Tree Physiology, 2026, **46**, tpag006
https://doi.org/10.1093/treephys/tpag006
Advance access publication date 2 February 2026
Research paper

Drought response of fire-adapted Mediterranean shrubs under elevated CO_2

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Handling Editor: Roberto Tognetti
Jordi Martinez-Vilalta holds the position of Editor for Tree Physiology and has not peer reviewed or made any editorial decisions for this paper.

The first summer after fire poses a high risk of drought mortality for species that rely on postfire recruitment for their persistence in fire-prone ecosystems (postfire seeders). However, little is known about how future conditions of elevated atmospheric CO_2 (eCO_2) and its interaction with severe drought will affect this key life history strategy in Mediterranean shrublands. We investigated the extent to which eCO_2 modifies plant traits and resource allocation, as well as how eCO_2 and drought intensity interact to modify plant water status, water fluxes and hydraulic behavior in 8-month-old seedlings of five seeder shrub species. Among the studied species, three regenerate postfire exclusively by seed (non-resprouting seeders), while two can also resprout after fire (resprouting seeders). We found that eCO_2 significantly reduced specific leaf area for all species and increased above- and belowground biomass for three species but had no species-specific effects on stomatal density or root-to-shoot ratios. When species were grouped by resprouting ability, eCO_2 reduced root-to-shoot ratios in resprouting species but not in non-resprouting species. Importantly, eCO_2 did not affect plant water relations under drought either at the species level or when grouped by resprouting ability. However, resprouting and non-resprouting seeders exhibited distinct water-use strategies under drought, independent of CO_2 treatment. Overall, eCO_2 is unlikely to mitigate drought stress for postfire seeders in future climates. Considering functional differences across life history strategies, as well as the variability of traits within them, is key to predicting future patterns of biodiversity in a drier and more fire-prone world.

Keywords: climate change, postfire seeder, resprouting, water relations.

Introduction

Global climate change is intensifying droughts and shifting fire regimes in Mediterranean ecosystems (Zittis et al. 2019, Grau-Andrés et al. 2024), thereby increasing the probability of compound disturbances (Batllori et al. 2017, Pausas and Keeley 2021, Richardson et al. 2022). One of the most iconic life history strategies in these ecosystems is the ability to recruit after fire from a persistent seed bank, that is, the postfire seeders (Pausas and Lamont 2022). This life history strategy is very distinct from co-existing sclerophyllous obligate resprouters that are more commonly studied (Pausas and Keeley 2014). Postfire seeders compose a large proportion of the Mediterranean biodiversity (Keeley et al. 2012), and are well suited to deal with both fire (Keeley and Pausas 2022) and drought (Galmés et al. 2007, Blanco-Sánchez et al. 2022). In postfire seeders, fire, via heat shock or smoke, breaks seed dormancy of the seeds stored in the seedbank; and germination typically occurs with the first rains after fire (Pausas and Lamont 2022). Seedling recruitment postfire (i.e., in open gaps) is subject to high incident sunlight and strong oscillations in water availability, and requires rapid growth to establish and develop rooting depth before the next summer drought (Frazer and Davis 1988, Parra and Moreno 2017), and to mature early to ensure seed production before the next fire (Pausas and Lamont 2022). The first summer after germination is thus a critical season with high risk of mortality (Quintana et al. 2004). As postfire environments increasingly coincide with more severe droughts, the recruitment of postfire seeders may become constrained (Enright et al. 2015, Nolan et al. 2021, Salesa et al. 2022).

One key uncertainty in predicting postfire seedling survival under future drought conditions is the role of elevated atmospheric CO_2 (eCO_2). Atmospheric CO_2 has increased by 47.3% since 1750, currently exceeding 420 p.p.m., and is projected to further double by the end of the century (Arias et al. 2021). Rising CO_2 levels may alter plant resource allocation, water-use and drought resistance of postfire seedlings. Under eCO_2 conditions, plants can potentially minimize leaf-level water loss through reduced stomatal conductance and by growing leaves with lower stomatal densities, while sustaining sufficient CO_2 uptake (Xu et al. 2016). This response increases water-use efficiency, leading to the assumption that plants growing under eCO_2 would experience an amelioration effect on drought stress (De Kauwe et al. 2021, Wang et al. 2022). However, scaling this effect from the leaf to the whole-plant level is complex, because eCO_2 typically increases plant growth (Bussotti et al. 2014). Increased root growth may positively impact water uptake (Uddin et al. 2018), but

Received: September 24, 2025. **Accepted:** January 17, 2026

increased leaf growth can expand the total area over which the plant loses water, increasing overall transpiration and possibly counteracting leaf-level water-use benefits (Osborne et al. 2000, De Kauwe et al. 2021).

Previous research has found a general trend of eCO_2 improving leaf water status under drought conditions, mediated by reduced stomatal conductance and increased root-to-shoot ratio (Wullschleger et al. 2002, Wang et al. 2022), but also note that this effect is not evident across all studies. The bulk of research into eCO_2 effects on plant water relations during drought is based on crop and tree species (Wullschleger et al. 2002, Wang et al. 2022). Studies of Mediterranean shrub species have typically focused on a few obligate resprouting Mediterranean species (e.g., *Quercus* spp.) (Tognetti et al. 1999, Vaz et al. 2012, Aranda et al. 2020) while very little is known on postfire seeders (but see Arp et al. 1998, Tognetti et al. 2000*a*, 2000*b*). It has been recognized that stomatal response to eCO_2 varies across biomes and plant species (Vicente-Serrano et al. 2022) and substantial differences have been found between growth forms of herbs, shrubs, and trees (Ainsworth and Long 2005, Ainsworth and Rogers 2007). Many climate change models include plant water-use responses to eCO_2 (Swann et al. 2016, Vicente-Serrano et al. 2022), underscoring the importance of avoiding oversimplifications and generalizations across and within growth forms (Temme et al. 2020). Thus, we still lack an understanding of the impact of eCO_2 on drought stress for postfire seeder shrubs that are a unique biodiversity component in Mediterranean ecosystems.

Within the postfire seeding strategy there is some important variability. Some species recruit solely from persistent seedbanks (non-resprouting or obligate seeders), while others can also resprout postfire (resprouting or facultative seeders). These contrasting postfire strategies are associated with distinct physiological and morphological traits related to drought response. Recruitment after fire is often associated with drought tolerance mechanisms for survival during early establishment (Ackerly 2004, Pratt et al. 2008, Pausas et al. 2016), such as high resistance to xylem cavitation (Jacobsen et al. 2007, Pratt et al. 2008, 2010), and structurally drought-resistant leaves (Paula and Pausas 2006), as well as shallow root systems that enable quick response to pulses of moisture (Moreno-Gutiérrez et al. 2012, Vilagrosa et al. 2014). In contrast, the ability to resprout after disturbance requires the allocation of resources belowground for reserve accumulation, leading to more extensive root systems that access deeper, more stable water sources and help maintain water potential as moisture declines (Schwilk and Ackerly 2005, Moreira et al. 2012). Facultative seeder species possess traits related to both postfire strategies and may therefore be expected to exhibit ecophysiological characteristics that are intermediate between those of obligate seeders and resprouters (Nefzi et al. 2023). Thus, we propose that the ability to resprout may be an important factor in understanding drought responses under eCO_2 among postfire seeder species.

Our study aimed to disentangle eCO_2 effects on plant traits, resource allocation, plant water status (i.e., drought stress), water-fluxes and hydraulic behavior for a key but understudied life history strategy (postfire seeder shrubs) of Mediterranean species, to improve predictions of fire-prone shrubland drought response in future eCO_2 conditions. We examined three specific questions. (i) For each species, does eCO_2 alter plant traits and resource allocation? (ii) For each species, during drought, does eCO_2 alter plant water status, water fluxes and hydraulic behavior? (iii) Does the ability to resprout modify the above eCO_2 effects and drought responses (resprouting vs non-resprouting seeders)? To answer these questions, we selected five species that represent a range of the functional trait diversity found in postfire seeders typical in Mediterranean shrublands of eastern Spain, including two resprouting (facultative) and three non-resprouting (obligate) seeder species. Plants were grown in greenhouses with either ambient or eCO_2 concentrations, and subjected to a three-phase, progressively intense drought treatment composed of controlled decreases in soil water content (from 50% to 10% gravimetric soil water content).

Materials and methods

Species

We selected five species from different genera: *Anthyllis cytisoides* L., *Dorycnium pentaphyllum* Scop. (synonym of *Lotus dorycnium* L.), *Lavandula stoechas* L., *Cistus albidus* L. and *Salvia rosmarinus* Schleid (synonym *Rosmarinus officinalis* L.) (Table 1). All species are common postfire seeders, belong to three of the most abundant families in fire-prone shrublands of eastern Spain (Fabaceae, Lamiaceae and Cistaceae), and thus account for a large proportion of trait variability of postfire seeders in the region. *Anthyllis cytisoides* and *D. pentaphyllum* have a strong ability to resprout after fire, while *L. stoechas*, *C. albidus* and *S. rosmarinus* do not (Table 1). Postfire seeders in general are typically highly tolerant to dehydration, but in Mediterranean basin shrublands, there are differences in leaf traits within this functional group as well. For instance, *A. cytisoides* is drought-deciduous with a defined, but temporally variable resting period (Haase et al. 2000). *Cistus albidus* is semi-drought deciduous and produces smaller leaves in summer (seasonal dimorphism) (Aronne and Micco 2001) to reduce transpiring surface area during prolonged periods of drought (Table 1). The other study species depend mainly on small or narrow leaves for drought tolerance (see Text S1 available as Supplementary Data at *Tree Physiology* Online for full descriptions of all species and Figure S1 available as Supplementary Data at *Tree Physiology* Online for photos). The distribution of *A. cytisoides*, *D. pentaphyllum* and *C. albidus* is limited to the western Mediterranean basin, while *L. stoechas* and *S. rosmarinus* cover most of the Mediterranean basin (Bolòs and Vigo 1995, Affouard et al. 2025). Latitudinally, all species range from southern France to northern Africa. We collected seeds of *A. cytisoides*, *D. pentaphyllum* and *C. albidus* in the Valencia region (eastern Spain) in 2018. Seeds of *S. rosmarinus* and *L. stoechas* were provided by Intersemillas plant nursery and were collected from wild populations in the Valencia region (2018), and in Andalucia (2017), respectively. All seeds of a given species came from the same population. The sampling area has a typical Mediterranean climate with high interannual rainfall and temperature variability, summer droughts and relatively frequent crown fires (Keeley et al. 2012).

Greenhouse experiment: eCO_2 and drought

Our experiment simulated levels of ambient (mean 415 mmol mol^{-1}) and of elevated (mean 825 mmol mol^{-1}) atmospheric CO_2 concentrations in six hermetic small greenhouses

Table 1. Fire and drought-related responses for the five species considered.

	Anthyllis cytisoides	*Dorycnium pentaphyllum*	*Lavandula stoechas*	*Cistus albidus*	*Salvia rosmarinus*
Species abbreviations	ACY	DPE	LST	CAL	SRO
Family	Fabaceae	Fabaceae	Lamiaceae	Cistaceae	Lamiaceae
Postfire strategy	Resprouting seeder	Resprouting seeder	Non-resprouting seeder	Non-resprouting seeder	Non-resprouting seeder
Postfire resprout ability	R+	R+	R−	R−	R−
Fire-related germination cue	Heat	Heat	Smoke	Heat	Smoke
Leaf drought response	Drought-deciduous	Small leaves, moderate leaf shedding	Small, narrow leaves	Semi drought-deciduous	Small, narrow leaves
Root characteristics	Deep roots	Deep roots	Shallow roots	Shallow roots	Shallow roots
P50	−1.79		−2.41, −2.46	−5.78	−9.4

Postfire strategy and resprouting ability (yes: R+, no: R−) was obtained from the BROT database (Tavşanoğlu and Pausas 2018). Fire-related germination cue was obtained from Moreira et al. (2010). Vulnerability to xylem cavitation (P50 MPa) was obtained from Pausas et al. (2016), except for P50 values of *L. stoechas*, which are based on *L. angustifolia* and *L. intermedia* (Lamacque et al. 2020). P50 values for *D. pentaphyllum* or closely related taxa were not available. Leaf and root characteristics for each species are described and referenced in Supplementary material S1. available as Supplementary Data at *Tree Physiology* Online.

(2 m × 2 m × 2 m) (Figure S2 available as Supplementary Data at *Tree Physiology* Online). The eCO_2 treatment comprised a set of three greenhouses with a shared CO_2 supply system and CO_2 control system (Techgrow) (Figure S2 available as Supplementary Data at *Tree Physiology* Online). The ambient treatment also comprised a set of three greenhouses and included a CO_2 sensor, but not a CO_2 supply system. All greenhouses were maintained under climate-controlled conditions (Figure S2 available as Supplementary Data at *Tree Physiology* Online). Each treatment set of greenhouses had a closed system of air circulation with air conditioning units for temperature moderation. In addition, each greenhouse had fans to circulate air and CO_2, and an automatic ventilation hatch that opened once a night for 30 min to refresh the air, balance CO_2 levels and reduce humidity. Each greenhouse had a supplemental lamp that was turned on during the day to compensate for reduced sunlight from the plastic roof and tunnel netting and for seasonal differences in natural light, and an automatic drip irrigation watering system (Netafim). Each set of greenhouses had a datalogger (Techgrow DL1) that recorded sensor data of CO_2 concentrations (p.p.m.), temperature (°C), relative light units and relative humidity (%) throughout the experiment (Figure S3 available as Supplementary Data at *Tree Physiology* Online).

In April 2019, seeds were sown in their respective greenhouse after applying corresponding pre-germination treatments needed to break seed dormancy (Table S1 available as Supplementary Data at *Tree Physiology* Online) and ensure germination. One seedling was grown per pot. Pots contained 10 L of substrate and were made of PVC pipes (16 cm diameter and 60 cm height; Figure S1 available as Supplementary Data at *Tree Physiology* Online) with dense Styrofoam caps fitted at the bottom that had four holes to allow drainage. The substrate was composed of 50% peat, 20% sand, 20% perlite and 10% soil collected from the field. Our substrate contained a higher percentage of peat than would be present in native soils, because without the water retention it provides, plants may not thrive in the greenhouse environment. Similarly, we included a higher percentage of perlite, to create a porous substrate and reduce the effect of the roots growing to the sides of the pots. Lastly, we mixed 30 g of NPK slow-releasing fertilizer (Planta Plus) into each pot. Plants were grown for 8 months, a period that corresponds to the typical growing season postfire before summer. During this growing period, plants were provided with sufficient water (equivalent to 22.88 mm of rainfall per week). Again, this amount of water is more than would be typically received in the Mediterranean environment, because plants in greenhouse pots dry out more quickly than in natural environments.

In January 2020, all plants were watered to soil saturation, then the water system was turned off and the soil in each pot was covered by plastic to prevent evaporation. To determine the drought level at which differences in drought responses between eCO_2 treatments occur, we subjected all plants to a three-phase progressive drought treatment over 12 weeks (Figure 1). Gravimetric soil water content was controlled by daily weighing pots with a digital floor scale, and individual hand watering (Figure S4 available as Supplementary Data at *Tree Physiology* Online). The first phase of the experiment (p1) involved maintaining soil moisture at ∼50% gravimetric water content (∼field capacity; see section 'Soil water availability' in the Materials and methods) for 3 weeks, representing well-watered conditions. This was followed by a controlled 1-week reduction to 30% (moderate drought), initiating the second phase (p2), where soil moisture was maintained at 30% for 3 weeks. A subsequent 2-week gradual decrease to 10% accounted for slowed soil water loss due to reduced plant transpiration, leading to the final phase (p3), where soil moisture was maintained at 10% (intense drought) for 3 weeks. While plants were weighed and watered daily in p1 and p2, this was reduced to every second day in p3 in response to the lower water demand under intense drought. Controlling soil water content for each plant partially removed the effect of plant size, thereby revealing the underlying mechanisms of water-use and drought response under eCO_2.

In total, 138 plants were measured throughout the entire experiment, with 12–15 plants of each species per CO_2 treatment (Table S1 available as Supplementary Data at *Tree Physiology* Online), evenly distributed between greenhouses. At the end of the drought period, six of those plants, all from the eCO_2 (3 *A. cytisoides;* 1× *D. pentaphyllum*, and 2× *L. stoechas*) were at their dehydration limits (i.e., loss of all photosynthetic area on a stem marked for sampling), and thus it was impossible to estimate their leaf biomass and calculate their leaf specific water-use traits (leaf specific transpiration, conductance and conductivity) (see section 'Water

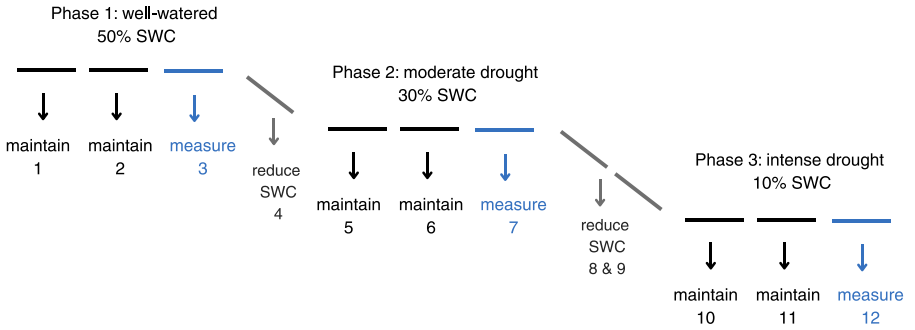

Figure 1. Schematic of the drought treatment experimental design showing a 12-week timeline divided into three phases with target gravimetric soil water content (SWC): well-watered (50%), moderate drought (30%) and intense drought (10%). Black segments indicate periods during which SWC was maintained at the target level, blue segments indicate plant measurement periods and grey segments indicate transition periods during which SWC was progressively reduced to the next target level. Each horizontal segment represents 1 week, with week numbers shown. Specific leaf area and stomatal density were measured during the well-watered phase 1. Transpiration was measured daily (p1 and p2) or every other day (p3) throughout the experiment. Water potential, osmotic potential and leaf loss were measured during all three measurement periods (p1, p2 and p3). Plant biomass, root NSC (resprouting species only), and gravimetric soil water content were measured during the measurement period in phase 3.

fluxes and hydraulic behavior, below–eCO2 and drought', below).

Soil water availability

Each pot (≈10 L soil) was weighed daily during phases 1 and 2 of the drought treatment, and every other day during phase 3. To guide the drought treatment, we used fresh and oven-dry weights from a 10 L test pot to estimate the pot weights corresponding to target gravimetric water contents (θg). At the end of the experiment (after all plant measurements were completed), we emptied each pot and recorded its total fresh soil mass. Because oven-drying all 10 L of soil was impractical, we collected a 1 L subsample from each pot. These subsamples were weighed fresh, oven-dried (105 °C, 72 h), and reweighed to calculate θg (water loss divided by dry mass). To correct the time series, we compared the estimated θg (based on pot weight) for the final day to the measured subsample value. The resulting difference was applied to all prior estimates, aligning them with the 'true' gravimetric water content. We then converted θg to volumetric water content (θv). Each pot's total dry soil mass was calculated by multiplying its total fresh mass by the final θg. Bulk density was obtained by dividing the total dry soil mass by the soil volume (10,000 cm^3). Finally, corrected θg values were multiplied by bulk density to calculate θv.

Soil water potentials for each plant pot throughout the drought were obtained by constructing a soil water retention curve (i.e., water retained at several pressure potentials), for our substrate. Due to having an artificial substrate, with a significant amount of perlite that absorbs water, estimations based on soil texture were avoided and instead laboratory methods were used (Parker and Patrignani 2023). For this, three replicate soil samples were measured at suction pressures from 0 to −1 kPa using a mini disk infiltrometer, and from −1 to −100 kPa using Richards' pressure plates (Richards 1948) (Figure S5 available as Supplementary Data at *Tree Physiology* Online). We fitted the Campbell model (1974) using the RETFIT program (LEACHM software; Hutson and Wagenet 1995) to predict soil water matric potential (h):

$$h = a(\theta_v/\theta_s)^{-b}$$

where θ_v is a given volumetric water content, θ_s is saturated volumetric water content, and a and b are constants. Constant a is sometimes regarded as air-entry value, but it is typically obtained by curve-fitting (Hutson and Wagenet 1995). Constant b is often regarded as an index for soil pore-size distribution (Moldrup et al. 2001).

Field capacity was determined from the soil water retention curve by identifying the point at which the decline in θ_v with increasing suction became much less steep, indicating a slowing of drainage. Across the three replicate curves, this transition consistently occurred at ∼−12 kPa (see Figure S5 available as Supplementary Data at *Tree Physiology* Online). For each replicate, θv at −12 kPa was obtained by interpolation, and these three values were then averaged to give a mean of 0.239 cm^3 cm^{-3}. This was converted to θg for each of the 138 pots using their individual bulk densities, and the resulting θg values were averaged, yielding a mean of 48.8%.

Plant traits and eCO_2

Leaf traits were measured during the first experimental phase (p1, no drought), as to ascertain the effect of CO_2. *Stomatal density* of each species was measured from leaf impressions made of the abaxial lamina of fully exposed mature leaves. Leaf impressions were obtained using nail varnish (*D. pentaphyllum, A. cytisoides*) or silicon (Aquasil ultra soft putty) for species with less uniform, delicate or hairy leaves (*S. rosmarinus, L. stoechas, C. albidus*) (Weyers and Johansen 1985, Galmés et al. 2007). We successfully made three to four leaf impressions for each of three plants of *L. stoechas* and *S. rosmarinus*, five plants of C. *albidus* and six plants of *A. cytisoides* and *D. pentaphyllum* per CO_2 treatment. The number of stomata was counted in three focal areas of each leaf impression at 200× magnification (*D. pentaphyllum*), 400× magnification (*C. albidus*, *A. cytisoides*) or 1000× magnification (*L. stoechas*, *S. rosmarinus*) with a stereo microscope (Olympus, field number 18) and then expressed as number of stomata mm^{-2} leaf. *Specific leaf area (SLA)* was measured for all individuals of all species, calculated as the ratio of green leaf area (mm^2) to leaf dry mass (mg). We collected 10–20 mature leaves from each plant (taken from leafy stems used for Scholander chamber measurements), that were scanned, and leaf areas determined using ImageJ software. Leaves were oven-dried for 48 h at 80 °C and weighed for dry mass (Pérez-Harguindeguy et al. 2013).

Plant resource allocation and eCO_2

At the end of the experiment all plants were removed from the pots, and leaves, stems (including those used for Scholander chamber measurements), main roots and fine roots were separated, oven-dried for 48 h at 80 °C and weighed to obtain dry mass (Pérez-Harguindeguy et al. 2013). *Plant belowground biomass* was calculated as the sum of oven-dried roots. Leaves and stems were combined to estimate *plant aboveground biomass*. *Root-to-shoot ratio* was estimated as the ratio between below- and aboveground biomass. At this point, drought-deciduous species (*A. cytisoides* and *C. albidus*) had already lost leaves in response to drought, and thus root-to-shoot ratio reflects the summer state of the plant after an intense drought (Pérez-Harguindeguy et al. 2013). Main root length and diameter were also measured for each plant. We had a range of root biomass and main root size values, confirming that they were not overly confined by the plant pot (Figure S6 available as Supplementary Data at *Tree Physiology* Online).

Root non-structural carbohydrates (NSC) (starch and soluble sugars) were analyzed only for the two resprouting seeders (*D. pentaphyllum* and *A. cytisoides*) (Agrolab Analitica, SL, Navarro), because the low values of NSC in non-resprouters is well known (Knox and Clarke 2005). In addition, we were interested in the changes with eCO_2 (and not in the comparison among species) and thus resprouters are the ideal species for this purpose. For this, on the last day of sampling (10–16 April 2020), fresh lateral root samples (less than 1 cm in diameter) were collected and immediately stored in a sealed plastic bag with silica gel and transported in a cooler with freezing packs (5 °C) to the laboratory. Root samples were immediately oven-dried at 80 °C for 24 h to stop root respiration. Samples were then stored in the freezer at −18 °C in hermetic bags with silica gel until analyzed (Moreira et al. 2012). Starch was determined by an enzymatic procedure and measured colorimetrically using a coupled glucose oxidase/peroxidase reaction and spectrometric analysis (Rasmussen and Henry 1990), and soluble sugars were measured through calorimetric reaction and spectrometric analysis (Green et al. 1989) (Agrolab Analitica, SL, Navarro).

To estimate *leaf biomass* in the previous drought phases, we counted the number of leaves on the same marked stem (identified with tape) during each measuring period (Figure 1). Leaf biomass in phases 1 and 2 was estimated using the formula: estimated leaf biomass (g) = leaf dry biomass (p3) × leaf count (p1 or p2)/leaf count (p3). *Total leaf area* per individual plant was then estimated for each drought phase as: leaf biomass (g) × SLA. Since leaf biomass was estimated at the end of each drought phase, *leaf biomass change* (%) during phase 2 was calculated as the difference between phase 1 and phase 2, and biomass change during phase 3 as the difference between phase 2 and phase 3.

Plant water status—eCO_2 and drought

Plant water status was measured for each individual plant at the end of each of the three drought phases (in weeks 3, 7 and 12 after the beginning of experimental phase 1, Figure 1). Plant water potential of leaf tissue is the standard measurement of the level of dehydration stress a plant is experiencing and an overall indicator of plant health (Giménez et al. 2013). We measured *midday water potential* (MPa) using a pressure Scholander chamber (Skye Instruments), within 2 h of midday (Pérez-Harguindeguy et al. 2013). As these Mediterranean species have small leaves with short petioles, measurements were performed using terminal leafy shoots with 10–20 leaves attached that were cut and immediately sealed into the chamber (Pérez-Harguindeguy et al. 2013). Although this included disturbance to the plant, the amount of biomass removed was minor compared with plant size (Figure S1 available as Supplementary Data at *Tree Physiology* Online). Plants in natural conditions also have similar small disturbances (wind, heat, drought, herbivory), and because it was similar for all plants we do not expect this to affect our results. Osmotic potential is one component of total water potential and decreases as solutes in cell sap concentrate during plant dehydration (Giménez et al. 2013). To measure *leaf osmotic potential*, a second leafy shoot was cut from each plant at midday and immediately frozen in liquid nitrogen. Frozen leaves were sealed into a small Eppendorf, then closed inside a larger Eppendorf, and introduced in the centrifuge at 10,000 r.p.m. for 30 s to extract sap. Solute concentration was measured in extracted sap using a Wescor 5500 vapor pressure osmometer (Wescor Inc., Logan, UT, USA), according to Gucci et al. 1991 and Bartlett et al. 2012. Osmometer readings in mmol kg^{-1} were converted to osmotic potential (MPa) using the Van't Hoff equation relating solute concentration to vapor pressure (Maréchaux et al. 2015).

Water fluxes and hydraulic behavior—eCO_2 and drought

Whole-plant transpiration (T) was calculated daily or every other day, throughout the drought, as pot weight loss (water loss, in grams) between two consecutive pot weighing measurements. Mean daily transpiration for each drought phase was then calculated. Plant hydraulic conductance refers to the rate of water flow ($g{\cdot}d^{-1}$) per unit pressure drop (kPa) and describes the ease with which water can move through the plant system. For each drought phase, mean daily whole-plant transpiration (T; $g{\cdot}d^{-1}$), midday water potential (Ψ_{md}; kPa), and soil water potential (Ψ_{soil}; kPa) were used to estimate *whole-plant hydraulic conductance (k)*, as $k = T/(\Psi_{soil} - \Psi_{md})$ (Jiang et al. 2021), with smaller values indicating reduced conductance. *Whole-plant conductivity (K)* was calculated considering plant height (h; cm), using the equation $K = (T \times h)/(\Psi_{soil} - \Psi_{md})$. Plant height was measured at the beginning of the first drought phase (i.e., ∼8 months after sowing). Note that Ψ_{md} is a truncated variable with a lower limit of −7500 kPa (the minimum value that could be measured with the Scholander chamber), while Ψ_{soil} is an estimated variable without a limit. Eight plants from eCO_2 had Ψ_{soil} estimations less than −7500 kPa and thus had negative conductance and conductivity values ($\Psi_{soil} < \Psi_{md}$). For analysis, we truncated our data to the lowest positive rounded value in our dataset: 0.01 ($g{\cdot}d^{-1}{\cdot}kPa^{-1}$) for whole-plant conductance, and 0.5 ($g{\cdot}d^{-1}{\cdot}cm{\cdot}kPa^{-1}$) for whole-plant conductivity.

Leaf-specific transpiration was calculated by dividing whole-plant transpiration (T) by leaf area (see section 'Plant resource allocation and eCO_2', above). *Leaf-specific conductance and conductivity* were calculated dividing k and K by the leaf area, respectively, for each drought phase.

Statistical analysis

Plant traits and resource allocation were measured once per plant. To assess the effect of CO_2, we constructed linear

mixed models (LMMs) for each of the following (dependent) variables: leaf stomatal density, SLA, plant total above- and belowground biomass, leaf biomass, stem biomass, root-to-shoot ratios (R:S) and root NSC. Each LMM included CO_2 (ambient/elevated), species and their interaction as fixed effects, and greenhouse as a random effect. Biomass and R:S variables were log-transformed to improve normality. The NSC model included only the two resprouting species (*A. cytisoides* and *D. pentaphyllum*).

Plant water status, water fluxes and hydraulic behavior were measured three times per plant (once in each drought phase). To assess the main and interactive effects of drought and CO_2, we fitted a series of LMMs, one for each species, for the following (dependent) variables: midday leaf water potential, leaf osmotic potential, transpiration (whole-plant and leaf-specific), hydraulic conductance (whole-plant and leaf-specific) and hydraulic conductivity (whole-plant and leaf-specific). Each LMM included drought intensity (drought phase p1, p2 or p3) and CO_2 (ambient/elevated), and their interaction as fixed effects, as well as plant identity nested in greenhouse as a random effect. Drought intensity was an ordered factor variable, and all dependent variables were log-transformed to improve normality; because water potential and osmotic potential are negative, these variables were transformed as log (−value).

We expected resprouting ability to modify resource allocation patterns, such as belowground storage and drought response strategies, due to differences in root characteristics and traits such as resistance to xylem cavitation (Pausas et al. 2016). Thus, we included resprouting ability as a predictor in additional models. For plant trait and resource allocation models (excluding NSC), we included CO_2 (ambient/elevated) and resprouting (yes/no), as fixed effects; and species and greenhouse as (crossed) random effects. For plant water status and hydraulic behavior models, we included drought intensity (p1, p2 or p3), CO_2 (ambient/elevated) and resprouting ability (yes/no) as fixed effects, plant identity nested in species and greenhouse as random effects.

Linear mixed models were constructed using the R-packages lme4 (Bates et al. 2015) and lmerTEST (Kuznetsova et al. 2017). We used a forward stepwise approach to select the most parsimonious model in all cases; each variable was added sequentially in order of their contribution to the explained variance, assessed by analysis of variance (ANOVA) and Akaike information criterion. We then used the 'anova' function for an analysis of variance with Satterthwaite's method and 'emmeans' function for pairwise comparative post-hoc tests from the emmeans package (Lenth and Piaskowski 2025). Normality of model residuals was tested with DHARMa (Hartig 2024).

Results

Effect of eCO_2

Plant traits and resource allocation exhibited variable responses to eCO_2. Specific leaf area significantly decreased under eCO_2 across all species (Figure 2, Table 2). Total aboveground and belowground biomass responses were species-specific, as indicated by a significant interaction between species and CO_2 treatment. Post-hoc tests revealed that both biomass components significantly increased under eCO_2 for *A. cytisoides* and *S. rosmarinus,* and aboveground biomass increased for *C. albidus* (Figure 2, Table S3 available as Supplementary Data at *Tree Physiology* Online). Examining each aboveground component separately showed that leaf biomass was significantly greater under eCO_2 in *A. cytisoides*, *C. albidus* and *S. rosmarinus*, whereas stem biomass was significantly greater under eCO_2 in *A. cytisoides* and *S. rosmarinus* (Tables S2 available as Supplementary Data at *Tree Physiology* Online and S3 available as Supplementary Data at *Tree Physiology* Online). eCO_2 had no significant effect on stomatal density (Table S2 available as Supplementary Data at *Tree Physiology* Online) or R:S (Table 2) across species. Similarly, there was no effect of eCO_2 on root NSC (i.e., allocation to storage) in resprouting species (Table S2 available as Supplementary Data at *Tree Physiology* Online). Descriptive statistics are provided in the supplementary material (Table S4 available as Supplementary Data at *Tree Physiology* Online).

Resprout ability was significant only for R:S, as part of an interaction term with eCO_2 ($P = 0.036$; Figure 2, Table S5 available as Supplementary Data at *Tree Physiology* Online). R:S of resprouting species significantly decreased under eCO_2 compared with the ambient CO_2 treatment, while R:S of non-resprouting species was not affected (Figure 2; Tables S5d and S6 available as Supplementary Data at *Tree Physiology* Online). That is, resprouting species tend to have marginally higher root-to-shoot ratio than non-resprouting species, but in eCO_2, the increased aboveground biomass is higher than the increase belowground and thus the differences in R:S allocation between resprouting and non-resprouting species is reduced.

Effect of drought under eCO_2

Plant water status, water flux and hydraulic models showed that drought intensity was the main determinant of plant water status and water-use during drought (Figure 3, Table S7 available as Supplementary Data at *Tree Physiology* Online). Leaf loss was observed predominantly in drought phase 3, with no significant differences between CO_2 treatments across any of the five species (Figure S7 available as Supplementary Data at *Tree Physiology* Online). Estimated total plant leaf area did not differ between CO_2 treatments for any species in any of the three drought phases (Figure S8 available as Supplementary Data at *Tree Physiology* Online; Supplementary Tables S7 available as Supplementary Data at *Tree Physiology* Online and S8). There was a clear threshold between drought phase 2 and 3 (moderate and intense drought), at which all variables significantly decreased in value for all species (Figure 3). We found no significant effect of eCO_2 for most variables and species (Figure S9 available as Supplementary Data at *Tree Physiology* Online; Table S7 available as Supplementary Data at *Tree Physiology* Online). There were a few exceptions, specifically *C. albidus* had lower leaf water potential in eCO_2 (Table S7 available as Supplementary Data at *Tree Physiology* Online), while post-hoc tests showed that *D. pentaphyllum* had lower osmotic potential under eCO_2 in drought phase 3 (Table S8 available as Supplementary Data at *Tree Physiology* Online). Because CO_2 was a primary treatment of interest, we retained it in all models and report its coefficients (Table S7 available as Supplementary Data at *Tree Physiology* Online), regardless of significance, to allow direct comparison across species.

Resprouting ability was found to be significant for plant water status, as part of an interaction term with drought

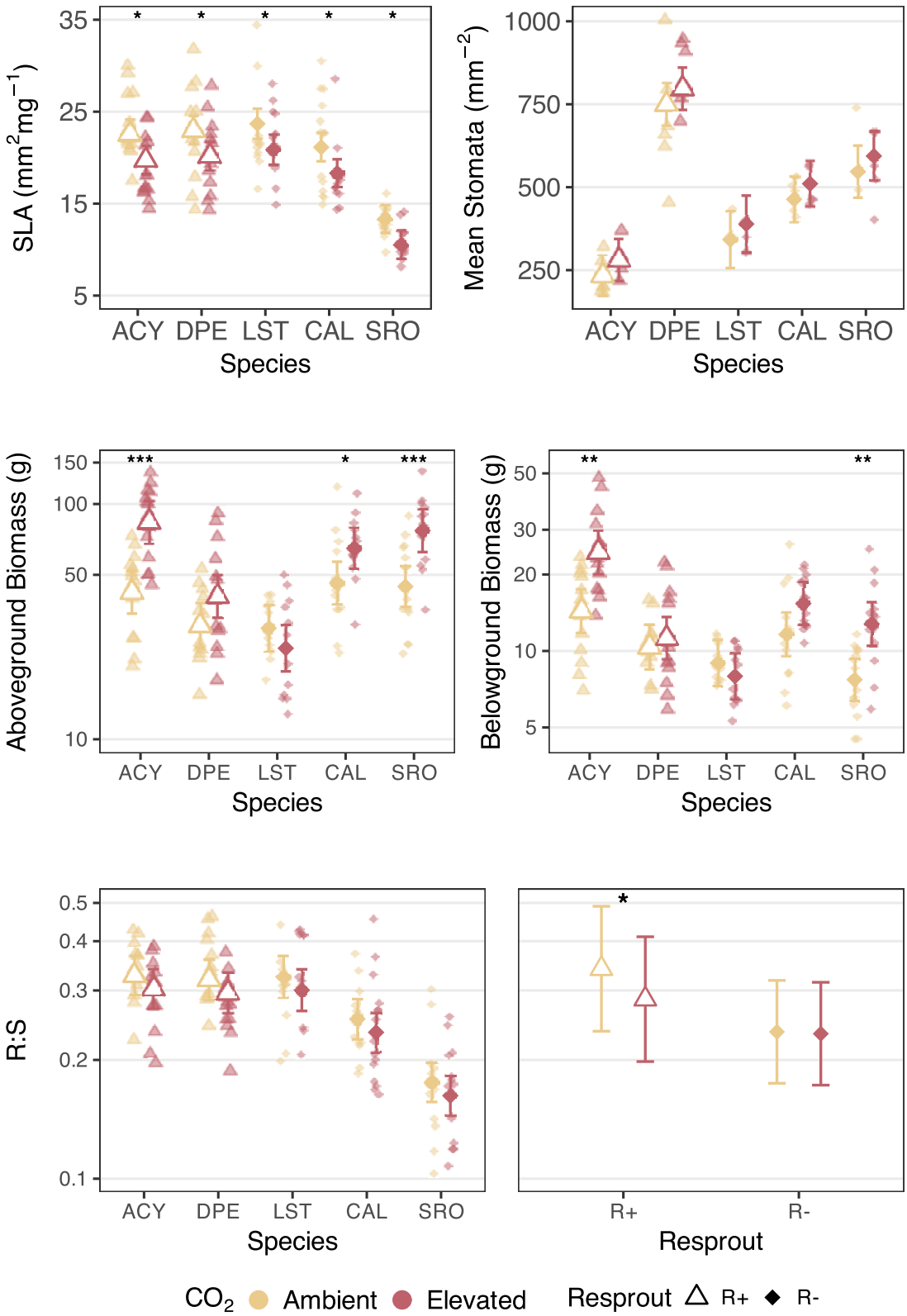

Figure 2. Effect of CO_2 treatment (ambient vs elevated) and species (*ACY, DPE, LST, CAL, SRO),* on plant traits and resource allocation. Response variables include SLA ($mm^2 \cdot mg^{-1}$), stomatal density (mm^{-2}) aboveground biomass (g), belowground biomass (g) and root-to-shoot ratio (R:S). The models for aboveground and belowground biomass include CO_2 × species interactions. A separate analysis of R:S examines the interactive effect of CO_2 and resprouting ability (R+ vs R−) (bottom-right plot). The y-axes for biomass and root-to-shoot ratio are on a log scale (log-transformed response variables), with axis labels back-transformed to original values for clarity. Large white or solid-filled symbols represent predicted mean values with 95% confidence intervals, while light-colored symbols represent raw data (n = 138, 137, 138, 137 and 137, respectively). Asterisks at the top indicate significant differences between CO_2 treatments for each species or resprouting group. For statistical analysis, see Table 2; for post-hoc contrasts, see Supplementary Tables S2 and S6. Significance codes: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

Table 2. Summary of LMMs for (a) SLA (mm^2 mg^{-1}); (b) plant aboveground biomass (g, log-transformed); (c) plant belowground biomass (g, log-transformed); and (d) root-to-shoot ratio (R:S, log-transformed).

Model	df	AIC	*P*	Estimate
(a) SLA				
Null		865.21		22.54
+ Species	4	776.34	**0**	−9.21 (SRO)
+ CO_2	**1**	**766.79**	**0.001**	−2.82 (e)
+ Species ∗ CO_2	4	770.14	0.326	
(b) Above biomass				
Null		221.65		3.74
+ Species	4	168.54	**0**	−0.33 (DPE); −0.35 (LST)
+ CO_2	1	158.63	**0.001**	0.69 (e)
+ Species ∗ CO_2	**4**	**148.03**	**0.001**	−0.88 (LST: e)
(c) Below biomass				
Null		176.62		2.67
+ Species	4	115.51	**0**	−0.33 (DPE); −0.47 (LST)
+ CO_2	1	110.42	**0.01**	0.53 (e)
+ Species ∗ CO_2	**4**	**99.15**	**0.001**	−0.45 (DPE: e); −0.65 (LST: e)
(d) R:S				
Null		96.04		−1.11
+ Species	4	0.92	**0**	−0.25(CAL); −0.62 (SRO)
+ CO_2	**1**	**0.94**	0.16	−0.08
+ Species ∗ CO_2	4	2.47	0.167	

Note that each row represents an individual model. Variables were selected by the stepwise procedure (variables added sequentially in order of their contribution to the remaining explained deviance), and models were compared with ANOVA test and the Akaike information criterion. Fixed effects included CO_2 treatment (ambient (a)/elevated (e)), and species and greenhouse was included as a random effect. If there are significant variables ($P < 0.05$), the coefficient estimates of the corresponding model are shown in the last column on the right (estimate for intercept is in the row of null model). N = (a) 138; (b) 137; (c) 138; (d) 137. The final model and statistically significant ($P < 0.05$) differences are highlighted in bold.

intensity (Figure 3; Table S9 available as Supplementary Data at *Tree Physiology* Online). Post-hoc tests revealed that in p1 and p2, plants of resprouting species had significantly lower leaf water status (i.e., more negative water and osmotic potentials) than non-resprouting species. However, in p3 (intense drought) differences in water status became non-significant between resprouting habits (Table S10 available as Supplementary Data at *Tree Physiology* Online). Note that osmotic potential had a small sample size in the final drought phase (N = 86 in p3 vs N = 138 in p1 and p2), because many plants lacked enough sap for measurement.

The interaction of resprouting ability and drought intensity was also significant for leaf specific transpiration, hydraulic conductance and conductivity (whole-plant and leaf specific). Post-hoc contrasts revealed that in general, resprouting and non-resprouting species did not differ within each drought phase (the only exception being that leaf specific hydraulic conductivity was lower for non-resprouting species in drought phase 3) (Table S10 available as Supplementary Data at *Tree Physiology* Online). Rather, the interaction demonstrated that non-resprouting species consistently had larger decreases of water-flux and hydraulic behavior variables between drought phases (Figure 3; Figure S9 available as Supplementary Data at *Tree Physiology* Online; Table S9 available as Supplementary Data at *Tree Physiology* Online), with significant changes often occurring between phase 2 and 3 (whole-plant hydraulic conductance, whole-plant hydraulic conductivity and leaf specific conductivity) (Table S10 available as Supplementary Data at *Tree Physiology* Online).

Discussion

Future postfire regeneration in Mediterranean shrublands will contend with increased drought and elevated atmospheric CO_2 concentrations (Meinshausen et al. 2011, Zittis et al. 2019). It has been proposed that, under drought, rising atmospheric CO_2 could reduce plant drought stress, via reduced stomatal conductance and increased root-to-shoot ratios (De Kauwe et al. 2021, Wang et al. 2022). Yet, drought response under eCO_2 is unlikely to be similar across growth forms (Ainsworth and Long 2005) and may vary depending on plant traits. In the current study, we show that eCO_2 had direct effects on plant traits and resource allocation but did not ameliorate drought stress for seedlings of five Mediterranean postfire seeder shrubs. Furthermore, we found evidence that the interaction between drought intensity and resprouting ability were significant determinants of plant drought response.

The observed decrease in SLA under eCO_2 (Figure 2) is in line with the general pattern across almost all C_3 plants and is likely due to the accumulation of NSC in leaves (Poorter and Navas 2003). Although stomatal density is often assumed to decrease under eCO_2, no significant change was observed in this study. This finding aligns with a recent meta-analysis (Poorter et al. 2025), which reported weak and inconsistent responses of stomatal density to CO_2 and identified light as a substantially stronger driver. An increase in plant biomass, both above- and belowground, is also expected under eCO_2 (fertilization effect) (Han et al. 2023), although in our study, this increase was significant for only three of the five species. Interestingly, while we did not find a significant

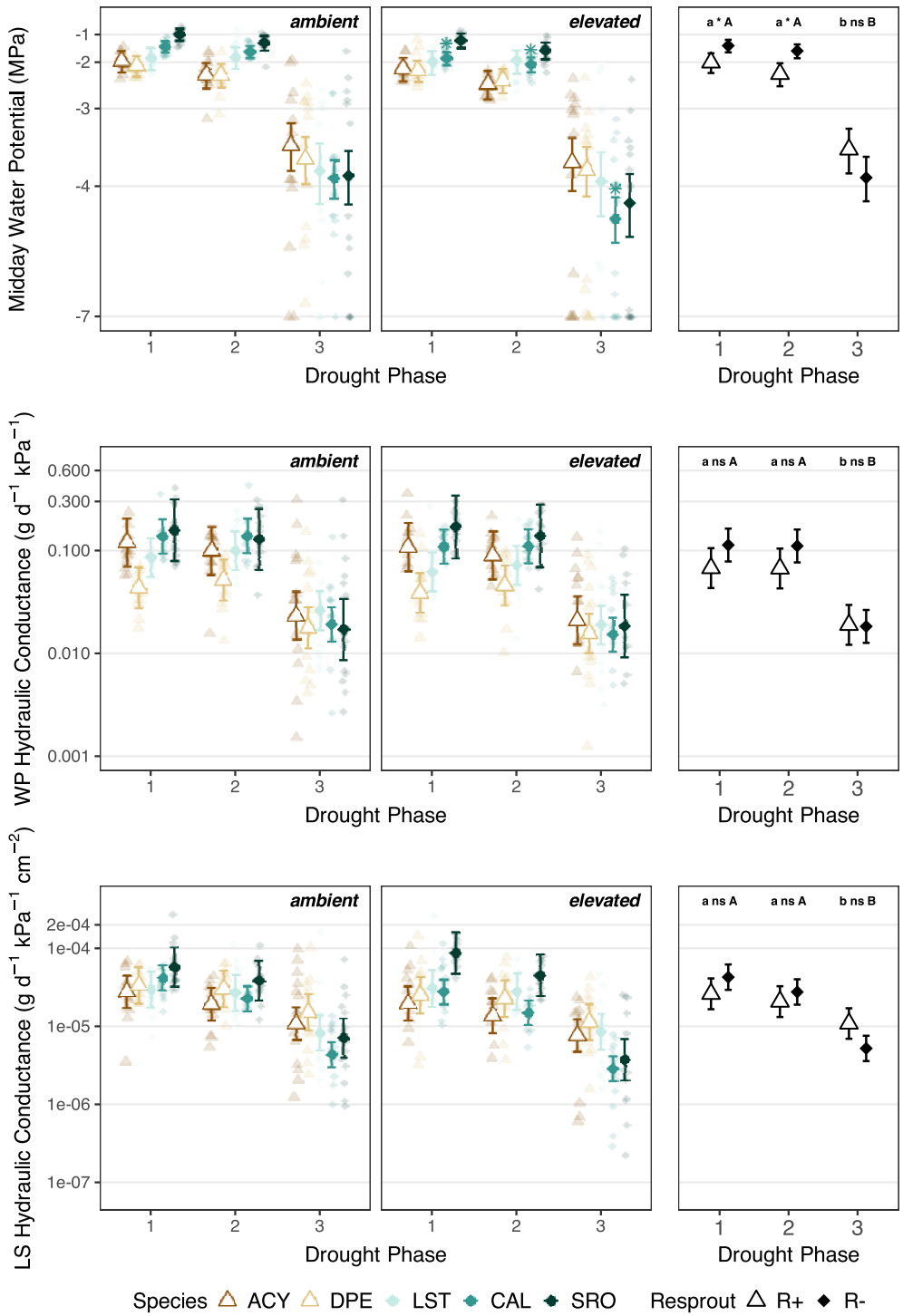

Figure 3. Effect of drought intensity (drought phases: p1 = 50%, p2 = 30%, p3 = 10% soil water content), CO_2 treatment (ambient vs elevated) and resprouting ability (R+ vs R) on plant water status and hydraulic behavior. Response variables include leaf midday water potential (MPa), whole-plant (WP) hydraulic conductance ($g \cdot d^{-1} \cdot kPa^{-1}$) and leaf-specific (LS) hydraulic conductance ($g \cdot d^{-1} \cdot kPa^{-1} \cdot cm^{-2}$). The left column plots show species-level responses, with models including drought intensity and CO_2 treatment (shown in facets), fitted independently for each species. Asterisks in the elevated CO_2 panels indicate significant CO_2 treatment contrasts within drought phases, with colors corresponding to species. See Table S7 available as Supplementary Data at *Tree Physiology* Online for full model statistics. Right column plots show the interactive effect of drought intensity and resprouting ability for the same variables, without CO_2 treatment included in the models. Lowercase letters denote significant differences among drought phases for R+ species, uppercase letters for R− species; shared letters indicate non-significance ($P > 0.05$). Asterisks indicate contrasts between R+ and R− within drought phases. For full statistical results, see Table S9 available as Supplementary Data at *Tree Physiology* Online; for post-hoc contrasts, see Table S10 available as Supplementary Data at *Tree Physiology* Online. Y-axes of all plots are on a log scale (log-transformed response variables), with axis labels back-transformed to original values for clarity. Large white or solid-filled symbols represent predicted mean values with 95% confidence intervals, while light-colored symbols represent raw data (n = 138, 138 in p1 and p2 and 86 in p3, 137 and 136, respectively). Significance codes: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, ns, non-significant.

change in R:S for any specific species, resprouting plants as a group exhibited a decrease in R:S under eCO_2 (Figure 2). Root NSC of *A. cytisoides* and *D. pentaphyllum* (resprouting species) were not significantly different between CO_2 treatments (Table S3 available as Supplementary Data at *Tree Physiology* Online). This indicates that resprouting species allocated a greater proportion of extra assimilated carbon to aboveground growth rather than belowground growth or storage. Plant leaf loss occurred primarily during drought phase 3 and was not significantly affected by CO_2 treatment for any species (Figure S8 available as Supplementary Data at *Tree Physiology* Online). Thus, R:S values measured at the end of the experiment reflect resource allocation rather than differences in leaf shedding patterns under eCO_2. Both the lack of a species-level effect and the decrease in R:S at the resprouting level contrast with Han et al. (2023), which found a general increase in R:S at a global scale. However, our results align with Clarke et al. (2016) that also found allocation of additional carbohydrates from eCO_2 primarily to aboveground growth in Mediterranean resprouting species of Australia. The ability to access stable water sources due to more extensive root systems is a key drought avoidance strategy for resprouting plants (Moreno-Gutiérrez et al. 2012) and the implications of a less favorable root-to-shoot ratio in eCO_2 may be detrimental for seedlings response to drought. Note that this effect of eCO_2 is possibly only relevant for the seedling recruitment stage (such as in our study), not for mature plants that resprout after disturbance as they maintain their pre-disturbance root system. A similar result of decreased root mass ratio (allocation of dry matter to roots in relation to total biomass) after 6 months of exposure to eCO_2 has been found for seedlings of obligate resprouter *Quercus suber* (Vaz et al. 2012), suggesting that it may be a general pattern in resprouters.

The trade-off between carbon uptake and water conservation is key in water-limited ecosystems (Chaves et al. 2016, McDowell et al. 2022). Improved water-use efficiency, via reduced stomatal conductance under eCO_2, could potentially ameliorate plant stress during drought (De Kauwe et al. 2021, Wang et al. 2022), yet our study finds extremely limited effects of eCO_2 on water status, water fluxes, hydraulic behavior or stomatal density of postfire seeder shrubs. Our experimental design specifically controlled soil water content during each drought phase, so we were able to compare the response of the five species under the same effective water availability, independently of the plant size. Additionally, despite increases in leaf biomass for some species, estimated total plant leaf area did not differ between CO_2 treatments for any species in any drought phase, likely due to the corresponding decreases in SLA, and explaining the absence of eCO_2 effects on whole-plant transpiration. The only species-level effects of eCO_2 observed in our study were a reduction in leaf water potential in *C. albidus* and a decrease in osmotic potential in *D. pentaphyllum*, both indicative of increased drought stress rather than alleviation. Indeed, evidence of a positive effect of eCO_2 buffering plant drought stress is highly variable in the literature. Previous studies have found increased drought resistance in seedlings of *Eucalyptus* and *Quercus* species under eCO_2 (Aranda et al. 2020, Jiang et al. 2021), no effect of eCO_2 on plant water-use in *Quercus* species (Vaz et al. 2012), or even increased vulnerability to drought stress due to shallow rooting habits in *Eucalyptus* (Duursma et al. 2011). Within Mediterranean shrublands specifically, drought response under eCO_2 has been found to differ between species and between seasons (Tognetti et al. 2000*a*). Our studied life history strategy, postfire seeders, has evolved in water-limited landscapes (Filella and Peñuelas 2003, Moreno-Gutiérrez et al. 2012), and has acquired an array of adaptive drought responses beyond stomatal control. The lack of a drought alleviation effect of eCO_2 in our study may reflect these broader adaptive traits, including root properties and hydraulic architecture, both of which strongly influence water potential dynamics (Martínez-Vilalta and Garcia-Forner 2017).

Species with differing resprouting abilities exhibited distinct drought responses. Specifically, we found a significant interaction between drought intensity and resprouting ability on plant water use. A clear threshold emerged between moderate and intense drought (30% vs 10% soil water content), marked by a sharp decline in all water-use variables under intense drought (p3). Non-resprouting species exhibited higher water and osmotic potential than resprouting species in p1 and p2, but these differences disappeared, with a tendency to reverse, by p3 (Figure 3), indicating that non-resprouting seeders experienced the steepest declines in water status under intense drought. Midday water and osmotic potential represent the lowest potentials a plant experiences during peak transpiration (Pérez-Harguindeguy et al. 2013). Species regulate this potential through distinct water-use strategies, ranging from restricting water loss to sustaining transpiration despite significant declines in water potential (Moreno-Gutiérrez et al. 2012). While within-species differences in midday water potential in previous analyses indicated variations in drought stress, differences across species here reflect contrasting water-use strategies. Postfire seeders generally exhibit opportunistic water-use patterns, maintaining high stomatal conductance during peak growing seasons and continuing gas exchange under drought, even at the cost of greater dehydration (Vilagrosa et al. 2014). Seeders typically have higher specific root length (Paula and Pausas 2011), greater xylem cavitation resistance (P_{50}, MPa; Table 1; Jacobsen et al. 2007, Pratt et al. 2010) and lower water-storage capacity than resprouting species (Pausas et al. 2016). As a result, they can tolerate greater declines in water potential but may also be more vulnerable to hydraulic failure under intense or prolonged drought (Pausas et al. 2016, Venturas et al. 2016). In contrast, resprouting species maintain more stable water potentials due to their conservative water-use strategies and larger root systems (Moreno-Gutiérrez et al. 2012, Vilagrosa et al. 2014). Although our study focused on young plants with relatively undeveloped root systems, resprouting seeders exhibited more stable water potentials under drought than non-resprouting seeders (Figure 3). A similar, but less strong pattern was found for leaf transpiration, whole-plant and leaf hydraulic conductance and conductivity. These results demonstrate that resprouting and non-resprouting seeders diverge in their drought responses, even under eCO_2 conditions. This life-history contrast is similar to findings from chaparral ecosystems, where non-resprouting seeders were more resistant to water stress than resprouting seeders (Pratt et al. 2008). More broadly, similar divergence in acclimation strategies among functional types has been reported in savanna tree species under elevated CO_2 across contrasting water conditions (Singini et al. 2025). Note that our dataset included a limited number of species and families in each resprouting strategy, thus, we strongly recommend further

research of drought response under eCO_2 conditions in additional postfire seeder shrub species, to better elucidate the general relationship between stress tolerance and resprouting ability.

Conclusions

Fire-prone shrublands cover large areas of Mediterranean-type ecosystems and are predicted to expand with the increase of drought occurrence (Batllori et al. 2017, Schwörer et al. 2024). The current study demonstrates that eCO_2 will not ameliorate drought stress of postfire seeder seedlings in Mediterranean shrublands. Furthermore, resprout ability was identified as a likely predictor of plant response during drought even in eCO_2 conditions, with resprouting seeders exhibiting more stable water potentials under drought, consistent with a more conservative water-use strategy. Our findings emphasize the importance of including varying responses to eCO_2 across life history strategies and key traits within them (such as resprouting) in simulation modeling of plant water-use under global change (Swann et al. 2016, Vicente-Serrano et al. 2022), as well as for future predictions of drought-related mortality, flammability, fuel availability and fire regimes (Nolan et al. 2018).

Acknowledgments

We thank Vicent Cerdan, Esperanza Campos Fuster and everyone at the Centro para la Investigación y Experimentación Forestal (CIEF) for facilities and invaluable advice of germination techniques and plant care, Juanjo Albert (CIDE) for the construction and maintenance of greenhouse chambers and water systems, and CIDE technician Guillermo Benítez López for his work throughout the experiment. We also thank the many CIDE students (Raul, Francisco, Alba, Clara) who helped with experimental data collection, Diego Intrigliolo for advice on methodology, Angeles Catalayud (IVIA) for use of osmometer and sharing technical advice, and Antonio Lidon (UPV) for his contribution of water retention curves. We also thank Ricardo Sorio and Enrique Paula (CIAS Valencia diving center) and the Oceanogràfic València for providing compressed air cylinders for the Scholander pressure chamber during the COVID-19 pandemic shutdown (2020). We extend special thanks to Guillermo Benítez López, Beatriz López Gurillo and Joan Nicolau Jiménez Osuna for intensive data collection during the pandemic shutdown period, which required substantial additional effort. This work was supported by the Generalitat Valenciana through the program Santiago Grisolia (GRISOLIAP/2017/176) and projects FOCSEC (PROMETEO/2016/021) and FOCSCALES (PROMETEO/2021/040). CIDE is a joint institute of the Spanish National Research Council (CSIC), the University of Valencia and the regional government of Valencia (Generalitat Valenciana). J.M.-V. benefited from an ICREA Academia award. Jordi Martínez-Vilalta holds the position of Editor for Tree Physiology and has not peer reviewed or made any editorial decisions for this paper.

Supplementary Data

Supplementary data for this article are available at *Tree Physiology* Online.

Author contributions

Maya A. Zomer: Conceptualization; investigation; methodology; formal analysis; writing—original draft; writing—review & editing. Bruno Moreira: Conceptualization; methodology; formal analysis; writing—review & editing. Jordi Martínez-Vilalta: Formal analysis; writing—review & editing. Juli G. Pausas: Funding acquisition; resources; conceptualization; methodology; formal analysis; writing—review & editing.

Conflict of interest

None declared.

Funding

None declared.

Data availability

The data that support the findings of this study are openly available in the Figshare digital repository: https://doi.org/10.6084/m9.figshare.26166589.

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