

Multiyear impacts of partial throughfall exclusion on *Buxus sempervirens* in a Mediterranean forest

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Abstract

Aim of study: We examined the impact of sustained partial throughfall exclusion on the functional performance of *Buxus sempervirens* L. in the understory of a Mediterranean evergreen forest. We further considered whether any impacts of throughfall exclusion were affected by light availability.

Area of study: The study was conducted in the south of France.

Material and methods: Several leaf physiological and branch structural traits were measured along a light gradient after seven years from the onset of a throughfall exclusion experiment (TEE). The results were analysed along with annual growth and survival data.

Main results: Plant mortality was nil in both the throughfall exclusion and control treatments. Stem diameter growth was reduced by 39% in plants subjected to throughfall exclusion, but this difference was only significant at the $p = 0.10$ significance level. Leaf physiology remained unaffected by the TEE, but small changes were evident in branch structural traits in high light microsites following throughfall exclusion; branches had lower wood density in the TEE plot, and more biomass was partitioned to leaves relative to stems.

Research highlights: These changes do not seem to reflect an acclimatory response that would enhance drought tolerance. Instead, we suggest that these drought effects might exacerbate vulnerability to xylem cavitation in the more open microsites. Reduced growth and increased vulnerability to drought may indicate an incipient decline in plant vitality following TEE. The extension of observations to the whole-plant level and longer periods will elucidate the consequences of these observations for plant fitness, and permit verification of the positive effect of shade on *Buxus sempervirens* under increased drought.

Key words: common box; drought susceptibility; shade; rainfall manipulation; abiotic stress; long-term study.

Introduction

Studies in Mediterranean forest ecosystems have recently suggested that periods of abnormally high temperatures and low rainfall have caused a decline in tree vigour and modified species composition by eliminating more drought sensitive species (Sarris *et al.*, 2007; Carnicer *et al.*, 2011). The ongoing worldwide increase in air temperatures is being paralleled by declining precipitation in the Mediterranean basin (Sarris *et al.*,

2007), with climate models predicting an intensification of this trend through the 21st century (Somot *et al.*, 2008). The consequences of eventual aridification for vegetation are difficult to anticipate. More research is needed on species responses to water deficit, especially with regard to long-term drought effects under field conditions on traits related to carbon balance, carbon allocation, water use and fitness. Over the long-term, trees tend to acclimate to enhanced water deficit by reducing leaf area (Limousin *et al.*, 2010a; Metcalfe

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et al., 2010), and/or by increasing leaf mass per unit area (Metcalf *et al.*, 2010), as well as cross-sectional sapwood area per supported leaf area (*i.e.* the Huber value; Cinnirella *et al.*, 2002). Overall, these changes promote xylem hydraulic safety. Leaf physiological traits may remain unchanged following long-term exposure to increased drought (Limousin *et al.*, 2010a) or reach an altered state, due to parallel shifts in nitrogen concentration, leaf density and other anatomical and hydraulic traits (Ripullone *et al.*, 2009; Metcalfe *et al.*, 2010).

To anticipate species responses to more frequent and intense drought, we must consider the interactive nature of co-occurring abiotic factors under field conditions. In Mediterranean-type habitats, both the extent of water deficit and the ability to tolerate it vary among microsites (Rodríguez-Calcerrada *et al.*, 2010a). The overstory canopy plays an important role in the modulation of abiotic stress and, hence, in the creation of favourable microsites for recruitment (Gómez-Aparicio *et al.*, 2005; Rodríguez-Calcerrada *et al.*, 2010b; Holmgren *et al.*, 2012). Many plant species preferentially establish in the forest understory, where moderate shade attenuates soil and air dryness relative to forest clearings. However, the beneficial effect of shade that takes place under average rainfall conditions can be cancelled out in exceptionally dry years (Valladares *et al.*, 2008). The idea of shade-induced facilitation of improved plant performance is challenged by the trade-off between tolerance to shade and drought (Smith and Huston, 1989), a trade-off that is most evident in drought-tolerant species (Holmgren *et al.*, 2012). Acclimatory responses to shade may occur at different organizational levels that render individuals less tolerant of drought. For example, shade plants may be more vulnerable to xylem cavitation, and allocate a greater proportion of carbon toward improving light capture by producing leaves with low mass per unit area and branches with high leaf area per unit stem mass or cross-sectional diameter (Barigah *et al.*, 2006; Letts *et al.*, 2012). These changes could render plants of shaded understories more susceptible to water deficit than those in higher light microsites.

Despite the apparent conflict between shade and drought tolerance, certain plant species spread abundantly, and often preferentially, in the understory of drought-prone Mediterranean forests. This has been demonstrated for certain broadleaved, evergreen woody species such as *Viburnum tinus* L., *Hedera helix* L., *Phyllirea latifolia* L. (Sack *et al.*, 2003), *Ilex aquifo-*

lium L. (Aranda *et al.*, 2008) and *Buxus sempervirens* L. (Quézel and Médail, 2003; García-Plazaola *et al.*, 2008). *Buxus sempervirens* is a tall-shrub commonly found in Mediterranean ecosystems of western Europe. It is abundant in *Quercus* forests, in large clearings, gaps, and closed understories, but little is known about its ecophysiology. In the context of the “leaf economics spectrum” (Wright *et al.*, 2004), *B. sempervirens* presents the slow-return suite of traits typical of conservative plants, with high leaf life span and leaf mass per area, but low nitrogen content and photosynthetic capacity per unit dry mass (Letts *et al.*, 2012). This combination of traits reflects high drought tolerance (Hallik *et al.*, 2009). Since a number of leaf traits that contribute to species drought tolerance are detrimental to shade tolerance, the ability of *B. sempervirens* to establish in the forest understory as well as in open sites might rely on the plasticity of some traits and the homeostasis of others, and/or the possession of some features conferring a low demand for resources (Sack *et al.*, 2003; Letts *et al.*, 2012).

Making use of a throughfall experimental manipulation in a *Quercus ilex* L. forest, our work was designed to address the following three questions: Does a sustained reduction in throughfall affect the performance of *B. sempervirens* in the forest understory? If so, do plant responses reflect an increased potential to tolerate drought? Thirdly, does understory light availability modify the impact of increased drought on plants? We compared mature individuals of *B. sempervirens* that had been subjected to either a c 33% throughfall exclusion or normal rainfall for seven years. We measured leaf and branch variables related to carbon gain, light use efficiency, and water use efficiency during one spring, when there was no drought, so that potential differences between treatments reflected long-term acclimation to previous drought history, rather than to current-year drought. We expected to see changes in the morphology of branches reflecting higher tolerance of water deficits. However, little change in leaf physiology is observed in response to long-term enhanced drought in certain Mediterranean species (Limousin *et al.*, 2010a), and coordinated shifts between leaves and branches (Cinnirella *et al.*, 2002; Carter and White, 2009) led us to expect a mild effect of throughfall reduction on leaf physiological variables. We further anticipated that the impact of the throughfall reduction would be greater in more shaded microsites, due to the trade-off between shade and drought tolerance.

Material and methods

Study area and experimental design

The study was conducted at the Puéchabon Experimental Site, near Montpellier, France (3° 35' 45" E, 43° 44' 29" N; 270 m.a.s.l.). This is an even-aged forest, dominated by the evergreen *Q. ilex* L., with 5,100 stems ha⁻¹ and a basal area of 27.4 m² ha⁻¹. There are many shrub species in the understory, including *Pistacia lentiscus* L., *Pistacia terebinthus* L. and *Viburnum tinus* L., but *B. sempervirens* is the most abundant. The climate of the region is Mediterranean sub-humid. Mean annual rainfall is relatively high (914 mm), but irregularly distributed, with about 80% of rainfall occurring between September and April. Mean annual temperature is 13.1°C, and ranges from 22.9°C in July to 5.5°C in January. Soils are extremely rocky, with 75% of the soil volume composed of stones in the top 0-50 cm layer and 90% through the whole profile. The fine fraction of the 0-50 soil layer is classified as a silty clay loam according to the USDA soil texture triangle.

In the spring of 2003, a stand of flat topography was subjected to a throughfall exclusion experiment (TEE). Three 10 × 10-m size plots were chosen, with each divided into two to apply either throughfall exclusion (Exclusion; E) or no throughfall manipulation (Control; C). The throughfall exclusion was achieved with a permanent system of PVC rain gutters installed below the tree canopy. Gutters covered 33% of the ground area and were mounted at a variable height, to carry water outside the plots. The control areas were also covered with upside down gutters to ensure similar light availability and microclimatic conditions at the soil level. The TEE has been continually operational since 2003. Gutters are regularly inspected for adequate water runoff and cleared of litter, which is returned to the soil. Soil water content has been measured in the 0-30 cm layer since 2004. The measurements were taken with three time-domain reflectometry (TDR) probes (MiniTrase 6050X3K1B, Soil Moisture Equipment Corp., USA), installed in one plot per treatment. Soil relative water content was calculated from these values and the field capacity of the soil in the C and E treatments. Field capacity was calculated after two days of free drainage, following rainfall events greater than 40 mm, during periods with low atmospheric demand.

In the spring of 2010, we selected 42 individuals of *B. sempervirens*, evenly divided between the control (C) and throughfall-exclusion treatments (E), including six to eight individuals per replicate plot. We exclusively sampled individuals located at least one metre from the border of the plot. We also chose individuals in contrasting light environments within each treatment, to evaluate whether relationships between functional traits and light availability differed between treatments (see data analysis). Sampling took place between May 7 and May 25, sequentially from block one to three. Daily mean values of air vapour pressure deficit (VPD) ranged from 0.7 ± 0.5 kPa to 1.4 ± 0.8 kPa during the measurement period. Predawn shoot water potential (Ψ_{pd}), measured with a pressure chamber (PMS 1000, PMS Instruments, Corvallis, Oregon, USA) on May 19 in a subsample of eight plants per treatment, was -0.65 ± 0.02 MPa in C and -0.66 ± 0.07 MPa in E. Sampling under near-optimal seasonal conditions facilitated assessment of plant acclimation to enhanced water stress, as imposed by long-term throughfall exclusion, but without the confounding effect of current-year summer drought. All leaf-level measurements were made on previous-year leaves, from branches with new growth with still immature leaves. Sampled leaves and branches were selected from the outer crown of individuals. Light availability was measured during completely overcast conditions at the leaf sampling height, using an LI-250 Light Meter (LI-COR Biosciences, Lincoln, NB, USA). Values were compared with those obtained over the tree canopy to calculate the percent photosynthetic photon flux density (%PPFD) as a measure of light availability of leaves and branches. Mean %PPFD was similar between treatments ($p_{t-test} > 0.10$).

Physiological variables

We measured chlorophyll fluorescence and photosynthetic gas-exchange simultaneously with two recently-calibrated LI-COR 6400XTR Portable Photosynthesis Systems (LI-COR Biosciences), equipped with a LI-COR 6400-40 Leaf Chamber Fluorometer. Measurements were made at ten different PPFD intensities to produce photosynthetic light curves. Leaves were allowed to equilibrate to a PPFD intensity of 1,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and then subjected to a decline in PPFD down to 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in nine steps (1,000, 650, 400, 200, 120, 80, 50 and 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The air tem-

perature into the leaf chamber was set at near 25°C and relative humidity near 50%. Mean actual leaf temperatures during measurements were $24.8 \pm 0.5^\circ\text{C}$ and $25.5 \pm 0.5^\circ\text{C}$ in the C and E treatments, respectively, while leaf-to-atmosphere vapour pressure deficit was 1.8 kPa in both treatments. Following measurement, the portion of the leaf enclosed in the chamber was cut with a borer, measured with a light table system (MK2 Area Meter; Delta-T devices Ltd., Cambridge, UK) and oven-dried to reference gas exchange rates to measured leaf area and dry mass. The light-saturated rate of net CO₂ assimilation was computed as the value of photosynthesis at $1,500 \mu\text{mol m}^{-2} \text{s}^{-1}$ and was expressed per unit leaf area (A_a), or leaf dry mass (A_m) after dividing A_a by leaf mass per unit area (LMA). Light-saturated rates of stomatal conductance to water vapour (g_s) and transpiration (E) per unit area were also taken as measured values at $1,500 \mu\text{mol m}^{-2} \text{s}^{-1}$. The intrinsic water use efficiency was calculated as the ratio of A_a to g_s . The apparent quantum yield (α) was calculated as the slope of the linear part of the photosynthesis light curve and the light compensation point (LCP) as the intercept of this portion of the curve with the light axis. Dark respiration was measured after 30 minutes at $0 \mu\text{mol m}^{-2} \text{s}^{-1}$ and was expressed on either an area (R_a) or a dry mass basis (R_m). The ratio of R_a to A_a was used as a surrogate of net leaf carbon gain.

At each PPF level of the curve, we measured steady-state, minimum, and maximum chlorophyll fluorescence after the last record of gas exchange. The electron transport rate (ETR) was calculated using the equation of Krall and Edwards (1992). Photorespiration (PR) was then calculated from A_a and ETR, following Valentini *et al.* (1995).

Biochemical variables

Dried leaves sampled for physiology were used for measuring stable carbon isotope composition ($\delta^{13}\text{C}$, an integrative surrogate of water use efficiency) and nitrogen concentration (N). Dried leaves, with the petioles removed, were powdered using a ball mill. $\delta^{13}\text{C}$ and N were then determined by combustion of samples in an elemental analyzer (Flash EA1112 Series, ThermoFinnigan, Milan, Italy), coupled to a gas isotope ratio mass spectrometer (Delta S, Finnigan, San Jose, CA, USA). LMA was used to convert mass-based nitrogen concentration (N_m) to area-based nitrogen concentration (N_a).

Architectural and morphological variables

Several architectural and morphological traits were measured in branches from the same individuals sampled for physiology. The percentage of terminal shoots with new growth was calculated on ten randomly-selected, southeast-oriented branches. The oldest growth unit with at least one apparently healthy leaf was counted on four of these branches as a surrogate of leaf lifespan (LLS). Mean stem and leaf inclination were measured relative to the horizontal in three growth units of the same four branches using a protractor. One of the branches of each plant was severed at the base of the eighth growth unit and taken to the laboratory. The length of the branch, the base diameter of the main stem and the area of all leaves were measured while plant material was still fresh. Stem diameter was measured after bark removal, and the Huber value was calculated as the ratio of the stem cross-sectional area to total leaf area (TLA). The branch leaf area ratio (LAR) was determined by dividing TLA by the total branch dry mass. The leaf mass fraction (LMF) was calculated by dividing total leaf dry mass by total branch dry mass. A leaf clumping index (LCI) was defined as the ratio of TLA to total branch length. A 2 cm-long segment stripped of bark was cut at the base of the stem to determine wood density. The dry mass of the segment was divided by its fresh volume, which was determined using an analytical balance (Sartorius CP224S, Germany) and a density determination kit (Sartorius YDK01 Germany) based on Archimedes' principle.

Fruit production

Fruit production was measured as a proxy for plant reproductive fitness. All fruits in the south half of the plants were collected, counted and weighed. Total fruit production was expressed on a per plant basis, by multiplying the number of fruits observed by two.

Plant diameter growth and survival

Starting one year after the onset of the TEE, stem diameter at breast height (DBH) and mortality were measured annually in the plants of C and E treatments. Only one plant from the C treatment and two from the E treatment that had been selected for leaf- and branch-level measurements were not previously monitored for

stem growth and mortality. Stem diameter increment was calculated over six years of the TEE and expressed relative to the initial DBH to account for initial differences in plant size.

Statistical analysis

We tested the effect of the throughfall exclusion treatment with general linear models (GLM). We included as continuous variables the percent photosynthetic photon flux density (%PPFD) for analyses of branch and leaf variables, and the vapour pressure deficit experienced by leaves for analyses of gas exchange. An interaction term between the factor throughfall exclusion and the %PPFD was specified to test whether the relationship of each dependent variable with %PPFD differed significantly between C and E plants. Variables were transformed when necessary to attain normality and make relationships linear. The plot was included as a random-effects term in the GLMs, to account for unknown spatial and temporal variability. In addition, a Principal Component Analysis was performed, to explore multivariate relationships between standardized leaf and branch variables. All analyses were carried out with Statistica 7.1 (StatSoft Inc., Tulsa, USA).

Results

Based on the model of rainfall interception developed by Limousin *et al.* (2008) for *Q. ilex* forests, we

estimated that the soil of C and E treatments received, respectively, 81% and 58% of total precipitation, during the seven years preceding this study (Table 1). Relative water content in the 0-30 cm soil layer (RWC), averaged over the period of May through August, was slightly lower in E than C except in the year 2004, but the difference was not significant for any year at $p < 0.05$. The mean number of days per year with RWC lower than 0.6 was 102 in treatment C and 116 in treatment E.

Plants of the C and E treatments showed similar values in a number of leaf and branch traits related with growth, biomass allocation, and resource-use efficiency. Leaf physiology was mostly unaltered by the TEE (Table 2). Of all physiological variables measured, only light-saturated rates of photosynthesis per unit area (A_a) and mass (A_m) were marginally significantly higher in the leaves of E than C plants. Light and VPD had significant effects on several variables. For example, light availability had a negative effect on N_m and A_m , and VPD had negative effects on g_s and A_a (Table 2), but their effects were similar for both treatments and so these results are not shown.

While the main effect of the treatment on every morphological variable was insignificant, significant interactions between the treatment and the light covariate indicated a light-dependent impact of throughfall reduction on several morphological branch characteristics (Table 3). The sensitivity of leaf area ratio (LAR) to light availability was reduced by the effect of throughfall exclusion (Fig. 1a), so that branches of E plants growing in higher light microsites had higher LAR

Table 1. Annual precipitation (P) in the study area. The following data are indicated for control (C) and throughfall exclusion (E) treatments: annual net precipitation (*i.e.* throughfall + stem-flow), mean relative water content (RWC \pm standard error) for the period from May through August and, in parentheses, the number of days per year for which RWC was lower than 0.6

Year	P (mm)	Net P (mm)		RWC (days RWC < 0.6)	
		C	E	C	E
2003	1,311	1,105	786	n.a.	n.a.
2004	989	781	553	0.53 \pm 0.11 (120)	0.54 \pm 0.12 (119)
2005	835	671	475	0.54 \pm 0.11 (91)	0.50 \pm 0.08 (132)
2006	940	774	550	0.52 \pm 0.06 (121)	0.51 \pm 0.07 (122)
2007	681	509	360	0.66 \pm 0.16 (75)	0.62 \pm 0.14 (83)
2008	1,249	1,050	746	0.72 \pm 0.18 (102)	0.69 \pm 0.18 (109)
2009	742	597	422	0.61 \pm 0.11 (105)	0.59 \pm 0.11 (130)
Mean	964	784	556	0.60 \pm 0.03 (102)	0.57 \pm 0.03 (116)

Source: Rodríguez-Calcerrada *et al.*, FS2013.

Table 2. Mean values (\pm standard error) of leaf physiological and biochemical variables in control (C) and throughfall-exclusion plants (E), and significance of effects of treatment (T), light availability (%PPFD), vapour pressure deficit (VPD) and the interaction between T and %PPFD on each variable and on the component scores of factors 1 (CS1) and 3 (CS3) derived from Principal Component Analysis. F-values are given and *p*-values are indicated as †: *p* < 0.10, *: *p* < 0.05, **: *p* < 0.01. Effects significant at *p* < 0.10 or lower are highlighted in bold. See Material and methods for the significance of abbreviations

Variable	Control (C)	Exclusion (E)	Treat (T)	%PPFD	VPD	T × %PPFD
A_a ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	4.23 \pm 0.22	4.44 \pm 0.23	2.88 †	0.07 ^{ns}	7.97 **	1.65 ^{ns}
A_m ($\text{nmol g}^{-1} \text{s}^{-1}$)	25.0 \pm 1.3	27.8 \pm 1.7	3.60 †	6.39 *	10.19 **	0.99 ^{ns}
α (mol mol^{-1})	0.033 \pm 0.003	0.035 \pm 0.002	0.02 ^{ns}	0.02 ^{ns}	5.53 *	0.35 ^{ns}
LCP ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	9.43 \pm 1.69	6.85 \pm 0.74	0.05 ^{ns}	0.18 ^{ns}	0.89 ^{ns}	0.67 ^{ns}
g_s ($\text{mol m}^{-2} \text{s}^{-1}$)	0.043 \pm 0.003	0.045 \pm 0.003	1.68 ^{ns}	0.07 ^{ns}	6.56 *	0.94 ^{ns}
E ($\text{mmol m}^{-2} \text{s}^{-1}$)	0.76 \pm 0.05	0.83 \pm 0.05	1.49 ^{ns}	0.05 ^{ns}	0.33 ^{ns}	0.77 ^{ns}
A_a/g_s ($\mu\text{mol mol}^{-1}$)	102 \pm 4	102 \pm 3	2.0 10^{-3} ^{ns}	1.02 ^{ns}	0.64 ^{ns}	0.03 ^{ns}
PR ($\text{mmol m}^{-2} \text{s}^{-1}$)	2.05 \pm 0.2	2.02 \pm 0.2	2.50 ^{ns}	0.01 ^{ns}	0.54 ^{ns}	2.64 ^{ns}
R_a/A_a (%)	6.60 \pm 0.62	5.32 \pm 0.58	0.13 ^{ns}	0.26 ^{ns}	0.06 ^{ns}	0.08 ^{ns}
R_a ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	0.27 \pm 0.02	0.23 \pm 0.02	0.12 ^{ns}	3.04 †		1.01 ^{ns}
R_m ($\text{nmol g}^{-1} \text{s}^{-1}$)	1.56 \pm 0.13	1.44 \pm 0.15	0.02 ^{ns}	0.06 ^{ns}		0.14 ^{ns}
N_a (g m^{-2})	2.55 \pm 0.10	2.34 \pm 0.09	0.13 ^{ns}	3.2 †		0.31 ^{ns}
N_m (%)	1.50 \pm 0.05	1.45 \pm 0.07	0.10 ^{ns}	4.22 *		0.1 10^{-3} ^{ns}
$\delta^{13}\text{C}$ (‰)	-28.7 \pm 0.2	-28.9 \pm 0.2	0.08 ^{ns}	6.38 *		0.1 10^{-3} ^{ns}
CS1			0.83 ^{ns}	0.24 ^{ns}		0.63 ^{ns}
CS3			0.01 ^{ns}	1.59 ^{ns}		1.47 ^{ns}

Source: Rodríguez-Calcerrada *et al.*, FS2013.

than those of C plants: 46.4 ± 1.8 vs 38.0 ± 2.4 $\text{cm}^2 \text{g}^{-1}$ in microsites with light > 30 %PPFD ($F_{1,13} = 6.92$, *p* < 0.05). The superior LAR at the higher end of the light

gradient in E plants was not due to an enhanced production of leaf area. In fact, although not significantly, TLA was lower in E than C plants at light > 30 %PPFD

Table 3. Mean values (\pm standard error) of morphological and architectural variables in control (C) and throughfall-exclusion plants (E), and significance of effects of treatment (T), block (not shown), light availability (%PPFD), and the interaction between T and %PPFD on each variable and on the component scores of factor 2 (CS2) derived from Principal Component Analysis. F-values are given and *p*-values are indicated as †: *p* < 0.10, *: *p* < 0.05, **: *p* < 0.01, and ***: *p* < 0.001. Effects significant at *p* < 0.10 or lower are highlighted in bold. See Material and methods for the significance of abbreviations

Variable	Control (C)	Exclusion (E)	Treat (T)	%PPFD	T × %PPFD
LMA (g m^{-2})	170 \pm 6	163 \pm 5	0.28 ^{ns}	33.48 ***	1.93 ^{ns}
LMF (g g^{-1})	0.69 \pm 0.01	0.73 \pm 0.01	0.03 ^{ns}	1.30 ^{ns}	2.23 ^{ns}
LAR ($\text{cm}^2 \text{g}^{-1}$)	41.7 \pm 1.5	45.8 \pm 1.1	0.40 ^{ns}	3.11 †	3.91 †
TLA (cm^2)	231 \pm 19	235 \pm 24	2.83 ^{ns}	0.08 ^{ns}	3.67 †
Branch mass (g)	1.85 \pm 0.21	1.44 \pm 0.19	2.17 ^{ns}	0.01 ^{ns}	6.87 *
Branch diameter (cm)	0.35 \pm 0.01	0.34 \pm 0.02	1.76 ^{ns}	0.2 10^{-3} ^{ns}	2.51 ^{ns}
Branch length (cm)	31.2 \pm 1.9	28.5 \pm 1.3	0.30 ^{ns}	0.67 ^{ns}	2.08 ^{ns}
Wood density (g cm^{-3})	0.85 \pm 0.01	0.85 \pm 0.01	0.59 ^{ns}	0.15 ^{ns}	2.81 †
Huber value ($\text{cm}^2 \text{m}^{-2}$)	2.35 \pm 0.12	2.04 \pm 0.10	0.01 ^{ns}	0.14 ^{ns}	1.24 ^{ns}
LLS	6 \pm 0	6 \pm 0	0.08 ^{ns}	4.45 *	0.10 ^{ns}
Shoot growth (%)	48.1 \pm 5.7	42.9 \pm 6.6	0.2 10^{-3} ^{ns}	0.01 ^{ns}	0.23 ^{ns}
Branch inclination (°)	1.55 \pm 2.65	-0.22 \pm 2.97	0.41 ^{ns}	5.48 *	0.33 ^{ns}
Leaf inclination (°)	36.4 \pm 5.0	30.6 \pm 5.0	0.02 ^{ns}	3.47 †	0.21 ^{ns}
LCI ($\text{cm}^2 \text{cm}^{-1}$)	7.47 \pm 0.52	8.17 \pm 0.71	2.14 ^{ns}	0.22 ^{ns}	1.50 ^{ns}
Fruit size (g)	0.097 \pm 0.009	0.092 \pm 0.006	0.76 ^{ns}	0.3 10^{-3} ^{ns}	0.74 ^{ns}
Production (g plant^{-1})	0.76 \pm 0.29	0.32 \pm 0.14	0.66 ^{ns}	1.21 ^{ns}	5.8 10^{-3} ^{ns}
CS2			1.11 ^{ns}	1.97 ^{ns}	5.16 *

Source: Rodríguez-Calcerrada *et al.*, FS2013.

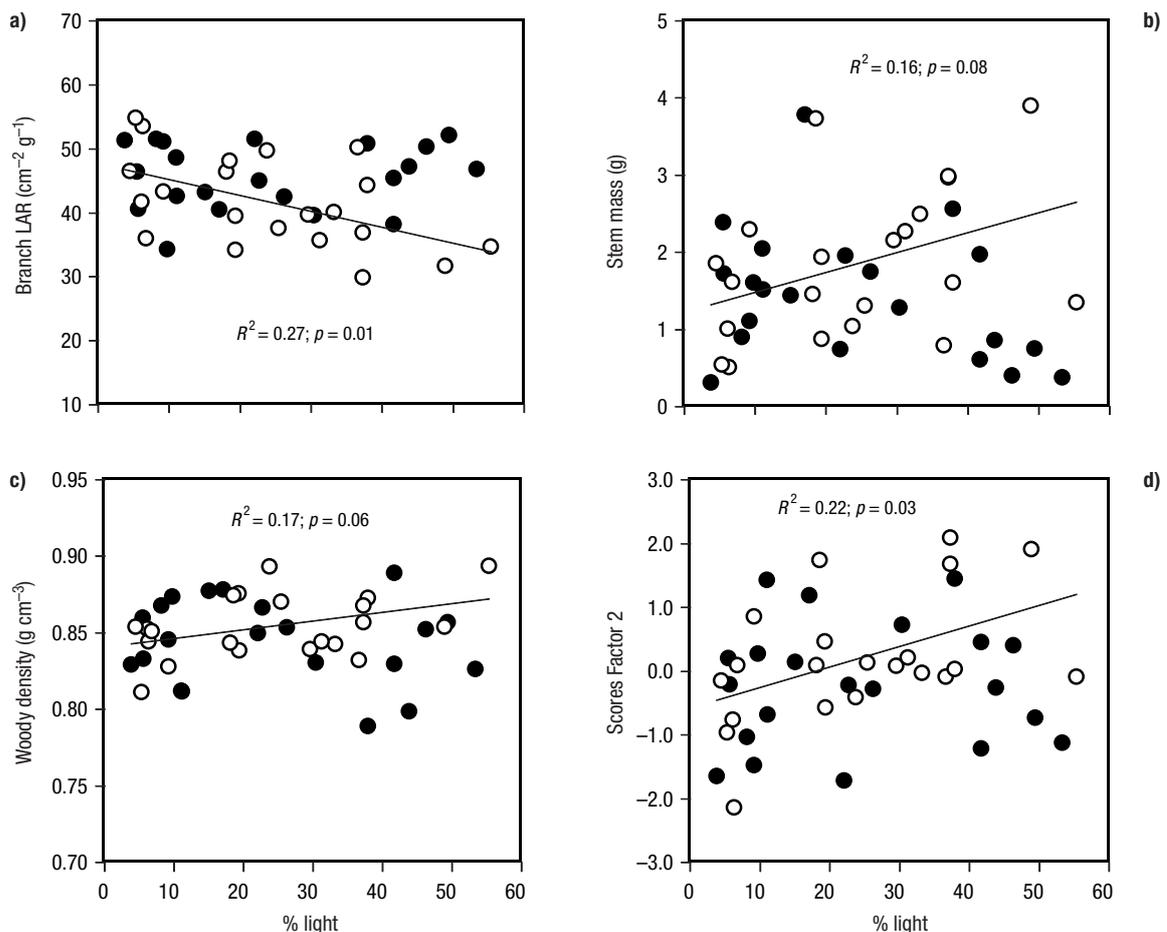


Figure 1. Relationship between understory light availability and a) branch leaf area ratio (LAR), b) stem mass, c) wood density, and d) component scores of *morphological* factor 2 obtained from Principal Component Analysis. Branches were severed at the base of the eighth growth unit. The coefficient of determination and significance level of the linear regressions are shown. White symbols correspond to control (C) plants and filled symbols to throughfall exclusion (E) plants. Regression lines significant at $p = 0.10$ were drawn for C plants, but no significant relationship was found for E plants. *Source:* Rodríguez-Calcerrada *et al.*, FS2013.

(197 ± 27 vs 273 ± 33 cm², respectively, $F_{1,35} = 2.29$, $p > 0.10$); however, stem biomass production was even lower (1.11 ± 0.28 g in E vs 2.30 ± 0.36 g in C at > 30 %PPFD, $F_{1,13} = 5.50$, $p < 0.05$; Fig. 1b), resulting in higher LAR in E than C plants in high light microsites. The lower stem dry mass in branches of E plants at the higher end of the light gradient was due to (i) lower wood density than C plants (0.83 ± 0.01 g cm⁻³ in E vs. 0.86 ± 0.01 g cm⁻³ in C at light > 30 %PPFD, $F_{1,13} = 4.63$, $p = 0.051$; Fig. 1c), and (ii) a trend for shorter length and narrower diameter of the main stem (e.g. diameter: 0.32 ± 0.03 cm in E vs 0.38 ± 0.02 cm in C at light > 30 %PPFD, $F_{1,35} = 2.20$, $p > 0.10$). Moreover, in light microsites with more than 30% PPFD, E plants had lower Huber values than C plants: 2.03 ± 0.13 vs. 2.42 ± 0.12 cm² stem m⁻² leaf, respectively; $F_{1,13} = 4.96$, $p < 0.05$.

A principal components analysis with most variables revealed three factors that together explained 49.6% of total variance. Photosynthetic and water use efficiency traits showed high loadings for the first factor, while morphological traits showed high loadings for the second factor, and respiratory traits showed high loadings for the third factor (Table 4). Tests of the effect of the treatment and light availability on the component scores of each plant for factors 1 to 3 (using GLM) confirmed that throughfall exclusion did not affect the physiology of leaves (CS1 and CS2) but did reduce the light plasticity of branch morphological features (CS3) (Table 4; Fig. 1d). Collectively, these results indicated a slight but significant trend for branches of C plants to exhibit higher growth rates, wood density and proportional biomass partitioning to stems than E plants at the higher end of the light gradient (Fig. 1).

Table 4. Loadings of variables for the first three factors obtained after Principal Components Analysis and the percentage of variance explained by each factor. Loadings >0.5 are shown in bold. See Material and methods for the significance of abbreviations

Variable	Factor 1	Factor 2	Factor 3
A _a	0.87	-0.07	-0.21
A _m	0.76	-0.35	-0.15
α	0.75	-0.14	0.14
LCP	-0.56	-0.07	-0.63
g _s	0.92	-0.13	-0.25
E	0.80	-0.09	-0.32
A _a /g _s	-0.64	0.14	0.23
PR	-0.31	0.17	-0.18
R _a /A _a	-0.57	0.02	-0.60
R _a	-0.19	-0.03	-0.78
N _a	0.27	0.31	-0.05
δ ¹³ C	-0.25	0.41	0.26
LMA	0.02	0.54	-0.06
LMF	-0.28	-0.56	0.29
LAR	-0.26	-0.64	0.50
TLA	0.13	0.70	0.36
Branch mass	0.22	0.88	0.01
Branch diam.	0.12	0.85	0.18
Branch length	0.24	0.54	0.08
Wood density	0.14	0.12	-0.35
Huber value	-0.02	0.48	-0.37
LLS	-0.06	-0.05	-0.05
Branch angle	-0.21	0.15	-0.36
Leaf angle	-0.26	0.35	-0.28
Leaf clumping	0.02	0.46	0.37
% Variance	20.39	17.61	11.56

Source: Rodríguez-Calcerrada *et al.*, FS2013.

The TEE had a greater impact on higher hierarchical levels of the plant (Fig. 2). From the winter of 2003 to the winter of 2009, stem diameter increased by 0.28 cm in the C treatment and 0.16 cm in the E treatment, on average ($F_{1,35} = 7.25$, $p < 0.05$). The difference between treatments was lower when initial differences in plant size were taken into account, but a marginally significant effect of the TEE was still observed, with relative stem growth reduced by 39% ($F_{1,35} = 3.30$, $p = 0.078$; Fig. 2b). Mean stem diameter at breast height in winter 2009 was 2.6 ± 0.2 cm in C and 2.1 ± 0.1 cm in E ($F_{1,35} = 6.32$, $p < 0.05$). None of the plants monitored for survival died in any treatment (data not shown).

Discussion

The response of plants to altered rainfall in their natural habitats is a question of global interest. In the present study, mature saplings of the Mediterranean shrub *Buxus sempervirens* established in the understory of a *Q. ilex* forest were experimentally subjected to seven years of sustained throughfall reduction and compared with control plants experiencing normal rainfall. Contrary to our expectations, plants were only slightly affected by the reduction in throughfall. Furthermore, the changes observed in some variables occurred only in high light microsites, and could exacerbate vulnerability to water deficit, rather than helping plants to overcome an increase in drought intensity.

The limited response of plants to throughfall exclusion was due, in part, to the low impact of the TEE on

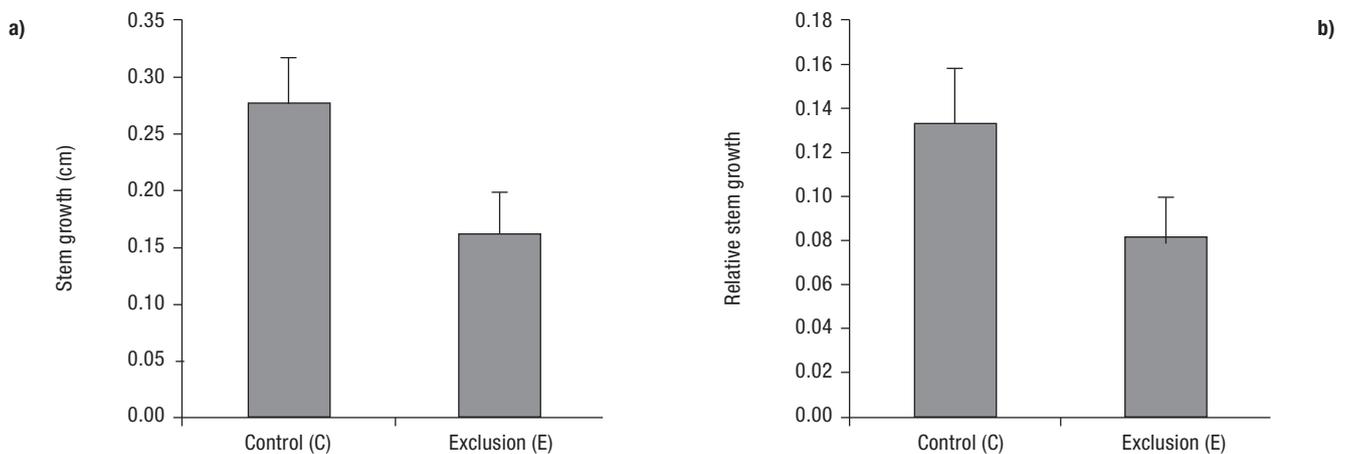


Figure 2. a) Stem diameter growth and b) stem diameter growth relative to initial diameter from 2004 through 2009. Control (C) plants were subjected to normal rainfall and Exclusion (E) plants to 33% less throughfall.

soil water content. The 33% throughfall reduction corresponded to a 23.5% reduction of incident precipitation because of stemflow water input. Furthermore, small rainfall events in summer in semi-arid forests contribute little to net precipitation and soil water storage, due to canopy interception and evapotranspiration (Huxman *et al.*, 2004). These factors reduce the effectiveness of throughfall exclusions (Limousin *et al.*, 2008; Cotrufo *et al.*, 2011). This needs to be taken into account when setting up this kind of experimental system in Mediterranean forests. Nonetheless, as an indicator of plant water availability over the whole soil profile, we measured the shoot predawn water potential (Ψ_{pd}) of five plants in each treatment on two occasions during the summer of 2011. Ψ_{pd} was -2.5 ± 0.1 MPa in C plants and -3.2 ± 0.1 MPa in E plants in the month of July, and -5.4 ± 0.1 MPa and -6.1 ± 0.1 MPa in C and E plants, respectively, in the month of August ($p_{t-test} < 0.01$ for both months). Therefore, the throughfall exclusion experiment (TEE) affected the availability of water to plants. Moreover, the observed difference in the number of days with RWC below 0.6 is important for plant function, because soil water potential and gross primary productivity drop steeply below this value at the study site (Rambal *et al.*, 2003).

In water-limited environments, it is technically difficult to detect a change in growth in response to a further increase in drought imposed by an experimental rainfall reduction unless observations are cumulated over several years (Miranda *et al.*, 2011). Here, stem diameter growth was reduced by throughfall exclusion, though differences between treatments were significant only at the $p=0.10$ significance level. The 39% reduction in growth for *B. sempervirens* contrasts with the similar stem diameter growth of *Q. ilex* trees in C and E treatments observed in the same TEE (Rodríguez-Calcerrada *et al.*, 2011). Ogaya *et al.* (2003) also observed a different response of stem growth to throughfall exclusion in three evergreen broadleaf trees, and concluded it was the result of their contrasting drought sensitivity. Species that are relatively tolerant to drought may be less susceptible to the impacts of rainfall or throughfall experimental manipulations. Both *B. sempervirens* and *Q. ilex* are tolerant of drought, which could partly explain the overall weak effect of the TEE relative to other ecosystems. However, *B. sempervirens* could be more sensitive than *Q. ilex* to further reductions in summer precipitation by the TEE because it has a shallower root system and, thus, experiences very low water potentials during summer (this study;

Aussenac and Valette, 1982). Furthermore, given the abundance of *B. sempervirens* in the understory, its sensitivity to throughfall exclusion could increase the pool of water and nutrient resources available to neighbouring plants, such as *Q. ilex*. This kind of indirect interaction between species was proposed by Seifan *et al.* (2010) to explain the counterintuitive positive effect of drought on recruitment of a Mediterranean shrub.

The seven years of throughfall exclusion had a greater impact on the morphology of branches than the physiology of leaves in the wet season. Changes in branch morphology suggested that plants toward the high end of the light gradient invested proportionally less biomass in stems than leaves, relative to control plants. Given that transpiration rates were similar in the two treatments, the higher LAR and lower Huber values observed in the exclusion plots in open microsites would exacerbate water loss relative to water supply at the branch level. Increased hydraulic conductivity could counteract this effect, though no change was observed in this variable in a previous study on long-term drought acclimation in *Q. ilex* (Limousin *et al.*, 2010b). Throughfall exclusion also reduced wood density in high light plants, as observed by Limousin *et al.* (2010b) within the same TEE. In a study with 36 angiosperms, Hacke *et al.* (2001) reported a non-linear relationship between wood density and water potential resulting in a 50% loss of hydraulic conductivity (P_{50}). If the pattern across species seen in Hacke *et al.* (2001) held within species, as it holds for the genus *Acer* (Lens *et al.*, 2011), the 0.03 g cm^{-3} lower wood density of E plants observed in the higher light microsites would translate into approximately 2MPa higher P_{50} . Although stem anatomical and hydraulic measurements are needed to quantify xylem conductivity and implosion resistance under drought (Fernández *et al.*, 2012), the structural adjustments observed in *B. sempervirens* branches do not appear to be consistent with acclimation to chronically enhanced drought. Instead, they seem indicative of higher drought vulnerability. This result contrasts with observations in species occupying dominant positions in the overstory (Cinnirella *et al.*, 2002; Limousin *et al.*, 2010b).

The higher impact of drought in more open understory microsites is consistent with the finding that shade facilitates plant performance under harsh abiotic conditions (Callaway, 1995; Sthultz *et al.*, 2006). However, it does not support the hypothesis, derived from field studies with seedlings in Mediterranean-type

habitats (Valladares and Pearcy, 2002; Rodríguez-Calcerrada *et al.*, 2010b; Prévosto *et al.*, 2011), that increasing shade would exacerbate sensitivity to increased drought. One explanation for this result is that we studied large, mature saplings well established in the understory. Large saplings have large pools of non-structural carbohydrates, which could make them less susceptible to the combined effects of shade and drought than seedlings (Niinemets, 2010). Plants in high light microsites were more affected by the throughfall exclusion probably because of higher evaporative demand and lower minimum water potential.

One caveat to the above interpretation is that our results are based on measurements of branches. Although we studied a large set of variables to account for potential adjustments at some levels of organization that might not be seen in others (*e.g.* a change in branch Huber value that may lead to homeostasis of leaf gas exchange; Carter and White, 2009), we cannot rule out the possibility that acclimation to light and drought occurred at the whole plant level through changes in carbon allocation, as suggested for *Q. ilex* in response to the same level of throughfall reduction (Limousin *et al.*, 2010b; Martin-StPaul *et al.*, 2013).

The reason for the marginally higher photosynthesis in leaves of E plants is unknown. In some studies, drought-induced reductions in leaf area have been related to higher rates of leaf-specific transpiration under optimal water conditions (Cinnirella *et al.*, 2002). The decline in leaf area can similarly cause remaining leaves to possess higher nitrogen content per unit leaf area (Limousin *et al.*, 2010a) and, thus, higher gas exchange rates. However, foliar nitrogen concentration did not differ between treatments in the present study.

In conclusion, seven years of partial throughfall exclusion appear to be causing an incipient decline in *B. sempervirens* vigour. In the absence of data on plant biomass allocation that might reveal an acclimatory response, our data suggest that there was no acclimation to long-term experimental throughfall exclusion. Instead, the TEE caused a reduction in stem growth and a mild, but significant, negative impact on branch traits suggesting increased vulnerability to xylem cavitation in the more open microsites of the forest understory. Longer-term observations will confirm whether more shaded microsites are safer places for *B. sempervirens* to face chronic decreases in soil water availability than more open sites, and whether survival is eventually compromised.

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