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Research paper

Acclimation of leaf cohorts expanded under light and water stresses: an adaptive mechanism of *Eucryphia cordifolia* to face changes in climatic conditions?

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Eucryphia cordifolia Cav. is a long-lived evergreen tree species, commonly found as a canopy emergent tree in the Chilean temperate rain forest. This species displays successive leaf cohorts throughout the entire growing season. Thus, full leaf expansion occurs under different environmental conditions during growing such as air temperature, vapor pressure deficit and the progress of moderate water stress (WS). These climate variations can be reflected as differences in anatomical and physiological characteristics among leaf cohorts. Thus, we investigated the potential adaptive role of different co-existing leaf cohorts in seedlings grown under shade, drought stress or a combination of the two. Photosynthetic and anatomical traits were measured in the first displayed leaf cohort and in a subsequent leaf cohort generated during the mid-season. Although most anatomical and photosynthetic pigments did not vary between cohorts, photosynthetic acclimation did occur in the leaf cohort and was mainly driven by biochemical processes such as leaf nitrogen content, Rubisco carboxylation capacity and maximal Photosystem II electron transport rather than CO₂ diffusion conductance. Cohort acclimation could be relevant in the context of climate change, as this temperate rainforest will likely face some degree of summer WS even under low light conditions. We suggest that the acclimation of the photosynthetic capacity among current leaf cohorts represents a well-tuned mechanism helping *E. cordifolia* seedlings to face a single stress like shade or drought stress, but is insufficient to cope with simultaneous stresses.

Keywords: foliar development, multiple stresses, polytolerance dynamics.

Introduction

In dry and warm ecosystems, water stress (WS) usually occurs concomitantly with high light (HL) levels, and thus plants have evolved effective mechanisms to endure this specific stress combination. (See Table 1 for abbreviation details.) These include finely tuned stomatal regulation and higher investment in root biomass to improve water balance and photosynthetic

water-use efficiency (Brodrigg et al. 2009, Markesteijn and Poorter 2009, Flexas et al. 2012). Since it is crucial to maintain the redox state in the cell, plants under HL levels growing under WS reach higher photochemical efficiency and lower Photosystem II (PSII) excitation pressure than those under shade (Ensminger et al. 2000, Obata and Fernie 2012). Efficient light energy partitioning is achieved by increased

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Table 1. Abbreviations and units.

Abbreviation	Parameter	Units
<i>Treatments</i>		
HL	High light	–
LL	Low light	–
WW	Well watered	–
WS	Water stress	–
LC1	First leaf cohort	–
LC2	Second leaf cohort	–
Ψ_{MD}	Midday xylem total water potential	MPa
<i>Environment</i>		
PAR	Photosynthetic active radiation	$\mu\text{mol m}^{-2} \text{s}^{-1}$
PPFD	Photosynthetic photon flux density	$\mu\text{mol m}^{-2} \text{s}^{-1}$
IQF	Integrated quantum flux	$\text{mol m}^{-2} \text{s}^{-1}$
RH	Air relative humidity	%
<i>Leaf morph-anatomy</i>		
LMA	Leaf mass area	g m^{-2}
LT	Leaf thickness	μm
UET; LET	Upper and lower epidermis thickness	μm
SMT; PMT	Spongy and palisade parenchyma thickness	μm
PLN	Number of palisade layers	Unit
f_{ias}	Porosity of the lamina	$\mu\text{m}^2 \mu\text{m}^{-2}$
<i>Photosynthetic traits</i>		
LSP	Light saturation point	$\mu\text{mol photons m}^{-2} \text{s}^{-1}$
g_s	Stomatal conductance	$\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$
g_c	Cuticular conductance	$\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$
A_n	Net CO_2 assimilation	$\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$
A_{max}	Photosynthetic capacity at CO_2 400 mmol mol^{-1} air	$\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$
$V_{c,max}$	Apparent maximal velocity of Rubisco carboxylation	$\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$
J_{max}	Maximal electron transport rate through PSII	$\mu\text{mol electrons m}^{-2} \text{s}^{-1}$
R_n	Dark respiration	$\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$
R_d	Day respiration	$\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$
IWUE	Intrinsic water use efficiency	$\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$
SL	Stomatal limitations	%
NSL	Non-stomatal limitations	%
SL + NSL	Total limitations	%
C_i	Sub-stomatal CO_2 concentration	mmol mol^{-1} air
C_a	Atmospheric CO_2 concentration	mmol mol^{-1} air
LNC	Light nitrogen content	% (g g^{-1} dry matter)
<i>Photosynthetic pigments</i>		
Chla+b	Chlorophyll a+b	mmol mol^{-1}
Chla/b	Chlorophyll a/b	mmol mol^{-1}
Neo/Chla+b	Neoxanthin	mmol mol^{-1}
Lut/Chla+b	Lutein	mmol mol^{-1}
β -Car/Chla+b	β -Carotene	mmol mol^{-1}
VAZ	Xanthophyll pool size	mmol mol^{-1}
EPS	Epoxidation state	mmol mol^{-1}

investment in nitrogen (N) to electron transport chain components and Rubisco (Niinemets 2007, Flexas et al. 2012), as well as by larger xanthophyll pool size and de-epoxidation capacity (Demmig-Adams and Adams 1992, García-Plazaola et al. 2012). Additionally, photorespiration is a safety regulator that opportunely consumes more electrons when the stomata are closed under drought or HL stress, preventing oxidative damage (Voss et al. 2013). However, an increase in photorespiration

implies a decrease in carbon assimilation efficiency in addition to biomass synthesis and consequently in plant productivity (Zelitch 1973, Aliyev 2012, Weber and Bauwe 2013). In summary, mechanisms to endure this particular stress combination (HL and drought) diminish both the risk of photoinhibition and the accumulation of reactive oxygen in species (García-Plazaola et al. 1997), but may result in a reduced carbon balance (Coste et al. 2010, Egea et al. 2011, Wyka et al. 2012).

While the co-occurrence of drought and HL has been intensively studied, especially in Mediterranean ecosystems (Pereira and Chaves 1993, Chaves et al. 2003, Valladares et al. 2005, Quero et al. 2006), the combination of drought and shade has received less attention. Commonly, under limited light conditions, plant responses are focused on light capture maximization by increasing their chlorophyll concentration, proteins in the antennas and their leaf area ratio (Kitajima 1994, Niinemets 2010), at the expense of leaf thickness (LT), leaf mass per area and root/shoot ratio (Markestijn and Poorter 2009). Consequently, shade acclimation responses are most likely impaired under drought conditions, due to the evident morpho-physiological trade-offs under deep-shade conditions (Smith and Huston 1989, Sack et al. 2003, Niinemets and Valladares 2006). Although species tolerant to shade and drought have been reported, they are generally restricted to warm or oceanic temperate forests, characterized by an extended growing season (Sack 2004, Niinemets and Valladares 2006, Valladares and Niinemets 2007). The Chilean temperate rainforest is no exception, exhibiting a long active growth period of 7–8 months, which can extend from September to April (Di Castri and Hajek 1976). Considering the long growth season available in this ecosystem type, late successional species have evolved deep-shade tolerance under well-watered conditions (Lusk et al. 2006, Valladares and Niinemets 2008). However, tolerance to drought under recurrent deep shade must be constrained by the restrictive carbon balance exhibited by deep-shade tolerant plants living close to their light compensation point (Niinemets and Valladares 2006).

In addition, predicted global change involves the modification of several environmental factors that will lead to new situations of stress for terrestrial plants. Expected lower precipitation rates and increasing temperatures in many parts of the world will result in more prolonged and severe droughts in many ecosystems (Meehl et al. 2007). Climate predictions agree with the marked tendency towards drier and warmer conditions reported for northwestern Patagonia over the last century 1912–2002. Over this interval, warm season temperatures have increased by 0.06 °C per decade, whereas cold season precipitation records (~73.5% of annual totals) have declined at a rate of 4.85% per decade (Masiokas et al. 2008). In particular, since 1861 to date an ~30% decrease in precipitation has been instrumentally recorded 30 km from the study site (on average 5.8 mm year⁻¹, data from the Chilean Meteorological Service, El Tepual Airport weather station, Puerto Montt, Chile). Predictions from several coupled atmosphere–ocean general circulation models (Cubasch et al. 2001, Bradley et al. 2004) suggest that these climatic trends will extend well into the 21st century. Hence, a 25–40% decrease in summer precipitations is expected for south-central Chile over the next 70 years (Fuenzalida et al. 2006, IPCC 2007). Therefore, this forest type could become one of the most susceptible to future climate change, given the

high-shade tolerance of long-lived species and the progress of drought under shade during the growing season.

Evergreen species' plant acclimation potential involves the coordination of physiological changes in existing preformed leaves with new leaf production and turnover (Katahata et al. 2007, Ishii and Ohsugi 2011). In species with single short leaf flush at the beginning of the growing season from preformed buds, photosynthetic acclimation is constrained by initial leaf anatomical determination, and the adjustment to stressful conditions throughout the growing season is restricted to biochemical acclimation, i.e., N partitioning among photosynthetic proteins and leaf cohorts (Sims and Percy 1992, Naidu and DeLucia 1998, Oguchi et al. 2003, Niinemets et al. 2006). Conversely, due to the conspicuous environmentally driven morphogenesis of expanding leaves (Abrams and Kubiske 1990, Wyka et al. 2008), species with continuous leaf production could achieve full photosynthetic acclimation (i.e., at both anatomical and biochemical levels) in leaf cohorts developed during the growing season from neoformed buds or leaf primordia expansion (Clane and Harrinton 2007). Although there is evidence that preformed and neoformed leaves differ in their developmental and functional attributes, most studies that deal with leaf flushing have focused on light acclimation (Guédon et al. 2006, Ishii and Ohsugi 2011, Varela et al. 2012). Few studies have proposed multiple leaf flushing as an early acclimation strategy to optimize photosynthetic functioning under drought conditions; these include one in a dry monsoon forest with a short wet growing season (Elliott et al. 2006), and another in the canopy of tropical trees species (Kitajima et al. 1997). To the best of our knowledge, this is the first study that addresses continuous leaf production as an adaptive strategy that promotes polytolerance dynamics in response to environmental changes throughout the growing season.

The long-lived *Eucryphia cordifolia* Cav. is a canopy emergent species growing in low elevation temperate rainforests. It is mainly found <700 m above sea level in the Chilean Coastal Range under warm conditions and humid soil (Escobar et al. 2006). *Eucryphia cordifolia* presents a broad latitudinal distribution in central-southern Chile (36° to 42°S), limited to the north by the Mediterranean region and to the south by the northern Patagonic forest (Veblen 1985). *Eucryphia cordifolia* is one of the most widespread and economically promising tree species in this ecosystem, due to its fast growth, highly appreciated wood (used as lumber and for construction and furniture) and melliferous flowers. Regrettably, these properties are the reasons for the high anthropogenic pressure that has led to the current vulnerable conservation state of most of its populations. *Eucryphia cordifolia* regenerates from seedling banks and by roots and stump resprouts after clearcutting or fire (Veblen et al. 1979). Nevertheless, its sexual recruitment is strongly influenced by the interaction between light availability and summer drought (Figuerola et al. 2010). Considering these regeneration

restrictions, recently root sproutings have been suggested as a niche extension mechanism towards more illuminated and higher vapor pressure deficit (VPD) sites (Escandón et al. 2013). This species is currently exposed to substantial climate variations and exhibits continuous leaf formation and expansion during the growing season, also referred to as 'indeterminate growth' by Clane and Harrinton (2007). Its leaf life-span is also at least 2 years (Lusk et al. 2008). Since multiple flushing generally declines with tree age and is most vigorous and noticeable in young trees (Harrington 1991, Battey 2003), successive leaf cohort generation in young plants is most likely associated with regeneration success and thus with forest dynamics under environmental conditions. Consequently, this species' seedlings are an excellent model to study the effect of simultaneous drought and shade on successively displayed leaf cohorts.

Specifically, we evaluated how this stress combination determines leaf structure, biochemistry and subsequent photosynthetic functioning and its overall impact on plant survival. We hypothesized that the survival of plants subject to stress is adaptively mediated by the acclimation level reached by the leaf cohort developed throughout the growing season. In addition, we expected shade and drought stress to have an interactive effect, due to a lower acclimation capacity of leaf cohorts simultaneously exposed to these stress factors when compared with plants subject to a single stress (shade or drought).

Materials and methods

Plant material and growth conditions

Seeds collected at a local coastal forest were used to grow *E. cordifolia* seedlings in a nursery for 1 year under optimal water, mineral nutrition and light conditions. We selected 320 homogeneous seedlings (mean height \pm SE = 19.7 \pm 0.3 cm), which were placed in 5.8 l pots with perlite: peat moss (4 : 1 v/v) and 3 g l⁻¹ of Basacote 3 M (COMPO, Münster, Germany) and maintained under optimal conditions for 1 month. After this and 1 month before bud sprouting, two light treatments combined with two water availabilities were applied and maintained for 180 days throughout the growing season (October–March 2012). Light treatments consisted of full and 7% sunlight irradiance (hereafter HL and low light, LL, respectively) under polyethylene greenhouse covering (52% photosynthetic active radiation transmittance at the plant canopy). Light was attenuated with neutral shade meshes. During the second week after bud-break, midday maximum photosynthetic photon flux densities (PPFD) on five sunny days (mean \pm SD) were 1108 \pm 63 and 46 \pm 5 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for HL and LL, respectively, while daily integrated quantum fluxes (IQF) were 26.6 \pm 4.2 and 1.2 \pm 0.2 mol m⁻² day⁻¹, for HL and LL, respectively. The IQF values used corresponded to percentiles 10 and 90 of *E. cordifolia* regeneration in a secondary forest located in central-southern Chile (Katalapi Park: 41°31'8"S, 72°45'2"W; Escandón et al. 2013). Under each light treatment,

half of the plants were well watered (WW) and the other half were subject to moderate WS. Each treatment combination of light–water treatment consisted of a block of 80 plants, where WWHL was considered as the control treatment. Moderate WS was defined as that which produced a 65% decrease in the stomatal conductance (g_s) with respect to values of WW plants, following Flexas and Medrano (2002). g_s was measured daily within its maximum period (9:00–13:00 h) in five plants per treatment using an IRGA (LI-6400XT, Li-Cor, Inc., Lincoln, NE, USA). Whole-plant evapotranspiration was determined by weighing the pots daily at 18:00 h. Thereafter, WS intensity was controlled daily by the restitution of the evapotranspired water to maintain g_s values in the range of 70–80 mmol H₂O m⁻² s⁻¹ (Figure 1c and d). As for the remaining plants, g_s was measured daily with a SC-1 porometer (Decagon Devices, Inc., Hixson, TN, USA). The relation between IRGA and porometer g_s was fit using a polynomial regression (curve finder function, Curve Expert Professional software v.1.2.0, <http://www.curveexpert.net/>, Hixson, TN, USA), which allowed for individual irrigation control using LI-6400 XT g_s estimated values (Figure S1 available as Supplementary Data at *Tree Physiology* Online).

The midday total xylem water potential (Ψ_{MD}) was determined with a pressure chamber (Model 600, PMS Instrument Company, Albany, OR, USA) on two sunny days after moderate WS was reached under both light treatments. Since *E. cordifolia* has stalkless leaves, Ψ_{MD} was evaluated in the nearest leaf-supporting stem of 10 plants per treatment.

Meteorological stations recorded air temperature, relative air humidity and PPFd every 10 s for each of the light treatments with H21-002 HOBO, connected to S-LIA-M003 and S-THA-M0xx sensors (Onset, Cape Cod, MA, USA). Integrated quantum flux was determined as the sum of PPFd during the photoperiod. To better represent the environmental conditions during photosynthetic functioning, daily average values of air temperature and relative humidity (RH) were calculated within the photoperiod at PPFds >100 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

The growth season was defined as the period of time in which leaf primordia expansion occurred (from spring bud break until autumn growth recess extending from late September to late April). Studies dealing with differences between preformed and neoformed leaves and their phenology are generally scarce (Ishii and Ohsugi 2011) and are nonexistent for Chilean temperate rainforest species, which present 'indeterminate growth' (Clane and Harrinton 2007). Thus, we incorporated the following empirical observations to provide a broader context of the studied species under its natural conditions: (i) foliar development in *E. cordifolia* occurs at different time periods during the growing season under different environmental conditions. (ii) The number of full-expanded leaf cohorts generated in one growing season ranges from approximately three to five and leaf flushing increases with more favorable environmental conditions. (iii) *Eucryphia cordifolia* presents a delayed

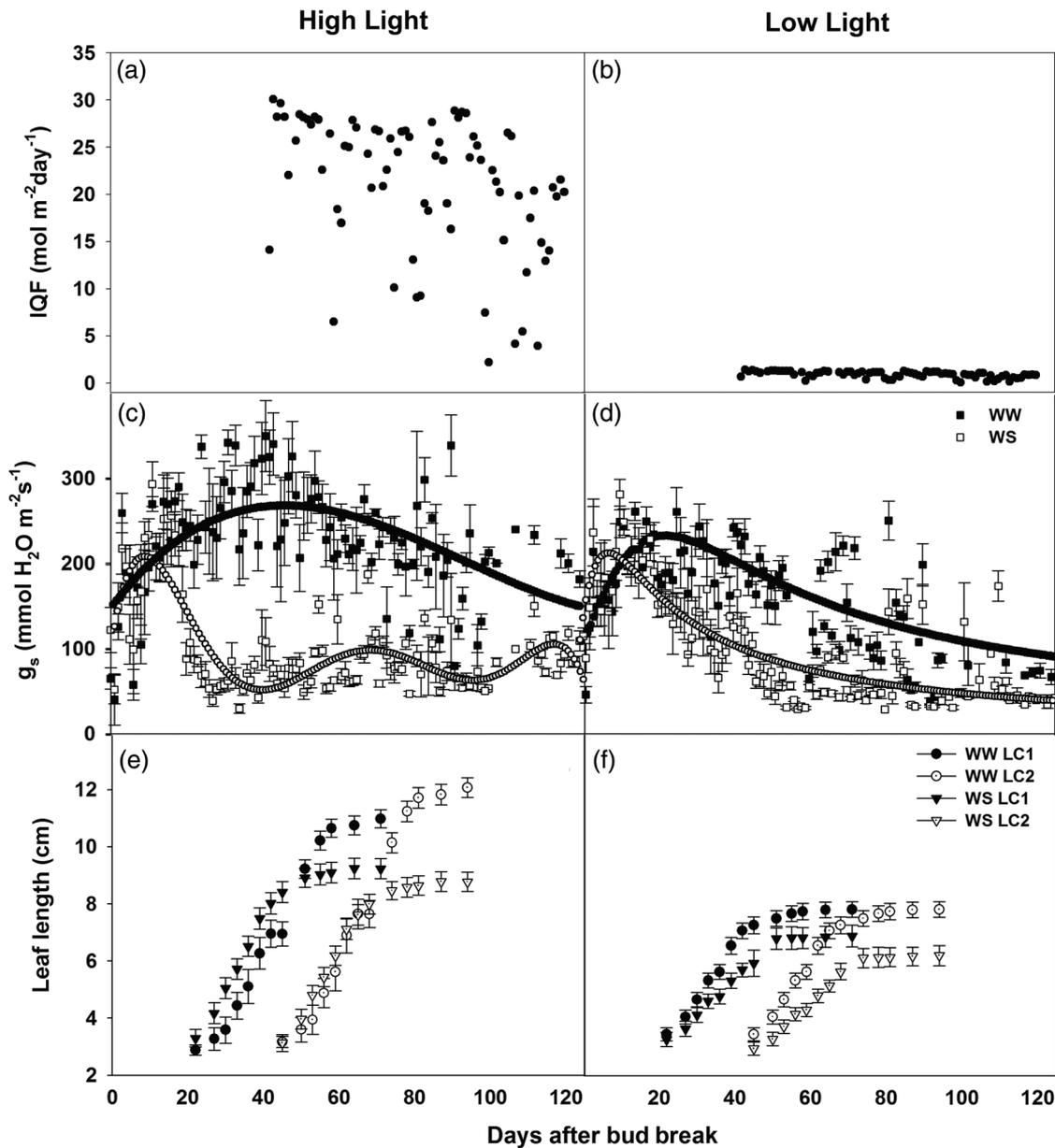


Figure 1. (a and b) Integrated quantum flux (IQF), (c and d) stomatal conductance (g_s) and (e and f) leaf length measured during the entire growing season in *E. cordifolia* plants grown under different light and water availabilities. Details of experimental setup are shown in Table 2. Symbols and treatments are as follows: in (c) and (d), full symbols represent well-watered (WW) and empty symbols water stress (WS). In panels, (e) and (f), filled circles, WW LC1; empty circles, WW LC2; filled triangles, WS LC1; empty triangles, WS LC2. Mean values \pm SE are shown. In (c) and (d), data were fit using: WWHL [polynomial regression in the form of $y = a + bx + cx^2 + dx^3$ ($R^2 = 0.61$)]; WSHL [polynomial regressions in the form of $y = (a + bx)/(1 + cx + dx^2 + ex^3 + fx^4 + gx^5 + hx^6)$, ($R^2 = 0.73$)]; WWLL [rational model $y = (a + bx)/(1 + cx + dx^2)$, ($R^2 = 0.82$)]; WSLL [rational model $y = (a + bx)/(1 + cx + dx^2)$, ($R^2 = 0.86$)] ($P < 0.001$ for all).

greening after full leaf expansion of ~ 1 or 2 months, and it is likely that the last elongated leaf cohort's full development is reached in the winter. (iv) The beginning of leaf detachment by senescence can occur at the end of the second growing season, concomitantly with evident self-shading.

To evaluate the differences between leaf cohorts, leaf-level measurements were performed in fully expanded leaf cohorts generated at different time periods during the growing season. The first leaf cohorts (LC1) developed 20–65 days after bud

break and the second leaf cohorts (LC2) 41–82 days after bud break. The number of leaves (mean \pm SE) generated in LC1 and LC2 under control conditions (WWHL) reached 51 ± 3 and 135 ± 7 , and under simultaneous water and LL stress (WSLL) they reached 21 ± 2 and 25 ± 2 , respectively.

Foliar anatomy and survival

Foliar anatomy was studied in the same leaves used for gas exchange (see below). Leaf sections ~ 1 mm thick were fixed in

p-formaldehyde (2%) and glutaraldehyde (4%) in a 0.1-M phosphate buffer solution (pH 7.2) in vacuum (Hanba et al. 2002). Afterwards, the samples were fixed in 1% osmium tetroxide for 1 h and dehydrated in a graded ethanol series, followed by three washings in propylene oxide. The dehydrated segments were embedded in Spurr's resin (Monocomp Instrumentación, Madrid, Spain) and cured in an oven at 60 °C for 48 h. Semi-thin (0.8 µm) cross-sections were cut with an ultramicrotome model Ultracut E (Reichert, Inc., NY, USA). Cross-sections were stained with 1% toluidine blue and viewed under an optical microscope (PrimoStar, Zeiss-Vision, Oberkochen, Germany) at 10 and 40× depending on the trait. Images were captured with a camera (AxioCam MR, Zeiss-Vision) coupled with a microscope. Total LT, upper and lower epidermis thickness, palisade and spongy parenchyma thickness, number of palisade layers and porosity of the lamina (estimated from the fraction of mesophyll tissue occupied by intercellular air space; f_{ias}) were determined following the methodology described by Tomás et al. (2013). All images were analyzed with image analysis software (ImageJ; Wayne Rasband/NIH, Bethesda, MD, USA). Leaf lengths of the two longer leaves of each cohort were recorded weekly in seven plants per treatment. Length measurements began when the leaves reached ~3 cm length and continued until they reached a constant length for at least 3 weeks. Mortality was determined by counting in each treatment dead individuals at the end of the experiment (plants were considered dead when all of their photosynthetic tissues were dry and brown, and no evidence of sprouting was observed for >2 weeks). The percentage of survival was calculated as the ratio (total plants by treatment at the beginning of the experiment minus the number of dead individuals)/(total plants by treatment at the beginning of the experiment) × 100.

Foliar nitrogen and photosynthetic pigments

Leaf N content (LNC, g g⁻¹ DM) per dry mass was determined in the same tissue used for LMA according to the Kjeldahl procedure (AOAC 1980). Photosynthetic pigments were quantified in three plants per treatment by collecting three leaf discs (3.8 mm diameter) from fully elongated leaves. Samples were collected at predawn and midday, immediately frozen in liquid N and stored at -80 °C. Discs (~30 mg fresh weight) were pulverized in a cold mortar with liquid N. The solution was added with the tip of a spatula with CaCO₃ (~1 mg) dissolved in 1 ml of 100% high-performance liquid chromatography (HPLC) grade acetone added before extraction, at 4 °C under LL. Pigments were separated and quantified by reverse-phase HPLC (García-Plazaola and Becerril 1999), equipped with a quaternary pump with an automatic degasification system and an automatic injector. Signals from a diode matrix detector were integrated and analyzed with Agilent Chem Station B.04.01 software (Agilent Technologies, Waldbronn, Germany). Chromatography was performed in a reverse-phase Spherisorb

ODS-1 column (5 µm particle size; 4.6 × 250 mm, Atlantil Hilit, Waters, Dublin, Ireland) and a Nova-Pack C-18 guard column (4 µm; 3.9 × 20 mm) (Waters). The mobile phase was binary: Solvent A, acetonitrile : methanol : Tris buffer (0.1 M, pH 8.0) (42 : 1 : 7) and Solvent B: methanol : ethyl acetate (34 : 16). Pigments were eluted using a linear gradient of 100% A to 100% B for the first 12 min, followed by isocratic elution with 100% B during the next 6 min. Absorbance was monitored at 445 nm. Retention time and response factors of Chl_a, Chl_b, lutein, β-carotene, violaxanthin (V), anteraxanthin (A) and zeaxanthin (Z) were determined by injecting pure standards (DHI, Hoersholm, Denmark). The epoxidation state (EPS) was determined as $V + 0.5A/VAZ$. To evaluate the impact of varying N investments in chlorophyll and pigment-binding proteins on leaf light-harvesting efficiency, the leaf chlorophyll content was expressed in N equivalents (C_B) as the ratio [mmol, Chl_a + b (g LNC⁻¹)] (Niinemets and Tenhunen 1997, Niinemets 2007).

Gas exchange and quantitative analysis of photosynthetic limitations

All gas exchange measurements were performed in fully expanded leaves during the morning hours (9:00–13:00 h) with an IRGA Li-6400XT (Li-Cor, Inc.). The flux was set to 300 ml min⁻¹. Block temperature was kept at 20 °C and air RH was maintained under ambient conditions (leaf temperatures and RH: 16–25 °C and 49–63%), with VPD of 1.33 ± 0.13 kPa.

Photosynthetic light response curves were plotted at a CO₂ concentration (C_a) of 400 µmol mol⁻¹, and varying PPFDs from 2000 to 0 µmol m⁻² s⁻¹ (90 : 10% red : blue light), with 12 different intensity levels. The light saturation point (LSP) was calculated with Photosynthesis Assistant software 1.1 (Dundee Scientific, Dundee, UK).

The response of net CO₂ assimilation (A_N) to varying C_a was conducted at PPFDs 20% higher than the LSP for each treatment, and consisted of 13 different CO₂ concentrations between 50 and 2000 µmol mol⁻¹, following the procedures described in Galmés et al. (2012). Carbon dioxide leakage into and out of the leaf cuvette was assessed as in Flexas et al. (2007) and the data corrected accordingly. The net CO₂ assimilation (A_N), the stomatal conductance (g_s) and the substomatal CO₂ concentration (C_i) were recorded from steady-state measurements at a C_a of 400 µmol mol⁻¹ and the intrinsic water-use efficiency (IWUE) was calculated as the ratio A_N g_s⁻¹.

Due to low g_s values under stressful conditions, cuticular conductance (g_c) could result in erroneous estimates of C_i (Boyer et al. 1997). Since *E. cordifolia* is hypostomatous, g_c was determined by measuring g_s under the same conditions for A_N–C_i curves at a C_a of 400 µmol mol⁻¹, but with the leaf abaxial surface covered with silicone grease and a polyethylene filter to prevent stomatal gas exchange. These data were used to recalculate g_s and C_i as described previously (Boyer et al. 1997, Flexas and Medrano 2002). Mitochondrial respiration (R_n) rates

were measured after the plants were kept in the dark for 120 min. Non-photorespiratory CO_2 released in the light (R_d) was considered as half of R_n (Niinemets et al. 2005, Gallé et al. 2011). Apparent $V_{c,\max}$ and J_{\max} were calculated by fitting the Farquhar et al. (1980) model to A_N-C_i curves. Temperature dependence on kinetic parameters of Rubisco described on a C_i basis by Bernacchi et al. (2001) was used as explained by Galmés et al. (2012). Accordingly, a quantitative limitation analysis of stomatal (SL) and non-stomatal limitations (NSL) of photosynthesis was performed using values of A_N , g_s and $V_{c,\max}$, assuming that the reference maximum assimilation rate is defined as a standard (Wilson et al. 2000). The maximum assimilation rate, along with g_s and $V_{c,\max}$, was reached under WWHL conditions; therefore, WWHL was used as the reference for the quantitative limitation analysis.

Statistical analyses

Generalized linear models (GLM) and analysis of variance were used to evaluate differences between light and water availability treatments, and foliar cohorts. An HSD test ($P < 0.05$) was used to carry out post hoc analysis. Normality and homogeneity of variance were evaluated with Shapiro–Wilk ($P < 0.05$) and Levene ($P < 0.05$) tests, respectively. When appropriate, variables were transformed to follow the former assumptions (Quinn and Keough 2006). Generalized linear models and deviance analyses were used to compare the number of layers in palisade parenchyma and survival, assuming Poisson and Binomial error distributions, respectively (McCullagh and Nelder 1989). A principal component analysis (PCA, correlation matrix) was used to study the relationships between morpho-physiological leaf cohorts' acclimation responses and survival. Thus, PCA was conducted exclusively with the average values of traits that showed significant differences among leaf cohorts. A linear regression analysis was conducted to assess the relationship between the photosynthetic capacity and PC1 factor scores. Finally, we defined the leaf cohort acclimation index as the absolute difference between LC2 and LC1 for each PC1 and PC2 factor score.

Results

Environmental conditions during leaf development

Under HL conditions the IQF ranged between 5 and 30 mol $\text{m}^{-2} \text{day}^{-1}$, with an average of 26.6 mol $\text{m}^{-2} \text{day}^{-1}$; for the LL treatment, IQF ranged between 0.1 and 1.5 mol $\text{m}^{-2} \text{day}^{-1}$ with an average of 1.2 mol $\text{m}^{-2} \text{day}^{-1}$. The lowest values in both light conditions corresponded to cloudy days (Figure 1a and b). Consequently, LL plants grew at 26% lower VPD compared with HL plants, due to a 6.6% lower mean air temperature and a 12.4% higher mean air RH (Table S1 available as Supplementary Data at *Tree Physiology* Online). Environmental conditions changed significantly during the growing season, exhibiting similar reduction rates in

both light treatments; air temperature and VPD decreased by 0.42 °C and 0.11 kPa week⁻¹, respectively (Table S1 available as Supplementary Data at *Tree Physiology* Online).

Stomatal conductance values during the LC1 expansion period of WW plants were on average higher at HL (32%; Figure 1c and d). After 60 days of water suppression, moderate WS levels were reached in the two light treatments, with average g_s values ranging between 70 and 90 mmol $\text{H}_2\text{O m}^{-2} \text{s}^{-1}$. Nevertheless, the higher evaporative demand under HL conditions determined that drought-induced reductions in g_s began 25 days earlier than under LL conditions (Figure 1c and d). On the contrary, LC1 in WSLL was almost totally expanded before they were exposed to WS, as revealed by g_s (Figure 1f). The previous situation determined that LC1 in WSHL was displayed under WS conditions generating a fully acclimated cohort. This allowed discrimination between partially and fully acclimated leaves to WS in LL conditions, which is similar to the progression of drought in the field. Midday xylem total water potential (Ψ_{MD} : mean \pm SE) of non-stressed plants (WWHL) was -0.31 ± 0.05 MPa, while WS plants under HL and LL conditions reached slightly, but significantly lower values than WWHL; -0.40 ± 0.03 MPa, $P < 0.016$ and -0.8 ± 0.09 MPa, $P < 0.001$, respectively. For the two light treatments, the first leaf cohort (LC1) was developed under similar temperatures, but lower air RH, and consequently under higher VPD. Low IQF during LC2 unfolding implies higher air RH%, but not changes in air temperatures, therefore the lower VPD must be attributed to an intra-seasonal increment in RH% (Table S1 available as Supplementary Data at *Tree Physiology* Online).

Leaf anatomy and plant survival

Fully expanded leaf length was modulated by resource availability. LC2 leaves were 10% longer than LC1 in the high-resource environment (WWHL), but 10% smaller under low resources (WSLL). Leaves developed under LL and WS were significantly smaller than those in other treatments, reflecting the interactive effect of both stresses (Figure 1e and f). The higher LMA values shown by HL plants were consistent with the increase in LT (Table 2), which indeed was determined by the large spongy and palisade parenchyma thickness reached under HL. There was a marginally significant interaction (water \times light \times LC: $P = 0.068$), which was reflected in the lower LMA of LC2 for WWLL plants (Figure 2b). Water availability did not affect either LT or leaf porosity (f_{ias}), while f_{ias} decreased with the interaction (light \times LC: $P = 0.002$) just as LMA did. This reflects that the lower LMA of LC2 for WWLL was completely driven by the decrease in f_{ias} in new fully expanded leaves in WWLL (Figure 2b, Table 2). Epidermis thickness (adaxial and abaxial) and number of palisade parenchyma layers did not change with light, water or LC (Table 2). We found 100% survival in WW treatments. Conversely, in WS treatments survival was 83.8% under HL and 67.5% under LL.

Leaf nitrogen content and photosynthetic pigments

The leaf N content (LNC) was higher in WS ($P < 0.001$), LL ($P = 0.005$) and in LC2 ($P < 0.001$). There was a significant interaction among the three factors ($P = 0.029$), due to the higher LNC reached in the LC2 of the WWLL treatment (Figure 2c and d).

Light was the main factor explaining pigment composition (on average, 63% for samples collected at predawn and 87% for samples collected at midday). Thus, at midday the total chlorophyll (Chla + b) content was lower in HL. However, the ratios of Chla/b and β -carotene/Chl did not change with

light treatments (Table S2 available as Supplementary Data at *Tree Physiology* Online). Water availability did not affect these variables in different leaf cohorts. The amount of chlorophyll corresponding to a unit of N invested in light-harvesting 'chlorophyll binding' (CB) was mainly affected by the light conditions ($P < 0.001$); it proved to be higher in leaves developed under LL regardless of water condition and leaf cohort. The changes in the ratios of neoxanthin and lutein to chlorophyll at predawn were independent of light and water availability in the different leaf cohorts. Lutein content increased with WS independently of light availability and leaf cohort. The

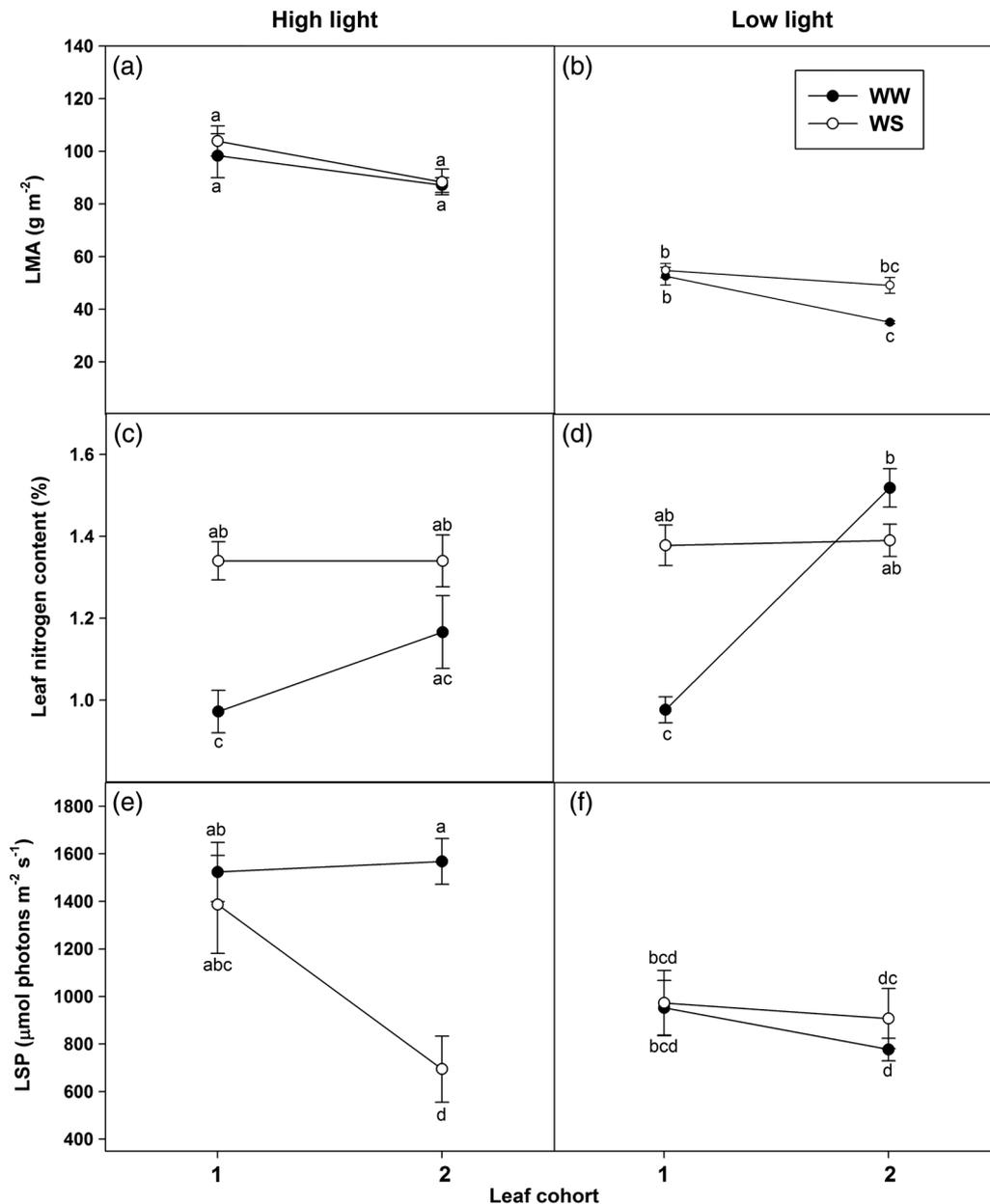


Figure 2. Leaf mass area (LMA: g m⁻², a and b), leaf N content (LNC: g g⁻¹, c and d) and light saturation point (LSP: μmol photons m⁻² s⁻¹, e and f) measured in the first displayed leaf cohort (LC1) and in a subsequent cohort generated during the mid-season (LC2) of *E. cordifolia* plants grown under different light and water availabilities [well watered (WW) and water stress (WS)]. Values are mean ± SE ($n = 5$). Different letters indicate significant differences between environmental treatments and leaf cohorts (HSD test, $P < 0.05$).

xanthophyll cycle pool size (VAZ) increased mainly with HL availability ($P < 0.001$) and to a lower degree with WS. These two factors explained 85 and 4% of this increment, respectively. This was determined by the increments of violaxanthin at predawn and of antheraxanthin at midday. No interactions were observed among light and water availability, and leaf cohort for any photosynthetic pigment (Table S2 available as Supplementary Data at *Tree Physiology* Online). The xanthophyll cycle's daily activity in HL plants was reflected in daily changes in EPS. Thus, light availability explained 29 and 85% of the increment in EPS at predawn and midday, respectively. However, VAZ-cycle activity was absent in LL. Overnight retention of de-epoxidized xanthophylls (A and Z), which leads to low predawn EPS, was observed only under WS ($P < 0.001$), explaining a 30% of the variation of this parameter (Table S2 available as Supplementary Data at *Tree Physiology* Online).

Gas exchange and quantitative limitation analysis of photosynthesis

The LSP was higher in HL and remained unchanged between water availabilities and leaf cohorts, with the exception of a 50% decrease under WS of the LC2 compared with LC1 under HL (Figure 2c and d). The assimilation rate (A_{\max}) measured at a C_a of $400 \mu\text{mol mol}^{-1}$ and saturating PPFs decreased by 40% in LL ($P < 0.001$) and 42% under WS ($P < 0.001$) (Figure 2a and b).

A_{\max} changed with leaf cohort ($P = 0.017$), with the exception of WSL where no differences among cohorts were found. Water stress in HL decreased A_{\max} by 39 and 57% in LC1 and LC2, respectively, but in LL WS decreased A_{\max} by ~30% in both cohorts (Figure 3a and b). The IWUE changed with water, light and leaf cohort ($P < 0.001$ respectively). The main differences between treatments were due to water condition; IWUE was 36% higher under WS, independent of the light and leaf cohort, with the exception of LC2 in LL where WW was 7.5% higher than WS. Stomatal conductance (g_s) changed with water and light treatments ($P < 0.001$). Water stress under HL decreased g_s by 68 and 78% in LC1 and LC2, respectively. In LL, WS decreased g_s by 63% in LC1 and by 24% in LC2 (Figure 3c and d). Cuticular conductance (g_c) was not affected by light, water or leaf cohort, reaching on average $0.012 \pm 0.007 \mu\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$. The maximum carboxylation rate ($V_{c,\max}$) was 96% higher in HL ($P < 0.001$). Under WS conditions, $V_{c,\max}$ decreased 19% in HL and 30% in LL. $V_{c,\max}$ was 30% lower in LC2 with the exception of WWHL and WSL, where there was no difference between leaf cohorts (Figure 3e and f). Maximum electron transport rate (J_{\max}) was 94% higher in HL ($P < 0.001$). Under WS conditions, J_{\max} decreased by 24% in HL and by 18% in LL. J_{\max} was 17% lower in LC2 (Figure 3g and h). R_d was 38% higher in LL and was affected by the interaction between water and LC (Figure 3i and j).

The quantitative limitation analysis of photosynthesis highlights the previously observed patterns between light–WS

treatments and leaf cohorts. Leaves grown under moderate WS (in either HL or LL) had higher stomatal limitations (SL), representing 47% in HL and 46 and 53% in LL in LC1 and LC2, respectively (Figure 4). Non-stomatal limitations were higher in WSL reaching 18% in LC2. Shaded leaves and fully expanded leaves under WW conditions demonstrated both types of limitations, 17–24% for LC1 and 24–42% for LC2. In general, the total limitations (SL + NSL) in LL were 23% higher in LC2. It is important to note that even new cohorts which developed under WWHL showed improved behavior. Stomatal limitation was the main limitation when WS was imposed, independent of the light treatment. Limitations between light treatments without WS showed a more compensated proportion between SL and NSL (Figure 4).

Principal component analysis was conducted with the six explanatory variables that changed among cohorts, while A_{\max} and survival were considered as supplementary variables. The first two principal components explained 79% of the total variance. PC1 explained 56% of the variance, showing that A_{\max} of leaf cohorts had a strongly positive covariate with $V_{c,\max}$, J_{\max} and LSP; these variables were the main contributors to PC1 with 27, 27 and 24%, respectively (Figure 5a). Conversely, A_{\max} showed a negative covariate with N in mass basis. The importance of the biochemical acclimation in determining the photosynthetic capacity was confirmed by the close and significant relationship between PC1 factor scores and A_{\max} [$\ln(A_{\max}) = 2.2097 - 0.4617 \text{ PC1}$; $R^2 = 76\%$, $P = 0.005$]. On the other hand, PC2 explained only 23% of the total variance, R_d and f_{ias} showed opposite covariation contributing 47 and 42% to PC2, respectively.

Leaf cohorts' acclimation exhibited different responses to each treatment, as noted by their distance on the factor plane with the exposition to single or multiple stresses (Figure 5b). The later-developed leaf cohort (LC2) in the control (WWHL) and multiple stress (WSLL) treatments showed few changes in PC1 and PC2, located in the same quadrant of LC1. On the contrary, LC2 in both single stress treatments (WSHL and WWLL) showed a substantial change in PC1 and PC2, indicating high acclimation responses (Figure 5b). Finally, the anatomical, biochemical and physiological acclimation capacity of the later-developed leaf cohort, estimated as the acclimation index, reflected a strong relationship with plant survival under single stress (shade or drought), but not under simultaneous stresses (Figure 6).

Discussion

Drought tolerance under shade

In recent years, a number of studies, principally in Mediterranean ecosystems (Chaves et al. 2003, Valladares et al. 2005, Quero et al. 2006), have been conducted to evaluate photosynthetic responses to WS under different light availabilities, focusing on

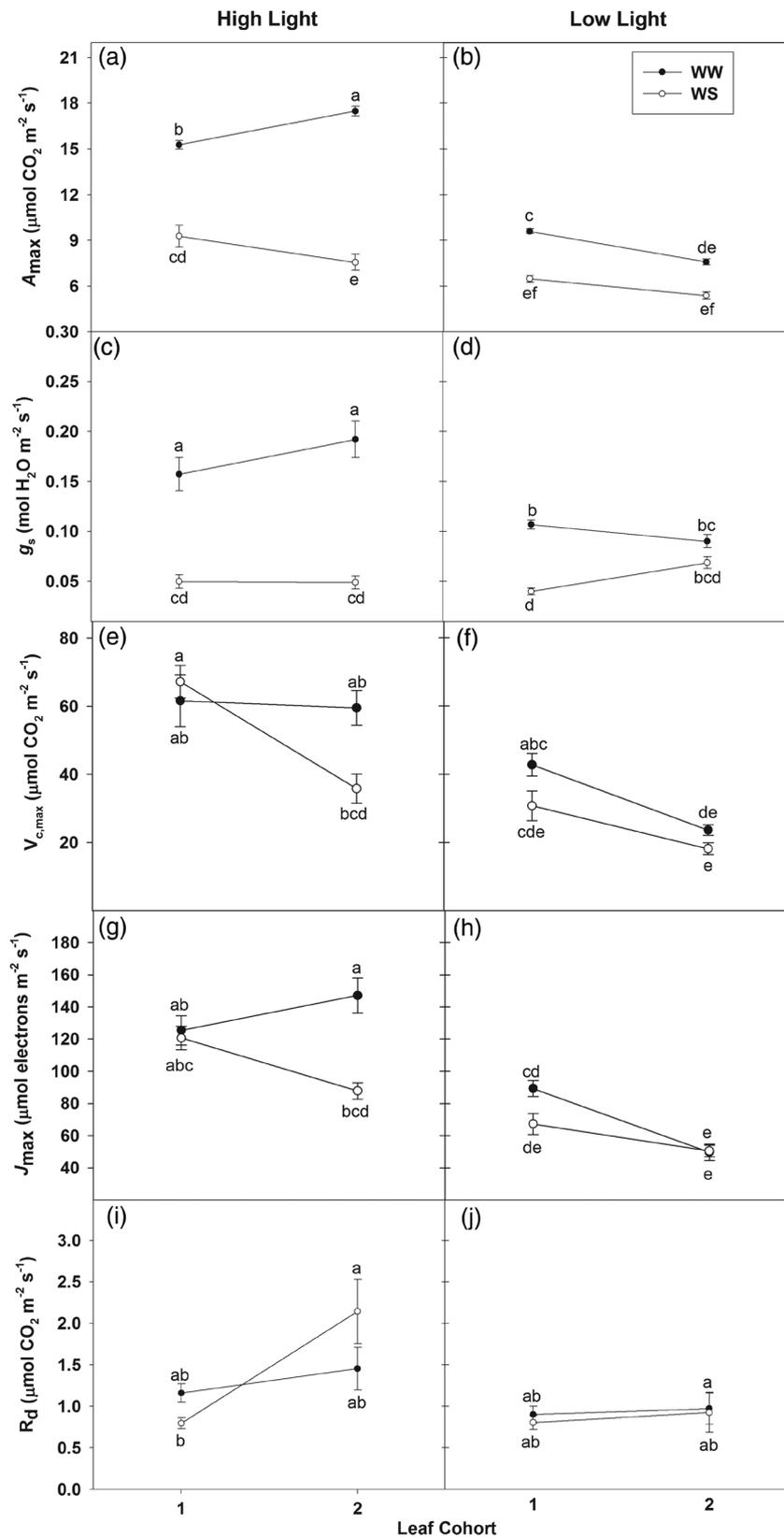


Figure 3. Photosynthetic parameters among leaf cohorts. Photosynthetic capacity (A_{max} , a and b), stomatal conductance (g_s , c and d), apparent maximal velocity of Rubisco carboxylation ($V_{c,max}$, e and f), maximal electron transport rate through PSII (J_{max} , g and h) and day respiration (R_d , i and j) measured in the first displayed leaf cohort (LC1) and in a subsequent cohort generated during the mid-season (LC2) of *E. cordifolia* plants grown under different light and water availabilities (WW and WS). Values are mean \pm SE ($n = 5$). Different letters indicate significant differences between environmental treatments and leaf cohorts (HSD Test, $P < 0.05$).

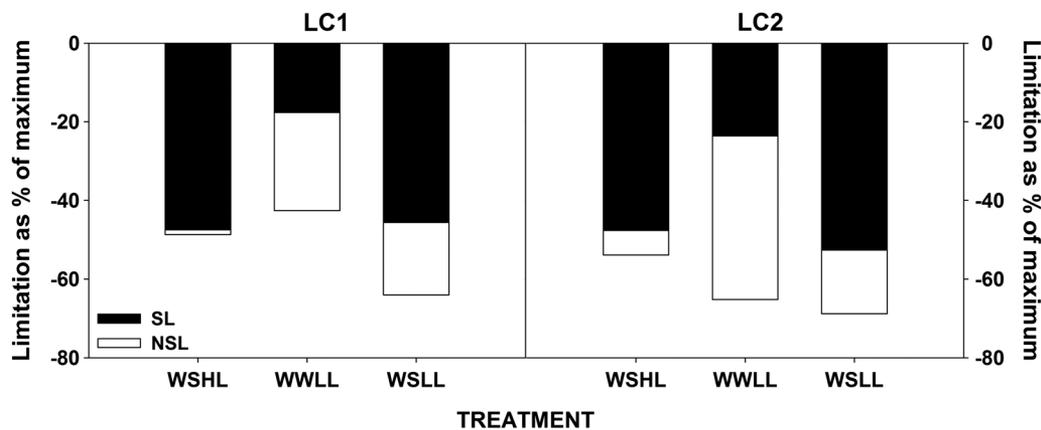


Figure 4. Quantitative limitation analysis of photosynthesis evaluated in the first displayed leaf cohort (LC1) and in a subsequent cohort generated during the mid-season (LC2) of *E. cordifolia* plants grown under different light and water availabilities (WW and WS). SL and NSL indicate stomatal and non-stomatal limitations. The maximum assimilation rate, concomitantly with g_s and $V_{c,max}$ was reached under WWHL conditions (Figure 3); therefore, WWHL was used as the reference.

the relevance of these responses in forest dynamics under current and predicted climate conditions (Niinemets and Valladares 2004, 2006). In particular, the mechanisms underlying drought tolerance under shade remain unresolved and differ among species and habitats (Valladares and Pearcy 2002). There are few studies, which show somewhat contradictory results (Valladares and Pearcy 2002, Quero et al. 2006, Markesteijn and Poorter 2009) that can be grouped into three hypotheses: (i) shade may lessen drought's impact on woody seedlings, by reducing overheating, VPD and oxidative stress (Valladares and Pearcy 1998, Holmgren 2000); (ii) independent effects of shade and drought have been reported in the relative growth rate and biomass allocation of some woody plants (Sack 2004, Markesteijn and Poorter 2009); and (iii) some authors propose a rather counterintuitive observation, affirming that drought provokes stronger photoinhibition in the shade than under full sunlight (Valladares et al. 2005). In our opinion, the origin of these discrepancies could be the inadequate comparison of experiments, which have studied from instantaneous to mid-term responses and have varied in terms of the magnitudes of environmental variations considered. For instance, some studies have shown similar magnitudes of photosynthetic down-regulation when exposed to single moderate drought or shade stress, while the simultaneous exposition to both stresses has provoked a larger down-regulation of photosynthesis (Quero et al. 2006, Guidi et al. 2008). In agreement, we found that shade or WS induced similar decreases in the photosynthetic rates in each case, i.e., ~50% when compared with WWHL as the control. In addition, the photosynthesis reduction under simultaneous stresses was ~64%, just 14% more than the reduction found under a single stress (Figure 3a and b). Nevertheless, this small reduction was accompanied by an 87% decrease of the total plant biomass (data not shown) and a 50% increase in mortality compared with WSHL (Figure 6).

Leaf cohorts acclimation as an adaptive mechanism to face changes in climate

Despite the fact that 'indeterminate growth' is fairly common in some temperate evergreen species, and that leaves are highly plastic depending on the prevailing environment during their expansion (Nobel et al. 1975, Jurik 1986, Mulkey et al. 1992), the functional significance of successive leaf generation as a plant acclimation mechanism is not well understood (Ishii and Ohsugi 2011). Our results provide new insights into acclimation responses to water and light stress, addressing the role of continuous leaf generation as a polytolerance dynamic mechanism. We found that the photosynthetic capacity of leaf cohorts which fully expanded during the growing season showed high acclimation in plants subjected to either shade or drought. This was reflected in structural and biochemical changes, resulting in the down-regulation of LSP and A_{max} (Figures 2e and f, 3a and b; Rijkers et al. 2000, Chaves et al. 2003, Warren et al. 2007, Coste et al. 2009). The reductions in LSP and A_{max} were explained by decreases in several key biochemical processes for CO_2 assimilation, such as RuBP regeneration and $V_{c,max}$ (among other biochemical traits), which are concomitant with the prolonged decrease in g_s caused by shade or drought (Figure 3c–h; Flexas and Medrano 2002). Conversely, newly fully expanded leaves under non-limiting supplies of light and water were able to funnel more energy through photochemistry and showed enhanced photo-protection capacity (i.e., higher xanthophyll pool size; Demmig-Adams and Adams 1992). Therefore, acclimation during the growing season implies photosynthetic up-regulation and increased resource use (Figure 3g, Table S2 available as Supplementary Data at *Tree Physiology* Online; Kitajima et al. 1997, Guédon et al. 2006, Ishii and Ohsugi 2011). Even though the mid-season foliage expansion was not restricted by the initial unstressed

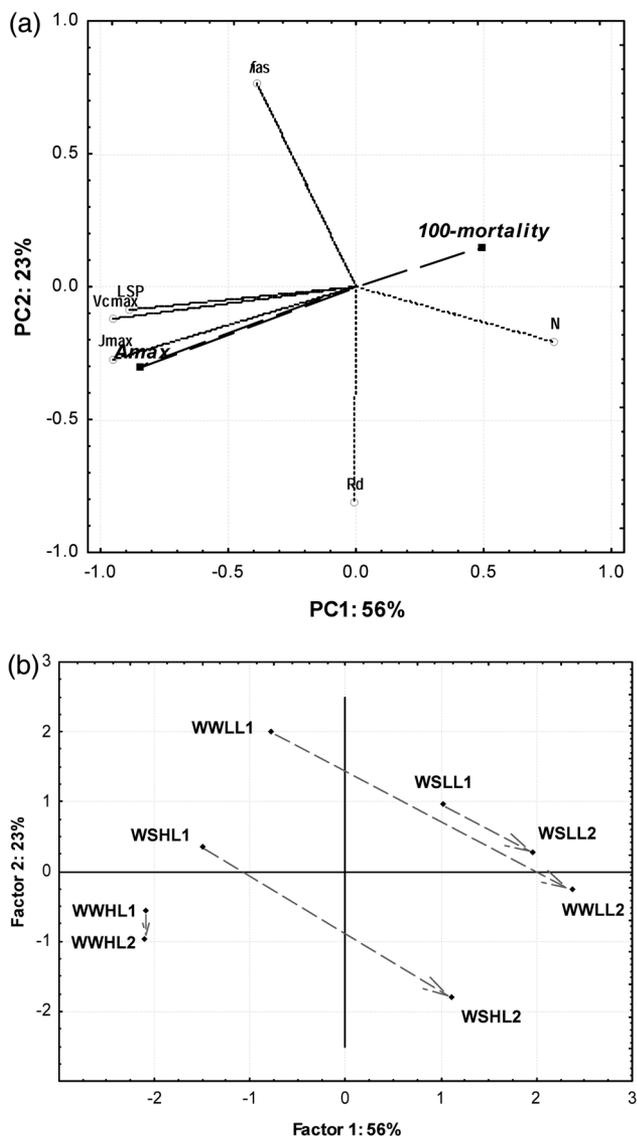


Figure 5. Principal component analysis considering the anatomical, biochemical and functional leaf traits that showed differences between leaf cohorts. (a) Projection of the leaf traits on the bi-dimensional space defined by the first two principal components. Active variables included were: porosity of the lamina (f_{ias}), leaf N content per dry mass (N), apparent maximal velocity of carboxylation of Rubisco ($V_{c,max}$) and maximal electron transport rate through PSII (J_{max}), light saturation point (LSP) and day respiration (R_d). Plant performance traits were included as supplementary variables (A_{max}) and plant survival (100-mortality). (b) Projection of leaf cohorts subjected to different combinations of light and water availabilities in the same bi-dimensional space.

anatomical determination, variations in leaves' biochemistry were the most responsive traits. Within the few anatomical traits that interact with light and water availability, the lowest LMA reached under shade and drought in mid-season fully expanded cohorts was driven by the decrease in leaf density and not by changes in leaf thickness (Figure 2b, Table 2). Similarly, increases in LMA induced by larger mesophyll tissue per unit leaf area have been reported by Kitajima et al. (1997), and these increases were more marked in leaves

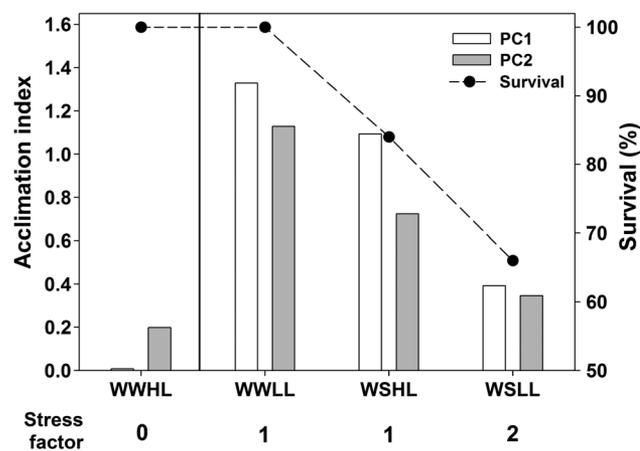


Figure 6. Acclimation of leaf cohorts as an adaptive mechanism to face changes in climatic conditions. Relationship between the leaf cohort acclimation index and plant survival: acclimation index was calculated as the absolute difference between leaf cohorts in the factor scores for each principal component axis. Stress factor denotes the number of stresses applied. Vertical solid line is used to separate the control from stress treatments. Dashed line indicates plant survival percentage under single and simultaneous stresses.

which extended at the end of the rainy season than in leaves which expanded at the beginning. In this study, late flushing was suggested as a pre-acclimation strategy preceding the coming dry season's conditions, like many plant phenological events which often precede the occurrence of optimal environmental conditions, and for which day length or temperature drop may be used as cues (Wright 1996).

Overall, for both of the leaf cohorts, total limitation of photosynthesis was higher in plants subjected to simultaneous stresses, supporting the interactive responses found by Valladares et al. (2005) and negating the independent responses to simultaneous stresses reported by Sack (2004) and Markesteijn and Poorter (2009). The processes limiting photosynthesis varied depending on the exposure to one or both stresses (Figure 4). As expected, photosynthesis was mostly constrained by SL under drought (Limousin et al. 2010, Misson et al. 2010, Gallé et al. 2011), while in the shade there was a more balanced proportion between SL and NSL. Thus, the interactive decrease in photosynthesis exerted by foliage developed during the mid-season and exposed to both stresses was attributed to higher NSLs, contrary to those in plants subjected only to drought. Nevertheless, total photosynthesis limitation alone did not explain the great differences in survival observed among stress treatments. While photosynthesis was almost equally reduced in plants subjected to drought or shade compared with control plants, mortality was only detected in water stressed plants. Noticeably, the mid-season's acclimation level of fully expanded leaf cohorts in stressed plants strongly correlated with plant survival (Figure 6). Under a single stress, plants were able to adjust their photosynthetic machinery (down-regulation at a biochemical level) to maintain plant homeostasis (Huner et al.

Table 2. Leaf anatomical properties measured in the first fully expanded leaf cohort (LC1) and in a subsequent fully expanded cohort during the mid-season (LC2) of *E. cordifolia* plants grown under different light and water availabilities. Light treatments consisted of full and 7% sunlight reaching integrated quantum fluxes of 26.6 ± 4.2 and 1.2 ± 0.2 mol m⁻² day⁻¹. Water treatments were well watered (WW) and moderate water stress (WS) defined as 0 and 65% decreases in g_s with respect to the control. Given are leaf thickness of (LT), upper and lower epidermis (UET; LET), spongy and palisade parenchyma (SMT; PMT), palisade layers number (PLN) and porosity of the lamina (f_{ias}). Mean \pm SE, $n = 3$ are shown. Different letters indicate significant differences between environmental treatments and leaf cohorts (HSD test, $P < 0.05$).

Treatment	LC	LT (μm)	UET (μm)	LET (μm)	SMT (μm)	PMT (μm)	PLN	f_{ias}
WWHL	1	227.3 \pm 10.9a	31.6 \pm 1.0a	11.8 \pm 0.6a	114.0 \pm 19.9a	68.0 \pm 10.2ab	2.3 \pm 0.3a	0.004 \pm 0.001ab
	2	216.1 \pm 34.0a	29.4 \pm 0.9a	12.1 \pm 0.3a	109.4 \pm 8.3a	56.6 \pm 17.2abc	3.0 \pm 0.0a	0.005 \pm 0.001abc
WSHL	1	228.3 \pm 25.4a	31.6 \pm 1.8a	12.6 \pm 0.8a	96.6 \pm 2.2abc	90.1 \pm 6.6a	4.3 \pm 0.9a	0.006 \pm 0.001ac
	2	247.5 \pm 32.5a	30.2 \pm 3.7a	12.3 \pm 0.6a	125.0 \pm 2.9a	80.6 \pm 6.7a	4.0 \pm 0.6a	0.004 \pm 0.001abc
WWLL	1	199.2 \pm 1.1ab	33.7 \pm 0.7a	14.6 \pm 0.8a	120.0 \pm 5.9ac	15.0 \pm 0.7c	2.0 \pm 0.0a	0.009 \pm 0.001a
	2	131.0 \pm 6.3b	29.8 \pm 1.2a	10.6 \pm 0.6a	75.2 \pm 4.7bc	33.4 \pm 3.8c	3.5 \pm 0.5a	0.003 \pm 0.000b
WSLL	1	147.6 \pm 8.6ab	29.8 \pm 2.4a	13.4 \pm 2.0a	83.7 \pm 5.0abc	25.6 \pm 2.3c	2.7 \pm 0.7a	0.006 \pm 0.001ac
	2	121.8 \pm 6.3b	27.0 \pm 0.8a	8.7 \pm 0.6a	66.7 \pm 2.9b	34.9 \pm 2.8bc	2.3 \pm 0.3a	0.004 \pm 0.000ab

1996, Flexas et al. 1999). Conversely, plants subjected to simultaneous stresses showed an additional down-regulation of A_{max} in comparison with single stressed plants. The decoupling between interacting stresses (the amount of stress) and the limited available range of acclimation, after single stress acclimation has occurred, could be the consequence of the lower survival under multiple stress conditions (Figure 5b). However, the magnitude of the acclimation reached by the mid-season leaf cohort was reflected in high survival under single stress exposition. In this context, the acclimation capacity of the photosynthetic machinery among successive fully elongated cohorts represents, at the whole-plant level, a well-tuned mechanism responsible for improved survival of *E. cordifolia* seedlings enduring prevailing and future climate conditions.

Despite the relevance of the present findings, we realize that leaf expansion and leaf physiological maturation do not progress simultaneously in many evergreen species, as the so-called 'delayed greening' occurs, which has been proposed to increase the acclimation potential of current-year leaves to changes in environmental conditions throughout the growing season (Yamashita et al. 2002, Muller et al. 2009, Ishii and Ohsugi 2011). In addition, metabolism at the leaf level is not enough to fully explain plant performance (Percy and Yang 1996, Valladares and Niinemets 2007). In this sense, several key crown-level ontogenetic considerations, such as the leaf area of each cohort, biomass allocation and the architectural determinants of light capture, strongly contribute to the carbon balance at the plant level (Percy and Yang 1996), and could help explain the decoupling between reductions in carbon assimilation at leaf level and survival under multiple stresses. Further experiments are thus needed to understand the role of continuous leaf expansion and development throughout the growing season as a whole-plant acclimation mechanism of evergreen tree species. Since multiple flushings are apparently most profuse in young trees and decline with ontogeny (Battey 2003), this could be an interesting trait to better predict seedlings' ability to cope with future climate changes, with studies

focusing on structural and physiological traits addressing the influences of ontogeny and interacting stress factors.

Concluding remarks

Important modifications in foliage anatomy and photosynthetic limitations occurred in fully expanded leaf cohorts throughout the growing season. Mid-season elongated leaves adopted a lower steady-state A_{max} under stress conditions. This down-regulation of photosynthesis resulted in a higher acclimation capacity, which was reflected in a higher survival rate. Conversely, under favorable growing conditions, the acclimation of the mid-season-developed cohort was reflected in an increased capacity for exploiting the available resources. In this way, the co-existence of leaf cohorts may improve the whole-plant acclimation potential by extending the growth period and architectural development during the growing season. This study increases current knowledge about the role of anatomy, biochemistry and photosynthetic functioning variations among leaf cohorts and their relation with survival of *E. cordifolia* seedlings. It also provides the framework for more accurate canopy gas exchange modeling by including differential leaf cohort functioning. Overall, our results indicate that morpho-physiological leaf cohort's acclimation will be more relevant when *E. cordifolia* faces a single shade or drought stress. However, under predicted future climate changes this evergreen temperate rainforest will most likely face moderate summer drought under shade, a stress combination to which leaf-level cohorts cannot apparently acclimate. Studies focused on assessing the different cohorts' contribution to the whole-plant carbon balance are needed to validate the conclusions stated in our study.

Supplementary data

Supplementary data for this article are available at [Tree Physiology Online](http://www.treephys.oxfordjournals.org/).

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Conflict of interest

None declared.

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