



Tree Physiology 32, 554–564
doi:10.1093/treephys/tps024



Research paper

Foliar anatomical and morphological variation in *Nothofagus pumilio* seedlings under controlled irradiance and soil moisture levels

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Received September 5, 2011; accepted March 2, 2012; published online April 11, 2012; handling Editor Guillermo Goldstein

Foliar anatomy and morphology are strongly related to physiological performance; therefore, phenotypic plasticity in leaves to variations in environmental conditions, such as irradiance and soil moisture availability, can be related to growth rate and survivorship, mainly during critical growth phases, such as establishment. The aim of this work was to analyze changes in the foliar internal anatomy (tissue proportions and cell dimensions) and external morphology (leaf length, width and area) of *Nothofagus pumilio* (Poepp. et Endl.) Krasser seedlings growing in a greenhouse under controlled irradiance (three levels) and soil moisture (two levels) during one growing season (measured three times), and to relate them to physiological traits. Three irradiance levels (4, 26 and 64% of the natural incident light) and two soil moisture levels (40 and 80% soil capacity) were evaluated during November, January and March. Internal foliar anatomy of seedlings was analyzed using digital photographs of histological cuttings, while leaf gross morphology was measured using digital calipers and image analysis software. Most internal anatomical variables presented significant differences under different irradiance levels during the growing season, but differences were not detected between soil moisture levels. Palisade parenchyma was the tissue most sensitive to irradiance levels, and high irradiance levels (64% natural incident light) produced greater values in most of the internal anatomical variables than lower irradiance levels (4–24% natural incident light). Complementarily, larger leaves were observed in medium and low irradiance levels, as well as under low soil moisture levels (40% soil capacity). The relationship of main results with some eco-physiological traits was discussed. Foliar internal anatomical and external morphological plasticity allows quick acclimation of seedlings to environmental changes (e.g., during harvesting). These results can be used to propose new forest practices that consider soil moisture and light availability changes to maintain high physiological performance of seedlings.

Keywords: irradiance, leaf external morphology, leaf internal anatomy, leaf phenotypic plasticity, soil moisture.

Introduction

Foliar anatomy and morphology are strongly related to physiological performance; therefore, plasticity in leaves (including physiological, biochemical and anatomical changes) can be related to growth rate and survivorship (e.g., Smith and Nobel 1978, Chazdon and Kaufmann 1993, Grassi and Bagnaresi 2001, Hanba et al. 2002). In a wide sense, plasticity is described as the responses of organisms to environmental con-

ditions, and it is particularly important for plants due to their limitations of motility (Novoplansky 2002). Plasticity is a key characteristic to define the survivorship and developmental success of a plant growing under a specific condition, like some particular irradiance or soil moisture level, and establishment is the most critical period for trees. Forest recovery after a disturbance event (e.g., forest management) depends on seedling acclimation to new environmental conditions. In this context, plasticity gives the potential to modify seedling functional and

structural characters, which define plant behavior when changes in resources occur (Aranda et al. 2004). Modifications in foliar anatomy and morphology in response to changes in irradiance were studied for different temperate tree species (e.g., *Populus*, *Fraxinus*, *Tilia*, *Nothofagus*, *Fagus*, *Quercus*) (Aranda et al. 2001, 2004, Niinemets 2001, Hovenden and Vander Schoor 2003), and in some of them, internal foliar anatomy could present greater changes than biochemistry (Niinemets et al. 1998). However, few of these works describe changes in internal foliar anatomy at the tissue level, such as tissue characteristics, proportions and cell dimensions (Rodrigues Marques et al. 2000, Hanba et al. 2002, Guarnaschelli et al. 2009).

Nothofagus pumilio (Poepp. et Endl.) Krasser (Fagaceae) is a broadleaved, monoecious and deciduous Southern Patagonian tree species, with a wide latitudinal (from 35°35' to 55°30' S) and altitudinal distribution (from sea level to tree-line up to 2000 m a.s.l.) (Massaccesi et al. 2008), which is commonly harvested for timber production (González et al. 2006). Natural forests of this mid-shade-tolerant species (Martínez Pastur et al. 2007) present an abundant seedling bank (Cuevas 2002), which often survives and grows slowly for a long period of time in the shaded understorey (Rebertus and Veblen 1993), and can quickly react after anthropogenic or natural disturbances (Heinemann et al. 2000, Anderson et al. 2006, Rosenfeld et al. 2006, Martínez Pastur et al. 2011a). Structural and physiological changes of seedlings growing in different environmental conditions have been registered for this species: (i) growth and biomass compartmentalization (Lencinas et al. 2007), (ii) tissue nutrient concentration and foliar pigment content (Soler Esteban et al. 2011) and (iii) eco-physiological processes, such as photosynthesis and respiration (Martínez Pastur et al. 2007, Peri et al. 2009). The aims of this work were to analyze changes in the foliar internal anatomy (tissue proportions and cell dimensions) and external morphology (leaf length, width and area) of *N. pumilio* seedlings growing in a greenhouse under controlled irradiance and soil moisture levels during one growing season, and relate them to physiological traits. Tested hypotheses were: (i) foliar internal anatomy and external morphology of *N. pumilio* seedlings change under different environmental conditions, e.g., irradiance and soil moisture levels; (ii) environmental conditions differentially affect foliar internal anatomy and external morphology during the growing season; and (iii) changes in foliar internal anatomy and external morphology are related to physiological traits.

Materials and methods

Plant material and growing conditions

Two- to three-year-old seedlings of 6–7 cm height were obtained from natural primary forests (54°06' S, 68°37' W) with complete canopy cover ($94 \pm 5\%$ standard deviation (SD) measured with a concave spherical crown densitometer,

'Forestry Suppliers' Manufacturer, Jackson, MI, USA) at the beginning of the spring before budburst occurred. Seedlings were transplanted into plastic pots of 14 cm diameter and 15 cm height, which were filled with a substrate of peat–sand–humic forest soil (1 : 1 : 1). This substrate corresponded to a clay loam soil (sand–silt–clay, 36–24–40%) with 7% organic matter, pH 4.99 and water field capacity of 81% (for more details see Martínez Pastur et al. 2007). Plants were kept in a greenhouse at Ushuaia, Tierra del Fuego Province, Argentina (54°46' S, 68°12' W) during one growing season (September 2005 to March 2006), under three irradiance conditions: (i) 4% (I4), 26% (I26) and 64% (I64) of the natural incident light, using different layers of commercially available black shade cloth, and a greenhouse covering of 100 μm of plastic commercial nylon to avoid natural rainfall. Average solar irradiance during the summer was $2085 \pm 534 \mu\text{mol m}^{-2} \text{s}^{-1}$ SD, with a maximum mean value of $2702 \mu\text{mol m}^{-2} \text{s}^{-1}$, recorded with a lux-meter using lux units (visible flux density) (Model Extech, Waltham, MA, USA). Compared with field measurements, experimental irradiances assayed in the greenhouse reflected natural conditions: the low irradiance treatment (I4) corresponded to a primary forest with a full closed canopy, the medium irradiance treatment (I26) was comparable to open areas inside primary forest or closed sectors in harvested stands (up to $30 \text{ m}^2 \text{ ha}^{-1}$ basal area) and the high irradiance treatment (I64) was comparable to post-harvesting conditions in managed forests with large open areas ($10\text{--}15 \text{ m}^2 \text{ ha}^{-1}$ basal area) (Martínez Pastur et al. 2007).

Temperature was controlled through forced ventilation, avoiding $>24^\circ\text{C}$ at plant canopy level. Air humidity, air and soil temperatures were measured in each irradiance treatment using data loggers through the study period (ONSET Computer Corporation, Bourne, MA, USA) (Table 1). Irrigation was done manually, maintaining half of the plants under soil moisture of 40–60% soil capacity (S40), while the other half was grown under 80–100% soil capacity (S80). The amount of irrigation was determined gravimetrically every 3 days. Similar to irradiance, soil moisture assayed levels mimic natural conditions, from medium soil moisture levels (S40) in primary forests to higher soil moisture levels (S80) in cut stands, since harvesting increases effective rainfall at the understorey level (Caldentey et al. 2005).

Sampling and microscopy studies

For each combination of irradiance and soil moisture, blocks of 20 plastic pots were individualized in the greenhouse for bimonthly samplings in November, January and March. During the first week of each selected month, three plants per block per treatment were randomly chosen for leaf extraction ($n = 3$ per treatment per month). First, non-senescent, fully expanded leaves were cut and fixed in FAA (formaldehyde, glacial acetic acid and ethanol, 5 : 5 : 90) for later processing.

Table 1. Air temperature and humidity, and soil temperature (average \pm standard error) registered in greenhouse under different irradiance levels during the growing season.

Month	Air temperature ($^{\circ}$ C)			Air humidity (%)			Soil temperature ($^{\circ}$ C)		
	I64	I26	I4	I64	I26	I4	I64	I26	I4
October	8.5 \pm 2.7	9.1 \pm 2.4	9.5 \pm 2.3	66 \pm 8	66 \pm 7	65 \pm 7	9.1 \pm 2.1	8.1 \pm 1.3	8.0 \pm 1.4
November	11.4 \pm 2.5	12.5 \pm 2.4	12.5 \pm 2.3	67 \pm 8	66 \pm 7	66 \pm 6	12.2 \pm 1.8	11.2 \pm 1.3	11.0 \pm 1.3
December	11.9 \pm 3.2	12.9 \pm 3.1	13.0 \pm 3.0	67 \pm 6	66 \pm 5	67 \pm 5	12.1 \pm 2.1	11.4 \pm 1.7	11.3 \pm 1.8
January	16.3 \pm 1.2	16.9 \pm 1.5	16.9 \pm 1.4	65 \pm 2	65 \pm 2	64 \pm 2	16.6 \pm 1.0	14.4 \pm 0.7	14.3 \pm 0.8
February	13.1 \pm 2.1	13.6 \pm 1.9	13.4 \pm 1.8	66 \pm 10	66 \pm 9	68 \pm 9	13.6 \pm 1.3	12.4 \pm 0.7	12.1 \pm 0.9
March	10.9 \pm 2.4	11.2 \pm 2.4	10.8 \pm 2.3	67 \pm 7	68 \pm 7	69 \pm 7	11.2 \pm 1.7	10.4 \pm 1.2	10.1 \pm 1.3

I64, high irradiance with 64% natural incident light; I26, medium irradiance with 26% natural incident light; I4, low irradiance with 4% natural incident light.

Leaf mesophyll samples were dehydrated in graded ethanol series for 24 h, followed by TBA (tert-butyl alcohol) and then embedded in Paraplast Plus tissue-embedding medium (Sigma Aldrich, Buenos Aires, Argentina) for 72 h at 57 $^{\circ}$ C. Transverse cross sections 40 μ m thick were stained in a mixture of equal volumes of 0.1% aqueous solutions of safranin O and fast green FCF and mounted for light microscopy observation (Ruzin 1999). For the analyses, only the entire transverse section of the central widest part of the leaf and major vein were selected. In each cutting, three digital photographs were obtained (Leica DFC-420C, Leica, Heerbrugg, Switzerland), using \times 630 magnification (\times 63 and \times 10 lenses), and a 2 mm grid was also used as a reference scale.

Digital photos were scaled and analyzed digitally (Martínez Pastur et al. 2011b) by ArcView GIS 3.2 software (Environmental Systems Research Institute-ESRI, Redlands, CA, USA) (ESRI 2000), measuring the following variables: (i) percentage area (%) occupied by tissues (UE = upper epidermis, PP = palisade parenchyma, SP = spongy parenchyma, ES = empty spaces and LE = lower epidermis), (ii) tissue thickness (μ m) (LT = lamina, PPT = palisade parenchyma, UCT = upper cuticle, UET = upper epidermis, LET = lower epidermis and LCT = lower cuticle), (iii) average cell sizes (μ m) of tissues (PPCW = palisade parenchyma cell width, PPCL = palisade parenchyma cell length and SPCW = spongy parenchyma cell width) and (iv) CFPP = number of cell files in palisade parenchyma (Figure 1).

Likewise, three fully expanded leaves were selected from six plants in each irradiance and soil moisture treatment during the growing season. In these leaves, leaf gross morphology was measured using digital calipers (cm): leaf length (L) from base of petiole to leaf tip, and leaf width (W) as maximum width of the blade. Also, each leaf area (LA) (cm^2) was measured using digital photos and image analysis software, and then averaged for each treatment (Hovenden and Vander Schoor 2003).

Statistical analyses

The final data base included six treatments (three irradiance and two soil moisture levels) \times three bimonthly samplings (November, January and March). For internal anatomical

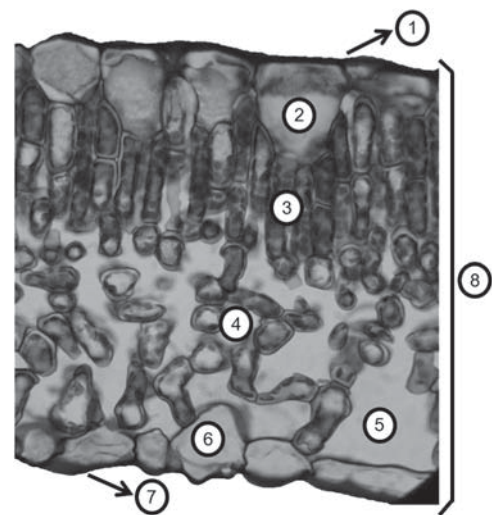


Figure 1. Tissue identification in slide cuttings of *N. pumilio* leaves. (1) upper cuticle, (2) upper epidermis, (3) palisade parenchyma, (4) spongy parenchyma, (5) empty spaces, (6) lower epidermis, (7) lower cuticle and (8) lamina.

comparison, three replicas were obtained, where the average of the three digital photo analyses of each transverse cutting was a replica. For external gross morphology comparison, six replicas were obtained, where the average of the three measured leaves per plant from each treatment was a replica. The following statistical analyses were conducted: (i) three-factorial analysis of variance (ANOVA) for internal anatomical and external morphology variables, considering month, irradiance and soil moisture as main factors, and where means were separated through a Tukey's multiple comparison test at $P \leq 0.05$; (ii) multivariate ordination, by principal component analysis (PCA) using a correlation matrix of the internal anatomical variables for the 3-month data, and a randomization test (Monte Carlo, with 999 randomization runs) to graphically analyze through the plot distribution pattern the variation among treatments and the influence of each variable in the expression of treatments; (iii) cluster analyses, using average linkage with a Euclidean distance matrix, to highlight similarities among irradiance and soil moisture treatments at the end of the growing season (March).

Results

Tissue proportions on *N. pumilio* leaves significantly varied through the growing season and with irradiance levels, but did not present differences between soil moisture treatments (Table 2 and Figure 2). Proportion of epidermis (UE and LE) and SP significantly diminished during the growing season (UE: 23 to 19%, LE: 10 to 8%, SP: 17 to 10%), while ES significantly increased (14 to 27%). Palisade parenchyma significantly diminished from November to January, but increased again during March. When irradiance levels were analyzed, the proportion of epidermis (UE and LE) and ES diminished when irradiance increased (UE: 24 to 19%, LE: 10 to 8%, ES: 26 to 16%), while parenchyma (PP and SP) increased (PP: 30 to 40%, SP: 10 to 17%) (Figure 2). Few interactions were detected (Table 2 and Figure 3) due to: (i) the proportion of UE presenting a different trend for each moisture level during March (Figure 3a) and (ii) the proportion of SP being higher for lower moisture levels (S40) during November compared with the other months (Figure 3b).

Differences in foliar anatomical parameters in slide cuttings were found during the growing season and with the irradiance gradient, but we did not detect differences between soil moisture treatments (Table 3 and Figure 2). Cuticle thickness

(UCT and LCT) significantly diminished through time (2.7 to 2.3 μm , and 1.9 to 1.4 μm , respectively), while the other thickness and cell sizes (LT, PPT, UET, PPCW and PPCL) increased their values during the growing season. When the irradiance gradient was analyzed, most variables increased with irradiance, except for PPCW, which decreased in size at higher light levels (Table 3). Most variables presented interactions (Table 3 and Figure 3) where: (i) some of them (LT, PPT, UCT, SPCW, CFPP and LCT) showed different responses among months for each irradiance level; (ii) other variables (UCT, PPCW and LCT) presented a similar trend but with different magnitudes in some irradiance level for each moisture level; and (iii) LCT presented different trends for each moisture level during March.

External gross morphology of *N. pumilio* leaves differed significantly among months, irradiance and soil moisture levels (Table 2). Length (*L*), width (*W*) and leaf area (*LA*) increased during the growing season, with maximum values for *L* and *W* since January, and for *LA* in March. With respect to irradiance levels, significantly larger leaves were observed in I26 > I4 > I64 for both *L* and *W*, while *LA* was similar in I26 and I4, and significantly higher than in I64. On the other hand, the three variables were higher in S40 than in S80. Interactions (Table 2 and Figure 3) were similar for *L* and *W*:

Table 2. Multiple ANOVA of tissue proportions in slide cutting and external gross morphology of *N. pumilio* leaves considering month, irradiance and soil moisture as main factors, and upper epidermis (UE) (%), palisade parenchyma (PP) (%), spongy parenchyma (SP) (%), empty spaces (ES) (%), lower epidermis (LE) (%), leaf length (*L*) (cm), leaf width (*W*) (cm) and leaf area (*LA*) (cm^2) as variables.

Treatment	Factor	UE	PP	SP	ES	LE	L	W	LA
Month (A)	November	22.5 b	36.4 b	16.9 b	14.3 a	10.0 b	1.6 a	1.2 a	1.2 a
	January	20.8 ab	33.4 a	10.7 a	27.1 b	7.9 a	2.4 b	1.8 b	2.0 b
	March	19.3 a	36.1 ab	10.3 a	26.6 b	7.8 a	2.4 b	1.8 b	2.9 c
	<i>F</i>	7.17	4.08	38.56	46.71	15.09	244.02	239.46	52.83
	<i>P</i>	0.002	0.025	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Irradiance (B)	I64	18.9 a	40.1 c	17.2 b	16.0 a	7.8 a	1.7 a	1.3 a	1.3 a
	I26	20.1 a	34.8 b	11.1 a	25.5 b	8.4 ab	2.5 c	1.8 c	2.5 b
	I4	23.5 b	30.1 a	9.6 a	26.4 b	9.5 b	2.2 b	1.7 b	2.3 b
	<i>F</i>	15.65	30.71	44.43	29.21	7.01	199.06	148.64	31.81
	<i>P</i>	<0.001	<0.001	<0.001	<0.001	0.003	<0.001	<0.001	<0.001
Soil moisture (C)	S80	21.0	35.5	12.7	22.3	8.5	2.0 a	1.5 a	1.8 a
	S40	20.7	35.1	12.6	23.1	8.6	2.2 b	1.7 b	2.2 b
	<i>F</i>	0.27	0.27	0.01	0.42	0.05	33.98	32.63	7.75
	<i>P</i>	0.610	0.610	0.961	0.523	0.817	<0.001	<0.001	0.007
A × B	<i>F</i>	0.70	1.15	2.42	2.33	1.70	27.75	16.46	4.08
	<i>P</i>	0.598	0.349	0.066	0.074	0.171	<0.001	<0.001	0.004
A × C	<i>F</i>	4.50	0.19	3.59	1.85	0.32	4.78	9.34	2.4
	<i>P</i>	0.018	0.828	0.038	0.170	0.730	0.011	<0.001	0.096
B × C	<i>F</i>	0.04	0.90	1.10	0.26	2.20	15.63	14.96	2.24
	<i>P</i>	0.958	0.417	0.344	0.776	0.126	<0.001	<0.001	0.113
A × B × C	<i>F</i>	1.68	0.24	0.63	2.75	1.86	1.36	2.63	3.51
	<i>P</i>	0.175	0.912	0.641	0.043	0.139	0.256	0.039	0.010

I64, high irradiance with 64% natural incident light; I26, medium irradiance with 26% natural incident light; I4, low irradiance with 4% natural incident light; S80, soils with 80–100% soil capacity; S40, soils with 40–60% soil capacity.

F = *F* test statistic; *P* = probability at 0.05.

Values followed by different letters in each column and for each factor are significantly different with Tukey's multiple range test at $P < 0.05$.

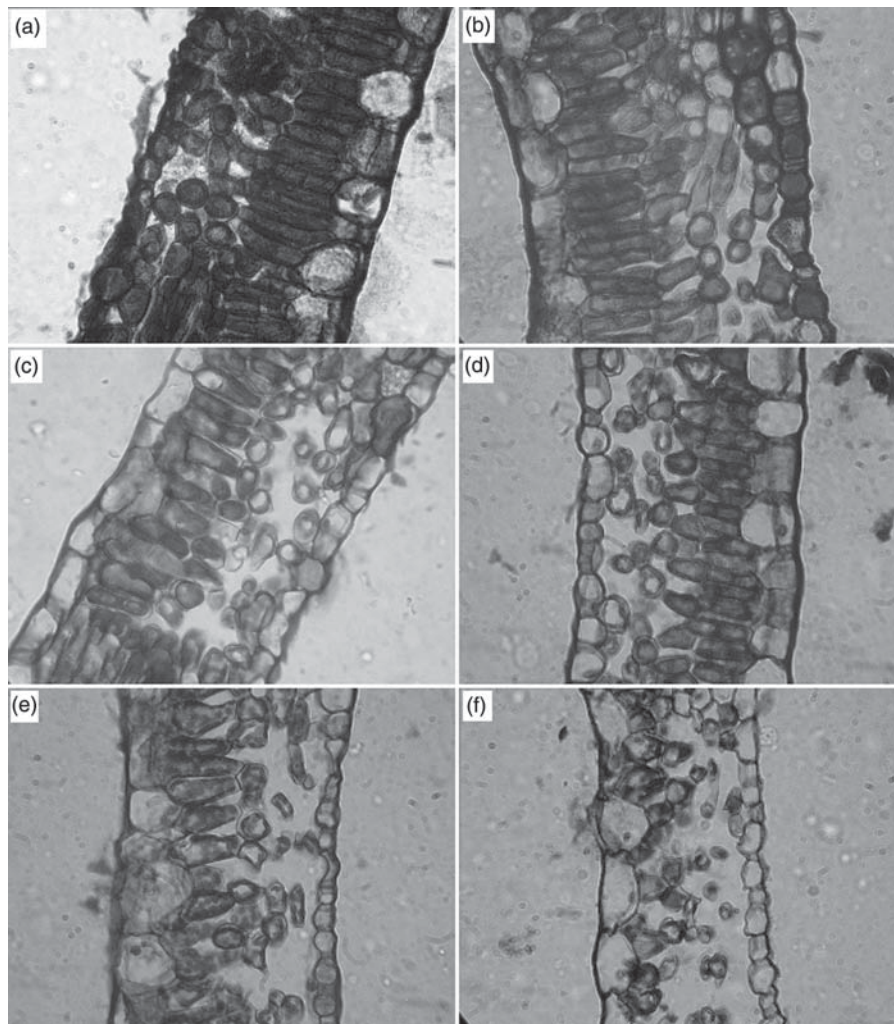


Figure 2. Tissue slide cuttings of *N. pumilio* leaves along irradiance and soil moisture gradients during January. (a) I64 and S80, (b) I64 and S40, (c) I26 and S80, (d) I26 and S40, (e) I4 and S80, and (f) I4 and S40. I64, high irradiance with 64% natural incident light; I26, medium irradiance with 26% natural incident light; I4, low irradiance with 4% natural incident light; S80, soils with 80–100% soil capacity; S40, soils with 40–60% soil capacity.

(i) I4 values were higher than I26 in November, but lower in January and March; (ii) similar values for both soil moisture levels were observed in November, and differences occurred since January; and (iii) soil moisture levels were different in I64, but very similar in I26 and I4. For LA, interaction occurred only between irradiance levels and months, because I4 and I26 were similar in November and March, but differed in January.

In PCA, the first two principal components (axes) were significant to interpret results according to the randomization test ($P = 0.001$ for both axes 1 and 2). The axis 1 (eigenvalue = 6.872) explained 46% of total variance, while the axis 2 (eigenvalue = 3.447) described 23% of the total variance. Plot ordination (Figure 4a) highlighted the multivariate response of each treatment, where both months and irradiance levels showed different patterns, but it was not the case for soil moisture levels, which did not present clear patterns.

Gradients were observed from left to right related to irradiance (I64–I26–I4), and from top to bottom related to months (November–January–March), although January and March from I26 and I4 treatments were mostly intermingled. The influence of each variable in the expression of treatments was represented in a vector graph (Figure 4b and Table 4), where axis 1 was negatively and mainly related to PPT, lamina thickness (LT) and CFPP, and axis 2 was mainly positively related to SP and LE proportion and negatively related to ES proportion. Complementarily, cluster analysis for March data (Figure 4c) highlighted the similarity among irradiance treatments at the end of the growing season, where irradiance levels were joined in three groups independently of their soil moisture level at lower Euclidean distance (3.11) for I64 treatments, medium distance (3.35) for I26 treatments and higher distance (4.24) for I64; I26 and I4 were also more similar to each other (4.44) than to I64 (7.21 Euclidean distance).

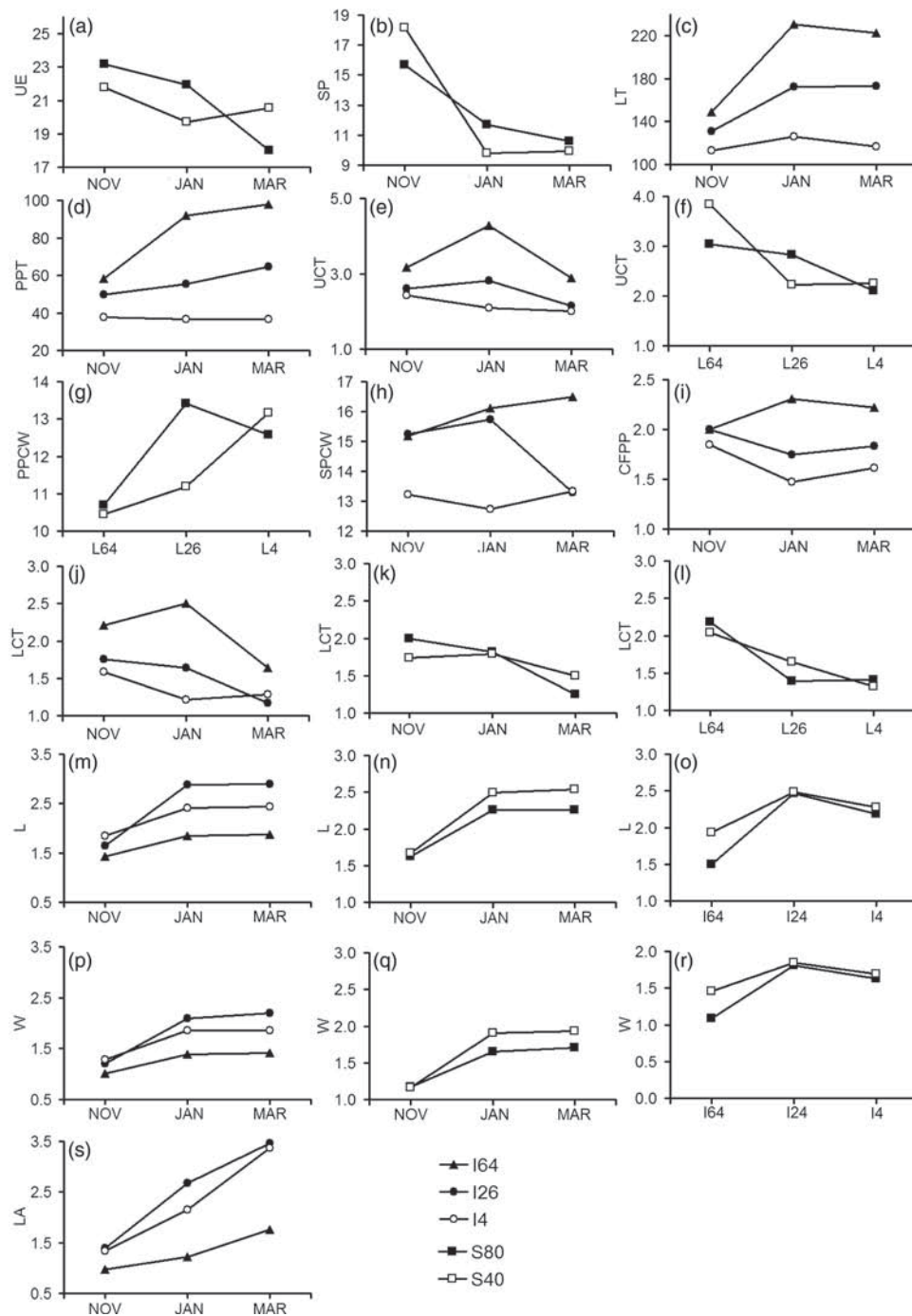


Figure 3. Graph of significant interactions of multiple ANOVAs described in Tables 2 and 3. I64, high irradiance with 64% natural incident light; I26, medium irradiance with 26% natural incident light; I4, low irradiance with 4% natural incident light; S80, soils with 80–100% soil capacity; S40, soils with 40–60% soil capacity. UE, upper epidermis (%); SP, spongy parenchyma (%); LT, lamina thickness (μm); PPT, palisade parenchyma thickness (μm); UCT, upper cuticle thickness (μm); PPCW, palisade parenchyma cell width (μm); SPCW, spongy parenchyma cell width (μm); CFPP, number of cell files in palisade parenchyma (n); LCT, lower cuticle thickness (μm); L, leaf length (cm); W, leaf width (cm); LA, leaf area (cm^2).

Discussion

Leaves of *N. pumilio* seedlings growing at different irradiance and soil moisture levels presented significant internal anatomical and external morphological variations as a consequence of their plasticity to the studied factors, and since the beginning of the growing

season. Irradiance and soil moisture are usually the most important factors that affect growth and survival of seedlings in Patagonian forests (Heinemann et al. 2000), and according to the observed results both factors significantly influence external gross morphology. However, only irradiance was significantly relevant in the morphological differentiation of leaf tissues.

Table 3. Multiple ANOVA of foliar internal anatomical parameters in slide cuttings of *N. pumilio* leaves considering month, irradiance and soil moisture as main factors, and lamina thickness (LT) (μm), number of cell files in palisade parenchyma (CFPP), palisade parenchyma thickness (PPT) (μm), upper cuticle thickness (UCT) (μm), upper epidermis thickness (UET) (μm), palisade parenchyma cell width (PPCW) (μm), palisade parenchyma cell length (PPCL) (μm), spongy parenchyma cell width (SPCW) (μm), lower epidermis thickness (LET) (μm) and lower cuticle thickness (LCT) (μm) as variables.

Treatment	Factor	LT	CFPP	PPT	UCT	UET	PPCW	PPCL	SPCW	LET	LCT
Month (A)	November	131.3 a	1.9	48.7 a	2.7 b	28.4 a	10.6 a	35.7 a	14.6	13.6	1.9 b
	January	176.3 b	1.8	61.3 b	3.1 b	35.8 b	12.0 b	48.6 b	14.9	13.7	1.8 b
	March	170.9 b	1.9	66.4 b	2.3 a	31.5 ab	13.2 c	51.2 b	14.4	12.5	1.4 a
	<i>F</i>	14.59	1.55	9.98	12.05	5.21	16.39	22.29	0.53	1.83	23.03
	<i>P</i>	<0.001	0.226	<0.001	<0.001	0.010	<0.001	<0.001	0.590	0.175	<0.001
Irradiance (B)	I64	200.8 c	2.2 c	82.7 c	3.4 b	36.0 b	10.6 a	53.5 b	15.9 c	15.6 c	2.1 b
	I26	159.0 b	1.9 b	56.6 b	2.5 b	31.1 ab	12.3 b	47.5 b	14.8 b	13.1 b	1.5 a
	I4	118.8 a	1.6 a	37.0 a	2.2 a	28.6 a	12.9 b	34.4 a	13.1 a	11.0 a	1.4 a
	<i>F</i>	40.57	39.02	62.84	39.65	5.33	13.45	30.74	17.80	20.12	51.54
	<i>P</i>	<0.001	<0.001	<0.001	<0.001	0.009	<0.001	<0.001	<0.001	<0.001	<0.001
Soil moisture (C)	S80	161.5	1.9	60.0	2.7	32.9	12.2	46.9	14.9	13.3	1.7
	S40	157.5	1.9	57.5	2.8	30.9	11.6	43.4	14.3	13.3	1.7
	<i>F</i>	0.30	0.01	0.58	0.97	1.14	2.84	2.96	1.75	0.01	0.03
	<i>P</i>	0.588	0.951	0.453	0.331	0.293	0.101	0.094	0.194	0.987	0.869
A × B	<i>F</i>	3.26	6.22	5.17	5.05	2.34	1.20	1.89	2.97	1.73	6.47
	<i>P</i>	0.022	<0.001	0.002	0.003	0.071	0.328	0.132	0.032	0.162	<0.001
A × C	<i>F</i>	0.11	0.36	0.42	2.43	1.69	1.60	0.14	2.15	0.19	5.01
	<i>P</i>	0.895	0.698	0.662	0.101	0.198	0.215	0.869	0.129	0.828	0.012
B × C	<i>F</i>	0.16	1.69	0.91	11.42	0.63	4.87	2.10	2.59	0.90	3.78
	<i>P</i>	0.852	0.197	0.411	<0.001	0.536	0.014	0.135	0.088	0.413	0.032
A × B × C	<i>F</i>	0.49	2.06	0.16	4.34	0.59	1.70	0.25	3.07	1.15	5.35
	<i>P</i>	0.745	0.107	0.955	0.006	0.672	0.172	0.911	0.028	0.351	0.002

I64, high irradiance with 64% natural incident light; I26, medium irradiance with 26% natural incident light; I4, low irradiance with 4% natural incident light; S80, soils with 80–100% soil capacity; S40, soils with 40–60% soil capacity.

F = *F* test statistic; *P* = probability at 0.05.

Values followed by different letters in each column and for each factor are significantly different with Tukey's multiple range test at $P < 0.05$.

External gross leaf morphology in *N. pumilio* seedlings increased from November to January accordingly with their phenological stages (leaf unfolding to leaf expansion) (Martínez Pastur et al. 2007), independently of irradiance and soil moisture levels. Although the largest leaves usually occurred at the relatively low irradiance characteristic of shaded habitats (e.g., James and Bell 2000, Guarnaschelli et al. 2009), and where appreciable soil water is available (e.g., Smith and Nobel 1978, Witkowski and Lamont 1991), *N. pumilio* produced the largest leaves (since January to March) at medium irradiance (I26) and low soil moisture levels (S40), but with statistically similar area in both medium and low irradiance treatments. This result contrasts with other studies where leaves with lowest absorptance and under high soil water potential produced greater leaves (Smith and Nobel 1978). Comparatively higher growth rates in *N. pumilio* leaves at medium irradiance and low soil moisture levels could be explained by higher photosynthetic efficiency under these conditions (Martínez Pastur et al. 2007), and also could be an indicator of mid-tolerance to shaded environments, according to Gutiérrez (1994).

Internal foliar anatomy changed with phenological stages too (Martínez Pastur et al. 2007), due to normal changes

during the morphological development of *Nothofagus* deciduous leaves. From November to March, increases in the ES proportion, leaf and PPT and PPCL and PPCW were observed, but greater differences were observed between November and January for all variables, as compared with differences between January and March. These variations can be related to a greater morphological differentiation of leaf tissues in the first stages of development, compared with final stages, near to senescence.

Assayed irradiance levels induced greater changes in the more sensitive tissues, which were mainly related to epidermis and parenchyma proportion, thickness and cell size. The increase in the parenchyma tissue proportion (PP and SP) under the highest irradiance treatment (I64) explained also the lower proportion of epidermis (UE and LE). Palisade parenchyma was the tissue with highest sensitivity to changes in irradiance. This sensitivity could be explained due to the highest chlorophyll content in the chloroplasts of these tissues (Strasburger et al. 1985, Terashima et al. 2001). Exposure to high irradiance stimulates the increase of chloroplast numbers to improve light absorption, and consequently of PPT (Terashima et al. 2001). The usual consequence is a greater

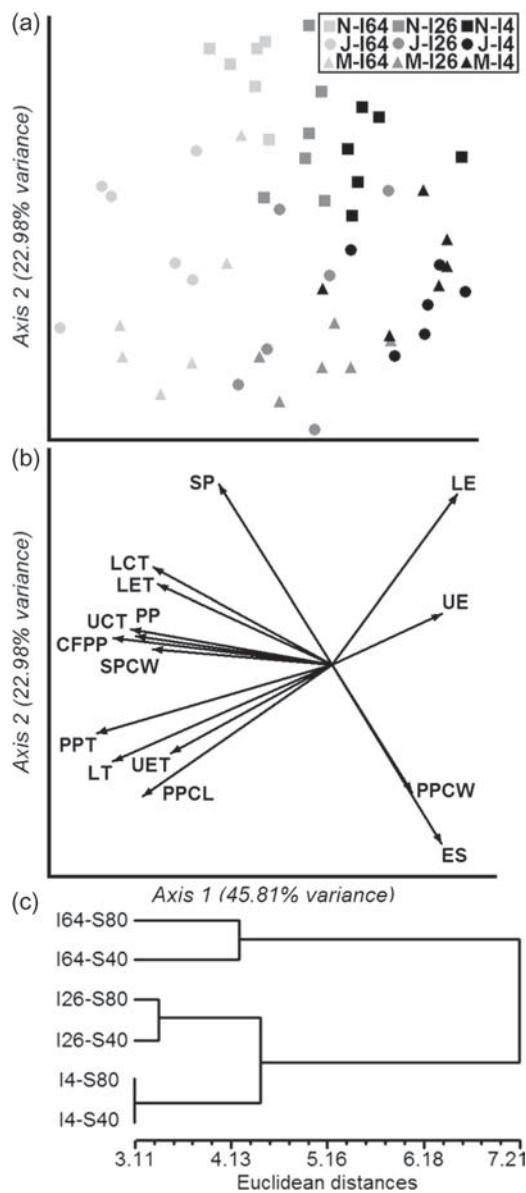


Figure 4. Principal component analysis (November, January and March) and cluster analysis (March) for foliar morphological variables of *N. pumilio* seedlings for different irradiance (I64, high irradiance with 64% natural incident light; I26, medium irradiance with 26% natural incident light; I4, low irradiance with 4% natural incident light) and soil moisture (S80, soils with 80–100% soil capacity; S40, soils with 40–60% soil capacity) levels.

lamina thickness and specifically, greater PPT on leaves growing under direct sunlight (Thompson et al. 1992, Rodrigues Marques et al. 2000, Aranda et al. 2001, Hanba et al. 2002, Guarnaschelli et al. 2009). However, some authors found greater thickness in shaded leaves (Abrams and Kubiske 1990). Spongy parenchyma cell size also increased in high irradiance treatments in our study, as was observed for *Miconia stenostachya* Schrank & Mart. Ex DC. in high irradiance environments by Rodrigues Marques et al. (2000). However, Puértolas et al. (2008) found greater responses to

Table 4. Correlation values of two first components of PCA for foliar internal anatomical variables of *N. pumilio* seedlings during the growing season.

Variables	C1	C2
UE	0.4321	0.2326
PP	−0.7904	0.1600
SP	−0.4435	0.8119
ES	0.4310	−0.8037
LE	0.4919	0.7659
LT	−0.8626	−0.4321
CFPP	−0.8592	0.1209
PPT	−0.9229	−0.3068
UCT	−0.7700	0.1311
UET	−0.6333	−0.3971
PPCW	0.3158	−0.5733
PPCL	−0.7437	−0.5904
SPCW	−0.7038	0.0704
LET	−0.6843	0.3631
LCT	−0.7014	0.4375

C1, first component; C2, second component; UE, percentage of upper epidermis; PP, percentage of palisade parenchyma; SP, percentage of spongy parenchyma; ES, percentage of empty spaces; LE, percentage of lower epidermis; LT, lamina thickness; CFPP, number of cell files in palisade parenchyma; PPT, palisade parenchyma thickness; UCT, upper cuticle thickness; UET, upper epidermis thickness; PPCW, palisade parenchyma cell width; PPCL, palisade parenchyma cell length; SPCW, spongy parenchyma cell width; LET, lower epidermis thickness; LCT, lower cuticle thickness.

high irradiance in *Quercus suber* L. seedling PP than on SP and epidermis tissue. Accumulation of photosynthetic compounds that enlarge parenchyma and therefore leaf thickness in *N. pumilio* leaves could be the explanation of the greatest foliar photosynthetic rates per unit area at medium and high irradiance levels (Martínez Pastur et al. 2007), as was also observed for Niinemets (2001) for other tree and shrub species. Complementarily, lower leaf thickness under low irradiances can be understood as a strategy of shade adaptation (Kolowski and Pallardy 1997).

Leaf surface structures (upper and lower cuticle and epidermis) affect whole-leaf photosynthesis performance, due to alterations in absorbed sunlight (Smith et al. 1997). It was suggested that upper epidermis and cuticle thickness (UET and UCT) may increase leaf reflectance (Chazdon and Kaufmann 1993). For example, a thicker epidermal layer, as was observed in high irradiance treatments (I64) in our study, alters light-scattering processes within mesophyll tissue, and protects photosynthetic tissues from excessive irradiance (Roth 1984). Finally, the higher proportion of ES found in lower irradiance treatments (I4 and I26) can also be understood as strategies to increase light absorption efficiency, and was also observed by Thompson et al. (1992) for sun- and shade-tolerant trees. Delucia et al. (1996) states that a larger fraction of air space in the leaf interior generates larger quantities of scattered light, increasing light absorption by chloroplasts within the mesophyll.

Likewise, decrease in ES within mesophyll, as was observed in medium and high irradiance treatments, can be related to a greater resistance to CO₂ diffusion inside the leaf (Hanba et al. 2002).

The lack of statistical significance observed in the soil moisture gradient could be due to a lower leaf phenotypic plasticity to changes in soil available water. On the other hand, it could be due to the fact that studied soil moisture levels, between 40 and 100% of soil capacity, were not low enough to generate responses in foliar anatomy of *N. pumilio* seedlings. Other authors also explain the lack of reaction of foliar anatomy to changes in soil moisture due to the application of extremely moderated levels of hydric stress in *Q. suber* (Aranda et al. 2004). However, interactions found in this study could mask some responses of the internal foliar anatomy to soil moisture levels, and can be explained by different acclimation strategies of *N. pumilio* seedlings under diverse environmental conditions. Some interactions occurred between soil moisture levels and months (e.g., for the UE proportion, SP and LCT) (Figure 3a, b and k). Upper epidermis proportion diminished from January to March in high soil moisture treatments (S80), as an anatomical response to enhance photosynthetic performance by focusing on the intercepted light (Markesteijn et al. 2007). Beside this, UE did not vary in low soil moisture treatments (S40) throughout the growing season, perhaps as a strategy to minimize evapotranspiration. The January to March period presented higher temperatures than the November to January period (Table 1), which increased stress conditions for plants growing under lower soil moisture levels. Spongy parenchyma also showed a steeper decrease under lower (S40) compared with those seedlings under higher soil moisture treatments (S80) throughout the growing season, which can be inferred as a xeromorphic characteristic oriented to limit water vapor diffusion to decrease evapotranspiration (Bosabalidis and Kofidis 2002) and thereby maintain turgidity (Terashima et al. 2001).

Another group of interactions occurred between irradiance levels and months during the growing season. For example, lamina thickness (LT) and PPT increased during the growing season (e.g. Figure 3c and d) under higher light intensities (I26 and I64), but remained stable under low irradiance (I4). PPT increases occurred due to cell elongation, but not by increasing the CFPP, which did not vary during the growing season. Hansen (1959) proposed that the state of the mesophyll structure depends on the irradiation at the time of bud break. However, greater leaf thickness can be explained by the increase of CFPP, PPT and cell sizes (PPCL and SPCW), as was stated by Aranda et al. (2001), who observed *Fagus sylvatica* L. seedlings in different irradiance gradients and described an increase in PPCW with a higher quantity of cells. Also, two *Miconia* species increased leaf width through the extension of

PP cells under full sun exposure (Rodrigues Marques et al. 2000).

Several authors have tried to classify leaves in shaded and sun categories, depending on the CFPP. Eschrich et al. (1989) categorized leaves with two prominent layers of PP, such as sun leaves, while those with only one layer of palisade cells are regarded as shade leaves. In *N. pumilio* leaves, differences in CFPP could also contribute to sun/shade leaf classification. Finally, interaction of UCT between irradiance and soil moisture gradients (e.g. Figure 3f) reflected leaf acclimation response to high light irradiance levels, mediated by widening of cuticle when soil moisture became a limiting factor. Rodrigues Marques et al. (2000) registered significant increasing in leaf cuticle thickness of two *Miconia* species growing under high light levels compared with low light levels. Likewise, lower thickness in the UCT under low irradiance and in soils without moisture restriction could be a mechanism to diminish reflectance, and then improve the efficiency in light interception (Chazdon and Kaufmann 1993).

The ordination analysis (PCA) allowed us to understand the distribution pattern of treatments in relation to the measured internal anatomic variables, which were highlighted for both irradiance levels and months. An inverse relation among ES and PPCW (associated with January and March values for I26 and I4 treatments) vs. SP (associated with November values for I64 treatments), as well as among leaf thickness, PPCL, PPT and UET (associated with January and March values for I64 treatments) vs. upper and LE proportion (associated with November values for I26 and I64 treatments) was observed. Likewise, cluster analysis showed that treatments at the end of the growing season were clearly differentiated by irradiance levels, with more similarities among I26 and I4 irradiance levels.

Nothofagus pumilio seedlings varied their foliar internal anatomy and external morphology according to the availability of light and soil moisture in a short period of time. Acclimation of seedlings to the new environmental conditions included great changes in palisade parenchyma size, epidermis thickness and tissue distribution inside mesophyll. This phenotypic plasticity may allow seedlings to quickly acclimate to several environmental changes, including those found in current silvicultural proposals which open the canopy to stimulate natural regeneration (Martínez Pastur et al. 2000, 2011a), in a wide range of stand types and growing conditions (Gea et al. 2004). Studies on morphological and anatomical responses in leaves, as well as about growth and eco-physiological performances (Lencinas et al. 2007, Martínez Pastur et al. 2007, Peri et al. 2009, Soler Esteban et al. 2011) allowed us to explain the previously observed ecological regeneration responses (e.g., Rosenfeld et al. 2006, Martínez Pastur et al. 2011a). According to these results, seedlings of *N. pumilio* could produce larger leaves, with a relatively higher proportion of photosynthetic

tissues, at moderate levels of natural incident irradiance and soil water availability, which are also the optimum conditions to maximize their photosynthetic performance (Martínez Pastur et al. 2007). These results support the proposal for modified silvicultural strategies within the context of new and more effective forest management, to achieve environmental conditions similar to those which could improve the growth rate of seedlings at the establishment phase.

Acknowledgments

The authors gratefully acknowledge Enrique Barrio and Emilce Gallo for their help during the greenhouse experiments and Christopher Anderson for his comments on the manuscript.

Conflict of interest

None declared.

Funding

This research has been supported by BOSAMCA-MIA (CATIE Costa Rica) and PAE2004 22428 (ANPCyT Argentina) projects.

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