Mediterranean Botany
ISSNe 2603-9109

EDICIONES
COMPLUTENSE

https://doi.org/10.5209/mbot.70622

Herbaria collections as cues of leaf trait adjustments in *Senecio pyrenaicus* subsp. *carpetanus* in response to environmental aggravation

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Received: 13 July 2020 / Accepted: 22 March 2021 / Published online: 21 March 2022

Abstract. The direct link of stomatal traits to fundamental physiological processes makes them critical attributes for defining leaves' physiological potential. Stomatal traits can serve as proficient ecophysiological proxies of plant responses to changing environments. Studying stomatal patterns and structures in leaves could be a particularly suitable approach to assess plant responses to the current climate crisis imperiling our ecosystems. The reconstruction of plant responses to past conditions could be attained by assessing stomatal and other foliar traits from preserved specimens in herbarium collections associated with primary strategies (i.e., growth, resource gaining, water usage). In combination with climatic records, studying these traits offers a promising and rarely explored venue in the context of changing climate. The assessment of herbarium specimens offers a powerful tool to reproduce long-term trait variations along shifting climates when monitoring programs are inexistent. This research aims to unveil the great potential in-built in herbaria specimens to discern long-term feedbacks at various scales (morphologic, physiologic) in the high-mountain herb *Senecio pyrenaicus* subsp. *carpetanus*. The latter to foresee its enduring capacity to the challenging conditions during its growing season in Sierra de Guadarrama and their expected aggravation due to the ongoing climate crisis. Analysis of conserved and recent specimens revealed *S. carpetanus* could adjust to the shift to drier growing seasons in these mountains and the aggravating climate over the last three decades by modifying leaf micromorphological traits (i.e., smaller, densely packed stomata) and leaf size.

Additionally, these results suggest a potential influence of the reportedly increasing O_3 levels in the study site on the observed increases in stomatal density and reduced stomatal size. These findings point out a potential improvement in the control of stomatal aperture and in minimizing the incoming rates of O_3 to plant cells. Overall, this research expects to draw attention to the valuable evidence in herbaria collections to assess plant distribution variations, morphology, and phenology across spatial and time scales. Similarly, it intends to open the gate for future studies incorporating herbaria collections to evaluate plant responses and predict their ensuing fitness under changing environmental conditions.

Keywords: herbaria, climate change, resilience, high-mountains, stomatal traits.

How to cite: Magaña Ugarte, R. 2022. Herbaria collections as cues of leaf trait adjustments in *Senecio pyrenaicus* subsp. *carpetanus* in response to environmental aggravation. Mediterr. Bot. 43, e70622. https://doi.org/10.5209/mbot.70622

Introduction

The autecology scheme provides a fundamental tool to understand an ecosystem's inner workings, focusing on the individual plant species integrating through the study of single traits or sets of concomitant traits (Díaz *et al*., 2004). Knowledge of these attributes, associated with primary strategies (i.e., growth, resource gaining, reproduction), could grant improved projections of the responses in constituent species and, in turn, that of plant communities to forthcoming disturbances in their habitats (Grime *et al*., 2007). Consequently, the assessment of leaf functional traits in an ecophysiological context can help identify adaptive or acclimating features on leaf architecture to discern the potential performance and assembling of plant communities to shifting environmental conditions. For instance, combining the study of macro- and micromorphological leaf traits (e.g., stomatal patterning, leaf area, leaf width) with physiological attributes can improve our understanding of the mechanisms driving the stomatal sensitivity in response to changing environments (Hetherington and Woodward 2003; Bertolino *et al*., 2019). Their evaluation turns out to be a particularly needful and convenient approach to determine the response to the foreseen warmer and drier growth seasons (Giorgi & Lionello, 2008).

It is widely known that stomatal density (number of stomata per mm^2, SD) and size (SS) play a crucial role in regulating gas exchange, photosynthesis, water loss, and evaporative cooling (McElwain & Chaloner, 1995; Hetherington & Woodward, 2003; Franks & Beerling, 2009; Franks *et al*., 2013). Even with the extensive knowledge available, the study of stomata remains remarkably attractive given their ability to react to local and global changes on timescales from minutes to millennia (Paoletti & Grülke, 2005). The latter is coupled to the superior responsiveness of stomatal behavior and morphology to a series of environmental and plant stimuli, such as intracellular

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and hormone signaling and changes in concentration of atmospheric pollutants (e.g., ozone). The high oxidative capacity of ozone (O_3) resulted in its acknowledgment as the most harmful air pollutant distressing vegetation (IPCC, 2007). Exposure to O_3 prompts alterations in plant physiology and biochemistry, mainly in carbon allocation and gas exchange processes, and leads to constraints in plant growth and reproduction (Grülke & Heath, 2020; Manninen *et al*., 2003; Paoletti & Grülke, 2005). Short-term elevated O_3 exposure is associated with visible injuries and a reduction of photosynthesis as a side-effect of increased internal leaf CO_2 concentration (c_i), derived from the inhibition of carbon assimilation (Grülke & Heath, 2020; Paoletti & Grülke 2005). Among the strategies activated in plants to counter O_3 stress are those leading to the tolerance of its products (*via* antioxidant activity) or those entailing stomatal limitation (i.e., exclusion of O_3; Levitt, 1980; Grülke & Heath, 2020). It is still essential to underline that stomata could fail to close completely and that O_3 exposure may compromise stomatal function (Paoletti & Grülke, 2005). Integrated multi-scale assessment of datasets and models has revealed a qualitatively com-parable sensitivity of plants to changing CO_2 levels (either enrichment or decline), marked by an adaptive response through adjustments on stomatal anatomy and chloroplast biochemistry (Hetherington & Woodward, 2003; Franks *et al*., 2013). However, most of the studies under controlled experiments and current global change scenarios reflect the response to short-term CO_2 exposure (<5 years). Consequently, these studies do not adequately describe prolonged exposure responses under current CO_2 levels, i.e., modification of resource constraints or adaptation.

Long-term studies (>5 years) or monitoring programs evaluating responses to moderate to high O_3 and CO_2 levels remain scarce, particularly for high-mountain flora. The latter, albeit the significant vulnerability of these species to the increasing levels of air pollutants in mountain areas, as an outcome of global change. Fittingly, the outstanding source of evidence available in herbarium collections can recreate long-term trait variations throughout fluctuating environments when monitoring programs are lacking. Several studies have accounted for climate gradients' influence on leaf morphology variations, especially in broad-leaved species, due to their higher liability to climatic extremes (Ackerly *et al*., 2002). Thus, studying the stomatal patterns and structures and leaf traits in conserved specimens in herbaria collections could inform the conditions at the specific time of collection, allowing for reconstruction of environmental scenarios, plant fitness, and behavior. The latter, seeing as these are deemed as well-preserved traits in ex-situ collections for extended periods. Furthermore, based on the strong link between stomata and water loss in plants, examining longstanding trends in stomatal patterning from conserved individuals could aid to unveil future plant responses to the hotter and drier growing seasons expected in water-limited environments like Mediterranean high-mountains (Xu & Zhou, 2008; Fraser *et al*., 2009; Carlson *et al*., 2016). The information accumulated in herbaria along years of systematic collection of wild populations has been hardly valued and considered for describing variations in stomatal and leaf traits along gradients, expressly in the context of shifting climate conditions (Magaña Ugarte *et al*., 2020). Hence, assessing foliar traits in broad-leaved species from herbaria collections could further our understanding of long-term feedback at the morphological and physiological levels in plants, coupled with improving their resilience predictions. In this sense, herbaria collections prove of great relevance in the construal of future plant responses to climate worsening in species from habitats subject to the detrimental effects of the climate crisis, such as high-mountains. The relatively recent and limited instauration of monitoring programs in high-mountain environments and their focus on following-up the assembly of plant communities and macro-morphological traits add to encouraging focusing research efforts to evaluate changes in the micromorphological and physiological traits of these plants (Magaña Ugarte *et al*., 2019).

High-mountain areas are ideal places to assess the ecological effects of the ongoing climate crisis (also known as climate change), given their high taxonomic diversity, lower human disturbance compared to lowland areas, and the presence of steep ecological gradients (Körner, 2003; Dullinger *et al*., 2007). Expressly underrepresented mountain ecosystems like the Mediterranean high-mountains, including the Spanish counterparts, deserve more significant consideration in these circumstances (Magaña Ugarte *et al*., 2019). The latter, seeing as these refuges of exceptional levels of taxonomic diversity (Pauli *et al*., 2003; Stanisci *et al*., 2016; Winkler *et al*., 2016), could see their viability limited due to the ongoing aggravation of climatic conditions, especially during the growing season of vegetation (summertime; Giorgi & Lionello, 2008). The elevated temperatures, intense solar radiation, and scarce rainfall, particular of the summer period in Mediterranean areas (i.e., summer stress), foster the attainment of maximum O_3 concentrations in summer by promoting photochemical O_3 generation, consequently posing risks to human health and natural ecosystems (Scebba *et al*., 2005; Meleux *et al*., 2007). Moreover, the foreseen warmer and drier summer conditions in this region (Giorgi & Lionello, 2008) indicate a potential upsurge in summer tropospheric O_3 levels, representing a significant challenge to ecosystems (Meleux et *al*., 2007).

In recent years, mean summer temperatures in the Sierra de Guadarrama mountain range (Central Spain) have registered readings up to 7ºC above the expected values and a drop in mean summer rainfall of ~6% (Figure 1), indicating a shift towards drier growing seasons as of the 1990s in these mountains (Ruiz-Labourdette *et al*., 2014; Magaña Ugarte *et al*., 2020). Furthermore, chronic tropospheric O_3 pollution has been ascribed in these summits, particularly during summer (Elvira *et al*., 2016), concurring with the growing season of vegetation. The elevated O_3 values in these summits, which exceed the thresholds established for vegetation (Air Quality Directive 2008/50/EC), underline the potential risk of exposure in plant communities in Sierra de Guadarrama to high O_3 levels. The latter is of the essence for species maintaining partially open stomata at night, such as our species of interest, *Senecio pyrenaicus* subsp. *carpetanus* (Elvira *et al*., 2011). Hence, among the variation in plant traits, one could foresee the adjustments in stomatal traits (e.g., SD, SS) in this species to reduce O_3 intake per stomata and the consequent oxidative pressure. To date, there are few existing studies on temporal changes in leaf anatomical features in *S. carpetanus*, along with those relating stomatal and foliar traits from conserved herbarium specimens with global change factors other than climate exacerbation, such as atmospheric pollutants. In this sense, the present work aims to assess the potential variations in macro- and micromorphological leaf features in *S. carpetanus* individuals inhabiting the highest summits of Sierra de Guadarrama through the last 71 years with changing climatic conditions and fluctuating values of atmospheric contaminants proven adverse for vegetation, i.e., O_3 (IPCC, 2007; Elvira *et al*., 2016). The goal is to determine whether these global change factors can be classified as drivers of variations in the leaf and stomatal morphology of *S. carpetanus* from Sierra de Guadarrama using as evidence herbaria collections and recent field samples.

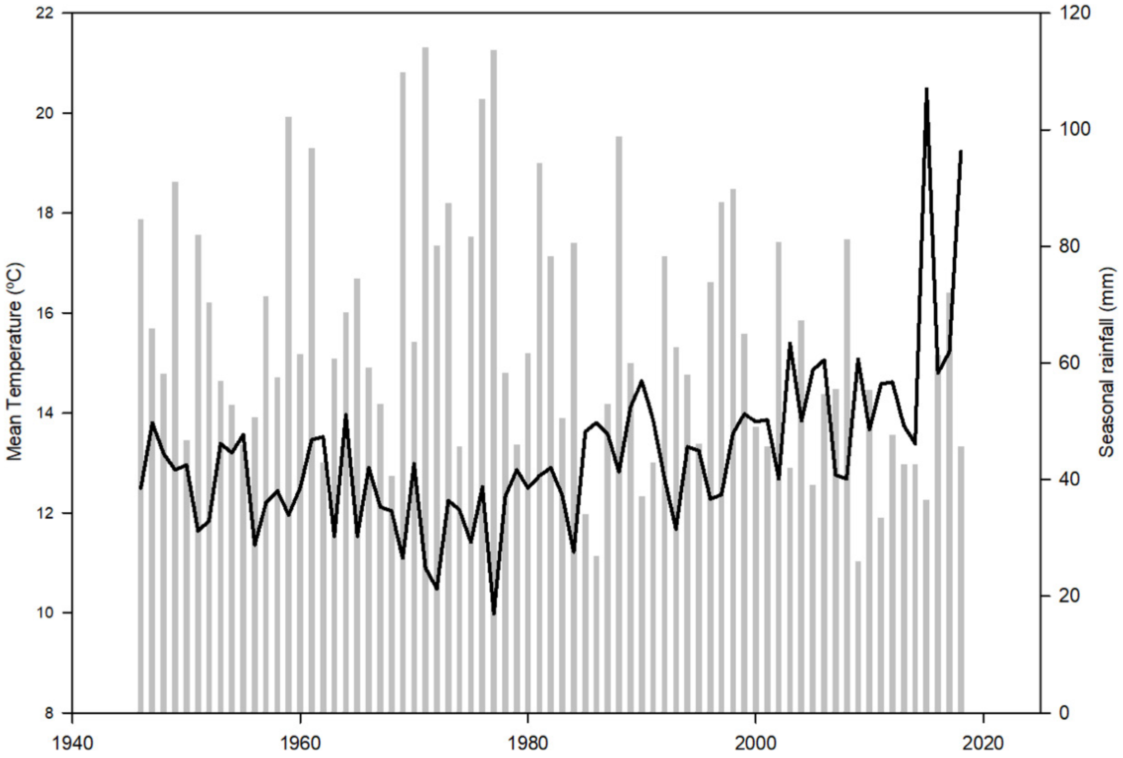

Figure 1. Climatic variation during the growing season (i.e., summertime) in Sierra de Guadarrama summits for the period 1945–2019. Data retrieved from the Spanish Meteorological Agency (AEMET).

Materials and methods

Study site and plant material

The selected study area is the Sierra de Guadarrama summits, part of the Sistema Central mountain range in central Spain. This mountain range experiences a Mediterranean-type climate: cold temperatures in winter and elevated temperatures with limited summer precipitation. Mean annual rainfall is ~1350 mm, with a dry period taking place from May to October (<10% total annual rainfall, topsoil moisture content <5% and <10% in August and September, respectively; Gutiérrez-Girón and Gavilán, 2013), which coincides with the active growing season of vegetation in these habitats. Thus, these high-mountain orophytes experience the simultaneous impact of high temperatures, intense solar radiation, and scarce rainfall, labeled as summer stress, which constitutes the primary constraint for plant performance and survival in Mediterranean-type regions (Larcher, 2000). Exposure to high tropospheric O_3 levels poses an additional stress factor for these orophytes, particularly the notably increased O_3 phytotoxicity potential during summertime. The elevated O_3 during their growing season could be exceeding the thresholds for human health and vegetation protection established in the European Air Quality Directive (2008/50/EC) and the surpassing of the CLRTAP thresholds for vegetation protection (CLRTAP, 2011; European Environment Agency, 2014; Elvira *et al*., 2016) during this period.

Senecio pyrenaicus subsp. *carpetanus* (Willk.) Rivas-Mart. (henceforth "*S. carpetanus*") from Sierra de Guadarrama is a frequent species of the pioneer plant community thriving in stony, siliceous, mobile screes of these high-mountain habitats. Its coverage surges in sun-exposed areas alongside increased soil consolidation and integrity in any stony soil, fostering the gradual establishment of species proper of the psicroxerophitic grasslands (Fernández-González, 1991). It grows up to 20–

45 cm tall, with erect, densely leafy stems that only branch in the inflorescence. *S. carpetanus* can also be found together with broom-shrubs in established grasslands, in areas not covered by dominant grass *Festuca curvifolia* (Fernández-González, 1991). The latter denotes its capacity to exploit temporal niches or skeletal habitats (Grime *et al*., 2007). This "wider" microhabitat amplitude could be attributed to the inferred long-term feedbacks in this species in response to the climate aggravation in Sierra de Guadarrama (Ruiz-Labourdette *et al*., 2014; Magaña Ugarte *et al*., 2020). The latter, seeking to optimize resource uptake, usage, and release via the adjustment of related leaf traits (e.g., leaf morphology, stomatal patterning). Nevertheless, and despite the fundamental role of this species in establishing high-mountain communities, its autecology, the plant traits, and selective advantages linked to its ecological specialization have been barely studied.

Herbarium collections of *S. carpetanus* were sourced for locations in the highest summits in Sierra de Guadarrama. The goal was to obtain the appropriate time-lapse representation corresponding with the period for which climatic records are available for Sierra de Guadarrama (data provided by the Spanish Agency of Meteorology, AEMET). However, due to the limited availability of conserved specimens, in most cases, only one individual per year was assessed. Field studies were performed from May to October in 2017 and 2018 to evaluate the phenology, occurrence, and performance of *S. carpetanus* throughout its entire growing season. Additionally, healthy individuals were collected during the growing season of 2018 within the optimum altitudinal range defined for *S. carpetanus* (above 1800 m asl; Fernández-González, 1991). These were subsequently dried and preserved as herbarium specimens for later analyses of leaf macro and micromorphological traits, the calculation of the SLA adjustment factor, and the accurate comparison of morphological leaf traits with older herbarium specimens.

Macro- and micromorphological leaf traits

Morphological traits were measured on *S. carpetanus* specimens, according to Cornelissen *et al*. (2003). Leaf area (LA) and specific leaf area (SLA) were calculated with the Image J free image analysis software (ImageJ, US National Institutes of Health, Maryland, USA) using dry and fresh leaves. The SLA adjustment factor was obtained from comparing fresh and dry SLA values from the 2018 samples. Leaf length and width were measured in all conserved specimens on fully developed leaves.

Stomatal features were assessed on fully developed leaves of mature, conserved specimens to disregard the substantial variation in the stomatal traits due to sampling, leaf initiation, or leaf expansion. Leaf imprints were taken following the silicone rubber technique described by Weyers and Johansen (1985), with impression material (A-Silicone Putty Fast Set. VANINI Dental Industry, Italy). Leaf rubber impressions were taken from the mid-portion on both leaf surfaces, avoiding the central vein, to determine possible changes in stomatal patterning and allocation through time. Image analysis of leaf imprints was carried out as described by Fanourakis *et al*. (2013). The SD and SS were determined in five non-overlapping fields of view per imprint using a Nikon (ECLIPSE 80i) digital microscope and camera control unit (DS Camera Control Unit DS-L2, Nikon Metrology Inc.). The SS was measured on 20 randomly selected stomata per leaf surface. Lastly, the stomatal pore index (SPI; guard cell length^2 x stomatal density) was calculated as a potential proxy of transpiration and g_s of conserved specimens under shifting climate and atmospheric pollutant concentrations (Sack *et al*., 2003; Holland & Richardson, 2009). Here the SPI represents a relative measure of the ratio of total stomatal pore area to leaf area. Given that stomatal densities and size differed between the abaxial and adaxial surfaces (if applicable), each side's measurements were recorded separately. The given value for SPI per plant refers to the averaged SPI from both surfaces.

Atmospheric pollutant data

The O_3 concentrations (µL/L) in the Sierra de Guadarrama summits were obtained from all available references. For the period between 2009–2011, we retrieved data from Elvira et al. (2016), including only the values from monitoring stations located at 2262 and 1850 m. asl. The selected data corresponded to the mean monthly values and the six-month AOT40 O3-exposure accumulation index, according to the proposed within the CLTRAP (2011) and the EU Air Quality Directive (2008/50/CE). For the remaining years of study (2012–2019), the data of O_3 concentrations were obtained from a personal communication by Elvira, S. (CIEMAT) and the Air Quality Network of the Madrid Autonomous Community (see Table S1).

Statistical analysis

The effect of the temporal variation on LA, SD, and SS was evaluated using Linear Mixed Models (LMMs) via restricted maximum likelihood (REML; (Patterson & Thompson, 1971). The data was analyzed considering the additive effect of elevation and year as explanatory variables (fixed factors) and elevation as a random factor to have a model unhindered to the elevations of the herbaria collections sourced. The response variables were modeled with Gaussian error distribution, using the function "lme" from package "nlme" (Pinheiro *et al*., 2018). The simplification of full models consisted of removing non-significant parameters in order to obtain more parsimonious models. Fixed factor significance was assessed via posteriori likelihood ratio tests.

The effect of atmospheric pollutants (period 2005–2018 for O_3) on the SD, SS, and SPI variations through time was assessed using multivariate ANOVAs. Previous tests for normal distribution and homogeneous variance using the Shapiro-Wilk test and Levene test with the functions "Shapiro.test" and "leveneTest" from packages "dplyr" and "car," respectively, were performed (Fox & Weisberg, 2011; Wickham *et al*., 2018). The factors tested were elevation, O_3, temperature, and rainfall (annual and growing season). Significant differences between means were determined with the least significant difference test calculated at the 5% level.

Generalized additive models (GAMs) were used to determine the long-term trend of changes in climatic parameters (period 1947–2018) and O_3 in LA, SD, and SS, using the "mgcv" package (Wood, 2011). The initial models for all the assessed foliar traits included the additive effect of mean annual temperature, mean annual rainfall, dry season average temperature, dry season average rainfall, and O_3 as predictors. In order to explore the responses to the confirmed shift towards drier, hotter conditions in Sierra de Guadarrama in the 90s (Ruíz-Labourdette *et al*., 2014; Magaña Ugarte *et al*., 2020), we performed GAMs on two subdivisions of the data: a) samples before 1990; and b) samples from 1990 and onwards; retaining the same factors as in the analysis as mentioned earlier. Multicollinearity among predictors was tested before performing the GAMs via the assessment of the variance inflation factor (VIF) with the "vif" function of the "car" package (Fox and Weisberg, 2011). Given that maximum VIF values were lower than three, all variables were included in the initial models.

All statistical analyses were performed using the R statistical package (R-Core-Team, 2018).

Results

Species timing and phenology

Follow-up of the seasonal development of *S. carpetanus* in the field throughout the entire growing season in consecutive and climatically contrasting years (2017–2018) allowed for identifying an atypical behavior in *S. carpetanus* in response to the environmental conditions taking place, unnoticed until now. This species displayed a recurring withdrawal pattern from its growing site in elevations above 2000 m when exposed to extreme climatic events (i.e., heatwaves in 2017) or acutely depleted soil water content. Generally, this was followed by a confirmed re-sprouting in the remaining of its growing season once the environmental conditions ameliorated. This re-sprouting entailed the emergence of new individuals, which maintained short stature and a considerably small SLA, in contrast to the initial individuals who faded under adverse conditions. A comparison of the mean SLA between the initial and subsequent (re-sprouted) individuals is given in Table 1.

Table 1. Values for specific leaf area (SLA) during the maximum of plant activity and the end of the growing season in *S. carpetanus* through the growing seasons of 2017 and 2018, climatically contrasting years.

Sampling date	Elevation (m asl)	SLA_{FRESH} ($mm^2 mg^{-1}$)	SLA_{DRY} (mm^2mg^{-1})
July 2017	2244	21.3	12.9
	1980	16.8	13.4
September 2017	2244	7.9	4.6
	1980	10.6	5.5
July 2018	2244	94.7	60.3
	1980	124.8	88.72
September 2018	2244	19.7	11.75
	1980	47.7	32.29

Adjustment factor for SLA

The evaluation of the recently collected field specimens, and its further treatment as herbarium accessions, allowed establishing an adjustment factor for SLA in *S. carpetanus* to estimate the fresh SLA in preserved specimens. This adjustment factor differed among phenological stages and species timing (i.e., maximum vegetative activity, re-sprouting, senescence; Table 2). The adjustment factor was calculated as the ratio between SLA fresh/SLA dry (mm^2 mg^{-1}) of 10 different individuals (5 leaves/individual). The estimation of fresh SLA should be calculated by multiplying the SLA of dry samples by the respective adjustment factor, depending on the specimens' phenological stage.

Micromorphological leaf traits

Similar to other *Senecio* species, these results revealed the stomata type in *S. carpetanus* is paracytic. Moreover, there was an absence of variation in the disposition of stomata in the leaves of *S. carpetanus* (both conserved and recent samples) since the highest rate of stomata remained in the abaxial surface of the leaves of the specimens from the entire period assessed while displaying negligible SD (<5) in the adaxial surface. In turn, the following SD and SS results refer only to stomata on the abaxial surface. The SD showed a significant increasing tendency in specimens from the period before the shift towards drier growing seasons (i.e., before 1990; Figure 2a), followed by a significant increase in SD in the last two decades (Figure 2b), all unrelated to elevation. Also, SD showed a positive correlation with temperature changes during the active growing season in both periods (before and after 1990; p=0.001; Figure S1).

Further analyses revealed the considerable reduction of SS through the period assessed, irrespectively of elevation. The SS shrinkage was particularly noticeable in specimens from the 21st century (i.e., 2007, 2009, 2011, 2015; Figure 3), in correspondence with both the substantial decline in accumulated rainfall and the temperatures above the mean monthly values befalling the active growing seasons in Sierra de Guadarrama.

Table 2. Specific leaf area adjustment factors for each phenological stage assessed in fresh and conserved specimens of *S. carpetanus*.

Species Timing/Phenological stage	SLA Adjustment factor	Parameters to consider (values per individual)
Maximum of plant activity	1.463	$LA_{DRY} > 900$ mm^2 Plant height > 35 cm Inflorescence emergence or presence
Early development or re-sprouting individuals	1.960	$LA_{DRY} < 800$ mm^2 Plant height < 30 cm No inflorescences present

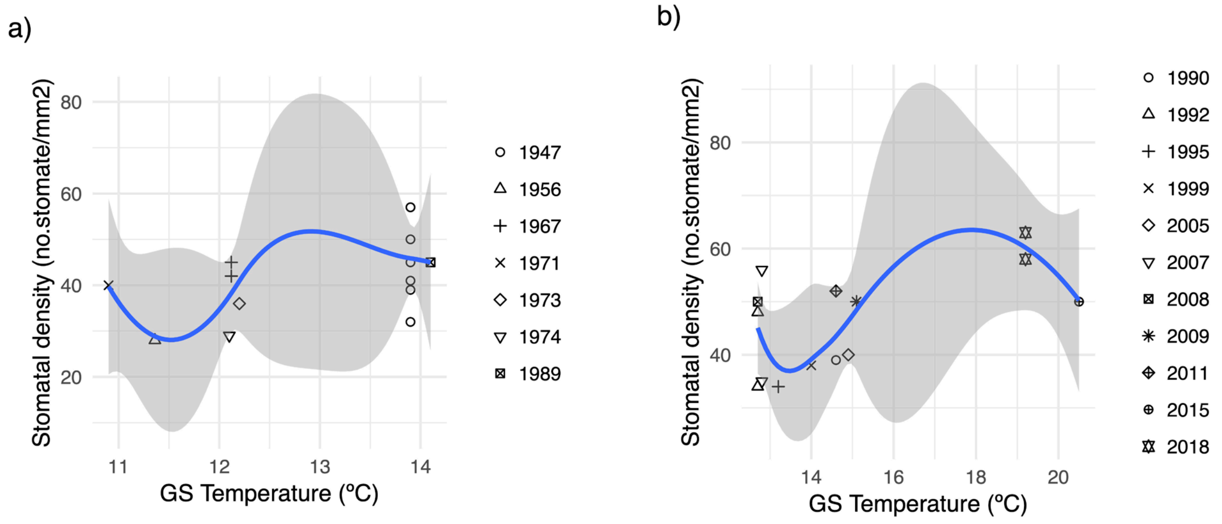

Figure 2. Changes in stomatal density in response to mean temperature during the growing season a) before the shift towards drier conditions (i.e., before 1990), and b) as of the years where this shift was documented (i.e., 1990). Plotted values are in means. The blue lines represent the modeled data, while the surrounding gray area represents the “smoothed” parameter estimation made by the generalized additive models (GAMs).

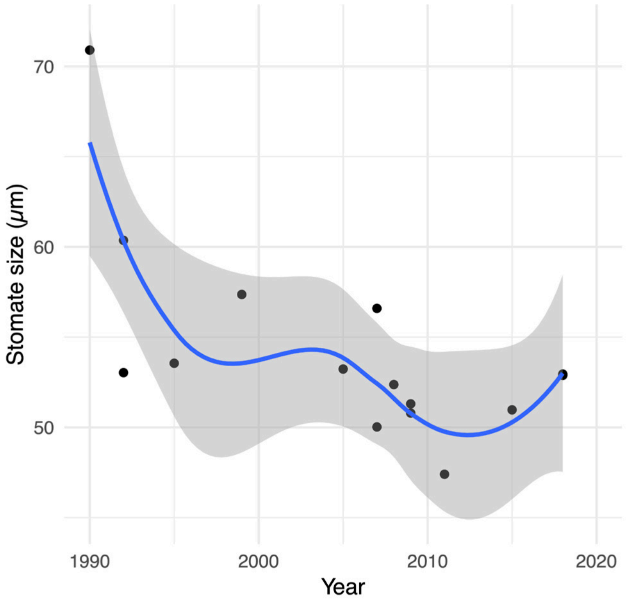

Figure 3. Changes in stomatal size (SS) in conserved *S. carpetanus* collected during the period 1990-2018. The blue line represents the modeled data, and the surrounding gray area represents the “smoothed” variable estimation made by the generalized additive models (GAMs).

Conversely, changes in the level of tropospheric O_3 showed marginal significance (*p-value*=0.056) over the variation in SD of *S. carpetanus* (Figure 4b). The lack of significance for O_3 could be due to the limited O_3 data included and not the triviality of this agent's damaging effect. Increasing levels of O_3 have been linked with drier and warmer summers in Sierra de Guadarrama by Elvira *et al*. (2016), thus suggesting the potentially significant effect of the simultaneous action of adverse environmental factors on stomatal patterning and related processes.

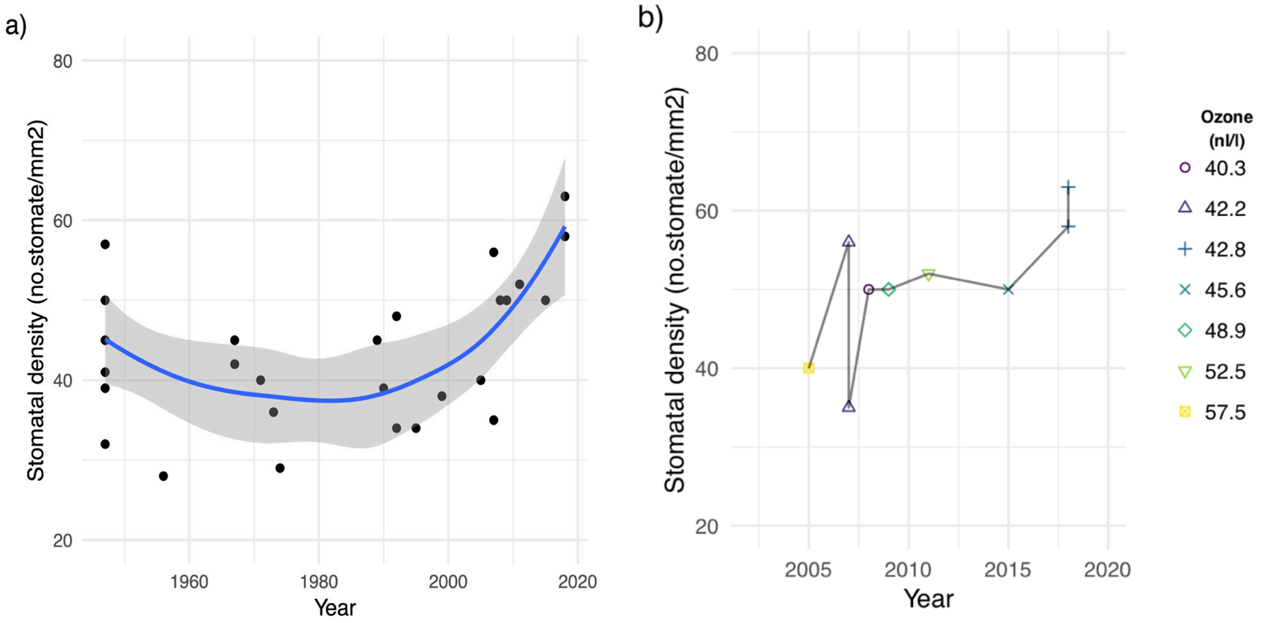

Figure 4. a, Temporal tendency in stomatal density through the past 71 years reported by Magaña Ugarte *et al*. (2020) The blue line represents the modeled data, and the surrounding gray area represents the "smoothed" parameter estimation made by the generalized additive models (GAMs); b, Stomatal density variation in *S. carpetanus* through time with varying tropospheric O_3 in Sierra de Guadarrama summits. O_3 data refers to the 6-month mean values (May-October), retrieved from Elvira *et al*. (2016) for the period 2005–2011, and personal communication by Elvira, S. for the period 2012–2018. All the data for stomatal density was obtained from preserved specimens.

Discussion

Assessing the adjustments in stomatal characteristics in response to more adverse climatic conditions and increasing atmospheric pollutants is crucial to understand the role of natural selection in plant adaptation and phenotypic response to the ongoing trend of more unfavorable conditions.

CO_2 increases will be a minor driver in leaf macro and micromorphological parameters in high-mountain vegetation under the current climate change scenario, compared to other environmental factors such as the ones considered in the present paper (i.e., climatic parameters, O_3), given the negligible effects of recent increases in global CO_2 on SD changes in several high-mountain species under natural environments (Körner, 1988; Körner, 2017). Herbarium and fossil records of vascular plants have provided evidence of evolutionary adaptations in SD and SS in several species in response to fluctuations in atmospheric CO_2 over geological time (Bertolino *et al*., 2019). However, implementing preserved specimens to study the effect of other atmospheric pollutants, such as O_3, is uncommon if not nonexistent. The latter highlights the added value of herbaria collections and underlines this research's merit for evaluating genuine longstanding changes in leaf traits in conserved specimens to the current climate aggravation in Sierra de Guadarrama.

Thus far, there is no consensus on the effect of O_3 on SD, with some studies reporting an increase in SD across different species following O_3 exposure (Matyssek *et al*., 1991; Wiltshire *et al*. 1996), while others report no SD changes in either sensitive or insensitive cultivars (Grulke *et al*., 2006). The observed absence of a significant effect of the recent increases (i.e., last 14 years) in tropospheric O_3 in Sierra de Guadarrama on the stomatal patterning of *S. carpetanus* individuals relates to the opinion suggesting the effect of ozone on SD could be related to species- dependent responses (Grülke & Heath, 2020; Grulke *et al*., 2006; Pääkkönen *et al*., 1997). Moreover, the high SD in individuals from the last 20 years could suggest acclimation to elevated O_3 exposures (Manninen *et al*., 2003; Pääkkönen *et al*., 1997). The increase of SD in the last years could be better explained by the increased stress conditions rather than by changes in O_3 concentrations. As the O_3 tendency in the last 15 years, the latter has been opposed to the increases in SD.

Nevertheless, the absence of significance of O_3 on this data does not rule out its established role as a phytotoxic pollutant for the vegetation in Sierra de Guadarrama (Elvira *et al*., 2016; Grülke & Heath, 2020 and references therein). Alternatively, the lacking significant effect of elevated O_3 levels on stomatal patterning could be a sign of an improved regulation capacity of g_s or an exceptional antioxidant response in *S. carpetanus*, an outcome of the SS shrinkage found in the corresponding specimens. The latter since smaller apertures have been suggested to lessen O_3 uptake per stoma, theoretically reducing the response-aperture time under unfavorable conditions coupled with cutting down the demand for antioxidant capacity in a given volume of apoplastic water (Grülke & Heath, 2020; Paoletti & Grulke, 2005). Additionally, the minor changes in SD in response to the variations in O_3 (Figure 4b) could indicate enhanced tolerance in *S. carpetanus* to high O_3 levels. Similar to the observed in *Vicia faba* L. under "chronic" O_3 exposure (Turcsányi *et al*., 2000), these results suggest the recurrent field exposure of *S. carpetanus* to high O_3 levels (>40 ppb) could have triggered a hardiness effect to later exposures, diminishing its reaction to subsequent high O_3 exposures (Grülke & Heath, 2020; McCool *et al*., 1988). Plants with higher growth rates often experience more detrimental effects at a given O_3 exposure level than plants from poor or unsuitable conditions (Grülke & Heath, 2020). Resembling the findings by Grülke *et al*. (2006) in O_3-nonsensitive coneflower, the absence of visible tissue damage and early senescence, and the maintenance of high g_s rates and high chlorophyll content (unpublished data) even in periods with elevated diurnal O_3 levels support the inferred O_3-hardiness of *S. carpetanus.* Nevertheless, further experiments involving antioxidant enzyme activity, g_s, and chlorophyll fluorescence are required to determine the manifestation of oxidative damage and the actual exposure-response at different levels of organization in this species. The latter since, as expressed by Körner (2017), stomatal traits are the outcome of a cascade of functional relations associated with microclimate and the actual climate a leaf is experiencing. Thus, stomatal traits should not be related to just one individual parameter, i.e., atmospheric pollutants, but rather account for the entire atmospheric conditions when analyzing these responses over prolonged periods to establish a link with plant ecophysiology. Other O_3 metrics could be used to assess the stomatal response.

These results confirm a shift towards narrower, smaller leaves in *S. carpetanus* with a higher SD and reduced SS due to the dryer, warmer growing seasons in Sierra de Guadarrama, an outcome of climate change. Similarly, Guerin *et al*. (2012) linked the morphological shifts in leaves of *Dodonaea viscosa* over 127 years to contemporary climate change, inferring climate aggravation as the critical driver of leaf morphology at regional and local scales. Accordingly, the studies mentioned above, coupled to the significant correlations found between temperature in the growing season and the leaf and stomatal traits assessed (Magaña Ugarte *et al*., 2020), further ratify the ongoing climate crisis as a significant driver of the observed changes in the leaf macro- and micromorphology in *S. carpetanus*.

The SD and their diffusive conductance are positively correlated with the net-photosynthetic rates and, by extension, biomass production (Woodward *et al*., 2002). SPI changes are mainly induced by SD changes throughout altitudinal gradients, while SS remains comparably constant within species (Körner *et al*., 1986; Bucher *et al*., 2016). Consistently, the present findings show minor SS changes throughout the entire optimal altitudinal range for *S. carpetanus*. At the same time, SPI showed a clear correlation with the increases in SD, particularly in samples from recent years (Table S2). The absence of a significant relationship between the changes in SD with elevation tentatively dismisses a possible sign of plastic responses in this case. Previous studies resemble our findings, attributing the increases in SD to local conditions or variations in light availability and moisture content, deeming them more significant drivers of changes in SD than elevation itself (Körner, 1988). Nevertheless, genetic variability and common garden experiments under controlled conditions are required to discern whether the reasons for this performance can be formally labeled as an adaptive response or merely as phenotypic plasticity in *S. carpetanus*.

The earliest sign of a plant's responses to environmental alterations manifests in adjusting its functional traits within the reaction norm (Tonin *et al*., 2019). One major drawback when incorporating herbarium specimens in ecophysiological studies to assess plant responses to climate change is that these specimens must be unspoiled to remain useful in future years. Thus, this restricts obtaining functional traits associated with primary strategies that could reconstruct resource acquisition efficiency and partitioning, such as SLA (Pérez-Harguindeguy *et al*., 2013). In turn, the calculation of an adjustment factor for SLA from recently collected individuals could enable estimating this trait in conserved specimens of a particular species with similar characteristics. In line with the latter, the adjustment factor for SLA calculated for *S. carpetanus* at different growing stages could entail an added piece of information to define the magnitude and the trend of progressive changes in its morpho-physiological responses. The latter may be useful to estimate its fate and the plant communities it it belongs to under the ongoing climatic aggravation in Sierra de Guadarrama (Nicotra *et al*., 2010). These results highlight the use of herbarium specimens as evidence of past plant behavior and their utility to assess trait adjustments in response to climate aggravation.

Overall, these findings allow attributing a reduced sensitivity of *S. carpetanus* to the current level of environmental aggravation in Sierra de Guadarrama. However, it further emphasizes the need to identify the response limits and critical levels of all the major abiotic factors proven to affect performance (soil water stress, temperature) for this species. In particular, more attention should focus on studying the effects of O_3 in this species, given its potential to increase sensitivity to climate stress in plants under sequential stresses (Brown *et al*., 1987; Alscher *et al*., 1989) and its significant increase in the area of study in recent years.

Acknowledgments

Thanks are due to Director, Prof. D. Sánchez-Mata, and curator of the MAF herbarium, Dr. J.M. Pizarro, for their assistance to acquire all herbaria material used for the present research. The author also acknowledges the staff of the collections of vascular plants from SALA Herbarium, the Herbarium of the Universitat de Valencia, the RJB Herbarium, and CIEMAT Madrid for providing the material required for this study. Additional thanks to Prof. Escudero for his assistance during data analysis. This research was funded by the Madrid Autonomous Region Government (REMEDINAL TE-CM, S2018/EMT-4338).

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Supplementary Material

Figure S1. Linear regression of the relationship between the stomatal density (no. stomata/mm^2) and the variation in temperatures during the growing season (ºC) of Senecio pyrenaicus subsp. carpetanus found in conserved specimens.

Table S1. Ozone threshold values and concentration-based ozone critical levels (CLe_c) for plant protection established within the CLTRAP (2011), and the ozone objective values designated by the EU Air Quality Directive (2008/50/CE).

Table S2. Herbaria data of the studied *S. carpetanus* specimens selected for the present study, coupled with the mean morphological parameters measured per herbarium record.