \( N_a \), was not significantly different. Values of \( N_a \) were significantly higher for tall trees compared with short trees (average difference 10%). Rates of photosynthesis at saturating irradiance and saturating CO₂ partial pressure, \( A_{\text{satQ}} \), and at saturating irradiance and ambient CO₂ partial pressure, \( A_{\text{satQ}} \), were significantly lower for Year 1 leaves (both by 25%) compared with values for Year 0 leaves in the stands of both tree sizes.

Stomatal and mesophyll conductance

Overall, the values of stomatal conductance to CO₂ transfer, \( g_s \), and mesophyll conductance, \( g_m \), were not significantly different between leaf ages. However, \( g_s \) and \( g_m \) were 24 and 19% lower, respectively, for leaves on tall trees compared with short

---

**Table 1.** Leaf characteristics and rates of photosynthesis for Year 0 (current-year) and Year 1 (1-year-old) *N. solandrii* var. *cliffortiodes* leaves on short (10-year-old) and tall (120-year-old) trees.

<table>
<thead>
<tr>
<th>Tree age (years)</th>
<th>10</th>
<th>10</th>
<th>120</th>
<th>120</th>
<th>ANOVA statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf age (years)</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>( S ) (m² kg⁻¹)</td>
<td>5.58 ± 0.21</td>
<td>5.25 ± 0.15</td>
<td>5.01 ± 0.12</td>
<td>4.80 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>( N_m ) (mmol g⁻¹)</td>
<td>0.87 ± 0.02</td>
<td>0.70 ± 0.01</td>
<td>0.84 ± 0.02</td>
<td>0.71 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>( N_a ) (mmol m⁻²)</td>
<td>158.0 ± 6.2</td>
<td>133.5 ± 4.5</td>
<td>168.9 ± 6.0</td>
<td>149.5 ± 6.0</td>
<td></td>
</tr>
<tr>
<td>( A_{\text{max}} ) (µmol m⁻² s⁻¹)</td>
<td>28.2 ± 1.4</td>
<td>20.3 ± 0.8</td>
<td>25.0 ± 0.8</td>
<td>19.6 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>( A_{\text{satQ}} ) (µmol m⁻² s⁻¹)</td>
<td>16.1 ± 1.1</td>
<td>12.1 ± 0.5</td>
<td>14.7 ± 0.7</td>
<td>10.9 ± 0.7</td>
<td></td>
</tr>
</tbody>
</table>

Data shown are mean ± standard error values for the leaf area to mass ratio, \( S \), nitrogen concentration on a mass, \( N_m \), and area, \( N_a \), basis, rates of photosynthesis at saturating irradiance and saturating CO₂ partial pressure, \( A_{\text{max}} \), and at saturating irradiance and ambient CO₂ partial pressure, \( A_{\text{satQ}} \). Significance of the main effects of leaf age, \( L \), tree size, \( T \), and their interaction, \( L \times T \), are shown with the \( P \) values or as not significant, ns, when \( P > 0.05 \).
Table 2. Estimates of stomatal and mesophyll characteristics for Year 0 (current-year) and Year 1 (1-year-old) *N. solandri* var. *cliffortiodes* leaves on short (10-year-old) and tall (120-year-old) trees.

<table>
<thead>
<tr>
<th>Tree age (years)</th>
<th>Leaf age (years)</th>
<th>0</th>
<th>1</th>
<th>0</th>
<th>1</th>
<th>ANOVA statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>10</td>
<td>120</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$g_s$ (mmol m$^{-2}$ s$^{-1}$)</td>
<td>89.9 ± 13.2</td>
<td>76.1 ± 9.3</td>
<td>67.4 ± 7.1</td>
<td>58.7 ± 6.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$g_m$ (mmol m$^{-2}$ s$^{-1}$)</td>
<td>73.7 ± 3.5</td>
<td>84.9 ± 6.4</td>
<td>56.3 ± 2.6</td>
<td>72.7 ± 7.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$g_m/g_s$ (mol mol$^{-1}$)</td>
<td>0.94 ± 0.15</td>
<td>0.92 ± 0.11</td>
<td>0.83 ± 0.11</td>
<td>1.00 ± 0.14</td>
</tr>
<tr>
<td>$\delta^{13}C$ (%)</td>
<td></td>
<td>−28.21 ± 0.18</td>
<td>−28.78 ± 0.17</td>
<td>−27.04 ± 0.26</td>
<td>−27.50 ± 0.23</td>
<td>L ns</td>
</tr>
</tbody>
</table>

Data shown are mean ± standard error values for stomatal conductance to CO$_2$ transfer, $g_s$, mesophyll conductance, $g_m$, and the $\delta^{13}C$ isotopic composition of leaves. Significance of the main effects of leaf age, $L$, tree size, $T$, and their interaction, $L \times T$, are shown with the $P$ values or as not significant, ns, when $P > 0.05$. Values for $g_s$ needed to be logarithmically transformed before the analysis of variance was undertaken.

trees (Table 2). The mean ratio of $g_m/g_s$ for leaves within each treatment was similar, with an overall average (±SE) value of 0.93 ± 0.06 mol mol$^{-1}$.

Analysis of the responses of photosynthesis, $A$, to CO$_2$ partial pressure in ambient air, $C_a$, in intercellular spaces, $C_i$, and at the chloroplasts, $C_c$, highlighted the higher rates of photosynthesis in Year 0 leaves compared with Year 1 leaves. Further, values of $C_c$ were higher for Year 0 leaves compared with Year 1 leaves (Figure 1). From these responses and Eqs (1) and (2), the limitation to photosynthesis by stomatal conductance, $L_s$, was similar for leaves of both ages from short and tall trees except for Year 1 leaves on short trees where $L_s$ was lower (Table 3). Limitations to photosynthesis by mesophyll conductance, $L_m$, were higher than $L_s$ for all leaves and values were higher for Year 0 leaves than for Year 1 leaves on trees of both sizes.

### Parameters describing photosynthesis

The mitochondrial CO$_2$ compensation point at the chloroplasts, $r^*$ (mean ± SE = 3.1 ± 0.23 Pa), and the rate of mitochondrial respiration in the light, $R_d$ (mean ± SE = 1.6 ± 0.17 μmol m$^{-2}$ s$^{-1}$), were very similar for leaves in both leaf age and tree size classes. The maximum rate of Rubisco carboxylation, $V_{\text{cmax}}$, calculated on the basis of $C_c$ was 44% higher for Year 0 leaves compared with Year 1 leaves and significantly higher within both tree size classes (Table 4), consistent with differences in $A_{\text{max}}$ and $A_{\text{sat}}$ (Table 1). There were no significant differences in $V_{\text{cmax}}$ with tree size for Year 0 or Year 1 leaves.

Values of $V_{\text{cmax}}$ expressed on the basis of $C_c$ were higher than those expressed on the basis of $C_i$, and differences between leaf age, but not tree size, were highly significant (Table 4). The mean (±SE) increase in $V_{\text{cmax}}$ calculated on the basis of $C_c$ rather than $C_i$ across all leaves was 60 ± 3%.

### Discussion

We have demonstrated that $S$, $N_m$, and $A_{\text{max}}$ were lower in Year 1 leaves compared with Year 0 leaves but we did not detect significant differences in $g_s$ or $g_m$. We could not detect significant differences in $g_s$ for leaves of the same age from tall trees compared with those on short trees, but values of $g_m$ were lower in leaves from tall trees. Values of foliar $\delta^{13}C$ were less depleted for leaves of the same age on tall trees compared with short trees, consistent with more pronounced limitation to photosynthesis from diffusive conductance, possibly attributable to lower $g_m$ associated with thicker leaves. However, lower values of $V_{\text{cmax}}$ in Year 1 leaves compared with Year 0 leaves suggest more pronounced biochemical limitation of photosynthesis, possibly associated with lower nitrogen concentrations.
in Year 1 leaves. Our results provide further support for the notion that there is more pronounced hydraulic limitation to photosynthesis with increasing tree height.

Our values of \( g_m \) were lower than maximum values reported for a wide range of species in earlier reviews (Manter and Kerrigan 2004, Flexas et al. 2008, Warren 2008), although Flexas et al. (2008) emphasize the dynamic nature of \( g_m \) and the high variability associated with different species, growing conditions and environmental variables. Warren (2008) pointed out that it is more useful to compare the drawdown in CO\(_2\) partial pressure between \( C_a \) and \( C_i \) and between \( C_i \) and \( C_c \) among species than absolute values of \( g_m \). For the leaves in our study (data not shown), the mean values of \( C_a - C_i \) and \( C_i - C_c \) were 15.6 and 9.8 Pa, respectively, and these are very similar to the mean values of 13.6 and 8.8 Pa reported for woody deciduous trees (Warren 2008). Further, the ratio of \( g_m/g_s \) was close to unity for all leaves, similar to the value of 0.82 mol mol\(^{-1}\) for deciduous trees (Warren 2008).

Variability in \( g_m \) with leaf age is likely attributable to both anatomical and biochemical factors (Warren 2008). Niinemets et al. (2005) attributed lower rates of photosynthesis in older leaves of shrub species to lower \( N_m \) and reduced amounts of photosynthetic proteins, but lower values of \( g_m \) were attributable

Table 3. Estimates of stomatal, \( L_s \), and mesophyll, \( L_m \), limitations to photosynthesis for Year 0 (current-year) and Year 1 (1-year-old) *N. solandrii* var. *cliffortiodes* leaves on short (10-year-old) and tall (120-year-old) trees calculated from analysis of the average response of photosynthesis, \( A \), to CO\(_2\) partial pressure in ambient air, \( C_a \), intercellular spaces, \( C_i \), and at the chloroplasts, \( C_c \), shown in Figure 1 and Eqs (1) and (2) for each leaf class.

<table>
<thead>
<tr>
<th>Tree age (years)</th>
<th>10</th>
<th>10</th>
<th>120</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf age (years)</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>( L_s )</td>
<td>0.31</td>
<td>0.18</td>
<td>0.35</td>
<td>0.33</td>
</tr>
<tr>
<td>( L_m )</td>
<td>0.52</td>
<td>0.22</td>
<td>0.55</td>
<td>0.39</td>
</tr>
<tr>
<td>( L_s/L_m )</td>
<td>0.60</td>
<td>0.82</td>
<td>0.64</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Figure 1. Graphical analysis of the response of photosynthesis, \( A \), to CO\(_2\) partial pressure in ambient air, \( C_a \), in intercellular spaces, \( C_i \), and at the chloroplasts, \( C_c \), for Year 0 (current-year) and Year 1 (1-year-old) *N. solandrii* var. *cliffortiodes* leaves on short (10-year-old) and tall (120-year-old) trees. Rates of photosynthesis, \( A_n \), at saturating irradiance (1000 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)) and ambient CO\(_2\) partial pressure (38 Pa) were estimated using the mean measurements of stomatal conductance, \( g_s \), and mesophyll conductance, \( g_m \), given in Table 2 with Eq. (1). Rates of photosynthesis at saturating irradiance when \( C_c = C_i \) were estimated using measured \( g_s \) and assuming \( g_m \) is infinite, \( A_m \), and rates of photosynthesis at saturating irradiance when \( C_i = C_a \) were estimated using measured \( g_m \) and assuming \( g_s \) is infinite, \( A_s \). Stomatal, \( L_s \), and mesophyll, \( L_m \), limitations estimated from Eq. (2) and the data in the figure are given in Table 3.
to higher cell wall fractions in older leaves. The increased cell wall fraction in older *Quercus ilex* leaves led to increased limitations to diffusion resulting from structural acclimation to the light environment and accumulation of structural compounds (Niinemets et al. 2006). In contrast to these findings, despite differences in *g*$_m$ and *g*$_s$ in needles ranging in age from current year to 3 years in *Pinus pinaster*, Warren (2006) found no differences in stomatal or mesophyll limitations to photosynthesis with increasing needle age. Lower rates of photosynthesis with increasing needle age were attributed to lower Rubisco activity. However, lower values of *g*$_m$ are generally associated with thicker leaves (Warren and Adams 2006). Although mechanistic explanations to account for differences in *g*$_m$ in relation to leaf structural properties are not known, data from a wide

<table>
<thead>
<tr>
<th>Tree age (years)</th>
<th>Leaf age (years)</th>
<th><em>V</em>$_{cmax}$, <em>C</em>$_i$ basis ($\mu$mol m$^{-2}$ s$^{-1}$)</th>
<th><em>V</em>$_{cmax}$, <em>C</em>$_c$ basis ($\mu$mol m$^{-2}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
<td>43.5 ± 2.4</td>
<td>69.0 ± 6.2</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>24.0 ± 1.2</td>
<td>29.0 ± 2.0</td>
</tr>
<tr>
<td>120</td>
<td>0</td>
<td>37.0 ± 1.9</td>
<td>58.7 ± 3.8</td>
</tr>
<tr>
<td>120</td>
<td>1</td>
<td>21.3 ± 2.0</td>
<td>42.3 ± 6.4</td>
</tr>
</tbody>
</table>

Table 4. Estimates of the maximum rate of Rubisco carboxylation, *V*$_{cmax}$, for Year 0 (current-year) and Year 1 (1-year-old) *N. solandrii* var. **clifforiodes** leaves on short (10-year-old) and tall (120-year-old) trees.

Data shown are mean ± standard error values for *V*$_{cmax}$ calculated on the basis of intercellular CO$_2$ concentration, *C*$_i$, and CO$_2$ concentration at the chloroplasts, *C*$_c$. Significance of the main effects of leaf age, *L*, tree size, *T*, and their interaction, *L* × *T*, are shown with the *P* values or as not significant, ns, when *P* > 0.05.
range of species suggest that leaf structure sets the upper limit for \( g_m \) in leaves with high values of \( S \) (younger leaves) but actual \( g_m \) varies in response to other environmental variables (Flexas et al. 2008).

Despite significantly higher values of \( S \) in Year 0 leaves compared with Year 1 leaves at our site, the lack of significant differences in \( g_s \) and \( g_m \), but marked differences in \( N_m \) and \( V_{cmax} \) between leaf ages support the conclusion that differences in the rate of photosynthesis are attributable to biochemical limitation of photosynthesis, possibly associated with low nitrogen availability. The values of \( N_m \) for *Nothofagus* were low when compared with other New Zealand native broad-leaved, nitrogen-fixing species (Dungan et al. 2003, Whitehead et al. 2005), non-nitrogen-fixing species (Whitehead and Walcroft 2005) and mature *Nothofagus fusca* (Hollinger 1996).

In an earlier study at our site, Clinton et al. (2002) suggested that low \( N_m \) in both the 10- and 120-year-old stands could be attributable to reduced soil nitrogen availability, possibly resulting from disturbance, compared with an adjacent mature (150+ years old) stand, leading to lower rates of productivity. We also found that \( N_m \) for the two leaf ages was not significantly different, suggesting that lower \( N_m \) in Year 1 leaves might be due to dilution of nitrogen with increased mass per unit area (lower \( S \)), as reported in the Mediterranean shrub species *Q. ilex* by Niinemets et al. (2006). Further support for biochemical limitation to photosynthesis is provided by reconstruction of the response curves of photosynthesis to ambient, intercellular and chloroplastic \( CO_2 \) partial pressure (Figure 1) and estimates of stomatal, \( L_s \), and mesophyll, \( L_m \), limitations to photosynthesis (Table 3). The rates of photosynthesis in Year 1 leaves were much lower than those in Year 0 leaves but \( L_m \) was lower in Year 1 leaves, suggesting that biochemical limitation to photosynthesis was higher for Year 1 leaves compared with Year 0 leaves.

Our data demonstrate that \( g_s \) and \( g_m \) were generally lower in the tall trees compared with the short trees for both leaf ages, but only the difference in \( g_m \) was significant. Evidence from several studies suggests that \( g_m \) declines with increasing water stress over periods from several days to weeks (Loreto et al. 1997, Flexas et al. 2008), but there have been few studies of changes in \( g_m \) with increasing height within tree canopies. Warren et al. (2003) showed an increase in \( g_m \) from 160 to 200 mmol m\(^{-2}\) s\(^{-1}\) between the lower and upper canopy in a 34-m-tall *P. menziesii* tree, but this difference was likely associated with higher levels of irradiance or higher leaf nitrogen concentration in the upper canopy. In contrast, Woodruff et al. (2009) found a decrease in \( g_m \) of 1.1 mmol m\(^{-2}\) s\(^{-1}\) per metre increase in height when measured in *P. menziesii* trees ranging in height from 5 to 55 m. They concluded that decreasing \( g_m \) with increasing tree height was associated with an increasing water potential gradient and resulting effects of turgor on leaf expansion in taller trees.

Enrichment of foliage \( \delta^{13}C \) with increasing tree height (McDowell et al. 2002, 2005) is consistent with a height-related increase in water use efficiency resulting from increased hydraulic limitation. Although this may be more pronounced in upper canopies where foliage is fully exposed to high irradiance, the effect may be obscured in the lower canopy because of shading effects on stomatal conductance and photosynthesis (Waring and Silvester 1994). In our study, lack of significant differences in \( g_s \), \( A_{max} \) and \( A_{i,n} \) between leaves of the same age on both short and tall trees suggests the absence of shading effects in trees of different heights. However, the highly significant enrichment in foliage \( \delta^{13}C \), the decrease in \( g_m \), and higher \( L_a \) and \( L_m \) for leaves of the same age on tall trees compared with those on short trees provide evidence to support the hydraulic limitation hypothesis (McDowell et al. 2002, 2005) and its effect on \( g_m \). This effect is independent of a biochemical limitation to photosynthesis as shown by the lack of differences in \( V_{cmax} \) with tree height.

The range in our data was not sufficient to allow us to reveal relationships between \( A \) and \( g_m \) but other studies have shown that these variables scale proportionally (Loreto et al. 1992, DeLucia et al. 2003, Warren et al. 2003, Bown et al. 2009), although the relationship varies with the magnitude of \( g_m \) (Ethier and Livingston 2004, Warren and Adams 2006, Warren 2008). Variability in \( g_m \) is often not explained well by differences in \( A_{max} \), partly because of errors in estimating \( g_m \) (Warren 2008) and because of differences in the relationship between \( g_m \) and \( A_{max} \) among species (Warren 2004). This results in differences in the magnitude of the gradient between \( C_i \) and \( C_c \) and thus differences in the interpretation of \( V_{cmax} \) and \( J_{max} \) from \( A/C_i \) rather than \( A/C_c \) curves. Most values of \( V_{cmax} \) reported in the literature are based on calculations from \( A/C_c \) curves (Farquhar et al. 1980), which assume that \( g_m \) is infinitely large and therefore there is no reduction in \( CO_2 \) partial pressure from \( C_i \) to \( C_c \), leading to underestimation of \( V_{cmax} \) (Ethier and Livingston 2004). In our study, calculations of \( V_{cmax} \) using \( A/C_c \) curves were 60% higher than those using \( A/C_i \) curves, which is exactly the same increase as the mean value for a range of species compiled by Warren (2008), noting the need to use appropriate kinetic constants (Bernacchi et al. 2002, Ethier and Livingston 2004).

We are aware that our estimates of \( g_m \) using the constant \( J \) method are insensitive to changes in \( R_{d} \) but very sensitive to errors in \( \Gamma^{*} \) (Harley et al. 1992). We minimized these errors using independent estimates of \( \Gamma^{*} \) (Pons et al. 2009), avoided sensitivity of \( \Gamma^{*} \) to temperature (Warren 2008) by making all measurements at the same temperature and maximized the number of measurements in the curvature region of the \( A/C_c \) curve (Ethier and Livingston 2004). Further, we minimized errors in \( R_{d} \) from leakage of \( CO_2 \) across the gasket in the leaf chamber (Pons and Welschen 2002) because leaves were fully contained within the chamber except for petioles passing...
through the gasket. Further, the flow rate was set to ensure a reasonable difference in CO₂ partial pressure between inside and outside the chamber (Pons et al. 2009).

Estimates of instantaneous water use efficiency obtained from measurements of A/g′, where g′ is stomatal conductance to water vapour, would not have been appropriate in our study because we were working with detached branches. However, estimates of water use efficiency from measurements of foliar δ¹³C can be used as an integrated measure for the life of the leaf, as described by Woodruff et al. (2009). Our measurements showing that δ¹³C values were less depleted for leaves of the same age on tall trees compared with short trees provide support for a height-related increase in water use efficiency, which is consistent with the observation of increasing hydraulic limitation of photosynthesis with increasing height (Ryan and Yoder 1997, McDowell et al. 2002, 2005, Woodruff et al. 2009). Fitting the model of carbon isotope discrimination using long-term integrated measurements of foliage δ¹³C and Eq. (4) to show the potential operating combination of A and gₘ highlighted the importance of gₘ in regulating photosynthesis. The surfaces showed that, when using measured values of gₘ, A was overestimated substantially when gₘ was excluded from the model. The model allows interpretation of the response of A to a wide range in gₘ and is not constrained to the values measured on the sampled shoots.

Independent estimates of gₘ combined with our model of carbon isotope fractionation provide a powerful approach to estimating actual, integrated operating values of A within set limits of gₘ. Incorporation of fractionation attributable to gₘ in the model highlighted the significance of limitation to photosynthesis by gₘ and could be used to analyse responses of gₘ to other environmental variables.

In conclusion, our data and model of carbon isotope fractionation that included discrimination attributable to gₘ provide further evidence for strong limitation to photosynthesis from mesophyll conductance that is more pronounced in tall trees compared with short trees. However, differences in photosynthesis between leaves of different ages on trees of the same height were more strongly attributable to differences in biochemical limitation than gₘ. Our findings also confirm the underestimation of Vₘₐₓ if the effects of gₘ are ignored, highlighting the need to incorporate responses of gₘ to environmental variables in models of canopy photosynthesis.

Acknowledgements

We are grateful to Gilbert Ethier for early discussion on data interpretation and to Markus Löw for undertaking statistical analysis. We thank Rob Allen and Murray Davis for permission to use the field plots and John Hunt for collecting the leaves using a shotgun.

Funding

Funding for this work was provided by the Foundation for Research, Science and Technology, New Zealand.

References


