Low temperature responses of *Nothofagus dombeyi* and *Nothofagus nitida*, two evergreen species from south central Chile

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**Summary** *Nothofagus dombeyi* (Mirb.) Blume and *Nothofagus nitida* (Phil.) Krasser are closely related evergreen trees native to south central Chile. *Nothofagus dombeyi* is a pioneer in habitats subject to high daytime irradiances and nighttime freezing temperatures and has a wider altitudinal and latitudinal distribution than *N. nitida*, which is restricted to more oceanic climates. We postulated that *N. dombeyi* has a greater cold-acclimation capacity, expressed as a greater capacity to maintain a functional photosynthetic apparatus at low temperatures, than *N. nitida*. Because cold-acclimation may be related to the accumulation of cryoprotective substances, we investigated relationships between ice nucleation temperature (IN), freezing temperature (FT), and the temperature causing injury to 50% of the leaf tissues (LT₅₀) on the one hand, and concentrations of total soluble carbohydrates (TSC), starch and proline on the other hand. Observations were made throughout a seasonal cycle in adults and seedlings in the field and in seedlings in the laboratory under cold-acclimation inductive and non-inductive conditions. In adults, LT₅₀ values were lower in *N. dombeyi* than in *N. nitida*, suggesting that *N. dombeyi* is the more frost tolerant species. Adults of both species tolerated freezing in autumn and winter but not in spring and summer. In the fall and winter, seedlings of *N. dombeyi* had a much lower LT₅₀ than those of *N. nitida*. *Nothofagus nitida* seedlings, in autumn and winter, exhibited freezing avoidance mechanisms. Although elevated TSC and proline concentrations may contribute to freezing tolerance in adults of both species, an increase in proline concentration is unlikely to be the dominant frost tolerance response in adults because proline concentrations were higher in *N. nitida* than in *N. dombeyi*. In seedlings, however, there were large differences in proline accumulation between species that may account for the difference between them in freezing tolerance. Starch concentration in both species decreased during winter. Chlorophyll fluorescence indicated that maximal photochemical efficiency (Fᵥ/Fₘ) remained at optimal values (~0.8) throughout the year. The effective photochemical efficiency of PSII (ϕPSII) and relative electron transport rates (ETRᵥ) decreased in winter in both species. In seedlings, fluorescence parameters were more affected in winter in *N. nitida* than in *N. dombeyi*. We concluded that adults and seedlings of *N. dombeyi* are harder than adults and seedlings of *N. nitida*, which is consistent with its wider latitudinal and altitudinal distribution.

**Keywords:** carbohydrates, chlorophyll fluorescence, cold acclimation, freezing tolerance, ice nucleation, photosynthesis, proline, starch.

**Introduction**

Evergreen *Nothofagus* species play an important physiognomic role in south central Chile, between 37 and 43° S (Alberdi et al. 1985, Alberdi 1987). *Nothofagus dombeyi* (Mirb.) Blume occurs most frequently on the humid slopes of the west side of the Andes. It is a pioneer tree in areas formed by volcanic scoria that are subject to high solar irradiance and low nighttime temperatures (McQueen 1977, Alberdi 1995, Veblen et al. 1995). *Nothofagus nitida* (Phil.) Krasser is the only *Nothofagus* species absent from the eastern slopes of the Andes (Veblen et al. 1996), regenerating only in coastal regions. *Nothofagus dombeyi* is a pioneer tree in areas formed by volcanic scoria that are subject to high solar irradiance and low nighttime temperatures (McQueen 1977, Alberdi 1995, Veblen et al. 1995). *Nothofagus nitida* (Phil.) Krasser is the only *Nothofagus* species absent from the eastern slopes of the Andes (Veblen et al. 1996), regenerating only in coastal regions. *Nothofagus dombeyi* is typically a shade-intolerant heliophyte (Donoso and Lara 1998), whereas *N. nitida* is more sciophytic (semi-shade-tolerant). Weinberger (1973) observed that *N. dombeyi* and *N. nitida* seldom grow together in their natural habitats. Weinberger et al. (1973) and Alberdi et al. (1974) reported that, although both species have a high dessication tolerance, dessication tolerance is highest in *N. dombeyi*. Some aspects of freezing resistance of both species have been reported by Alberdi et al. (1985, 1989); however, little is known about their cold acclimation capacity and the mechanism(s) by which they resist low temperatures.

Plants able to withstand freezing temperatures either avoid ice formation in the tissues (freezing avoidance) or tolerate the effects of extracellular (apoplastic) ice formation, such as dehydration or cell shrinkage (freezing tolerance) (Levitt 1980, Alberdi and Corcuera 1991, Larcher 2000, 2003). Plants commonly avoid freezing injury by supercooling during periods of high metabolic activity (Levitt 1980, Sakai and Larcher 1987).

The maximum freezing tolerance of plants is not a constitutive trait, being induced in response to low, nonfreezing temperatures (< 10 °C): a phenomenon known as cold acclimation.
or cold hardening (Levitt 1980, Alberdi and Corcuera 1991). Compatible solutes accumulate during cold acclimation and it is thought that this accumulation serves a cryoprotective function in some plants (Alberdi and Corcuera 1991, Livingston 1996). Proline, which accumulates in a variety of plants subjected to cold, has been correlated with freezing tolerance (Bravo et al. 1998, Wanner and Juntilla 1999). Soluble carbohydrates and free proline may be involved in freezing point depression of cell sap, prevention of plasmolysis during cell dehydration caused by freezing, and membrane stabilization (Strauss and Hauser 1986, Alberdi et al. 1991, Santarius 1992).

We studied cold acclimation responses of adults and seedlings of N. dombeyi and N. nitida grown in a common garden in an N. nitida stand in south central Chile and in the laboratory. Because N. dombeyi occupies harsher habitats than N. nitida, we postulated that N. dombeyi has a greater freezing tolerance than N. nitida, expressed as a greater capacity to maintain a functional photosynthetic apparatus, reflecting a greater ability to accumulate cryoprotective substances.

Materials and methods

Characteristics of the study site

This study was performed in an N. nitida forest stand in Pichiquilla, by Cordillera de Quillaipe, X Región, in south-central Chile (41°31’07.5” S, 72°45’2.2” W), where N. nitida grows naturally. Ten years earlier, in anticipation of the study, 2-year-old plants of N. dombeyi and N. nitida were planted at the site. Nothofagus dombeyi plants were obtained from a forest located in San Pablo de Tregua in Panguipulli (Province of Valdivia at 39°38’ S, 72°09’ W) south central Chile. Its climate is characterized by short and relatively dry summers and long, wet and rainy winters (Lara et al. 2002). Mean annual air temperature is 11 °C. Mean minimum monthly air temperature (5 °C) occurs in August, and the mean monthly maximum temperature (20 °C) occurs in February. Air temperatures are similar at Pichiquilla, the site of the common garden study. The climate at Pichiquilla is considered temperate and strongly humid with an oceanic tendency (Di Castri and Hajek 1976), although winter frosts occur. Annual precipitation is around 2200 mm or more (Armesto et al. 1995). Daily maxima and minima air temperatures and precipitation were recorded at the study site with a data logger LI-1400 using LI-1400-104 and LI-1400-106 sensors, respectively (Li-Cor, Lincoln, NE).

Field and laboratory experiments

Field experiments were performed with 2-year-old seedlings and adult trees (about 12 years old) of both species. Both species flower after 10 years (Rodríguez et al. 1995). Physiological and biochemical measurements of mature leaves from 10 selected adult trees were made seasonally and, for seedlings, were made at the beginning of autumn and winter. These seasons were selected to estimate natural cold acclimation in seedlings.

For the laboratory experiments, 2-year-old seedlings of both species were taken from the field in winter, transferred to pots filled with soil from their respective locations and maintained outdoors for about 6 months. Before beginning the experiments, the pots were placed in controlled-environment chambers and maintained for 1 month at 15 °C in a 16-h photoperiod, with a photosynthetic photon flux (PPF) of 100–120 mmol m–2 s–1 at the top of the canopy. The light source was cool white fluorescent tubes. Seedlings were irrigated once a week and fertilized with 0.2 g l–1 Photrogen (with a mineral composition of 14,10,22 (N,P,K) plus Mg, S and chelated trace elements) (Solaris, Buckinghamshire, U.K.) every 2 weeks. To study the cold acclimation process, the plants were then assigned to an 8- or 16-h photoperiod at 4 °C.

Thermal analysis

One expanded mature leaf was removed and attached to a thermocouple (Gauge 30 cooper-constantan thermocouples; Cole Palmer Instruments, Vernon Hills, IL), and immediately enclosed in a small, tightly closed cryotube to avoid changes in tissue water concentration. Temperature was monitored once per second with a Personal Daq/56 multi-channel thermocouple USB data acquisition module (IOtech, Cleveland, OH). The tubes were placed in a cryostat and the temperature lowered from 0 to −15 °C at a rate of about 2 °C h–1. Temperature records were made and the freezing exotherms analyzed. The temperature at the initiation of the exotherm corresponds to the ice nucleation temperature (IN), and the highest point of the exotherm represents the freezing temperature (FT) of the water in the apoplast (including symplastic water driven outwards by the water potential difference caused by apoplastic ice formation) (Larcher 2003). A second exotherm was not observed.

Freezing tolerance

Samples of twigs with mature leaves were randomly collected and introduced into separate, hermetically sealed steel cells, and incubated in a cryostat at freezing temperatures from 0 to −20 °C for 2 h. The cooling rate was about 2 °C h–1. To ensure homogeneous cooling, the sample was kept at the desired temperature for 2 h. Then, the steel cells were removed from the cryostat and left at 4 °C overnight for sample thawing. This temperature was selected based on the FT of leaves determined by thermal analysis, ensuring a difference of at least 5 °C to achieve fast thawing, which prevents ice recrystallization, giving rise to possible cellular damage (Griffith et al. 2001, Mazur 2004). The twigs were then transferred, with their stems in water, to a controlled-environment chamber providing diffuse light at 15 °C for 7 days, when the extent of visible leaf damage was assessed (Lange 1961), and corroborated by the 2,3,5-triphenyl tetrazolium chloride method according to Larcher et al. (1969) and Alberdi and Rios (1983). The temperature causing injury to 50% of the leaf tissues (LT50) was determined (Levitt 1980, Larcher 2000). Assays on twigs or detached leaves were performed to control for sample size in
the freezing test. No differences between the LT50 of detached leaves and leaves on detached twigs were observed.

**Freezing resistance mechanism**

By comparing the LT50 and the IN temperatures obtained by thermal analyses, the mechanism of freezing resistance can be evaluated (Squeo et al. 1991). When damage occurs at a lower temperature than the IN temperature, the plant tissue can tolerate extracellular freezing. When damage occurs near the IN temperature or higher, the tissue cannot tolerate extracellular ice formation.

**Proline**

Free proline was determined colorimetrically according to Bates et al. (1973). Leaves (0.2 g fresh mass) were ground in liquid nitrogen and extracted with 5 ml of 3% sulfosalicylic acid. After filtration through Whatman No. 1 filter paper, 2.0 ml of extract was assayed by the acid ninhydrin reaction. Ninhydrin–proline complexes were extracted in 4 ml of 1,2-dichloroethane and the A520 was compared with a standard curve obtained with pure proline (Merck KGaA, Darmstadt, Germany).

**Carbohydrate extraction and determination**

Carbohydrates were extracted from fresh leaf tissue (0.1 g) in 86% (v/v) ethanol:water with overnight agitation. The samples were depigmented in a 1:3 (v/v) chloroform:water mixture. The aqueous fraction was lyophilized overnight and the carbohydrates resuspended in 500 µl of methanol. Total soluble carbohydrates (TSC) concentration was determined spectrophotometrically by the Resorcinol method (Roe 1934) at a wavelength of 520 nm, with sucrose as the standard.

**Starch determination**

Starch was quantified from the residues of the ethanolic extractions of TSC, after acid hydrolysis with 52% (v/v) perchloric acid, by the Resorcinol method (Roe 1934) at a wavelength of 520 nm, with starch as the standard.

**Fluorescence measurements**

Fluorescence of attached unshaded leaves was measured by a pulse-amplitude modulated fluorimeter (FMS 2, Hansatech Instruments, U.K.). Leaves of both species were dark adapted for 30 min (to obtain open centers) with leaf-clips from a mobile shutter plate. The fiber-optic and its fiber-optic adapter were then attached to a ring located above the clip about 10 mm from the sample. Different light pulses (see below) were applied following standard routines programmed within the machine. Signal recording and calculation were performed on a personal computer using data analyses and control software (Hansatech Instruments). According to the terminology of Van Kooten and Snel (1990), minimal fluorescence (Fm) was determined by applying a weak modulated light (0.4 µmol m⁻² s⁻¹) and maximal fluorescence (Fm') was induced by a short pulse (0.8 s) of saturating light (9000 µmol m⁻² s⁻¹). After 10 s, actinic light (480 µmol m⁻² s⁻¹) was turned on to obtain fluorescence parameters during steady-state photosynthesis. Saturating pulses were applied after steady-state photosynthesis had been reached in order to determine maximal fluorescence in light-adapted leaves (Fm') and steady-state fluorescence (Fv). Finally, the actinic light was turned off and immediately a 2-s far-red (FR) pulse was applied to obtain minimal fluorescence in light-adapted leaves (Fm). In this paper, Fv/Fm (where variable fluorescence Fv = Fm – Fo), and effective photosynthetic efficiency (ϕPSII) (where ϕPSII = Fm' – Fv/Fm'), were used as indicators of the potential and effective quantum yield (photosynthetic efficiency) of the PSII, respectively (Genty et al. 1989). Relative electron transport rate was calculated as:

\[
ETR = PPF \times 0.5 \times \phi_{PSII} \times 0.84
\]

Photochemical quenching (qP) was calculated as:

\[
qP = \frac{F_{m'} - F_v}{F_{m'} - F_o}
\]

where Fm' is maximal fluorescence in light-adapted leaves, and Fo is steady-state fluorescence.
Non-photochemical quenching (NPQ) was calculated as (Bilger and Björkman 1990):

\[
NPQ = \frac{F_{m} - F_{m}'}{F_{m}'}
\]

**Statistics**

Reported values correspond to the means of 10 replicates for each species for freezing resistance experiments, fluorescence parameters and metabolites. Data were subjected to a two-way ANOVA (where the factors were species and seasons), with repeated measurement for one factor (species). A Tukey test was used to identify those values with significant differences. Both analyses were performed with Sigma Stat 2.0 software (SPSS, Chicago, IL). Differences between the values were considered significant at P ≤ 0.05.

**Results**

**Microclimatic measurements**

Precipitation was highest in winter (around 700 mm), and June and August of 2003 and April and June of 2004 were the rainiest months (292, 246 mm and 361, 352 mm, respectively) (Figure 1). Rainfall was low in autumn and summer, but no month was precipitation-free. Air temperature was lowest in winter (2.9 °C in June), but subzero temperatures also occurred during autumn and at the beginning of spring (Figure 1). Mean maximum air temperature ranged between 10 and 22 °C in July and February, respectively.

**Seasonal course of freezing resistance**

Adult plants of both species exhibited seasonal changes in freezing resistance under field conditions, indicating that they
tolerated freezing in autumn and winter and avoided it in spring and summer (Table 1). In both species, LT50 was around –7 °C in spring and summer, whereas in winter, it was significantly lower (P ≤ 0.05), reaching –14.3 and –10.2 °C in *N. dombeyi* and *N. nitida*, respectively (Table 1), indicating that cold acclimation occurred in both species, but that *N. dombeyi* had a higher freezing tolerance than *N. nitida* in winter. In *N. dombeyi*, FT was highest in summer (P ≤ 0.05) and lowest in spring. Seasonal changes in FT in *N. nitida* were small. Ice nucleation temperature values showed smaller seasonal fluctuations than LT50 values (Table 1). The IN temperatures of both species were lower in spring and summer than in winter and autumn, and lower in *N. dombeyi* than in *N. nitida* (P ≤ 0.05).

As observed in adult trees, LT50, IN and FT decreased from autumn to winter in seedlings growing in the field (Table 2A) and were significantly lower in *N. dombeyi* than in *N. nitida* (P ≤ 0.05). The freezing resistance mechanism of the seedlings differed between species. *Nothofagus dombeyi* leaves tolerated extracellular freezing in the autumn and winter, whereas *N. nitida* leaves were damaged by freezing. A similar difference in freezing resistance was observed between species when seedlings were cold acclimated at 4 °C for 21 days in the laboratory in a 16-h photoperiod with *N. dombeyi* seedlings exhibiting freezing tolerance and *N. nitida* seedlings exhibiting freezing avoidance (Table 2B). Acclimation capacity was significantly (P ≤ 0.05) higher in *N. dombeyi* seedlings than in *N. nitida* seedlings (a 3.2 °C decrease in LT50 in cold-acclimated *N. dombeyi* seedlings compared with non-acclimated seedlings, versus a 0.8 °C decrease in LT50 in cold-acclimated *N. nitida* seedlings) (Table 2B). The extent of the cold acclima-

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Table 1. Freezing resistance of leaves of adult plants of *Notofagus dombeyi* and *N. nitida* growing in a common garden in south central Chile. The temperature causing damage to 50% of the tissue (LT50), ice nucleation temperature (IN) and freezing temperature (FT) were determined in detached leaves. Values are means of 10 replicates ± SE. Values for LT50 lower than values for IN indicate freezing tolerance and LT50 values higher than IN values indicate freezing avoidance. Different lower case letters indicate statistically significant differences (P ≤ 0.05) between the seasonal values for the same plant species (columns). Different upper case letters indicate statistically significant differences (P ≤ 0.05) between LT50 and IN of each species in the same season. Asterisks indicate statistically significant differences (P ≤ 0.05) between species for each parameter in the same season.

<table>
<thead>
<tr>
<th>Species</th>
<th>Season</th>
<th>FT (°C)</th>
<th>IN (°C)</th>
<th>LT50 (°C)</th>
<th>Freezing resistance mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. dombeyi</em></td>
<td>Autumn</td>
<td>–3.5 ± 0.3 a*</td>
<td>–5.2 ± 0.3 aA*</td>
<td>–9.8 ± 0.2 aB*</td>
<td>Tolerance</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>–3.8 ± 0.2 a</td>
<td>–5.4 ± 0.3 aA*</td>
<td>–14.3 ± 0.1 bB*</td>
<td>Tolerance</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>–4.1 ± 0.3 a*</td>
<td>–6.7 ± 0.2 bA</td>
<td>–7.3 ± 0.2 cA</td>
<td>Avoidance</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>–2.5 ± 0.2 b</td>
<td>–6.5 ± 0.3 bA</td>
<td>–7.0 ± 0.1 cA</td>
<td>Avoidance</td>
</tr>
<tr>
<td><em>N. nitida</em></td>
<td>Autumn</td>
<td>–2.8 ± 0.2 a</td>
<td>–4.1 ± 0.1 aA</td>
<td>–8.0 ± 0.2 aB</td>
<td>Tolerance</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>–3.0 ± 0.2 b</td>
<td>–4.8 ± 0.2 bA</td>
<td>–10.1 ± 0.2 bB</td>
<td>Tolerance</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>–3.0 ± 0.1 b</td>
<td>–6.2 ± 0.4 cA</td>
<td>–7.25 ± 0.2 cA</td>
<td>Avoidance</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>–3.0 ± 0.2 b</td>
<td>–5.8 ± 0.2 cA</td>
<td>–6.78 ± 0.2 dA</td>
<td>Avoidance</td>
</tr>
</tbody>
</table>
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Table 2. Freezing resistance mechanisms in leaves of seedlings of *N. dombeyi* and *N. nitida* grown under field conditions in a common garden in South Central Chile (A) and cold acclimated at 4 °C (CA) under laboratory conditions (B). Temperature causing damage to 50% of the tissue (LT50), ice nucleation temperature (IN) and freezing temperature (FT) were determined on detached leaves. Values are means of 10 replicates ± SE for field conditions and five replicates ± SE for laboratory conditions. Values for LT50 lower than values for IN indicate freezing tolerance and LT50 values close to or higher than IN values indicate freezing avoidance. Different lower case letters indicate statistically significant differences (*P* ≤ 0.05) between the seasonal values for the same plant species (columns). Different upper case letters indicate statistically significant differences (*P* ≤ 0.05) between LT50 and IN of each species in the same season. Asterisks indicate statistically significant differences (*P* ≤ 0.05) for each parameter in the same season.

<table>
<thead>
<tr>
<th>Species</th>
<th>Season/CA</th>
<th>FT (°C)</th>
<th>IN (°C)</th>
<th>LT50 (°C)</th>
<th>Freezing resistance mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(A) Field conditions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. dombeyi</em></td>
<td>Autumn</td>
<td>−2.9 ± 0.3 a*</td>
<td>−5.5 ± 0.2 a*</td>
<td>−8.4 ± 0.2 aB*</td>
<td>Tolerance</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>−4.0 ± 0.2 b*</td>
<td>−6.7 ± 0.2 bA*</td>
<td>−12.8 ± 0.6 bB*</td>
<td>Tolerance</td>
</tr>
<tr>
<td><em>N. nitida</em></td>
<td>Autumn</td>
<td>−1.9 ± 0.1 a</td>
<td>−3.9 ± 0.1 aA</td>
<td>−4.2 ± 0.2 aA</td>
<td>Avoidance</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>−3.0 ± 0.1 b</td>
<td>−5.1 ± 0.2 bA</td>
<td>−6.2 ± 0.2 bA</td>
<td>Avoidance</td>
</tr>
<tr>
<td><strong>(B) Laboratory conditions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. dombeyi</em></td>
<td>0</td>
<td>−2.7 ± 0.1 a</td>
<td>−4.8 ± 0.1 aA</td>
<td>−9.6 ± 0.1 aB*</td>
<td>Tolerance</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>−3.0 ± 0.2 a</td>
<td>−5.3 ± 0.2 abA</td>
<td>−10 ± 0.3 abB*</td>
<td>Tolerance</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>−3.7 ± 0.1 b</td>
<td>−5.7 ± 0.1 bA</td>
<td>−10.3 ± 0.3 abB*</td>
<td>Tolerance</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>−2.9 ± 0.1 a</td>
<td>−5.3 ± 0.1 abA</td>
<td>−10.6 ± 0.1 bB*</td>
<td>Tolerance</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>−2.7 ± 0.1 a</td>
<td>−5.1 ± 0.2 aA</td>
<td>−12.8 ± 0.1 cB*</td>
<td>Tolerance</td>
</tr>
<tr>
<td><em>N. nitida</em></td>
<td>0</td>
<td>−4.1 ± 0.1 a*</td>
<td>−5.5 ± 0.2 aA*</td>
<td>−5.7 ± 0.4 aA</td>
<td>Avoidance</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>−3.8 ± 0.0 a*</td>
<td>−5.2 ± 0.1 aA</td>
<td>−4.8 ± 0.3 bA</td>
<td>Avoidance</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>−4.0 ± 0.1 a*</td>
<td>−5.5 ± 0.1 acA</td>
<td>−5.6 ± 0.2 aA</td>
<td>Avoidance</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>−4.4 ± 0.1 b*</td>
<td>−6.2 ± 0.1 bA*</td>
<td>−6.2 ± 0.1 cA</td>
<td>Avoidance</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>−4.7 ± 0.1 b*</td>
<td>−5.9 ± 0.1 bcA*</td>
<td>−6.5 ± 0.2 cA</td>
<td>Avoidance</td>
</tr>
</tbody>
</table>

Freezing response of LT50 in seedlings in the field (autumn–winter) and in seedlings cold-acclimated for 21 days in an 8-h photoperiod (LT50 = −12.6 ± 0.4 °C in *N. dombeyi* and −6.1 ± 0.2 °C in *N. nitida*) was similar to that in seedlings cold-acclimated for 21 days in a 16-h photoperiod, indicating that the factor that induced cold acclimation in both species was the decrease in temperature from summer to winter, not the shortening of the photoperiod.

**Compound analyses**

Total soluble carbohydrate concentrations in leaves of field-grown adult plants of *N. dombeyi* were higher in winter than in the other seasons, and about twice as high as in the autumn (Figure 2A). In all seasons, TSC concentrations in *N. dombeyi* were significantly higher (about 33%) than in *N. nitida* (*P* ≤ 0.05). Starch concentrations of both species were lower in winter than in other seasons (*P* ≤ 0.05) (Figure 2B). With the exception of summer, starch concentrations were higher in *N. nitida* than in *N. dombeyi* (Figure 2B).

Proline concentrations in adult plants were significantly higher (*P* ≤ 0.05) in *N. nitida* than in *N. dombeyi*, except during summer. In both species, proline accumulation was greater in winter than in the other seasons (*P* ≤ 0.05) (Figure 2C). In adult plants, LT50 values were inversely correlated with increases in TSC and proline (*r* = −0.80 and −0.93, respectively for *N. dombeyi*, and −0.66 and −0.88, respectively for *N. nitida*).

In *N. dombeyi* seedlings growing under field conditions, TSC concentrations were higher in winter than in autumn (Figure 3A). The TSC concentrations in seedlings of both species were higher under laboratory conditions than under field conditions. Significantly higher TSC accumulation (*P* ≤ 0.05) was observed in the laboratory on Day 7 of cold acclimation in *N. dombeyi* seedlings (Figure 3D). Conversely, starch concentrations were higher in seedlings in the field than in the laboratory (Figures 3B and 3E), with no significant differences between species in the field. Cold acclimation in the laboratory slightly decreased starch concentrations, more significantly in *N. dombeyi* seedlings than in *N. nitida* seedlings (*P* ≤ 0.05), but only until Day 14 of cold acclimation. Proline concentrations were higher in *N. dombeyi* seedlings than in *N. nitida* seedlings under field and laboratory conditions (Figures 3C and 3F). Although solute accumulation may cause freezing point depression, no significant correlations were observed between FT and metabolite concentrations (*r* = ~−0.1).

**Fluorescence parameters**

Highest maximal photochemical efficiency (*Fm*/*Fm′*) of adult plants of both species was obtained in autumn (*P* ≤ 0.05), decreasing by about 5% in the other seasons (Table 3). Nonetheless, *Fm*/*Fm′* values were around 0.86 throughout the year, indicating an optimal physiological state (Björkman and Demmig 1987). Effective photochemical efficiency of both species was lower in winter than at other times of the year. The highest ϕPSII was in *N. dombeyi* in autumn (*P* ≤ 0.05) (Table 3). Both ETR, qP decreased in winter in both species. The highest
NPQ in *N. dombeyi* was observed in autumn, whereas NPQ remained constant in all seasons in *N. nitida*. In seedlings, $F_v/F_m$ was maintained between 0.83 and 0.86 (Table 3). Values of $\Phi$PSII, ETR, qP and NPQ were higher in *N. dombeyi* seedlings than in *N. nitida* seedlings in both studied seasons ($P \leq 0.05$).

**Discussion**

To our knowledge, this is the first study in which LT$_{50}$ and thermal analyses have been combined to dissect the components of freezing resistance of leaves of woody evergreens from the Chilean temperate rain forest and to analyze their seasonal and growth stage fluctuations. Freezing resistance in seedlings and adult plants of *N. dombeyi* was significantly greater than in *N. nitida*. This is consistent with the wider altitudinal and latitudinal distribution of *N. dombeyi* and its success as a pioneer species in harsh cold environments; by comparison, *N. nitida* has a more restricted distribution (Weinberger 1973, McQueen 1977). Because the lowest minimum recorded temperature at the study site was $-2.9 \degree C$, it is apparent that both species are well equipped to cope with the lowest likely habitat temperature. The temperature at the site of the common garden would thus not impose severe frost damage on adults. Freezing tolerance is usually observed in plants from cold and high regions with seasonal climates, where the temperature can drop below zero at any time of year (Larcher 2000, 2003). The adult plants of both species studied tolerated ice formation in autumn and winter (lower LT$_{50}$ than IN), indicating that, following cold acclimation, they were able to tolerate apoplastic ice nucleation and freezing in their tissues and the concomitant desiccation without suffering severe damage (Bravo et al. 2001). The adult plants also exhibited limited supercooling capacity according to the IN temperature, which was about $-5 \degree C$ (see Table 1). The highest supercooling capacity was observed in spring. In the seedling stage, however, *N. dombeyi* showed freezing tolerance, whereas *N. nitida* exhibited avoidance through supercooling. Freezing avoidance by limited supercooling appears to be the main freezing resistance component that allows survival of *N. nitida* seedlings during winter. Freezing avoidance by supercooling is frequently observed during periods of rapid development and high metabolic activity (Levitt 1980, Sakai and Larcher 1987). We observed freezing avoidance by supercooling in adult plants of both species during spring and summer, which is when woody evergreen plants in south Chile experience most of their development and growth (Romero et al. 1987).

Our field and laboratory studies on freezing resistance were performed with detached twigs. Studies reported by Neuner et al. (1995) and Taschler et al. (2004) demonstrated that small but significant differences are obtained between frost resistance measurements determined in situ on attached and detached leaves. For example, in *Picea abies* (L.) Karst., incipient frost damage in detached leaves was 1.2 $\degree$C higher than for attached leaves. Even if this were the case with *Nothofagus* species, the differences we observed between IN and LT$_{50}$ would still be significant. Nonetheless, in situ freezing resistance studies would be preferable for measuring precise LT$_{50}$ values.

Accumulation of compatible solutes (e.g., soluble carbohydrates and proline) is a common response to low temperature stress in plants (Alberdi and Corcuera 1991, Gusta et al. 2004). Higher carbohydrate concentrations were found in seedlings and adults of *N. dombeyi* at the time of the highest freezing tolerance. In adults of *N. dombeyi* grown in the field, LT$_{50}$ and TSC were inversely correlated ($r = -0.80$). In adults of both species grown in the field, proline concentration was inversely correlated with cold acclimation ($r = -0.93$ and $-0.88$ for...
Figure 3. Seasonal course of metabolite accumulation in leaves of seedlings of *Nothofagus dombeyi* and *N. nitida* growing in a common garden in south central Chile and during cold acclimation in the laboratory. Total soluble carbohydrate (TSC) (A, D), starch (B, E) and proline (C, F) concentrations. For field conditions (A–C), values are the means of 10 replicates ± SE. For laboratory conditions (D–F), values are means of five replicates ± SE. Different lower case letters indicate statistically significant differences ($P \leq 0.05$) between the seasonal values for *N. dombeyi*. Different upper case letters indicate statistically significant differences ($P \leq 0.05$) between the seasonal values for *N. nitida*. Asterisks indicate statistically significant differences ($P \leq 0.05$) between species for each parameter in the same season.

Table 3. Seasonal changes in optimal photochemical efficiency ($F_v/F_m$), effective photosynthetic efficiency ($\phi$PSII), relative electron transport rate (ETR_r), photochemical quenching (qP) and non-photochemical quenching (NPQ) of leaves of *Nothofagus dombeyi* and *N. nitida* grown in a common garden in south central Chile. Values are means of 10 replicates ± SE and correspond to measurements performed in the morning (1000–1100 h). Different lower case letters indicate statistically significant differences ($P \leq 0.05$) between the seasonal values for the same species (columns). An asterisk indicates a statistically significant difference ($P \leq 0.05$) between species for each parameter ($F_v/F_m$, $\phi$PSII, ETR_r, qP and NPQ) in the same season.

<table>
<thead>
<tr>
<th>Species</th>
<th>Season</th>
<th>$F_v/F_m$</th>
<th>$\phi$PSII</th>
<th>ETR_r</th>
<th>qP</th>
<th>NPQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult plants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. dombeyi</em></td>
<td>Autumn</td>
<td>0.90 ± 0.006 a</td>
<td>0.22 ± 0.01 a*</td>
<td>53.3 ± 4.1 a*</td>
<td>0.32 ± 0.03 a*</td>
<td>5.15 ± 0.34 a*</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>0.86 ± 0.007 b</td>
<td>0.07 ± 0.001 b</td>
<td>18.1 ± 2.5 b</td>
<td>0.15 ± 0.02 b</td>
<td>3.52 ± 0.25 b</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>0.89 ± 0.01 ab</td>
<td>0.13 ± 0.01 c</td>
<td>31.1 ± 3.2 c</td>
<td>0.20 ± 0.03 b</td>
<td>3.40 ± 0.31 b</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>0.86 ± 0.01 b</td>
<td>0.13 ± 0.02 c</td>
<td>30.7 ± 2.0 c</td>
<td>0.28 ± 0.03 a</td>
<td>4.28 ± 0.24 b*</td>
</tr>
<tr>
<td><em>N. nitida</em></td>
<td>Autumn</td>
<td>0.91 ± 0.002 a</td>
<td>0.16 ± 0.02 a</td>
<td>36.8 ± 3.8 a</td>
<td>0.24 ± 0.02 a</td>
<td>3.83 ± 0.33 a</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>0.85 ± 0.02 b</td>
<td>0.09 ± 0.02 b</td>
<td>14.5 ± 2.4 b</td>
<td>0.15 ± 0.03 b</td>
<td>3.38 ± 0.17 a</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>0.89 ± 0.004 a</td>
<td>0.15 ± 0.02 a</td>
<td>29.1 ± 3.4 c</td>
<td>0.21 ± 0.03 a</td>
<td>3.30 ± 0.28 a</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>0.84 ± 0.02 b</td>
<td>0.16 ± 0.01 a</td>
<td>34.3 ± 3.5 ac</td>
<td>0.30 ± 0.03 c</td>
<td>3.01 ± 0.26 a</td>
</tr>
<tr>
<td>Seedlings</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. dombeyi</em></td>
<td>Autumn</td>
<td>0.83 ± 0.01 a</td>
<td>0.10 ± 0.01 a*</td>
<td>23.5 ± 2.1 a*</td>
<td>0.22 ± 0.02 a*</td>
<td>4.31 ± 0.31 a*</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>0.83 ± 0.01 a</td>
<td>0.23 ± 0.02 b*</td>
<td>56.7 ± 4.5 b*</td>
<td>0.50 ± 0.02 b*</td>
<td>3.68 ± 0.23 a</td>
</tr>
<tr>
<td><em>N. nitida</em></td>
<td>Autumn</td>
<td>0.84 ± 0.007 a</td>
<td>0.04 ± 0.006 a</td>
<td>8.1 ± 1.3 a</td>
<td>0.07 ± 0.009 a</td>
<td>1.91 ± 0.23 a</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>0.86 ± 0.01 a</td>
<td>0.08 ± 0.01 b</td>
<td>18.7 ± 3.2 b</td>
<td>0.14 ± 0.02 b</td>
<td>3.02 ± 0.32 b</td>
</tr>
</tbody>
</table>
Nothofagus dombeyi and N. nitida, respectively); however, this correlation was lower in seedlings grown under laboratory conditions ($r = -0.57$ and $-0.68$, N. dombeyi and N. nitida, respectively). In contrast, there was no significant correlation between FT and proline and TSC concentrations. This may be explained by either the small contribution of freezing point depression to the decrease in LT50 in these species, as indicated by the small variations in FT from summer to winter in the field and under laboratory conditions for N. dombeyi (about 1 °C) and N. nitida (0.2 °C), or by the non-colligative cryoprotective effects of sugars and proline on membrane stabilization (Strauss and Hauser 1986, Anchorduguy et al. 1987).

In adult plants, increased TSC accumulation in winter correlated with decreased starch concentrations in N. dombeyi and N. nitida ($r = -0.65$ and $-0.82$, respectively). This decrease could be explained by hydrolysis of starch at low temperature, as reported in leaves of some evergreens in the Northern Hemisphere (Biebl 1962, Boldingh et al. 2000). Accumulation of leaf carbohydrates in winter may also be explained by insufficient metabolic sinks. Low respiration and growth rates are reported to lead to inhibition of secondary processes of photosynthesis in evergreen sclerophyll leaves (Rhizopoulou et al. 1989). Starch concentrations were significantly higher in N. nitida than in N. dombeyi, except in summer ($P \leq 0.05$), suggesting perhaps that N. nitida has a greater constitutive ability to form starch than N. dombeyi. Field-grown seedlings of both species showed increased starch accumulation during winter, unlike the cold-acclimated plants in the laboratory. This difference may be a result of the 5-fold higher irradiance in the field compared with the growth chamber, favoring photosynthesis and supporting the accumulation of soluble sugars and starch. It is likely that soluble carbohydrate accumulation in seedlings under laboratory conditions relies partly on starch hydrolysis. Reserves and accumulation of sugars favor proline accumulation during cold acclimation in some plants, because of a decrease in proline oxidation (Stewart 1978), which could explain, at least partially, the higher accumulation of proline in N. dombeyi seedlings compared with N. nitida seedlings.

Chilean woody evergreens from the families Proteaceae, Myrtaceae and Podocarpaceae show increased leaf TSC in winter and during cold acclimation (Alberdi 1995). In addition to the cryoprotective activity of sugars, their accumulation in specific regions of the plant is also important because of their influence on freezing point depression, which could increase plant survival (Levitt 1980, Sakai and Larcher 1987, Livingston and Henson 1998, Larcher 2000, 2003, Li et al. 2002).

Comparing our results with those observed in leaves of native Nothofagus species of New Zealand, some similarities in low temperature response were found. New Zealand Nothofagus species showed moderate cold acclimation (1–6 °C) during winter, with LT50 values between $-2$ and $-15$ °C. This attribute is characteristic of freezing-tolerant species (Neuner and Bannister 1995). Leaves from adult trees of N. dombeyi and N. nitida under field conditions showed an acclimation capacity of 4.5 and 2.2 °C respectively, and their LT50 values ranged between $-4.2$ and $-14.3$ °C, depending on species, season and developmental stage. Thus, Nothofagus species from the Southern Hemisphere are characterized by similar freezing resistance behavior. In contrast to the evergreen N. solandri var. cliffortioides from New Zealand (Wardle and Campbell 1976), we observed no evidence that short photoperiod had an effect on cold acclimation of Chilean evergreen Nothofagus. Our result is consistent with reports that cold acclimation of Eucalyptus and other woody angiosperms of the Southern Hemisphere is insensitive to photoperiod (Sakai and Larcher 1987).

Thylakoid membranes of chloroplasts are primary sensors of environmental changes (Anderson et al. 1997) and are highly susceptible to low temperature. Hence, the photochemical process of photosynthesis is often the first to be affected by low temperatures (Krause and Weis 1991, Demmig-Adams and Adams 1992). In vivo fluorescence is a useful intrinsic indicator of thylakoid organization and changes in membrane fluidity (Terzaghi et al. 1989). Unlike the behavior of Mediterranean sclerophyllous leaves, where $Fv/Fm$ decreased from autumn (around 0.8) to winter (around 0.6), we found that $Fv/Fm$ was maintained around normal physiological values, between 0.83 and 0.9 ($P \leq 0.05$) during all seasons.

Larcher (2000) calculated a winter photooinactivation degree ($\Phi i$) in Mediterranean evergreen woody plants by the formula $\Phi i = 1 - (Fv/Fm)_{max}/(Fv/Fm)_{opt}$, where $Fv/Fm$ is the potential efficiency of PSII photochemistry in the actual state under winter conditions and $Fv/Fm_{opt}$ is the highest mean value in the optimal state during the most favorable season. He reported $\Phi i$ values ranging from 0.27 to 0.07. The calculated $\Phi i$ values of 0.07 and 0.04 for N. nitida and N. dombeyi, respectively, indicated that there was no significant inactivation of PSII in leaves of either species in winter. These low $\Phi i$ values are consistent with the milder climatic conditions in south central Chile compared with the Mediterranean climate (Di Castri and Hajek 1976, Sakai and Larcher 1987).

Effective $\phi$PSII, ETR, and qP of adult plants of both species were lower in winter than in the other seasons ($P \leq 0.05$), indicating that winter conditions decreased the photochemical efficiency of PSII and the photochemical process, as has been reported by others (Demmig-Adams and Adams 1992, Savitch et al. 2002, Larcher 2003). Thermal dissipation of excess energy (NPQ) was higher in N. dombeyi than in N. nitida, probably reflecting adaptation of these species to different light environments. It is well established that N. dombeyi is a shade-intolerant plant, whereas N. nitida is semi-shade-tolerant (Donoso and Lara 1998). All of the fluorescence parameters differed between N. nitida and N. dombeyi seedlings, consistent with the regeneration patterns of these species: N. dombeyi regenerates mainly in open spaces, whereas N. nitida regenerates in canopy-protected areas (Veblen and Schlegel 1982). Seedlings of N. dombeyi exhibited higher $\phi$PSII, ETR, and qP during winter than adults, which is probably related to a higher demand for photoassimilates in leaves of N. dombeyi seedlings in order to sustain growth during winter. This is a typical response of cold hardy plants that do not become dormant during winter (Savitch et al. 2002).

We concluded that the main freezing resistance mechanism for N. dombeyi was tolerance. Nothofagus nitida tolerated
freezing in autumn and winter only at the adult stage. Both species underwent cold acclimation, but *N. dombeyi* underwent greater acclimation than *N. nitida*. Accumulation of TSC and proline may contribute to the winter survival capacity of these species. The higher freezing resistance and performance of the photosynthetic apparatus of *N. dombeyi* in winter, especially at the seedling stage, likely contributed to the successful adaptation of this species as a pioneer in harsh and cold environments. Overall, seedling mortality caused by frost or excess light appears to determine the regeneration patterns of these species and therefore their geographical distribution.

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**References**


