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Contrasting seasonal abiotic stress and herbivory incidence in *Cistus albidus* L. plants growing in their natural habitat on a Mediterranean mountain

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ABSTRACT

Mediterranean climate mountain areas are characterized by stressful abiotic conditions for plants. Drought, high photosynthetically active photon flux density and extreme temperatures are the main abiotic factors that drive shrub biodiversity in these areas. However, little is known of the extent to which herbivory may additionally influence the physiology of these shrub communities. We studied the incidence of abiotic and biotic stresses in a representative Mediterranean shrub, *Cistus albidus* L., growing under natural conditions, with an emphasis on the seasonal occurrence of abiotic stress and herbivory, and the possible influence of mountain aspect and plant ageing on the stress response. Reductions in maximum quantum yield of photosystem II (PSII) occurred during early summer and coincided with a combination of drought, high light and high temperatures (above 35 °C in July). East-facing plants experienced greater reductions in PSII quantum yield during July and leaf water content during September than west-facing ones. However, east-facing plants also showed less herbivory-related damage during winter than west-facing plants, particularly from goats. Plant age did not influence herbivory pressure or the stress response to abiotic factors. It is concluded that *C. albidus* is a highly tolerant shrub to both abiotic and biotic stress factors in Mediterranean mountain areas. It only shows slight photoinhibition during early summer and mild symptoms of herbivory attack in winter.

1. Introduction

Plant response to the environment is a complex process tiggered by a combination of climatic variables. Both abiotic and biotic factors converge in space and time to finely modulate plant life. Environmentally extreme seasons in the Mediterranean are summer and to a lesser extent winter due to extreme temperatures, elevated solar irradiance and drought. The typical Mediterranean climate (*Csa* in the Köppen classification, Köppen, 1936) is temperate. It is characterized by dry summers (with less than 30 mm rainfall in the driest month) and relatively low temperatures during winter (although they can reach below 0 °C as elevation and distance from the sea increase). In Mediterranean-type ecosystems, aside from drought, high light and extreme temperatures, major pressure factors for plant diversity include herbivory and common perturbations such as fire.

Shrubland communities are essential elements of Mediterranean ecosystems. Their biodiversity is crucial to confer plasticity to these vulnerable ecosystems and thus alleviate the negative effects of climate change, such as increased water scarcity (Cowling et al., 1995; Cabal et al., 2017; Rodríguez-Ramírez et al., 2017). *Cistus albidus* L. is considered an ancient, well-adapted shrub of these ecosystems. It has a plethora of adaptations to the common stresses of Mediterranean-type ecosystems. As an obligate seeder, it is involved in primary succession after a fire event (Roy and Sonié, 1992). In addition, several of its features, such as its semi-deciduous strategy, its high phenotypic plasticity to water scarcity, and several morphological and physiological acclimation responses, such as the modulation of photoprotective mechanisms or leaf orientation, allow this shrub to overcome stressful seasons (Correia et al., 1992; Grant et al., 2005; Correia and Ascensão, 2017; Oliveira and Peñuelas, 2000; 2004; Munné-Bosch et al., 2003; Pérez-Llorca et al., 2019).

Although the molecular mechanisms remain relatively unknown, it has been shown that those underlying plant response to abiotic stresses change as the plant ages (Rankenberg et al., 2021). Plant ageing at organism level has garnered great interest in recent decades, but it is still quite an enigmatic biological process. Several unresolved questions remain, such as its relationship with the senescence of single organs, mechanisms of senescence avoidance at organism level or the potential

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of plants to attain near immortality (Thomas 2002, 2013; Munné-Bosch 2020; Cannon et al., 2022). Plant ageing is associated with several metabolic and physiological changes that occur over time, many of them associated with plant maturity and increased size that may not necessarily lead to organism senescence (i.e., increased mortality as plants age), including changes in leaf morphology, changes in redox homeostasis, reductions in photosynthetic rates or alterations in plant reproductive strategy (Thomas 2013; Mencuccini and Munné-Bosch, 2018; Munné-Bosch 2007, 2020). However, plant age-related effects on susceptibility to pathogen infection have been described in some plant species and commonly reduce susceptibility to pathogen infection as the plant ages. This is called age-related resistance (Kus et al., 2002; Barton and Koricheva, 2010; Yang et al., 2020). In *C. albidus*, plant ageing has been shown to affect reproductive traits such as flower bud vigour, flower production, rates of embryo abortion and seed vigour. This shows that ageing does not negatively influence overall fecundity through compensatory effects (Oñate and Munné-Bosch, 2010; Müller et al., 2014). However, as with other perennials, very little is known about age-related changes in plant response to abiotic and biotic stress factors in this plant species.

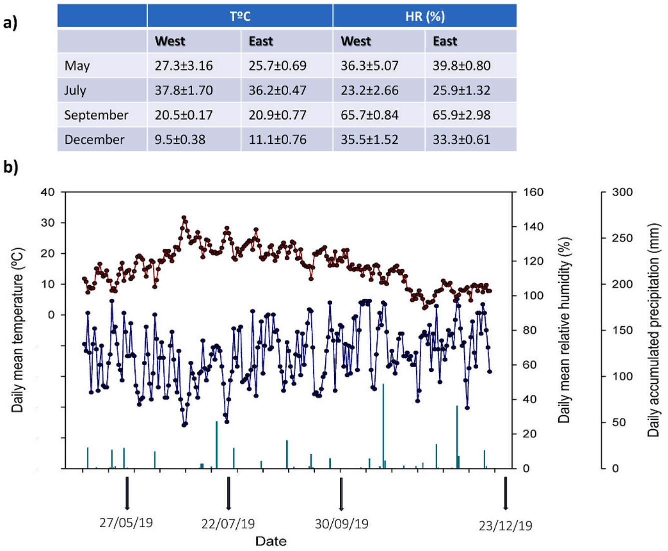
Here, we aimed to investigate the response of *C. albidus* to abiotic and biotic stresses under natural conditions in a Mediterranean mountain area, with an emphasis on the occurrence of abiotic stress and herbivory in relation to seasonal environmental variations, mountain aspect and plant ageing. This is important to (i) explore the limits in stress tolerance of an important shrub in Mediterranean-type ecosystems where abiotic stress is commonly present and where herbivory usually converges with it, (ii) study to what extent environmental conditions (both seasonal and microclimatic) could modulate this stress convergence and plant response, and (iii) assess the effect of plant ageing in terms of stress response and/or susceptibility. Overall, it helps to better understand the functioning of these vulnerable ecosystems and thus better implement management strategies. We hypothesized that when stresses converge, herbivory damage may superimpose abiotic stress factors and cause major impacts on plant performance under natural conditions. The effects were expected to be more evident as plants age.

2. Methods

2.1. Study site and experimental design

The study was performed from May to December 2019 in a natural population of *Cistus albidus* growing at 1100 m.a.s.l. under natural conditions in Montserrat Natural Park (Spain, 41.586N 1.830E). We randomly selected 100 individuals from the population, 50 growing on the east-facing slope and 50 on the west-facing slope of the mountainside. Monitoring of environmental data was provided by a weather station from the Meteorological Service of Catalonia (MeteoCat) situated on a south-facing slope at 916 m.a.s.l. at Monistrol de Montserrat (NE Spain). The temperature and relative humidity of each mountainside were recorded using a portable thermohygrometer (DO9847 Multifunction Meter, Delta Ohm, Padova, Italy, Fig. 1).

Surveys were undertaken on 27 May (mid-spring), 22 July (midsummer), 30 September (early autumn), and 23 December 2019 (early winter). Environmental conditions were typical of Mediterranean mountain areas. Monthly mean temperatures during winter ranged between 5 and 10 °C with the lowest daily minimum temperature close to 0 °C, and we registered temperatures around 10 °C the day of the survey. In summer, the monthly average was over 20 °C, with highest daily maximum temperatures occasionally above 30 °C and we registered temperatures above 35 °C the day of the survey. During the study period, most of the rainfall was recorded in late autumn (i.e., 105 mm in October) while September was the driest month with a total accumulated precipitation of 33 mm (Fig. 1). All surveys were performed at midday on sunny, clear days, except on 30 September, when small high clouds formed. From a phenological perspective, we observed flowering in May while fruiting and major leaf shedding occurred in July.



a)

	TºC		HR (%)	
	West	East	West	East
May	27.3±3.16	25.7±0.69	36.3±5.07	39.8±0.80
July	37.8±1.70	36.2±0.47	23.2±2.66	25.9±1.32
September	20.5±0.17	20.9±0.77	65.7±0.84	65.9±2.98
December	9.5±0.38	11.1±0.76	35.5±1.52	33.3±0.61

Fig. 1. Environmental conditions in the Montserrat mountains during the experimental period. (a) Air temperatures (T°C) and relative humidity (HR%) during measurement days comparing the two sun orientations, and (b) mean daily temperatures and relative humidity, together with precipitation, recorded at the closest meteorological station (see material and methods for details). Arrows indicate the days of surveys of plants during the study.

2.2. Abiotic stress markers

Two fully expanded, sun-exposed, undamaged apical leaves were sampled for each individual and survey time to study abiotic stress markers. One of these leaves was analysed for leaf water content and chlorophyll fluorescence measurements, which are commonly used as abiotic stress markers in this species (Jubany-Marí et al., 2009; Brossa et al., 2015), while the other was immediately frozen in liquid nitrogen and kept at −80 °C until analysis for chlorophylls. Leaf hydration and relative water content of leaves were measured as described by Munné-Bosch et al. (2003) based on fresh weight, turgid weight (after 24 h of soaking the leaf in distilled water), and dry weight (after drying the leaf in the oven at 65 °C to constant weight). Maximum quantum yield of photosystem II in the dark-adapted state (F_v/F_m), which was used as an indicator of photoinhibition, was measured based on chlorophyll fluorescence measured using a fluorimeter Mini-PAM II (Photosynthesis yield analyser, Walz, Germany) after 1 h of dark adaptation of leaves. To estimate the chlorophyll content, which is commonly used as an abiotic stress marker in this species (Oliveira and Peñuelas, 2000), 50 mg of leaf were extracted in 500 μl of methanol containing 0.01% butylated hydroxytoluene using ultrasonication for 30 min (Branson 2510 ultrasonic cleaner, Bransonic, Danbury, CT, USA). The extract was centrifugated at 13,000 rpm at 4 °C and the supernatant was collected. This process was repeated twice with 250 μl of solvent. Absorbance was read at 470 nm, 653 nm, 666 nm and 750 nm using a UV/Visible double beam CE Aquarius spectrophotometer (Cecyl Instruments Ltd, Cambridge, UK). Chlorophyll *a and b* contents were estimated using the equations described by Lichtenthaler and Wellburn (1983).

2.3. Biotic stress markers

Each plant was meticulously examined for herbivore pressure. Photographs of all damaged leaves were taken to subsequently count and classify insect-damaged and goat-damaged leaves. The type of damage was easily identified by the marks left on leaves. Then, the total number of affected leaves per individual was counted and the percentage of affected leaves estimated.

2.4. *Plant ageing*

Plant height and trunk circumference was measured in all individuals, and plant age was estimated as described by Siles et al. (2017) based on a logarithmic regression between the trunk circumference and plant age in individuals of *C. albidus* growing in Montserrat Mountains. Furthermore, two fully expanded, sun-exposed, undamaged apical leaves were sampled for each individual and survey time to study oxidative stress indicators as additional potential plant ageing markers. For the measurement of these additional markers (vitamin E and lipid hydroperoxides), leaves were immediately frozen in liquid nitrogen and kept at −80 °C until biochemical analyses. Vitamin E analysis was performed by HPLC as described by Cela et al. (2011) using a normal phase column (Inertsil 100A, 5 μμ, 30 × 250 mm, GL Sciences, Torrance, CA, USA) and fluorescence detection at an excitation wavelength of 295 nm and emission at 330 nm. Standards of α-tocopherol and γ-tocopherol (Sigma–Aldrich) were used for quantification. Lipid peroxidation was estimated by measuring the amounts of lipid hydroperoxides using the FOX assay as described by Bou et al. (2008).

2.5. *Statistical analyses*

A two-way ANOVA for repeated measures considering the plant ID as a random factor was used to determine the effect of "orientation" or "age" and "time" using the LME model to deal with the heterogeneity of data. Multiple comparisons were tested with Tukey's post hoc test. All differences were considered significant when $P \leq 0.05$. The normality and homoscedasticity of residues were checked as described by Zuur et al. (2009). Correlation analyses were performed using Spearman's rank correlation test. All statistical tests were performed using R statistical software (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. *Enhanced abiotic stress during summer and herbivory pressure during winter*

Abiotic stress symptoms were observed in all surveyed plants during the summer, in July and to a lesser extent in September. A depression in the maximum quantum yield of PSII, as indicated by reductions in the F_v/F_m ratio under 0.75, occurred in *C. albidus* plants during the summer (July). This reduction in PSII efficiency was observed in parallel with 21% reductions in leaf water content (mainly in leaf hydration [H]) and a 34% reduction in chlorophyll content, with values that dropped to around 2 mg/g DW. However, reductions in the maximum quantum yield observed in summer were found particularly in east-facing plants, which showed F_v/F_m values of 0.70, concomitant with a 16% reduction in the chlorophyll *a/b* ratio compared to the west-facing plants (Fig. 2). While the maximum abiotic stress suffered by plants was observed in summer, maximum herbivory pressure occurred in winter and included insect and goat herbivory, in terms of the number and the percentage of leaves affected (Fig. 3a and b). The predominant type of herbivory was that caused by insects, which caused between 72% and 94% of the total herbivory in terms of the number of leaves affected. Herbivory due to wild goats affected 28% of total damaged leaves at the highest maximum values observed in December in west-facing plants (Fig. 3c). A 5-fold and

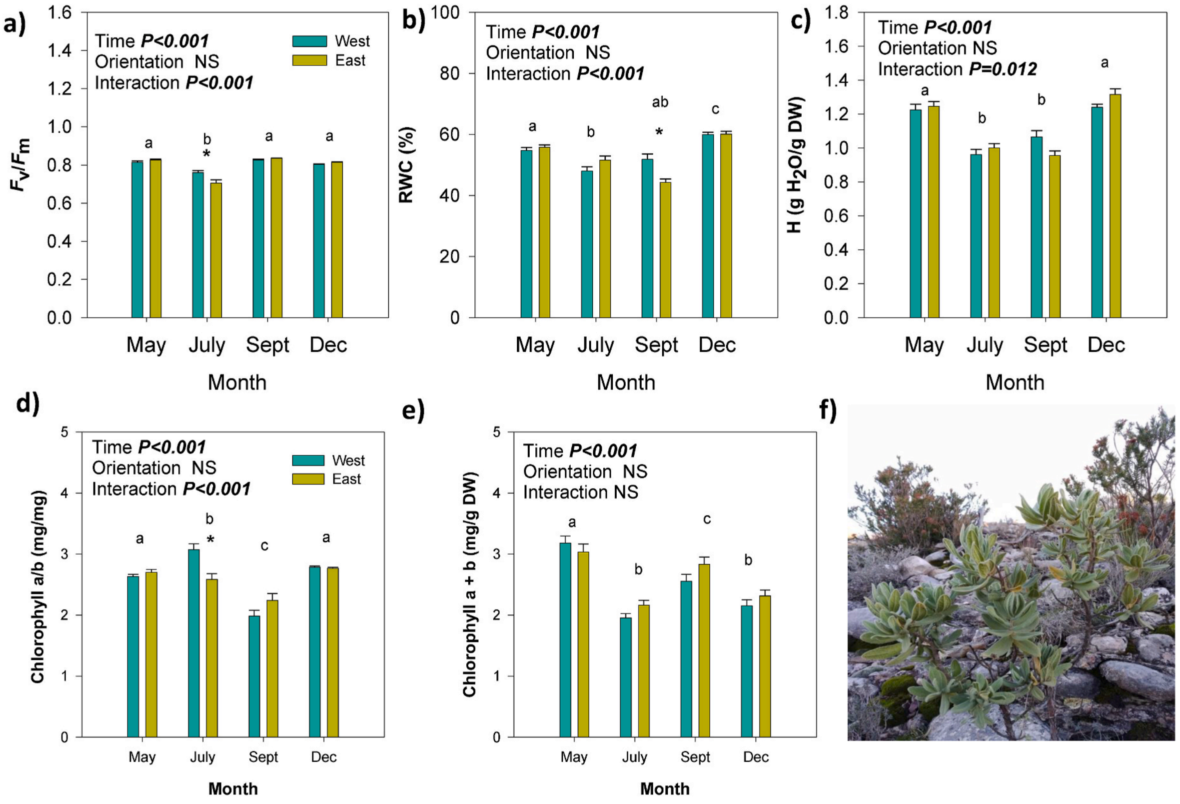


Fig. 2. Abiotic stress markers in leaves of the *Cistus albidus* population during the year of study as affected by sun orientation of the growing site. (a) Maximum efficiency of photosystem II photochemistry (Fv/Fm), (b) relative water content (RWC), (c) hydration (H), (d) chlorophyll *a*/b ratio (mg/mg) and (e) total chlorophyll content. Data are mean of $n = 50 \pm$ SE. *P* values of two-way ANOVA are in the inlets and *P* values > 0.05 were considered not significant (NS). Different letters represent differences between seasons and an asterisk represents differences between sun orientation groups of the season. (f) picture of an individual specimen of *C. albidus* from the population studied.

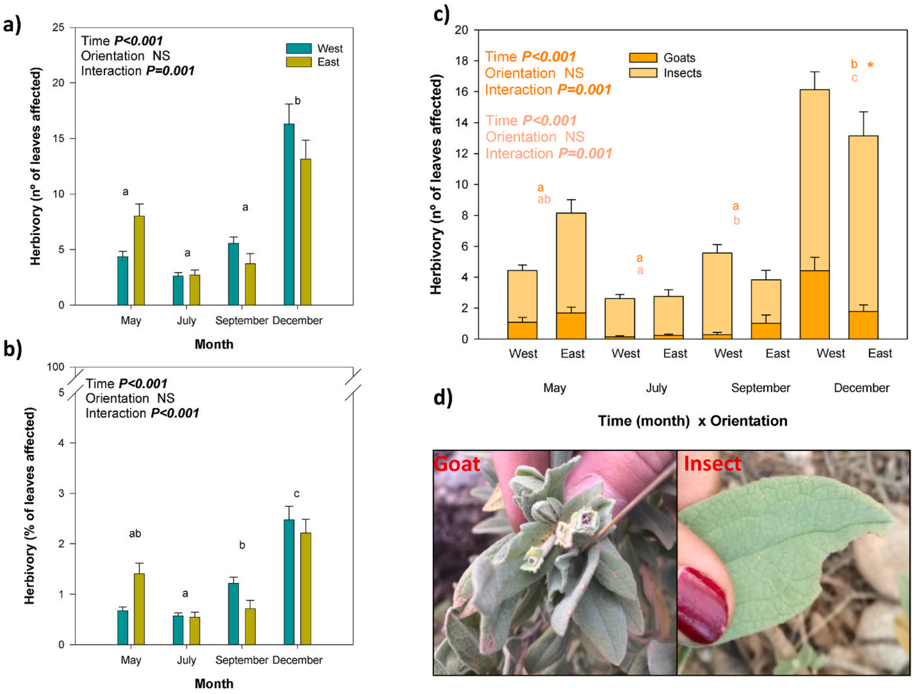
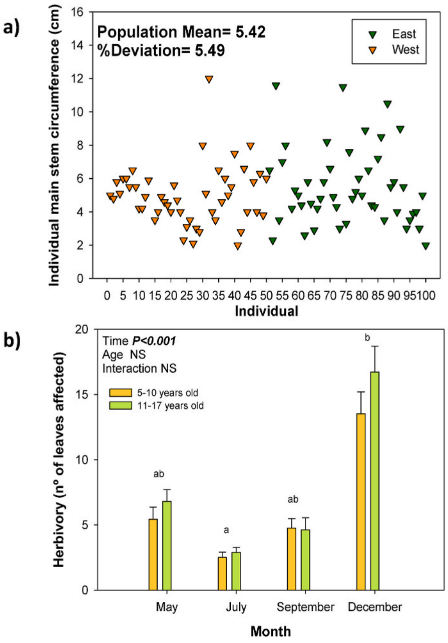


Fig. 3. Herbivory damage recorded during the study as affected by sun orientation of the growing site and expressed as (a) the number of leaves affected by herbivory, (b) the percentage of herbivory-damaged leaves out of the total leaves of the plant and (c) the number of leaves affected by goats or insects. Data are mean of $n = 50 \pm$ SE. P values of two-way ANOVAs are in the inlets and P values > 0.05 were considered not significant (NS). Different letters represent differences between seasons and asterisks represent differences between sun orientation. (d) Pictures of the type of herbivory damage caused by goats and insects recorded during the study.

3-fold increase in herbivory, in terms of the number of leaves affected compared to autumn, was caused by goats and insects, respectively, in winter compared to autumn.

3.2. Plant ageing does not increase stress sensitivity

The present study showed no effects of plant ageing on stress sensitivity when mature plants of different ages were compared. Plant age was not found to negative affect the plant response to abiotic or biotic stress in any of the variables studied (Fig. 4). The average trunk diameter of the studied population was 5.4 cm, which corresponded to an average age of 10.6 years. Therefore, we selected two groups for analyses: one below and the other above this average. The oldest individual was 17 years old. Two groups with ages 5–10 years and 11–17 years were compared. Neither herbivory pressure nor abiotic stress markers (F_v/F_m ratio, leaf water content, chlorophyll content, vitamin E leaf content and lipid hydroperoxides) differed in plants from these age groups (Fig. 4). Thus, stress sensitivity was similar. Indeed, Spearman's rank correlation analyses (considering plant age as a continuous independent variable in the X axis and using no age groups) confirmed that plant ageing did not have any significant effect on any of the studied parameters (P > 0.05).



c)

Variable	Time	Age	Interaction
Fv/Fm	P<0.001	NS	NS
RWC	P<0.001	NS	NS
H	P<0.001	NS	NS
Chl a/b	P<0.001	NS	NS
Chl a+b	P<0.001	NS	NS
α-Toc	P<0.001	NS	NS
α-Toc/chl	P<0.001	NS	NS
γ-Toc	P<0.001	NS	NS
γ-Toc/chl	P<0.001	NS	NS
vit.E	P<0.001	NS	NS
vit.E/chl	P<0.001	NS	NS
LOOH	NS	NS	NS

Fig. 4. Influence of plant age on abiotic stress markers and herbivory damage during the study. (a) Individual main stem circumference distribution of the population studied. Population mean and percent deviation are in the inlet. (b) Herbivory damage of the *Cistus albidus* population during the study as influenced by the season and age groups, expressed as the percentage of damaged leaves. P values of two-way ANOVA are in the inlets ($P > 0.05$ were considered not significant [NS]). Different letters represent differences between seasons and asterisks represent differences between age groups. (c) Results of the two-way ANOVAs for each variable studied with factors "Time" and "Age". Data are mean of $n = 48 \pm$ SE for the 5–10 years old group and $n = 50 \pm$ SE for the 11–17 years old group. RWC: leaf relative water content, H: leaf hydration, Chl a/b: chlorophyll a/b ratio, chl a+b: total chlorophyll content, α-Toc: α-Tocopherol content, γ-Toc: γ-Tocopherol content, α-Toc/chl: α-Tocopherol content per unit of chlorophyll, γ-Toc/chl: γ-Tocopherol content per unit of chlorophyll, vit.E: vitamin E content, vit.E/chl: vitamin E content per unit of chlorophyll, LOOH: lipid hydroperoxides content.

4. Discussion

4.1. East-facing plants show more abiotic stress during summer

It is known that seasonality strongly affects the stress susceptibility of native flora in Mediterranean-type ecosystems, with summer and winter being the most stressful seasons of the year (Oliveira and Peñuelas, 2000; Morales et al., 2016). Summer drought is becoming more common in the Mediterranean region under the current global change scenario and this condition triggers potential damage to the photosynthetic apparatus (Werner et al., 1999; Oliveira and Peñuelas, 2001; Munné-Bosch et al., 2003; Grant et al., 2005; Jubany-Marí et al., 2009; Alderotti et al., 2020). The impact of small differences in microclimate conditions, such as those experienced by east- and west-facing plants, on plant stress responses is still very little explored. A depression in the maximum quantum yield of PSII occurred in *C. albidus* plants during the summer (July), in parallel with reductions in leaf water and chlorophyll contents. However, this photoinhibition was particularly notable in east-facing plants, concomitant with reductions in the chlorophyll *a/b* ratio (Fig. 2). The asymmetry in microclimatic conditions during summer might have resulted in more stressful conditions in plants growing on the east-facing than on the west-facing slope of the mountain. Given that the chlorophyll *a/b* ratio, but neither total chlorophyll nor leaf water contents, differed between east and west individuals, it is likely that these slight variations may be triggered more by differences in incident light than by changes in air temperature. In other plant species that grow on the east aspect of the mountain and receive high amounts of sunlight in the morning, plant growth was higher than in plants that receive high amounts of light in the afternoon (Rodríguez-López et al., 2014). This indicates that the plant response to mountain aspect is strongly species-specific, since each plant species will undoubtedly have its specific optimal environmental requirements for growth.

It has been previously suggested that PSII efficiency is closely linked to plant performance (Adams III et al., 2013). However, the interpretation of any mechanisms responsible for levels of maximum PSII efficiency observed in the present study, or whether depressions in this parameter have important consequences for plant performance, requires additional measures. Moreover, the causes of the observed reductions in the maximum quantum yield of PSII may vary. Different levels of PSII efficiency might be attributed to causes such as (i) different ratios of PSI to PSII and the relative contribution of fluorescence emitted from the chlorophyll associated with each group of centres, (ii) state transitions, (iii) chloroplast movements (positioning of chloroplasts for maximum exposure to incident light versus minimum exposure to incident light on different sides of the mesophyll cells), (iv) engagement of zeaxanthin (and antheraxanthin) in photoprotective energy dissipation, (v) transient inactivation of PSII centres or (vi) disassembly of PSII centres (Demmig-Adams and Adams, 2018; Malnoë, 2018). Many of these may occur when the source to sink ratio of the plant increases (i.e., when the rate of photosynthesis is downregulated as the supply of photosynthate exceeds the needs of the plant, such as when plants cease to grow in response to drought and high temperature, Adams III et al., 2013). When this is the case, reductions in PSII efficiency are an appropriate regulatory response of the photosynthetic apparatus to the source-sink imbalance and may not be considered damaging. Since all data were collected at midday, it could be that the greater level of depression in PSII efficiency in plants on the east-facing slope resulted from the fact that the plants received direct sunlight all morning (since the sun rises in the east) whereas the plants on the west-facing slope would only receive the greatest level of direct sunlight as the sun set in the west in the late afternoon. Therefore, although further research is needed to understand the underlying causes of such differences in *C. albidus* plants growing in their natural habitat, it is very likely that variations in maximum quantum yield of PSII may respond to acclimatory mechanisms and probably have no major negative consequences for plant performance, given that this plant species is very well adapted to the Mediterranean climate.

4.2. East-facing plants show reduced herbivory pressure during winter

Herbivory modulates shrub dynamics in Mediterranean ecosystems by exerting pressure on specific species, individuals and tissues. The most common herbivory types in Mediterranean ecosystems are those exerted by insects, small mammals and wild or domestic ungulates, such as goats (Hódar and Zamora, 2004; Focardi and Tinelli, 2005; Miranda et al., 2011). While maximum abiotic stress was suffered in summer in the present study, maximum herbivory pressure was observed in winter, including that caused by insect and wild goat herbivory (Fig. 3). We observed hole feeding and margin feeding behaviour, commonly attributed to Coleoptera, Orthoptera, Lepidoptera and Hymenoptera (Carvalho et al., 2014). Although it is well known that insect herbivory in Mediterranean-type shrublands ecosystems is driven by seasonality and leaf phenology, little or nothing is known about insect herbivory in *Cistus* species. General insect phenology determines that spring is the most active season for insects. However, some insects are active during winter in Mediterranean ecosystems (i.e., Coleoptera and Lepidoptera, Sarto, 1982; Jaskula and Soszyńska-Maj, 2011). Furthermore, herbivore selectivity is a key point to understand plant-herbivore relationships. It has been demonstrated that plant stress can affect selectivity by insect herbivores and the nutritional value and secondary compound accumulation (Meyer et al., 2006; Staley et al., 2006; Ribeiro-Neto et al., 2012). For instance, some herbivores chose drought-stressed plants instead of non-stressed individuals. In addition, the semi-deciduous strategy of *Cistus albidus* (most leaf shedding in summer) could affect herbivory susceptibility, since leaf shedding has been proven to reduce herbivory pressure in evergreen shrubs with a large variation in leaf retention (Karban, 2007; 2008). Goats have great seasonal adaptability in Mediterranean ecosystems and select particular plant species, mostly woody species, in each season, probably depending on nutritional aspects such as the crude protein percentage, bit mass, nitrogen and secondary compounds or trichome density, and not necessarily depending on availability (Papachristou, 2000; Molina-Montenegro et al., 2006; Mancilla-Leytón et al., 2012, 2013, 2014; Cheblil et al., 2020). It is known that winter leaves of *Cistus albidus* are richer in nitrogen than summer leaves, so this might be related to the selection of this species particularly in winter by goats (Correia et al., 1992).

4.3. Plant ageing does not increase stress sensitivity

Plant ageing has been demonstrated to influence stress susceptibility and response in some species (reviewed by Rankenberg et al., 2021). However, very few studies have been performed in Mediterranean shrubs growing in their natural habitat. In a previous study using *Cistus albidus*, we showed that reductions in plant size under natural conditions is an effective strategy to prevent the wear and tear of aging, and that plants develop compensatory mechanisms at reproduction level (including changes in flowering, fruit and seed production) that prevent senescence in terms of seed yield and viability (Müller et al., 2014). The present study showed no effects of plant ageing on stress sensitivity when mature plants of different ages were compared. It adds to current knowledge by investigating plant age-dependent herbivory pressure. Interestingly, it was found that plant aging did not negatively affect the plant response to abiotic stress or biotic stress in any of the variables studied (Fig. 4). Altogether, it seems that *C. albidus* may have compensatory strategies that reduce age-related effects on plant stress response, as they do in reproductive terms.

5. Conclusion

It is concluded that *C. albidus* is a highly tolerant shrub to both abiotic and biotic stresses in Mediterranean mountain areas, showing only slight photoinhibition during early summer and mild symptoms of herbivory attack in winter. Furthermore, seasonal and microclimatic driven variability revealed an antagonistic relationship between the occurrence of abiotic stress and herbivory-induced damage. Plant ageing did not negatively influence the plant stress response (to abiotic stress factors or herbivory). Therefore, we rejected the hypothesis that herbivory may superimpose abiotic stress factors and negatively impact plant performance under natural conditions, and that these effects are more evident as plants age. Indeed, the results show the extraordinary capacity of this plant species to adapt to Mediterranean climatic conditions in mountain areas.

Author contributions

AC and SMB conceived and designed the study. AC and RB performed experiments and biochemical analyses. AC and SMB wrote the manuscript. AC prepared all figures and analysed the data. All authors revised and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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