Science of the Total Environment 831 (2022) 154797

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Low acclimation potential compromises the performance of water-stressed pine saplings under Mediterranean xeric conditions

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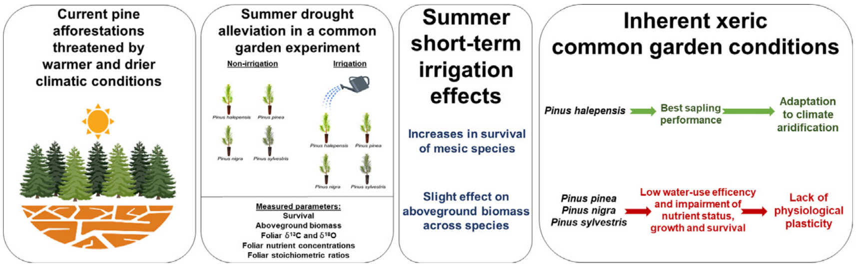
HIGHLIGHTS

- Performance of pine saplings determines future afforestation success.
- Short-term irrigation increased survival of the more mesic-adapted pines.
- Leaf isotopic composition and nutrient concentrations did not change after irrigation.
- The more mesic-adapted pines suffered from carbon and nutrient starvation.
- Water-stressed pine species lacked of physiological plasticity to tolerate water shortage.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Elena Paoletti

Keywords:
Afforestation
Irrigation
Physiological plasticity
Reforestation
Resource-use strategies

ABSTRACT

Predicted hotter and drier climatic conditions in the Mediterranean Basin will probably hamper current afforestations and reforestations by negatively influencing tree performance. Understanding how saplings can adjust their physiology to shortages in water availability is essential to predict early-stage success of forest ecological restoration. Pines are common target species used in afforestations and reforestations; however, the capacity of their saplings for physiological plasticity to promote drought tolerance remains largely unexplored. In this study, we evaluated the demographical and resource-use consequences of short-term irrigation among four pine species (*Pinus halepensis*, *Pinus pinea*, *Pinus nigra* and *Pinus sylvestris*) growing under water-limiting conditions in a common garden experiment. Summer irrigation increased the survival rate of those pines that were suffering from hydric stress under the xeric conditions of the common garden (i.e. *P. pinea*, *P. nigra* and *P. sylvestris*). Moreover, short-term water supplementation slightly enhanced aboveground biomass production across species. However, leaf isotopic composition and nutrient concentrations did not change after summer irrigation. Independently of water supplementation, *P. halepensis* was the best adapted species to water scarcity and showed the best physiological and growth performance. By contrast, *P. pinea*, *P. nigra* and *P. sylvestris* saplings exhibited drought-induced reductions in stomatal conductance and low water-use efficiency, nutrient deficiency, and severe N:P and N:K stoichiometric imbalances, leading to impaired growth. We conclude that the lack of physiological plasticity of water-stressed pine saplings to withstand the impacts of climate aridification will likely cause severe impairment of their nutrient status, growth and survival, with dire implications for the successful establishment of Mediterranean afforestation and reforestation programs.

1. Introduction

Increasingly frequent and more severe drought episodes have been predicted for the Mediterranean Basin under future climate change scenarios (Guiot and Cramer, 2016; Samaniego et al., 2018; Tramblay et al., 2020). Many afforested and reforested pine stands in the Mediterranean region

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http://dx.doi.org/10.1016/j.scitotenv.2022.154797
Received 11 February 2022; Received in revised form 17 March 2022; Accepted 20 March 2022
Available online 25 March 2022

are already suffering the negative impacts of climate change and some of their key ecosystem functions (e.g. carbon storage capacity and nutrient cycling) are expected to be seriously compromised in the near future (Valladares et al., 2014; Peñuelas and Sardans, 2021). Plant carbon assimilation strategies may be severely affected by low water availability, and species with an isohydric behavior (i.e. closing their stomata earlier at relatively high plant water potentials during drought stress) are more prone to carbon starvation than species with an anisohydric behavior (i.e. with a looser stomatal control of leaf gas exchange at low water potentials; McDowell et al., 2008; McDowell, 2011). Moreover, drought-induced foliar nutrient deficits and imbalances resulting from poorer nutrient uptake during prolonged dry periods may intensify the detrimental effects of low water availability by further impairing intrinsic water-use efficiency (Gessler et al., 2017; Salazar-Tortosa et al., 2018a). In this context of changing abiotic conditions, early success of afforestation and reforestation plans is heavily dependent on the functional attributes, stress tolerance and physiological performance of the target species under water shortage (Andivia et al., 2021).

Plant-water use strategies in water-limited environments can be assessed by measuring the stable carbon and oxygen isotope ratios of leaves ($\delta^{13}C$ and $\delta^{18}O$, respectively; Dawson et al., 2002). Leaf $\delta^{13}C$ depends on the ratio of intercellular to atmospheric CO_2 concentrations (c_i/c_a) during the period in which C is fixed and leaf material is produced. Leaf c_i is controlled by the net photosynthetic rate (i.e. the rate of CO_2 fixation by RuBisCO, A) and stomatal conductance (g_s) (Farquhar et al., 1989). Hence, foliar $\delta^{13}C$ is a suitable proxy for time-integrated intrinsic water-use efficiency (where WUE_i is the ratio between A and g_s) (Seibt et al., 2008). However, it is difficult to disentangle to what extent the interspecific differences in foliar $\delta^{13}C$ are determined by differences in A, g_s or both factors (Scheidegger et al., 2000; Werner and Máguas, 2010). Leaf $\delta^{18}O$ is inversely linked to the ratio of intercellular and ambient water vapor pressure (e_i/e_a) (Barbour and Farquhar, 2000), and is determined by the isotopic composition of the source water, the evaporative isotopic enrichment of the water in transpiring leaves and the isotopic exchange between oxygen atoms in organic molecules and source water (Barbour, 2007). Foliar $\delta^{18}O$ is strongly influenced by g_s, thereby being a good proxy measure of time-integrated stomatal conductance and cumulative transpiration over the growing season (Wang and Yakir, 2000; Barbour, 2007). Using both isotopes in combination (i.e. the dual isotopic approach) gives us time-integrated information about plant responses to changes in abiotic factors because foliar $\delta^{18}O$ is influenced by g_s but is independent of variations in A. Consequently, this allows us to separate the distinct effects of A and g_s on foliar $\delta^{13}C$ and WUE_i (Scheidegger et al., 2000; Grams et al., 2007).

The species-specific resistance of vascular plants to projected climate change conditions in the Mediterranean region has been shown to depend on their stomatal regulation and water-use efficiency, but also on their intricate links with plant water potentials, photochemical efficiency and foliar macro- and micro-nutrient concentrations (Moreno-Gutiérrez et al., 2012; Prieto et al., 2018; León-Sánchez et al., 2018, 2020). Moreover, saplings rather than adult trees are expected to suffer most from stressful abiotic conditions in drought-prone environments due to shallower root systems (Herrero et al., 2013b). Field studies in natural plant communities and common garden experiments assessing inter-specific differences in performance under the same abiotic limiting factors can improve our understanding of species resource-use strategies and local adaptation (Moreno-Gutiérrez et al., 2012; Prieto et al., 2018). They can also help in selecting the most suitable species for the ecological restoration of forests, especially when explicitly considering the earliest stages of the plant life cycle (Gibson et al., 2016; Ramírez-Valiente et al., 2021). This gains relevance under the ongoing, unprecedented efforts to increase forest cover at global scale, making particularly necessary to know the response of juveniles to current and future climatic conditions (Andivia et al., 2021; Albrecht et al., 2022; Castro et al., 2021).

It has been documented that drought-induced mortality particularly affects boreal and mesic pine species which have their southernmost rear-edge populations growing under xeric conditions (Martínez-Vilalta and Piñol, 2002; Lesser and Jackson, 2012; Herrero et al., 2013a; Clark et al., 2016; Férriz et al., 2021). However, some on-going afforestation and reforestation programs still consider some of these mesic pines as suitable for dry environments (Vadell et al., 2016; Fargione et al., 2021). Investigating the comparative ecophysiology of pine species and the ability of their juveniles to thrive in water-limited contexts can provide important information about their degree of physiological plasticity for tolerating abiotic stress and, hence, about possible future shifts in species distributions and suitability for reforestation projects under climate change (Lévesque et al., 2014; Conlisk et al., 2018; Matías et al., 2017; Andivia et al., 2020). For example, for saplings of four different pines growing under the same water stress conditions, the multiple interplays between carbon starvation and hydraulic failure led to severe growth impairment and mortality through foliar nutrient deficits in mesic species with strict isohydric behavior and tight stomatal regulation even at relatively high plant water potentials (Salazar-Tortosa et al., 2018a). In this sense, field studies involving experimental manipulation of soil water availability through supplemental irrigation may provide further insights into the actual impacts of drier climatic conditions on the feedback loops between drought stress, stomatal behavior, plant nutrient status, water-use efficiency, growth and survival. These studies can also help unravel how these feedbacks and interactions may ultimately influence the demography and establishment of different pine species under a warmer and drier climate.

We conducted a 2-year summer drought alleviation experiment to evaluate the impacts of water supplementation on the demography, resource-use strategies and ecophysiology of pines growing in a common garden under xeric conditions. As targets, we selected four species that are native to the Mediterranean region. We considered two pine species with strict isohydric behavior and tight stomatal regulation (*Pinus nigra* Arnold and *Pinus sylvestris* L.) and another two pine species with less strict stomatal control of transpiration flux even at lower plant water potentials and more anisohydric behavior (*Pinus halepensis* Mill. and *Pinus pinea* L.) (Villar-Salvador et al., 2013; Salazar-Tortosa et al., 2018b). We aimed at obtaining a comprehensive assessment of their responses to changes in soil water availability in terms of survival, growth, leaf nutrient status and time-integrated leaf gas exchange as inferred from their isotopic composition. We hypothesized that irrigation would (H1) promote sapling survival and growth across pine species. Moreover, the beneficial effect of water supplementation would (H2) be greater for species with tighter stomatal control (isohydric) when compared with those with looser stomatal control (anisohydric), thereby reducing the water stress that the former species face under xeric conditions and ameliorating their time-integrated stomatal conductance, transpiration and water-use efficiency. In turn, summer drought alleviation would (H3) lead to smaller macro- and micronutrient deficiencies and stoichiometric imbalances among pine species growing under Mediterranean xeric conditions. With this study, we seek to explore the species-specific capacity of pines to acclimate to water-limiting conditions (i.e. physiological plasticity) and whether this capacity is linked to improved sapling performance under drought stress. Ultimately, this could help forest managers in selecting the most suitable pine species for afforestation and reforestation programs, thereby maximizing restoration success in the face of climate change.

2. Materials and methods

2.1. Study site, selected species and experimental design

A common garden experiment was established at the “Huerta de La Paloma” farm (37° 10′ 03.43″ N, 3° 36′ 58.80″ W; Granada, Southern Spain), a flat (slope ca. 2%) and agricultural terrain located at 649 m a.s.l. The climate in the area is Mediterranean, characterised by hot, dry summers and with most of the rainfall occurring in autumn and spring. The mean annual rainfall is 389 mm and the mean annual temperature is 15.3 °C, with July being the warmest month (mean temperature of 25.9 °C) and January the coldest (mean temperature of 6.2 °C; period 2006–2020, climatic data collected from the meteorological station of

IFAPA Research Field Station, located 1.5 km away). Soil type is calcaric Fluvisol and the soil is deep with a loamy texture (44.48% sand, 41.8% silt and 13.3% clay). Soil water content was 13% at wilting point and 33% at field capacity (−1.5 and −0.033 MPa, respectively; means for the profile down to 1 m deep and without marked horizons in soil profile) (Salazar-Tortosa et al., 2018a).

Edaphic and climatic characteristics at the common garden experiment (i.e. calcareous soil with hot and dry summer conditions) are optimal for *P. halepensis* (Mauri et al., 2016). By contrast, *P. pinea* prefers narrower annual temperature variation and nutrient-poor sandy soils with low-water holding capacity (Villar-Salvador et al., 2013; Abad-Viñas et al., 2016), but can also be found in reforestations nearby the common garden site. *P. nigra* can tolerate wide seasonal temperature fluctuations (Enescu et al., 2016), whereas *P. sylvestris* is found in colder and wetter areas (Houston-Durrant et al., 2016). In addition, previous studies found that *P. halepensis* and *P. pinea* exhibit lower water stem potentials under the xeric Mediterranean conditions of the experimental site (−2.37 ± 0.04 MPa and −2.06 ± 0.06 MPa, respectively) than *P. nigra* and *P. sylvestris* (−1.88 ± 0.05 MPa and −1.84 ± 0.04 MPa, respectively) (Klein et al., 2013; Villar-Salvador et al., 2013; Salazar-Tortosa et al., 2018b).

Seeds of the four selected pine species were collected from four different certified provenance regions of the Iberian Peninsula: Alcarria (40° 24′ 52″ N, 2° 24′ 33″ W) for *P. halepensis*; La Mancha (39° 11′ 25″ N, 1° 56′ 25″ W) for *P. pinea*; Sistema Ibérico (40° 15′ 16″ N, 1° 58′ 22″ W) for *P. nigra*; and Montes Universales (40° 28′ 09″ N, 1° 38′ 42″ W) for *P. sylvestris*. They were stored at 4 °C until seeding time, and seeding was performed using 300-ml trays filled with fertilized peat (White 420 F6 Kekkilä, Finland; pH 4.7) containing 0.8–1 kg/m^3 of a slow-release fertilizer NPK 16-10-20 in a greenhouse at El Serranillo Field Station (Spanish National Centre of Forest Genetic Resources; Guadalajara, Spain; 40° 39′ 56.14″ N, 3° 10′ 15.20″ W). Afterwards, seedlings were moved outdoors and grown under optimal forest nursery conditions. On 27 April 2018, all seedlings were collected from the nursery. We chose 2-year-old individuals of *P. nigra* and *P. sylvestris*, and 1-year-old individuals of *P. halepensis* and *P. pinea* to minimize the differences in size across species (see Table S1 for initial values of leader shoot length and stem-root collar diameter at the moment of transplanting). From now on, the term “seedlings” is used only for the young individuals grown under optimal forest nursery conditions until the moment of transplanting in the field, whereas the term “saplings” is used for referring to transplanted individuals that were harvested at the end of the experiment, when they were 3 (*P. halepensis* and *P. pinea*) or 4 (*P. nigra* and *P. sylvestris*) years old (see below).

On 29 April 2018, seedlings were transplanted to the common garden experimental site following a randomized-block design. Specifically, three blocks of 450 m^2 were established side by side and separated by a 2.5-m buffer zone. A total of 48 seedlings of each species were planted in each of the three blocks (48 individuals × 3 blocks × 4 species = 576 individuals in total). Pines were planted evenly at 1.25 m distance from each other, and were distributed randomly within each block. A total of 288 individuals were randomly selected for the present experiment (50% of those planted; the rest were used for other purposes). Of those, half of them were subjected to irrigation during the growing season (I+), whereas the other half were not irrigated (I−). Starting on 20 June 2018, half of the pines were watered weekly until 29 August 2018 (i.e. 11 experimental irrigation applications in total in 2018). Analogously, the same amount of water was applied weekly from 13 May 2019 until 14 July 2019 (i.e. 9 experimental irrigations in total in 2019). For each irrigation application, 4 l of water were added in a surface of 30 × 30 cm (i.e. equivalent to 44.44 mm rainfall) at each experimental irrigation. However, it should be noted that the real amount of irrigation water available for the plants should be lower due to irrigation bulb expansion and movement (i.e. horizontal diffusion and vertical percolation). The amounts of rainfall during the irrigation periods in 2018 and 2019 were 1.8 mm and 0.2 mm, respectively. Fig. S1 shows the irrigation dates, and the values of average daily temperature and daily rainfall throughout the whole field experimental period. Weeds surrounding the saplings were periodically removed, both manually and with a cultivator, to prevent herbaceous competition.

2.2. Soil water content

Soil water content was measured at 10, 20, 30 and 40-cm depth during three consecutive weekly periods in summer 2019 by installing PR-2 Soil Moisture Profile Probes (Delta T, Cambridge, UK). Specifically, 4 PR-2 Probes were installed at each block, with two of them located beside irrigated individuals and the other two beside non-irrigated individuals ($n = 12$). Measurements were taken at each of the three periods at 6 h, 1 day, 2 days, 3 days and 7 days after watering. Measurement periods were 10th–17th June 2019, 17th–24th June 2019 and 24th–31st June 2019.

2.3. Sapling survival and growth

We monitored survival 4 times from 29 April 2018 to 30 August 2019 in the common garden experiment. The initial leader shoot length and stem-root collar diameter were measured for all transplanted plants as a baseline to calculate growth increment for all the surviving saplings at the end of the experiment (October 2019). Stem volume was calculated assuming a conical shape for the stem, and the stem volume increment was determined taking into account the initial and final measurements of leader shoot length and stem-root collar diameter (Table S2; see Salazar-Tortosa et al., 2018a for a similar procedure).

2.4. Destructive harvesting, nutrient analysis and leaf isotopic composition

In October 2019, all surviving pine saplings were harvested. For each harvested individual, a subsample of leaves produced during the year 2019 was taken (i.e. the collected foliar material had been produced in the field during the last growing season in all cases, and thus was 4–5 months old across species). Leaves were oven-dried at 60 °C to constant weight and ground to a fine powder for nutrient and leaf isotopic composition analyses. The remaining plant material was similarly oven-dried and weighed in order to obtain aboveground biomass measurements.

Across all species, aboveground biomass production at final harvest was tightly correlated with the final measurements of leader shoot length (ρ: 0.8555; p-value <0.0012) and stem-root collar diameter (ρ: 0.9255; p-value <0.001), as well as with stem volume increment (ρ: 0.9630; p-value <0.001). Hence, aboveground biomass at final harvest was used as the most representative growth variable for subsequent analyses.

The foliar concentrations of eleven nutrients considered as essential for plant growth in vascular plants (Marschner, 1995) were analysed. Specifically, six macronutrients (N, P, K, Ca, Mg and S) and five micronutrients (Fe, Cu, Zn, Mn and B) were measured on finely ground leaf samples. Leaf P, K, Ca, Mg, S, Fe, Cu, Zn, Mn and B concentrations were measured by inductively coupled plasma emission spectrometry (ICP- OES; Thermo Elemental Iris Intrepid II XDL, Franklin, MA, USA) at the CEBAS-CSIC (Murcia, Spain), and leaf N concentrations were measured using an automatic CN analyser as described below.

Four mg of finely ground and powdered leaf material were weighed in tin capsules for leaf $\delta^{13}C$ measurements, and 0.7–0.8 mg of the same material were weighed in silver capsules for leaf $\delta^{18}O$ measurements. All isotopic samples were analysed at the Center for Stable Isotope Biogeochemistry of the University of California-Berkeley (USA). A CHNOS Elemental Analyser interfaced to an IsoPrime100 mass spectrometer was used to determine leaf $\delta^{13}C$ and N concentrations by continuous flow dual isotope analysis. Leaf $\delta^{13}C$ isotope composition was expressed in delta notation (‰) relative to international standard V-PDB, with a long-term external precision of ±0.10‰. An Elemental PYRO Cube interfaced to a Thermo Delta V mass spectrometer was used to determined leaf $\delta^{18}O$. Leaf $\delta^{18}O$ isotope composition was expressed in delta notation (‰) relative to V-SMOW for $\delta^{18}O$, with a long term-external precision for IAEA-V-9 (cotton cellulose) of ±0.20‰.

Aboveground biomass, leaf isotopic composition and nutrient concentrations before transplanting to the experimental field site in late April

2018 were similarly analysed in 18 seedlings per species directly harvested from the forest trays, reflecting therefore their physiological status under optimal nursery conditions.

2.5. Statistical analyses

Repeated measures ANOVA was used to test whether the irrigation treatment showed a significant effect on soil water content in the common garden experiment. We tested for significant differences between time (6 h, 1 day, 2 days, 3 days and 7 days after watering; within-subject variable), irrigation treatment (I+ and I−; between-subject variable) and soil depth (10 cm, 20 cm, 30 cm and 40 cm; between-subject variable).

Cox regressions models were performed to test for differences in sapling survival in the 1st and 2nd years and at the end of the experiment between species and between irrigated and non-irrigated individuals. Linear mixed models were used to test for differences in sapling traits among pine species and between irrigated and non-irrigated individuals. The analysed response variables were aboveground biomass, $\delta^{13}C$, $\delta^{18}O$, N, P, K, Ca, Mg, S, Fe, Cu, Zn, Mn, B, N:P ratio and N:K ratio with “species”, “irrigation” and their interaction as fixed factors and “block” as the random factor. Aboveground biomass, N:P and N:K ratios were log-transformed prior to analyses. Moreover, stepwise multiple regression models were performed to test which combinations of predictor variables better explained the variance in aboveground biomass production.

Pearson correlation coefficients were performed to explore links between traits related to growth (aboveground biomass), leaf isotopic composition ($\delta^{13}C$ and $\delta^{18}O$) and leaf nutrient status (N, P, K, Ca, Mg, S, Fe, Cu, Zn, Mn, B, N:P and N:K ratios) at the end of the experiment. Moreover, general linear models were used to test for differences in seedling traits among pine species under optimal nursery conditions. The response variables were leader shoot length, stem-root collar diameter, aboveground biomass, $\delta^{13}C$, $\delta^{18}O$, N, P, K, N:P ratio and N:K ratio. Residuals of all models were assessed for normality and homogeneity of variances. Model analyses were followed by Tukey's post hoc tests to analyse significant differences for the measured variables. Models were performed using “survival” (Therneau, 2015), “lme4” (Bates et al., 2015) and “lmerTest” (Kuznetsova et al., 2017) packages. All statistical analyses were performed in R (R.3.6.2., R Core Team, 2018).

3. Results

3.1. Effect of summer irrigation on soil water content at the common garden site

Soil water content during the summer dry period was significantly higher in irrigated than in non-irrigated soils and it decreased significantly over time after watering (Table 1 and Fig. 1). However, differences were significant at 10 cm and 20 cm depth, but not at 30 cm or 40 cm depth (Table 1 and Fig. 1). Moreover, we did not find any significant differences in soil water content between the three different measurement periods (i.e. 10th–17th June 2019, 17th–24th June 2019 and 24th–31st June 2019).

Table 1
Summary of the results of the repeated measures ANOVA testing the effects on soil water content of irrigation (I+ and I−), soil depth (10 cm, 20 cm, 30 cm and 40 cm), time after watering (6 h, 1 day, 2 days, 3 days and 7 days) and their interactions (p-value <0.05).

	d.f.	F value	p-value
Between-subjects			
Irrigation	1	26.80	<0.001
Depth	3	86.76	<0.001
Irrigation ∗ depth	3	9.31	<0.001
Within-subjects			
Time	4	5.86	<0.001
Irrigation ∗ time	4	3.10	0.015
Depth ∗ time	12	6.77	<0.001
Irrigation ∗ depth ∗ time	12	5.22	<0.001

3.2. Survival and aboveground biomass production after 2 years of summer irrigation

A total of 184 saplings (65%) survived at the end of the experiment. Final survival rate differed significantly both among species and between non-irrigated and irrigated saplings (Table S3; Fig. 2a). Accordingly, *P. halepensis* exhibited the highest final survival percentage (93%^{a}), followed by *P. pinea* (80%^{b}), whereas *P. nigra* (52%^{c}) and *P. sylvestris* (37%^{c}) showed the lowest values (different superscript letters indicate significant differences among species; p-value <0.005, Tukey post-hoc test). *P. pinea*, *P. nigra* and *P. sylvestris* exhibited significantly higher final survival percentages for irrigated than for non-irrigated plants, whereas *P. halepensis* did not show differences between irrigation treatments (Fig. 2a).

When considering the survival rates separately by growing season, the pattern was the same for the first year of the study (Fig. 2b): *P. halepensis* had the highest survival rate regardless of irrigation treatment, and there were higher survival values for irrigated saplings of *P. pinea*, *P. nigra* and *P. sylvestris*. However, this pattern changed for the second year (Fig. 2c): *P. halepensis* and *P. pinea* showed the highest survival values and without differences between irrigation treatments, whereas for *P. nigra* differences still persisted between irrigated and non-irrigated saplings. In the case of *P. sylvestris*, survival values were the lowest, and differences between irrigation treatments were not significant despite a trend towards a higher survival for irrigated plants. It should be noted that sample size at the end of the experiment was very low for this species (Fig. 2c), which might have masked differences between treatments. Moreover, drought-related mortality occurred largely during the summer dry season, with only 8 individuals (evenly distributed across species and irrigation treatments) dying during the winter (winter mortality sampled in March 2019; data not shown).

At the end of the experiment, aboveground biomass differed significantly among species and between irrigation treatments (Table 2). *P. halepensis* exhibited the highest aboveground biomass value, followed by *P. pinea*, and *P. nigra,* whereas *P. sylvestris* showed the lowest values (Table 2). Across species, the irrigated plants exhibited 18% greater mean aboveground biomass than the non-irrigated ones (Table 2).

3.3. Leaf isotopic composition, nutrient concentrations and stoichiometric ratios after 2 years of summer irrigation

Leaf isotopic composition differed significantly among species, but not between irrigated and non-irrigated saplings (Table 2). Moreover, the interaction between species and irrigation treatment was not significant (Table 2). Specifically, *P. halepensis* showed the highest foliar $\delta^{13}C$ values (indicative of highest WUE_i), followed by *P. sylvestris*, *P. nigra* and *P. pinea* (Table 2). By contrast, *P. nigra* and *P. sylvestris* showed significantly higher foliar $\delta^{18}O$ values (indicating lower time-integrated stomatal conductance) than *P. halepensis* and *P. pinea* (Table 2).

All concentrations of foliar nutrients, except Cu, were significantly different among pine species, indicating idiosyncratic and distinct biogeochemical niches in the different species. Leaf P (Table 2), Zn (Table S4) and S (Table S4) concentrations were highest for *P. halepensis*, with this species showing on average 1.60-fold (P), 1.57-fold (Zn) and 1.37-fold (S) higher mean values than *P. pinea*, *P. nigra* and *P. sylvestris*. Moreover, leaf Mg (Table S4) and Mn (Table S4) concentrations were highest for *P. pinea,* and this species exhibited 1.34-fold (Mg) and 1.33-fold (Mn) higher mean values than the other three pines (Table S4). Leaf Fe (Table S4), Ca (Table S4) and B (Table S4) concentrations were highest for *P. sylvestris*, with this species showing 2.50-fold (Fe), 1.72-fold (B) and 1.68-fold (Ca) higher mean values than the others. Foliar N (Table 2) and K (Table 2) concentrations were less variable among species.

In addition, irrigated and non-irrigated saplings only showed significant differences for foliar Ca and Mn concentrations in *P. nigra* and *P. sylvestris* (Table S4). Average Ca concentrations were 1.32-fold higher and 1.36-fold higher for non-irrigated than for irrigated individuals in *P. nigra* and in *P. sylvestris*, respectively (Table S4). Similarly, average Mn concentrations were 1.37-fold and 1.54-fold higher for non-irrigated than for irrigated individuals in *P. nigra* and in *P. sylvestris*, respectively (Table S4).

Regarding stoichiometric relations between essential macronutrients, the foliar N:P ratio was significantly lower for *P. halepensis* than for *P. pinea*, *P. nigra* and *P. sylvestris* (Table 2), whereas the foliar N:K ratio was significantly higher for *P. sylvestris* than for the other three pine species (Table 2).

3.4. Relationships between traits across pine species

All pairwise Pearson correlations between key traits related to aboveground biomass, leaf isotopic composition and leaf nutrient status are shown in Table S5. Across pine species, foliar δ^{18}O was significantly positively correlated with leaf N, Ca, Fe and B concentrations and N:K and N:P ratios, revealing increasing P and K deficit and nutrient imbalance with lower time-integrated stomatal conductance. In addition, foliar δ^{13}C was significantly positively associated with leaf N, P, S and Zn concentrations and N:K ratio, indicating increasing WUE$_i$ with higher leaf nutrient concentrations across species. In contrast, foliar δ^{13}C was negatively associated with Mg, Mn, B and N:P ratio, reflecting a negative impact of P deficit and nutrient imbalance on WUE$_i$. Across species, aboveground biomass production showed significant positive correlations with leaf δ^{13}C, P, S and Zn, and significant negative correlations with foliar δ^{18}O, N:P ratio, Ca, Mg, Fe and B. Overall, these correlation patterns indicated increasing biomass productivity with increasing stomatal conductance, phosphorus uptake and water-use efficiency across pine species. Stepwise multiple regression models showed that the combination of foliar N:P ratio (−), δ^{18}O (−) and S concentration (+) as predictor variables explained over one-third ($R^2 = 0.34$) of the total variance in aboveground biomass production across all species and individuals.

3.5. Optimal nursery conditions versus xeric common garden conditions

After transplanting to the common garden site under Mediterranean xeric conditions, average values of aboveground biomass of *P. halepensis* and *P. pinea* were substantially higher than those of *P. nigra* and *P. sylvestris* (Table 2), despite the fact that the former showed smaller sizes than the latter under optimal nursery conditions (Fig. 3a; Table S1; note in any case that *P. nigra* and *P. sylvestris* individuals were one year older than *P. halepensis* and *P. pinea*). In addition, sharp increases under

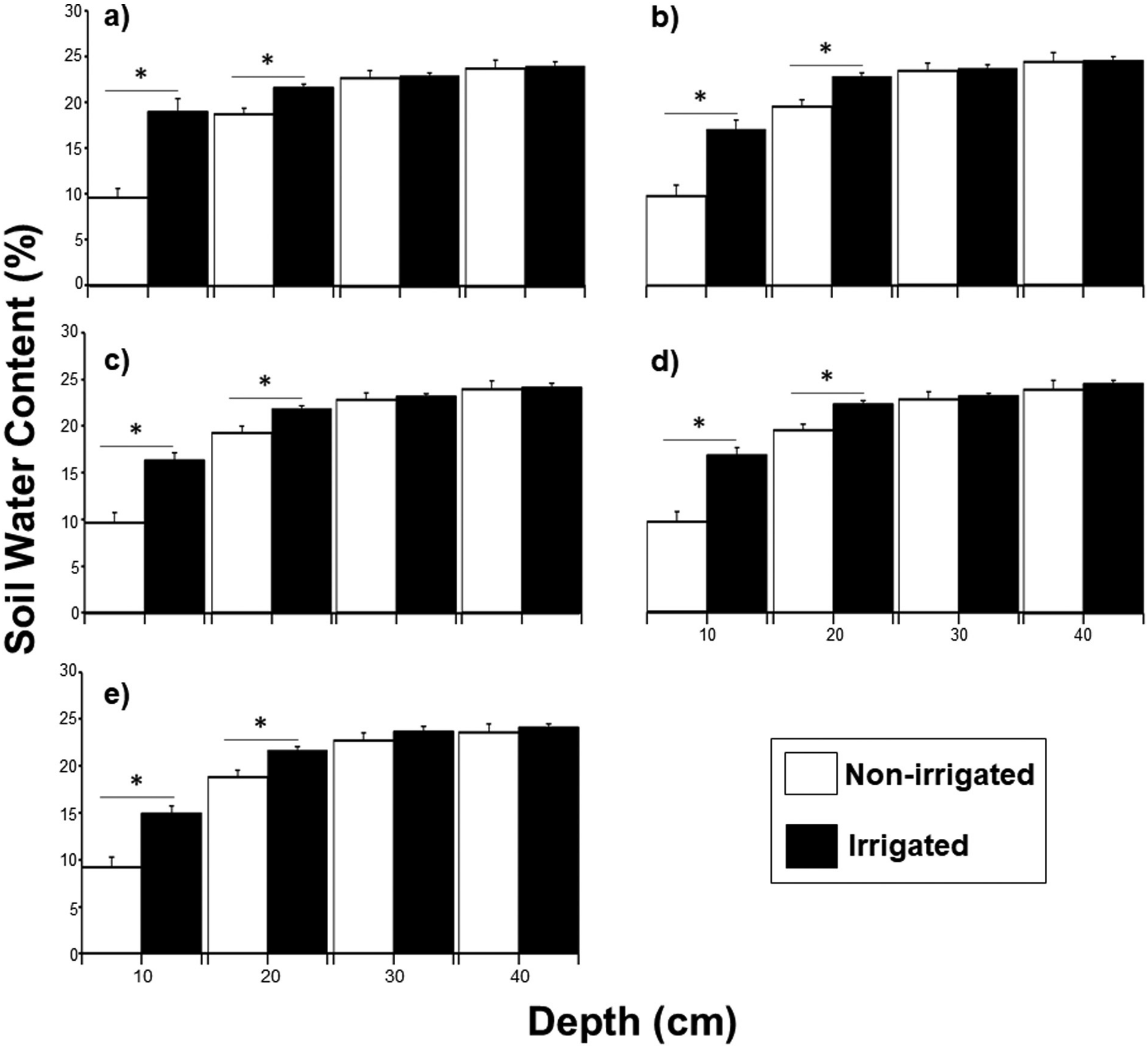


Fig. 1. Mean values ± SE of soil water content for non-irrigated and irrigated soils along the soil profile (10 cm, 20 cm, 30 cm and 40 cm) along the different times at which measurements were collected after watering: (a) 6 h, (b) 1 day, (c) 2 days, (d) 3 days and (e) 7 days. n = 12 probes were installed along the field experiment. Measurement periods were: 10th–17th June 2019, 17th–24th June 2019 and 24th–31st June 2019. Significant differences between irrigation and non-irrigation treatments for each depth are highlighted with an asterisk (p-value <0.05).

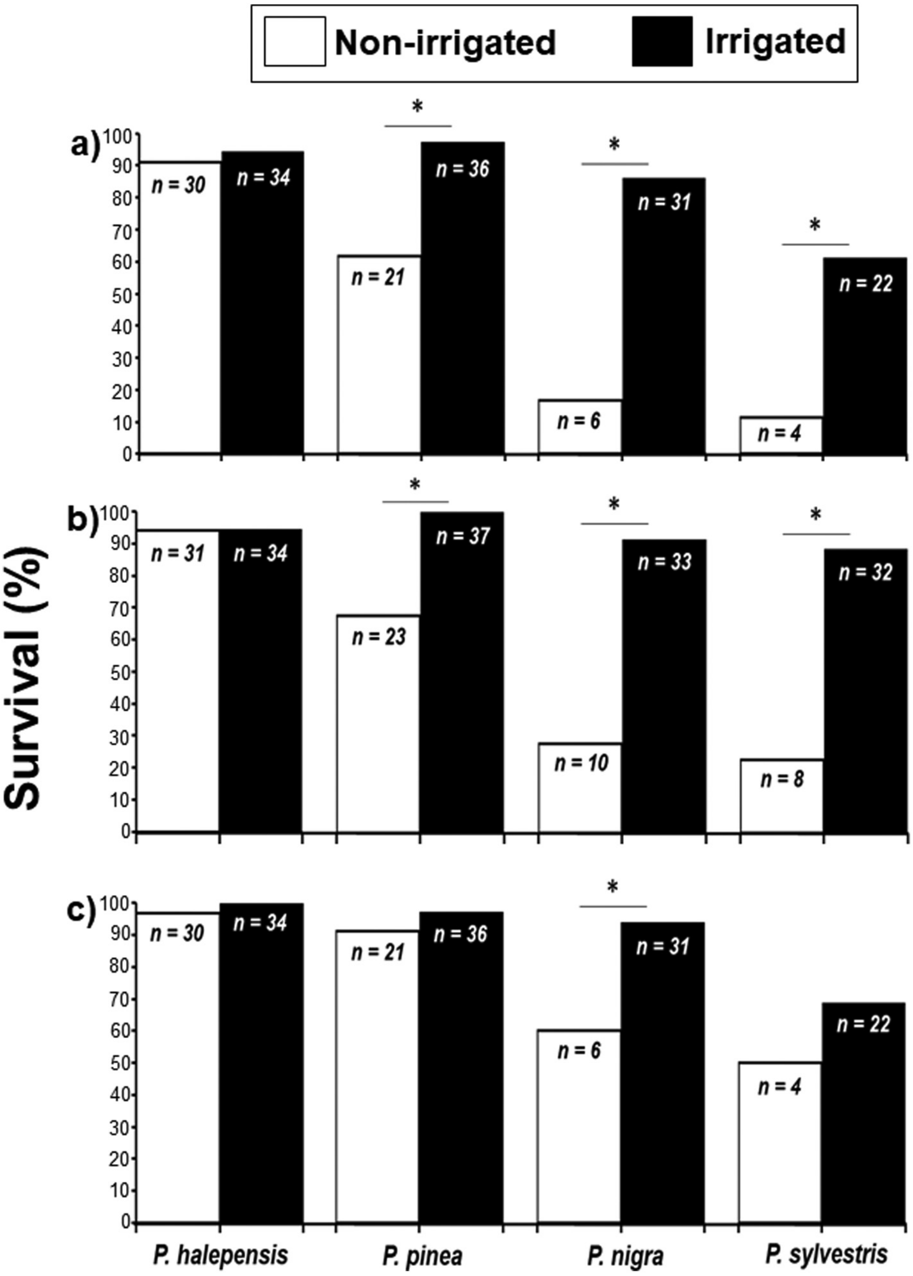


Fig. 2. Survival (%) of non-irrigated and irrigated saplings of each investigated species (a) at the end of the experiment, (b) in October 2018 (first year of the experiment) and (c) October 2019 (second year of the experiment). Significant differences between irrigated and non-irrigated individuals for each species are highlighted with an asterisk (p-value <0.05).

xeric common garden conditions were observed for foliar $\delta^{18}O$ (Fig. 3b) and $\delta^{13}C$ values (Fig. 3c), N concentrations (Fig. 3d), N:P ratios (Fig. 3g) and N:K ratios (Fig. 3h) in all four pine species. In contrast, leaf P concentrations decreased significantly in *P. pinea* and *P. sylvestris* (Fig. 3e), whereas leaf K concentrations decreased in all the pine species (Fig. 3f).

4. Discussion

4.1. Moderate positive effect of summer irrigation on pine performance under xeric Mediterranean conditions

In partial support with our first hypothesis, the drought-survival rate of pine saplings increased with summer irrigation, especially for those species whose native distribution areas are more mesic than the experimental site (i.e. *P. pinea, P. nigra* and *P. sylvestris*). This effect was particularly evident during the first summer season of the field experiment because abiotic stresses are known to exacerbate mortality at the earliest plant life stage under xeric conditions (Roldán et al., 1996; Castro et al., 2004; McDowell et al., 2008; Tíscar and Linares, 2014; Tíscar et al., 2017), but still persisted during the second year for the most drought-sensitive species. By contrast, *P. halepensis* showed the highest survival rate and was not significantly affected by summer drought stress alleviation through irrigation, further confirming that this pine species is the best adapted to water scarcity in terms of survivorship (Matías et al., 2017). Moreover, the initial age difference between species (1-year-old for *P. halepensis* and *P. pinea* vs. 2-year-old for *P. nigra* and *P. sylvestris* at the time of field transplanting) was not a confounding factor under common garden conditions because saplings of *P. halepensis* and *P. pinea* at the end of the field experiment were actually taller and had greater above-ground biomass than those of *P. nigra* and *P. sylvestris*. In this respect, we can infer that it was clearly the inherent

Table 2

Results of the linear mixed models (χ^2 values with p-values within brackets) for aboveground biomass, leaf isotopic composition, N, P and K concentrations, and stoichiometric ratios across species and between non-irrigated and irrigated saplings. Mean values ± SE of all measured variables in the four selected species and irrigation treatments are shown. Significant differences between species are shown with different letters (p-value <0.05).

	Linear mixed models results			*P. halepensis*		*P. pinea*		*P. nigra*		*P. sylvestris*	
	Species	Irrigation	Species × irrigation	I−	I+	I−	I+	I−	I+	I−	I+
Aboveground biomass (g)	174.950 **(<0.001)**	4.406 **(<0.001)**	3.702 (0.296)	c 197.14 ± 21.80	212.69 ± 20.02	b 48.18 ± 6.48	77.11 ± 8.52	a 20.65 ± 2.68	27.99 ± 2.43	a 21.84 ± 1.19	21.59 ± 2.29
Foliar $\delta^{18}O$ (‰)	48.097 **(<0.001)**	0.315 (0.574)	0.170 (0.982)	a 28.31 ± 0.30	28.56 ± 0.17	a 27.58 ± 0.46	27.75 ± 0.32	b 29.53 ± 0.78	29.49 ± 0.29	b 30.56 ± 0.53	30.50 ± 0.47
Foliar $\delta^{13}C$ (‰)	42.330 **(<0.001)**	0.595 (0.440)	5.179 (0.159)	c −26.65 ± 0.11	−26.72 ± 0.13	a −27.68 ± 0.23	−27.79 ± 0.19	ab −27.65 ± 0.42	−27.31 ± 0.17	bc −27.76 ± 0.70	−26.76 ± 0.18
N (%)	73.957 **(<0.001)**	0.303 (0.582)	1.341 (0.720)	c 1.85 ± 0.03	1.88 ± 0.05	a 1.33 ± 0.04	1.42 ± 0.05	b 1.77 ± 0.19	1.69 ± 0.06	bc 1.84 ± 0.11	1.80 ± 0.08
P ($g \cdot 100\ g^{-1}$)	125.700 **(<0.001)**	0.321 (0.571)	0.479 (0.923)	c 0.091 ± 0.003	0.093 ± 0.003	a 0.052 ± 0.004	0.072 ± 0.002	b 0.068 ± 0.010	0.078 ± 0.002	ab 0.057 ± 0.002	0.087 ± 0.004
K ($g \cdot 100\ g^{-1}$)	17.779 **(<0.001)**	0.004 (0.95)	1.209 (0.751)	c 0.37 ± 0.01	0.38 ± 0.01	ab 0.34 ± 0.02	0.32 ± 0.02	bc 0.33 ± 0.03	0.36 ± 0.01	a 0.30 ± 0.02	0.30 ± 0.01
N:P ratio	50.608 **(<0.001)**	0.047 (0.829)	0.613 (0.829)	a 21.07 ± 0.74	20.63 ± 0.60	b 29.06 ± 2.09	30.33 ± 1.77	b 27.85 ± 3.10	29.47 ± 2.28	b 32.24 ± 1.11	31.24 ± 2.55
N:K ratio	20.594 **(<0.001)**	0.136 (0.713)	2.244 (0.523)	a 5.17 ± 0.18	5.15 ± 0.21	a 4.35 ± 0.30	4.87 ± 0.27	a 5.48 ± 0.67	4.93 ± 0.25	b 6.36 ± 0.75	6.54 ± 0.62

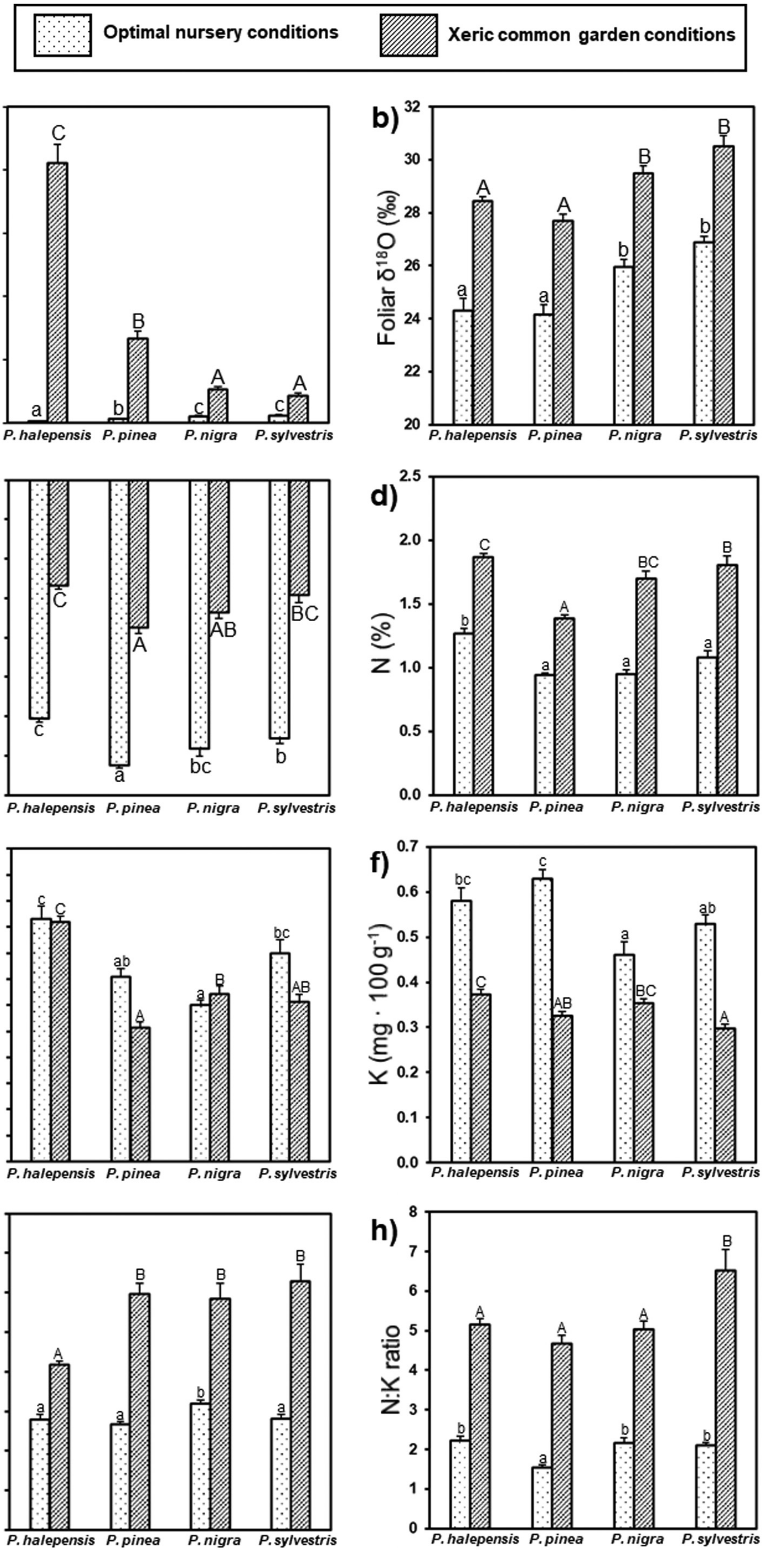
Significant fixed factors ($p < 0.05$) for each model are highlighted in bold. Degrees of freedom are 3 for "Species" and "Species ∗ Irrigation" and 1 for "Irrigation".

species-specific tolerance to the xeric conditions of the field site, rather than the initial age, that was driving pine performance in this 2-year experiment. However, the average aboveground biomass of the irrigated saplings was only 18% higher than that of non-irrigated ones at time of final harvest, indicating that water supplementation during two consecutive summers was not very effective to enhance aboveground growth despite its positive impact on survival. Hence, coping with water-limiting conditions in the Mediterranean region remains challenging for drought-stressed pines and does not only depend on the survival success during the first years of sapling development (Vilà-Cabrera et al., 2018).

In a context of low water availability, leaf-level intrinsic water-use efficiency tends to increase when stomatal conductance and carbon uptake are reduced to minimize water loss (Farquhar et al., 1989; Martin-StPaul et al., 2017). This typical response to drought stress is well illustrated by the sharp increases of both leaf $\delta^{18}O$ and $\delta^{13}C$ values in all the pine species after transplanting from the mesic nursery conditions (with ample irrigation) to the xeric field site (Fig. 3), which indicate much tighter stomatal regulation of plant water flux under field conditions (Scheidegger et al., 2000). However, our 2-year summer drought alleviation treatment did not induce any changes in foliar carbon and oxygen isotopic composition in October 2019. More specifically, contrary to what we expected with our second hypothesis, we did not observe a greater reduction of leaf $\delta^{18}O$ and increase in $\delta^{13}C$ values after irrigation in species with tight stomatal control (i.e. *P. nigra* and *P. sylvestris*) in comparison with those with looser stomatal regulation (i.e. *P. halepensis* and *P. pinea*). It is important to remark that any potential transient changes in WUE_i, stomatal conductance, and cumulative transpiration cannot be discarded in our study because we did not measure the isotopic composition of soluble carbohydrates just after irrigation and at different intervals, and only collected foliar material for isotopic composition analysis a single time at the end of the experiment. In this sense, leaf $\delta^{18}O$ and $\delta^{13}C$ may largely reflect plant physiological activity during the main growing season (spring) when new leaves are produced in pines. Hence, the isotopic signal of the foliar material collected in October 2019 might be diluted in the bulk saplings biomass. Apart from this, we cannot discard that carbon assimilation from the end of the summer to the time of plant harvest (early October) could also have reduced across-treatments differences in the isotopic signal. Therefore, it would be necessary to study intra- and inter-annual changes of leaf isotopic composition of irrigated and non-irrigated saplings to unravel the exact timing of the positive effects of short-term water supply under Mediterranean xeric conditions.

In contrast to our findings, an immediate response of early- and latewood $\delta^{13}C$ to water supply was found in another short-term irrigation experiment with *P. sylvestris* conducted under wetter and cooler climatic conditions (Eilmann et al., 2010). These contrasting results may be due to the fact that pine juveniles have difficulty in adjusting their hydraulic systems and leaf gas exchange activity in response to relatively small water pulses causing short-term changes in soil water availability, particularly under the stressful heat and drought conditions prevailing during Mediterranean summers (Oliveras et al., 2003; Ripullone et al., 2007). Moreover, non-structural carbohydrates produced during the dry summer may include mobile soluble sugars and sugar alcohols that are primarily used for plant stress resistance against drought, but they may not be allocated to aboveground growth (Chapin et al., 1990; Herms and Mattson, 1992; Hartmann, 2015). Thus, it is possible that carbon allocation under xeric summer conditions was prioritized towards drought-defence mechanisms rather than to aboveground growth increases, which might explain the modest aboveground biomass increases and the absence of changes in foliar isotopic composition with irrigation at the end of the experiment. Additionally, this supports the need for longer-term water supplementation of pine saplings to achieve significant ameliorations in growth and storage processes (von Arx et al., 2017; Feichtinger et al., 2017; Schönbeck et al., 2018).

In addition, an increase in soil nutrient availability to roots was expected after water supplementation to dry topsoil during the summer, thereby improving plant nutrient uptake and foliar nutrient concentrations (Lambers et al., 2008). However, our third hypothesis was not supported as


Optimal nursery conditions
Xeric common garden conditions
a)
Aboveground biomass (g)
250
200
150
100
50
0
a
C
b
B
c
A
c
A
P. halepensis
P. pinea
P. nigra
P. sylvestris
b)
Foliar δ18O (‰)
32
30
28
26
24
22
20
a
A
a
A
b
B
b
B
c)
Foliar δ13C (‰)
-24
-25
-26
-27
-28
-29
-30
-31
-32
C
c
A
a
AB
bc
BC
b
d)
N (%)
2.5
2.0
1.5
1.0
0.5
0.0
b
C
a
A
a
BC
a
B
e)
P (mg · 100 g-1)
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
c
C
ab
A
a
B
bc
AB
f)
K (mg · 100 g-1)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
bc
C
c
AB
a
BC
ab
A
g)
N:P ratio
40
35
30
25
20
15
10
5
0
a
A
a
B
b
B
a
B
h)
N:K ratio
8
7
6
5
4
3
2
1
0
b
A
a
A
b
A
b
B

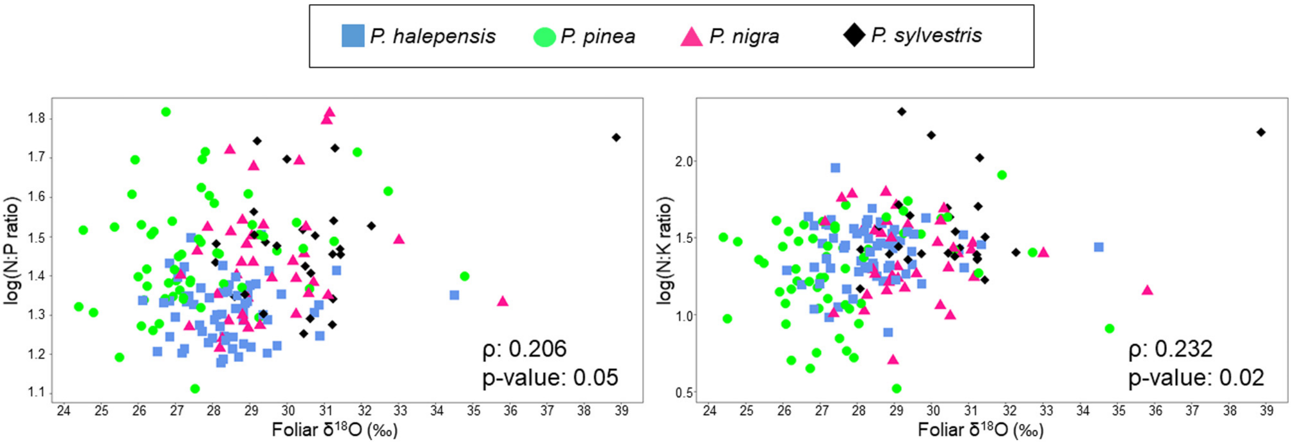


Fig. 4. Significant Pearson correlation coefficients (p-value <0.05) of foliar δ^{18}O with N:P ratio and N:K ratio across species. Low values of foliar δ^{18}O indicate high time-integrated stomatal conductance.

we found similar values of leaf nutrient concentrations and stoichiometric ratios between irrigated and non-irrigated saplings across pine species. In any case, it should be noted that moderate biomass growth stimulation by irrigation necessarily must have been accompanied by moderately enhanced nutrient uptake to maintain similar foliar nutrient concentrations in irrigated plants. On the other side, water supplementation only had an effect in the upper 20 cm of the soil profile, but saplings could probably be taking up nutrients from deeper parts of the soil, reducing therefore the possible interaction between irrigation and nutrient acquisition. Moreover, the potential benefit of short-term irrigation on plant nutrient uptake may have been masked by the high soil nutrient availability existing at the common garden site, which is located on former agricultural land (see Appendix S1, Table S2 in Salazar-Tortosa et al., 2018a for more information about soil characteristics of the common garden site). In agreement with our results, other studies conducted in nutrient-rich soils following 5 (Dobbertin et al., 2010) and 10 years (Schönbeck et al., 2018) of experimental irrigation did not document changes in leaf nutrient concentrations before and after drought stress release.

4.2. Poor acclimation to xeric conditions in mesic-adapted pine species

Under optimal nursery conditions, leaf N, P and K concentrations and N:P and N:K ratios fell within the typical ranges found for these pine species in the Mediterranean region (see Table S2 in Sardans et al., 2011). However, after transplanting to the common garden site under Mediterranean xeric conditions, we found dramatic increases in leaf N:P and N:K ratios, especially in the more mesic-adapted species (i.e. *P. pinea*, *P. nigra* and *P. sylvestris*). Hence, this ultimately led to severe drought-induced nutrient (P, K) deficiencies and stoichiometric imbalances that further impair photosynthesis and growth (Güsewell, 2004; He and Dijkstra, 2014; Yuan and Chen, 2015; Peng et al., 2019; Mariotte et al., 2020).

Under common garden conditions and regardless of water supplementation, the higher time-integrated stomatal conductance and cumulative transpiration of *P. halepensis* (inferred from lower leaf δ^{18}O values) enhanced soil nutrient uptake and allowed for a better and more balanced NPK status, as reflected by the significant correlations of foliar δ^{18}O with N:P and N:K ratios (Fig. 4). Moreover, δ^{18}O, N:P and S explained the highest percentage of variance in aboveground biomass, highlighting the key roles of adequate stomatal conductance, sufficient P and S uptake and balanced N:P ratios in enhancing pine growth under xeric conditions. In turn, a more balanced leaf NPK status along with higher foliar concentrations of other essential nutrients such as S and Zn in *P. halepensis*, allowed for a better water-use efficiency, as shown by the significant links of δ^{13}C with N, P, N:P and N:K ratios across species (Fig. 5). The much larger aboveground biomass production of *P. halepensis* when compared with the other species was therefore related to higher values of foliar δ^{13}C and P concentrations combined with lower values of δ^{18}O and N:P ratios under drought stress (Fig. 6). Hence, higher time-integrated stomatal conductance, transpiration and cumulative nutrient uptake were combined with higher photosynthetic rates and water-use efficiency in *P. halepensis,* relative to the other species (Farquhar et al., 1989; Scheidegger et al., 2000). This is in concordance with previous findings and indicates that *P. halepensis* can withstand xeric conditions without suffering from any significant nutrient starvation or imbalance, thanks to a looser stomatal regulation of transpiration that enhances nutrient uptake under water shortage, while at the same time achieving high photosynthesis and WUE$_i$ thanks to adequate leaf nutrient status (Voltas et al., 2008; Salazar-Tortosa et al., 2018a).

The inherent xeric conditions of the common garden site were the main factor determining the poor performance of the other three pine species. Specifically, *P. pinea*, *P. nigra* and *P. sylvestris* exhibited lower δ^{13}C values combined with poorer and more imbalanced leaf nutrient status than *P. halepensis* (Fig. 5). Lower carbon assimilation, WUE$_i$ and growth in these species may be linked to low transpiration-driven mass flow of nutrient to roots due to their tighter stomatal regulation of plant water flux under drought (Villar-Salvador et al., 2013; Salazar-Tortosa et al., 2018a, 2018b). Furthermore, the high Mg and Mn concentrations found in *P. pinea* leaves were not related to higher biomass production nor better water-use efficiency, which may indicate that these nutrients were taken up wastefully, thereby suggesting luxury consumption and possibly reflecting drought-induced inhibition of growth dilution effects for these particular nutrients (Knecht and Göransson, 2004; De la Riva et al., 2018). Moreover, non-irrigated saplings of *P. nigra* and *P. sylvestris* exhibited higher values of Ca and Mn than irrigated ones, reflecting that these nutrients were not limiting aboveground biomass production at the experimental site. Similarly, the high concentrations of B, Ca and Fe found in *P. sylvestris* needles may be attributable to strong inhibition of the normal growth dilution effects due to drought-induced growth impairment. Additionally, the high average values of foliar δ^{18}O in *P. nigra* and *P. sylvestris* indicate low stomatal conductance and transpiration combined with poor biomass growth (Fig. 6), which confirmed the inherent inability for adequate carbon assimilation under xeric conditions in these pine species. Their strict isohydric behavior likely led to rapid stomatal closure and carbon assimilation inhibition even under relatively mild drought stress (Flexas and Medrano, 2002;

Fig. 3. Mean values ± SE of (a) aboveground biomass, (b) foliar δ^{18}O, (c) foliar δ^{13}C, (d) N, (e) P, (f) K, (g) N:P ratio and (h) N:K ratio under optimal nursery conditions (i.e. seedlings before transplanting to the experimental field) and xeric common garden conditions (i.e. saplings two years after transplanting). Different lower-case letters indicate significant differences between species under optimal nursery conditions and different upper-case letters indicate significant differences between species under xeric common garden conditions (p-value <0.05).

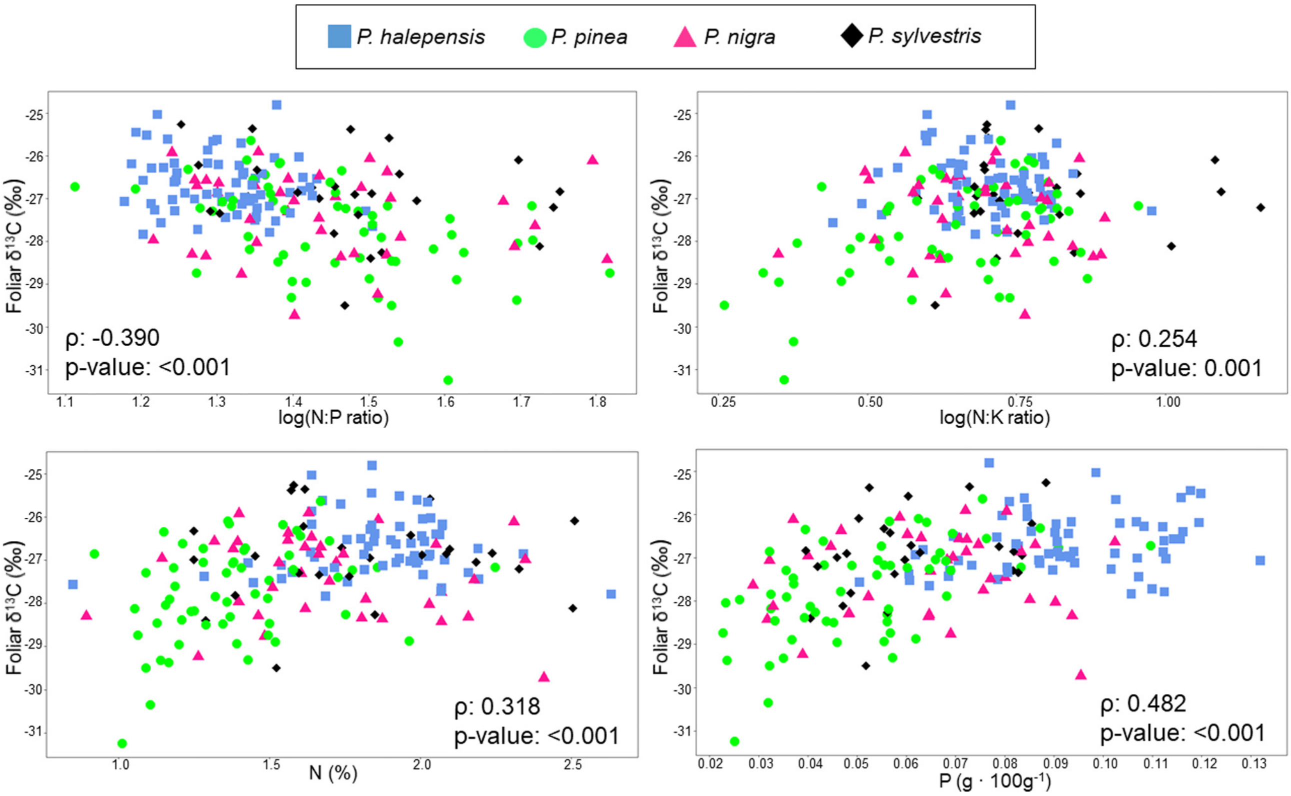


Fig. 5. Significant Pearson correlation coefficients (p-value <0.05) of N:P ratio, N:K ratio, N and P with foliar δ^{13}C with across species. High values of foliar δ^{13}C indicate high time-integrated water-use efficiency.

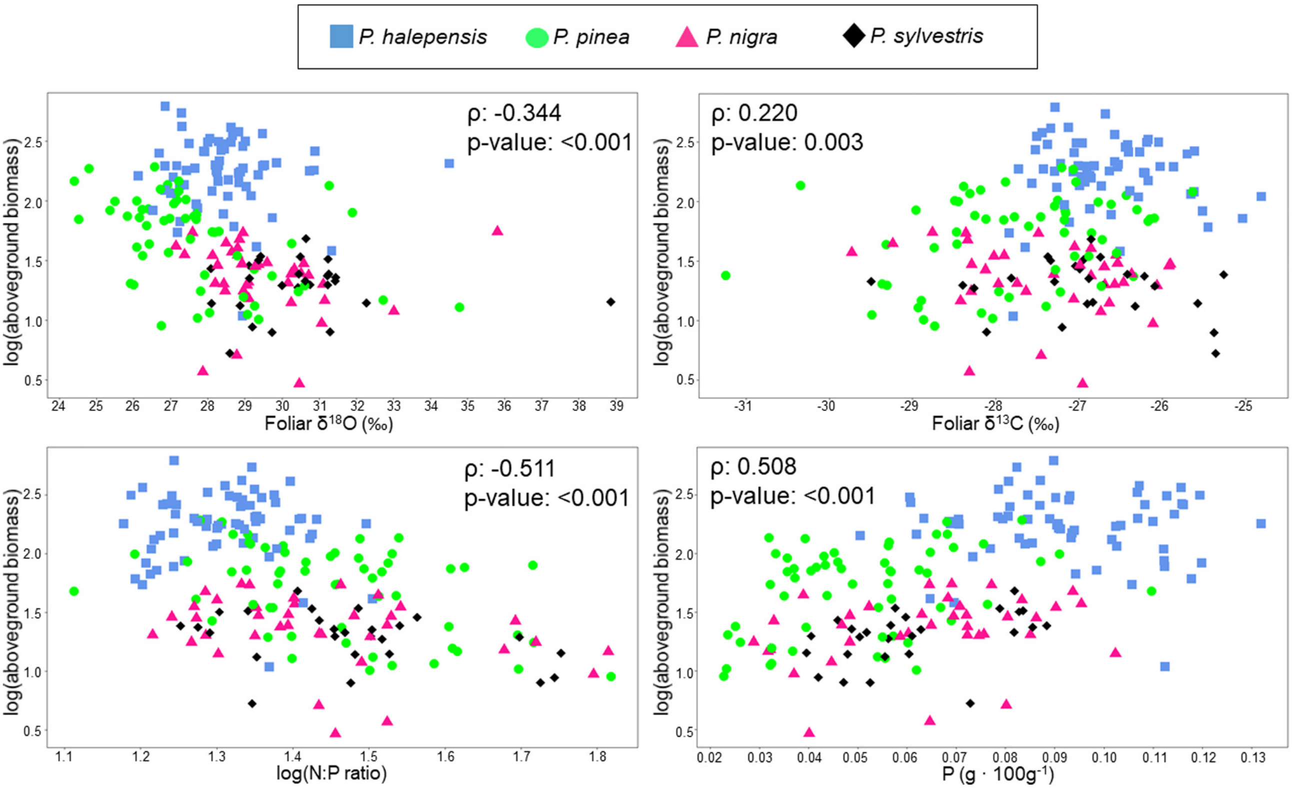


Fig. 6. Significant Pearson correlation coefficients (p-value <0.05) of foliar δ^{18}O, foliar δ^{13}C, N:P ratio and P with aboveground biomass across species. Low values of foliar δ^{18}O indicate high time-integrated stomatal conductance and high values of foliar δ^{13}C indicate high time-integrated water-use efficiency.

Manzanera et al., 2017; Salazar-Tortosa et al., 2018a). Therefore, the narrow physiological plasticity of saplings of mesic-adapted pine species in response to dry environmental conditions severely constrains the flexibility of their resource-use strategies during drought, thereby leading to severe nutrient deficit and imbalances that further exacerbate the negative impacts of drought stress on water relations, photosynthesis and growth (Güsewell, 2004; Yuan and Chen, 2015; Salazar-Tortosa et al., 2018a).

5. Conclusions

Short-term irrigation increased drought survival and moderately enhanced growth but did not alter the leaf isotopic composition and nutrient concentrations of water-stressed pine saplings. Overall, our findings are in concordance with the home-field advantage hypothesis (Kawecki and Ebert, 2004), because sapling performance was optimal when local environmental conditions were similar to those of the species native range (i.e. the drought-tolerant *P. halepensis*). In contrast, the strongly water-limiting conditions of the common garden site led to reductions of stomatal conductance and transpiration, severe nutrient deficits and imbalance and inhibition of photosynthesis and water-use efficiency in the saplings of pines growing beyond their ecological optimum (i.e. *P. pinea*, *P. nigra* and *P. sylvestris*). Accordingly, on-going pine afforestations and reforestations in the Mediterranean Basin with mesic pine species may lack the physiological plasticity and adaptations necessary to tolerate increasingly xeric conditions in a climate change scenario in drier areas. Thus, clarifying the underlying mechanisms that link the vulnerability to drought-induced nutrient deficits with plant-water use strategies at early life stages can help select the most suitable and locally adapted pine species for future afforestation and reforestation programs in a context of climate aridification.

CRediT authorship contribution statement

Raquel Juan-Ovejero: Data curation, Formal analysis, Visualization, Writing – original draft. **Jorge Castro:** Conceptualization, Methodology, Funding acquisition, Writing – review & editing. **José I. Querejeta:** Conceptualization, Methodology, Writing – review & editing.

Declaration of competing interest

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

Acknowledgements

We are grateful to the Centro Nacional de Recursos Genéticos Forestales “El Serranillo” (MITECO) for cultivating the plants during the nursery stage. Moreover, we want to thank two anonymous reviewers for improving the manuscript with their comments. This study was supported by the projects RESISTE (P18-RT-1927) of the Consejería de Transformación Económica, Industria, Conocimiento y Universidades from the Junta de Andalucía, and PID2019-107382RB-I00 of Ministerio de Ciencia e Innovación from Gobierno de España. Funding for open access charge: Universidad de Granada / CBUA.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.154797.

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