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Physiological and morphological short-term responses to light and temperature in two *Nothofagus* species of Patagonia, South America

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Abstract

The study of plant responses to environmental stress factors is essential for management of plant systems and for anticipating their response to climate change. The main goal of this study was to determine morphological and physiological responses of *Nothofagus obliqua* and *N. nervosa* seedlings to light and temperature, two of the main stress factors acting in their current natural distribution in NW Patagonia. Responses to light were evaluated analyzing growth and survival, as well as morphological and physiological traits related to them, in seedlings subjected to three contrasting light conditions (full-sun conditions, 50% of sunlight and 20% of sunlight) during one growth season. Temperature photosynthetic responses were evaluated in seedlings subjected to temperature treatments between −5 and 40°C for 2 and 4 h. Growth rate and biomass partition were similar between light treatments in both species. High apical meristem damage and decreased photosynthetic capacity of preformed leaves were observed under full-sun conditions, suggesting that high light levels have a deleterious effect on plant yield. Both species produced neoformed leaves during the growing season with better photosynthetic capacity than preformed leaves under full sun conditions, contributing to plant acclimation. Almost no plasticity was observed in morphological traits in response to shade. Both species differed in optimum temperature for photosynthesis, with a wider temperature range at which high photosynthesis is maintained in *N. obliqua*. In both species the higher values of net photosynthetic rate were found at higher temperatures than the mean annual temperature of its current natural distribution range. Under no water-stress conditions, future higher temperatures could increase carbon fixation of these species, with a little advantage of *N. obliqua* if temperature variance is high. Synergy effect of various environmental stress factors, particularly considering cultivation of these species outside their current natural distribution sites require further studies.

Additional key words: light acclimation; neoformation; photoinhibition; preformation; thermal stress.

Introduction

Among the environmental factors affecting plant development, light and temperature are the most variable on a temporal scale; even during a single day plants are exposed to rapid environmental fluctuations in these factors, conditioning plant capacity to cope with such variability (Gaspar *et al.* 2002). Light is one of the most heterogeneous factors affecting plants, varying by more than three orders of magnitude both in time and

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Abbreviations: AB – aboveground dry biomass; AMD – apical meristem damage; D – diameter at stem base; E – leaf transpiration rate; ER – estimate ratio; F_0 – minimum fluorescence of the dark-adapted state; FC – field capacity; F_m – maximum fluorescence of the dark-adapted state; FS – full-sun light treatment; F_v/F_m – maximum quantum yield of PSII photochemistry; g_s – stomatal conductance; H – seedling height; INTA – National Institute for Agricultural Technology of Argentina; LCP – light compensation point; LRTs – likelihood ratio tests; MCMC – Markov Chain Monte Carlo Simulations; NL – number of leaves per plant; P_{max} – maximum net photosynthetic rate; P_N – net photosynthetic rate; PPFD – photosynthetic photon flux density; P_{sat} – net photosynthetic rate at saturating PPFD; Q – apparent quantum yield; R_D – dark respiration rate; RB – root dry biomass; RID – relative increase in diameter; RIH – relative increase in height; SH/R – shoot/root relationship; SLA – specific leaf area; T – pretreatment temperature; T_{op} – temperature in which P_{sat} is maximum; WUE – instantaneous water-use efficiency.

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space; light can be a limiting factor at low intensities and a stress factor at excessive levels (Valladares 2003, Gómez-Aparicio *et al.* 2006). During the life cycle of a woody plant, light environment may change dramatically as the plant grows taller (Coopman *et al.* 2008, 2011). To cope with this, plants may exhibit a remarkable ability to modify their physiology and morphology according to prevailing light conditions.

At the leaf level, adjustments are made in leaf longevity, morphology, biochemistry and photosynthetic capacity (Valladares and Niinemets 2008, Ishii and Ohsugi 2011). For species with determinate growth, leaf emergence may occur in a single, short flush from the preformed bud or in multiple flushes from neoformed buds during the growing season. In multiple flushing species, newly produced leaves can acclimate to abrupt changes in light environment during the growing season (Guedon *et al.* 2006, Ishii and Ohsugi 2011).

There is an evidence that preformed and neoformed leaves of the same shoot may differ in their developmental and functional attributes (Seleznyova and Greer 2001). Such dissimilarities may be explained by the fact that these two types of leaves differentiate and/or extend at different times (different leaf ages) and, therefore, under different environmental conditions. The contribution of each leaf type to plant future development and population productivity may indeed be different. Analysis of the variability of organogenesis and extension (two basic processes of primary growth) and its contribution to environment acclimation is needed to improve our understanding of adaptability in trees (Taugourdeau and Sabatier 2010).

On the other hand, human-induced climate change has the potential to alter the prevalence and severity of extreme climatic events such as heat waves, cold waves, storms, floods and droughts. The International Panel on Climate Change (IPCC 2008) assumes that changes in the relationship between mean temperature and standard deviation are of potential importance to climate change and suggests that in the future the frequency of extreme temperature events and their magnitude will increase (Rusticucci and Barrucand 2004, Mundo *et al.* 2010). Under this scenario, events of short scale duration but high intensity can be deleterious principally in juvenile and seedling stages of many tree species. Though predicting changes in these types of events under a changing climate is difficult, understanding vulnerabilities to such changes is a critical part of estimating vulnerabilities and future climate change impacts on the environment and vegetal species (IPCC 2008). This emphasizes the need for studies in order to understand the short-term morphological and physiological mechanisms in response to environmental factors (Valladares *et al.* 2002).

Climate of NW Patagonia is highly variable; plants are exposed to a high temporal and spatial unpredictability of environmental conditions that constraints survival, regeneration and productivity of vegetation systems in this region (Paruelo *et al.* 1998). Species of the genus *Nothofagus* (*Nothofagaceae*) represent about 80% of the tree component of Patagonian Andean forests (Veblen *et al.* 1996). In Argentina, this genus includes six species with high ecological and commercial importance. Two of these species are the deciduous *N. nervosa* (Phil.) Krassen and *N. obliqua* (Mirb.) Oerst., both included in breeding and domestication programs (Gallo *et al.* 2009). Their productive use in commercial plantations outside their natural distribution range is currently considered by technical, private and decision-maker sectors. Both for planting the species outside their natural distribution area as well as to predict their response to climatic variability, knowledge is needed about the species general responses to environmental variables with a particular interest in the early development phases (*e.g.* seedlings or juvenile stages).

Considering the ecophysiological available information (*see* below), there are some studies analyzing their environmental requirements in terms of water, light and temperature; however, several important questions are still open. Both species are tolerant to shade during juvenile stages and show the capacity of producing neoformed leaves within the growth season (Puntieri *et al.* 2007). Knowledge about the physiological differences related to light acclimation between preformed and neoformed leaves is scarce (Ishii and Ohsugi 2011) and null for Patagonian species.

Regarding their response to temperature, the available information suggests a more thermophilic habit of *N. nervosa* compared to *N. obliqua* (Alberdi 1987, Alberdi 1996, Weinberger and Ramirez 2001). Several studies have determined the resistance to low temperature of these and other *Nothofagus* species (*e.g.* Sakai *et al.* 1981, Alberdi 1987, Greer *et al.* 1989, Neuner and Bannister 1995), however no study has documented their photosynthetic capacity under high temperature or abrupt changes in temperature such as those predicted under the future climate scenarios for the region (IPCC 2008).

The objective of the study was to describe the physiological and morphological responses of *N. nervosa* and *N. obliqua* to different light and temperature conditions during the seedling stage. Whereas the mechanisms for rapid response in this stage are those that influence the further development and growth of plants (even more in deciduous species), we focused the study on this type of short term (within only one growing season) or instantaneous responses (after a few hours of treatment). In addition to analyzing different leaf and whole plant variables, we were particularly interested in the acclimation capacity to light environment of different leaf types. We hypothesized that light acclimation capacity of neoformed leaves is higher than that of preformed ones. In relation to temperature response the main goal of this study was to characterize the short-term responses to low as much high temperatures in both species in order to determinate differential susceptibility of both species.

Materials and methods

Light responses

Plants, experimental design, and environmental conditions: Ninety four-year-old seedlings of *N. nervosa* and *N. obliqua* were bred in 265 cm^3 containers under greenhouse conditions, using a fertigation regime (Azpilicueta *et al.* 2010) during the first year (2003–2004), and in 3-L pots during the subsequent years, without fertilization. The substrate was a sandy clay loam soil composed of 54% sand, 24% silt and 22% clay. During 2003 to 2007, the seedlings were kept under greenhouse conditions (20 ± 10°C; 200 to 700 μmol (photon) m^{-2} s^{-1} of photosynthetic photon flux density; 50 ± 20% of relative air humidity) and were periodically watered (2 to 3 times a week) during the growing season (November to April). In order to avoid the influence of initial plant size on responses to different light levels, we selected plants of fairly homogeneous sizes (approximately 30 cm of height and 3 mm of diameter) within each species prior to bud break (October 2007), moment at which seedlings were placed in the experimental area, outside the greenhouse. The experiment was performed at the Bariloche Experimental Station of INTA (National Institute for Agricultural Technology of Argentina; 41°08′00″S, 71°18′37″W; 893 m a.s.l.) from November 2007 to April 2008.

Three light treatments (50% and 80% of coverage and full sunlight called 50%, 80% and FS, respectively) were randomly assigned to nine 8 × 2.5 m plots (3 replicates per light treatment). Five seedlings per species were randomly assigned to each plot. Inside the plots, the seedlings were placed at a distance of 0.5 m between plants. In each plot, we used black commercial shade cloths (of 100% virgin high density polyethylene) to provide a 1.5 m high roof structure. Average values of midday photosynthetic photon flux density (PPFD) were 1,011 ± 743; 566 ± 366 and 313 ± 227 μmol m^{-2} s^{-1} for FS, 50% and 80%, respectively. Light measurements were performed in several (10) dates along the duration of the experiment with *LI-9901-013* quantum sensors (*LI-COR*, Lincoln, Nebraska, USA) under all the light treatments at the same time (between 9:00 and 12:00 h).

Soil moisture within the pots was kept close to field capacity (FC) by automated daily irrigation. Throughout the study pot volumetric soil water content was 37.2 ± 5.7% (v/v), *i.e.* always higher than the values of permanent wilting point (26.4%, v/v).

Morphology and biomass allocation: At the beginning and at the end of the 2007–2008 growing season we measured seedling height (H) and diameter at stem base (D) in order to calculate the relative increase (to the initial size) in height (RIH) and relative increase in diameter (RID).

In order to quantify the short-term variation in specific leaf area (SLA), three leaves of 5 to 7 plants per species and treatment were harvested weekly, scanned, oven-dried at 70ºC for 48 h and weighed. Leaf area of green leaves (prior to being oven-dried) was measured using the software *HOJA 1.0* (*Verga 2000*, Cordoba, Argentina). The removal of three leaves per date represented a small proportion (< 3%) of the seedling leaf area and did not cause any effect over meristem health. At the end of the growing season, the seedlings were harvested to measure the root (RB) and aboveground dry biomass (stem + leaves – AB) and the number of leaves per plant (NL). These records were used to establish the shoot/root relationship (SH/R). Percentages of seedling survival and apical meristem damage were calculated using binary measurements performed in April 2008 (end of the growing season). Number 1 was assigned to living plants, 0 to dead plants, and 1 was assigned to plants with any type of meristem damage and 0 to plants without visible damage. Percentages were calculated as the number of dead or damaged plants divided by the total number of seedlings, and then multiplied by 100. In all cases plant survival and damage of apical meristem were quantified observationally.

Physiological variables: Net photosynthetic rate at saturating PPFD (P_{sat}), stomatal conductance (g_s), and leaf transpiration rate (E) were measured using a *LI-6400* portable photosynthesis measuring system (*LI-COR*, Lincoln, NE, USA) with a *6400-02B LED* source. Measurements were performed weekly on one fully expanded apical or subapical leaf of 5 to 7 seedlings per species and treatment. Instantaneous water use efficiency (WUE) was calculated as P_{sat}/E, per date.

Net photosynthetic rate (P_N) *vs.* PPFD curves was plotted twice, at the beginning and the middle of the growing season, using at least 5 plants per species and treatment per date. These measurement periods were chosen in order to record the variation between different phenological leaves stages: preformed (November to December 2007) and neoformed leaves (December 2007 to April 2008). The classification of leaf types was assessed *via* morphological measurements (in neoformed leaves the relation between leaf length and width needs to be equal or higher to 3; J. Puntieri, CONICET-Univ. of Río Negro, Argentina, pers. com.). Light levels of P_N *vs.* PPFD curves were 0, 50, 100, 500, 750; 1,000; and 1,200 μmol(photon) m^{-2} s^{-1}. The atmosphere in the *LI-6400* chamber was maintained at 18°C, 60% relative humidity, with a CO_2 concentration of 400 ppm and a flow rate of 300 mL min^{-1}, using minimum times at each light level of 3 min. Measurements were performed between 9:00 and 12:00 h.

Additionally, we measured the modulated fluorescence of chlorophyll (Chl) *a* expressing the results as the quantum efficiency of open PSII (ratio of variable to maximum fluorescence; F_v/F_m; Maxwell and Johnson

2000). For this purpose, leaves of seedlings growing under different light treatments were measured weekly during November and December 2007 with a *LI-6400-40* fluorometer added to the equipment *LI-6400* (*LI-COR*, Lincoln, NE, USA). Prior to fluorescence measurements, 7 seedlings per species and treatment were subjected to darkness for a period of 30 min, and after applying a pulse of low-intensity light, we registered the minimum fluorescence yield (F_0). Then we applied a saturating high-intensity light pulse to induce the temporary closing of PSII, recording the maximum fluorescence yield (F_m). Maximum quantum yield of PSII (F_v/F_m) was then estimated. Measurements were recorded only during November and December (preformed leaves) due to posterior breakage of the fluorometer equipment.

Chl fluorescence parameters for the *LI-COR 6400-40* (*LI-COR*, NE, USA) were defined as follows and as suggested in the *LI-COR* manual 5 (2004). Minimum fluorescence of the dark-adapted state (F_0) was determined applying a weak modulated light [0.4 μmol(photon) m^{-2} s^{-1}] and maximum fluorescence of the dark-adapted state (F_m) was induced in dark-adapted leaves by a short pulse (0.8 s) of saturating light [around 8,000 μmol (photon) m^{-2} s^{-1}]. Measurements were performed between 9:00 and 12.00 h.

Data analyses: For the variables NL, RHI; RDI, RB, AB, and SH/R, we fitted linear mixed effect models:

$$y_{ijk} = \mu + Tr_i + Sp_j + (Sp \times Tr)_{ij} + e_{ij} + d_{ijk} \qquad (1)$$

where y_{ijk} is the value of the response variable for the kth seedling under the ith treatment from the jth species, μ is the overall mean of the response variable; Tr_i is the effect of the ith light treatment (i = 1, 2, 3); Sp_j is the effect of the jth species (j = 1, 2); e_{ij} is the experimental error and d_{ijk} is the sub sampling error.

For the response variables P_{sat}, g_s, E, WUE, F_v/F_m, and SLA we fitted linear mixed effect models for longitudinal data (same type of information on the same subjects at multiple points in time), assuming correlated random effects for intercepts and slopes, in order to account for the responses over time within subjects and for the time variation trends between subjects. The complete model was:

$$y_{ijklmt} = \mu + Tr_i + Sp_j + (Sp \times Tr)_{ij} + L_k + (\alpha + a_1) + (\beta + b_1) \times \mathrm{Time}_{ijkt} + e_{ijkl} + d_{ijklm} + s_{ijklmt} \qquad (2)$$

where y_{ijklmt} is the value of the response variable for the lth seedling under the ith light treatment for the jth species; μ = overall mean of the response variable; Tr_i is the effect of the ith light treatment (i = 1, 2, 3); Sp_j is the effect of the jth species (j = 1, 2); L is the effect of the leaf type (1, 2); α = random intercept for the effect of the ith seedling measured over time (Time), β = slopes for the effect of the ith seedling measured over time (Time); a_1 = average intercept, b_1= average slope; e_{ijkl} = principal error and d_{ijklm} = sampling error and s_{ijklmt} the time error.

We used the 'lmer' function in the 'lme4' package (Bates and Maechler 2010) with the REML algorithm, in R 2.10.1 (R Development Core Team 2009), for fitting all the mixed models. The significance of both the random and fixed effects was assessed *via* likelihood ratio tests (LRTs), testing the null hypotheses that simpler models fitted after removing the effect of interest from the Eq. 1 and Eq. 2 were sufficient. In order to test for the contribution of the fixed effects, we refitted the models using Maximum Likelihood instead of REML (Faraway 2006). For each variable, we report which of our fixed factors significantly contributed to the observed variation (*i.e.* the likelihood of the general model was higher than the likelihood of the corresponding restricted model; *e.g.* Table 1). For the response variables without a periodical measurements (*e.g.* RID, RIH), after selecting the most parsimonious model for each response variable, we fitted Markov Chain Monte Carlo Simulations (MCMC) samplings in order to asess the significance of the differences among the levels of the fixed effects at the end of the growing season. This was performed with the 'language R' package (Baayen 2010).

For seedling survival and meristem apex damage (binary responses), we performed an analysis of variance using the logit link function and full maximum likelihood estimation of the models' parameters with the Laplace approximation to the marginal likelihood, in the 'lme4' package.

We fitted a rectangular hyperbola for each data set, describing the response of P_N to PPFD (Landsberg 1977), for this purpose we used nonlinear regression in *Prism4* (*GraphPad*, San Diego, CA, USA):

$$P_N = P_{max} \times Q \times (PPFD - LCP)/[P_{max} + Q \times (PPFD - LCP)] \qquad (3)$$

where P_{max} – maximum net photosynthetic rate; Q – apparent quantum yield (slope of the first part of the curve); LCP – light compensation point, and PPFD – photosynthetic photon flux density (independent variable). The accuracy of the fitted parameters was examined *via* the ratios between the standard errors of estimate (SEE) and the best fitted values (Zar 1999). We used global fitting (Motulsky and Christopoulos 2004) to compare the fitted parameters of the Landsberg equations between the species, and between the light treatments within species. In each case, we report the evidence ratio (ER) in favor of the better model (*i.e.*, global *vs.* separate fitting to the data; Motulsky and Christopoulos 2004).

Response to temperature level

Plants and experimental design: The effect of temperature on carbon fixation was evaluated in two-year-old seedlings of each species using two growth chambers (*SAD-9000 CA, Consultar Group*, Rosario, Argentina). Prior to measurements, five seedlings of each species were assigned to one chamber, where they were maintained at 18ºC (close to the reported optimal temperature for other species of the genus; Zuñiga *et al.* 2006). Those measurements were used as a control for phenological effects during the whole measurement period. Other 45 seedlings of each species were used for all temperature treatments, assigning five plants per treatment. The temperature treatments were −5, 0, 5, 10, 25, 30, 35, or 40°C during 2-h periods. Additionally, for temperature values of 40 and 0°C, we also tried 4-h treatment in order to detect a combined effect of intensity and duration of the stress factor. Light and relative air humidity conditions in both chambers were kept at 500 µmol(photon) m^{-2} s^{-1} (using *VIALOX NAV-T* high pressure sodium vapor lamps, *OSRAM*, Munich, Germany) and 40%, respectively. All the seedlings were in 3-l pots and were irrigated daily to field capacity. Greenhouse conditions, prior to growth chamber experiments, were similar to those described in the light experiment.

Variables measured and data analysis: At the end of each temperature treatment, P_N was measured under dark conditions (dark respiration rate – R_D) and under a PPFD of 1,000 µmol(photon) m^{-2} s^{-1} using the *LI-6400* equipment with a *6400-02B LED* source in all 5 seedlings per species and treatment. We adjusted the conditions of the *LI-6400* chamber to a CO_2 concentration of 400 ppm, a flow of 300 ml min^{-1} and a temperature of 18°C, using a minimum measuring time of 3 min between 9:00 and 12.00 h.

Differences in R_D between seedlings of each species under 18°C treatment and for the different pretreatment temperatures were tested using one-way *ANOVA*.

The relationship between P_{sat} and T° was described with three types of models using a Bayesian approach:

(*1*) A common asymmetric logistic model (Eq. 4) applied to a pooled set of both species data

(*2*) Asymmetric logistic models (Eq. 4) applied to separate data sets for both species

$$\text{if } T < T_{op} : P_{sat} = \frac{4}{\left(e^{\frac{(T-T_{op})}{h_0^2}} + e^{\frac{-(T-T_{op})^2}{h_0^2}}\right)^2}$$

$$\text{if } T > T_{op} : P_{sat} = \frac{4}{\left(e^{\frac{(T-T_{op})}{h_1^2}} + e^{\frac{-(T-T_{op})^2}{h_1^2}}\right)^2} \qquad (4)$$

where T is the pretreatment temperature and T_{op} is the temperature in which P_{sat} is maximum, h is a scale factor of P_{sat}.

Models 1 and 2 were proposed because in Bayesian statistics there is no hypothesis testing, and to prove that the response of P_{sat} to T° of the two species differs, we proposed a model in which both species can be explained by a single curve, and another in which a different curve was fitted for each species. If the second model best represents the information contained in the data (Deviance Information Criteria; *see* below), then we say that both species models differ in their parameters.

(*3*) For model simplicity, the P_{sat} to T° relationship was also described using a three-stage "Broken stick" function (Eq. 5; piecewise linear regression model; Draper and Smith 1998). This type of model has been used for this purpose in previous studies (van Keulen and Seligman 1987, Peri *et al.* 2002, Caballé *et al.*, 2011), and allows the incorporation of structural breaks into the regression model. In this way, the determination of the optimal thermal range for P_{max} as the percentage of reduction of P_N outside of this range was estimated for each species. The optimal thermal range for photosynthesis was estimated as the range of temperatures in which the values of photosynthesis represent 90% of the mean maximum value of each species.

$$\begin{aligned}
&\text{if } T \le T_0 : P_{sat} = 0 && (5)\\
&\text{if } T > T_0 \wedge T \le T_1 : P_{sat} = P_m \times (T - T_0)/(T_1 - T_0)\\
&\text{if } T > T_1 \wedge T \le T_2 : P_{sat} = P_m\\
&\text{if } T > T_2 \wedge T \le T_3 : P_{sat} = P_m \times (T_3 - T)/(T_3 - T_2)\\
&\text{if } T > T_3 : P_{sat} = 0
\end{aligned}$$

where T is the pretreatment temperature; T_0, T_1, T_2 and T_3 are the temperatures at which the model generates the break points (see below) and P_m is the net photosynthetic rate in the optimal range of temperature (between T_1 and T_2).

Each species model was chosen on the basis of Deviance Information Criteria (Gelman *et al.* 2003). The parameters of the equations were calculated using MCMC methods, with normal likelihood between the observed and expected errors. The *a priori* distributions were noninformative positive half normal with $\sigma = 10$ for the parameters in which the variable is positive (such as the amplitude and the scale parameters of the asymmetric logistic models) and noninformative normal with $\sigma = 10$ for the others. The first 100,000 iterations were always treated as burn in (Gelman *et al.* 2003) and the parameters were calculated using the last 500,000. All the MCMC simulations were performed using the pymc library for Bayesian estimation (Patil *et al.* 2010) for the Python programming language (Python Software Foundation 2009). The convergence diagnostics were performed by inspecting plotted traces and histograms of the observed MCMC sample, together with Geweke's, and Raftery's methods (Fonnesbeck *et al.* 2008).

Results

Response to light level

Morphology and biomass allocation: At the end of the growing season, the number of leaves (NL), the absolute and relative increases in RIH and RID, RB and AB were similar between seedlings of each species under different light treatments (Table 1).

Average RIH was 2.11 ± 2.08 and 2.65 ± 2.79 cm for *N. nervosa* and *N. obliqua*, respectively. Averge RID was 1.43 ± 0.64 and 2.00 ± 0.87 mm for *N. nervosa* and *N. obliqua*, respectively.

SH/R was similar between all the treatments of *N. obliqua,* while for *N. nervosa* the FS treatment showed higher values than the 80% treatment; 50% treatment was similar to FS and 80% treatment (0.693; analysis based on MCMC). The light treatment had an interaction with the species; under the 80% treatment, *N. obliqua* showed higher SH/R values than *N. nervosa*, an opposite pattern than that observed at 50% and FS treatments.

Seedling survival was 100% in all treatments; however, the percentage of apex meristem damage (apex mortality) followed the order: FS > 80% > 50% for both species (Table 1; p=0.0002).

Specific leaf area (SLA) was similar in the different treatments within species (Table 2) along all the growing season (preformed and neoformed leaves).

At the interspecific level, *N. nervosa* showed a higher RIH than *N. obliqua* independently of the light treatment, while NL, RID, RB, and AB showed the opposite pattern (Table 1). Additionally, *N. nervosa* had a higher average SLA than *N. obliqua* (Table 2, Fig. 1) independently of the considered leaf type.

Gas exchange: P_{sat} of each species was similar within species considering the different light treatments. However a trend of lower values of P_{sat} in preformed leaves under the FS treatments was observed, mainly in *N. nervosa*. Comparing both species, seedlings of *N. obliqua* showed higher P_{sat} values than *N. nervosa* (Table 2, Fig. 1) independently of the considered treatment. In addition, within each species, neoformed leaves showed higher P_{sat} than preformed ones (Table 2, Fig. 1). F_v/F_m of preformed leaves differed between the different light

Table 1. Seedling morphological and allometric variables at the end of light experiment with *Nothofagus nervosa* and *N. obliqua*. Number of leaves (NL ± SD; n = 15), relative increase in height (RIH ± SD; n = 15), relative increase in diameter (RID ± SD; n = 15), root biomass (RB ± SD; n = 15), aerial biomass (AB ± SD; n = 15) and apical meristem damage (AMD) Significant estimates of the parameters per species (Sp), treatment (Tr) and their interaction (Sp × Tr) in leaves of *N. nervosa* and *N. obliqua* grown under different light treatments (FS – full sun conditions, and 50% and 80% of coverage). *N. n – N. nervosa*; *N. o – N. obliqua*; ns –statistically nonsignificant.

Species/Significance	Tr	NL	RIH	RID	RB [g]	AB [g]	AMD [%]
N. nervosa	50%	21 ± 8	0.12 ± 0.10	0.78 ± 0.41	2.07 ± 1.61	1.32 ± 0.99	0
	80%	18 ± 10	0.15 ± 0.19	0.62 ± 0.40	2.58 ± 1.41	1.18 ± 0.31	7
	FS	26 ± 10	0.08 ± 0.09	0.52 ± 0.31	1.15 ± 0.40	1.10 ± 0.34	53
Sp Avergae		22 ± 10	0.12 ± 0.14	0.64 ± 0.38	1.90 ± 1.33	1.20 ± 1.33	20
N. obliqua	50%	86 ± 29	0.10 ± 0.08	0.89 ± 0.41	6.11 ± 2.57	2.79 ± 0.99	7
	80%	62 ± 18	0.04 ± 0.06	0.59 ± 0.18	4.98 ± 2.59	2.66 ± 1.15	20
	FS	75 ± 24	0.06 ± 0.07	0.95 ± 0.56	4.44 ± 1.24	2.98 ± 0.36	33
Sp Avergae		74 ± 26	0.07 ± 0.07	0.81 ± 0.43	5.19 ± 2.30	2.80 ± 2.30	21
Sp sig.		*N.o > N.n*	*N.n > N.o*	*N.o > N.n*	*N.o > N.n*	*N.o > N.n*	ns
Tr sig.		ns	ns	ns	ns	ns	FS > 80% = 50%
Sp × Tr sig.		ns	ns	ns	ns	ns	ns

Table 2. Significance of the fixed factors species (Sp), treatment (Tr), Sp × Tr interaction and leaf type. We report the degree of freedom (DF) of the likelihood ratio test, the χ^2 value and the associated p-values for the response variables net photosynthetic rate at saturating PPFD (P_{sat}); stomatal conductance (g_s); transpiration rate (E); instantaneous water-use efficiency (WUE) and specific leaf area (SLA); ns –statistically nonsignificant.

Factor	DF test	P_{sat}		g_s		E		WUE		SLA	
		χ^2	p	χ^2	p	chi^2	p	χ^2	p	χ^2	p
Sp	3	8.6824	0.0338	2.4509	ns	1.6263	ns	18.857	< 0.001	10.865	0.0125
Tr	4	4.4145	ns	6.6698	ns	4.6195	ns	30.727	< 0.001	7.2753	ns
Sp × Tr	2	3.1234	ns	2.2131	ns	1.4436	ns	17.013	< 0.001	1.2436	ns
Leaf type	1	4.0305	0.0477	1.9743	ns	2.1655	ns	0.4014	ns	0.1673	ns

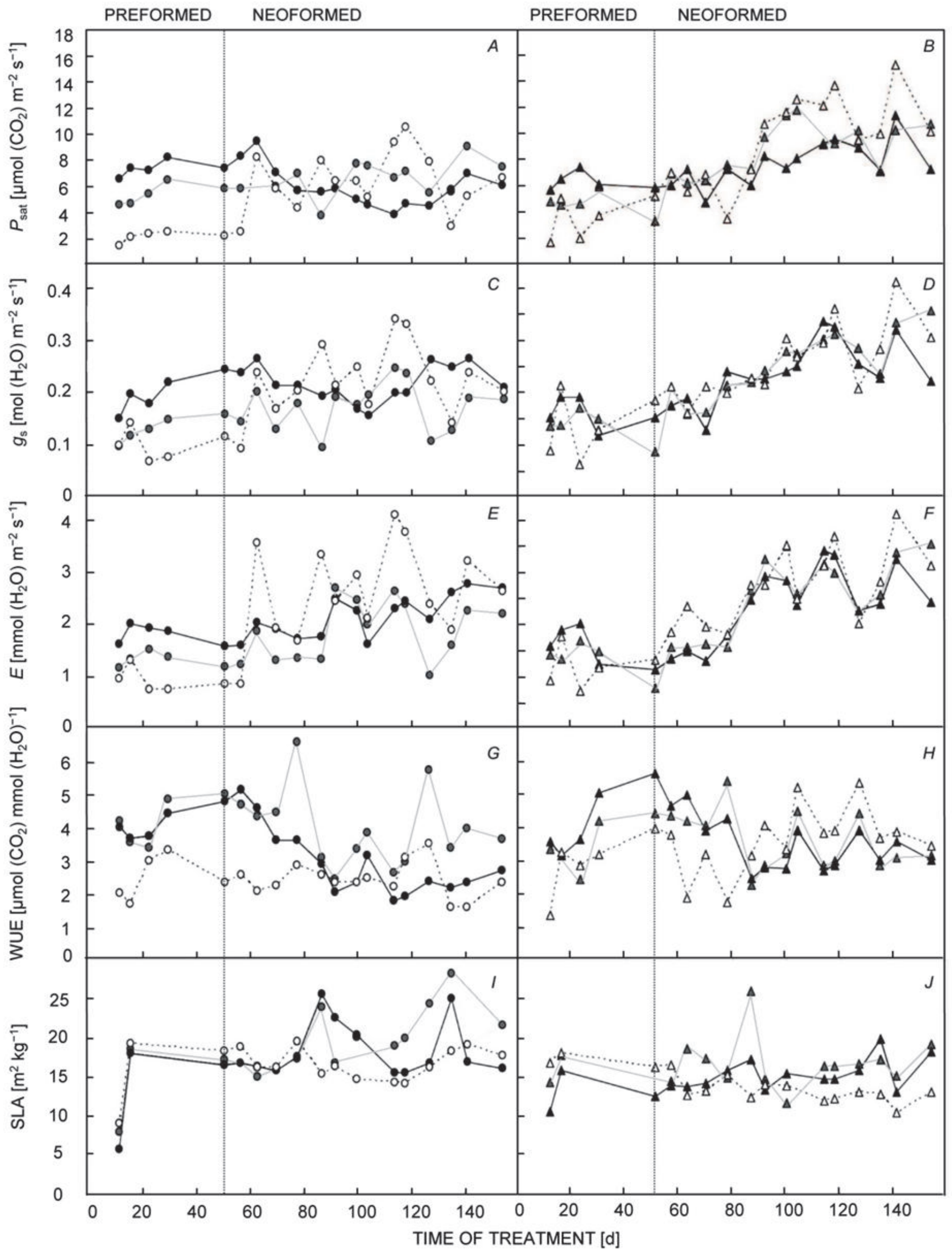

Fig. 1. Mean physiological parameters (P_N, g_s, E, and WUE; *A–H*) and SLA (*I,J*) for *N. nervosa* (*A,C,E,G,I*) and *N. obliqua* (*B,D,F,H,J*) throughout the growing season per species and treatments (n = 7). *Vertical dotted line* in the graph shows the division between preformed and neoformed leaves. Gray lines in the graphs show the values for seedlings under 50% treatment, the black lines show the values for seedlings under 80%; the dotted lines show the values for seedlings under FS.

treatments in both species, following the order 80% > 50% > FS with average values of 0.74; 0.70 and 0.53 respectively ($p_{50,\,80}$=0.0468; $p_{50,\,\mathrm{FS}}$= 0.0002).

g_s and E did not differ between treatments, species or leaf type (Table 2, Fig. 1). WUE presented an interaction effect between species and treatment (Table 2). Seedlings under 50% and 80% treatments showed higher WUE than in FS treatment in both species (Table 2). However, seedlings of *N. obliqua* showed higher WUE than *N. nervosa* in 80% and FS treatments, whereas the opposite pattern (WUE of *N. nervosa* > WUE of *N. obliqua*) was observed in 50% treatment (Table 2; Fig. 1).

Comparing the results between treatments within each species, preformed leaves (October measurements) showed different P_N *vs*. PPFD curves basically due to differences in P_{max} or a combined effect of P_{max} and Q (Table 3). The whole models were different between each pair of treatments combination in *N. nervosa* (Table 3;

Table 3. Parameters (SE and standard error of the estimate) of the fitted Landsberg model (net photosynthetic rate-PPFD; P_{max}, Q, LCP) in preformed (Oct-07) and neoformed (Jan-08) leaves of *N. nervosa* and *N. obliqua* grown under full sun (FS) and 50% and 80% of coverage (n = 7 per species, treatment and date of register). *Capital letters* show significant differences between species within each treatment. *Lowercase letters* show differences between treatments within species.

			Radiation treatment		
Date/Type of leave	Species	Parameter/Goodness of Fit	FS	50%	80%
Oct-07 Preformed leaves	*N. nervosa*	P_{max} [μmol(CO$_2$) m^{-2} s^{-1}]	2.2 ± 0.7^{cA}	4.9 ± 0.5^{bA}	6.3 ± 0.2^{aA}
		Q [μmol(CO$_2$) μmol(photon)$^{-1}$]	0.014 ± 0.016^{aA}	0.039 ± 0.017^{aA}	0.047 ± 0.006^{aA}
		LCP [μmol(photon)]	14 ± 32^{aA}	16 ± 10^{aA}	15 ± 3^{aA}
		R^2	0.34 (1.39)	0.97 (0.47)	0.71 (1.46)
	N. obliqua	P_{max} [μmol(CO$_2$) m^{-2} s^{-1}]	7.2 ± 5.9^{aA}	6.5 ± 0.8^{bA}	6.7 ± 0.6^{bA}
		Q [μmol(CO$_2$) μmol(photon)$^{-1}$]	0.005 ± 0.003^{bA}	0.032 ± 0.015^{aA}	0.050 ± 0.018^{aA}
		LCP [μmol(photon)]	23 ± 84^{aA}	14 ± 13^{aA}	11 ± 8^{aA}
		R^2	0.59 (1.36)	0.81 (1.38)	0.84 (1.29)
Jan-08 Neoformed leaves	*N. nervosa*	P_{max} [μmol (CO$_2$) m^{-2} s^{-1}]	9.1 ± 1.1^{aA}	4.2 ± 0.3^{bB}	6.7 ± 0.8^{aA}
		Q [μmol(CO$_2$) μmol(photon)$^{-1}$]	0.062 ± 0.024^{aA}	0.055 ± 0.019^{aB}	0.087 ± 0.047^{aA}
		LCP [μmol(photon)]	23 ± 12^{aA}	9 ± 7^{aA}	6 ± 10^{aA}
		R^2	0.67 (2.68)	0.67 (1.09)	0.55 (2.34)
	N. obliqua	P_{max} [μmol(CO$_2$) m^{-2} s^{-1}]	6.9 ± 1.2^{aA}	8.6 ± 1.9^{aA}	6.5 ± 0.6^{aA}
		Q [μmol(CO$_2$) μmol(photon)$^{-1}$]	0.046 ± 0.027^{aA}	0.059 ± 0.046^{aA}	0.075 ± 0.026^{aA}
		LCP [μmol(photon)]	23 ± 17^{aA}	13 ± 22^{aA}	5 ± 7^{aA}
		R^2	0.48 (3.00)	0.36 (4.43)	0.71 (1.61)

Table 4. Estimated parameters of the Model 1: Common asymmetric logistic model (Common Asym. Logstc.); Model 2: Asymmetric logistic (Asym. Logstc.) model per species and Model 3: piecewise linear regression model (Broken stick) and DIC values. T_{op} – temperature in which P_{sat} is maximum; T_0, T_1, T_2, T_3 – temperatures in which the Model 3 generates the brake points. n = 5 per treatment.

Model	Amplitude	Estimated deviance	T_{op}	T_0	T_1	T_2	T_3	DIC
Common Asym. Logstc.	10.37 ± 0.63	6.84 ± 1.08	22.97 ± 1.56	-	-	-	-	417.88
N. nervosa Asym. Logstc.	12.53 ± 0.70	4.81 ± 1.21	24.77 ± 1.39	-	-	-	-	400.65
N. obliqua Asym. Logstc.	8.68 ± 0.80	6.67 ± 1.48	20.82 ± 2.15	-	-	-	-	
N. nervosa Broken stick	12.48 ± 0.80	4.45 ± 0.87	-	–8.46 ± 2.15	23.12 ± 2.10	26.04 ± 1.46	45.15 ± 1.98	398.33
N. obliqua Broken stick	7.74 ± 0.81	5.66 ± 0.91	-	–9.43 ± 3.69	18.54 ± 2.82	24.08 ± 3.50	18.54 ± 2.82	

$ER_{50,\ FS}$= 569.51; $ER_{80,\ FS}$ = tending to infinity; $ER_{50,\ 80}$= 1.59). Seedlings of *N. obliqua* showed similar models for 50% and 80% treatments ($ER_{50,\ 80}$= 25.66; Table 3) and both were different to the FS model ($ER_{FS,\ 50}$= 411.68 and $ER_{FS,\ 80}$= tending to infinity). In the first comparison (50% *vs*. FS), differences between models were due to differences in Q. In the second case, differences between models were due to differences in P_{max} (Table 3). Finally, within each light treatment, no differences were observed between P_N *vs*. PPFD models of preformed leaves of both species (Table 3).

In contrast to preformed leaves results, P_N *vs*. PPFD models for neoformed leaves were similar between all light treatments in *N. obliqua* (ER= 164.76; Table 3), but differed between treatments in *N. nervosa* (ER= 8,265.09; Table 3). Differences were observed between 50% and 80% models and between 50% and FS models ($ER_{50,\ 80}$= 113.11; $ER_{50,\ FS}$= tending to infinity), and similar models were observed between 80% and FS treatments ($ER_{80,\ FS}$ = 3.14). Differences between *N. nervosa* models were due to differences in P_{max} ($ER_{80,\ 50}$= 2.82; $ER_{FS,\ 50}$= 2.9). Plants growing in the 50% treatment had lower values of P_{max} than plants in the 80% and FS treatments (Table 3). Within each light treatment, both species models of neoformed leaves differed only in 50% treatment (ER = 8.16). The observed differences were due to an interaction between the parameters P_{max} and Q. Models of seedlings growing in 80% and FS treatments were similar between both species (ER_{80} = 34.07; ER_{FS} = 3.19).

Finally, the comparison of the P_N *vs*. PPFD curves of preformed and neoformed leaves of each species and treatment showed that the curves of seedlings under 50% and 80% coverage, independently of the considered species, did not differ. Contrarily, curves of seedlings

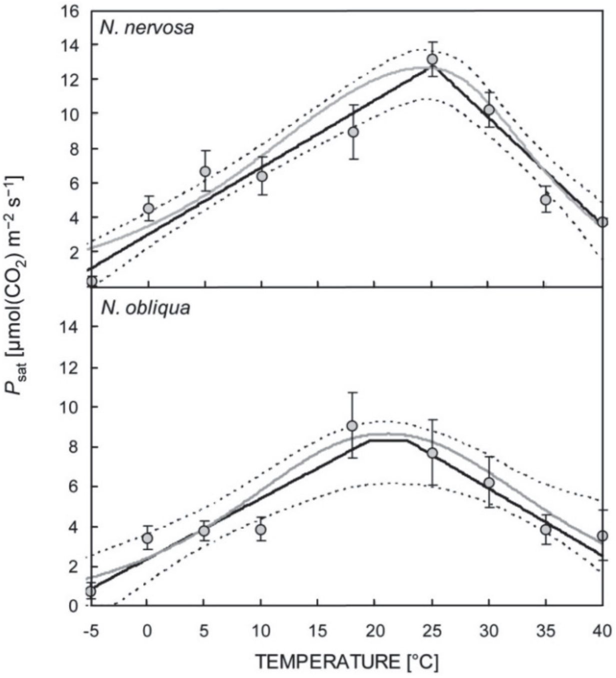

Fig. 2 Net photosynthetic rate at saturating PPFD (P_{sat}) in relation to pretreatment temperature (2 h) for *N. nervosa* and *N. obliqua*. The gray full lines show the asymmetric logistic model fits. The full black lines show the "Broken stick" models fits and the dotted lines show the respective 0.25 and 0.975 Bayesian interval of confidence. The circles in the graphs shows the mean average value of five measurements per species (n = 5) and error bars show the SE.

under FS treatment do differ (*N. nervosa* ER – tending to infinity; *N. obliqua* ER – 10.48). *N. nervosa* showed higher values of P_{max} (ER – tending to infinity) and Q (ER – tending to infinity) in neoformed than in preformed leaves and similar values of LCP. Differences in *N. obliqua* were due to an interaction between the parameters P_{max}, Q and LCP.

Response to temperature: The data described by the Model 1 (common asymmetric logistic model; Table 4) present a higher DIC value than Model 2 (different asymmetric logistic models for each species; Table 4). For this reason Model 2 was preferred in order to analyze the response of P_{sat} to temperature.

The temperature at which P_{sat} achieved the mean maximum value estimated using Model 2 (T_{opt} = optimal temperature) was 24.8 ± 1.4°C for *N. nervosa* and 20.8 ± 2.1°C for *N. obliqua* (Table 4; Fig. 2). The "broken stick" model described a plateau (optimal temperature range) between 23.1 ± 2.1 and 26.0 ± 1.5°C for *N. nervosa* and between 18.5 ± 2.8 and 24.1 ± 3.5ºC for *N. obliqua.* Then, P_{sat} of *N. nervosa* decreased by an average of 0.40 ± 0.05 µmol(CO$_2$) m^{-2} s^{-1} ºC^{-1} from 23.1 to –8.4 ± 2.1ºC and at a rate of 0.67 ± 0.16 µmol(CO$_2$) m^{-2} s^{-1} ºC^{-1} from 26.0ºC to 45.1ºC. In the case of *N. obliqua*, P_N decreased at a rate of 0.29 ± 0.07 µmol(CO$_2$) m^{-2} s^{-1} ºC^{-1} from 18.5 to −9.4 ± 3.7ºC and 0.28 µmol(CO$_2$) m^{-2} s^{-1} ºC^{-1} from 24.1 ºC to 56.0ºC (Fig. 2). Broken-stick model presented a lower DIC than Model 2 (Table 4).

R_D was similar throughout the whole temperature range for both species, ranging from −0.6 to −1.6 µmol(CO$_2$) m^{-2} s^{-1} for *N. nervosa* and from −0.5 to −1.6 µmol(CO$_2$) m^{-2} s^{-1} for *N. obliqua*.

Discussion

Response to light: For both species seedlings grown under different light treatments showed similar height, diameter increase and biomass allocation. This quite surprising result, particularly considering the high differences in light levels between the most contrasting treatments, may be explained at least in part by a lower carbon fixation in seedlings under FS treatments during the first part of the growing season. Additionally, comparing seedlings under 50% and 80% treatments we found a trend to higher values of Q in 80% seedlings, suggesting a potentiality of higher carbon fixation rate in seedlings under lower light treatment. In addition, seedlings under FS treatment may allocate a proportion of the fixed carbon to other sinks than root or stem growth. In this regard, carbon can be used for photoprotection compounds production (Demmig-Adams and Adams 1992) or lost in respiration. These carbon sinks need to be quantified in future studies.

In spite of the similarities in biomass accumulation, under FS conditions leaves showed photoinhibition and damage on its photosynthetic apparatus and lower values of WUE. The high apical meristem damage recorded at FS and 50% treatment (Table 1) indicates the existence of a deleterious effect of light producing a loss of apical dominance, in agreement with results reported by Davel *et al.* (2003) and Peyrou (2002). Additionally, in *N. nervosa* species the higher SH:R ratio observed in FS seedlings could indicate that these plants can support a higher leaf area than plants in shaded treatments, even with a cost of higher photoinhibition. This point could be explained at least in part because plants had a suitable water supply during the entire experiment, and thus there was not a signal to increase root biomass. Additionally, this lack of plasticity in biomass allocation patterns may simply result from short acclimation time and to low extent from investment into photoprotective mechanisms.

SLA of preformed and neoformed leaves of both species was similar under different light treatments (Table 2), which contrasts to that measured in several studies showing high plasticity in this trait (*e.g.* Valladares *et al.* 2002). These studies showed a negative relationship between SLA and light level, allowing plants to increase light capture and preventing photoinhibition damage under high light levels (*e.g.* Le Roux *et al.* 2001).

The shade tolerance of *N. obliqua* has been previously mentioned in the literature (Rosenfeld 1972). In addition, it has been reported that this species can suffer photoinhibition when is planted under conditions of full sunlight (Peyrou 2002, Azpilicueta *et al.* 2010). The latter author reported 100% mortality of one-year-old seedlings under full-sun conditions. Those results differ with ours probably due to differential vulnerability to high light at different plant ages.

Contrary to the general trends given by biomass allocation models, our results suggest that the studied species have no plasticity in allocation patterns in response to light reduction, in agreement with studies conducted in *N. obliqua* and *N. dombeyi* (Dezzoti 2008).

Regarding physiological variables, P_{sat} of preformed leaves showed a trend to higher reduction under FS treatment compared to 50% and 80%, accompanied by PSII damage (fluorescence values below those corresponding to excellent healthiness *sensu* Mohammed *et al.* 2003; $F_v/F_m = 0.83$ to 0.76). The average values of F_v/F_m of 0.74 in 80% treatment and 0.70 in 50% treatment could be related with photoprotective processes (Osmond *et al.* 1999). This reduction in photosynthetic capacity during the beginning of the growing season could lead to a reduction in carbon fixation, compared to the potential based on the light availability under FS conditions, resulting probably in similar rates of C fixation under different light environments. Notwithstanding, it is interesting to note that P_{sat} of neoformed leaves indicates a potential recovery of photosynthetic capacity in both species under the FS treatment (Table 2; p=0.0447). This result suggests an acclimation capacity to high irradiance achieved as the growing season progresses, through the development of new leaves that probably have a greater resistance to photoinhibition than preformed leaves. Particularly for *N. nervosa*, reductions of P_{sat} in FS treatment at the end of the growing season (Fig. 2*A*) could be due to nutrient retranslocation (in agreement with Diehl *et al.* 2003). This differential acclimation capacity has been reported for other species with preformed and neoformed leaves (Ishii and Ohsugi 2011), including another species of *Nothofagus* genus (*N. nitida,* Coopman *et al.* 2008).

Although leaf turnover was not quantified in this study, recurrent leaf fall and quickly replacement by new leaves was observed in FS in both species. A high leaf turnover along the growing season could serve as a mechanism of acclimation to light stress conditions (Guedon *et al.* 2006), but also implies an energetic cost with a probably effect on root or stem biomass production. Moreover, this acclimation mechanism might be reflected in similar P_{max} recorded in neoformed leaves of both species under different light treatments at the middle of the growing season. Seedlings under FS treatment reached similar or even higher P_{max} values than seedlings under 50% and 80% treatments (Table 3), opposite to what was observed in preformed leaves. Particularly for *N. nervosa*, seedlings under 80% treatment could have higher values of P_{max} than seedlings under 50% treament due to light requirements of this species.

Similar results of acclimation to high irradiance were found by Valladares *et al.* (2002) for *Quercus robur* and the previously mentioned study of Coopman *et al.* (2008) in *N. nitida*. Our results suggest a lower physiological and morphological plasticity in both species in relation to low-light conditions and a physiologically based acclimation capacity under high-light conditions due to leaf neoformation process (in agreement with Ishii and Ohsugi 2011). More studies are needed to elucidate the relative contribution of morphological *vs*. physiological plasticity acclimation in response to different environmental cues and the importance of neoformation process in this response.

Response to temperature: The optimum temperature estimated by both the Asymmetric logistic model and the Broken stick model (optimum range), showed a mean value of 25°C for *N. nervosa* and 21°C for *N. obliqua* that agreed with Kohout and Read (2006) study on the same species. On the contrary, Weinberger and Ramirez (2001) found an optimum temperature for photosynthesis at 16°C for *N. nervosa* and 19°C for *N. obliqua.* Differences between studies can be attributed to measurements done on different ontogenetic stages and/or different moments within the growing season.

Mean annual temperature under natural current distribution for both species varies between 5 to 9°C (Gaitan *et al.* 2011, Sabatier *et al.* 2011). Under no water-stress conditions, future higher temperatures could increase carbon fixation of these species, with a little advantage of *N. obliqua* if temperature variance is high.

Comparing species, *N. obliqua* showed a wider temperature range at which P_{sat} was close to the maximum value (90% of P_{max}) respect to *N. nervosa* ("Broken stick" model, Fig. 2). This suggests a higher capacity of *N. obliqua* to cope with changes in temperature. Additionally, *N. nervosa* showed a greater reduction rate in P_{sat} both at high and low temperatures compared to *N. obliqua*.

Notwithstanding, considering the absolute values of P_{sat}, *N. nervosa* presented a higher carbon fixation capacity than *N. obliqua,* contrarily to what was observed in the previous experiment (light experiment). This result is important for understanding the opposite patterns found in different studies discussed in the first paragraph of this section. In addition, if phenological related trends in P_N values are different between species, then this kind of experiment should be repeated at different moments in the growing season. However, at least considering the whole period of measurements (one month), we did not find any phenological effect on control plants suggesting that *N. obliqua* seedlings had a reduction in its carbon fixation rate.

Both species showed high and similar photosynthetic susceptibility to freezing temperatures (−5°C). Under this temperature mortality of all leaves was registered (data not shown). Similar results have been reported by Greer *et al.* (1989) for *N. solandri*. However, Alberdi (1996) and Weinberger and Ramirez (2001) concluded that *N. nervosa* leaves are easily damaged during freezing events, and their repair is unusual, being this species more susceptible to freezing temperatures than *N. obliqua*.

From a methodological point of view, this study can be useful to highlight the capabilities of applying different model types (asymmetric logistic model *vs.* "broken stick" model) to evaluate the relationship P_{sat} *vs.* T°. Broken-stick model showed a lower DIC (Table 4), thus suggesting a "better description" of data behavior. Despite its simplicity, this model would serve to describe differences in optimum temperature values, as well as in the range of temperatures at which P_N is maintained around a maximum level. This important information is not clearly provided with a symmetric or an asymmetric logistic model; therefore broken-stick models could represent a valuable tool to describe the response of photosynthesis to temperature taking into account biological meaningful parameters.

The values of dark respiration (R_D) were similar for all temperature treatments. It is important to note that R_D was measured (as in the case of P_{sat}) at 19 ± 1°C after treating plants with different temperatures. Therefore, R_D probably reflects the current temperature effect with a very low influence of the previous thermal condition. In this regard it is interesting to note the differential influence of premeasurement temperature on P_{sat} and R_D. This would have important implications for the net carbon fixation capacity of plants, which requires specific modeling to quantify the magnitude of this differential effect. It would also be interesting to asess the influence of different exposure times to different temperatures over the photosynthetic rate.

Conclusion: Results of the light experiment suggest that both species can grow and survive under a wide range of light conditions, despite having low morphological and physiological plasticity in relation to low light, with high and low apical meristem damage under full sun and intermediate light condition, respectively. Leaf neoformation promotes an acclimation process in response to high light during the growing season, but even when total growth is similar than under shade conditions, high apex mortality suggests that this FS condition has to be avoided if the plant form is a part of the desirable product. These results may be useful to guide management of natural regeneration, as well as for planting these species.

If mean temperature does increase in the context of global change, an increase in average levels of CO_2 fixation of both species may be expected. Additionally, *N. obliqua* may probably have an advantage over *N. nervosa* in relation to varying temperature conditions (higher variance). However, these results must be treated with caution, particularly for predicting the performance of both species outside their natural distributional range. Information presented in this article allows us to better understand the response of both species to environmental stress factors when they act separately, which is important to know as a first step in ecophysiological species characterization. However, stress factors act together in nature; therefore it is important to consider the synergy between them. In this regard, expected combinations are water stress and high light or water stress and low light (dry shadow *sensu* Valladares *et al.* 2004). Similarly, high temperatures combined with drought conditions (low precipitation) could have a detrimental effect on photosynthesis of the studied species. More studies are needed about these particular topics, trying also to identify, if possible, genotypes with a better performance under single or multiple stress conditions.

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