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Morpho-physiological responses of *Nothofagus obliqua* to light intensity and water status, with focus on primary growth dynamics

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Abstract

Key message **Intra-individual variations of plant responses to environmental conditions are related to axis differentiation. Plastic responses include variations in primary growth, shoot allometry, leaves' chlorophyll content and water use efficiency.**

Abstract Primary growth affects plant plasticity by influencing the number, spatial arrangement and time of differentiation of the leaves. For *Nothofagus obliqua*, we evaluated if primary shoot growth dynamics determines short-term responses to environmental conditions, and intra-individual variations in such responses. Before bud-break, saplings were located in experimental conditions, combining two light treatments with two water supply treatments. For each individual, morphology, relative extension rate (RER), and relative leaf chlorophyll content (SPAD units) were registered for the annual shoot derived from the most distal bud of two axis categories: the main axis (trunk) and a main branch. For trunk shoots, we analysed the net photosynthetic rate at saturating photosynthetic photon flux density (P_{sat}) of proximal and distal leaves. Shaded trees had higher RER, longer and more slender shoots, longer internodes, and higher leaf chlorophyll content than unshaded trees. The number of nodes was affected by water condition in trunk shoots, and by light condition in main branch shoots. Full sunlight stimulated the development of a branch following the length and growth direction of its parent shoot (relay branch). Shade would have promoted the development of neoformed leaves. Under shade and low water availability, water use efficiency was higher in distal than proximal leaves. Under high sunlight and water availability, leaves of relay branches presented slightly higher P_{sat} with higher water loss than both proximal preformed leaves and distal leaves under the other tested conditions. This study highlights the need of assessing intra-individual variations in shoot acclimation in relation to axis differentiation.

Keywords Acclimation · Annual shoots · Axis differentiation · Plasticity · Primary growth

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Introduction

The capacity of organisms to tune their phenotypes to changing environmental conditions is widely recognised (Matesanz et al. 2010; Nicotra et al. 2010). The plastic responses

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of plants to environmental conditions involve architectural, morphological and/or physiological traits (Fisher and Hibbs 1982; Van Hees 1997; Valladares et al. 2002). Such responses have usually been studied by analysing the morphological and/or physiological attributes of different structural units, such as leaves, metamers, shoots, and axes (Uemura et al. 2000; Delagrange et al. 2004; Valladares and Niinemets 2008). Woody plants most often consist of axes that may be categorised according to their morphological and functional features (e.g. Puntieri et al. 2000, 2002, 2007; Souza et al. 2000; Sabatier and Barthélémy 1999, 2001; Stecconi et al. 2010; Charles-Dominique et al. 2012). Thus, within the same plant, long axes and short axes may co-exist; the former may be described as less differentiated and express an earlier ontogenetic stage than the latter (Barthélémy and Caraglio 2007). Among the attributes characterising different axes within a plant are extension dynamics and growth phenology, both of which may be related to leaf physiology (Ishii and Ohsugi 2011; Varela et al. 2012; Morales et al. 2014). As a consequence of axis differentiation, morphological and/or physiological responses to environmental pressures could exhibit intra-individual variations, which deserve further investigations (Fisher and Hibbs 1982; Kawecki 2008; Banta et al. 2012; Osada et al. 2014; Valladares et al. 2014).

The primary growth of the structural units making up plant axes, known as annual shoots, is linked with the times of leaf differentiation and expansion, and contributes to the spatial arrangement of leaves and the morphological and physiological adjustments of a plant to environmental conditions (Sabatier et al. 2003; Taugordeau and Sabatier 2010; Morales et al. 2014; Magnin et al. 2014). In many species, the annual shoots extended in one growing season are mostly composed of organs which have spent a period as primordia in buds, and are thus referred to as *preformed organs* (Gordon et al. 2006; Barthélémy and Caraglio 2007). Among temperate species, organ differentiation in buds often occurs between late summer and early autumn (Souza et al. 2000; Puntieri et al. 2002; García et al. 2006; Taugordeau and Sabatier 2010). In these species, shoot development in one growing season would depend on environmental conditions both in the current as well as in the previous growing seasons (Kimura et al. 1998; Uemura et al. 2000; Taugordeau and Sabatier 2010; Alla et al. 2013). After spring bud-break, the development of organs additional to those that were preformed in the winter buds has been shown to take place in many cases. This addition of organs may occur either by the break of buds initiated after preformation extension (i.e. recurrent bud-break, as in vigorous shoots of *Quercus* spp.; Heuret et al. 2003), or by the simultaneous differentiation and extension of organs after the extension of preformed organs (i.e. neoformation, as in vigorous shoots of

Nothofagus spp.; Guédon et al. 2006; Barthélémy and Caraglio 2007).

For temperate trees in which annual shoots consist exclusively of preformed organs, it has been shown that shoot morphology, as well gross morphological traits of the leaves, are affected by previous-year irradiance conditions, whereas acclimation to current-year light environment includes biochemical and physiological features (Kimura et al. 1998; Uemura et al. 2000). Therefore, recurrent bud-break and neoformation may be considered morphogenetic expressions that improve plant plasticity due to environmental conditions during shoot extension (Guédon et al. 2006; Barthélémy and Caraglio 2007; Magnin et al. 2016). As both differentiation and extension of these additional organs take place during the same growing season, higher levels of acclimation would be expected in these organs compared to preformed organs (Varela et al. 2012). In plants that are able to develop organs additional to those that were preformed, it may be assumed that proximal leaves within a shoot are highly differentiated before bud-break, whereas distal leaves differentiate partially or totally during the current growing season. Then, the higher the number of leaves of an annual shoot, the more contrasting the acclimation capabilities of proximal and distal leaves. Since growth dynamics is one of the traits that contribute to the definition of axis categories within a species (Puntieri et al. 1998; Sabatier and Barthélémy 2001), the evaluation of this topic may help in understanding plasticity at the plant level.

The performance of plants of the same species under different light conditions, particularly at early ontogenetic stages, is closely linked to the aptitude of these plants for surviving and growing in forest understories. Plant responses to light conditions have been broadly assessed for a number of species (see Valladares and Niinemets 2008 for a review). In tree species, intra-individual variations of such responses should be expected, since through the crown there are shoots and leaves subject to contrasting micro-environmental conditions, resource allocation and morphogenetic gradients. In support to this idea, for adult trees of *Fagus crenata*, it has been observed that the relationship between nitrogen content per leaf area and light availability varies among shoots at different position within the crown (Osada et al. 2014). Nevertheless, intra-individual variations in the responses of trees to different light conditions have been little investigated in connection with axis differentiation (e.g. Charles-Dominique et al. 2012; Puntieri et al. 2013). In addition, environmental variables such as water availability and/or temperature should be considered when assessing plant responses to different light conditions. Climate models for Patagonia predict a trend towards decreasing water inputs (Bates et al. 2008), with more frequent and/or intense drought periods, which may have negative impacts on the survival, regeneration

and productivity of some vegetation systems in this region (Suarez et al. 2004).

Nothofagus obliqua (Mirb.) Oerst. is a deciduous tree species native to temperate forests of north Patagonia and is highly valued because of its wood quality. This species has been considered a moderately shade-tolerant or even shade-intolerant tree (Donoso et al. 2006). However, the morphological responses recorded for this species under low light conditions (Varela et al. 2012; Puntieri et al. 2013) are not those typically associated with low shade tolerance (e.g. longer internodes, slender stems). For this and for closely-related species, different axis categories have been described in adult trees. The least vigorous shoots in these species consist exclusively of preformed organs, whereas vigorous shoots may include additional organs, i.e. issue of neoformation or recurrent bud-breaks (Puntieri et al. 2006, 2007). In the present study we evaluated, for *N. obliqua*, the plastic short-term responses of two major axis categories to environmental conditions after bud-break. Moreover, we assessed the relationship between morphological and physiological responses by analysing the link between the extent of leaf production per shoot and physiological traits of proximal leaves and distal leaves for plants under different environmental conditions. The following hypotheses were tested: (1) the morphogenetic expressions associated with axis differentiation affect the aptitude of different axes to respond to variations in environmental conditions by modifying annual shoot growth and morphology as well as leaf functioning; (2) the number of leaves produced by an annual shoot is related to the physiological differentiation between distal leaves and proximal leaves, and affects the annual shoot's capacity to acclimate to environmental conditions.

Materials and methods

Experimental design

Five-year-old saplings (mean basal diameter = 0.83 cm, SD = 0.09; mean height = 86.6 cm, SD = 9.1), produced at a nursery of *Dirección de Bosques de la Provincia de Río Negro*, located at San Carlos de Bariloche city, Argentina (41°07′36″S, 71°25′31″W, 825 m a.s.l.), were utilised. These saplings had been obtained from seeds collected in natural forests at Lanín National Park (40°09′S, 71°23′–71°34′W), Argentina, and were grown under more than 70% canopy openness and daily irrigation during spring and summer. Before bud-break in late winter (August 2014), 216 saplings were transferred to a nearby nursery (40°09′S, 71°23′–71°34′W), and arranged in a 30-m^2 area divided into 12 plots of 1.2 m × 1.2 m. Three plots, each one consisting of 18 randomly selected plants, were assigned to each of the following treatments: (1) high sunlight and high water supply (hereafter L+W+), (2) high sunlight and low water supply (L+W−), (3) shade and high water supply (L−W+), and (4) shade and low water supply (L−W−). For the shading treatments, a black commercial shade cloth (known as "80% shade cloth") was utilised. Photosynthetically active radiation (PAR) was measured for both light treatments using a quantum lineal sensor (ceptometer BAR-RAD DUAL USB, Cavadevices®, Buenos Aires, Argentina). Regarding water availability, plots corresponding to W+ received 20 litres of water per week (= 16.7 mm rain) between September and November, and 40 litres of water per week (= 33.3 mm rain) between December and April. In each of these periods, natural rain was 22.5 and 3.1 mm/week, respectively (*Subsecretaría de Recursos Hídricos de la Nación*, Argentina). Plants under W− condition received no water supply between September and November, and 15 litres of water per week (= 12.5 mm rain) between December and April, because of the extremely low natural rain during the latter period. Soil volumetric water content (VWC) and air temperature, relative humidity and vapour pressure deficit (VPD) for each treatment during the experiment are provided as Figs. S1 and S2 in the Supplementary material.

Primary growth dynamics

For each plant, we selected the apical or sub-apical bud of the trunk (i.e. the main axis with vertical growth; hereafter *trunk*) and the apical or sub-apical bud of one main branch (i.e. one of the largest branches arising from distal nodes of a trunk shoot; hereafter *MB*). The selected MB of each individual derived from the distal portion of the trunk, so intra-individual self-shading effects were considered negligible. After bud-break, weekly measurements of the shoots derived from the selected buds were performed: shoot length, number of nodes, apex condition (persistent or dead) and the occurrence of intra-annual branching were recorded. Regarding intra-annual branching, the distinction was made between branches that followed the length growth direction of their parent shoot, i.e. relay branches, and branches that developed in lateral directions. Whenever a relay branch was produced, we considered the annual shoot as composed by two growth units and measurements included both units. At the end of the growing season, we registered the basal diameter of each shoot, which was measured just above the scars left by the bud scales, with digital callipers (precision = 0.1 mm).

Leaf chlorophyll content and physiological measures

For each individual, weekly nondestructive measurements of the chlorophyll (chl) content were taken using a portable chlorophyll meter (Clorofilio, Cavadevices®, Bs. As., Argentina)

on three leaves located in proximal to intermediate position (thus avoiding partially unfolded leaves) of the trunk shoot and the MB shoot. This chlorophyll meter registers the transmittances of infrared light (940 nm) and visible light within the range of use of chl (660 nm), and, through the ratio of both transmittances, provides an arbitrary index (SPAD units) proportional to the amount of chl in the leaf; no estimation of the absolute chl content is provided by this technique (Richardson et al. 2002; Coste et al. 2010). For adult trees of *N. obliqua*, we found a high correlation between SPAD units and the chl content per unit area obtained by extraction with acetone/tris buffer (80:20 vol:vol, pH = 7.8; Sims and Gamon 2002; Fig S3 in the Supplementary material). Chlorophyll content is closely related to leaf nitrogen and phosphorous content, so it allows a rapid assessment leaf biochemical status (Niinemets 1997; Koike et al. 2004; Bojović and Sojanović 2005).

In late November 2014 (3 months after transferring the plants to the experimental conditions), net photosynthetic rate (P_N) vs. PAR curves were obtained for a proximal (thus preformed) leaf of three plants per treatment, taking one plant per plot, using an infrared gas analyser (IRGA, Li-Cor 6400; Li-Cor, Lincoln, Nebraska, USA) with a 6400-02B LED source. Light levels were 0, 50, 100, 500, 750, 1000 and 1500 μmol photon m^{-2} s^{-1} based on Varela et al. (2010, 2012). Air conditions within the IRGA's light chamber were maintained at 24 °C, 28% relative humidity, 400 ppm CO_2 concentration and 500 mL min^{-1} flow rate; exposure time to each light condition was between 120 and 180 s according to stability level. Measurements were performed between 9:00 and 12:00 h. At the end of the growing season (mid-March, 2015), the potential photosynthetic capacity of proximal and distal leaves was assessed for six saplings of each treatment (two per plot). From the trunk shoot of each of these saplings, we selected one of the three most proximal leaves and one of the three most distal ones, avoiding partially necrotic leaves. Whenever the selected shoot presented a relay branch, the distal leaf was selected from such a portion of the annual shoot. After setting the IRGA at saturating PPFD (1000 μmol m^{-2} s^{-1}, based on the previously performed P_N vs. PAR curves), net photosynthetic rate at saturating light (P_{sat}; μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s; mol H_2O m^{-2} s^{-1}) and leaf transpiration rate (E; mmol H_2O m^{-2} s^{-1}) were measured for each leaf. Instantaneous water use efficiency (WUE; μmol CO_2 mmol H_2O^{-1}) was calculated as the ratio between P_{sat} and E.

Data analysis

For each selected shoot the weekly relative extension rate (RER) was calculated as:

$$RER = \frac{\log(L2) - \log(L1)}{t2 - t1} \times 7,$$

where $L1$ and $L2$ are shoot lengths at times $t1$ and $t2$, respectively. Mean RER (RERm), maximum RER (RERmax) and duration of the growth period were computed for each treatment. For each shoot, RERm was calculated by averaging all RER values above zero. Statistical comparisons of RERm among treatments were performed separately for two periods: early, from mid-September to mid-November, and late, from late November to late February. RER for the early period reflected the extension of preformed organs, and RER for the late period could reflect either the extension of neoformed organs or that of relay branches. Also, at each date during the growing season, RER was compared among shoots of different treatments. In addition to relative extension rates, at the end of the growing season, the following variables of shoot morphology were compared among treatments for each axis type: length, number of nodes, basal diameter, and the ratios length/number of nodes (i.e. mean internode length), length/basal diameter (stem slenderness) and basal diameter/number of nodes (i.e. the basal stem conduction surface per leaf). In each comparison, a mixed-effects model (GLMM) was applied, assuming normal data distribution, and considering two fixed factors (light and water supply) and one random factor (plot). Differences between axis categories regarding growth rates, shoot morphology and allometry have been shown in a previous studies on *N. obliqua* (Puntieri et al. 2003; Torres et al. 2009) and were obvious in the present case; thus, so as highlight variations of equivalent axis categories under contrasting conditions, statistical analyses regarding shoot growth were performed independently for trunk shoots and MB shoots.

The degree of differentiation between trunk and MB was compared among treatments by means of the following ratios: trunk shoot length/MB shoot length, number of nodes of trunk shoot/number of nodes of MB shoot, and trunk shoot basal diameter/MB shoot basal diameter. Each ratio was compared among treatments with a two-way mixed-effect model as described above, including plot as random factor. Logarithmic transformations of variables were applied whenever necessary so as to comply with the normal distribution of residuals and/or homoscedasticity.

To analyse variations regarding the probability of production of intra-annual relay branch and lateral branches, a mixed model was applied including three fixed factors (light, water supply and axis category) and two random factors (plot and individual), assuming binomial error distribution with a log-link function in the package lme4 (Bates et al. 2009) of R software (R Development Core Team 2014).

Chlorophyll content of proximal leaves was compared between treatments and axis categories. Within the growing season, statistical analyses were performed separately for three different periods: $t1$ (from bud-break to early November), $t2$ (from early November to mid-January) and $t3$ (from mid-January to the end of April). Such a division was based

on the variations observed regarding RERm throughout the growing season. The mean chl content per plant and axis within each of these periods were analysed by means of a mixed-effects model (GLMM), assuming normal error distribution and considering three fixed factors (light, water supply and axis category), and three random factors (date of measure, plot, and individual).

To assess saturating values of PPDF for preformed leaves of plants of each treatment, P_N vs. PAR curves were fitted to a rectangular hyperbola (Landsberg 1977) by means of a non-linear regression in *Prism4* (*GraphPad*, San Diego, CA, USA):

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

where P_{max} is the maximum net photosynthetic rate, Q is the apparent quantum yield, LCP is the light compensation point and PPFD is the photosynthetic photon flux density. The accuracy of the fitted parameters was examined via the ratios between the standard errors of estimated and best-fitted values (Zar 1999). We used global fitting (Motulsky and Christopoulos 2004) to compare the fitted P_{max} parameter between treatments. Whenever differences among treatments were found, we reported the evidence ratio (ER) in favour of the better model (i.e., global vs. separate fitting to the data; Motulsky and Christopoulos 2004). Values of Q and LCP were not compared among treatments since the used intervals of PPFD were too wide to allow precise estimations of these parameters.

The values of P_{sat}, g_s, E and WUE measured at the end of the growing season were compared between proximal and distal leaves of trunk shoots by means of non-parametric Wilcoxon paired tests. Comparisons among treatments were analysed separately for proximal and distal leaves by applying Kruskal–Wallis tests (Sokal and Rohlf 1981).

For those lineal models in which normal error distribution was assumed, analysis of variance was applied so as to assess the effects of each fixed factor and/or the interaction between each pair of factors, considering a 5% significance level. Whenever binomial error distribution was assumed, model fitting was performed by means of Akaike's information criteria (AIC), and chi-squared test of sequential deviance was applied to evaluate significant improvements of verisimilitude associated with the inclusion of each fixed factor (and their interactions) with respect to the null model (Zuur et al. 2009).

Results

Experimental conditions

Mean PAR for plants under L+ and L− treatments was, respectively, 730 (standard error, SE = 109.1) and 183.5 (SE = 36.5) $\mu mol\ m^{-2}\ s^{-1}$; i.e. 76% mean PAR reduction from L+ to L−. Except for 1 week in early December, soil VWC of W− treatments between late October and mid-February was close to or below the permanent wilting point (PWP; Supplementary material 1). From December to March, VPD at midday was higher than 1.5 KPa. Soil VWC was mostly above 10%, which, in *N. obliqua*, would be a threshold below which plant water status is significantly reduced (Varela 2010). On the other hand, for W+ treatments, soil VWC was mostly above the PWP. The VPD at midday was slightly higher in L+ than in L− treatments from December to March (Supplementary material 2).

Primary growth dynamics

Significant variations in RERm were caused by different light conditions but not by different water supplies (Fig. 1). During the first period of the growing season, the RERm of trunk shoots and MB shoots were significantly higher for L− plants than for L+ plants. Light had no effect on the RERm for the second period of the growing season. Regarding the effect of light, these results are consistent with differences in RERm between treatments for each weakly measure for both axes (Fig. 1). For trunk shoots, W+ plants had, compared to W− plants, a higher RERm in the third measure and a slightly lower RERm in the following measure. For MB shoots a slightly significant interaction between light and water availability was observed for two summer measures; in late December, L−W+ plants presented higher RER than L+W+ plants, and the opposite trend was observed in late January. Regarding RERmax, higher values were observed for L− than for L+ plants, whereas neither water supply (ws) nor the light × water supply interaction affected this variable: trunk shoots: $F_{light} = 8.8$, $p = 0.02$; $F_{ws} = 2.1$, $p = 0.19$; $F_{light \times ws} = 0.4$; $p = 0.53$; MB shoots: $F_{light} = 11.4$, $p = 0.01$; $F_{ws} = 0.0$, $p = 0.85$; $F_{light \times ws} = 0.3$; $p = 0.59$. The period of trunk shoot extension was, on average, slightly longer for L+ plants ($\bar{x} = 14.7$ weeks) than for L− plants ($\bar{x} = 13.9$ weeks; $F_{light} = 5.4$, $p = 0.04$), but was not affected by water supply or by the light × water supply interaction ($F_{ws} = 0.3$, $p = 0.61$, $F_{light \times ws} = 0.6$; $p = 0.47$). Similarly, for MB shoots, the period of extension was longer for L+ plants ($\bar{x} = 14.3$ weeks) than for L− plants ($\bar{x} = 12.8$ weeks; $F_{light} = 19.6$, $p < 0.01$) and similar for plants under different water supplies ($F_{ws} = 1.2$, $p = 0.30$; $F_{light \times ws} = 1.1$; $p = 0.32$).

Shoot structure

Trunk shoots were notably longer for L− plants than for L+ plants, whereas MB shoots were similar in length for both light conditions (Table 1; Fig. 2a). Shoot length did not vary between water supply treatments, neither for trunks nor for MBs (Table 1; Fig. 2a). The number of nodes composing

Fig. 1 Mean relative extension rate per week (RERm ± SE) through the growing season for trunk and MB shoots of each treatment. Statistical analyses (Fisher's *F* and its significance, *p* value) of the effects of environmental factors upon mean RER for each axis category at two periods of the growing season are presented. Symbols above error bars indicate, for each date, significant differences between light treatments (*), water supply treatments (×) and their interaction (X). Three symbols $p < 0.001$; two symbols $p < 0.01$; one symbol $p < 0.05$. L+W+: high sunlight and high water supply; L+W−: high sunlight and low water supply; L−W+; shade and high water supply; L−W−; shade and low water supply
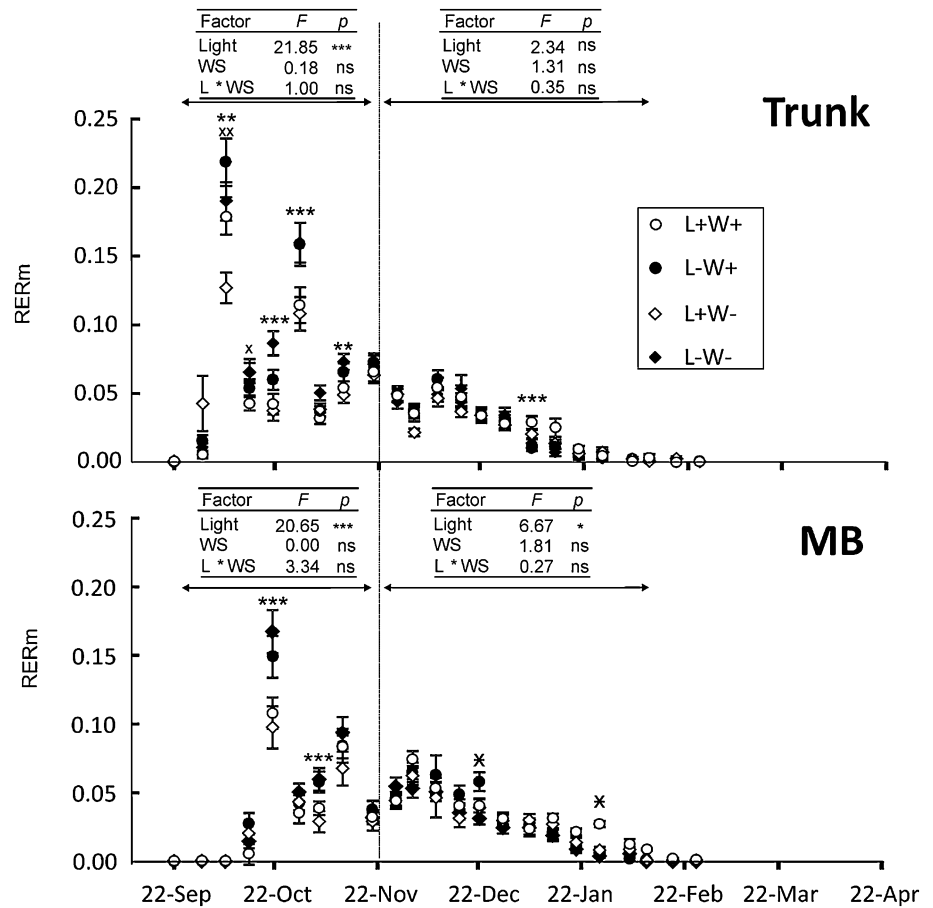

Table 1 Statistical analyses (Fisher's *F* and its significance, *p* value) for the effects of light availability (L), water supply (WS) and their interaction (L×WS) on morphological variables of trunk shoots and main branch (MB) shoots of *Nothofagus obliqua* plants

Factor	Length[a]		N° nodes		IN length[a]		ø[a]		Length/ø[a]		ø/N° nodes[a]	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Trunk												
Light	19.9	**	1.5	ns	48.7	***	0.3	ns	23.8	**	7.3	*
Water supply	2.3	ns	5.5	*	2.6	ns	2.7	ns	0.3	ns	2.2	ns
L×WS	0.7	ns	0.1	ns	0.7	ns	0.3	ns	1.4	ns	0.4	ns
MB												
Light	0.6	ns	12.9	**	7.6	*	3.1	ns	2.0	ns	16.7	**
Water supply	0.6	ns	1.4	ns	0.1	ns	1.1	ns	0.2	ns	0.8	ns
L×WS	0.0	ns	0.0	ns	0.0	ns	1.3	ns	0.2	ns	1.6	ns

N° nodes number of nodes, *IN length* mean internode length, *ø* basal stem diameter, *ns* not significant

***$p < 0.001$; **$p < 0.01$; *$p < 0.05$

[a]log-transformed variables for statistical analyses

trunk shoots was affected by water supply (W+ > W−), but not by light condition; the reversal was true for the number of nodes of MB shoots (L+ > L−; Table 1; Fig. 2b). Mean internode length of trunk and MB shoots did not vary with water supply, and was higher for L− plants than for L+ plants, especially in the case of trunk shoots (Table 1; Fig. 2c). Basal diameter was not affected by light or water supply (Table 1; Fig. 2d). Stem slenderness of trunk shoots

was higher for L− plants than for L+ plants (Table 1; Fig. 2e). The shoot diameter/number of nodes ratio was higher for L− plants than for L+ plants, more notably so for MB shoots (Table 1; Fig. 2f). There was no significant interaction between light and water supply for any morphological variables of either trunk or MB shoots.

The ratio between trunk shoot length and MB shoot length tended to be higher in L− plants than in L+ plants

Fig. 2 Average (±95% confidence interval) values of morphological variables for trunk and MB shoots of plants under different experimental conditions. **a** length, **b** number of nodes, **c** mean length of internodes, **d** basal diameter, **e** length/basal diameter ratio and **f** basal diameter/number of nodes ratio. Capital letters indicate statistical differences among trunk shoots of different treatments, and lowercase letters those among MB shoots ($p < 0.05$, two-way ANOVA; see Table 1). Treatment codes as in Fig. 1
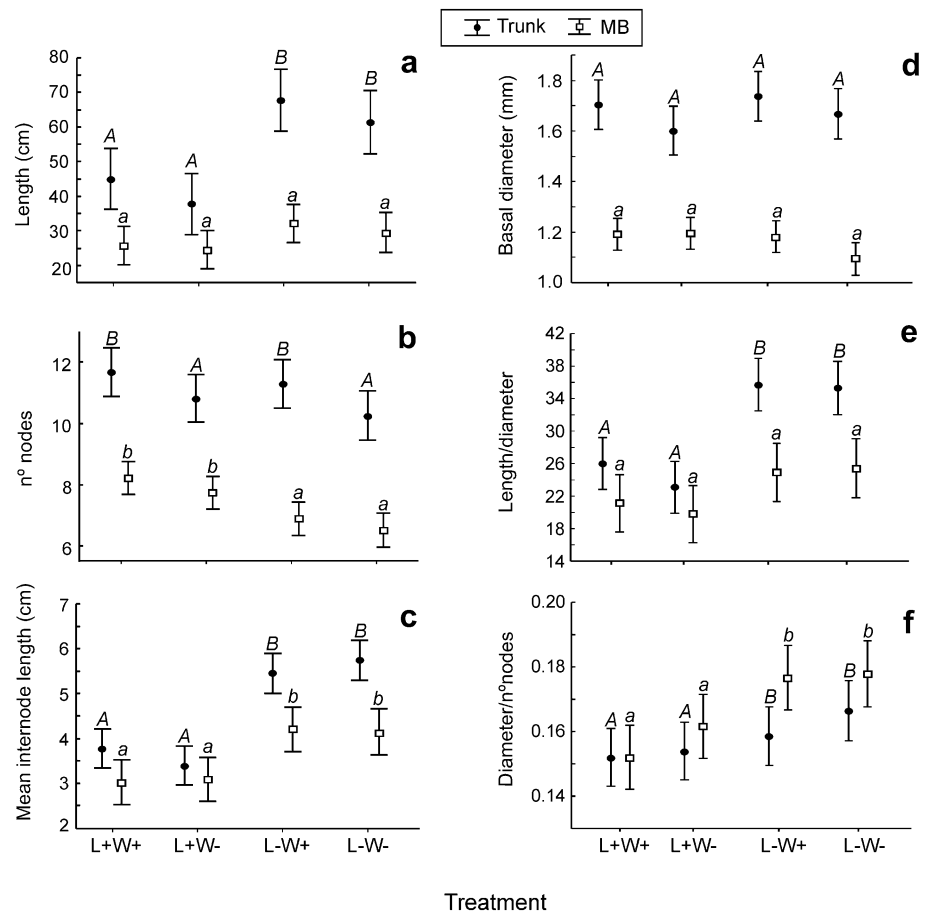
($F_{light} = 8.0$, $p = 0.02$), but was not affected by water supply or by the interaction between both factors ($F_{ws} = 0.02$, $p = 0.89$; $F_{light \times ws} = 0.87$, $p = 0.38$). Similar results were found regarding the ratio between the number of nodes of trunk shoots and MB shoots ($F_{light} = 8.0$, $p = 0.02$; $F_{ws} = 0.03$, $p = 0.86$; $F_{light \times ws} = 0.00$, $p = 0.95$). Conversely, the ratio of basal diameter between trunk shoot and MB shoot did not vary in response to light or water supply ($F_{light} = 3.3$, $p = 0.11$; $F_{ws} = 0.1$, $p = 0.79$; $F_{light \times ws} = 1.7$, $p = 0.23$).

Relay branches were more frequent in shoots of L+ plants than in those of L− plants, especially in the trunk (Tables 2, 3). A significant interaction among all three fixed factors was found (light × water supply × axis category), as W+ increased the occurrence of relay branches only in MB shoots of L+ plants (Tables 2, 3). The frequency of lateral branch production was higher in trunk shoots than in MB shoots. The production of lateral branches was higher in L+ plants than in L− plants, but was not affected by water supply or by the interaction between light condition and water supply (Tables 2, 3).

Table 2 Residual deviance analyses comparing binomial models for the probabilities of relay branch and lateral branch production, including light and water supply treatments and axis category

Probability		df	AIC	Residual deviance	p
Relay branch	Null model	3	363.5	357.5	
	Light	4	353.3	345.3	***
	Water supply	5	355.3	345.3	ns
	L × WS	6	357.1	345.1	ns
	Axis	7	333.7	319.7	***
	L × WS × Axis	10	317.4	297.4	***
Lateral branch	Null model	3	273.1	267.1	
	Light	4	368.8	260.8	*
	Water supply	5	270.7	260.7	ns
	L × WS	6	272.5	260.5	ns
	Axis	7	160.1	146.1	***
	L × WS × Axis	10	174.6	154.6	ns

df degrees of freedom, *AIC* Akaike's information criterion, *ns* not significant

***$p < 0.001$; **$p < 0.01$; *$p < 0.05$

Table 3 Proportion of trunk shoots and MB shoots with relay branch and lateral branch production for each treatment

Treatment	Proportion			
	Relay branch		Lateral branch	
	Trunk	MB	Trunk	MB
L+W−	0.34	0.09	0.22	0.02
L+W+	0.31	0.17	0.26	0.03
L−W−	0.11	0.06	0.10	0.10
L−W+	0.14	<0.001	0.10	0.01

L+W+ high sunlight and high water supply, L+W− high sunlight and low water supply, L−W+ shade and high water supply, L−W− shade and low water supply

Leaf chlorophyll content and photosynthetic yield

Leaf relative chl content estimated by SPAD values was higher for L− plants than for L+ plants (Table 4; Fig. 3). On the other hand, the chl content tended to be higher in leaves of W− plants than in those of W+ plants, independently of axis category; this tendency was more notable in L− plants than in L+ plants. For L+ plants, the effect of water supply was significant only at $t3$. Differences in chl content between trunk shoots and MB shoots depended on light condition (significant interaction between axis category and light condition; Table 4; Fig. 3). At $t1$, and only for plants under the L− treatment, chl content was higher in the leaves of trunk shoots than in those of MB shoots. At $t2$ and $t3$, the chl content of trunk leaves was slightly higher than that of MB leaves for L+ plants; in L− plants, differences between axes were not significant. For each condition, the chl content of leaves tended to increase along the growing season (Fig. 3).

In late November, P_N vs. PPFD curves performed on preformed leaves showed maximum P_N values at PPFD between 1000 and 1500 μmol m^{-2} s^{-1}. Leaves of plants of different treatments differed regarding P_{max} (μmol(CO$_2$)m^{-2} s^{-1}; ER = tending to infinity), which was highest for L+W− and

Table 4 Statistical analyses (Fisher's F and its significance, p value) of the effects of environmental factors, axis category, and their interactions upon the chlorophyll content of leaves (as estimated by SPAD values)

Factor	$t1$		$t2$		$t3$	
	F	p	F	p	F	p
Light (L)	42.4	***	317.5	***	226.1	***
Water supply (WS)	3.1	ns	15.9	***	6.5	*
Axis	31.3	***	0.3	ns	9.5	**
L×WS	6.5	**	6.7	**	0.3	ns
L×Axis	8.6	**	17.5	***	4.7	*
WS×Axis	0.5	ns	2.3	ns	0.0	ns
L×WS×Axis	1.5	ns	0.5	ns	1.7	ns

Different analyses are presented for each sub-period of the growing season ($t1$, $t2$ and $t3$),

ns not significant, ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$
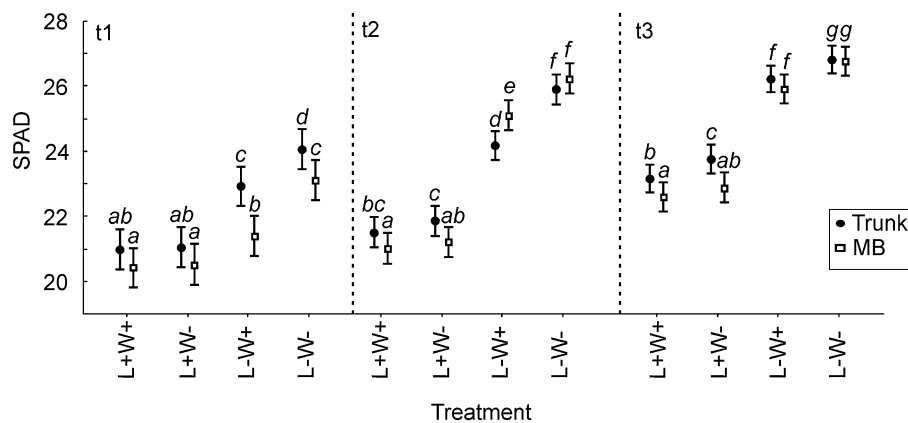
Fig. 3 Mean (±95% confidence interval) chlorophyll content (SPAD units) of leaves of trunk shoots and main branch shoots (MB) of plants of *N. obliqua* under contrasting light and water conditions throughout the growing season: t1 = from bud-break to early November; t2 = from early November to mid-January; t3 = from mid-January to the end of April. Treatment codes as in Fig. 1. Different letters above error bars indicate significant differences among treatments and axis types ($p < 0.05$, three-way ANOVA followed by *a posteriori* Tukey tests; see Table 4)
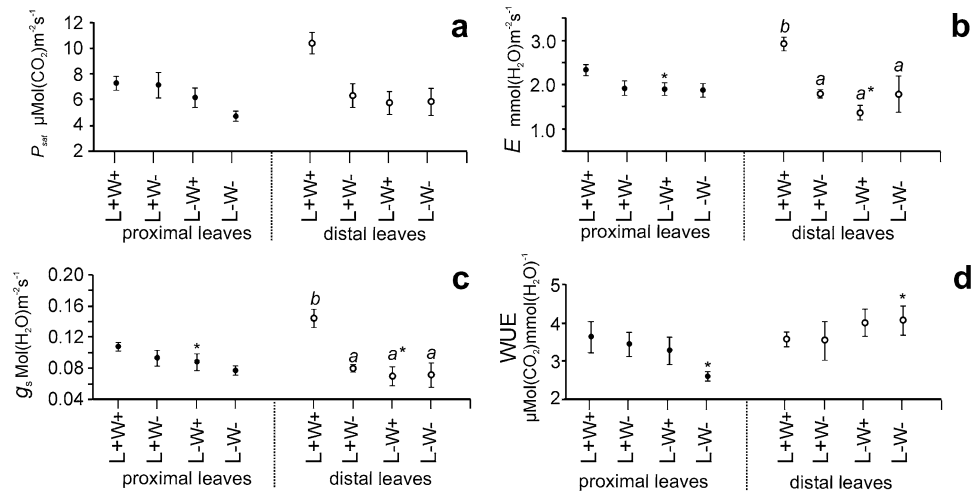
Fig. 4 Mean (±SE) values of physiological parameters for proximal and distal leaves of trunk shoots of six plants for each combination of light (L+ and L−) and water supply (W+ and W−) condition. **a** P_{sat} = net photosynthetic rate at saturating PPFD; **b** E = leaf transpiration rate; **c** g_s = stomatal conductance; **d** WUE = water use efficiency. Asterisks above error bars indicate treatments in which signifi-

cant differences between proximal and distal leaves were confirmed ($p < 0.05$, Wilcoxon paired comparisons); statistical differences among plants of different treatments for leaves in equivalent position (proximal or distal) are indicated by letters above error bars, whenever significant variations were detected ($p < 0.05$, Kruskal–Wallis tests followed by Mann–Whitney paired comparisons)

L+W+ plants ($\bar{x}$ = 9.1 and 8.8, SE = 0.93 and 0.53 respectively), intermediate for L−W− plants ($\bar{x}$ = 5.9, SE = 0.48) and lowest for L−W+ plants ($\bar{x}$ = 3.5, SE = 0.33). At the end of the growing season, P_{sat}, E, g_s and WUE presented no significant variations between proximal and distal leaves of the trunk shoot of L+ plants (Wilcoxon test, $p > 0.05$; Fig. 4a–d). However, a trend toward higher P_{sat}, E and g_s values in distal leaves (which were located in relay branches) than in proximal leaves of L+W+ plants was observed (Fig. 4a–c). For L−W− plants, significantly higher WUE in distal leaves than in proximal leaves was found ($p = 0.04$; Fig. 4d); for L−W+ plants, g_s and E were higher for leaves in proximal positions than for those in distal positions ($p = 0.03$ for both variables; Fig. 4b, c). Significant differences among treatments regarding leaf functioning were observed only for distal leaves: those of L+W+ plants presented higher g_s ($p = 0.05$) and E ($p = 0.02$) than distal leaves of plants under all other treatments (Fig. 4).

Discussion

Shoot growth and plastic responses

The present study contributes to the understanding of plant responses to variations in environmental conditions by showing that different axes of the same plants may react differently to the same conditions. As *N. obliqua* plants face significant changes in environmental conditions after budbreak, two of their main constituting axes, trunk and main

branches, may exhibit different responses in their primary growth (Fig. 5). For both axes, shading promoted higher stem extension rates early in the growing season, i.e. at the time of preformation extension. High water availability favoured the extension of trunk shoots only for a short period in spring. Peaks in RER were simultaneous for plants of all treatments, and the highest absolute differences in RER between L− and L+ plants occurred at that time. Therefore, factors other than light and water availabilities (e.g. temperature) would determine weakly fluctuations in RER, and light could induce quantitative variations. These results are related to the production of longer internodes in a shorter period in shaded compared to unshaded plants. For the second half of the growing season, the effect of light availability was less notable. In the case of trunk shoots, a brief period of positive light influence on RER was detected, probably associated with the development of organs additional to those that were preformed. The significant light × water supply interaction in the RER of MB shoots could be partly related to the fact that the morphogenetic responses of MBs (relay branching) was associated to specific conditions (L+W+). Overall, the shade effect was more pronounced in trunk shoots than in MB shoots, which resulted in longer trunk shoots in shaded plants than in unshaded plants.

Whereas light availability was the factor that most notably affected the number of nodes of MB shoots (L+ > L−), water availability was the only factor affecting the number of nodes of trunk shoots (W+ > W−). As all plants included in this study were growing under similar conditions before budbreak, similar numbers of preformed leaves may be assumed

Fig. 5 Overall results showing independent and mixed effects of light and water availability treatments upon different traits of trunk shoots (bold letters) and MB shoots (italics). See text for other abbreviations
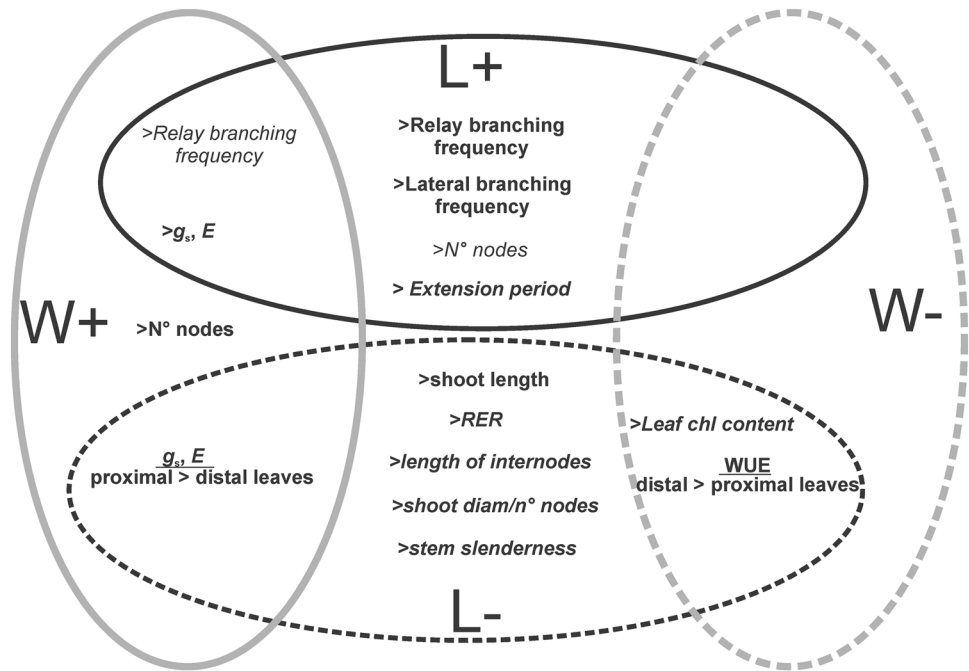
for buds in equivalent position (trunk or MB). Therefore, the development of leaves additional to those that were preformed in the buds, which has been reported for this and other *Nothofagus* species (Souza et al. 2000; Guédon et al. 2006; Puntieri et al. 2006), explains the observed results. The fact that some shoot morphological traits (e.g. internode length) varied in response to shade even though preformation occurred under high irradiation, contrasts with the results obtained for the related species *Fagus japonica*, in which the morphology of annual shoots was greatly affected by light conditions in the previous growing season (Kimura et al. 1998). In the case of *N. obliqua*, morphological responses to current conditions also include changes in the production of leaves additional to those that were preformed in the winter buds.

Intra-annual branching, i.e. a relay branch or lateral branches, was observed in plants under the L+ treatment. This result explains the higher mean number of nodes in the MB shoots of L+ plants compared to L− plants. In contrast, the number of nodes of trunk shoots in L+ and L− plants was alike, which suggests that a proportion of L− plants produced neoformed leaves in this axis. Thus, our results provide clear evidence that relay branch production is a frequent response of *N. obliqua* trees to high irradiation during the growing season, in accordance with previous studies (Puntieri et al. 2006; Varela et al. 2012). Interestingly, this response concerned mostly the trunk. It is possible that light exposure caused the death of the distal portion of shoots, and triggered the break of axillary buds which, depending on their position within the shoot, gave rise to a relay branch or a lateral branch (Collet et al. 1997; Puntieri et al. 2006).

The development of longer internodes is a frequent response to shading in plants species with low to intermediate shade tolerance (Valladares and Niinemets 2008; Charles-Dominique et al. 2012; Watari et al. 2014). Our present results agree with this, although a previous study on young-adult *N. obliqua* trees showed the opposite: shorter internodes in understory trees than in trees under canopy gaps (Puntieri et al. 2013). This within-species contrast may be explained by the possibility that those shoots that were measured in the shaded plants of the present study had been at least partially preformed in the previous growing season, i.e. under high light irradiation. The longer extension of internodes promoted by shade could, in this case, be interpreted as a short-term acclimation response, probably at the expense of carbohydrate reserves (see Kobe 1997), rather than an expression of the usual growth pattern of *N. obliqua* trees growing under permanently shaded conditions.

Shoot basal diameter was similar among plants under different experimental conditions, which could imply that the conductive area at the base of the shoots would be a conservative trait in *N. obliqua*. Thus, structural differences among shoots under different treatments were based on variation concerning shoot length and number of nodes. Stem slenderness was higher in shaded than in unshaded plants for trunk shoots but not for MB shoots. This contrast may be related with differences in growth direction: the horizontal direction of main branches could mean that stem length and slenderness are more limited by stem diameter than in the vertical trunk shoots (Telewski and Pruyn 1998; Meng et al. 2006). Higher values of the ratio between diameter and

number of nodes were registered for L− than for L+ plants, which is explained by the increase in the number of nodes due to the production of relay branches in L+ plants. These results suggest that the production of organs in addition to those that were preformed in the winter buds would not be paralleled by increases in the surface of water-transport tissues. Under this assumption, carbon assimilation by additional organs could be more limited by water transport in shoots with neoformation and/or relay-shoot production than in entirely preformed shoots.

The different responses to light that were observed for the two main axes of *N. obliqua* plants were reflected in higher levels of axis differentiation in L− than in L+ plants, as stem extension under shade increased more in trunk shoots than in MB shoots. In *Nothofagus*, both trunk and MBs may be described as exploratory axes (Puntieri et al. 2003). The present study indicates that, under shade conditions, the exploratory function of the trunk is prioritised over that of MBs in young *N. obliqua* trees. Contrasting plastic responses of different axis categories to particular environmental conditions is an interesting outcome of the present study. A noteworthy reference in this regard is the study by Charles-Dominique et al. (2012), in which different architectural strategies of a shrub species under different light environments are presented.

Leaf functioning and plasticity

The significant increases of chl content found here for L− *N. obliqua* leaves compared to L+ leaves agree with the frequent biochemical response in species exhibiting low to intermediate shade tolerance (Uemura et al. 2000; Valladares and Niinemets 2008). Leaf chl content was less variable than shoot growth between axis categories. Consistently higher chl content in trunk than in MB leaves was found only for the earliest stage of shoot growth (Fig. 3) which may be explained by the earlier bud-break of trunk shoots compared to MB shoots. Since chl synthesis depends on leaf nutrient content, mainly nitrogen (Niinemets 1997; Cate and Perkins 2003; Bojović and Sojanović 2005), and assuming that the faster growing trunk shoots would be stronger resource sinks than MB shoots, a higher chl content may have been expected in trunk than MB leaves, especially under shade conditions. In support of this idea, a previous study on *Juglans regia* found a positive relationship between leaf nitrogen concentration on an area basis and shoot vigour (Le Roux et al. 1998). Under the conditions of the experiment reported here, the similar chl content observed in trunk and MB shoots suggest that nutrient allocation to individual leaves would be homogeneous between both axis categories in *N. obliqua* saplings.

In shaded *N. obliqua* plants, leaf chl content was slightly but significantly higher in W− than in W+ leaves, suggesting

an interactive effect of shade and drought on leaf chl content. Mixed effects of shade and drought have been shown to vary depending on the species (Sack 2004). Under shade, higher chl content might help increasing water uptake. Variations in chl content with light and/or water availability have also been reported for both deciduous and evergreen *Quercus* species (Quero et al. 2006), some of which exhibited responses similar to that recorded here for *N. obliqua*. As pointed out by Quero et al. (2006), shading could mitigate the negative effects of water shortage, and changes in chl concentration could be one of the mechanisms through which this may be achieved. Nevertheless, chl losses under more severe drought conditions could not be discarded, since water deficiency during our experiment may not have reached a threshold value for significantly affecting chl synthesis. Previous studies on *N. obliqua* seedlings found significant losses in the water status of plants when soil VWC is below 10%, and showed that stomata closure is promoted by the combination of soil VWC lower than 10% and VPD higher than 2 KPa (Varela 2010; Varela et al. 2010). Such conditions were reached in a few days during the growing season in the L− treatment of this experiment, so that plants in this condition would have gone through short periods of water stress.

In late November, a positive effect of light on the $P_{\max}$ of proximal leaves was detected. At the end of the growing season, the only evidence supporting a higher acclimation of distal compared to proximal leaves of L− plants concerned WUE. In contrast, g_s and E were higher in proximal than in distal leaves of L−W+ plants. For L+ plants, no statistical differences between proximal and distal leaves were observed, although it should be remarked that, in L+W+ plants, the distal leaf of each shoot, borne by a relay branch, tended to present higher P_{sat}, E and g_s than the proximal leaf, issue of preformation. For leaves in equivalent position in the shoots, physiological differences between treatments were found only for distal leaves: those of L+W+ plants tended to have higher values of g_s, E and P_{sat} than those of plants in the other treatments. The overall results suggest that leaves located in relay branches of W+ plants could have higher photosynthetic rate and higher water loss than distal leaves that are not issued by relay branches (as in L− plants). Moreover, WUE of distal leaves was similar for all treatments. Based on this study, it may be proposed that the functional attributes of non-preformed leaves of *N. obliqua* depend on light and water conditions. On the one hand, in L+ conditions, leaves of relay branches would improve carbon fixation at the end of the growing season if water is available. On the other hand, in L− conditions, distal (presumably neoformed) leaves would improve WUE compared to proximal leaves when water availability is restricted. Since measurements were performed at the end of the growing season,

it should be considered that individual leaf ontogeny may have affected this result. A deep comparative knowledge of the physiology of preformed and non-preformed leaves, and their ontogenies would be necessary so as to clarify this issue. Our results complement the previous study by Varela et al. (2012) who reported, for *N. obliqua* seedlings, higher maximum photosynthetic rate in distal than in proximal leaves at advanced stages of the growing season for plants growing under non-limiting water conditions and high sun radiation. For other temperate trees, distal leaves have also been shown to be more able to acclimate to single-stress conditions than their proximal counterparts (Morales et al. 2014). Thus, the present work provides partial support to the idea that, in *N. obliqua*, leaves developed distally as a result of organ differentiation in addition to preformed organs should present contrasting acclimation mechanisms to conditions different from those that prevailed in the preceding growing season, whenever single-stress conditions prevail.

Conclusions

This study supports the hypothesis that short-term plastic responses of trees may vary depending on the axis category concerned. More interestingly, the morphogenetic events involved in such responses may differ among axis categories, and be related to the environmental factor/s involved. In the case of *N. obliqua*, short-term responses to light conditions were more notable than those promoted by water availability. Plastic responses of annual shoots to unpredicted environmental conditions may include variations in primary growth, allometric traits, leaf chl content and WUE. Other trends are worth mentioning, such as the higher photosynthetic rate of leaves of relay branches in sunlit plants with high water availability compared with both distal leaves in the same light conditions but low water availability and distal leaves of shaded plants.

Author contribution statement CT and JP proposed the idea and experimental design. The experimental device was mounted by CT, AM, JP, MS and JG. Data collection was performed by CT, JG, AM and MS. SV advised measurements and analysis of physiological data. CT performed data analysis and wrote the initial draft of the manuscript which was revised by JP, SV, AM, MS and JG.

Compliance with ethical standards

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