

# Response of photosynthesis and respiration to temperature under water deficit in two evergreen *Nothofagus* species

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## Abstract

Respiration and photosynthesis were studied in two *Nothofagus* species with different drought tolerance in order to evaluate the effect of water deficit on foliar carbon balance and the possible role of the alternative pathway on respiratory adjustment. We propose that under severe water deficit the more drought-tolerant species *N. dombeyi* is able to decrease its respiration more than the less drought-tolerant species *N. nitida*, thus carbon gain could be maintained when photosynthesis is suppressed by drought. Dark respiration ( $R_d$ ) and carbon assimilation under saturating light ( $A_{sat}$ ) were evaluated under seasonal field conditions and during drying and re-watering cycles under glasshouse. In addition, respiratory pathway changes were evaluated by oxygen isotope fractionation. In the field, *N. dombeyi* displayed greater light-saturated photosynthetic capacity than *N. nitida*, but  $R_d$  did not differ between species during summer. In the glasshouse, *N. dombeyi* displayed an unchanged rate of  $R_d$  and increased carbon loss under severe water deficit. *Nothofagus nitida* displayed a more flexible respiratory response to water deficit, with a lower thermal sensitivity of respiration (decrease in  $Q_{10}$ ) and a decrease in  $R_d$ . This contributed to maintaining leaf carbon balance during the water deficit period. Respiratory electron flow was mainly via the cytochrome pathway for both species and under all treatments, indicating no strong participation of alternative respiration. Our results suggest that under severe water stress, *N. dombeyi* could be more injured than *N. nitida* and that the lack of control in the carbon loss under prolonged periods of drought could be limiting for its survival.

**Keywords:** alternative oxidase, carbon balance, drought tolerance, isotopic discrimination, temperate forest.

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## Introduction

Water stress has been considered one of the most important factors limiting plant performance, especially in Mediterranean ecosystems where summer drought is considered the main environmental constraint on plant growth and survival (Galmes *et al.* 2007). The limitation on plant growth imposed by low water availability is mainly due to reductions in plant carbon balance (Lambers *et al.*

1998; Turnbull *et al.* 2001; Flexas *et al.* 2005, 2006). It is well known that drought has typically an inhibitory effect on photosynthesis, primarily as a result of stomatal closure (Chaves *et al.* 2002). Photosynthesis may be temporally and spatially limited, while respiration occurs continuously in every plant organ. Therefore, respiration is also an important factor controlling plant carbon balance, particularly when photosynthesis is largely depressed under drought conditions (Galmes *et al.* 2007). The maintenance of respiration could serve several positive roles for plant survival under water-deficit conditions, such as reoxidation of substrates from photorespiration,

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maintenance of cell-energy balance and redox status, and supplying chloroplasts with adenosine triphosphate (ATP) (Atkin & Macherel 2009). Although the initial stages of water stress may inhibit respiration, sustained exposure to severe water stress often results in leaf respiration increasing, probably in response to an increase in energy demand as leaves cope with drought (Flexas *et al.* 2006). However, the available experimental evidence does not support a clear pattern of respiration response to water stress, because different studies show increased, unaffected or decreased rates of respiration (Galmes *et al.* 2007).

Respiration rates are strongly influenced by temperature (Atkin *et al.* 2002; Atkin & Tjoelker 2003; Bolstad *et al.* 2003). Short-term changes in temperature may result in rapid changes in respiration and hence in  $Q_{10}$  (the proportional change in respiration rate with a 10°C rise in temperature). This parameter is an indicator of thermal sensitivity at the biochemical level, and may vary in response to environmentally induced changes in substrate supply and/or energy demand (Slot *et al.* 2008; Atkin & Macherel 2009). Only in recent years have studies evaluated the effect of water stress on the response of respiration to temperature (Turnbull *et al.* 2001; Martinez Pastur *et al.* 2007; Slot *et al.* 2008; Zaragoza-Castells *et al.* 2008; Gimeno *et al.* 2010; Rodriguez-Calcerrada *et al.* 2010; Varela *et al.* 2010; Ayub *et al.* 2011; Crous *et al.* 2012). In addition, despite its potential importance, regulation of respiration by water deficit at the metabolic level is also not well understood.

Plant mitochondria display remarkable flexibility in electron transfer under environmental changes. For this reason, it is important to know how electron partitioning between the cytochrome pathway (CP) and the cyanide-resistant alternative pathway (AP) changes under water stress. The AP increases in response to various types of stressful conditions (e.g. cold and hot temperatures, drought, pathogen infection, high light), likely functioning to maintain a flux of electrons through the electron transport chain when the CP is limited (Millenaar *et al.* 2000; Atkin *et al.* 2002; Flexas *et al.* 2006; Florez-Sarasa *et al.* 2007; Kumar *et al.* 2007). It may also divert excess nicotinamide adenine dinucleotide phosphate from chloroplasts during high light conditions, thus avoiding damage to the photosynthetic apparatus (Bartoli *et al.* 2005). In general, it appears that the AP contributes to maintenance processes rather than growth, in particular preventing oxidative damage. It has been reported that the AP increases as the CP decreases in response to stress, and that the AP increased in soybean leaves subjected to drought (Ribas-Carbo *et al.* 2005b). However, this response is not universal, and no AP response was detected in tobacco leaves subjected to water deficit (Guy & Vanlerberghe 2005).

Evergreen *Nothofagus* trees are an important feature of the ecology of forests in the Mediterranean-humid climate of Chile. They are able to inhabit sites where sporadic drought periods and high irradiance occur during summer (Di Castri & Hajek 1976). These sites may also be exposed to low temperature and frost during winter, spring, and even in summer. This may significantly decrease the available soil water (Weinberger *et al.* 1973). Moreover, these species are able to inhabit sites of edaphic stress, such as the nadi soils, moraines, and lahars (Read & Farquhar 1991). They dominate the upper canopy throughout their distribution ranges, often forming almost pure stands (Wardle 1984). *Nothofagus dombeyi* (Mirb.) Oerst. and *Nothofagus nitida* (Phil) Krasser are evergreen species with different latitudinal and altitudinal distribution (Zuñiga *et al.* 2006). *Nothofagus nitida* is facultatively shade-tolerant (Coopman *et al.* 2011 and references therein) and has been reported as a species with relatively limited drought tolerance (Piper *et al.* 2007). *Nothofagus dombeyi* shows greater resistance to low temperatures and drought than *N. nitida* (Reyes-Diaz 2006; Piper *et al.* 2007). During the drier season, *N. dombeyi* is capable of using light energy more efficiently than *N. nitida*, with a higher rate of CO<sub>2</sub> assimilation (Zuñiga *et al.* 2006; Piper *et al.* 2007).

In this study we evaluated the effect of water deficit on photosynthesis and foliar carbon balance in the two aforementioned evergreen *Nothofagus* species. We also studied the response of respiration to temperature under water deficit of both species and the possible role of the alternative pathway in the adjustment of foliar respiration. We hypothesized that, under a severe water-deficit treatment, the drought-tolerant species *N. dombeyi* is able to maintain or decrease foliar respiration rate, and hence maintain or improve its foliar carbon balance, in comparison to the less drought-tolerant *N. nitida*. This response would provide one physiological mechanism that would allow *N. dombeyi* to cope with lower water availability in its habitat, especially during the summer. We also hypothesized that, under water deficit, adjustments in respiration would involve changes in AP with respect to CP, and help to maintain redox status and restore cellular metabolism after the water-deficit period. Evergreen species are excellent models for these studies, because they have long-lived leaves that allow us to perform measurements over extended periods and determine structural and biochemical changes in pre-existing leaves under new growth environments (Zaragoza-Castells *et al.* 2007).

## Materials and methods

### *Plant material and treatments*

*Field experiments* The field study was undertaken in Katalapi Park, located in Pichiquillaiepe, Region de Los

Lagos, in South Central Chile (41°31'0.7"S; 72°45'2.2"W) on 20-year-old individuals of *N. dombeyi* (Coigüe) and *N. nitida* (Coigüe de Chiloé) growing in open sites or at the edge of the forest. During 2009, we selected five individuals of each species of similar sizes, with heights between 4 and 6 m and stem diameter between 0.30 and 0.35 m. In order to characterize seasonal variations in plant water status we measured the xylem water potential (MPa) of both species using a pressure chamber (Scholander *et al.* 1965; model PSI System 1100, Corvallis, OR, USA). Seasonal measurements of gas-exchange parameters (see details below) were conducted during January (summer), April (autumn), August (winter), and October (spring). Weather data from the study site were obtained from the Katalapi climate station (LI-1400 data logger connected to LI250, LI 1400-104 and LI 1400-106 sensors; Li-Cor Inc., Lincoln, NE, USA).

**Glasshouse experiments** This experiment was carried out during April and September 2011 in a glasshouse at the Universidad de Concepción, Concepción, Chile (36°49'39"S; 73°2'20"W). We used five saplings from 10-year-old *N. dombeyi* and *N. nitida* with heights between 1.6 and 1.9 m and stem diameter between 0.065 and 0.087 m, obtained from the natural range of distribution. To minimize the effect of genetic variation among individuals (Premoli *et al.* 2012) all plants used experienced the same procedure. Individuals were transplanted into 5-L black plastic bags filled with a mixture of organic soil with pine-bark compost (1:1 v/v). Once a week, plants were fertilized with 0.2 g/L of a commercial fertilizer solution (14, 10, 22 N, P, K, respectively, plus Mg, S and chelated trace elements). Following planting, plants were grown for two weeks to allow roots to develop. Immediately prior to the experiment, plants were maintained for approximately two weeks in an open nursery house to homogenize the conditions of both species before the drying treatment. Temperature (°C) and relative air humidity (RH%) were measured every 1 h and stored in a Micro Station Data Logger (HOBO Pro RH/Temp, Onset Computer Corporation, Bourne, MA, USA). Light intensity was measured with a LI-190SA quantum sensor (Li-Cor Inc.). In the open nursery house, maximum temperatures fluctuated between 10 and 20°C; the external photosynthetic active radiation (PAR) at midday fluctuated between 500 and 800  $\mu\text{mol}/\text{m}^2/\text{s}$  and plants were watered every other day to field capacity.

To evaluate physiological responses under severe water deficit, all the plants were moved to a glasshouse. For 15 days the irrigation regime was maintained, maximum temperatures fluctuated between 15 and 24°C (midday) and minimum temperatures between 7 and 11°C (night), RH ranged between 50 and 70% and PAR inside the glasshouse was 60% of the external value. Since medium size

pots were used, xylem water potentials were closely monitored to compare species at a similar water-stress level. The water-deficit treatment was then applied by suspending irrigation. The midday xylem water potential (MPa) was periodically monitored during the drought period, using a pressure chamber (Scholander *et al.* 1965). The measurements were made in groups of five plants according the stress level reached as follows: control (C; irrigated; -0.6 and -1.4 MPa for *N. dombeyi* and *N. nitida*, respectively), mild water stress (Mild WS; -1.42 and -1.50 MPa for *N. dombeyi* and *N. nitida*, respectively), moderate water stress (Mod WS; -2.43 and -2.41 MPa for *N. dombeyi* and *N. nitida*, respectively), severe water stress (Sev WS; -3.22 and -3.70 MPa for *N. dombeyi* and *N. nitida*, respectively), recovery 1 (REC1; 3 days after re-watering; -1.48 and -1.30 MPa for *N. dombeyi* and *N. nitida*, respectively), and recovery 2 (REC2; 10 days after re-watering; -1.19 and -1.06 MPa for *N. dombeyi* and *N. nitida*, respectively). Gas-exchange measurements and isotopic discrimination analysis were performed at different levels of water stress during the water-deficit treatment in individuals of both species in order to compare the response between species.

**Photosynthesis and respiration measurements** Field measurements of gas exchange were conducted on pre-existing, fully expanded sun-lit leaves (from the leaf cohort that emerged in the spring) from excised secondary branches. Cut branches were re-cut under water to prevent embolism. We used a portable infrared gas analysis system (IRGA; Ciras-2, PP-Systems, Hitchin, UK) with a LED-based light unit providing a PAR from 0 to 2000  $\mu\text{mol}/\text{m}^2/\text{s}$ . Glasshouse gas-exchange measurements were also made on pre-existing fully expanded leaves (see above) on branches from the upper third of each plant using an IRGA (GFS-3000, Heinz Walz GmbH, Effeltrich, Germany) with an LED light source 3040-L from 0 to 2000  $\mu\text{mol}/\text{m}^2/\text{s}$ . Niinemets (2012) suggests that any system can be used to track plant gas-exchange fluxes if they are made using equivalent chamber environmental responsiveness and accurate leaf-area estimation. For measurements of photosynthesis the light intensity was gradually applied to avoid photoinhibition and carbon assimilation was recorded to 1700  $\mu\text{moles photons}/\text{m}^2/\text{s}$  (above the saturation point for these species) corresponding to carbon assimilation at saturating light ( $A_{\text{sat}}$ ,  $\mu\text{mol CO}_2/\text{m}^2/\text{s}$ ). The IRGA chamber was maintained at 20°C, 60% RH, and 400 ppm of CO<sub>2</sub>. The rate was normalized by the cuvette area when leaves did not fully cover the cuvette. Stomatal conductance ( $g$ ;  $\text{mol H}_2\text{O}/\text{m}^2/\text{s}$ ) and leaf transpiration rate ( $E$ ;  $\text{mmol H}_2\text{O}/\text{m}^2/\text{s}$ ) were also registered. Water-use efficiency (WUE) was calculated as  $A_{\text{sat}}/E$  ( $\mu\text{mol CO}_2$  fixed per mmol of transpired H<sub>2</sub>O). In addition, the maximum quantum yield of photosystem II ( $F_v/F_m$ )

$F_m$ ) was measured in leaves previously dark adapted for 20 min (Maxwell & Johnson 2000) using a Mini-PAM fluorescence meter (Pulse Amplitude Modulation; Heinz Walz GmbH) applying a saturating pulse  $> 5500 \mu\text{mol}/\text{m}^2/\text{s}$ . Minimal ( $F_o$ ) and maximal ( $F_m$ ) fluorescence yields were used to calculate the variable fluorescence in order to obtain  $F_v/F_m$ .

Dark respiration ( $R_d$ ;  $\mu\text{mol CO}_2/\text{m}^2/\text{s}$ ) was measured in leaves that were previously dark-adapted for 30 min. For field and glasshouse measurements, the IRGA was maintained at 60% RH and 400 ppm of  $\text{CO}_2$ . In the field,  $R_d$  was measured at 20°C (no temperature–response curves were performed). This temperature was selected because previous determinations indicated that around 20°C is in the range of the optimum temperature for photosynthesis in *Nothofagus* species (Martinez Pastur *et al.* 2007; Varela *et al.* 2012). For glasshouse measurements,  $R_d$  was measured at temperatures of: 12, 16, 20, 24, and 28°C to plot temperature–response curves. Measurements were made at each temperature when gas exchange had equilibrated (i.e. taken to be when the coefficient of variation for the  $\text{CO}_2$  partial pressure differential between the sample and reference analyzers was below 1%). The measurements of photosynthesis and respiration were taken about 10 min after the stable temperature set point had been reached. The response of respiration to temperature for glasshouse plants was modeled using a modified Arrhenius equation (Lloyd & Taylor 1994; Turnbull *et al.* 2003; Kruse & Adams 2008):

$$R = R_{10} \cdot e^{\frac{E_o}{g} \left( \frac{1}{T_o} - \frac{1}{T_a} \right)}$$

where  $R$  is the respiration rate,  $R_{10}$  is the respiration rate at a reference temperature ( $T_o$ ) of 10°C,  $T_a$  is the measurement temperature of  $R$ ,  $g$  is the ideal gas constant (8.314 J/mol/K), and  $E_o$  is a parameter related to the overall energy of activation. Nonlinear curve fitting was performed using the Marquardt–Levenberg algorithm (Sigma Plot, v8; SPSS Inc., Chicago, IL, USA). The parameter  $Q_{10}$ , which is describing the proportional change in respiration with a 10°C increase in temperature, was also determined as described Atkin *et al.* (2005) for comparison with previous studies. Estimation of the foliar carbon balance was obtained from the ratio of dark respiration to carbon assimilation at saturating light, both measured at 20°C ( $R_d/A_{\text{sat}}$ ).

**Oxygen isotope measurements** Electron partitioning between the CP and AP was evaluated *in vivo* by determining the discrimination against the heavier isotope of oxygen ( $^{18}\text{O}$ ) during respiratory  $\text{O}_2$  consumption (Guy *et al.* 1989; Ribas-Carbo *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. dombeyi* and 1 g for *N. nitida* of fresh leaf tissue was placed in a 12 mL septum-topped Exetainer (Labco, High Wycombe, UK) with a pellet of potassium hydroxide (KOH) to reduce  $\text{CO}_2$  build-up. Four Exetainers were filled with varying amounts of leaf tissue 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 analyzed on a Thermo Scientific 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  $\text{O}_2/\text{N}_2$  was used to determine the rate of oxygen consumption. Isodat software was used to calculate the area under the oxygen and nitrogen peaks, and the  $^{18}\text{O}/^{16}\text{O}$  ratio. The change in the  $\text{O}_2/\text{N}_2$  ratio, relative to the  $\text{O}_2/\text{N}_2$  ratio of the air samples taken on the sampling day, was used to calculate the fraction of oxygen consumed, or negative natural logarithm ( $-\ln(f)$ ). The  $^{18}\text{O}/^{16}\text{O}$  ratio is reported by the Isodat software. Calculations of oxygen-isotope fractionation were made as described by Guy *et al.* (1989) and Ribas-Carbo *et al.* (2005a), with modifications to yield a value of discrimination against  $^{18}\text{O}/^{16}\text{O}$ . The change in this ratio relative to the  $^{18}\text{O}/^{16}\text{O}$  ratio in air samples taken on the sampling day was used to calculate  $\ln(R/R_o) \times 1000$ . The slope of a regression between  $-\ln(f)$  and  $\ln(R/R_o) \times 1000$  can be interpreted as the discrimination value expressed in parts per mil ( $D$ , ‰). Changes in  $D$  are used to indicate the relative changes in electron partitioning through the cytochrome and alternative pathways (Searle *et al.* 2011; Kornfeld *et al.* 2012) due to the difficulty in determining the precise end-points of oxygen isotope fractionation for AOX and COX in *Nothofagus* species (Sanhueza *et al.* 2013).

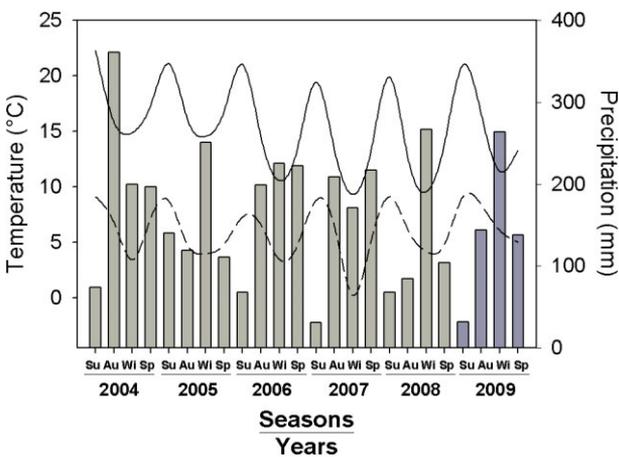
### Statistical analysis

Statistically significant differences in water potential,  $g_s$ , WUE,  $A_{\text{sat}}$ ,  $R_d$ ,  $R_d/A_{\text{sat}}$ ,  $E_o$  and  $Q_{10}$ . Reported values of all measurements and analyses correspond to the means of 5 replicates in each treatment. Data of field experiments were subjected to two-way ANOVA (factors: species and season). Due to the different water-stress levels reached by the two species, it was not possible to use two-way ANOVA for glasshouse experiments; therefore data were tested with repeated-measure ANOVA using water potential as a factor. The Tukey honestly significant difference (HSD) test ( $P \leq 0.05$ ) was applied for comparison between treatments. All the statistical analyses were performed using STATISTICA v6.0 (Statsoft, Tulsa, OK)

**Results**

*Seasonal study under field conditions*

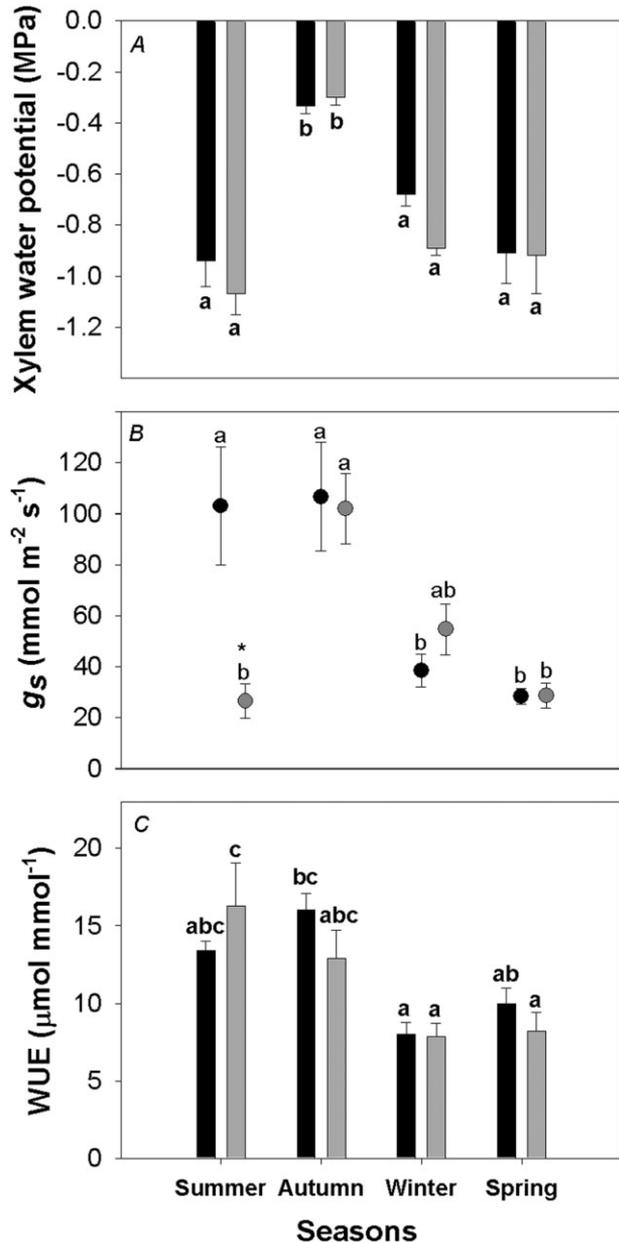
Compared with the previous four years, 2009 was very stable in terms of maximum and minimum temperatures during summer (Fig. 1). However, during winter the minimum temperature was slightly higher than the previous year (4.2°C during August 2008). The rainfall during summer was 50% lower compared with the average rainfall for the previous four years (mean ± SE = 61.6 ± 21.2 mm/month). Water potential (WP) tends to be lower during the driest season (summer) than winter (Fig. 2A). Nevertheless, it was not statistically different between species throughout the year ( $P = 0.237$ ) (Fig. 2A). The  $g_s$  value was higher for *N. dombeyi* than *N. nitida* during summer (Fig. 2B) but no differences in  $g_s$  were observed in the other seasons. Both species displayed similar WUE in each season ( $P = 0.948$ ) (Fig. 2C). During summer,  $A_{sat}$  was 50% higher for *N. dombeyi* than *N. nitida* ( $P = 0.018$ ) (Fig. 3A), but did not differ between species during the other seasons. For both species, the  $A_{sat}$  value was directly related to stomatal conductance during summer. At 20°C,  $R_d$  was also higher for *N. dombeyi* than *N. nitida* during summer (Fig. 3B). In autumn and spring no significant differences in  $R_d$  were registered between species, however, *N. dombeyi* tends to have lower  $R_d$  than *N. nitida* during winter. The  $R_d/A_{sat}$  ratio was not different between species during the year ( $P = 0.477$ ): it tended to be higher in winter than in summer, but these differences were not significant (Fig. 3C). The highest  $R_d/A_{sat}$  ratio for both species was reached during the spring season.



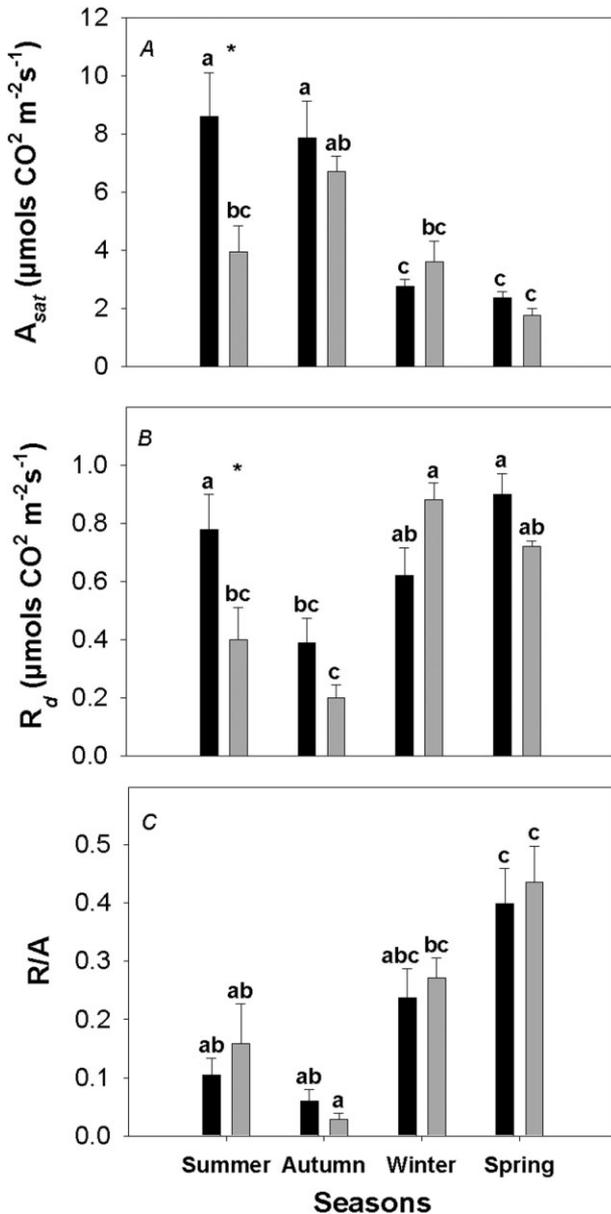
**Fig. 1** Maximum and minimum absolute air temperatures (solid and dashed lines respectively) and precipitation (gray bars) for the study site between 2004 and 2009. Darker bars correspond to the month in which the physiological measurements were performed, corresponding to January (summer), April (Autumn), August (winter) and October (Spring). The data were obtained from a climatic monitoring station.

*Water-stress study under glasshouse conditions*

The  $g_s$  value was generally higher for *N. dombeyi* than *N. nitida* (mean ± SE = 74.71 ± 11.06 and 26.77 ± 5.81 mmol H<sub>2</sub>O m<sup>2</sup>/s, respectively) (Fig. 4A) and



**Fig. 2** (A) Xylem water potential, (B) stomatal conductance and (C) water-use efficiency during the year for *N. dombeyi* (black bars) and *N. nitida* (gray bars) in the field seasonal study. Values shown are mean ± SEM. Different lower case letters show differences between seasons and the asterisk differences between species. Statistical differences were evaluated with Tukey honestly significant difference (HSD) test ( $P \leq 0.05$ ). ●, *Nothofagus dombeyi*; ●, *Nothofagus nitida*.



**Fig. 3** Variation in gas-exchange parameters in *N. dombeyi* and *N. nitida* in the field seasonal study. (A)  $A_{\text{sat}}$  represents the maximum  $\text{CO}_2$  assimilation measured at  $20^\circ\text{C}$  and light saturation. (B) Dark respiration was measured at  $20^\circ\text{C}$ . (C) Foliar  $R/A$  is an indicator of the carbon balance at the foliar level. Values shown are mean  $\pm$  SEM. Different lower case letters show differences between seasons and the asterisk differences between species. Statistical differences were evaluated with Tukey honestly significant difference (HSD) test ( $P \leq 0.05$ ). ■, *Nothofagus dombeyi*; ▒, *Nothofagus nitida*.

increased in *N. nitida* plants subjected to mild water stress (Fig. 4B). The WUE value was unchanged in *N. dombeyi* during treatment ( $P = 0.109$ ) (Fig. 4C), but in *N. nitida* it decreased as the drought treatment progressed (Fig. 4D). The values of  $F_v/F_m$  ranged between 0.82 and 0.87 in both

species at each level of the water-deficit treatment. Changes in  $A_{\text{sat}}$  were similar for both species under each level of water deficit (Fig. 5A,B): *N. dombeyi* was not able to recover  $A_{\text{sat}}$  after 10 days of re-watering, while *N. nitida* did (Fig. 5B).

The  $R_d$  value at the reference temperature of  $10^\circ\text{C}$  ( $R_{10}$ ) was calculated from temperature response curves (see materials and methods section). The  $R_{10}$  value did not change significantly ( $P = 0.178$ ) for *N. dombeyi* during the water-deficit treatment or the re-watering period (Fig. 5C). In *N. nitida*,  $R_{10}$  tended to increase from Mild WS to Mod WS (Fig. 5D), but was then significantly suppressed at Sev WS, and recovered to its original value during REC1 and REC2. The thermal sensitivity of respiration, shown by  $E_o$  in control plants, was lower in *N. dombeyi* than *N. nitida* under control conditions (mean  $\pm$  SE =  $55214 \pm 6616$  and  $81172 \pm 3328$  J/mol/K; respectively):  $E_o$  increased from Mild to Sev WS in *N. dombeyi* (Table 1), however, in *N. nitida*, it decreased at the start of the water deficit and was not recovered after treatment. The  $Q_{10}$  value was unchanged in *N. dombeyi* ( $P = 0.078$ ), while for *N. nitida* it decreased during the water deficit and re-watering (Table 1). The foliar  $R_d/A_{\text{sat}}$  ratio increased significantly ( $P < 0.001$ ) in *N. dombeyi* under Sev WS (Fig. 5E). The increase in  $R_d/A_{\text{sat}}$  in *N. nitida* was maintained from Mod WS to REC2 ( $P = 0.002$ ). Finally, under severe water deficit,  $R_d/A_{\text{sat}}$  was higher in *N. dombeyi* than *N. nitida* (Fig. 5E,F).

The  $D\%$  values obtained were in the range 18.5–19.7‰ for *N. dombeyi* and 19.1–19.9‰ for *N. nitida*, indicating no significant change throughout the gradual water-deficit treatment and recovery for either species.

## Discussion

### *Changes in photosynthesis and respiration under field conditions*

Despite differences in water availability between seasons, the two *Nothofagus* species did not differ in their xylem water potential. Similar response was found by Zuñiga *et al.* 2006, reporting similar water content in both species in a field study. We postulate that the similar water status during summer (Fig. 2) is achieved by different mechanisms associated with different strategies to cope with water deficit. *Nothofagus dombeyi* possesses a stomatal conductance approximately five times greater than *N. nitida* during summer (Fig. 2B), which allows it to reach higher photosynthetic activity (50% higher  $A_{\text{sat}}$ ). Other authors have also reported higher photosynthetic rates for *N. dombeyi* than *N. nitida* in the field during summer (Reyes-Diaz 2006; Zuñiga *et al.* 2006), which has been related with its higher drought tolerance. A sustained higher stomatal conductance under nonsevere drought may also indicate greater specific hydraulic conductivity

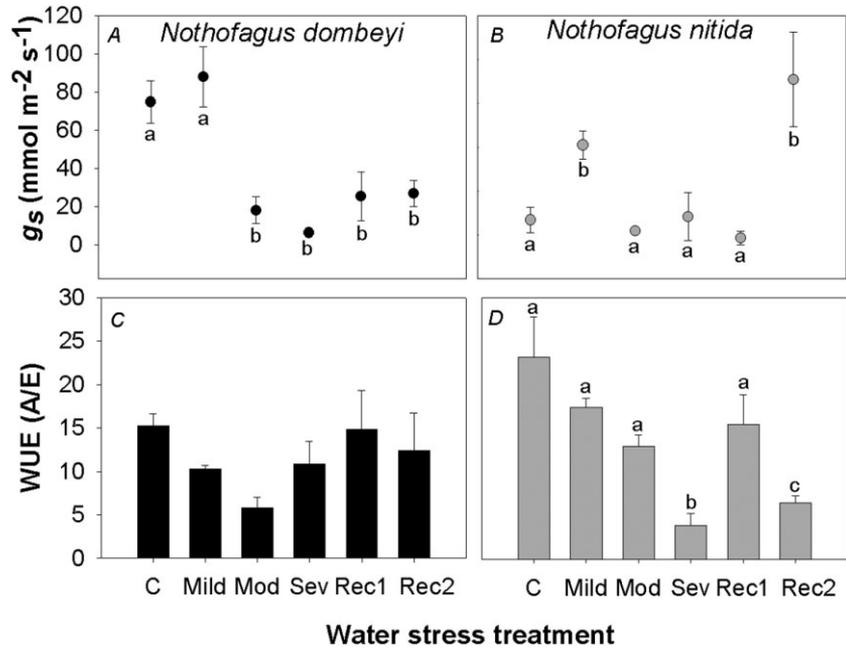


Fig. 4 Changes in (A,B) stomatal conductance and (C,D) water-use efficiency for *N. dombeyi* and *N. nitida* during drought treatment (mild, moderate, and severe water stress) and recovery (3 and 10 days of re-watering). Values shown are mean  $\pm$  SEM. Statistical differences were evaluated with Tukey honestly significant difference (HSD) test ( $P \leq 0.05$ ).

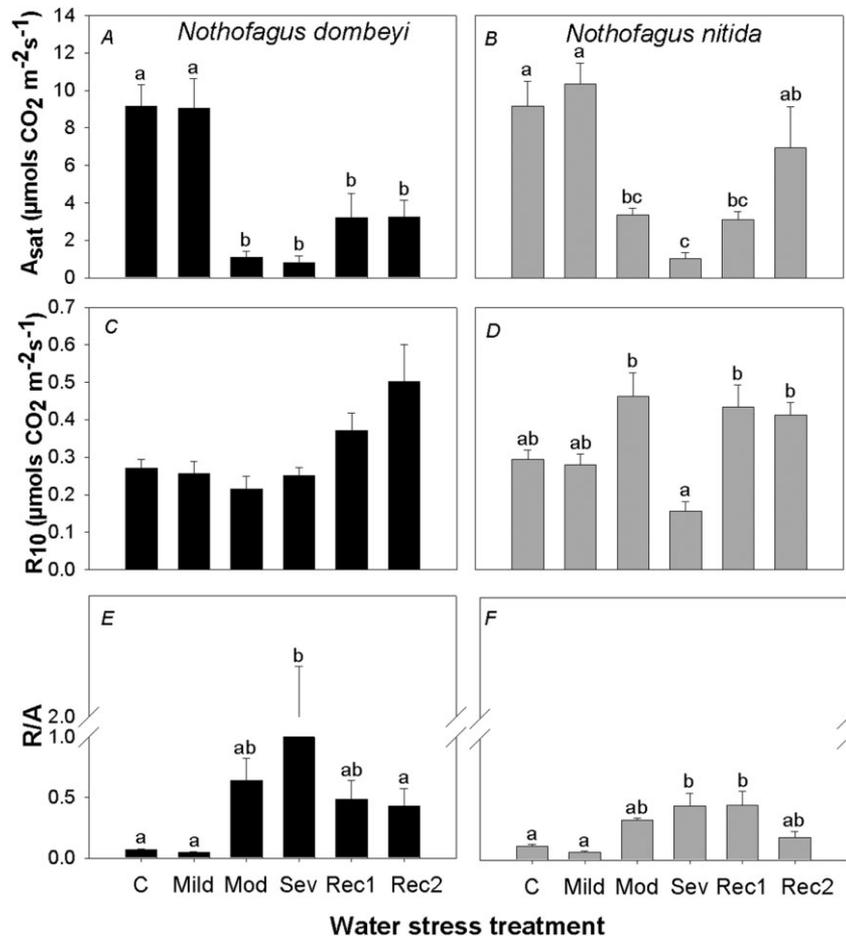


Fig. 5 Variation in gas-exchange parameters in *N. dombeyi* and *N. nitida* during drought treatment (mild, moderate, and severe water stress) and recovery (3 and 10 days of rewatering). (A,B)  $A_{sat}$  represents the maximum CO<sub>2</sub> assimilation measured at 20°C and light saturation. (C,D) Dark respiration was calculated from fitted temperature response curves. (E,F) Foliar R/A is an indicator of the carbon balance at the foliar level. Values shown are mean  $\pm$  SEM. Statistical differences were evaluated with Tukey honestly significant difference (HSD) test ( $P \leq 0.05$ ).

Variable	$E_o$ (J/mol/K)		$Q_{10}$	
	<i>N. dombeyi</i>	<i>N. nitida</i>	<i>N. dombeyi</i>	<i>N. nitida</i>
Control	55214 ± 6616ab	81172 ± 3328a	2.19 ± 0.22	3.08 ± 0.14a
Mild	37779 ± 4718a	58158 ± 3824ab	1.70 ± 0.11	2.25 ± 0.12b
Moderate	62113 ± 7797ab	53831 ± 4255b	2.41 ± 0.25	2.12 ± 0.12b
Severe	74125 ± 7105b	62015 ± 7119ab	2.81 ± 0.28	2.40 ± 0.22ab
REC1	60859 ± 5735ab	63489 ± 7901ab	2.34 ± 0.18	2.46 ± 0.28ab
REC2	51003 ± 9347ab	53842 ± 4354b	2.10 ± 0.30	2.12 ± 0.13b
ANOVA	$P = 0.034$	$P = 0.021$	$P = 0.078$	$P = 0.012$

**Table 1** Dark respiration parameters calculated from fitted temperature–response curves using a modified Arrhenius equation for leaves of *N. dombeyi* and *N. nitida* during water-deficit treatment. Values shown are mean ± SEM. One-way ANOVA ( $P \leq 0.05$ ) was performed where the factor was xylem water potential. Different letters show significantly different means (Tukey HSD test)

and lower vulnerability to cavitation, as suggested for *Nothofagus obliqua* (Varela *et al.* 2010). In addition to the greater stomatal conductance (Zuñiga *et al.* 2006), we suggest that the higher temperature during summer and greater optimal temperature also contribute to enhance metabolic processes relating to carbon fixation in *N. dombeyi*. This response has also been reported in the Mediterranean *Quercus suber* under similar water-stress conditions (Garcia-Plazaola *et al.* 1997).

Both *Nothofagus* species showed high WUE during summer (driest season) compared to winter (wettest season), which is a common response to water deficit (Piper *et al.* 2007). Despite that, both species show similar WUE according to Zuñiga *et al.* (2006). The slightly lower value reached by *N. dombeyi* during summer could be explained by higher transpiration rates than *N. nitida* (mean ± SE =  $0.65 \pm 0.12$  and  $0.16 \pm 0.03$  mmol H<sub>2</sub>O, respectively), which is consistent with the greater stomatal conductance. To avoid loss of water during the driest season, *N. nitida* closes the stomata thereby considerably reducing carbon assimilation. Under complete stomatal closure the maintenance of a constant minimum transpiration rate has been reported as an important strategy to avoid high water loss in dry periods (Burghardt & Riederer 2003).

The response of  $R_d$  was variable through the year and differed between species (Fig. 3). Contrary to our expectations, summer leaf respiration was not lower relative to photosynthesis in either species. Low  $R_d/A_{sat}$  during summer and autumn indicates a weak effect of summer drought on carbon gain. However, the increase in  $R_d$  in *N. nitida*, at least during spring, was associated with a decrease in  $A_{sat}$  producing a significant reduction in carbon gain during this season. We postulate that the increase in  $R_d$  during spring may be related to shoot growth and other processes, such as flowering and fruiting. During winter, when temperature is lower with respect to summer and there is not restriction in water availability,  $R_d$  in *N. nitida* increased despite very little change in  $A_{sat}$ . Some authors have suggested that increases in leaf respiration during winter are indicative of thermal acclimation (Atkin *et al.* 2000). The latter could be gener-

ally associated with an increase in ATP production to cope with metabolic maintenance when photosynthesis is reduced by low temperature. Although in the present study we did not do full determinations of the thermal response of  $R_d$  between seasons, the changes we did measure in  $R_d$  at 20°C are indicative of changes in respiratory capacity (Type II acclimation; Atkin & Tjoelker 2003). The increase in  $R_d$  during winter and spring initiates change in carbon gain (i.e. increased  $R_d/A_{sat}$ ; Fig. 3C), which seems to be related more with seasonal variations in temperature than changes in water availability.

#### *Photosynthetic and respiratory response to gradual water deficit and the effect on carbon balance*

The summer drought did not generate suppression of photosynthesis and subsequent respiration in these evergreen species. This response is strongly influenced by the development of the root system and/or age of the tree. Others studies with these species have reported lower rates of photosynthesis (Piper *et al.* 2007; Coopman *et al.* 2008) because they have studied seedlings (2–3 years old) and here we have worked with saplings of approximately 10–12 years of age. This increase in photosynthetic rate with developmental stage may be related to an increase in light-acclimation capacity of photosynthesis with ontogeny, as reported previously by Coopman *et al.* (2008) for *N. nitida*. Here we attempted to induce photosynthetic suppression under gradual water deficit in glasshouse conditions, where it is possible to maintain a more stable and controlled environment. Under these conditions we worked with 10-year-old saplings transplanted into pots, and these are clearly more sensitive to water deficit than adults trees in the field. However, the main objective of this work was to compare the response between species under different scenarios of water availability, and not to investigate the effect of ontogeny, for which it is difficult to extrapolate the responses from glasshouse to field. Piper *et al.* (2007) have previously found that a decline in net photosynthesis in response to drought was gradual in *N. nitida*, but

unlike our findings, *N. dombeyi* only decreased photosynthesis when the soil-water stress was more severe (Fig. 5A). In our work, complete inhibition of  $A_{\text{sat}}$  was evident for both species under severe water limitation. We assume that the water deficit applied was more extreme than in other studies (e.g. Zuñiga *et al.* 2006; Piper *et al.* 2007). It is also possible that the saturating light levels we used ( $1700 \mu\text{mol}/\text{m}^2/\text{s}$ ) may have induced the reduction in  $A_{\text{sat}}$ . However, values of  $F_v/F_m$  did not indicate damage to the photosynthetic machinery as a result of the water stress. In addition, previous studies have reported that both species have efficient mechanisms to deal with excess energy. The better photosynthetic performance in the field of *N. dombeyi* has been accompanied by a higher accumulation of photoprotective pigments of the xanthophylls cycle under high light and dry conditions (Zuñiga *et al.* 2006). In *N. nitida*, the maintenance of a high photochemical capacity is considered as one of the most important mechanisms of energy dissipation and photoprotection (Coopman *et al.* 2011). Probably if these species had been exposed to high light intensity and dehydration for a long time they would have shown photoinhibition, as reported in other works (Chaves *et al.* 2003; Gimeno *et al.* 2010). During the recovery period, only *N. nitida* was able to re-establish its photosynthesis after 10 days of re-watering, while *N. dombeyi* was able to recover only 80% of its initial photosynthetic rate after 120 days of re-watering (measurement performed after the water-deficit experiment). We found more shed leaves in pots of *N. dombeyi* than of *N. nitida* after the water-stress deficit, which would indicate that *N. dombeyi* suffered greater damage after the severe water-stress treatment. Limited recovery from imposed water stress has previously been observed in *Nothofagus* species from New Zealand, where drought tolerance was evaluated. Specifically, leaves of *Nothofagus menziesii* individuals were more damaged than those of *N. solandri* under conditions of severe water stress (Sun *et al.* 1995). These authors suggest that the lack of recovery was probably due to cavitation and embolism of xylem vessels, which adversely affected the movement of water from soil to leaves (Sun *et al.* 1995).

The changes in  $g_s$  in both species during the water-stress treatment (Fig. 4) indicate that the main mechanism regulating photosynthesis under water deficit in these species seems to be the regulation of stomatal closure. The ability to maintain opened stomata for a longer time permits not only water-vapor exchange, but also the uptake of  $\text{CO}_2$ , which maintains photosynthesis (Zuñiga *et al.* 2006). Our results, and other previous reports (Piper *et al.* 2007), confirm that for evergreen *Nothofagus* the maintenance of stomata openness under mild water deficit contributes significantly to the maintenance of carbon assimilation.

However, *N. dombeyi* and *N. nitida* showed differences in WUE in control plants (well-watered), which thus appeared to be driven primarily by a different degree of stomatal opening (Fig. 4A). Read and Farquhar (1991) found that Australasian *Nothofagus* species from sites with low rainfall in summer had surprisingly low WUE under well-watered conditions associated with maximum stomatal conductance. This was attributed to selection for opportunistic carbon gain under conditions of mild to low drought stress. Besides these differences in the control treatment, WUE of both species responded differently to water deficit and, in contrast to other reports (Piper *et al.* 2007), was unaltered by water deficit in *N. dombeyi*. This response may indicate that assimilation decreased at the same rate as transpiration, which may be an important mechanism for this species to cope with water stress.

The unchanged respiration of *N. dombeyi* under water-deficit experiment indicates low flexibility in the response of respiration to temperature under drought, or a strong metabolic need to maintain respiration even in the face of reduced C fixation. The temperature coefficient  $E_0$  was increased under severe water. This parameter indicates change in the energy of activation for respiration as an overall reaction, and sheds light on possible biochemical/physiological sensitivity to drought treatment at the biochemical level. Atkin and Macherel (2009) suggested that no change in respiration and no change in the mitochondrial electron partitioning (as we found here) are indicative of a constant rate of ATP synthesis. However, maintaining a constant respiratory metabolism may be adequate at low levels of water deficit, but when photosynthesis is suppressed under a severe water deficit, the result is in a progressively greater  $R_d/A_{\text{sat}}$  ratio, seriously affecting foliar carbon balance over extended periods of drought stress (Flexas *et al.* 2005, 2006). Increases in  $R_d/A_{\text{sat}}$  in response to decreasing soil-water availability were significant in *N. dombeyi*, reaching a value of 1.5, indicating a strong increase in the relative carbon cost of maintaining metabolism (Fig. 5E).

*Nothofagus nitida* was more flexible in its respiratory response. The increase in  $R_d$  at Mod WS and during recovery may indicate increased ATP synthesis needed to cope with metabolic demand (if adenylate restrictions are reduced) and for repairing protoplasmic damage caused by water loss (Slot *et al.* 2008). However, when WS was more severe, the reduction in respiration is indicative of downregulation of the activity of metabolic components, such as photosynthetic enzymes, nitrate reductase or sucrose phosphate synthase (Galmes *et al.* 2007). 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 (Gulias *et al.* 2002). This flexibility in respiration of *N. nitida* was combined with low thermal sensitivity of respiration

(decrease in  $E_o$  and  $Q_{10}$ ) in response to water deficit, which undoubtedly contributed to the lower relative carbon cost of maintaining metabolism at the foliar level than in *N. dombeyi*.

Our previous study performed on two New Zealand *Nothofagus* species (*N. solandri* and *N. menziesii*) with different levels of drought tolerance (Sanhueza *et al.* 2013) found that the less tolerant species (*N. menziesii*) displayed an unchanged  $R_{10}$ , which we concluded was a trait associated with drought sensitivity. However, the results of the present study suggest that the higher drought tolerance for *N. dombeyi* shown under mild water stress in the field (ca  $-1.5$  MPa) cannot be extrapolated to severe water-deficit conditions, because under these conditions a strong decrease in photosynthesis and low flexibility of respiration cause a significant increase in  $R_d/A_{\text{sat}}$ . Other works studying the effect of drought in *Nothofagus* species postulate low phenotypic plasticity in *N. dombeyi*, because of its high vulnerability to cavitation, which has previously resulted in severe drought-induced dieback during the most severe drought of the 20th century in northern Patagonia (Argentina) (Bucci *et al.* 2012).

The maintenance of a higher relative gain of carbon in *N. nitida* during water deficit may be associated with morphological and physiological advantages. We postulate that the maintenance of photosynthesis under mild and moderate water deficit may be related to the higher specific leaf area of *N. dombeyi* than *N. nitida* (mean  $\pm$  SE =  $7.66 \pm 0.66$  and  $4.83 \pm 0.48$  m<sup>2</sup>/Kg, respectively). Thicker leaves usually have a higher density of chlorophyll and proteins per unit leaf area and have a greater photosynthetic capacity than thinner leaves (Liu & Stützel 2004; Cooper *et al.* 2006; Ow *et al.* 2010). Moreover, it has been proposed that the higher leaf mass fraction of *N. nitida* likely promotes a higher whole-plant carbon gain (Piper *et al.* 2009).

#### Effect of water deficit on the alternative pathway

The reported  $D$  for both species ranged from 18.5 to 19.8%. Considering as control values for leaves the previously reported 26.7, 19.0, and 22.1% for potassium cyanide (KCN)-inhibited, salicylhydroxamic acid (SHAM)-inhibited and control treatments, respectively (Noguchi *et al.* 2001), we interpret our  $D$  values as indicative of a relatively constant electron flux maintained primarily through the CP. In our previous study on *Nothofagus* from New Zealand (Sanhueza *et al.* 2013) we also found no changes in partitioning between both pathways during a water-deficit treatment and recovery. However, an increase in AOX/COX protein content during recovery suggested that AP may be involved in modulating respiratory metabolism during the recovery after drought, potentially by controlling excess reactive

oxygen species (Searle *et al.* 2011). The AOX has been proposed to be involved in the response of plants to several environmental factors (Searle *et al.* 2011), including cold (Gonzalez-Meler *et al.* 1999; Armstrong *et al.* 2008) and drought (Ribas-Carbo *et al.* 2005b). Our results suggest that we are not able to resolve this role at the timescale of the discrimination measurements we made. In our previous work (Sanhueza *et al.* 2013) we suggested that the increase in the amount of AOX protein indicates participation of the AP: the proportion of AOX protein found by Western blot analysis might have been inactivated. Several studies have suggested post-translational regulation of AOX protein (Grant *et al.* 2008; Vanlerberghe *et al.* 2009). Further research including discrimination and activity of the alternative respiration with Western and Northern blots analysis are needed in order to identify the mechanisms responsible for regulation of this pathway and to determine if the AP is playing some role under water deficit and/or recovery.

#### Conclusions

Ours results indicate that the higher drought tolerance of *N. dombeyi* than *N. nitida* operates only under mild water stress, where the former is able to maintain greater carbon assimilation. However, under severe water stress, photosynthesis is largely suppressed and respiration becomes more important as water stress develops. Under this condition *N. dombeyi* showed lower flexibility in  $R_d$ , while *N. nitida* has the capacity to decrease  $R_d$  to reduce the net loss of foliar carbon. These results emphasize the important role that respiration may play in determining leaf and, subsequently, whole-plant carbon balance. These species inhabit southern areas of Chile characterized by a Mediterranean-climate where global climates models predict an increase in water deficit and drought risk caused by a reduction of rainfall. Under these predicted conditions, *N. dombeyi* could be more negatively affected than *N. nitida* because of its limited ability to limit carbon loss. Prolonged periods of drought could be limiting for survival of *N. dombeyi*, which could eventually lead to changes in the relative distribution of these important tree species.

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