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RESEARCH ARTICLE

The response of leaf respiration to water stress in *Nothofagus* species

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_Nothofagus solandri_ is regarded as more tolerant to drought than _Nothofagus menziesii_ in the field. However, the physiology of responses to water limitation in these species is not well understood. In this study, the thermal sensitivity of leaf respiration and its underlying metabolism in response to drought were investigated in mature trees and saplings. Respiration (_R_\(_d\)) and photosynthesis (_A_\(_\text{max}\)) were measured during drying and re-wetting cycles. In addition, respiratory pathway changes were evaluated by oxygen isotope fractionation and protein analyses. Under drought treatment in the glasshouse, both species showed similar photosynthetic performance, but under mild water stress _N. solandri_ was able to increase _A_\(_\text{max}\). Under moderate water deficit (around −2 MPa), _N. solandri_ increased respiration at a base temperature of 10°C (_R_\(_{10}\)) but then decreased it to initial values after re-watering. In _N. menziesii_, _R_\(_{10}\) did not respond significantly to water-stress treatment. The temperature sensitivity of _R_\(_d\) (_Q_\(_{10}\) and _E_\(_o\)) was unchanged for both species during the gradual deficit water treatment in the glasshouse. Although respiratory electron flow was mainly via the cytochrome pathway under all conditions, an increase in alternative oxidase/cytochrome oxidase protein content suggests that the alternative pathway is involved in modulating respiratory metabolism during the recovery after drought.

**Keywords:** alternative oxidase; cytochrome oxidase; drought; metabolism; New Zealand; trees

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**Introduction**

Approximately half of daily net photosynthetic carbon fixation is released through respiration (_R_) the following evening (Turnbull et al. 2001; Flexas et al. 2006). Respiration by leaves plays an important role in determining growth and survival of plants as it produces energy and carbon skeletons required for biosynthesis and cellular maintenance. In spite of its recognized importance, the effects of water stress on respiration at the physiological level are poorly understood. The available experimental evidence does not support a clear pattern of _R_\(_d\) response to water stress, with different studies showing increased (Zagdanska 1995; Bartoli et al. 2005; Galmés et al. 2007), unaffected (Lawlor & Fock 1975; Flexas et al. 2005) or decreased (González-Meler et al. 1999; Ribas-Carbó et al. 2005b) rates of respiration. Irrespective of how respiration responds to water stress, the ratio of _R_ to assimilation (_A_) generally increases in response to increasing water stress, and decreases in response to re-watering (Atkin & Macherel 2009).

Plant respiration comprises a suite of biochemical reactions that are sensitive to temperature. This thermal response may vary in response to environmentally induced changes in substrate supply (e.g. due to shade-induced or drought-induced changes in photosynthesis) and energy demand (e.g. resulting from water-stress-induced
increases in protein turnover or maintenance of ion gradients; Slot et al. 2008; Atkin & Macherel 2009). In spite of its well-recognized importance to plant carbon balance, regulation of respiration by drought at the metabolic level is not well understood. It has been indicated that in dry sites, respiration increases in some tree species while photosynthesis decreases—this shifts the balance of carbon gain and loss, limiting carbon acquisition in drier sites with respect to wetter sites (Turnbull et al. 2001).

The action on plants of various adverse factors, including drought, disturbs electron transport along the main cytochrome-mediated oxidation pathway (CP) and so reduces ATP synthesis. The disturbance of electron transport dramatically increases the potential for the generation of superoxide and other reactive oxygen species (González-Meler et al. 1999; Noguchi et al. 2005; Ribas-Carbó et al. 2005b; Armstrong et al. 2006; Shugaeva et al. 2007). Fortunately, plants possess an alternative non-phosphorylating pathway (AP) that channels the electrons directly from ubiquinone to O₂ through the alternative oxidase protein (AOX). Several positive roles are attributed to the AP: it may assist in the maintenance of redox balance in electron transport when the CP is restricted (Millenaar et al. 2000; Florez-Sarasa et al. 2007), allow carbon flux through the tricarboxylic acid cycle when ADP supply limits CP activity, or may protect against harmful reactive oxygen generation (Atkin et al. 2002; Bartoli et al. 2005; Kumar et al. 2007; Flexas et al. 2006). It has been reported that severe water stress induces a sharp decrease in CP activity, concomitantly with a similar increase in the AP rate, so that total respiration rate remains relatively constant (Ribas-Carbó et al. 2005b). Increases in AP respiration have been shown to be accompanied by increases in AOX protein abundance under stress conditions (Taylor et al. 2002; Bartoli et al. 2005).

Beech forest in New Zealand usually grows on hilly or mountainous terrain, where the weather is colder and wetter, growing seasons are shorter and soils are less fertile than low altitudes, where mixed conifer–broadleaf forest dominates (Orwin 2008). *Nothofagus solandri* commonly dominates the forests at high altitudes in the drier inland mountain regions of the South Island, while *Nothofagus menziesii* dominates or co-dominates with *N. solandri* at timberlines with high rainfall and improved soil conditions (Wardle 1984). Sun et al. (1995) relate this difference in distribution of *N. solandri* and *N. menziesii* to differences between the two species in tolerance to water stress, with *N. solandri* much more tolerant of water stress than *N. menziesii*. In this study, we conducted studies quantifying changes in leaf respiration (and oxidase activity) in response to the gradual onset of water deficit and during recovery from drought. An overarching notion was that the drought-tolerant *N. solandri* is capable of greater adjustment in respiration in response to drought, being able to maintain or decrease its overall respiration with respect to photosynthesis in comparison to its less drought-tolerant counterpart *N. menziesii*. This would allow *N. solandri* to cope with lower water availability in its habitat, especially during the summer. We further hypothesize that *N. solandri* responds to severe drought by increasing the respiratory flux through the AP and decreasing the flux through the CP. In contrast, *N. menziesii* has limited ability to change electron partitioning between both pathways under severe water stress.

**Materials and methods**

**Plant material and treatments**

**Mature trees** of both study species (500–700 cm in height) growing under open conditions were selected to determine the respiratory response to seasonal variation in temperature and water availability. We selected individuals of *Nothofagus solandri* var. cliffortioides (Mountain Beech) and *Nothofagus menziesii* (Silver Beech) growing on the campus of the University of Canterbury, New Zealand (43°32’S; 172°37’E) with heights between 500 and 700 cm growing in open sites or at the edge of other
trees. Seasonal measurements of respiration, photosynthesis and shoot water potential were made during April 2010 (late summer, the drier season) and in mid-August 2010 (mid-winter). Temperature data were obtained from the weather station of the Geography Department, University of Canterbury.

Saplings of *N. solandri* (100–130 cm high) and *N. menziesii* (160–190 cm high) were maintained in 5 L pots that contained a mixture of bark and chip (coarse organic material) with a ratio of 8 : 2, respectively. The potting mix contained a base level of slow release fertilizer (N : P : K in the proportions 8.8 : 5.5 : 10.6 plus trace elements). Individuals of both species were maintained for approximately 2 months at temperatures between 10 and 20°C, light intensities between 500 and 800 mol photons m⁻² s⁻¹ and watered every other day, maintaining water potentials of 0.5–0.9 MPa for *N. solandri* and 0.8–0.9 MPa for *N. menziesii*. To evaluate the respiratory response under severe water deficit, groups of individuals of both species were moved to a glasshouse facility at the University of Canterbury, where they were subjected to gradual water deficit treatment by suspending irrigation. Temperature and light intensity variations inside the glasshouse were measured every 10 min and stored in a data logger (Campbell Scientific CR21X; Logan, UT, USA). The levels of water deficit were characterized at different times during the imposition of the drought treatment by measurements of shoot xylem water potential at midday using a pressure chamber (Scholander et al. 1965; model PSI System 1100). The drought treatment was applied until plants reached a water potential of around −3 MPa (severe water deficit) and then the plants were re-irrigated. Recovery was measured 3–4 and 10 days after re-watering.

**Photosynthesis and respiration measurements**

*Aₘₐₓ* and *Rₐ* were measured using a portable infrared gas analysis system (Li-Cor 6400; Li-Cor, Lincoln, NE, USA). For mature trees, gas exchange was measured from excised secondary branches that were re-cut under water and kept in the dark. Previous studies have found no difference in respiration rates between in situ leaves and leaves from detached branches (Turnbull et al. 2003, 2005). In the glasshouse, gas exchange was measured in leaves on branches from the upper third of each plant.

Photosynthesis was measured as maximum assimilation rate (*Aₘₐₓ*). For excised branches from mature trees, the *Aₘₐₓ* was measured at 600 ppm of CO₂ (we used more CO₂ to avoid photosynthetic limitations by stomatal closure in excised branches) while potted saplings in the glasshouse were measured at 400 ppm CO₂. The photon flux density (*Q*) used was 1700 μmol photons m⁻² s⁻¹. In addition, chlorophyll fluorescence yield (*Fᵥ/Fₘ*) was measured on dark-adapted samples during each measurement period according to Maxwell & Johnson (2000) with a Mini-PAM (Pulse Amplitude Modulation; Walz, Effeltrich, Germany). For *Rₐ* measurements, leaves were dark-adapted for 30 min before measurement. Measurements were made at 15, 18, 21, 24 and 27°C for the seasonal study and at 12, 16, 20, 24 and 28°C for the glasshouse drought experiment. Temperature responses of *Rₐ* were measured inside growth cabinets at the University of Canterbury, which allowed us to control instantaneous measurement temperature of both the plant and the foliage within the gas exchange cuvette. The temperature response of respiration was modelled using a modified Arrhenius equation (Lloyd & Taylor 1994; Turnbull et al. 2003; Kruse & Adams 2008):

$$R = R_{10} \cdot e^{\frac{-E_0}{g \cdot (T_a - T_0)}}$$  \hspace{1cm} (1)

Where *R* is the respiration rate, *R₁₀* is the respiration rate at a reference temperature (*T₀*) of 10°C (273K), *Tₐ* is the measurement temperature of *R*, *g* is the ideal gas constant (8.314 J mol⁻¹ K⁻¹) and *E₀* is a parameter related to the overall energy of activation. Non-linear curve fitting was performed using the Marquardt–Levenberg algorithm (SIGMA PLOT, v8. SPSS Inc. Chicago, IL, USA).
**Oxygen isotope measurements**

Electron partitioning between the CP and AP was evaluated *in vivo* by determining the discrimination against the heavier isotope of oxygen (\(^{18}\)O) during respiratory O\(_2\) consumption (Guy et al. 1989; Ribas-Carbó et al. 2005a). We used an incubation method previously adopted by Searle et al. (2011) and more recently described in detail by Kornfeld et al. (2012). Approximately 0.7 g for *N. solandri* and 1 g for *N. menziesii* of leaf material was placed in a 12 mL septum-topped Exetainer® (Labco, High Wycombe, UK) with a pellet of KOH to reduce CO\(_2\) build-up. Four Exetainers were filled with varying amounts of leaf material from each sample to achieve a range of oxygen consumption. The Exetainers were incubated at 20 °C for 4 h and gas samples were taken by displacing the air in the Exetainer with water using gas-tight syringes. The samples were analysed on a ThermoScientific Delta V Plus Isotope Ratio mass spectrometer with a Finnigan Gas Bench II and a Varian fused silica 5A molecular sieve gas chromatography capillary column. The ratio of O\(_2\)/N\(_2\) was used to determine the rate of oxygen consumption. ISODAT software (Thermo Scientific, Bremen, Germany) was used to calculate the area under the oxygen and nitrogen peaks, and the \(^{18}\)O/\(^{16}\)O ratio. The change in the O\(_2\)/N\(_2\) ratio, relative to the O\(_2\)/N\(_2\) ratio of the air samples taken on the sampling day, was used to calculate the fraction of oxygen consumed, or – ln(f). Calculations of oxygen isotope fractionation were made as described by Guy et al. (1989) and Ribas-Carbó et al. (2005a) with modifications to yield a value of discrimination against \(^{18}\)O/\(^{16}\)O. The change in this ratio relative to the \(^{18}\)O/\(^{16}\)O ratio in air samples taken on the sampling day was used to calculate ln(\(R/R_o\))*1000. The slope of a regression between – ln(f) and ln(\(R/R_o\))*1000 can be interpreted as the discrimination value (\(D,\%\)).

We were unable to determine the end points of oxygen isotope fractionation for AOX and cytochrome oxidase (COX) as we could not sufficiently inhibit *N. solandri* or *N. menziesii* tissue with salicylhydroxamic acid and KCN, despite using a range of concentrations and incubation times. Despite exhaustive efforts, none of the methods used for infiltrations of inhibitors resulted in <30% residual respiration rate when both inhibitors were applied. As a consequence, in this investigation, changes in \(D\) are used to indicate the relative changes in electron partitioning through the CP and AP (Searle et al. 2011; Kornfeld et al. 2012).

**Western blot analysis of respiratory proteins and carbohydrate analysis**

Freeze-dried ground leaf samples were mixed with Laemmli buffer. Proteins were separated by sodium dodecyl sulphate—polyacrylamide gel electrophoresis using 4–12% gradient polyacrylamide gels and then transferred to a nitrocellulose membrane using the iBLOT system (Invitrogen, San Diego, CA, USA). Immunoblotting was performed using the Snap i.d.™ system (Millipore, Billerica, MA, USA). The AOX protein was detected using an anti-AOX monoclonal antibody (Elthon et al. 1989) at a dilution of 1 : 300 and then using an anti-mouse horseradish peroxidase conjugate and non-commercial enhanced chemiluminescence solution (Haan & Behrmann 2007). The same blots were then incubated in a 15% H\(_2\)O\(_2\) solution to deactivate the anti-mouse horseradish peroxidase conjugate before an application of anti-COX antibody at a dilution of 1:600 (Agrisera, Vannas, Sweden). The blots were photographed with a cryo-cooled digital camera (Chemigenius, Syngene, Cambridge, UK) and the bands were quantified using densitometry (Image, National Institutes of Health, Bethesda, MD, USA). The intensities of the bands were corrected for the background. A standard sample of each species was run on each blot and all samples are reported normalized to this standard. Protein abundance for each sample was then divided by the mean abundance of all analysed samples for each protein and species to equalize the distribution for AOX and COX (Searle et al. 2011). Relative
protein abundances for AOX and COX were divided to calculate the AOX/COX ratio—when this value is 1 it represents the mean AOX/COX ratio for each species (N. solandri and N. menziesii). For analysis of carbohydrates, leaves were dried and ground in a ball mill. Soluble sugar and starch were analysed following the method of Tissue & Wright (1995).

Statistical analysis
Reported values of all measurements correspond to the means of five replicates in each treatment. The results of the experiments were subjected to two-way analysis of variance (ANOVA) for mature trees (factors: species and season) and one-way ANOVA for glasshouse experiments (factor: water potential). Before ANOVA analysis, data were checked for normality and homogeneity of variances. Fisher least significant difference test was applied for comparison between treatments. Differences between the values were considered significant at $P \leq 0.05$. All the statistical analyses were performed with STATISTICA v6.0 (Statsoft, Tulsa, OK).

Results
Seasonal study
During April, which corresponded to the driest period and the end of the growing season, maximum temperatures ranged between 20 and 25 °C (Fig. 1). During winter (mid-August) the maximum temperatures in the study site were around 10 °C during the period of measurements. The midday shoot xylem water potential was statistically different between seasons ($P < 0.05$) and species ($P < 0.001$) (Table 1). Both species showed lower water potentials in April (N. solandri, $-1.86 \pm 0.18$ MPa and N. menziesii, $-1.43 \pm 0.13$ MPa) than in August (N. solandri, $-0.74 \pm 0.05$ MPa and N. menziesii, $-0.44 \pm 0.04$ MPa). Maximum carbon assimilation was considerably higher for N. solandri than N. menziesii during April (14.40 ± 1.03 and 6.08 ± 1.75 μmol CO$_2$ m$^{-2}$ s$^{-1}$, respectively) and August (14.97 ± 2.59 and 4.80 ± 0.80 μmol CO$_2$ m$^{-2}$ s$^{-1}$, respectively) without a significant seasonal effect ($P = 0.837$). Nothofagus solandri also displayed higher respiratory rates than N. menziesii during April (0.96 ± 0.09 and 0.24 ± 0.03 μmol CO$_2$ m$^{-2}$ s$^{-1}$, respectively) and August.

Figure 1 Maximum and minimum daily temperatures at the University of Canterbury study site (Geography Department, University of Canterbury). The values correspond to late summer (April 2010) and winter (mid-August 2010). The hatched band indicates the days of measurement.
respectively). *Nothofagus solandri*, which had higher $A_{\text{max}}$ and $R_{10}$ throughout the year, was able to maintain a lower foliar $R/A$ ratio (0.07±0.01 and 0.03±0.00 for April and August, respectively) than *N. menziesii* (0.14±0.04 and 0.10±0.03, respectively) (Table 1).

Table 1 Physiological, respiratory and photosynthetic parameters for leaves of *Nothofagus solandri* and *Nothofagus menziesii* during late summer and winter.

<table>
<thead>
<tr>
<th>Parameter</th>
<th><em>Nothofagus solandri</em></th>
<th><em>Nothofagus menziesii</em></th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylem water potential (MPa)</td>
<td>-1.86±0.18</td>
<td>-0.74±0.05</td>
<td>Sp &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>-1.43±0.13</td>
<td>-0.44±0.04</td>
<td>Se &lt; 0.05</td>
</tr>
<tr>
<td>$A_{\text{max}}$ ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$)</td>
<td>14.40±1.03</td>
<td>14.97±2.59</td>
<td>Se × Sp ns</td>
</tr>
<tr>
<td></td>
<td>6.08±1.75</td>
<td>4.80±0.80</td>
<td>Sp &lt; 0.001</td>
</tr>
<tr>
<td>$R_{10}$ ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$)</td>
<td>0.96±0.09</td>
<td>0.48±0.05</td>
<td>Se &lt; 0.005</td>
</tr>
<tr>
<td></td>
<td>0.24±0.03</td>
<td>0.38±0.04</td>
<td>Se × Sp &lt; 0.005</td>
</tr>
<tr>
<td>Foliar $R/A$</td>
<td>0.07±0.01</td>
<td>0.03±0.00</td>
<td>Sp &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>0.14±0.04</td>
<td>0.10±0.03</td>
<td>Se &lt; 0.05</td>
</tr>
<tr>
<td>$E_o$ (J mol$^{-1}$ K$^{-1}$)</td>
<td>39,875±1229</td>
<td>67,720±6571</td>
<td>Se &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>57,782±6204</td>
<td>53,684±4355</td>
<td>Se × Sp &lt; 0.05</td>
</tr>
<tr>
<td>$Q_{10}$</td>
<td>1.65±0.09</td>
<td>2.58±0.24</td>
<td>Sp ns</td>
</tr>
<tr>
<td></td>
<td>2.25±0.20</td>
<td>2.12±0.13</td>
<td>Se &lt; 0.05</td>
</tr>
<tr>
<td>Discrimination (%$\text{oo}$)</td>
<td>19.89±0.18</td>
<td>20.36±0.19</td>
<td>Sp &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>17.34±0.64</td>
<td>17.97±0.85</td>
<td>Se × Sp &lt; 0.05</td>
</tr>
<tr>
<td>Soluble sugars (%)</td>
<td>7.30±0.33</td>
<td>8.05±0.36</td>
<td>Sp ≤ 0.05</td>
</tr>
<tr>
<td></td>
<td>6.26±0.35</td>
<td>7.65±0.31</td>
<td>Se &lt; 0.05</td>
</tr>
<tr>
<td>Starch (%)</td>
<td>4.87±0.39</td>
<td>5.27±0.28</td>
<td>Sp &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>3.36±0.31</td>
<td>3.52±0.30</td>
<td>Se × Sp ns</td>
</tr>
</tbody>
</table>

Dark respiration parameters were calculated from fitted temperature curves, where $R_{10}$ is respiration at 10°C, $E_o$ is a modelled parameter related to the energy of activation and $Q_{10}$ denotes the relative change in respiration with a 10°C increase in temperature. $A_{\text{max}}$ indicates the maximum assimilation of carbon measured at 20°C and light saturation. Foliar $R/A$ is an indicator of carbon balance at the foliar level. The table also shows the respiratory $^{18}$O discrimination value for both species and the percentage of total non-structural carbohydrate (soluble sugars and starch). Values shown are means (± standard error of the mean) where $n$ = 5. Two-way analysis of variance was performed where the factors were species and season. Significance of treatment effect for species (Sp), season (Se) and the interaction between species and season (Sp × Se) are indicated as the $P$-value or as non-significant (ns).

Respiratory thermal-sensitivity parameters obtained from the temperature response curves, $E_o$ and $Q_{10}$, were significantly higher in August ($67,720±6571$ and $2.58±0.24$, respectively) than April ($39,875±1229$ and $1.65±0.09$, respectively) for *N. solandri*, whereas *N. menziesii* did not display any difference between seasons.
for either parameter (Table 1). Discrimination (D), as measured by oxygen isotope fractionation, was higher for *N. solandri* than *N. menziesii* in April (19.89 ± 0.18 and 17.34 ± 0.64, respectively) and August (20.36 ± 0.19 and 17.97 ± 0.85, respectively) (Table 1). The D values obtained for both species indicate that the mitochondrial electron flow is primarily through the CP. However, when we analysed the abundance of proteins, we did not find any relationship between partitioning of electrons to the CP and COX protein. During April, both species showed a similar AOX/COX protein ratio (Fig. 2). *Nothofagus menziesii* increased significantly the amount of AOX relative to COX during August, whereas *N. solandri* increased COX protein abundance relative to AOX. Total non-structural carbohydrate contents were significantly different between species. Soluble sugar percentage was similar for both species during August (Table 1) but decreased in *N. menziesii* in April. Starch concentrations where higher for *N. solandri* than *N. menziesii* but did not change with the season (*P* = 0.402).

**Glasshouse water stress study**

Plants were maintained in the glasshouse for 3 months (from May to July). During this period the temperature fluctuated between 24 and 28 °C (midday) and between 14 and 16 °C (night). The maximum natural light received during this period was 400–700 μmol photons m⁻² s⁻¹ at midday. Over 9 days, plants of both species were irrigated every other day (Control). Then the drought treatment was applied by suspending irrigation. Different lengths of treatment reflected the different rates at which actual tissue water potential responded to the removal of watering. Hence, we used tissue response as a measure of actual drought impact, and waited until each species had attained a similar level. Maximum water stress was reached in *N. solandri* (−3.02 ± 0.10 MPa) after 33 days and in *N. menziesii* (−3.11 ± 0.14 MPa) after 12 days. Changes in specific leaf area and water use efficiency (data not showed) during the treatment indicated that *N. solandri* had better control in the regulation of water status than *N. menziesii* and therefore the different drought periods were mainly attributed to their differential drought tolerance. After the plants reached the maximum water stress they were re-irrigated thereafter and recovery was assessed after 3 days (R1) and 10 days (R2) days in *N. solandri* and after 4 days (R1) and 10 days (R2) days in *N. menziesii*.

Maximum quantum yield of PSII (*Fvp/Fm*) was measured during the drought treatment.

**Figure 2** Relative abundance of alternative oxidase (AOX) and cytochrome oxidase (COX) proteins during summer and winter for *Nothofagus solandri* and *Nothofagus menziesii* in the seasonal study. Values shown are mean ± SEM. Two-way analysis of variance was performed including the factors species (*P* < 0.001) and season (*P* ≤ 0.05). Different lower case letters show statistical differences (Fisher least significant difference test).
period for both species. The values were within the range 0.83–0.85, indicating that there was no damage in the photosynthetic machinery as a result of water stress treatment (data not shown). For *N. solandri*, \( A_{\text{max}} \) increased significantly initially under mild water deficit (−1.6 MPa) but then decreased significantly as more severe water stress developed (Fig. 3C). In *N. menziesii*, \( A_{\text{max}} \) was highly sensitive to water deficit, decreasing significantly under moderate water stress (−2.4 MPa, 7 days from the start of the treatment) and then decreasing further to near zero under severe water stress, 12 days from suspension of irrigation (Fig. 3D). After re-watering, both species showed a similar capacity to recover their photosynthesis to initial values within 10 days (Fig. 3).

As in the mature trees, *N. solandri* showed higher leaf respiration rates than *N. menziesii* (Fig. 3D,E). In *N. solandri* the respiration rate (\( R_{10} \)) increased in response to moderate water deficit (−2.1 MPa). Under severe water stress the initial rate was re-established, but declined significantly during the first recovery period (\( R_{1} \); Fig. 3A). Ten days after re-watering, *N. solandri* partially recovered initial respiration rates (Fig. 3A). On the other hand, *N. menziesii* did not change \( R_{10} \) during the drought treatment or the recovery period (Fig. 3B). The relationship between \( R_{d} \) and \( A_{\text{max}} \) \((R/A; \text{indicating foliar carbon balance})\) for both species started to increase under moderate water deficit (around −2 MPa) and the maximum imbalance was reached under severe water deficit (Fig. 3E,F). Values in *N. menziesii* reached considerably higher than in *N. solandri*, indicating a greater loss of carbon (Fig. 3F). However, after re-watering, both species were able to restore the foliar carbon balance close to initial values (0.10 ± 0.01 and 0.07 ± 0.00 for *N. solandri* and *N. menziesii*, respectively). The thermal sensitivity of \( R \), as shown by the parameter \( E_{\text{a}} \), did not show a clear response during water stress treatment and recovery in either *N. solandri* or *N. menziesii*. Similarly, \( Q_{10} \) was unchanged for *N. solandri* \((P = 0.060)\) and *N. menziesii* \((P = 0.439)\) despite the severe water stress (Table 2).

The results obtained from the gradual water deficit treatment suggest that electron flow for both species was primarily and consistently through the CP rather than through the AP, even under the most severe water stress (Fig. 4A,B). The AOX/COX protein abundance indicates that in *N. solandri* the amount of AOX relative to COX increased under severe water stress and then in late recovery (10 days after re-watering) (Fig. 4C). However, in *N. menziesii*, the AOX/COX protein ratio did not change across the drought treatment and recovery period (Fig. 4D). Concentrations of soluble sugars and starch were maintained within a fairly narrow range (data not shown) and were not related to changes in respiration and photosynthesis.

**Discussion**

The response of respiration to temperature under drought was studied in two *Nothofagus* species with differential drought tolerance (*N. solandri* more tolerant to drought than *N. menziesii*). This enabled us to examine the extent to which changes in \( R_{d} \) allow the two species to maintain a positive carbon balance under water deficit conditions. Moreover, we evaluated the thermal sensitivity of leaf respiration and a possible role for changes in metabolism (i.e. the alternative pathway) in the respiratory adjustment under drought. Our results show that the responses of respiration at a basal reference temperature of 10 °C (\( R_{10} \)) and \( A_{\text{max}} \) differ significantly between the two species when they are submitted to a gradual water deficit. The more drought tolerant *N. solandri* showed more flexibility in its respiratory response (both in mature trees and in glasshouse-grown plants) and the alternative pathway appears to have some role during the recovery of metabolism after a drought period. This work is significant because there are very few studies that have evaluated drought affects on the physiology of respiration. Moreover, few investigations have reported the respiratory
response of *Nothofagus* species in New Zealand to seasonal variations in water availability including a controlled water deficit under glasshouse conditions. Finally, to the best of our understanding this is the first investigation evaluating the respiratory response under water deficit in two tree species with differing drought tolerance.
Table 2  Dark respiration parameters calculated from fitted temperature response curves using a modified Arrhenius equation for leaves of *Nothofagus solandri* and *Nothofagus menziesii* during water deficit treatment and recovery.

<table>
<thead>
<tr>
<th>WP (MPa)</th>
<th>$E_o$ (J mol$^{-1}$ K$^{-1}$)</th>
<th>$Q_{10}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$-0.8$ (C)</td>
<td>$39814 \pm 9979$ ab</td>
<td>$1.78 \pm 0.27$</td>
</tr>
<tr>
<td>$-1.0$</td>
<td>$54565 \pm 4880$ b</td>
<td>$2.14 \pm 0.14$</td>
</tr>
<tr>
<td>$-1.6$</td>
<td>$43047 \pm 4216$ ab</td>
<td>$1.83 \pm 0.11$</td>
</tr>
<tr>
<td>$-2.1$</td>
<td>$26582 \pm 3350$ a</td>
<td>$1.46 \pm 0.07$</td>
</tr>
<tr>
<td>$-3.0$</td>
<td>$46766 \pm 6745$ ab</td>
<td>$1.94 \pm 0.18$</td>
</tr>
<tr>
<td>$-1.0$ (R1)</td>
<td>$30472 \pm 9051$ a</td>
<td>$1.57 \pm 0.20$</td>
</tr>
<tr>
<td>$-1.0$ (R2)</td>
<td>$56718 \pm 8527$ b</td>
<td>$2.25 \pm 0.25$</td>
</tr>
</tbody>
</table>

ANOVA  $P < 0.05$ ns

<table>
<thead>
<tr>
<th>WP (MPa)</th>
<th>$E_o$ (J mol$^{-1}$ K$^{-1}$)</th>
<th>$Q_{10}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$-0.6$ (C)</td>
<td>$61151 \pm 3905$</td>
<td>$2.34 \pm 0.12$</td>
</tr>
<tr>
<td>$-1.6$</td>
<td>$61476 \pm 4458$</td>
<td>$2.36 \pm 0.15$</td>
</tr>
<tr>
<td>$-2.4$</td>
<td>$52979 \pm 8053$</td>
<td>$2.13 \pm 0.24$</td>
</tr>
<tr>
<td>$-3.1$</td>
<td>$56892 \pm 3737$</td>
<td>$2.21 \pm 0.11$</td>
</tr>
<tr>
<td>$-1.2$ (R1)</td>
<td>$47717 \pm 3490$</td>
<td>$1.95 \pm 0.09$</td>
</tr>
<tr>
<td>$-1.4$ (R2)</td>
<td>$51510 \pm 6517$</td>
<td>$2.07 \pm 0.19$</td>
</tr>
</tbody>
</table>

ANOVA  ns

WP is water potential, $E_o$ is a modelled parameter related to the energy of activation and $Q_{10}$ denotes the relative change in respiration with a 10 °C increase in temperature. Values shown are mean $\pm$ SEM. One-way analysis of variance ($P \leq 0.05$) was performed where the factor was xylem water potential during treatment being C (control) and R1–R2 (recovery). Different lower case letters show significantly different means (Fisher least significant differences test).

Figure 4  Changes in oxygen isotope discrimination (A, B) and protein abundance ratio of alternative oxidase and cytochrome oxidase (AOX/COX) (C, D) during water deficit treatment and recovery for *Nothofagus solandri* and *Nothofagus menziesii*. Values shown are mean ($\pm$ SE). One-way analysis of variance ($P \leq 0.05$) was performed where the factor was xylem water potential during treatment being C (control) and R1–R2 (recovery). Different lower case letters show significantly different means (Fisher least significant difference test).
Response of assimilation and respiration to drought

In mature trees, *N. solandri* experienced more negative water potentials than *N. menziesii* during April (−1.86 versus −1.43 MPa), which could indicate greater water stress. Despite this, rates of photosynthesis in *N. solandri* were higher than in *N. menziesii* and did not differ significantly between April and August. *Nothofagus menziesii* also did not display a significant seasonal response in \( A_{\text{max}} \). These responses could indicate that the late-summer ‘drought’ in the study site was not strong enough to suppress photosynthesis in these evergreen species. For this reason, the gradual water deficit treatment was applied in the glasshouse and this enabled us to induce a more severe water deficit. Both species possessed similar \( A_{\text{max}} \) values (around 6 μmol CO₂ m⁻² s⁻¹) under control conditions. These rates of photosynthesis were similar to those reported by Sun & Sweet (1996), who studied the responses of photosynthesis to light and temperature in individuals of both species from three different origins. The more drought-tolerant species was able to increase \( A_{\text{max}} \) under moderate water deficits (from 5.54 to 8.56 μmol CO₂ m⁻² s⁻¹). This increase in photosynthesis under moderate water deficit has been reported in other species (Schaedler & Gould 2005) and could indicate that a slight drought may improve carbon gain. An almost complete inhibition of photosynthesis was evident by day 12 of the drought treatment in *N. menziesii* and after 33 days in *N. solandri*. Both *Nothofagus* species were able to fully recover initial photosynthetic rate 10 days after re-watering. Several authors have reported that plants subjected to severe water stress recover only 40–60% of the maximum photosynthetic rate during the day after re-watering, and maximum photosynthesis rates are not always regained (Flexas et al. 2006). Our results show that, despite the difference in their drought tolerance, both evergreen *Nothofagus* species are able to recover their \( A_{\text{max}} \) despite the occurrence of a severe drought event.

During April, there was a significant increase in \( R_{10} \) for *N. solandri* compared with August, but lower temperature sensitivity (lower \( E_o \) and \( Q_{10} \)). This increase in respiration during the active period may reflect reductions in the adenylate restrictions of respiratory metabolism, via increased rates of ATP turn-over or increased ADP (Campbell et al. 2007). Also, under the gradual drought treatment, *N. solandri* was more flexible in its respiratory response than *N. menziesii*. *Nothofagus solandri* maintained its \( R_{10} \) during the 15 days in which it was under mild water deficit (from −1.3 to −1.6 MPa), but under moderate water deficit \( R_{10} \) increased significantly. A similar response was found in *Quercus humilis*, which showed an initial increase under mild water stress, followed by a large decrease under severe water stress (Gulisas et al. 2002). Other authors have reported that this increase of respiration under water stress is probably in response to an increase in energy demand as leaves cope with drought (Flexas et al. 2006; Zaragoza-Castells et al. 2008). Ten days after re-watering, *N. solandri* was able to re-establish its initial respiratory rates. The thermal sensitivity of respiration under the glasshouse drought treatment was similar in both *Nothofagus* species (Table 2) and \( Q_{10} \) was unchanged during the water deficit treatment. Maintenance of \( Q_{10} \) through rapid and adenylate control or enzymatic regulation may be important for the regulation of respiration under drought (Ribas-Carbó et al. 2005b; Campbell et al. 2007; Searle et al. 2011).

The balance between photosynthesis and respiration determines leaf carbon balance, and here we use the ratio of net carbon assimilation to dark respiration (\( R/A \)) as a simple estimate of leaf carbon balance that allows a qualitative comparison between species (Pattison et al. 1998; Galmés et al. 2005). Here, as in other studies, development of water stress resulted in a progressively increased \( R/A \), affecting the foliar carbon balance (Flexas et al. 2005, 2006). The higher photosynthetic rate in *N. solandri* mentioned above contributed to
maintaining a positive foliar carbon balance over the year, despite the increased $R_{10}$ during summer. Under the glasshouse drought treatment, the $R/A$ ratio of *N. menziesii* increased to a value of 6.5. When compared with the maximum value of 1.25 in the $R/A$ ratio in *N. solandri*, this indicates that *N. menziesii* pays a significant cost for its sensitivity to water stress—this is a cost that could clearly underpin its lower competitive ability in drier sites in the field. In addition, the ability of *N. solandri* to increase its photosynthetic performance under mild drought and maintain its respiratory activity even under severe water stress contributes to the maintenance of a more positive carbon balance in comparison to *N. menziesii*. These findings clearly support our hypothesis in this work.

**Changes in COX and AOX in response to drought**

Environmental changes such as low temperature (Watanabe et al. 2008) and drought (Ribas-Carbó et al. 2005a,b) may generate changes in the electron partitioning between each of the mitochondrial respiratory pathways. In this investigation, we evaluated electron partitioning by the isotopic discrimination technique to determine if, during a drought period, there was an increase in electron flux toward the AP. The oxygen isotope discrimination values reported here range from 17 to 20$\%$. It has been reported that $D$ values lower than 19$\%$ may be uncommon (Ribas-Carbó et al. 2005a). However, values of 17$\%$ and lower have been previously reported for various plant tissues (Guy et al. 1989; Millar et al. 1998; Nagel et al. 2001; Armstrong et al. 2006, 2008; Searle et al. 2011). In both species and in both mature trees and in the gradual water deficit experiment, under drought lower values of $D$ obtained were interpreted as relatively constant electron flux and maintained primarily through the CP.

Although the apparent partitioning between the oxidative pathways (as measured by $D$) did not change, protein abundance did (AOX/COX ratio). During winter, mature *N. menziesii* trees synthesized considerably more AOX—although in this case it was not associated with low water availability. In the glasshouse, in *N. solandri* the changes in $R_{10}$ during the gradual water deficit treatment were accompanied by changes in protein contents. An increase in AOX protein content (relative to COX) was displayed during severe water stress and 10 days after re-watering (Fig. 4c). The results found here and in other studies confirm that there is no clear positive correlation between the AOX concentration and its activity (Lennon et al. 1997; Guy & Vanlerberghe 2005; Ribas-Carbó et al. 2005b; Vidal et al. 2007; Grant et. al. 2008; Florez-Sarasa et al. 2011). We suggest that this increase in the amount of AOX protein indicates participation of the AP, but probably could not be determined by isotopic discrimination because AOX might have been inactivated. Several studies suggest that post-translational regulation of AOX protein is responsible for regulating activity, either through the reduction status of a disulphide bond or via an effector, such as pyruvate or other $\alpha$-ketoacids (Grant et al. 2008; Vanlerberghe et al. 2009). AOX protein has a slow turnover rate and a half-life estimated at 18 h (Millenaar et al. 2000). This suggests that the protein is not generated *de novo* but may rather be biochemically activated. Armstrong et al. (2008) postulated that an increase in the activation of pre-existing AOX protein is very fast, occurring within minutes to hours. They showed that in *Arabidopsis thaliana*, an increase in AP activity is a short-lived response that subsides once the plant is able to re-establish flux via the CP. Assuming that AOX protein is not generated *de novo* but is biochemically activated, we suggest that this activation may occur during the night. In the present study, $R_d$ and associated metabolism were measured during the day, but some authors have postulated that respiration measured in the dark during the day might change substrate supply and sink demand compared with nocturnal
conditions (Ribas-Carbó et al. (2005b). This leaves open the question of how electron flow via the alternative pathway might change during the night period. For this reason we suggest that the AP may be activated during the night period, when respiration dominates plant carbon metabolism. This would be a fruitful line of study in future investigations on the regulation of the AOX. We suggest making $R_{d}$ measurements with isotopic discrimination and protein analyses during the night period.

The AP may play an important role both during and after a drought period, working together with the CP to increase the energy, regenerating and osmoregulatory mechanisms that may have been damaged during the water stress. Under stress conditions, such as drought, the activity of the AOX pathways allows the tricarboxylic acid cycle to continue providing carbon skeletons for metabolism and this is particularly important in the synthesis of compatible solutes (Bartoli et al. 2005). Moreover, severe drought lowers photosynthetic rate, decreasing carboxylation efficiency. Under these conditions, high light intensity may result in photoinhibition and photo-oxidative destruction of the photosynthetic apparatus. Photosynthetic activity may be inhibited through an imbalance between light capture and use which may produce excessive reduction of the electron transport chain generating reactive oxygen species ($O_{2}$, $O_{2}$, $H_{2}O_{2}$, $OH^{-}$) leading to oxidative damage (Gulias et al. 2004). During recovery, when the plant begins to readjust its metabolism after severe water stress, the AP will probably act to sustain the redox state of the ubiquinone pool, limiting oxygen radical species production (Millar et al. 1998; Florez-Sarasa et al. 2007) and also maintaining chloroplast function by avoiding the over-reduction of electron carriers (Bartoli et al. 2005).

Concluding remarks
The aim of the present study was to evaluate the effect of drought on the temperature response of respiration in two species with differing drought tolerance. When saplings of each species were submitted to a gradual water deficit period, the capacity of $N. solandri$ to adjust respiration under the progressive water deficit and maintain photosynthetic activity even under severe drought contributed to a better carbon balance compared with the less tolerant species $N. menziesii$. A similar response has been found in $Nothofagus dombeysi$, which also has been described as a drought-tolerant and cold-tolerant species (Zuñiga et al. 2006; Pipper et al. 2007) and for other evergreen species from Mediterranean regions with seasonal droughts (Galmés et al. 2007; Atkin & Macharel 2009). Regarding the mechanisms underlying the respiratory response, we postulate that in $N. solandri$, variations in respiration may be associated with activation of the AP (reflected in an increase in the amount of AOX during severe water stress and the recovery period) which is post-translationally regulated and probably activated during the night. Our results suggest that these differential respiratory and photosynthetic responses are a consequence of their differential drought tolerance, which together with other factors will be determining the ecological distribution of these two tree species in New Zealand forests.

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