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RESEARCH

CO_2 enrichment does not alter the relative physiological performance of seedlings of two close pine species under water stress

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Abstract

Rising atmospheric CO_2 ($[CO_2]$) may partly alleviate the drought stress that threatens forests worldwide. Plasticity of functional traits in response to drought and $[CO_2]$ may influence future plant performance. We analyzed the performance of seedlings of two Mediterranean pine species with close ecological niches, but different drought tolerance (*Pinus pinaster* is less drought-tolerant than *Pinus pinea*), under two $[CO_2]$ (380 ppm vs 800 ppm) and two watering regimes (well-watered vs water stressed; 75% vs 40% field capacity) for 161 days. We quantified biomass allocation patterns, net photosynthetic rates (A_n), stomatal conductance to water vapor (g_{wv}), and water-use efficiency at plant (WUE) and leaf levels (intrinsic, WUEi). In both species, elevated $[CO_2]$ increased total biomass, A_n, WUE and WUEi, but reduced g_{wv}. Most functional traits were similar between species with ample water but differed under water stress. Under water stress, *P. pinaster* had lower shoot:root ratios, A_n, g_{wv} and WUEi than *P. pinea*, but higher biomass when well-watered. Species' WUE and WUEi responses to water stress differed. The effects of water stress on these traits were more negative under low $[CO_2]$. Under enriched $[CO_2]$, functional leaf adjustments benefited the carbon budget of both species by stimulating higher A_n despite lower g_{wv}, particularly in *P. pinea*. Overall, *P. pinea* performed better under water stress than *P. pinaster*. This relative performance between species was not modified by elevated $[CO_2]$. Thus, under future more arid climate and higher $[CO_2]$, *P. pinea* seedlings would be expected to maintain their relative competitive advantage over *P. pinaster*.

Keywords Drought tolerance · Plasticity · Morphological traits · Water use efficiency · Global change

Introduction

Atmospheric CO_2 concentration ($[CO_2]$) has risen from pre-industrial 280 ppm to current concentration of over 400 ppm, and it could achieve up to almost 1000 ppm by the year 2100 (Pörtner et al. 2022). Rising global mean temperature will increase evapotranspiration rates, under climates with more frequent and severe droughts in many areas of the world, including the Mediterranean Region (Lionello and Scarascia 2018; Pörtner et al. 2022). The combination of increased $[CO_2,]$, increased temperature, and drought, among other conditions, will potentially alter forest distribution, composition and dynamics. One of the most concerning impacts of increased drought is the aggravated forest vulnerability under increasing hot-droughts which results in increased mortality rates of adult trees (Allen et al. 2010; Anderegg et al. 2015; Choat et al. 2018; Brodribb et al. 2020) and negatively impacts juvenile phases of forest regeneration and recruitment success (Vilà-Cabrera et al. 2013; Martínez-Vilalta and Lloret 2016; Redmond et al. 2018). Increased adult mortality and regeneration failure may ultimately compromise species persistence or even induce biome shifts. Complementarily to the effects of climate change and drought on adult tree mortality, understanding the complex interactions between $[CO_2]$ and water stress on seedling performance is crucial to assess future forest dynamics under climate change (Mohan et al. 2007; Maschler et al. 2022).

Plants adjust different functional and morphological traits to optimize their performance under water stress towards maximizing carbon uptake rates while minimizing water

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losses and maintaining functional integrity (Niinemets 2010; Sala et al. 2010; Forner et al. 2018b). Key plant functional traits concerning drought tolerance are associated with their effective water management, including water use efficiency, root development and architecture, carbon allocation patterns (Sánchez-Gómez et al. 2010), and stomatal regulation to reduce water losses preventing tissues dehydration and maintaining hydraulic safety (Martínez-Vilalta et al. 2004; McDowell et al. 2008). The responses of hydraulic and carbon related traits are intimately interlinked and influenced by atmospheric [CO_2] jointly shaping plants' ability to withstand drought. The direct and indirect influence of atmospheric [CO_2] on plants' drought-tolerance may be modified under a CO_2 enriched atmosphere (Wullschleger et al. 2002; Wertin et al. 2010; Duursma et al. 2011; Becklin et al. 2017; Ainsworth and Long 2021).

There is yet no consensus on the global effect of the interaction between [CO_2] and drought on future forest performance (Medlyn et al. 2001; Gimeno et al. 2016; Bachofen et al. 2018; Wang et al. 2020). Under elevated [CO_2], plants commonly increase photosynthetic rates by increasing carboxylation (Nowak et al. 2004; Ainsworth and Rogers 2007) typically accompanied by a reduction in stomatal conductance to water vapour (g_{wv}) mediated by complex hydraulic acclimation and changes in soil water availability (Ainsworth and Rogers 2007; Assmann and Jegla 2016). Reduced stomatal conductance generally leads to a decrease in transpiration and plant water consumption, unless an increase in total leaf area occurs (Tissue et al. 1997; Wullschleger et al. 2002; Cernusak et al. 2019). Higher net photosynthetic rate (A_n), lower g_{wv} or both commonly result in increased intrinsic water use efficiency (WUEi) (Beer et al. 2009) i.e. water use efficiency at the leaf level (Wertin et al. 2010; Aranda et al. 2020; Birami et al. 2020). However, response of water use efficiency at the plant or ecosystem levels are more uncertain and may not agree with leaf-level WUEi (Xu et al. 2016; Sánchez-Gómez et al. 2017) mainly because of differences in leaf biomass allocation (Beer et al. 2009; Perry et al. 2013; Lavergne et al. 2019). Higher leaf-level WUEi generally does not result in increased growth or biomass (Cernusak et al. 2019; Lavergne et al. 2019; Peñuelas et al. 2011). In response to elevated [CO_2] and drought, plants generally increase biomass allocation to roots which improves their access to belowground resources (Curtis and Wang 1998; Norby et al. 2004; Iversen 2010; Jiang et al. 2020; Prescott et al. 2020) but this response is not ubiquitous (Tingey et al. 2000; Biel et al. 2004). The lack of a general pattern in WUEi and growth response to water stress may be due to different species-specific responses (Stulen and den Hertog 1993; Leakey et al. 2009; Dawes et al. 2011). Higher A_n under elevated [CO_2] may lead to a higher accumulation of non-structural carbohydrates (NSCs) in different plant organs if there is a carbon surplus (Poorter et al. 1997; Fernández de Simón et al. 2022). The potential net fertilization effect of enriched [CO_2] on plant productivity may be further conditioned by reduced nutrient availability, such as N or P (Norby and Zak 2011; Jiang et al. 2020; Wang et al. 2020). Thus, the net effects of the interaction between water stress and [CO_2] on plant growth performance and survival depend on complex linkages between different interconnected processes.

Climate change has increased mortality rates of different forest tree species worldwide, including the Mediterranean region (Senf et al. 2020; Gea-Izquierdo et al. 2021; Hartmann et al. 2022). Additionally, drought stress may differentially affect species regeneration dynamics and induced vegetation shifts (Martínez-Vilalta et al. 2016). In central Spain, *Pinus pinaster* Ait. shows decline (defoliation and infection by the mistletoe *Viscum album* L.) and diffuse drought-induced mortality in its current xeric distribution limit. In these mixed stands, adult trees of co-occurring and more drought-tolerant species like *Pinus pinea* L. do not show decline symptoms and have more abundant regeneration even in *P. pinaster*-dominated stands (Gea-Izquierdo et al. 2019; Férriz et al. 2021). The distribution of these two pine species in the Western Mediterranean has been highly influenced by human activities favoring *P. pinaster* stands for resin extraction and *P. pinea* for seed collection (Barberó et al. 1998; López-Sáez et al. 2014; Rodríguez-García et al. 2016). Although both species can co-occur in mixed stands, *P. pinea* displays a high phenotypic plasticity and a water-spending strategy (high A_n and low WUEi) under water shortage (Sánchez-Gómez et al. 2011) and is considered more thermophilous and drought-tolerant than *P. pinaster* (Picon et al. 1996; Martínez-Vilalta et al. 2004; Chambel et al. 2007; Perdiguero et al. 2015; Férriz et al. 2023). The regeneration patterns observed show low *P. pinaster* regeneration compared to that of *P. pinea* and other more drought-tolerant species like *Quercus ilex* L. and *Juniperus oxycedrus* L. (Gea-Izquierdo et al. 2019; Férriz et al. 2021). The relative performance of seedlings of these species may be further affected by future water stress and elevated [CO_2] which could ultimately drive species composition in these mixed forests.

Elevated [CO_2] can potentially alleviate the negative drought effects on pine seedlings and accelerate their recovery after drought, as observed in *P. pinaster* seedlings (Sánchez-Gómez et al. 2017; Fernández de Simón et al. 2020). Elevated [CO_2] can reduce *P. pinaster* inter-genotypic differences in drought tolerance in comparison with those observed under current [CO_2] (Sánchez-Gómez et al. 2017) suggesting that elevated [CO_2] can induce shifts in drought tolerance rankings across genotypes within the same tree species. In a previous study, we evaluated the functional plasticity in terms of leaf and xylem traits in seedlings of *P. pinaster* and *P. pinea* under elevated [CO_2] × water stress

(Férriz et al. 2023). In that study, we showed that, despite the positive effect of elevated [CO_2] on plants acclimation to water stress, the higher xylem phenotypic plasticity of *P. pinea* provided it with a higher drought tolerance over *P. pinaster*. In the present study, we complement our previous work studying the differences between both pine species by focusing on the response of morphological and physiological traits related to the carbon and water dynamics that ultimately drive plant performance. We tested four specific hypotheses: (i) *P. pinea* will show higher net photosynthetic rates and lower water use efficiency under water stress than *P. pinaster*; (ii) interspecific differences of studied traits in response to water stress will decrease under enriched [CO_2]; (iii) growing at enriched [CO_2] will partly buffer the negative effect of water stress in both species by increasing water use efficiency and modifying plant carbon allocation patterns to favor greater investment to belowground organs.

Materials and methods

Plantmaterial and experimental design: [CO_2] and waterstress levels

We tested the concomitant effects of [CO_2] and water stress in two pine species (*P. pinaster* and *P. pinea*) in a 2 × 2 factorial design: [CO_2] x watering regime. Seedlings were grown from seeds placed in perlite inside a climatic chamber at 25° C for germination. Approximately 25 days after germination, when radicles length reached 1.5 cm, we selected 136 individuals of each species to be planted in pots (two plants per pot) containing a mixture of blond peat and sand (2:1, v:v) and a slow-release fertilizer of NPK + micronutrients (Nutricote). The pot size for the experiment was 2.5 L. We randomly assigned 34 pots per species to one of two identical walk-in growth chambers (Fitoclima 10000EHHF, Aralab Ltd, Sintra, Portugal) where the experiment was conducted. Growing conditions were identical in both chambers, except for the [CO_2] levels. We set the photoperiod at 14/10 h light/dark. Daytime parameters were 24.8 ± 0.7 ºC, 64.7 ± 1.3% relative humidity and 762 ± 127 mol photons m^2 s^{-1}, and dark phase parameters were 20.0 ± 0.4ºC and 60.0 ± 1.1% relative humidity. Atmospheric CO_2 were maintained at 383 ± 32 ppm at the ambient [CO_2] chamber (hereafter a[CO_2]) and at 808 ± 15 ppm at the chamber with enriched [CO_2] (hereafter e[CO_2]). We set the elevated [CO_2] at 800 ppm because it is the atmospheric concentration predicted by year 2100 according to the model RCP 6.0 (Capellán-Pérez et al. 2016). After two weeks in the chamber, we removed one seedling from each pot to homogenize plant size. Within each chamber, pots were evenly placed and their position shifted randomly once a week. To minimize any potential chamber effect, we rotated plants and conditions between chambers every 60 days.

We set up two watering treatments in the experiment (Fig. 1, Table S1). During the initial phase of the experiment (97 days), all pots were maintained at 75% field capacity (FC), equivalent to 20% of volumetric soil water content

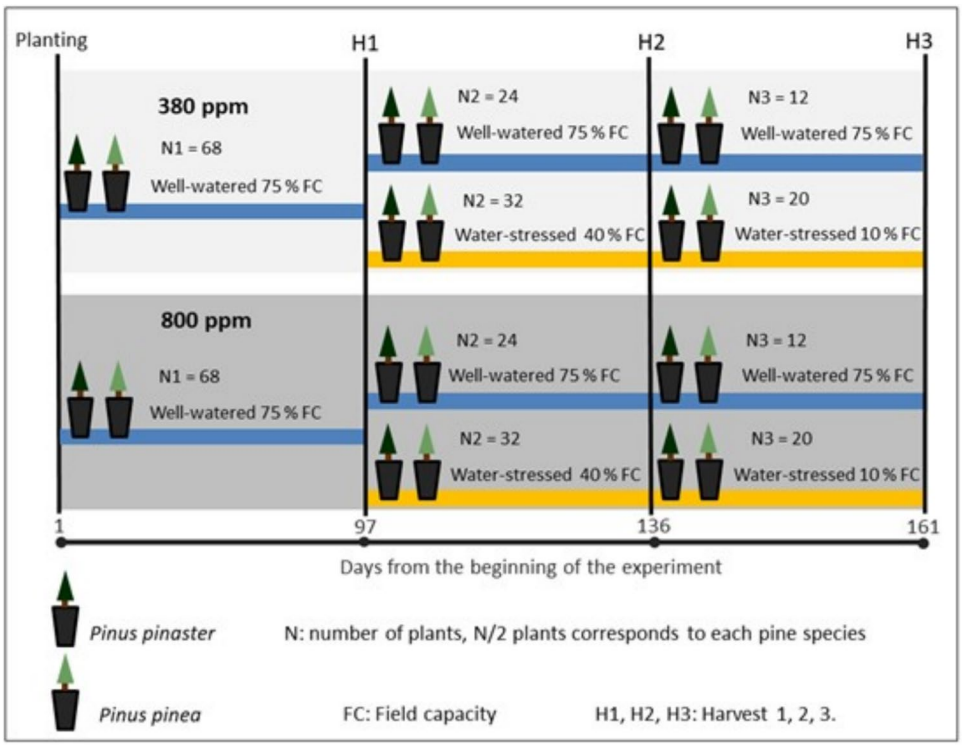


Fig. 1 Description and outline of the experimental design. Sampling accounts for plant harvest at three different dates. H1: Harvest at day 97 from the beginning of the experiment, 6 plants per species and CO_2 treatment were collected. H2: Harvest at day 136 from the beginning of the experiment, 6 plants per species, CO_2 treatment and water regime were collected. H3: Harvest at day 161 from the beginning of the experiment, 10 water-stressed and 6 well-watered plants species and CO_2 treatment were collected

(VWC) measured with a soil moisture probe (ProCheck, Decagon Devices). Three times a week, pots were individually weighed and watered up to a target weight defined for the specific VWC. Evapotranspiration was estimated for each plant as the difference of pot weight immediately after and before watering. Well-watered (WW) plants were grown at 75% FC for the total length of the experiment. After 97 days, we conducted the first harvest (Harvest 1, H1; Fig. 1). Additionally, that day 12 plants per species and [CO_2] treatment were assigned to the WW treatment and 16 plants to the water stress (WS) treatment. WS treatment was imposed in two phases differing in the minimum % FC reached in the pot substrate. After H1, the watering in WS plants was reduced gradually during a week to reach 40% FC (10% VWC). WS plants were grown at 40% FC during 39 days until Harvest 2 (H2). After H2, we further reduced the watering in the WS plants to 10% FC (5% VWC). These water conditions were maintained for 25 days until the end of the experiment in day 161 (Harvest 3, H3). The number of harvested plants changed during the experiment: at H1, 6 plants per species and [CO_2] (12 plants total); at H2, 6 plants per species and watering regime and [CO_2] (48 plants total), and at H3, 6 WW plants and 10 WS plants for each species and [CO_2] (64 plants total). From the beginning of the experiment, we had extra plants in each chamber. After H1 (day 97), these extra plants were assigned to WS treatment in order to compensate for possible plant mortality. In the end, no mortality happened during the experiment, thus these extra seedlings were excluded from the analysis.

Growth related functional traits

To estimate radial and height growth, every two weeks we measured collar diameter of all plants using a calliper (± 0.01 mm) and plant height monthly with a ruler (± 1 mm). We estimated diameter and height relative growth rates (RGR) as RGR = (log(G2)–log(G1))/(t2–t1), where log is the natural logarithm, and G1 and G2 are either plant diameter or height growth at days t1 and t2 (t1 < t2) (Hunt and Cornelissen 1997). To estimate biomass partitioning, at each harvesting date (97, 136 and 161 days into the experiment), we separated roots, stems and needles from each harvested plant individually. All parts were oven-dried (48 h, 60 °C) for dry biomass determination. The following biometric indexes were estimated for each plant: leaf mass fraction (LMF g g^{-1}), calculated as total leaf mass divided by the total plant mass; root mass fraction (RMF g g^{-1}) calculated as total root mass divided by total plant mass; stem mass fraction (SMF g g^{-1}) calculated as stem mass divided by total plant mass; shoot: root ratio (SRR g g^{-1}) calculated as aboveground mass (stem plus leaf) divided by the root mass; specific leaf area (SLA m2 kg^{-1}) as the ratio of leaf area to leaf mass; and leaf area ratio (LAR, m2 kg^{-1}) calculated as the total leaf area divided by the total plant mass.

Water potentials (ψ) were measured in one twig per harvested plant with a pressure chamber (PMS Instrument Co. 7000, Corvallis, OR, USA) twice during the experiment (at H2 and H3) in all harvested seedlings just before the beginning of the light period ($\psi_{predawn}$) and four hours after the beginning of the daytime conditions (ψ_{midday}). Gas exchange at midday was measured in H2 on one selected upper twig of each harvested plant using a Li-6400 with the 6400–07 conifer chamber (Li- Cor Inc., Lincoln, NE, USA). Net photosynthetic rate (A_n) and stomatal conductance to water vapour (g_{wv}) were measured five times on each plant and averaged to estimate plant-level values. In a[CO_2], the CO_2 concentration set as reference was 380 ppm, the CO_2 flow rate was 600 mol s^{-1} and the relative humidity was 61%. In e[CO_2], the CO_2 reference concentration was 800 ppm, the flow rate was 600 mol s^{-1} and the relative humidity was 60%. Gas exchange measurements were recalculated considering the total leaf area of each measured twig. Total leaf area was estimated as leaf dry mass multiplied by SLA (m^2 kg^{-1}). To estimate SLA, we measured the area of 10 fresh needles from each twig with a scanner and ImageJ program (Schneider et al. 2012). These 10 needles were later oven-dried and weighed. All other needles were oven-dried and weighed to estimate total twig leaf dry mass. Intrinsic water-use efficiency at leaf-level (WUEi) was estimated with two complementary methods and data: (i) directly from leaf-level gas exchange measurements as the ratio between photosynthetic rate (A_n) and stomatal conductance (g_{wv}) as WUEi = A_n/g_{wv} (µmol CO_2 mol^{-1} H_2O) which represents a measure of instantaneous WUEi; and (ii) indirectly by analysing stable carbon isotopic rates ($\Delta^{13}C$) in pine needles (see below) which integrates WUEi for the time of needle tissue development. These estimates of WUEi are henceforth referred to gas-exchange-derived WUEi and $\delta^{13}C$-derived WUEi, respectively. In addition, plant-level WUE (mg biomass g^{-1} H_2O) was calculated as the ratio between total plant dry biomass and total plant transpiration. Different indicators of water use efficiency are time-dependent (Beer et al. 2009). In the case of plant-level WUE in WS plants, this measurement integrates the water use during the first three months of the experiment, when plants were watered at 75% FC, and the two last months of water stress treatment. For determining $\delta^{13}C$-derived WUEi, however, needles were collected from the seedling apex. These terminal needles formed during the last weeks of the experiment and were representative of plant performance in this last phase.

Stable carbon isotopes in needles

We determined carbon stable isotopic ratios in the ten needles used to estimate SLA for each plant. These needles, sampled

from the terminal, main twig of the seedling, were formed during the last weeks of the experiment. Once dried (60 °C for 48 h), we grounded the needles in a mixer mill MM 300 (Retsch, Haan, Germany) and encapsulated 3.5 mg of sample in tin capsules. Samples were analyzed by continuous flow Isotope Ratio Mass Spectrometer (IRMS) (Stable Isotope Facility, UC Davis, LA, USA) to estimate $^{13}C/^{12}C$ content. Values of $\delta^{13}C_{needle}$ were estimated in relation to the standard Vienna Pee Dee Belemnite (VPDB):

$$\delta^{13}C_{needle} = \left[\frac{\left(^{13}C/^{12}C\right)needle}{\left(^{13}C/^{12}C\right)VPDB} - 1\right] \times 1000.$$

Injecting CO_2 may change the isotopic composition of source air ($\delta^{13}Ca$) in the enriched chamber (e[CO_2]) with respect to the ambient (a[CO_2]) chamber. To account for this potential artifact, we analyzed C isotopic ratios from well-water maize (*Zea mays*) plants ($\delta^{13}C_{maize}$) grown simultaneously with pine seedlings (9 plants per chamber) because $\delta^{13}C_{plant}$ from C4 plants is considered to correlate with $\delta^{13}C_{air}$ (Marino and McElroy 1991; but see del Toro et al. 2024). We thus estimated the isotopic composition of source air ($\delta^{13}C_a$) on each chamber $\delta^{13}C_a = 3.276 + \delta^{13}C_{maize}$ (Marino and McElroy 1991), where $\delta^{13}C_{maize}$ was −13.73‰ and −10.98‰ in the 380 ppm and 800 ppm chamber, respectively. Pine carbon isotopic discrimination was estimated following (Farquhar et al. 1982, 1989) as $\Delta^{13}C_{needle} = \frac{\delta^{13}C_a - \delta^{13}C_{needle}}{1-\delta^{13}C_{needle}/1000}$ and intrinsic water use efficiency as

$$WUE_i = \frac{c_a - (b - \Delta)}{1.6 - (b - a)}$$

where c_a is [CO_2] of the atmospheric air (here, 380 ppm or 800 ppm), *a* the fractionation induced by the diffusion of CO_2 through the stomata (~ 4.4‰), and b the discrimination against $^{13}CO_2$ by Rubisco during carboxylation (~ 27‰). Carbon isotopic ratios in needles are expected to be linearly related to gradient between atmospheric [CO_2] (c_a) and intercellular [CO_2] (c_i) to as.

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$$\delta^{13}C_{needle} = {}^{13}\delta C_a - a - (b - a)\frac{c_i}{c_a}$$

From the previous equation, we calculated the ratio (ci/ca) as

$$\frac{c_i}{c_a} = \frac{\delta^{13}C_{needle} - {}^{13}\delta C_a + a}{-(b - a)}$$

Statistical analyses

We used general linear models to test for differences in the effects of [CO_2] (a[CO_2] vs e[CO_2]), watering regime (well-watered, WW, vs water-stressed, WS), species (*P. pinea* vs *P. pinaster*) and their interactions on the different functional traits assessed on harvesting dates H2 and H3. We used Shapiro–Wilk's and Levene's tests to test for normality and homogeneity of variances, respectively. Variables were log-transformed to meet the assumptions of normality and homoscedasticity when needed. Pairwise contrasts between groups were evaluated using Tukey–Kramer post-hoc test (function *TukeyHSD* in the *stats* package in R) which accounts for slightly unbalanced sample sizes. All analyses were performed in the R statistical software (R Development Core Team 2010).

Results

The water stress treatment reduced $\psi_{predawn}$ and ψ_{midday} in plants of both species compared to well-watered plants (Fig. 2). Water potentials differed between species, lower in *P. pinea* than in *P. pinaster* in both $\psi_{predawn}$ (− 1.66 ± 0.31 MPa in *P. pinea* vs −1.31 ± 0.34 MPa in *P. pinaster*) and ψ_{midday} (− 1.69 ± 0.34 MPa vs − 1.20 ± 0.35 MPa) (Fig. 2, Table S2). At the end of the experiment (H3, day 161), water stressed plants reached lower $\psi_{predawn}$ values under e[CO_2] than under a[CO_2] (CO_2 × water, p = 0.008; Fig. 2; Table S2). However, ψ_{midday} values were similar between the two CO_2 treatments. The volume water content (VWC) immediately before H2 was 16.70 ± 5.76% in WW pots and 10.05 ± 3.08% in WS, and it decreased to 5.97 ± 1.06% in WS plants before H3.

Plant growth and biomass partitioning

In both species, plant biomass was significantly higher under e[CO_2] than a[CO_2], but consistently lower in WS plants than in WW plants (Fig. 3, Table S2 and Table S3). As the experiment progressed, the differences in biomass growth increased both between different [CO_2] and between watering treatments. At the end of the experiment, WW plants under e[CO_2] accumulated the highest biomass and WS plants at e[CO_2] yielded biomass similar to WW plants under a[CO_2] (Fig. 3). There were no overall differences in total biomass between species in the CO_2 and watering treatments. However, differences in biomass between watering treatments were larger in *P. pinaster* (77% more biomass in average in WW than WS plants) than in *P. pinea* (50% higher in average in WW than WS plants Fig. 3). Diameter and height growth were significantly enhanced by e[CO_2] (Table S2 and Table S3) only

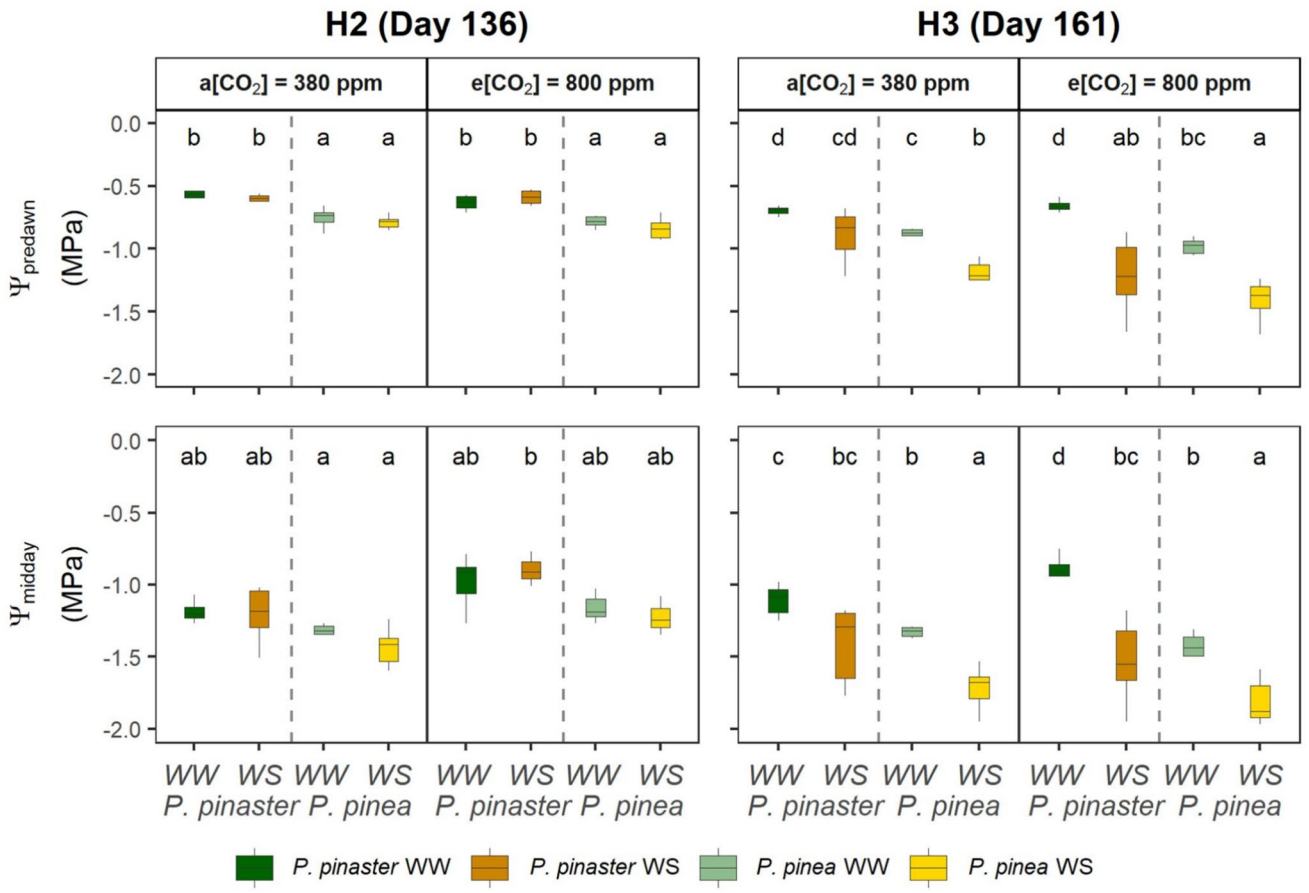


Fig. 2 Water potential at predawn ($\Psi_{predawn}$) and water potential at midday (Ψ_{midday}) from plants collected in Harvest 2 (H2, 136 days after start of the experiment) and in Harvest 3 (H3, 161 days after the start of the experiment). WW: Well-watered plants; WS: water-stressed plants. Different letters indicate significant differences between groups separated for each harvest H2 or H3 and the two different functional traits shown here. N = 6 plants in all treatments and species from H2, 6 plants from WW treatment in H3 and 10 plants from WS treatment and species in H3

under water stress (Fig. S2). Diameter and height RGR decreased in both species since day 140 with water stress (Fig. 4). Along the experiment, *P. pinea* plants were taller and exhibited greater diameters than *P. pinaster* (Table S2, Table S3 and Fig. S2).

Plants of both species growing under e[CO_2] had significantly lower leaf area ratio (LAR) and specific leaf area (SLA) than plants under a[CO_2]. In contrast, e[CO_2] increased the shoot:root ratio (SRR) in *P. pinea* (Fig. 5, Table S3). Lower SRR and higher RMF in WS plants of both species (Fig. 5, Table S3) evidenced greater investment in roots than in aboveground growth in response to water stress, particularly in *P. pinaster*. Overall, SRR, LMAF and SMF increased with plant biomass while RMF, LAR and SLA decreased (Fig. S3). During the experiment, only *P. pinaster* formed brachyblasts (Fig. S1), with a higher amount under e[CO_2] contributing to increase total leaf area in those plants (Fig. S5).

Water use efficiency at different levels of organization

Plants yielded differences in WUE at different organizational levels in response to [CO_2] and water stress levels. Lower soil water availability reduced g_{wv} ($p < 0.001$) and A_n ($p = 0.008$) in both species with a g_{wv} reduction of around 30% in WS compared to WW plants. Lower g_{wv} in WS plants resulted on an 41% increase in gas-exchange-derived WUEi in a[CO_2] and 20% in e[CO_2] compared to WW plants (Fig. 6) but a decrease in plant-level WUE of around 23% in a[CO_2] and 10% in e[CO_2] (Fig. 3) in WS plants. Averaged across water treatments and species, A_n, gas-exchange-derived WUEi and plant-level WUE were around 10%, 30% and 35% higher, while g_{wv} was 20% lower in e[CO_2] than under a[CO_2]. Plant-level WUE was significantly higher under e[CO_2] than a[CO_2] (3.11 ± 0.61 vs 1.98 ± 0.56 mg biomass g^{-1} H_2O) (Fig. 3, Table S2 and

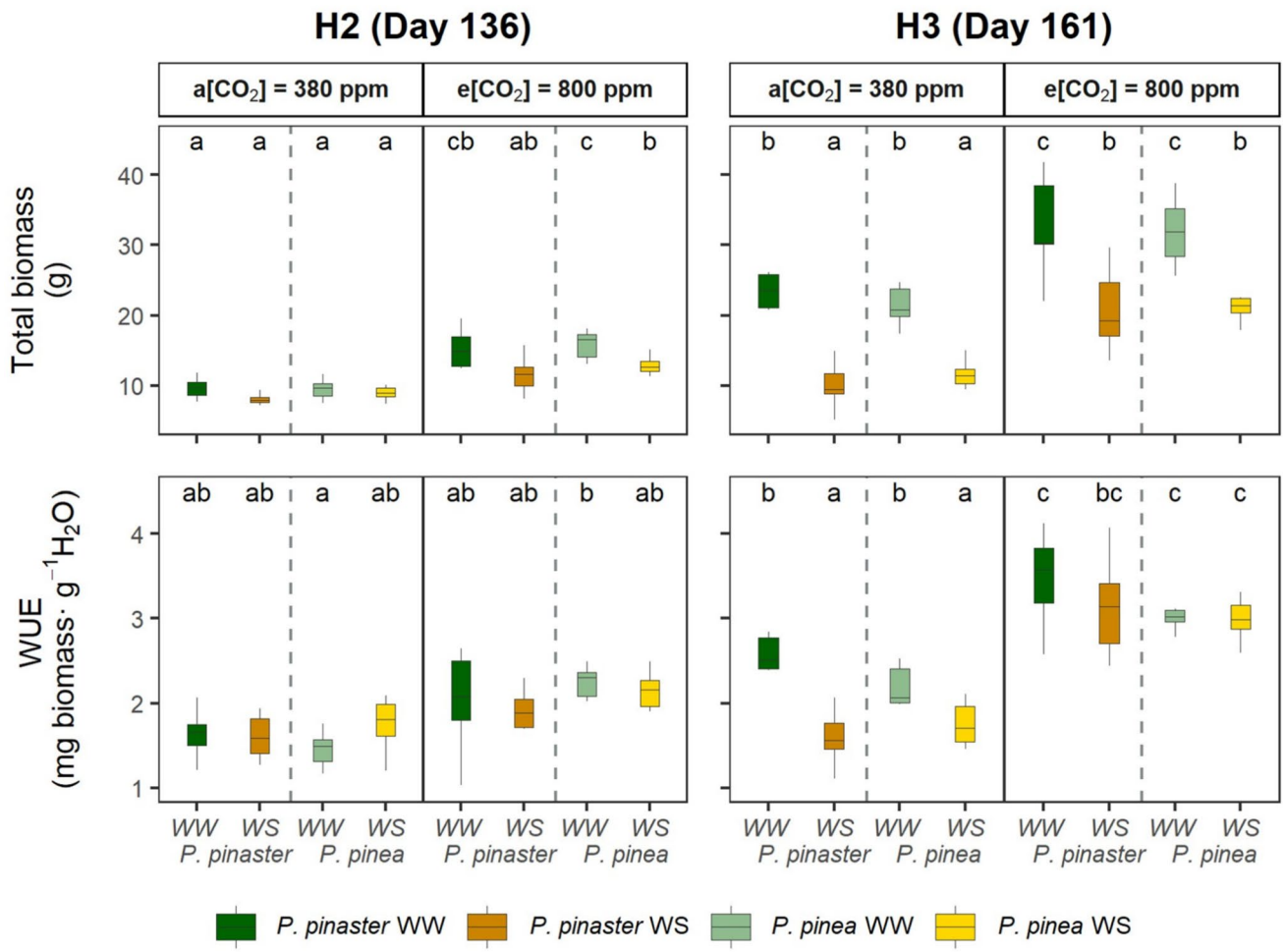


Fig. 3 Total biomass and plant water use efficiency (WUE) from plants collected in Harvest 2 (136 days after start of the experiment) and in Harvest 3 (161 days after the experiment start). WW: Well-watered plants; WS: water-stressed plants. Different letters indicate significant differences between groups separated for each harvest H2 and H3 and the two different functional traits shown here. N = 6 plants in all treatments and species from H2, 6 plants from WW treatment in H3 and 10 plants from WS treatment and species in H3

Table S3). Additionally, higher A_n and lower g_{wv} in both species at e[CO_2] resulted also in higher gas-exchange-derived WUEi than plants growing at a[CO_2] ($p < 0.001$ in all cases; Fig. 6, Table S2).

We also observed different responses in WUE at different organizational levels under different water treatments and [CO_2] between species (Fig. 6). Differences in gas-exchange-derived WUEi between species were lower under e[CO_2], due to larger differences in g_{wv}. Although the two species exhibited similar A_n for both water treatments under a[CO_2], A_n was higher in *P. pinea* than *P. pinaster* both under water stress and e[CO_2] (Fig. 6). Additionally, g_{wv} was lower and A_n higher under e[CO_2] than a[CO_2] in *P. pinea* than *P. pinaster* (Fig. 6). Following these leaf-level adjustments in A_n and g_{wv} at e[CO_2], gas-exchange-derived WUEi increased under water stress more in *P. pinaster* (38%) than in *P. pinea* (20%) compared to well-watered plants. Similarly, the integrative measure $\delta^{13}C$-derived WUEi was higher in *P. pinaster* than *P. pinea* and for WS plants than for WW plants of both species (Fig. 7; Table S2), in agreement with gas-exchange-derived WUEi (Fig. S7B). As expected, leaf $\delta^{13}C$-derived WUEi was linearly correlated with instantaneous (c_i/c_a), with lower (c_i/c_a) and higher WUEi in WS plants of both species (Fig. S7B). However, $\delta^{13}C$-derived WUEi increased more between WW and WS under e[CO_2] than gas-exchange-derived WUEi and there was no significant relationship between the two measures of WUEi (Fig. S7B). Importantly, plant-level WUE was similar between species, meaning that species-specific differences were not the same for leaf-level WUEi and plant-level WUE. WUE increased under e[CO_2], but, in contrast with WUEi, decreased in response to water stress under a[CO_2] in both species.

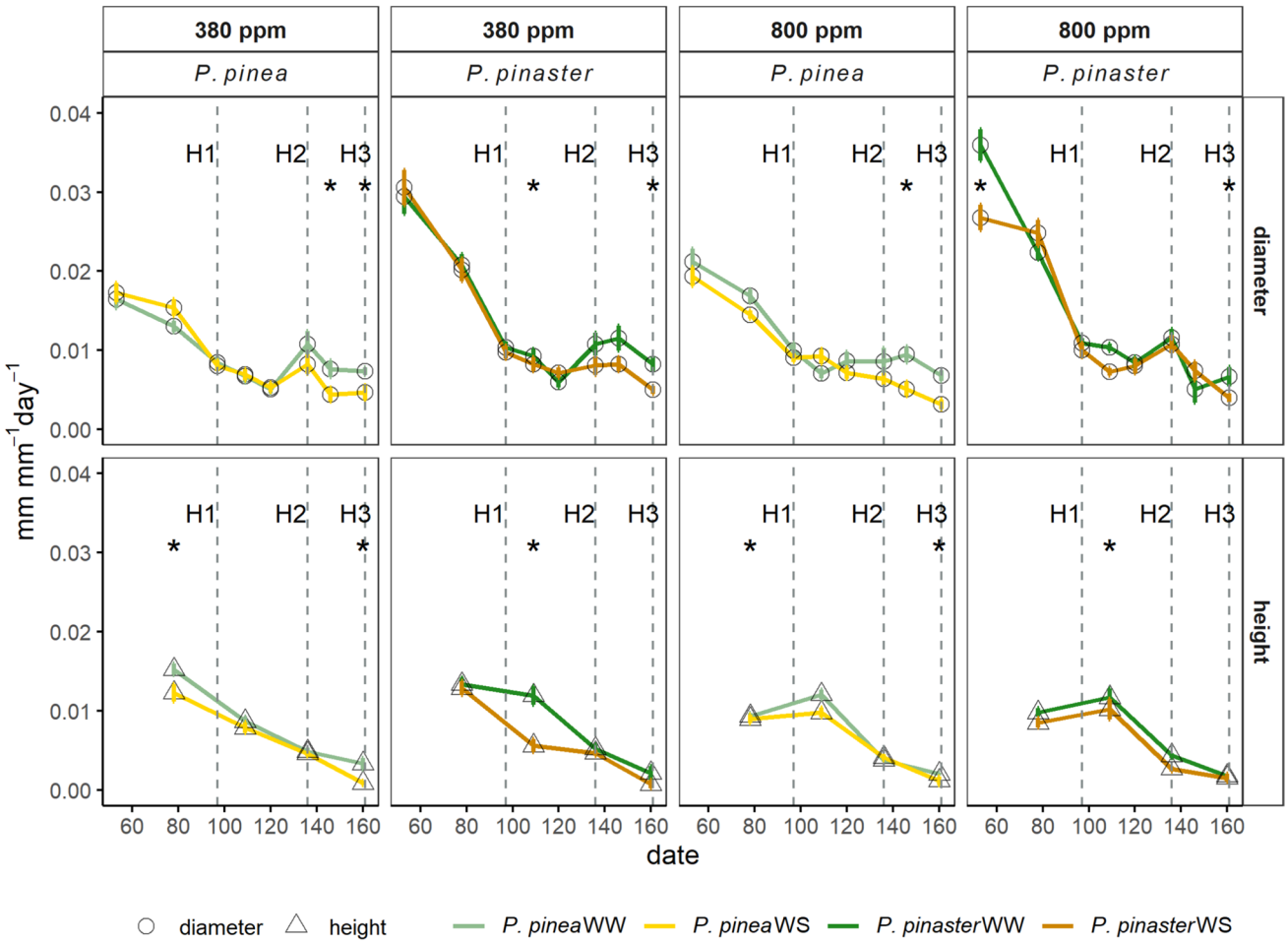


Fig. 4 Relative growth rates (RGR; $mm \cdot mm^{-1} \cdot day^{-1}$) in diameter (measured biweekly) and height (measured monthly) with standard errors along the experiment. Vertical dashed lines indicate harvest dates 1, 2 and 3 (96, 136 and 161 days after the start of the experiment, respectively). Asterisks (*) indicate significant differences between water treatments at specific dates. WW: Well-watered plants; WS: water-stressed plants. N = 6 in all treatments and species from Harvest 2 and WW plants in Harvest 3; N = 10 in WS treatment and species in Harvest 3

Discussion

In this study, we tested the combined effect of elevated [CO_2] and water stress on seedlings of two Mediterranean pines. As expected, low water availability reduced plant total biomass and increased carbon allocation to roots. Growing at e[CO_2] improved performance of water-stressed (WS) plants reducing the negative effect of water stress by increasing photosynthetic rates, WUEi, plant WUE and ultimately stimulating biomass growth. Seedling performance improved at e[CO_2] in both species, with a larger effect in the more drought-tolerant species *P. pinea*. These results suggest that, even if elevated [CO_2] can potentially mitigate the effects of water stress in seedlings of both species analyzed, higher photosynthetic rates observed in water-stressed *P. pinea* under [CO_2] may make *P. pinea* more drought tolerant than *P. pinaster* under future, xeric, [CO_2] enriched scenarios.

Water stress and CO_2 effects on carbon assimilation and biomass allocation

Elevated [CO_2] significantly enhanced total biomass production on plants of both species (Fig. 3). This is in line with a general CO_2 fertilization effect on biomass growth observed in studies including seedlings of different Mediterranean pines (Biel et al. 2004; Birami et al. 2020, but see Sánchez-Gómez et al. 2017; Bachofen et al. 2018). Increased biomass growth under e[CO_2] in both species could directly result from the enhanced net photosynthetic rates (A_n) observed (Tissue et al. 1997; Curtis and Wang 1998; Tingey et al. 2000; Biel et al. 2004; Perry et al. 2013). Higher A_n observed in *P. pinea* than in *P. pinaster* under e[CO_2] × water stress suggests that increased [CO_2] may partly buffer the negative impact of drought in *P. pinea* relatively more than in *P. pinaster*, at least under the moderate levels of water

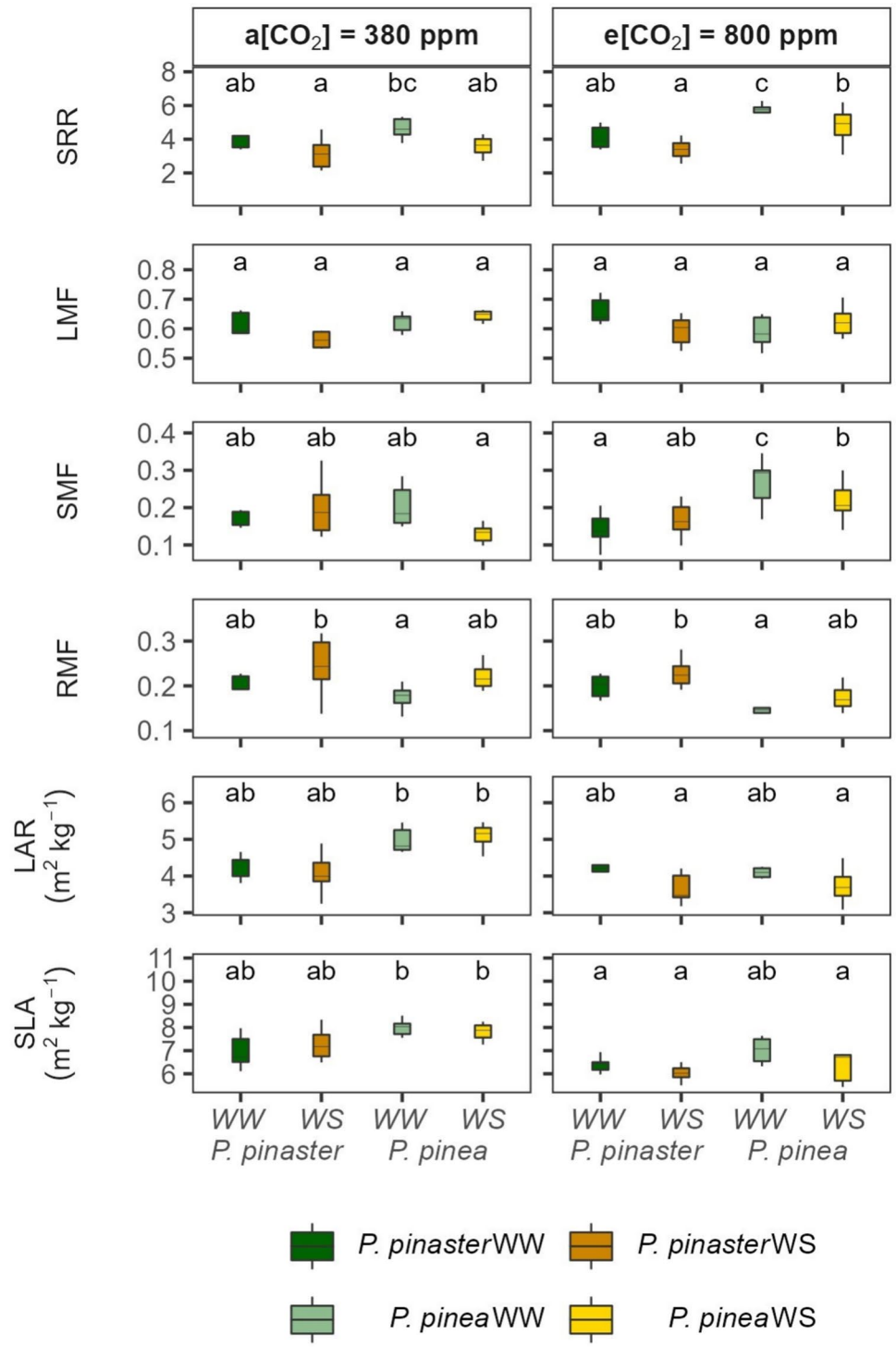


Fig. 5 Biomass allocation from data collected at H3 (161 days after the start of the experiment). SRR, Shoot: root ratio = (leaf and stem mass)/root mass; LMF (Leaf Mass Fraction) = leaf mass/total biomass; SMF (Stem Mass Fraction) = stem mass/total biomass; RMF (Root Mass Fraction) = root mass/total biomass; LAR (Leaf Area Ratio) = total leaf area/total biomass, and SLA (Specific Leaf Area) = total leaf area/leaf biomass (including juvenile needles and brachyblasts). Different letters indicate significant differences among groups with the two species and all treatments analysed together for each functional trait. For clarity outliers were not drawn in the plots but were maintained for all analyses. WW: Well-watered plants; WS: water-stressed plants. N = 6 WW plants and 10 WS plants per species and CO_2 treatment

stress reached in our experiment (Gea-Izquierdo et al. 2023). However, final total biomass was similar for both species probably due to differences in leaf development. The higher total leaf area in *P. pinaster*, including young needles and brachyblasts (Fig. S4), may have compensated for more constrained gas exchange rates under water stress in this species. Ultimately, CO_2 fertilization could partly ameliorate the negative effects of drought stress on growth of seedlings of both pines species studied (Picon et al. 1996; Biel et al. 2004; Wertin et al. 2010; Jiang et al. 2020).

Biomass allocation in plants commonly follows a functional equilibrium to reinforce the acquisition of the most limiting resource for growth (Poorter et al. 2012). In our study, seedlings responded to water stress by increasing

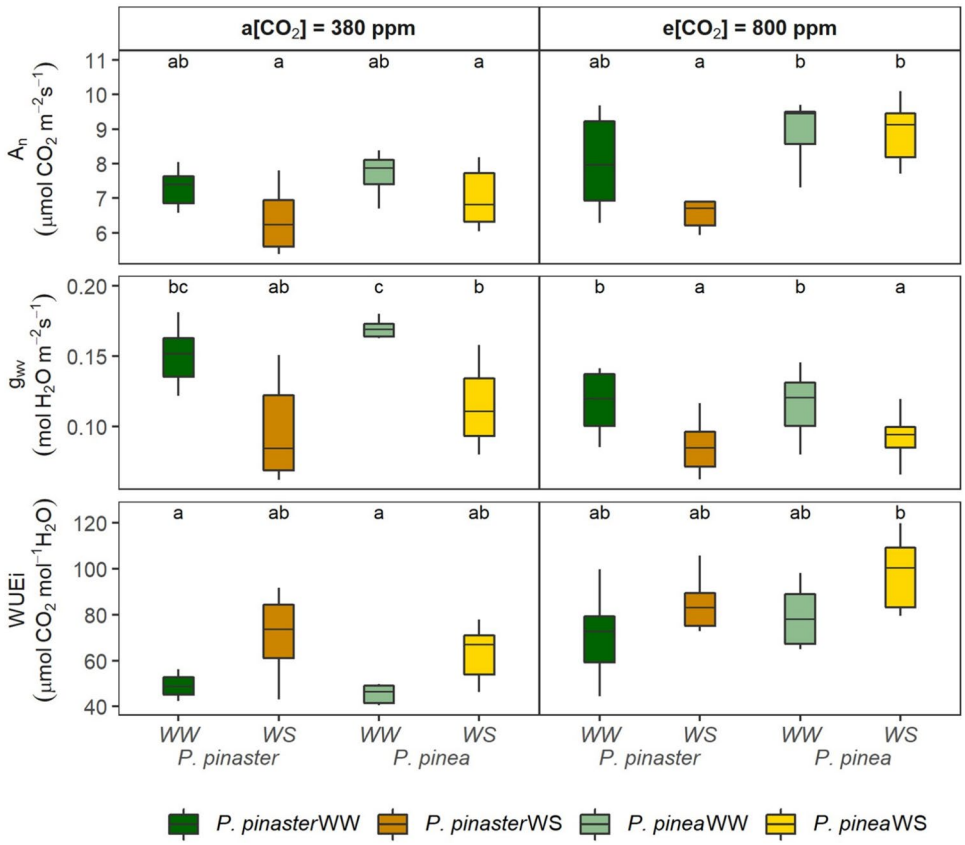


Fig. 6 Gas exchange measurements in H2 (136 days of experiment). A_n = photosynthetic rate (µmol CO_2 m^{-2} s^{-1}); g_{wv} = Stomatal conductance (mol H_2O m^{-2} s^{-1}); WUEi = Intrinsic water use efficiency (gas-exchange-derived WUEi) calculated as A_n/g_{wv} (µmol CO_2 mol^{-1} H_2O). WW: Well-watered plants; WS: water-stressed plants. Different letters indicate significant differences among groups with the two species and all treatments analysed together for the different functional traits shown. For clarity, outliers were removed from the plot, but maintained in the analysis. N = 6 plants and species in all treatments

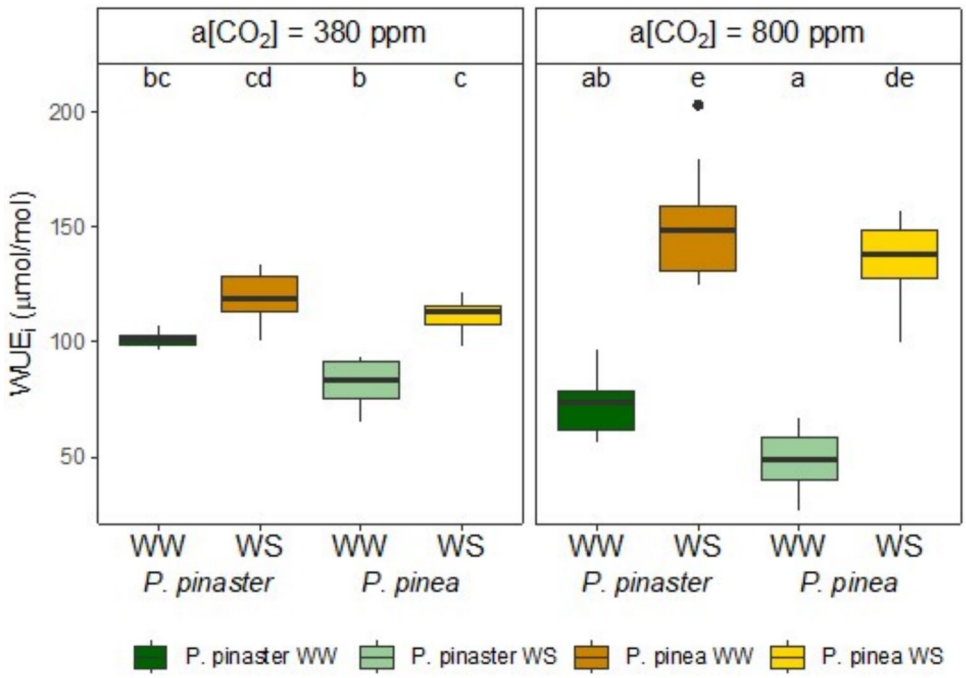


Fig. 7 Intrinsic water-use efficiency (WUEi) derived from carbon isotopic discrimination ($\delta^{13}C$-derived WUEi) in needles of the two species at the different water stress and CO_2 treatments applied at H3. Black dots shown represent outliers. Different letters indicate significant differences among groups with the two species and all treatments analysed together for the different functional traits shown. N = 6 WW plants and 10 WS plants per species and CO_2 treatment

the proportion of carbon allocated to belowground organs with respect to aboveground biomass (lower SRR) in both species, and particularly in the comparatively less drought-tolerant *P. pinaster* (lower SRR) (Fig. 5). In response to drought, plants commonly favor root allocation as a primary strategy for reducing the adverse impacts of water scarcity by enhancing their access to soil moisture (Padilla and Pugnaire 2007; Ramírez-Valiente et al. 2018). Contrary to our

hypotheses, high CO_2 levels did not modify the general allocation patterns in response to drought either by significantly promoting root development, decreasing the SRR or increasing the root mass fraction (RMF) (Table S3).

Biomass allocation patterns in both species were strongly size related (Tissue et al. 1997) as higher leaf mass sustained faster plant growth at e[CO_2] (Fig. S3). Comparing CO_2 treatments, plants growing in e[CO_2] allocated more carbon towards aboveground woody tissues and the formation of thicker and denser needles (lower LAR and SLA) (Tissue et al. 1997; Cha et al. 2017) with greater differences in *P. pinea* than in *P. pinaster*. Lower SLA would imply a higher protection against desiccation (Valladares and Sánchez-Gómez 2006; Greenwood et al. 2017). In addition, the higher proportion of brachyblasts in *P. pinaster* observed at e[CO_2] (Fig. S1) was independent of plant size, in line with previous reports of accelerated plant phenological and ontogenic changes from juvenile to mature needles under enriched [CO_2] (Navas et al. 1997; LaDeau and Clark 2006). Different metabolic responses to drought between needle types under high [CO_2] (Fernández de Simón et al. 2020) and higher resistance to desiccation of brachyblasts than young needles (Kuusk et al. 2018) suggest an advantage for plants of accelerating ontogenic changes in response to [CO_2] enrichment to better cope with drought stress.

Seedlings under stress had tighter stomatal control than WW plants, as expected (Klein 2014; Martínez-Vilalta and Garcia-Forner 2017; Yi et al. 2018; Gea-Izquierdo et al. 2021). Plants did not increase water potentials under [CO_2], with the only exception of WW *P. pinaster*. Despite g_{wv} reductions in *P. pinaster* under elevated [CO_2], an increase in transpiring total plant leaf biomass likely prevented a further amelioration of ψ_{midday}. The lowest ψ_{midday} observed were above those reported to produce by 50% xylem hydraulic conductivity loss in *P. pinea* and *P. pinaster* (Martínez-Vilalta and Piñol 2002; Oliveras et al. 2003) suggesting that there was no major loss of conductivity under the water stress levels imposed in our experiment as shown in Férriz et al. (2023). Important species-specific water relation strategies to cope with water stress were evidenced by differences in water potentials and stomatal control. Lower $\psi_{predawn}$ and ψ_{midday} in *P. pinea* with similar levels of stomatal control (Fig. 6, Fig. S6) suggest a less isohydric behavior than *P. pinaster* also evidenced by lower $\delta^{13}C$-derived WUEi in *P. pinea* compared to *P. pinaster* as shown here and other studies (Choat et al. 2012; Roman et al. 2015; Férriz et al. 2023). The comparatively more isohydric *P. pinaster* exhibited higher carbon investment to roots than *P. pinea*. Our results are in agreement with a previous study describing a water-spending strategy in different clones of *P. pinea* (Sánchez-Gómez et al. 2011). This relatively more water-spending strategy of *P. pinea* would be advantageous under enriched [CO_2] scenarios as it would contribute to buffer the negative impacts of water stress on growth, especially in xeric environments (Zhang et al. 1997; Nguyen-Queyrens et al. 1998; Forner et al. 2018b). Different studies conducted in the Mediterranean region have highlighted that in the case of isohydric species, prolonged periods of near-zero carbon uptake may increase the risk of tree mortality (Plaut et al. 2012) and limit plant recovery after a drought event, in comparison to lower mortality rates and higher drought-recovery observed in more anisohydric species (Forner et al. 2018a; Ulrich and Grossiord 2023; Aranda et al. 2024).

Effects of drought and CO_2 on water use efficiency at different plant scales

Reductions of stomatal conductance (g_{wv}) and increased photosynthetic rates (A_n) under e[CO_2] observed in both species independently of the watering treatment (Fig. 6) is a common response to e[CO_2] (Kellomäki and Wang 1996; Picon et al. 1996; Medlyn et al. 2001; Ceulemans et al. 2002). Opposite responses in A_n and g_{wv} resulted in significant increases of gas-exchange-derived WUEi (An/g_{wv}) in e[CO_2], particularly in water-stressed *P. pinea*, with mean WUEi increasing up to 55% over WUEi in a[CO_2]. Reductions of similar magnitude in g_{wv} are common for conifers under [CO_2] enrichment (Tissue et al. 1997; Medlyn et al. 2001; Wertin et al. 2010; Birami et al. 2020). Stomatal closure reduces transpiration rates and limits CO_2 diffusion into leaves (Cornic 2000) reducing A_n (Ainsworth and Rogers 2007). Under high [CO_2], however, plants may increase A_n by increasing carboxylation with similar or lower g_{wv}, particularly under higher water stress (Ainsworth and Rogers 2007), as shown by our results. Large increases of $\delta^{13}C$-derived WUEi in water stressed plants at a[CO_2], integrative of the needle response for the duration of the last part of the experiment (since the beginning of water stress treatment), reinforce evidence of the increased gas-exchange-derived WUEi (Fig. 7). Despite the common responses of both estimates of WUEi to water stress and [CO_2], representing two different time scales of integration (the instantaneous gas-exchange-derived WUEi vs longer-term $\delta^{13}C$ -derived WUEi), the low relationship between them, as shown in previous studies (Beer et al. 2009), could be due to the different temporal responses of the driving processes, such as A_n and g_{wv}, or different effects of leaf-to-air vapor concentration difference or temperature (Gallé and Feller 2007; Beer et al. 2009; Dawes et al. 2011; Liang et al. 2023). Nevertheless, the ability of seedlings of both pine species to increase both WUEi estimates, under elevated [CO_2] and under water stress, would result in similar or larger productivities with lower water consumption than at lower [CO_2]. However, plant transpiration in relation to leaf area was similar in plants in a[CO_2] and plants in e[CO_2] (Fig. S5) indicating that lower leaf g_{wv} may not necessarily reduce

canopy transpiration if leaf area concurrently increases in plants at e[CO_2] (Tor-ngern et al. 2015; Jiang et al. 2021). In contrast, whole-plant water use efficiency (WUE) differed from both estimates of leaf-level WUEi in response to water stress, suggesting differences in WUE adjustment at different organizational scales. Whole-plant WUE remained similar between water stress levels, except under a[CO_2], where plant-level WUE decreased in plants of both species under water stress, in contrast with WUEi. Increased WUEi in needles under water stress was not enough to compensate for lower leaf area which resulted in reduced WUE (Beer et al. 2009). Similar whole-plant WUE in both species resulted from a compensatory effect between their WUEi and leaf area ratios: fewer, but more efficient needles in *P. pinea* vs less efficient but more numerous needles in *P. pinaster*. Such a decoupled response in WUEi and WUE in plants growing under e[CO_2] × water stress conditions show that other factors such as leaf area, root fraction and plant respiration may further impact whole-plant WUE and ultimately performance at the plant individual level (Kelly et al. 2016; Medlyn et al. 2017; Duan et al. 2018).

Seedling performance, as expressed by plant total biomass and WUE, under the water stress and e[CO_2] levels tested, was similar for the two studied species suggesting that seedlings of both species may equally benefit from increased [CO_2]. Our results agree with observed differential regeneration occurrence in mixed forests between the two analyzed species (Ledo et al. 2014; Gea-Izquierdo et al. 2019; Vergarechea et al. 2019; Férriz et al. 2021). However, extrapolating results from controlled experiments to field conditions is not straightforward. Drought duration and intensity, along with the temperature and relative humidity in our experiment were less severe for plants than drought conditions endured by the species during Mediterranean summers (Martínez-Vilalta and Piñol 2002; Gea-Izquierdo et al. 2023). Additionally, the effects of [CO_2] and drought on seedlings may differ in time and with developmental stage (ontogeny) and limitations due to spatial (LAI or root development saturation), structural (e.g. canopy competition) characteristics (Norby et al. 1999) or other environmental factors such as light intensity (Herrick and Thomas 1999).. As a crucial process of forest dynamics, further research is needed to evaluate how seedlings of co-occurring species respond to more intense drought conditions under climate change and elevated CO_2 levels in order to infer potential compositional changes in forest ecosystems.

Conclusions

In this study, we investigated the functional adjustments related to carbon allocation and water use efficiency at different plant scales for two Mediterranean pine species (*P. pinea* and *P. pinaster*) in response to water stress and different atmospheric [CO_2]. Water stress consistently reduced photosynthetic rates and stomatal conductance in both species, with *P. pinaster* being more sensitive to water stress than *P. pinea*. Elevated [CO_2] partly mitigated those negative effects by maintaining photosynthetic activity despite tighter stomatal control, resulting on a higher needle-level WUEi, plant-level WUE, and biomass growth. Species-specific responses of WUE to water stress differed at organizational scales from the leaf- to the plant-level. While leaf-level WUEi increased with water stress, plant-level WUE remained either unchanged or decreased with water stress and a[CO_2]. Contrary to expectations, carbon allocation patterns were unaltered in response to e[CO_2], except for increases in leaf fraction and SLA. However, the species partly differed in their strategies for coping with water stress and [CO_2] changes, despite their close functional and phylogenetic relationships, and a common isohydric behaviour. Greater carbon investment to underground organs under drought stress and higher WUEi in *P. pinaster* evidenced a more isohydric response than *P. pinea*. At plant level, however, WUE was similar in both species probably due to compensatory mechanisms between needle-level WUEi responses and total plant needle production. Overall, e[CO_2] improved seedling performance, particularly in the more drought-tolerant *P. pinea* suggesting that e[CO_2] is unlikely to reverse the relative performance of these two species under water stress. If our findings hold true in field conditions, *P. pinea* may retain its current competitive advantage in maintaining essential physiological processes and potentially replace *P. pinaster* under future, more xeric conditions, even at elevated [CO_2]. Our results emphasize the importance of considering the interactive effects of CO_2 and water availability when assessing the performance and resilience of co-occurring species in the context of global change.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10342-025-01779-0.

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Author contributions M.F.: Data curation, Formal analysis, Writing, Review & editing. I.A.: Conceptualization, Methodology, Review & editing. G.G.I.: Conceptualization, Methodology, Review & editing. D.M.B.: Conceptualization, Methodology, Review & editing. All authors reviewed the manuscript.

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Data availability The data that support the findings of this study are not openly available, but they will be available from the corresponding author upon reasonable request. Data are located in controlled access data storage at Indiana University.

Code availability The authors will make the code available upon reasonable request.

Declarations

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

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