

Species-specific responses of tree saplings to herbivory in contrasting light environments: An experimental approach¹

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Abstract: Light availability and herbivory are 2 major factors affecting the regeneration of woody species, and thus a better perception of how light and herbivory interact to shape the sapling performance of cohabitant tree species emerges as a key issue. The purpose of this work is to experimentally analyze the influence of light conditions on tree responses to browsing. We evaluated the responses to browsing (simulated by mechanical clipping of 50% of current-year shoots) of saplings from 2 deciduous (*Acer opalus* ssp. *granatense* and *Quercus pyrenaica*) and 2 evergreen (*Quercus ilex* and *Pinus nigra* ssp. *salzmannii*) late-successional tree species, measuring an array of morphological, physiological, and biochemical traits. The experiment was performed with saplings grown for 2 y under 3 experimental light environments emulating natural microhabitats: full (open microhabitats), 80% (below pioneer shrub canopy), and 13% (below tree canopy) sunlight. Clipping affected biomass distribution, growth, and sapling size, while only slight physiological and biochemical effects were detected. Species characteristics and the light environment in which saplings grow determine their capacity to recover biomass lost after herbivore damage. Black pine was found to be the least tolerant species to clipping, whereas the broadleaf species displayed greater recovery after clipping. Light scarcity increased the nutritional quality of plants and negatively affected herbivory tolerance of 3 of the 4 species.

Keywords: clipping, compensation capacity, light availability, regeneration niche, sapling.

Résumé : La disponibilité de la lumière et l'herbivorie sont 2 facteurs principaux influençant la régénération d'espèces ligneuses. Ainsi, une meilleure compréhension de la façon dont la lumière et l'herbivorie interagissent pour modéliser la performance des gaules d'espèces coexistantes d'arbres ressort comme un élément clé. Le but de ce travail est d'analyser expérimentalement l'influence des conditions de lumière sur la réponse des arbres au broutement. Nous avons évalué après réponses au broutement (simulé par une coupe mécanique de 50 % des pousses de l'année) de gaules de 2 espèces d'arbres à feuilles caduques (*Acer opalus* ssp. *granatense* et *Quercus pyrenaica*) et de 2 autres à feuilles persistantes (*Quercus ilex* et *Pinus nigra* ssp. *salzmannii*), des espèces de fin de succession, pour un ensemble de traits morphologiques, physiologiques et biochimiques. L'expérience a été menée sur des gaules cultivées durant 2 ans dans 3 environnements lumineux expérimentaux simulant des microhabitats naturels : plein soleil (microhabitats ouverts), 80 % (sous une canopée d'arbustes pionniers) et 13 % (sous une canopée d'arbres). La coupe a eu un effet sur la distribution de la biomasse, la croissance et la taille des gaules, alors que les effets physiologiques et biochimiques détectés étaient faibles. Les caractéristiques de l'espèce et l'environnement lumineux dans lequel croissent les gaules déterminent leur capacité à récupérer la biomasse perdue après les dommages liés à l'herbivorie. Le pin de Salzmann était l'espèce la moins tolérante à la coupe, alors que l'espèce latifoliée présentait une plus grande récupération après coupe. Le manque de lumière causait une augmentation de la qualité nutritionnelle des plantes et avait un impact négatif sur la tolérance à l'herbivorie pour 3 des 4 espèces.

Mots-clés : capacité de compensation, coupe, disponibilité de la lumière, gaule, niche de régénération.

Nomenclature: Castroviejo, 1986; Pérez-Raya *et al.*, 2001.

Introduction

Plants respond to herbivory in a variety of ways, including changes in their biochemical, physiological, and morphological characteristics. They are also strongly affected by light availability, which determines the carbon resources for growth as well as the synthesis of chemicals. Many studies have analyzed herbivory's effects on plants (Rosenthal & Kotanen, 1994; Strauss & Agrawal, 1999;

Nykänen & Koricheva, 2004; Baraza *et al.*, 2007), and still more describe plant responses to light (King, 1994; Valladares, 2003; Percy, 2007; Valladares & Niinemets, 2008). However, less information is available on the interaction of these 2 factors (but see Lentz & Cipollini, 1998; Harmer, 1999; Roberts & Paul, 2006; Vandenberghe, Freléhoux & Buttler, 2008). In addition, contradictory results have been reported regarding the effect of light availability on the recovery of plants after browsing or grazing (Hawkes & Sullivan, 2001; Wise & Abrahamson, 2005; Baraza *et al.*, 2007). The purpose of this work is to analyze the influence of light conditions on tree responses

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to browsing in 4 late-successional trees in order to improve understanding of the effect of mammalian herbivory on forest structure and dynamics.

Plant biochemical, physiological, and morphological changes following herbivory perform 2 main functions: tolerance by means of compensation for the damaged tissues (Strauss & Agrawal, 1999) and reduction of the probability of future damage by induced defence (Karban & Baldwin, 1997). Tolerance includes mechanisms such as regrowth from undamaged meristems, biomass reallocation, and increased photosynthesis rate (Rosenthal & Kotanen, 1994; Strauss & Agrawal, 1999). Potential future damage may be reduced by increasing the concentration of secondary compounds in tissues after damage, thereby lowering their quality and/or appeal as food for herbivores (Karban & Baldwin, 1997). These processes depend on several factors, some of which are inherent to the plant species, such as growth rate (Chen, Kolb & Clancy, 2001), nutrient-storage sites (Millard *et al.*, 2001), and leaf lifespan (Nykänen & Koricheva, 2004). However, other factors are external, such as the amount, pattern, timing, and frequency of herbivory and overall resource availability (Rosenthal & Kotanen, 1994; Hawkes & Sullivan, 2001; Koricheva *et al.*, 1998; Baraza *et al.*, 2007).

The compensatory-continuum hypothesis (CCH) predicts that herbivory tolerance of a plant should be greater in resource-rich than in resource-poor environments (Maschinski & Whitham, 1989). By contrast, the growth-rate model (GRM) of Alward and Joern (1993) predicts greater plant tolerance to herbivory in low-resource situations relative to those higher in resources. An alternative model recently proposed by Wise and Abrahamson (2005), the limiting-resource model (LRM), predicts differing effects of resource shortage on plant response to herbivory. These effects depend on which resource limits plant performance, which resources are affected by the damage of different herbivores, and whether this damage affects the acquisition of limiting or other resources (Wise & Abrahamson, 2005; 2007). For instance, when plant growth is limited by light, plant tolerance to herbivory is expected to decline relative to a non-limited environment, since defoliation or browsing removes light acquisition tissue (leaves). Moreover, light limitation constrains the production of carbon-based secondary defence compounds following herbivore damage (Nabeshima, Masashi & Hiura, 2001), restraining the plant biochemical response to herbivory (Roberts & Paul, 2006). Thus, understanding the response of tree saplings to herbivory in contrasting light environments is critical for understanding how herbivores affect the forest, since their consequences scale up at the population and community levels, affecting specific composition and vegetation dynamics (Gill, 1992; Liang & Seagle, 2002; Baraza *et al.*, 2007). As a result, better understanding of how light and herbivory interact to build the regeneration niche of cohabitant tree species emerges as a key issue.

In this study, we analyze the response to browsing (simulated by mechanical clipping) of saplings growing under 3 levels of light intensity, measuring a wide array of morphological, biochemical, and physiological plant traits.

We carry out this study utilizing 4 Mediterranean tree species: 2 deciduous (*Acer opalus* ssp. *granatense* and *Quercus pyrenaica*) and 2 evergreen (*Quercus ilex* and *Pinus nigra* ssp. *salzmannii*). These species are common in the Mediterranean mountain forests of southwestern Europe, where they are subject to browsing from wild ungulates and domestic livestock (Le Houérou, 1981; Papanastasis & Peter, 1998). These species exhibit different levels of shade tolerance (Gómez-Aparicio, Valladares & Zamora, 2006) and palatability (Baraza, 2004; Baraza, Zamora & Hódar, 2006), making them ideal for comparative studies from both a research and a management perspective. We addressed the following specific questions: 1) What are the main mechanisms of tolerance and defence against herbivory? 2) Can species-specific characteristics (evergreen *versus* deciduous, coniferous *versus* broadleaved) explain the particular responses to browsing? 3) Does light availability affect plant response to herbivory equally for all species analyzed?

Methods

STUDY SITE AND SPECIES

The experiment was conducted in a 12.4-ha fenced area, La Cortijuela Botanical Garden (Junta de Andalucía, 2001), located at elevation 1650 m (asl) in Sierra Nevada National Park (southeastern Spain, 37° 5' N, 3° 28' W). The climate is mountain Mediterranean. The mean temperatures of the coldest (January) and hottest (July) months are 3.6 and 21.5 °C, respectively, with an annual mean of 11.5 °C. Rainfall concentrates in spring and autumn, with an annual mean of 818 ± 86 mm (average 1990–2006; data from climate station located in the study site). The main ungulate herbivore in the area is Spanish ibex (*Capra pyrenaica*), although herds of domestic goat (*Capra hircus*) and sheep (*Ovis aries*) are also common during summer. The presence of free-ranging cows (*Bos taurus*) and horses (*Equus caballus*) is occasional.

The tree species studied were representative of the woody community appearing in the native forest of the study area. Holm oak (*Quercus ilex*), a Mediterranean evergreen sclerophyllous species, represents about 13% of the forested cover of Sierra Nevada National Park (Pérez-Raya *et al.*, 2001). Pyrenean oak (*Q. pyrenaica*), which shows eco-physiological traits intermediate between nemoral and Mediterranean oaks (Corcuera, Camarero & Gil-Pelegrin, 2002), dominates the deciduous forest of Sierra Nevada (Pérez-Raya *et al.*, 2001). Spanish maple (*Acer opalus* ssp. *granatense*) is an Iberian–Mauritanian endemic with a distribution composed of small patches scattered over several mountains, including Sierra Nevada (Gómez-Aparicio, Zamora & Gómez, 2005). The fourth species, black pine (*Pinus nigra*), is a high-mountain Mediterranean pine, and in Sierra Nevada it is one of the main species used in reforestations, but there are also some natural patches restricted to small areas (Alejano & Martínez, 2006).

The experiment was performed under semi-natural conditions, with the advantage of control over certain variables (*e.g.*, light, water) while maintaining an altitude, temperature, and photoperiod equivalent to those of the natural habitats of these species. Seedlings came from seeds

collected from several trees per species situated in the surroundings of La Cortijuela. Holm oak acorns were collected in fall 1998 and stratified and sown in January 1999, with seedlings emerging in late May and June. Seeds of Spanish maple and black pine and acorns of Pyrenean oak were collected in fall 1999 and sown in December 1999, with seedlings emerging during spring 2000. Seeds were sown in cylindrical pots (0.3 L), which were watered to saturation and weeded periodically. In April 2001, 144 seedlings (> 1 y in age; mean height (mm): Spanish maple, 86.2; Holm oak, 156.9 black pine, 59.3; Pyrenean oak, 99.7) per species were randomly selected and transplanted to 2.5-L individual plastic pots containing a mixture of peat, vermiculite, and top soil (1:1:2, v/v). This date marked the initiation of the experiment.

EXPERIMENTAL DESIGN

The experiment encompassed the following 2 factors:

- 1) *Light*; with 3 levels: *i*) full sunlight, which represented the light environment of open interspaces, naturally found between shrubs and trees in a Mediterranean forest; *ii*) 80% sunlight, similar to the light environment found under the canopy of the more abundant, pioneer shrubs in the area; and *iii*) 13% sunlight, replicating the deepest shade generally found in the natural mature forests of the study area (Valladares, 2004).
- 2) *Clipping*; with 2 levels: *i*) clipped, where 50% (or 50% + 1 in the case of odd numbers) of the current-year shoots of each sapling were removed with pruning shears; and *ii*) unclipped, where the plants were left intact as a control. The clipping experiment emulated browsing damage, and consequently main and higher branches were chosen to simulate the form and amount of damage that ungulates typically cause to natural saplings in the area (Gómez *et al.*, 2001; Zamora *et al.*, 2001).

All pots were located outdoors, where irradiance was controlled with shade cloth. Microclimatic conditions of each treatment were characterized by measuring photosynthetic photon flux density (PPFD) every minute on 7 clear days in the middle of the summer (mean \pm 1 SE $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of 6 sensors per treatment: Full sunlight, 1513.4 ± 69.6 ; 80% sunlight, 1210.9 ± 107.7 ; 13% sunlight, 180.2 ± 27.7). PPFD was recorded with Ha-li light sensors (EIC SL, Madrid, Spain) cross-calibrated with a LI-190SA quantum sensor (Li-Cor, Lincoln, Nebraska, USA) connected to data loggers (HOBO 8, Onset, Bourne, Massachusetts, USA). A more detailed description of the microclimatic conditions for each light treatment can be found in Gómez-Aparicio, Valladares, and Zamora (2006).

Experimental seedlings were randomly grouped in 6 blocks per light treatment; each block contained 20 seedlings per species, which were moved periodically within the block to reduce possible effects of within-block light variability. Random assignment resulted in neither between-treatment nor between-block differences in seedling initial height for any species ($P > 0.05$ in all cases, nested two-way ANOVA). Throughout the experiment, pots were watered to saturation with a variable frequency depending on the season and the light treatment (from no watering in winter to 3 times per week in mid-summer), so that water was never a

limiting factor. To check homogeneity between light treatments and blocks in water availability, volumetric soil-water content was measured in July 2001 in 10 pots per treatment and block combination (total number of 180 pots) using ThetaProbe sensors (Delta-T Devices Ltd., Cambridge, UK). No significant differences in soil-water content were recorded among either treatments or blocks ($P > 0.05$ in all cases; nested two-way ANOVA). Pots were regularly weeded to avoid competition from weeds. Levels of natural insect herbivory on the plants were also monitored during the experiment. Since insect attack was negligible, no insecticide was applied, and insect effects were considered to be of no consequence to the experimental results.

In August 2002 (> 2 y in age), for each block, 8 saplings per species (the minimum even number of live saplings of any species in any block) were randomly set aside for the experiment, for a total of 576 experimental plants (8 saplings \times 4 species \times 3 light treatments \times 6 blocks). In each block, half of the selected saplings per species were randomly assigned to the clipping treatment, with the other half assigned to the unclipping treatment. There were no initial significant differences in height or number of buds ($P > 0.05$ in all cases, nested ANOVA). The clipping treatment was applied in the first week of September 2002, mimicking the time of the year with the highest ungulate damage to natural tree saplings (Baraza, 2004). Plant responses *in vivo* were measured during the next growth period after clipping (June–July 2003). In the final week of August 2003 (> 3 y in age), saplings were harvested, carefully washed to avoid losing the fine roots, and placed in plastic bags for transport to the laboratory.

MORPHOLOGICAL VARIABLES

Annual growth of the leader shoot was measured at the end of the 2003 growing season (the apical growth is easily recognizable by its different colour) with a digital calliper to the nearest 0.1 mm. For dry-weight determinations, saplings were separated into stem, roots, and leaves and dried in an oven for 1 week at 45 °C (at temperatures below 40 °C enzymes may still function, leading to oxidation, while at temperatures above 60 °C heat damage and polymerization may occur; Reed, 1995). The leaf area of each sapling of the 3 broadleaf species was calculated using scanned reproductions of the leaves in a computer imaging system (Medidor de Objetos 1999–2000, R. Ordiales Plaza®, Almería, Spain). To calculate the leaf areas in pines, we first measured length and diameter of 3 needles from buds of different growth years (2002–2003) and from clipped and unclipped buds (6–9 needles per sapling; total of 996 needles), then calculated their areas. Dry mass and projected areas of the individual needles were used to calculate the leaf mass per unit area (LMA) for each pine sapling. LMA and total needle mass were used to calculate the total leaf area per pine sapling.

Roots, stem and twigs, and leaves were weighed on an electronic spring balance (\pm 0.0001 g) to measure the following variables: Total biomass (g), ratio of leaf dry mass to total leaf area (leaf mass per unit area, LMA, $\text{g}\cdot\text{m}^{-2}$), ratio of leaf dry mass to total sapling dry mass (leaf-mass ratio, LMR, $\text{g}\cdot\text{g}^{-1}$), and ratio of root dry mass to total sapling dry mass (root-mass ratio, RMR, $\text{g}\cdot\text{g}^{-1}$).

PHYSIOLOGICAL VARIABLES

During July 2003, net CO₂ assimilation rates (A) were measured for 36 saplings per species, 1 per species in each light and clipping treatment in each block, with a portable open gas-exchange system (LCi, ADC BioScientific Ltd., Great Amwell, UK). Measurements were conducted under high light conditions (PAR > 900 μmol·m⁻²·s⁻¹). Gómez-Aparicio, Valladares, and Zamora, (2006) reported a light-saturation point around 900 μmol·m⁻²·s⁻¹ for all the studied species. Thus, it was assumed that *in situ* rates of photosynthesis would be at or near maximum level. We measured an arbitrarily selected, mature, undamaged leaf/needle produced after clipping from the middle part of a lateral branch. Leaves/needles used in the CO₂-assimilation analysis were marked in order to measure their individual area following harvest, to permit calculation of photosynthesis in a projected area (A_{area}).

In July 2003, variable (F_v) and maximum (F_m) fluorescences were measured, and the maximum photochemical efficiency of photosystem II (PSII) (F_v/F_m) in darkness was calculated as a measurement of the physiological condition of the saplings. Measurements were made at 0100, after more than 2 h of darkness on 2 consecutive nights.

PHOTOSYNTHETIC PIGMENTS

Also in July 2003, we collected samples of 36 saplings per species, 1 per species and treatment in each block, to analyze the chlorophyll content. Total chlorophyll (Chl *a* plus Chl *b*) content and carotenoids were determined spectrophotometrically (Spectronic 2000, Bausch & Lomb, Rochester, New York, USA) by incubating leaf fragments (5-mm-diameter for broadleaf species; pieces of needles 5- to 10-mm-long for black pine) in dimethyl sulfoxide for 3 h in the dark at 65 °C, following Barnes *et al.* (1992). To estimate dry mass of pine samples, a subsample of 10 pine saplings per treatment were weighed before and after drying in an oven at 65 °C for 10 d. With these data, we established a regression between fresh and dry mass ($R^2 = 0.97$) to calculate dry mass. In the case of broadleaf species, total leaves dry mass was estimated using the data of each leaf mass per area (LMA) of each sapling.

BIOCHEMICAL CHARACTERISTICS

Two chemical variables traditionally related to herbivore damage were analyzed on 5 to 7 randomly selected saplings per treatment and species (total number of 144 saplings). Before chemical analysis, dry leaves were ground to a particle size of ≤ 1 mm. The following chemical variables were analyzed:

- 1) *Nitrogen concentration*, as a nutrient content estimator. The N concentration, expressed as a percentage of dry weight, was quantified by sulphuric acid digestion of 0.5 g dry leaf/needle powder, distillation in a semi-automatic Kjeldahl distiller (Büchi B-324, Flawil, Switzerland), and subsequent titration.
- 2) *Phenolic compounds*, as chemical-defence estimators (Waterman & Mole, 1994). The oxidative capacity of total phenols and condensed tannins were analyzed by the Folin-Ciocalteu method (Hódar & Palo, 1997) and

proanthocyanidin assay (Waterman & Mole, 1994), respectively. These procedures are considered acceptable for relative comparisons of the oxidative capacity of phenolic compounds (Reed, 1995; Yu & Dahlgren, 2000; Baraza *et al.*, 2004). Concentrations of phenolic compounds were statistically analyzed using direct values of absorbance, without transformation to standard equivalents (see Waterman & Mole, 1994 for a full explanation of this procedure).

STATISTICAL ANALYSIS

Differences in percentage of mortality between treatments were analyzed using the Wilcoxon test. Given that our data were unbalanced because of sapling mortality, we used a mixed-effects model approach for split-plot design to test the effect of factors on each analyzed characteristic of the saplings (Crawley, 2007). The whole-plot factor was light, while the split-plot factors were species and herbivory treatments, with block as the random factor (*i.e.*, split-plot layout). The *lme* function (method set to restrict maximum likelihood from the *nlme* R package; Pinheiro *et al.*, 2008) was used to fit the linear mixed-effects models. Significance of fixed effects and their interactions were assessed by means of likelihood-ratio tests of reduced models lacking the factor being tested against the full model (likelihood-ratio test of a nested-model comparison; Zuur *et al.*, 2009). LMA, oxidative capacity of phenols, chlorophyll *a:b* ratio and the carotenoids:chlorophyll ratio were analysed with a Generalized Least Squares (GLS) model with light, species, and treatment as fixed factors. This model included a variance estimate for each species with varIdent variance function and a Compound Symmetry Correlation Structure for data of the same block, both from the *nlme* R package (Pinheiro *et al.*, 2008; Zuur *et al.*, 2009). Significance of fixed effects and their interactions were determined with the ANOVA function of the fitted GLS objects.

To improve homoscedasticity, when necessary, logarithmic, arcsine, or square root transformations were performed on variables. Standard model-checking plots were used to verify that there was no obvious inequality of their variance residuals and that they had an approximately normal distribution. Statistical analyses were carried out using the R statistical environment for statistical computing and graphics (R Development Core Team, 2008).

Results

Saplings of the studied species tolerated some degree of tissue removal, since the percentage of sapling mortality was not affected by clipping ($Z = -1.33$, $P = 0.18$, Wilcoxon test). Nevertheless, the differences between clipped and unclipped saplings varied from scant to highly significant, depending on the set of variables measured, the species, and the light environment (Tables I–III).

We found different morphological responses of saplings to herbivory depending not only on the trait analyzed, but also on species and light availability (Table I). In general, clipped plants showed a reduced (undercompensation) or

equal (exact compensation) total biomass and growth with respect to control plants, except for Pyrenean oak in full sunlight, which registered a greater total biomass after clipping (overcompensation; Figure 1). Moreover, biomass allocation varied after clipping only for some species and light treatments. Spanish maple, showed a significant reduction of total area and total biomass only for saplings growing in 13% sunlight (Figure 1), while Holm oak showed a significant decrease of total area, total biomass, LMR, and LMA when growing under full sunlight (Figure 1). In the case of Pyrenean oak, total area significantly increased after clipping in saplings growing in full sunlight, while clipped saplings in 13% sunlight showed a slight decrease. Pyrenean oak growing in 13% sunlight also experienced a significant reduction of LMR after clipping. Black pine was the species most affected by clipping, showing a significant effect for all morphological variables. Independently of the light treatment, clipping reduced total biomass, total area, and LMR of black pine saplings. A decrease of RMR was significant only for saplings growing at 80% and 13% sunlight, whereas reduction of leader-shoot growth was significant only for saplings growing at 13% (Figure 1).

Clipped plants did not show a significant change in the studied physiological properties in comparison to controls (Table II). However, clipped saplings tended to show an increased A_{area} under low light availability (clipped $5.15 \pm 0.84 \mu\text{mol CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$; control $3.16 \pm 0.64 \mu\text{mol CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) but reduced A_{area} in full sunlight (clipped $5.88 \pm 0.66 \mu\text{mol CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$; control $7.40 \pm 0.88 \mu\text{mol CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). Saplings of the 3 broadleaf species grown in full and 80% sunlight suffered mild photoinhibition, measured as F_v/F_m (values lower than the theoretical optimum 0.8 but higher than 0.7; Demmig-Adams & Björkman, 1987), whereas black pine in no case showed photoinhibition. Clipping effect on photoinhibition depended on the species, since Spanish maple saplings showed higher F_v/F_m after clipping (clipped 0.791 ± 0.007 ; control 0.778 ± 0.005), whereas Pyrenean oak presented the opposite tendency (clipped 0.799 ± 0.006 ; control 0.808 ± 0.003).

Clipping increased the total chlorophylls (clipped $4.61 \pm 0.27 \text{ g} \cdot \text{kg}^{-1}$; control $3.87 \pm 0.21 \text{ g} \cdot \text{kg}^{-1}$) and carotenoids:Chl ratio (clipped 0.206 ± 0.008 ; control 0.199 ± 0.007) (Table III). The chlorophyll $a:b$ ratio decreased with light availability for control saplings (full sunlight 2.42 ± 0.20 ; 80% sunlight 1.96 ± 0.11 ; 13% sunlight 1.85 ± 0.12) but remained similar between light treatments for those that were clipped (full sunlight 2.12 ± 0.13 ; 80% sunlight 1.80 ± 0.12 ; 13% sunlight 2.24 ± 0.21) (Table III). The effect of clipping on the carotenoids:Chl ratio varied with light: no differences were found in full sunlight (clipped 0.23 ± 0.01 ; control 0.23 ± 0.01), a slight decrease appeared on clipped saplings at 80% sunlight (clipped 0.21 ± 0.007 ; control 0.23 ± 0.01), and a larger increase was seen on clipped saplings at 13% sunlight (clipped 0.16 ± 0.01 ; control 0.14 ± 0.01). On the other hand, we found no significant changes in the biochemical properties measured in clipped plants for any of the species or light levels (Table III).

Light had a significant effect on the morphology, physiology, and biochemistry of saplings (Tables I–III). All morphological variables tended to decrease when light availability diminished, with the exception of leader-shoot growth and LMR, which increased, and total area, which diminished for black pine but increased for the 3 broadleaf

TABLE I. Values of level of significance (P value) obtained by likelihood-ratio test of reduced nested GLMM model comparison. The morphological variables analyzed are leader-shoot growth in 2003 (G.03), total biomass (Biomass), total leaf area per sapling (Area), leaf mass per area (LMA), leaf-mass ratio (LMR), and root-mass ratio (RMR). For LMA, significance was determined with the ANOVA function of the fitted GLS object. Significant values are in boldtype. P values correspond to the critical P value = 0.0328 calculated with the False Discovery Rate (FDR) at the 5% level, following the Ventura, Paciorek, and Risbey (2004) criteria.

	G.03	Biomass	Area	LMA	LMR	RMR
Species	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Light	< 0.0001	< 0.0001	0.3374	< 0.0001	< 0.0001	< 0.0001
Clipping	0.0325	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0087
S × L	0.0001	< 0.0001	< 0.0001	0.0013	0.0328	0.0049
S × C	< 0.0001	< 0.0001	< 0.0001	0.0004	0.0007	0.0007
L × C	0.0009	0.0255	0.0086	< 0.0001	0.1241	0.3034
S × L × C	0.0004	0.4964	0.0546	< 0.0001	0.0792	0.013

TABLE II. Values of level of significance (P value) determined by the likelihood-ratio test of reduced nested GLMM model comparison. The variables analyzed are net CO_2 assimilation rates, expressed on a leaf-area basis (A ; $\mu\text{mol CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), and photochemical efficiency of photosystem II (F_v/F_m), according to the factors species (S), light (L), and clipping (C) treatments. Significant values are in boldtype.

	A ($\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$)	F_v/F_m
Species	0.0524	< 0.0001
Light	0.0085	0.0007
Clipping	0.0829	0.1102
S × L	0.0007	0.0005
L × C	0.0098	0.5648
S × C	0.9726	0.0214
S × L × C	0.8345	0.1194

TABLE III. Values of level of significance (P value) determined by the likelihood-ratio test of reduced nested GLMM model comparison for total chlorophyll (Chl $a+b$) ($\text{g} \cdot \text{kg}^{-1}$), nitrogen concentration, and oxidative capacity of condensed tannins. For chlorophylls $a:b$ (Chl $a:b$) ratio, carotenoids:Chl ratio, and oxidative capacity of total phenols, significance was determined with the ANOVA function of the fitted GLS objects, according to the factors species (S), light (L), and clipping (C) treatments. Significant values are in boldtype. Critical P value ≤ 0.0222 was calculated with the False Discovery Rate (FDR) at the 5% level, following the Ventura, Paciorek, and Risbey (2004) criteria.

	Chl $a+b$	Chl $a:b$	Carotenoids:Chl	N (%)	Phenols	Tannins
Species	0.0001	0.0001	0.0001	< 0.0001	< 0.0001	< 0.0001
Light	0.0001	0.0038	0.0001	0.0003	0.0002	0.0521
Clipping	0.0031	0.4301	0.0075	0.8823	0.1414	0.4218
S × L	0.2172	0.2944	0.3382	0.0065	0.0049	0.8671
L × C	0.3726	0.0222	0.0091	0.0656	0.7318	0.8238
S × C	0.1279	0.8904	0.1285	0.8048	0.2685	0.8442
S × L × C	0.4689	0.8131	0.9961	0.6755	0.1170	0.0589

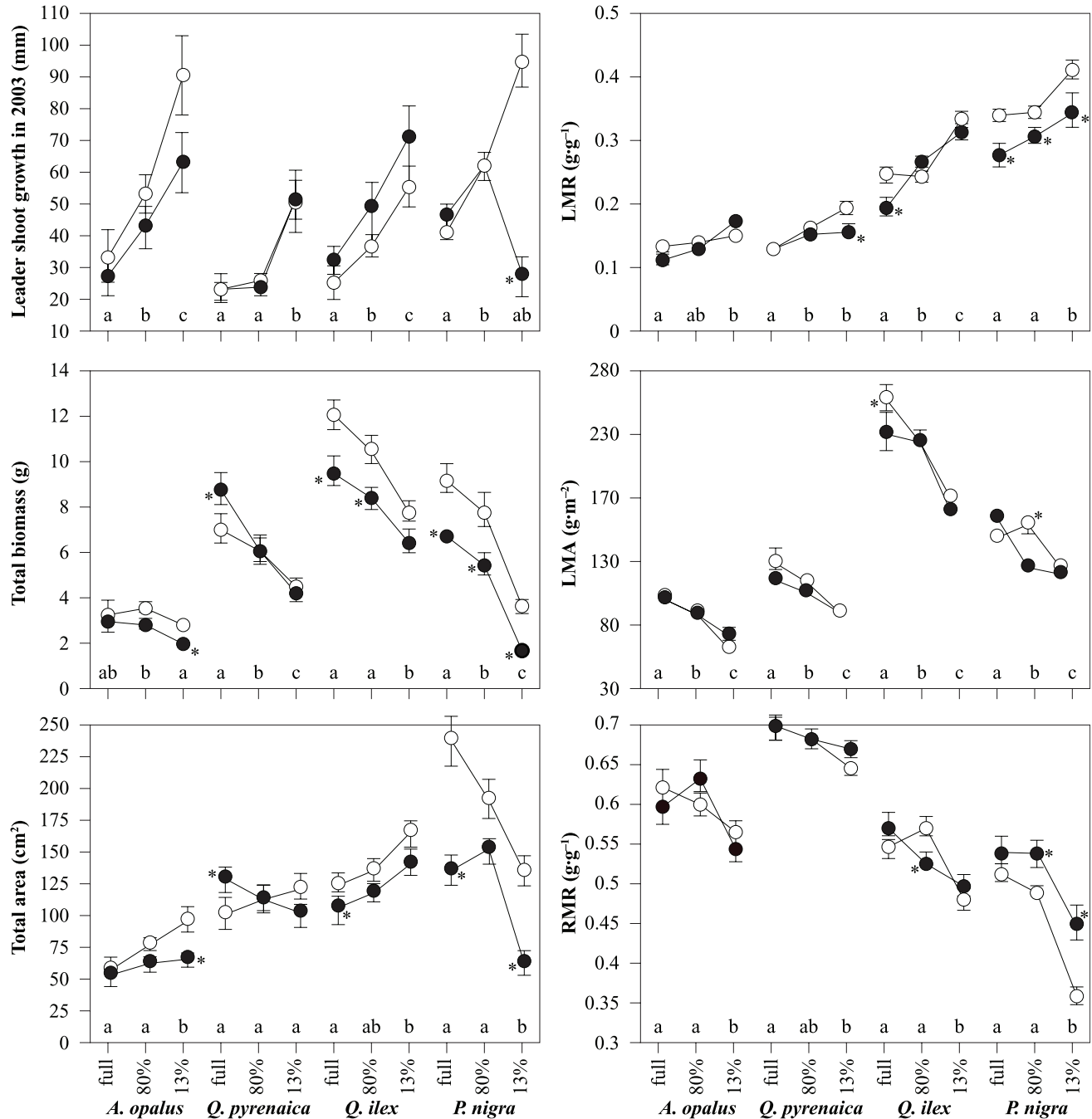


FIGURE 1. Mean \pm SE values among species, light treatments (full sunlight, 80%, and 13% of full sunlight), and clipping treatments (white: control; black: clipped) for the morphological variables leader-shoot growth in 2003 (mm), total biomass (g), total leaf area per sapling (cm^2), leaf:mass ratio (LMR, $\text{g}\cdot\text{g}^{-1}$), leaf mass per area (LMA, $\text{g}\cdot\text{m}^{-2}$), and root:mass ratio (RMR, $\text{g}\cdot\text{g}^{-1}$). Asterisks indicate significant differences between clipping treatments, and different letters indicate significant differences between light treatments within a species (*a posteriori* Tukey–Kramer HSD test).

species (Figure 1). That is, biomass allocation to leaves increased, and these leaves expanded their surface area per biomass unit. Moreover, shade diminished A_{area} (full sunlight $6.66 \pm 0.56 \mu\text{mol CO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; 80% sunlight $4.04 \pm 0.36 \mu\text{mol CO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; 13% sunlight $4.19 \pm 0.55 \mu\text{mol CO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), chlorophyll *a*:*b* (full sunlight 2.28 ± 0.12 ; 80% sunlight 1.88 ± 0.08 ; 13% sunlight 2.04 ± 0.12), and carotenoids:Chl ratios (full sunlight

0.23 ± 0.01 ; 80% sunlight 0.22 ± 0.01 ; 13% sunlight 0.15 ± 0.01) but increased total chlorophyll (full sunlight 3.24 ± 0.19 ; 80% sunlight 3.83 ± 0.23 ; 13% sunlight 5.56 ± 0.34). Similarly, nitrogen increased, but oxidative activity of total phenols and tannins decreased when light availability declined (Figure 2). Deciduous species showed more nitrogen than the evergreen ones, and showed a minor nitrogen increase as light decreased.

Discussion

Our results clearly show a species-specific effect of clipping in saplings, with a predominantly morphological

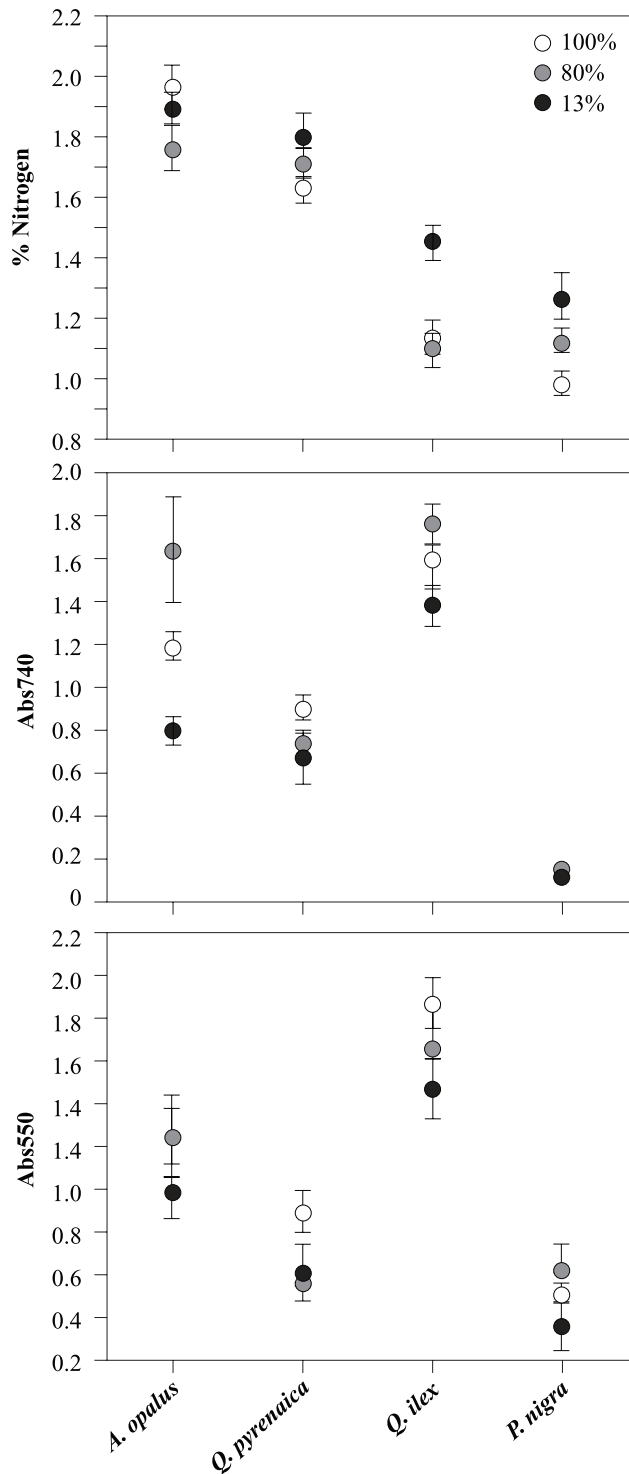


FIGURE 2. Mean \pm SE values among species and light treatments (full sunlight, 80%, and 13% of full sunlight) in nitrogen concentration, oxidative capacity of total phenols (Abs740), and oxidative capacity of condensed tannins (Abs550). For the sake of simplicity, the clipping treatment was not included due to the lack of significant effects in these variables (see Table III).

response to herbivory (variation in growth and biomass distribution). The slight physiological response or biochemical modification after clipping underlines the importance of other tolerance mechanisms, such as regrowth from undamaged meristems or biomass reallocation, for the ability of woody plants to recover the biomass lost (compensation capacity) (Millard *et al.*, 2001; Stevens, Kruger & Lindroth, 2008). Of most interest, this study experimentally demonstrates the importance of the light environment as a key factor shaping the defence and compensation capacity of tree saplings after herbivory.

MORPHOLOGICAL RESPONSE PREDOMINATES OVER PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES

Clipping affected several morphological traits of the saplings of the 4 species, which were not always able to compensate for the removed biomass. Depending on the species and the light treatment, we found a reduction of total biomass and total area on clipped saplings, indicating incomplete recuperation of the above-ground biomass lost and a decreasing ratio between above-ground and root mass (reduction of LMR and increase of RMR). Also, the clipping tended to decrease LMA and increase total chlorophylls, which could be interpreted as an effort by the plant to increase light absorption (Reich *et al.*, 1998). By contrast, we found no significant increase of the photosynthetic rate after clipping (Kotanen & Rosenthal, 2000; Haukioja & Koricheva, 2000), nor any significant effect on maximum photochemical efficiency of photosystem II, indicating a lack of sapling physiological response. This suggests that other tolerance mechanisms allow saplings to compensate for the removed tissues (Strauss & Agrawal, 1999), emphasizing the importance of measuring a variety of plant traits for a more accurate evaluation of plant tolerance to herbivory (Haukioja & Koricheva, 2000).

Light can influence physiological responses to clipping: there were increments of A_{area} , Chl *a:b*, and carotenoids:Chl ratios only among saplings growing in shade, whereas the opposite trend was found in saplings growing in full sunlight. A possible explanation of this pattern lies in the lack of reserves in plants growing in shade, as shown by their lower total biomass, lower RMR, and higher LMR; thus, the only compensation mechanism available is the increased photosynthetic rate. This would be consistent with recent studies showing an increment of photosynthetic rate as a dynamic response after herbivory that could be activated only under specific conditions (Ozaki, Saito & Yamamuro, 2004; Retuerto, Fernández-Lema & Obeso, 2006). Moreover, response to herbivory in contrasting light environments depends on the light-adaptation state of plants (Nabeshima, Masashi & Hiura, 2001; Retuerto, Fernández-Lema & Obeso, 2006).

After the clipping, we found no significant biochemical change in any of the species studied. This result is consistent with the meta-analysis review of Nykänen and Koricheva (2004), which suggested a low probability of increases in phenolic compounds after herbivory in saplings undergoing damage later in the season, as in our case. Similarly, Massei, Hartley, and Bacon (2000), in a study of 4 evergreen woody species growing in a Mediterranean

area, found no difference in phenolic content between browsed and unbrowsed plants. A long history of intensive exploitation of Mediterranean vegetation, by both human activity and heavy grazing, may have played a crucial role in the development of plant chemical defence, promoting constitutive defences more than inducible ones (Perevolotsky, 1994; Massei, Hartley & Bacon, 2000). In short, the most responsive variables in the clipping experiment were those related to architecture, growth pattern, and size.

RESPONSES TO BROWSING ARE SPECIES-SPECIFIC

We found marked differences between species in their capacity to recover the biomass lost (compensatory capacity). Black pine was the species most affected by clipping, whereas the rest of the species compensated depending on the light treatment. Compensatory capacity is not well developed in conifers, largely due to their more fixed, monopodial growth patterns, slower turnover of plant parts, and lack of nutrient translocation to stem and roots for winter storage (Millard *et al.*, 2001; Palmer & Truscott, 2003; Hódar *et al.*, 2008). By contrast, broadleaf species, which store nutrients in their woody tissues (lignotuber, wood, bark), are able to compensate or even overcompensate (as Pyrenean oak in full sunlight), presumably utilizing resources from the years prior to herbivory (Millard *et al.*, 2001; Hester *et al.*, 2004; Stevens, Kruger & Lindroth, 2008).

With respect to leaf lifespan, the evergreen Holm oak had herbivory tolerance unexpectedly similar to that of the deciduous species. This is presumably due to the accumulation of reserves in the lignotuber in this species. Such reserves can provide a high capacity to resprout after biomass losses (Espelta, Habrouk & Retana, 2006 and references therein). The presence of a storage site for reserves in tree species may therefore be crucial in providing a browsing-tolerance capacity, with leaf lifespan and growth rate characteristics being relatively less important (Millard *et al.*, 2001; Hester *et al.*, 2004; Stevens, Kruger & Lindroth, 2008).

LIGHT AVAILABILITY AFFECTS PLANT COMPENSATION AFTER HERBIVORY

There were species-specific responses for an interaction between clipping and light treatment (see Roberts & Paul, 2006 for similar findings). Light scarcity led to a strong negative effect of herbivory in black pine saplings (see Lentz & Cipollini, 1998; Harmer, 1999; Saunders & Puettmann, 1999 for other examples). The negative effect of shade on herbivore tolerance also occurred for Spanish maple, since there was a significant reduction in total area and total biomass only in 13% sunlight. In the case of Pyrenean oak, saplings growing in full sunlight showed total biomass and area overcompensation, whereas the other light treatments resulted in total compensation (see Baraza *et al.*, 2004 for similar results). Conversely, Holm oak saplings appeared to be negatively affected by clipping only when growing in full sunlight.

Clipping alters the plant's capacity to capture light by changing the leaf area available, both by removing light acquisition tissues during clipping and by conditioning the new photosynthetic tissue produced in the next growing season in damaged saplings (Figure 1). Moreover, apical-

meristem herbivory promotes competition for apical dominance, activating axillary meristems and growth of additional branches, enabling the plant to take advantage of the higher resource availability, but no such benefit is likely in a low-level resource situation (Wise & Abrahamson, 2005). Under both conditions, LMR predicts greater tolerance in environments in which light is not limited (Wise & Abrahamson, 2005; 2007). In our case, 3 of the 4 species analyzed corroborate the limiting-resource model (Wise & Abrahamson, 2005), showing a higher tolerance (even overcompensation in the case of Pyrenean oak) in the high-resource environment (Wise & Abrahamson, 2005; 2007), while the fourth species, Holm oak, offered inconclusive results. This suggests that further analysis of a broad spectrum of plant species would be desirable for a full understanding of the complex reality of light effects on plant responses to herbivory.

We have found that the light environment under which saplings grow could mediate their interaction with herbivores by affecting the constitutive defences of the saplings to a higher degree than the induced defences. Shade-grown saplings of the 4 studied species showed more nitrogen concentration and less oxidative capacity of phenolic compounds (Covelo & Gallardo, 2001; Baraza *et al.*, 2004; Estiarte, De Castro & Espelta, 2007), presumably making them more attractive to herbivores (Berteaux *et al.*, 1998; Nabeshima, Masashi & Hiura, 2001; Tripler *et al.*, 2002). In addition, 3 of our 4 species declined in compensation capacity after clipping in 13% sunlight, suggesting that shade-grown saplings are less able to compensate following herbivory (see Lentz & Cipollini, 1998; Saunders & Puettmann, 1999; Hódar *et al.*, 2008 for similar results).

Thus, light scarcity could influence the probability of herbivore damage (for both clipped and unclipped plants), and, after herbivory, shade affects compensation capacity. On the other hand, several studies have demonstrated that shade provided by pre-established pioneer shrubs is a requirement for the establishment of many woody species in Mediterranean environments (Hastwell & Facelli, 2003; Maestre *et al.*, 2003; Gómez-Aparicio *et al.*, 2004; 2005; 2006). Our work reveals for the first time that when light availability and herbivory are considered in combination, moderate shade, 80% sunlight in our case, does not limit saplings' compensation after herbivory in a sample of disparate sapling species such as *Quercus*, *Acer*, and *Pinus*. Thus, shrubs, which supply moderate shade, proved to be the most probable regeneration niche under high levels of stress, both abiotic (high irradiance) and biotic (herbivory). This reinforces the importance of considering environmental effects on plant tolerance to herbivory in order to understand the spatial organization of the bank of recruits in Mediterranean forest ecosystems.

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