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Light intensity as a growth limiting factor for the relict broadleaved spindle *Euonymus latifolius* in Mediterranean refugia. The need to preserve the forest canopy structure.

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Abstract. *Euonymus latifolius* is a paradigmatic nemoral-subMediterranean species of the relict flora from the Würm glaciation in the Iberian Peninsula. Due to its transitional nature, taxa of this plant group could be used as a sensitive indicator to evaluate the effects of climate change on plants in the near future. In the Iberian Peninsula, *E. latifolius* is scattered in relict, isolated small populations, and is catalogued as critically endangered. Previous studies of this taxon indicated the strong dependence of recruitment on canopy cover, which was attributed to the shade-mediated maintenance of soil humidity in the juvenile microhabitat. However, no information is available on the effects of light radiation on this nemoral species. In this study, we investigated the growth response of *E. latifolius* to photosynthetically active radiation by producing and transplanting saplings along a broad light intensity gradient, i.e., from direct illumination to dense shaded canopy, in a nemoral mixed deciduous forest stand in the Botanic Garden of Castilla-La Mancha. Our results confirmed the marked sciophilous nature of *E. latifolius*, which is particularly uncommon in the Mediterranean flora, by demonstrating a drastic reduction in growth with the increase of exposure to light intensity, which became critical at high values and even lethal when plants were directly exposed to light. The opening of the canopy in the natural habitat of this species due to simplification of the structure of nemoral communities promoted by climate change in southern latitudes of Europe comprises a real and short-term risk for *E. latifolius*, and probably for other relict nemoral-subMediterranean species. Conservation actions are recommended.

Keywords. Light intensity, Growth limiting factor, Euonymus latifolius, Mediterranean refugia, Relict flora, Climate change, Shade tolerance, Conservation.

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Introduction

The broadleaved spindle tree, *Euonymus latifolius* L. (Mill.) (Celastraceae), is a paradigmatic species of the relict flora from the Würm glaciation in the Iberian Peninsula (Agea *et al.*, 2021). *Euonymus latifolius* is a deciduous shrub or small tree with a wide distribution over Europe, and it can be found from eastern Spain to the Caucasus, as well as the Irano-Turanian region and North Africa, in the Middle Atlas, Tell Atlas, and Kabilia (Benedí, 1997). Southern European peninsulas have repeatedly played notable roles as refuges in the natural history of flora during periods of glaciation (Médail *et al.*, 2009). Indeed, the Iberian Peninsula is the southernmost European distribution limit for the broadleaved spindle tree (Agea *et al.*, 2021; GBIF, 2023) as a result of migration displacements during the last glaciation and subsequent loss of habitats caused by glacial retreat (López, 2001; Gutiérrez *et al.*, 2004). In the Iberian Peninsula, *E. latifolius* lives in remnants of relict nemoral-subMediterranean forests and thorny shrublands largely dominated by broadleaved deciduous species (Bolòs, 1985; Ozenda, 1994). This vegetation is recognized as a transitional flora between the Mediterranean and Euro-Siberian types in Europe (Sánchez de Dios *et al.*, 2009), which lives under intermediate climate conditions that characterize both regions, with a combination of species from the two phytogeographical zones (Bolòs, 1985; Morla & Pineda, 1985; Moreno *et al.*, 1990).

Due to their relict (Meusel & Jäger, 1989) and biogeographically transitional nature between cool/wet and warm/dry weather types, nemoral-subMediterranean species should be particularly vulnerable to current climate change, thereby providing with a sensitive

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indicator to evaluate the consequences of warming for plants in the near future. In particular, the broadleaved spindle tree is abundant in temperate central Europe (GBIF, 2023), but the Iberian relict populations are particularly scarce (Agea *et al.*, 2021) and distributed in small areas (Gutiérrez *et al.*, 2004; Peña *et al.*, 2018) with low recruitment rates and sizes in the minimum viable population (*sensu* Flather *et al.*, 2011) in most cases (Gutierrez *et al.*, 2003; Agea *et al.*, 2021). Due to its relict character and scarcity at these latitudes, *E. latifolius* is considered one of the rarest shrubs in the Iberian Peninsula (López, 2001), and thus it is catalogued as *Critically Endangered* (CR) in the *Atlas and Red Book of the Threatened Vascular Flora of Spain* (Gutiérrez *et al.*, 2004), and in the *Red List of Vascular Flora of Spain* (IUCN criteria: B2ab(iii,iv) C2a(i); Bañares *et al.*, 2008; Moreno *et al.*, 2011).

In addition, the predictions of the effects of climate change are pessimistic. Warming and low rainfall are expected to be intensified in the Iberian Peninsula in the short to middle term (Nogués-Bravo *et al.*, 2007; Sanz & Galán, 2020), which will make subMediterranean conditions rarer in Mediterranean mountains (Sánchez de Dios *et al.*, 2009), thereby reducing the presence of this transitional vegetation in the territory (Herranz *et al.*, 2009). Furthermore, evidence suggests that the harshening of climate conditions could progressively simplify the diversity and structure of these plant communities, with reduced plant cover (Heady & Child, 1994; Sanz & Galán, 2020). Moreover, grazing, especially that by wild ungulates, will contribute to the opening of the canopy in *E. latifolius* habitats (Tíscar-Oliver, 2015; García-Cardo, 2021). Agea *et al.* (2021) found a very strong dependence of *E. latifolius* recruitment on the canopy density (the abundance of seedlings was associated with high cover values, i.e., over 80%), thereby suggesting that this plant community feature is a limiting factor for population maintenance. They concluded that such a strong reliance may be due to detrimental changes in the juvenile microhabitat leading to the loss of shade, with a consequent increase in the soil temperature and decreased soil moisture. However, despite this strong causal relationship between the canopy density and seedling recruitment, no information is available regarding the effects of light radiation on the performance of the broadleaved spindle tree. *Euonymus latifolius* typically forms part of the understory in nemoral broadleaved hygrophilous plant communities (Gutierrez *et al.*, 2004; García-Cardo, 2021), so it is expected to be a shade tolerant species, and thus able to survive and complete its life cycle at low light levels (Valladares and Niinemeets, 2008). However, what are the specific light requirements of this unusual nemoral-subMediterranean plant in the Iberian Peninsula? This question should be particularly critical at low latitudes within the distribution range of *E. latifolius*, where populations are rare and subject to higher light irradiance than the average for the species (Roberts & Paul, 2005). The effects of the intensity of photosynthetically active radiation (PAR; i.e., light within the wavelength range of 400–700 nm) on plants have been studied widely in cultivated species (Cherbiy-Hoffmann *et al.*, 2013; Pinto *et al.*, 2011), tropical species (Zang *et al.*, 2021; Chao *et al.*, 2022), and even fern associations (Saldaña *et al.*, 2005; Rünk *et al.*, 2006). However, few studies have investigated wild forest species, particularly threatened species. The light-use efficiency is important for understanding biomass production, and thus several photosynthesis models that consider the light absorption profile have been proposed based on the optimal use of PAR photons (Farquhar, 1989; Hikosaka & Terashima, 1995; Terashima *et al.*, 2016). However, very high irradiances can damage photosystems and a range of mechanisms are known to bypass photoinhibition or photo-oxidation (Yamori, 2016). Thus, the safe use of direct solar radiation is a key concept for understanding light absorption by the leaves of terrestrial plants (Kume, 2017), particularly for shade-loving species with a nemoral habit because different capacities for the safe utilization of sunlight could limit the growth of this type of plants. Due to the realistic risk of community structure simplification and canopy opening for sensitive vegetation under progressive aridification in the Iberian Peninsula, what might be the response of critically endangered and presumably sciophilous species to eventual increases in exposure to light?

The main goal of this study was to understand the effect of PAR on *E. latifolius* plant growth to determine how the expected increase in light exposure due to canopy structure simplification under aridification might affect recruitment in endangered, small relict Mediterranean populations in the Iberian Peninsula. To achieve so, we collected and germinated seeds of this species from populations in the Sistema Ibérico Mountain range (central–eastern Spain) and cultivated saplings for 5 years. The saplings were then transplanted into a nemoral mixed deciduous forest stand at the Botanic Garden of Castilla-La Mancha along a broad PAR intensity gradient, and the plant growth responses were monitored. The findings obtained in this study will contribute to improving the selection criteria for locations and environments in restitution programs based on the light conditions. We realize that our experimental design based on a single location where the natural habitat of the species has been simulated may produce some biased, small-scale conditioned results, so caution should be paid in their interpretation, particularly when referring to the numerical dimension. In any case, this study should represent a critical step forward in the exploration of an unknown response trait in the very few remaining Iberian populations of *E. latifolius*, which may significantly contribute to improving the selection criteria for locations and environments in restitution programs based on the light conditions.

Materials and methods

Study species

Euonymus latifolius is a deciduous shrub or a small tree, which lives in shady, humid, inaccessible limestone rocky environments between 720–1900 m asl in the

Iberian Peninsula (Gutiérrez *et al.*, 2003), where it forms part of thorny deciduous scrublands (Rhamno-Prunetea Rivas Goday & Borja ex Tüxen 1962) and Euro-Siberian mixed forests (Tilio-Acerion Klika 1955) (Gutierrez *et al.*, 2004; García-Cardo, 2021) as a representative member of the nemoral-subMediterranean vegetation. These communities are largely dominated by broadleaf deciduous species, including trees such as *Tilia platyphyllos* Scop., *Corylus avellana* L., *Acer monspessulanum* L., *Sorbus aria* (L.) Crantz, *Sorbus aucuparia* L., *Ulmus glabra* Huds., *Fraxinus excelsior* L., and *Prunus avium* L., and shrubs such as *Rhamnus cathartica* L., *Prunus mahaleb* L., *Viburnum lantana* L., *Cornus sanguinea* L., *Lonicera xylosteum* L., and *Amelanchier ovalis* Medik. In the Iberian Peninsula, *E. latifolius* is distributed in two separate relict mountain metapopulations (Cazorla-Segura and central–eastern Spain; see Agea *et al.*, 2021) comprising 23 populations with around 320 mature plants overall, scattered in very small localities (Gutiérrez *et al.*, 2004; Peña *et al.*, 2018; Agea *et al.*, 2021), and thus it is considered critically endangered at these latitudes (Gutiérrez *et al.*, 2004; Moreno *et al.*, 2011). The main threats include climate change (Herranz *et al.*, 2009; Agea *et al.*, 2021; García-Cardo, 2021), herbivory (Agea *et al.*, 2021; García-Cardo, 2021), outdoor recreation activities (Gutiérrez *et al.*, 2003; García *et al.*, 2017; Agea *et al.*, 2021), population isolation (Rivera *et al.*, 2018), and low reproductive rates (Agea *et al.*, 2021).

Source of plant material

On September 25, 2009, we harvested *E. latifolius* fruits from shady, inaccessible, chalky boulders at the Natural Monument of Palancares and Tierra Muerta (Sistema Ibérico Meridional, Cuenca, central Spain) at a height of 1215 m asl (UTM: 30TWK889318). The climate in the area is nemoroMediterranean (Allúe, 1990), with annual rainfall of around 700 mm, slight summer droughts, and cold winters (monthly mean temperature of 2.5°C in the coldest month, January). In the laboratory, the seeds were extracted from the fruit flesh within the first 24 h after collection and preserved at ambient temperature in the laboratory for five days (for details, see Santiago *et al.*, 2023).

Germination and nursery

In early October 2009, we germinated 150 seeds to produce plants for our experiment. Apparently healthy *E. latifolius* seeds were subjected to a seed "move along" stratification treatment (according to Baskin & Baskin, 1998) in germination chambers (Ibercex, model F-4, Madrid) equipped with digital temperature and light control. Breaking seed dormancy in this species (i.e., intermediate physiological level; Santiago *et al.*, 2023) requires seed stratification at moderately warm temperatures (20/7°C + 15/4°C) for 10 weeks (i.e., simulation of autumn temperatures), followed by 15 weeks of cold stratification (5°C and/or 1.5°C; i.e., winter temperatures). The seeds are then germinated at 5°C or 15/4°C (i.e., temperatures in late winter/early spring; for details, see Santiago *et al.* 2023).

When the radicles emerged, a set of three germinated seeds were planted in each 350 cc (4,7 cm x 4.7 cm x 15.8 cm deep) sockets of plastic seedling trays, which were filled with a mixture of sterile black peat (80%), river sand (10%), and vermiculite (10%).The trays were kept in a shade-house (with no artificial temperature regulation) at the Higher Technical School of Agricultural and Forestry Engineering and Biotechnology (ETSIAMB) experimental facilities (UTM: 30SWJ98801452; University of Castilla-La Mancha, Albacete, Spain). The plants were watered to field capacity with well water (pH 7.84) once each week from May 31 to October 1, and twice each month for the rest of the year, except in the winter because the soil remained frozen, which simulated the rainfall conditions in the natural habitat of *E. latifolius* (i.e., humid mountain areas). Six months after planting (i.e., late April 2010), we retained the most vigorous seedling in each cell and removed the other two seedlings. Those seedlings were grown in the trays until their roots filled the whole container (i.e., for two years). The plants were then individually transferred to pots with a width of 22 cm and depth of 18 cm containing a mixture comprising 60% of the seedling substrate described above and 40% topsoil (consisting of marl and clay, with a pH of 8.9). The pots were kept in the shade-house under the watering regime described above for three more years. Every two weeks, any other competing plants that emerged in the pots were removed. Finally, we obtained 36 healthy, well-developed plants.

Experimental site design, plantation and soil analysis.

In September 2015, all plants reached maximum pot capacity for root growth (refer to Figure 1). Subsequently, they were transplanted into a 400 m^2 stand within the Botanic Garden of Castilla-La Mancha (UTM: 30SWJ99561410), located in Albacete city, approximately 2.08 km away from the greenhouse where they were initially cultivated five years earlier. The site was designed to simulate the climatic conditions and flora ensemble from the natural habitat of *E. latifolius*, as well as homogenizing the new plantation soil conditions to integrate those aspects into a pre-existing plant collection.

While temperature conditions exhibited comparability between the natural population of *E. latifolius*, from which the seeds used for this study were obtained, and the experimental areas in terms of average data over the study period, this was not the case for precipitation data (Table 1). Consequently, due to the divergence in precipitation levels between the natural and experimental areas, an automatic combined sprinkling and drip irrigation system was implemented to maintain consistent moisture levels for the experimental *E. latifolius* plants. Furthermore, to mitigate the impacts of evapotranspiration, synchronized irrigation initiation at dawn across all points via timers ensured optimization of light exposure until peak noon intensity.

Figure 1. A, *Euonymus latifolius* individuals as they were planted at the beginning of the test in September 2015; notice that at that time, the root system of plants filled completely pot capacity; B, Groups of three plants arranged in an equilateral triangle inscribed within a circular tree pit of 1 m in diameter; C, Stand of *E. latifolius*' natural habitat in living plant collections at the Botanic Garden of Castilla-La Mancha simulating a Euro-Siberian mountainous mixed deciduous temperate forest relict in southern Europe peninsulas, where saplings were planted for the experiment.

Table 1. Meteorological data over the study period (averages) including mean precipitation (mm), and temperatures (ºC) as absolute maximum temperature (TMA), absolute minimum temperature (tma), mean temperature of the mean temperatures (Tmm), mean minimum temperature (Tmm), and mean maximum temperature (TMM). Data provided by SIAR (Service of the Ministry of Agriculture, Water, and Rural Development, in collaboration with the University of Castilla-La Mancha) obtained from the meteorological stations of Albacete at 2.39 km from the experimental área (JBCLM) and Cañete (natural location) at 9.64 km, in a straight line from the population of *E. latifolius* from which the plant material originates.

	Precipitation (mm)	TMA (°C)	tma (°C)	Tmm (°C)	tmm (°C)	TMM (°C)
Albacete	331,7	38,7	-10,425	12,875	4,8	21,2
Cañete	574,475	37,875	-11,875	11,05	2,825	20,15

The volume of water applied remained uniform across all test plants, reflecting the maximum average value recorded under the subhumid ombroclimate in Spain (1000 mm year–1 distributed uniformly from May to October, approximately 167 mm per month). This water volume corresponded to the natural water levels available in the relict Euro-Siberian forests where E. latifolius typically thrives in Castilla-La Mancha (Central Spain) (Martín *et al.,* 2003).

The plant species responsible for providing shade were a pre-existing plant collection designed to mimic a Euro-Siberian mountainous mixed deciduous temperate forest found in the southern European peninsula, native to the area where *E. latifolius* thrives, which is a relic from the Würm glaciation stage. The community structure aimed to emulate dense nemoral-broadleaf forests (Martín *et al.,* 2003), characterized by distinct vertical plant strata (see Figure 1). Vegetation establishment began in 2003 with the introduction of 561 plants representing 66 species, within a *Brachypodium phoenicoides* grassland.

However, the canopy layer, responsible for shading *E. latifolius*, comprised a heterogeneous array of 140 plants from 37 species. The most abundant species included *Cornus sanguinea* subsp. *sanguinea* (8.7%), *Euonymus europaeus* (7.8%), *Fraxinus excelsior* (7.14%), *Ulmus glabra* (7.14%), *Prunus mahaleb* (6.4%), *Rosa canina* (6.4%), *Acer monspessulanum* (5%), *Sorbus aria* (5%), *Corylus avellana* (4.28%), and *Ilex aquifolium* (4.28%).

Individuals of *Euonymus latifolius* were planted within the aforementioned pre-existing plant collection, standing in groups of three, arranged in an equilateral triangle, with each plant situated within a circular tree pit measuring 1 meter in diameter (see Figure 1). In total, 12 test plant groups were established. The decision to employ three individuals per measurement point was guided by the necessity to mitigate potential variability arising from genotypic disparities (Breitling *et al.,* 2004; Kile & Debener, 2011; Schurch *et al.,* 2016).

Throughout the experiments, efforts were made to standardize the distribution of macro and micronutrients across all locations, with adjustments tailored to each plant's installation point. Each individual plant was allocated a planting hole with a volume of 40 cm^3. The soil within these holes was supplemented with a mixture comprising 50% commercial natural sifted mulch (particle size: 0–10 mm, pH 8.22, total nitrogen content 1.32%, organic nitrogen content 0.8%, potassium content 1.1%, phosphorus content 1.15%, organic carbon content 13.9%, copper concentration 61 mg/kg, zinc concentration 650 mg/kg, organic matter content 37.3%, fulvic acid content 13.9%) and 50% topsoil (consisting of marl and clay, with a pH of 8.9). Additionally, during the planting of the study specimens, all pre-existing roots within a 1-meter diameter planting hole and up to 0.5 meters deep were removed from the surrounding area.

Table 2. Average photosynthetically active radiation (PAR) and soil parameters (soil bulk density –SBD–, pH, and electric conductivity –EC–) for each PAR range. *P*-values denoting the significance of differences obtained from ANOVA comparisons are also indicated.

		PARm level				
		< 100	100–300	>300		
Parameter†	n††	4	4	4	F-Snedecor	*P*
PAR (mmol m^{-2} s^{-1})		62^{a}	149^{b}	424^{c}	11.57	0.001
SBD (g cm^{-3})		1.09^{d}	1.30^{d}	1.30^{d}	0.93	0.43
pH		8.68^{e}	8.75^{e}	8.70^{e}	0.35	0.72
EC (mS m^{-1})		0.46^{f}	0.29^{f}	0.30^{f}	1.78	0.23

†Different letters denote significant differences at 95% confidence interval (Fisher's least significant difference test). †† Number of samples (tree pits).

Furthermore, during the autumn of 2016, one-year post-planting, soil conditions were evaluated within each group of plants (i.e., within each tree pit) to assess potential spatial variations between planting sites. Soil samples were obtained by collecting three randomly selected subsamples from the top 15 cm of soil within the 1-meter diameter tree pit, which were then combined to create a homogeneous composite sample. Soil bulk density was determined by collecting soil subsamples using a 5-cm diameter Eijkelkamp soil sampler (Agrisearch Equipment BV) after carefully removing surface litter from the forest floor. pH and electrical conductivity were measured in a soil-water solution at a 1:5 ratio using a pH meter (Navi Horiba model, HORIBA Ltd, Kyoto, Japan) (Table 2).

Selection of different light intensity exposure sites and determination of PAR

The tree pits where we planted the *E. latifolius* groups were spatially distributed to cover the broadest possible range of plant exposure to light intensity within the forest stand by selecting sites that encompassed a light gradient from direct illumination to the densest shade produced by canopy cover.

To achieve conditions of minimal irradiation within the canopy, the pits were randomly positioned beneath broad-leaved deciduous tree specimens characterized by wide and flat leaves. *Ulmus glabra* and *Tilia platyphyllos* emerged as the predominant representatives of broad-leaved species in this environment. Additionally, comprising the species assemblage were *Populus nigra*, *Populus tremula,* and *Tilia cordata* in the tree stratum, along with *Cornus sanguinea, Corylus avellana, Ilex aquifolium, Sambucus nigra, Rosa canina, Ribes alpinum*, and *Rosa canina* in the shrub layer. Together, they collectively created a dense shade with their canopies, effectively filtering photosynthetically active radiation (PAR) across all strata.

Medium shaded pits were situated within the canopy of broad-leaved trees, where broad-leaved species were replaced by narrow-leaved deciduous trees such as *Fraxinus excelsior, Fraxinus angustifolia, Prunus mahaleb, Betula pendula*, and *Acer campestre*. To attain lower levels of shade, another group of pits was placed within the shrubby ensemble, where shade trees were substituted by small trees and bushes including *Euonymus europaeus, Ligustrum vulgare, Prunus spinosa, Rhamnus alpina, Rhamnus cathartica,* and *Berberis vulgaris* subsp. *seroi*, among others. Finally, one pit served as a control, and was situated directly under full sun exposure.

After planting the *E. latifolius* saplings, we evaluated and characterized the PAR at each site by measuring the light every 10 min (in mmol photons m^2 s^{-1}) for four days during the final week of August 2018 under clear sky conditions. Eleven quantum sensors were used to measure the photosynthetic photon flux density (model SQ-110; Apogee Instruments, Logan, UT, USA) at the center of each group of plants and 20 cm above the ground. The average PAR (PARm) from 5 am to 7 pm (solar time) over the four days of measurements was calculated for each test plant group (Table 3).

Table 3. Data displays the minimum, maximum, and average photosynthetically active radiation (PAR) levels (mmol photons m^{-2} s^{-1}) recorded for each plant location group (pits) throughout the experiment.

Tree pit	PAR min	PAR max	PARm
1	9	517	118
2	9	525	156
3	9	860	372
4	8	86	48
5	8	76	39
6	9	1333	327
7	8	267	98
8	8	299	117
9	9	525	205
10	9	1389	325
11	9	1751	671
12			

Plants growth and biometric measurements

Plant development was evaluated by measuring the two main parameters linked to growth comprising the maximum plant height (H, cm) and stem diameter (D, mm) at root crown (Trubat, 2004). H was measured considering the distance between the tallest limit of the photosynthetic main tissues in the plant (excluding inflorescences) and ground level. H is associated with the growth form, position of the species relative to the vertical gradient of light through vegetation, and competitive vigor (Matteucci & Colma, 1982). Both D and H were measured in all plants immediately after planting (October 2015) and then once each year in late October until 2018. Thus, both the absolute and relative increases in D and H were obtained for each plant during three growth periods (years).

In order to integrate the simultaneous growth in H and D for each plant, we defined a new variable called volume (Eq. 1) by simplifying a plant into a cylindrical trunk defined by D and H. Thus, we could consider both the diameter and height growth as one variable:

$$V = \frac{\pi}{4}\frac{D}{10}^{2} H \qquad \text{Eq. 1}$$

where V is the volume in cm^3, D is the diameter at the root crown in mm, and H is the maximum plant height in cm.

Thus, the mean annual increases in volume, diameter, and height (RVi, RDi, and RHi, respectively) relative to the initial volume, diameter, and height (i.e., in 2015) during the three years of measurements were calculated using (Eqs. 2–4).

$$RVi(\%) = 100\,\frac{V_{2018}-V_{2015}}{3\,V_{2015}} \qquad \text{Eq. 2}$$

$$RDi(\%) = 100\,\frac{D_{2018}-D_{2015}}{3\,D_{2015}} \qquad \text{Eq. 3}$$

$$RHi(\%) = 100\,\frac{H_{2018}-H_{2015}}{3\,H} \qquad \text{Eq. 4}$$

Statistical analysis

The standardized skewness and kurtosis were calculated for the data to confirm whether continuous variables conformed to a normal distribution. Analysis of variance (ANOVA) (Neter *et al.*, 1996) was conducted to compare mean growth values between groups (n = 3 saplings per tree pit) depending on PARm. Fisher's least significant difference (LSD) test with a 95% confidence interval was used to test whether mean values differed significantly from others. Significant differences were accepted at $P < 0.05$. The possible effects of soil characteristics on plant growth were also evaluated by classifying the PARm levels according to three classes (< 100, 100–300, and > 300 mmol photons m^{-2} s^{-1}), and differences in soil characteristics using this PAR classification were assessed by ANOVA. Soil parameters that differed significantly between PAR levels were considered in the growth models in addition to PAR.

A multiple regression analysis model (Neter *et al.*, 1996) was used to check effects of the predictive variables (PARm and soil variables) on the relative growth volume. Finally, a nonlinear regression model was used to define the final model for predicting growth as a function of PARm.

The 75th and 90th percentiles of mean annual relative growth variables (Rvi, Rdi, and RHi) were used to infer the optimal PARm interval based on the regression models describing the relationship between relative growth with PARm.

All statistical analyses were performed using Statgraphics Centurion XVIII® software (Statgraphics Technologies Inc., Virginia, USA).

Results

The singular group of plants subjected to full sun exposure experienced mortality within the initial month of the study. Consequently, only results from the remaining 11 experimental groups of *E. latifolius*, planted within the deciduous nemoral broadleaf forest stand at the Botanic Garden of Castilla-La Mancha, ranging from 39 to 671 mmol photons m^{2} s^{-1}, will be presented to evaluate the effects of PARm in this context. In addition, soil characteristics did not differ significantly between tree-pit groups defined by PARm classes ($P > 0.05$, Table 3). Thus, the soil bulk density, pH, and electric conductivity within PARm classes were similar and homogeneous. Consequently, the effect of PARm on sapling growth could not be confounded with the pure soil effects or interactions, and PARm alone could explain the sapling growth patterns.

Responses of biometric measurements to different PAR levels

Since the initial *E. latifolius* plant materials were germinated simultaneously and planted on the same day, utilizing identical substrate and under controlled conditions of shading, temperature, and humidity within a greenhouse, the initial height and diameter measurements were uniform. Consequently, in biometric terms, the initial *E. latifolius* plant materials were homogeneous, with morphological dimensions of all plants significantly similar, except for those in tree pit 6, where saplings had a slightly thicker diameter (D), resulting in a higher initial volume (V_0) (Table 4).

Table 4. Initial biometric sapling characteristics in 2015: diameter (D_0, mm), height (H_0, cm), and volume (V_0, cm^3); absolute mean annual increases in diameter, height, and volume (Di, mm; Hi, cm; and Vi, cm^3, respectively); and relative mean annual increases in the same parameters (RDi, %; RHi, %; and RVi, %, respectively) during the three years of the experiment (2015–2018). The average PARm is also shown (n = 3 saplings in all tree pits). ANOVA comparison outputs: H_0: F = 0.8, $P = 0.63$; D_0: F = 2.3, $P = 0.049$; V_0: F = 2.8, $P = 0.035$. Measurements are not shown for plants in the tree pits exposed directly to light because these individuals died early in the experiment.

Tree pit	PARm	D_0†	H_0†	V_0†	Di	Hi	Vi	RDi (%)	Rhi (%)	Rvi (%)
1	118	6.3^{a}	20.7^{a}	6.3^{a}	2.9	18.2	42.3	49	93	720
2	156	7.0^{a}	22.7^{a}	8.7^{a}	2.4	20.1	43.3	35	90	488
3	372	8.3^{ab}	22.7^{a}	13.1^{ab}	0.4	2.1	3.8	5	10	25
4	48	7.0^{a}	19.7^{a}	7.6^{a}	3.7	25.7	80.4	53	131	1090
5	39	8.0^{a}	21.0^{a}	10.6^{a}	4.9	28.8	142.8	61	137	1344
6	327	11.3^{b}	23.3^{a}	23.5^{b}	0.4	4.7	8.0	4	20	34
7	98	7.7^{a}	21.7^{a}	10.1^{a}	4.3	29.6	119.6	57	137	1218
8	117	8.7^{ab}	21.3^{a}	12.8^{ab}	2.2	24.4	54.1	28	115	486
9	205	9.7^{ab}	23.3^{a}	17.8^{ab}	0.9	11.6	18.5	9	50	102
10	325	8.3^{ab}	22.3^{a}	12.5^{ab}	0.7	8.6	9.5	8	38	76
11	671	6.7^{a}	21.7^{a}	7.6^{a}	–0.1	0.6	–0.1	–2	3	–1

†Different letters for D15, H15, and V15 denote significant differences at 95% confidence interval (Fisher's least significant difference test).

Both the soil conditions and the initial biometric traits were homogeneous in the experimental plant groups, so it was assumed that sapling growth patterns could be consistently explained by the effects of PARm. Indeed, we found a highly significant relationship between this light parameter and all three relative growth biometrics (RDi, RHi, and RVi; Figure 2).

The regression models in Figure 2 indicate that the optimal PARm range for *E. latifolius* growth was between 39 and 90, but critical over 370 mmol photons m^{-2} s^{-1}, and incompatible with plant growth over 670 mmol photons m^{-2} s^{-1}. Indeed, the plants that were exposed directly to sunlight (i.e., three in one of the tree pits used in the experiment) died during the first month after transplanting.

Discussion

Euonymus latifolius forms part of the nemoral-subMediterranean deciduous flora in Mediterranean mountains in the Iberian Peninsula as relict remnants from the dominant Euro-Siberian vegetation spread over this region during the Würm glaciation (Gutiérrez *et al.*, 2004; Agea *et al.*, 2021; García-Cardo, 2021). These shade-loving communities settled in enclaves with stable environmental humidity may be expected to be negatively affected by progressive warming and the increasing prevalence of drought conditions under the current climate change context (Sánchez de Dios *et al.*, 2009). Indeed, it is assumed that severe contractions will occur in their Mediterranean distribution range, which may eventually lead to local extinctions (Herranz *et al.*, 2009). Therefore, exploring how changes in abiotic environmental factors due to climate change can influence the biology of vulnerable Mediterranean plants should be a priority for conserving *E. latifolius* and other nemoral relict species.

In the present study, we focused on the ecological concept of shade tolerance, which refers to the ability of a given plant to survive and complete its life cycle at low light levels (Valladares and Niinemeets, 2008). The scarcity of light is a strong mediating factor that affects the richness of plant species (Dormann *et al.*, 2020). Shade tolerance is particularly important for determining the fundamental niches of plant species but also their competitive ability (Courbier & Pierik, 2019), and thus their responses to environmental conditions in the context of coexistence and interactions with other species (i.e., their realized niche; Schoener, 2009). In addition, differences in the competitive potential of co-occurring species mediated by shade tolerance are critical for predicting ecosystem responses to global change drivers, such as elevated CO_2, climate change, and the spread of invasive species (Valladares and Niinemets, 2008). Our results clearly demonstrated a reduction in the growth of *E. latifolius* as the light intensity increased, which became critical at high values and even lethal when plants were directly exposed to light. These results highlight the importance of shady environments for the survival and growth of this species: the optimal PARm value for vegetative development ranged between 39 and 90 mmol photons m^{-2} s^{-1}, and plant growth decreased dramatically with greater radiation. By contrast, the minimum PARm under direct light exposure at the study locality (i.e., the Botanic Garden of Castilla-La Mancha) was 9.3 times higher (i.e., 365 mmol photons m^{-2} s^{-1}; data not shown), which is close to the amount of irradiation at the Equator at midday in midsummer (Roberts & Paul, 2005), and the maximum irradiation in the European distribution range of *E. latifolius* (López, 2001). In general terms, shade tolerance is a trait defined by a gradient along which most plant species occupy an intermediate position skewed to a greater or lesser extent toward either end, i.e., sciophilous vs. heliophilous (Givnish, 1988). The high shade preference of *E. latifolius*, which is equivalent to that of sciophilous, northern species such as *Abies alba* Mill. (Cescatti and Zorer, 2003) or *Fagus sylvatica* L. (Košvancová-Zitová *et al.*, 2009), is particularly rare among the central and southern flora of the Iberian Peninsula, where few species are as tolerant of extremely low light conditions (Damascos and Rapoport, 2002; Wright *et al.*, 2003; Valladares *et al.*, 2005). Agea *et al.* (2021) stressed the maintenance of soil humidity as an important effect of canopy cover for the recruitment of the broadleaved spindle tree in Mediterranean populations. However, our results also identified protection against direct light as an additional, non-exclusive key condition for recruitment. Thus, the shade provided by the canopy could be crucial for the persistence of this relict nemoral species at Mediterranean latitudes.

The possible evolutionary origin of shade tolerance in *E. latifolius* may have been promoted by adaptive functional advantages in shady environments by maximizing the net carbon gain under low light (Givnish, 1988), as well as maximizing the resistance to biotic and abiotic stresses in the understory (i.e., the stress tolerance hypothesis; Kitajima, 1994). Clearly, these benefits have been obtained for as long as the abiotic and biotic conditions that promoted them in the past persisted. However, since the end of the last Würm glaciation 80,000 years ago, climate conditions have been changing, especially increased global warming in recent centuries (Weart, 2008). The natural habitats of *E. latifolius* in the Mediterranean mountains of the Iberian Peninsula, i.e., thorny deciduous scrublands and Euro-Siberian mixed forests (Martín *et al.*, 2003; García-Cardo, 2021), are largely dominated by nemoral broadleaf species, which are all hygrophilous to a greater or lesser degree. The general decrease in rainfall associated with climate change over the Iberian Mediterranean region is currently altering the structure and composition of many ecosystems (Piervitali and Colacino, 2003; Herrero and Zavala, 2015; Sanz and Galán, 2020). The predictions for ombrophilous deciduous vegetation in the Mediterranean mountains suggest a bleak future for broad-leaved spindle as a simplification of the vegetation structure is expected to open the canopy as the local atmospheric conditions become drier (Herranz *et al.* 2009; Sánchez de Dios *et al.*, 2009). This habitat change process may exacerbate the relict condition of *E. latifolius* in Mediterranean latitudes since the light conditions will become too harsh for its growth.

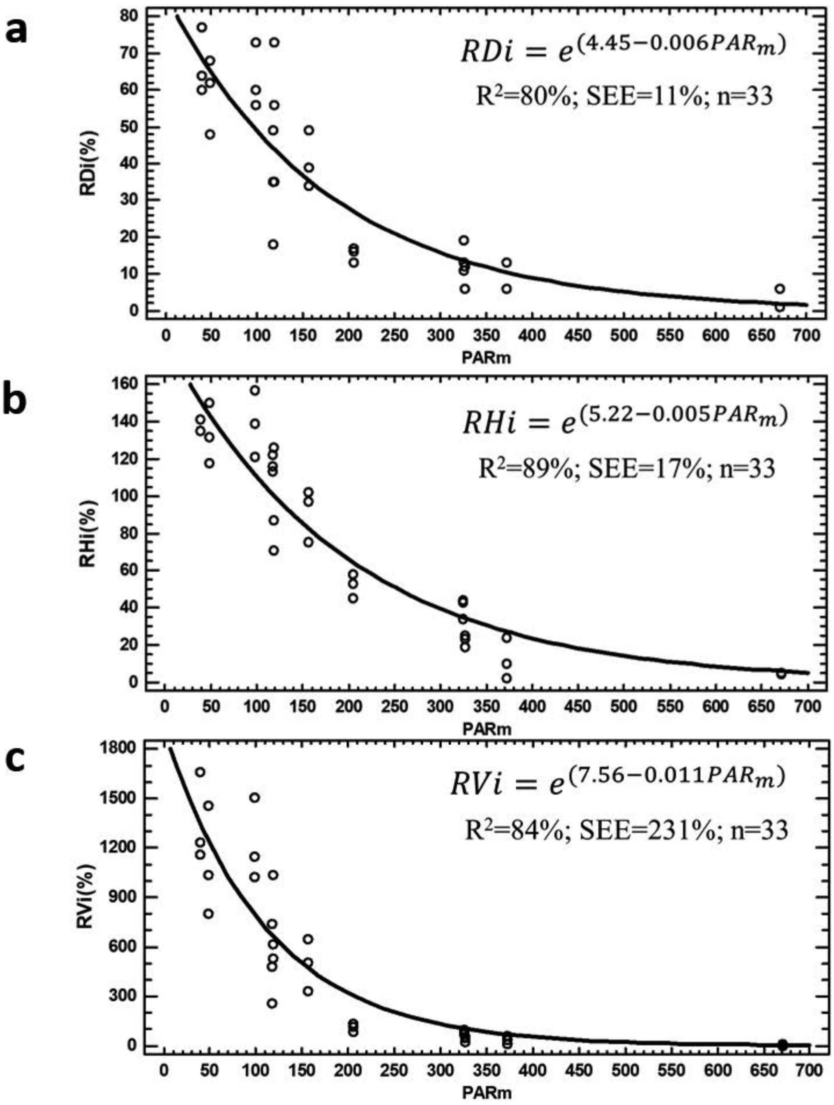

Figure 2. Relationships between mean PAR (PARm, mmol photons m^{-2} s^{-1}). A, relative mean annual diameter increase (RDi, %); b, relative mean annual height increase (RHi, %); c, relative mean annual volume increase (RVi, %). The figure shows the regression models and goodness-of-fit statistics. The valid range for the models was PARm = 39 to 671 mmol m^{-2} s^{-1}. Measurements of plants in the tree pits exposed directly to light were not considered in the regression models because these individuals died early in the experiment.

In addition, other problems associated with climate change are currently affecting the Iberian populations of *E. latifolius*. In particular, the seeds of this plant require a particularly intense cold and long winter period (i.e., 20 weeks at 5°C ± 1.5°C) to overcome physiological dormancy (i.e., intermediate level, *sensu* Baskin and Baskin, 2014) and germinate (Santiago *et al.*, 2023). This trait was probably selected under past periglacial conditions to favor germination in mildly cool springs, thereby protecting sensitive seedlings from exposure to extremely cold winter temperatures. However, under the current global warming scenario, which is particularly evident in the Mediterranean area (Sánchez-Salguero *et al.*, 2016), the temperature conditions and germination requirements could critically decouple to compromise recruitment in the relict Iberian populations. It is not surprising that *E. latifolius* plants in the Iberian mountains are concentrated in ravine beds and at the bottom of deep dolines where they are protected against herbivory (another important threat to the species in its Mediterranean range; Agea *et al.*, 2021; García-Cardo, 2021), but also where solar radiation rarely reaches directly throughout the day and warm temperatures are buffered (Estevo *et al.*, 2022). This restriction in the site selection pattern of this plant may indicate that climate change is now making the conditions fatal for Iberian *E. latifolius* populations, putting this and probably other relict nemoral-subMediterranean taxa in a critical endangered position in southern latitudes (García-Cardo *et al.*, 2021; Santiago *et al.*, 2019; Santiago *et al.*, 2023).

This critical situation is unlikely to be naturally remedied via sexual processes of genetic recombination. Indeed, the period of 80,000 years since the end of the expansion of *E. latifolius* over Mediterranean peninsulas may not have been sufficiently long to develop new adaptations in addition to those already present in the original genetic pool of the species, as also observed in *Chaerophyllum aureum* L., which is another relict nemoral taxon that shares a habitat with the broad-leaved spindle in the Iberian mountains (Santiago *et al.*, 2019). Therefore, the results obtained in the present study as well as the protocols for *E. latifolius* seed germination defined by Santiago *et al.* (2023) provide important information to facilitate the design of urgent and effective management, conservation, and restitution programs for this relict, critically threatened plant in the Iberian Peninsula. The light conditions are not important for *E. latifolius* seed germination (Santiago *et al.*, 2023) but special care is required in the subsequent phases of plant production. In particular, in any nursery stage, it is essential to protect the plants from direct exposure to solar radiation. Ideally, they should be placed under dense shaded meshes to reproduce the optimal light intensity range (i.e., PARm = 39 to 90 mmol photons m^{-2} s^{-1}) throughout the nursery season. In the selected habitat, saplings must be planted in shady sites under protection of the canopy and even accompanied by an artificial mesh structure if necessary. Previously obtained in-situ sensor measurements of PAR at potential plant sites may contribute to ensuring successful planting. Moreover, the structure of the plant community must be preserved, preventing any factor which results in the reduction of the canopy cover. In particular, planting key tree species may be important as well as managing ungulate populations if the herbivory pressure becomes high, as suggested by Agea *et al.* (2021). However, in addition to these recommendations, it is desirable to perform comparative genetic analyses of the relict Iberian populations of *E. latifolius* and broadly distributed central and north European populations to assess their biological uniqueness and accurately determine the effort required to conserve the rarest shrub in the Iberian Peninsula (López, 2001) under the current climate change context.

Authorship information

AS: Management of the project, Conceptualization, Research, Methodology, Writing.
FLS: Data curation, Formal analysis.
JMH: Resources.
GGS: Writing.
EC: Visualization.
PF: Fundraising, Writing.

Conflict of interest

None.

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