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THE IMPACT OF DROUGHT ON THE LEAF ANATOMY AND HYDRAULIC TRAITS OF *PINUS PINASTER AITON*, *PINUS PINEA L.* AND *PINUS HALEPENSIS MILL.*

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"Hold on to the memories, they will hold on to you."
Taylor Swift

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Resumo

Num cenário de mudanças climáticas, com um aumento dos eventos de seca severos, entender as estratégias adaptativas das plantas para lidar com a escassez de água é crucial. A região mediterrânica é particularmente vulnerável a condições climatéricas extremas, como ondas de calor e períodos de seca intensa. Durante os primeiros estágios de desenvolvimento, a mortalidade das árvores é alta e, portanto, é cada vez mais importante entender o impacto da seca nas plântulas. *Pinus pinaster, P. pinea e P. halepensis* são espécies importantes nas florestas mediterrâneas, cada uma com nichos ecológicos distintos e diferentes características adaptativas. Fizemos uma experiência de exclusão total de água com plântulas de *P. pinaster* (duas proveniências), *P. pinea* e *P. halepensis*, e quantificamos a sua resposta medindo o crescimento (diâmetro do caule, altura, biomassa), ponto de perda de turgescência (TLP) e características anatómicas das agulhas e traqueídos.

Durante a estação de crescimento, a primavera foi extremamente seca, diminuindo as diferenças entre o controlo e o tratamento de seca, levando a uma alta mortalidade das plântulas em ambos os tratamentos. No entanto, o *P. halepensis* apesar de ser mais tolerante à seca do que o *P. pinaster* e o *P. pinea*, teve uma mortalidade mais precoce e elevada no tratamento de seca. O valor de TLP, antes da aplicação do tratamento de seca, foi muito maior em *P. halepensis* em comparação com o *P. pinaster*, com o *P. pinea* apresentando valores intermédios. Embora as plântulas de o *P. halepensis* tivessem alocado mais biomassa às raízes, comparando com o *P. pinaster* e o *P. pinea*, apresentou uma redução da biomassa radicular no tratamento de seca, enquanto as outras duas espécies não apresentaram diferenças entre os tratamentos. Além disso, o *P. halepensis* no tratamento de seca mostrou um aumento da espessura da epiderme das agulhas, e uma redução da relação lúmen/espessura da parede celular dos traqueídos para valores <2, que define as células do tipo lenhoso tardio. Aparentemente, o tratamento de seca teve impacto diferencial nas respostas do *P. halepensis*. *Pinus pinaster* e *P. pinea* não apresentaram diferenças nos parâmetros medidos entre o tratamento controlo e seca, embora a taxa de sobrevivência do *P. pinaster* tenha sido maior no controlo. Uma exceção foi o tamanho das agulhas do *P. pinaster* Selected, uma das proveniências utilizadas, que foi menor no tratamento de seca, reduzindo assim a área foliar exposta à evapotranspiração. Também mostrou um aumento do ratio entre a biomassa da parte aérea e da raiz no tratamento de seca, um resultado contraintuitivo de acordo com a teoria de partição ótima. Embora as diferenças entre o tratamento controlo e de seca tenham sido reduzidas devido à primavera muito seca,

as três espécies parecem ter respondido de maneira diferente à exclusão de água, com o *P. halepensis*, e de alguma forma o *P. pinaster*, diferenciando a sua resposta em relação aos tratamentos, e o *P. pinea* não mostrando alteração de nenhum dos parâmetros medidos em resposta aos mesmos tratamentos.

Palavras-chave: agulhas, Mediterrâneo, ponto perda de turgescência, potencial hídrico, xilema

Abstract

In a scenario of climate change conditions and the associated risk of more frequent and severe drought events, understanding the adaptive strategies of plants to cope with water scarcity is crucial. The Mediterranean region is particularly vulnerable to extreme weather conditions, such as heatwaves and intense drought periods. During the first stages of development, tree mortality is high and thus it is increasingly important to understand the impact of drought on seedlings. *Pinus pinaster*, *P. pinea*, and *P. halepensis* are important species in Mediterranean forests, each with distinct ecological niches and adaptive traits. We have made a total water exclusion experiment with seedlings of *P. pinaster* (two provenances), *P. pinea* and *P. halepensis*, and quantified their response by measuring growth (stem diameter, height, biomass), turgor loss point (TLP) and anatomical traits of the needles and tracheids.

During the growing season, the spring was extremely dry, diminishing the differences between the control and drought treatment, and leading to a high mortality of the seedlings in both treatments. Nonetheless, *P. halepensis,* despite being more drought-tolerant than *P. pinaster* and *P. pinea*, experienced an earlier and higher mortality rate in the drought treatment. The TLP, before the application of the drought treatment, was much higher in *P. halepensis* compared to *P. pinaster*, with *P. pinea* showing intermediate values. Although the seedlings of *P. halepensis* allocated more biomass to the roots, compared with *P. pinaster* and *P. pinea*, it showed a reduction of the root biomass in the drought treatment, while the other two pine species showed no differences between the treatments. Also, *P. halepensis* in the drought treatment showed an increase of the needle epidermis thickness and a reduction of the ratio lumen/cell wall thickness of the tracheids to values <2, that defines latewood-like cells. Apparently, *P. halepensis* was reacting to the drought treatment. *Pinus pinaster* and *P. pinea* showed no differences in the parameters measured between the control and drought treatment, although the survival rate of *P. pinaster* was higher in the control treatment. One exception was the needle size of the *P. pinaster* Selected, one of the provenances used, that was lower in the drought treatment, thus reducing the leaf area exposed to evapotranspiration. It also showed an increase of the shoot/root biomass in the drought treatment, a counterintuitive result according with the optimal partitioning theory. Although the differences between the control and drought treatment were reduced by the very dry spring, the pine species have responded differently to the treatments, with *P. halepensis*, and *P. pinaster*, responding differently to the water exclusion, and *P. pinea*

showing no differential response of the measured parameters between the control and drought treatments.

Introduction

In a scenario of climate change conditions and the associated risk of more frequent and severe drought events, understanding the adaptive strategies of plants to cope with water scarcity is crucial. Drought is a significant environmental stressor that affects plant growth, development, and survival. This dissertation aims to investigate the impact of drought on the leaf anatomy and hydraulic traits of three important Mediterranean pine species: *Pinus pinaster*, *Pinus pinea*, and *Pinus halepensis*.

Climate change in the Mediterranean ecosystems

Climate change is a growing threat to global biodiversity and ecosystem functioning. Numerous studies, including long-term monitoring, have been conducted to investigate the impact of climate change on plant physiology, carbon and nutrient cycling among others (Aud *et al*., 2020). Comparing the preindustrial and present day, land surface global average air temperature increased about 1.53°C (Jia *et al*., 2019). The main causes for this increase are related to human activities, like burning fossil fuels, deforestation and agricultural activities, that lead to an increase in atmospheric CO_2, a greenhouse gas (Hegerl *et al*., 2019).

Extreme weather conditions are increasing with more frequent heatwaves and intense drought periods in the Mediterranean basin (Jia *et al*., 2019). For the Iberian Peninsula, since 1970 it is already observed an average increase in temperature close to 2 °C and a reduction of rainfall up to 20% (Allard *et al*., 2013). With 3°C global warming in 2100 drought stress could get up to 5x increase in comparison to the current situation, with the Mediterranean regions expected to be the most significantly affected (Figure 1, Cammalleri *et al*., 2020).

Trees and forests are crucial to mitigate climate change conditions. More than half of the carbon stored in soils and vegetation worldwide, 662 billion tons, is found in forests (FAO, 2022). Forests absorbed more carbon than they emitted between 2011–2020 due to replanting, better forest management, and other factors, despite the decreasing trend of forest area in certain countries (FAO, 2022). Forest areas can also alter the albedo, atmospheric water vapor, and aerosols, like dust and microparticles, that lead to a reduction in heat trapped (FAO, 2022).

The Mediterranean region is characterized by hot dry summers and mild wet winters. Summer lasts ~3 months, and rain is concentrated in the cooler months. It is known for its diverse and unique vegetation, including evergreen sclerophyllous forests (e.g., holm oak, cork

oak, and Aleppo pine), maquis (dense shrubland), garrigue (low-growing shrubland), and phrygana (sparse, herbaceous vegetation) (Allard *et al*., 2013). Although adapted to water stress, under drought conditions Mediterranean forests have lower productivity and growth, that can increase the mortality rates (Hevia *et al*., 2020). Drought can have severe impacts on tree growth and survival, as demonstrated on species such as *Pinus halepensis* (Camarero *et al*., 2015) and *Pinus sylvestris* (Garcia-Forner *et al*., 2016). Even when recovery from drought is possible it might impact the development for the following years (Camarero *et al*., 2015).

Mediterranean forests face significant challenges such as the increasing temperature, forest fires, poor land management, overexploitation, pollution as highlighted in the "State of Mediterranean Forests 2013" report by Allard *et al*. (2013).
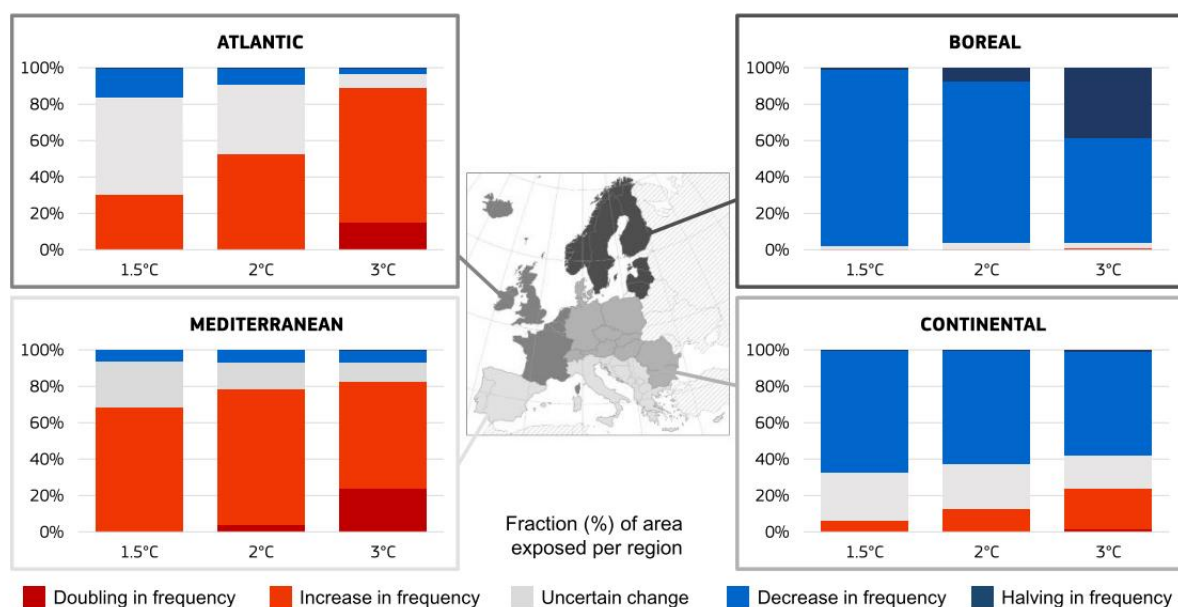

Figure 1. Frequency of European sub-regions areas exposed to drought occurrence, according to the Technical Report "Global warming and drought impacts in the EU" by Joint Research Centre (JRC). From Cammalleri *et al*. (2020).

Mediterranean pine species

Mediterranean pines have developed strategies to withstand summer water scarcity. Different species can have different strategies to cope with drought conditions but their long lifespan might prevent them from adapting quickly to environmental changes (Lindner *et al*., 2010). In fact, the increasing severity of droughts have been associated with forest dieback of several conifers in the Mediterranean, from the more Eurasian pine such as *P. sylvestris* L., to the most drought tolerant *P. halepensis* Mill. (Gracia *et al*., 2002).

The Mediterranean pines *P. pinaster*, *P. pinea* and *P. halepensis* are important species of the Mediterranean forests, and they have different ecological niches and ecophysiological characteristics. Thus, it is important to increase our knowledge on their ability to cope with the climate change conditions.

Pinus pinaster Aiton, commonly known as maritime pine, belongs to the family Pinaceae and is native to the western Mediterranean basin, including Portugal, Spain, France, and Morocco (Castroviejo, 1993). It is commercially important for its high-quality wood and resin, which is used in various industrial applications (Figueiredo *et al*., 2014). Maritime pine is well adapted to Mediterranean climate, where it is an important species of the forest ecosystem (Portoghesi, 2006), although it grows better in areas with ocean influence, which are still warm but wetter than typical Mediterranean conditions. The growth of *P. pinaster* is highly influenced by environmental factors such as temperature and precipitation, with summer rainfall influencing the germination and survival of seedlings (Ruano *et al*., 2009).

Maritime pine is adapted to a wide range of soil types, including sandy and nutrient-poor soils, (Alía & Martin, 2003). This pine species exhibits a range of morphological and physiological adaptations to water stress, including changes in stomatal density and needle anatomy (Maestre *et al*., 2021). *Pinus pinaster* exhibits high plasticity in its needle anatomy, which allows for better water-use efficiency and higher resistance to embolism under water scarcity (Bert *et al*., 2021). It is a drought-avoiding species known for its high stomatal sensitivity to water stress (Picon *et al*., 1996). The species has a high genetic diversity, and different provenances have been identified with varying degrees of drought tolerance (Moran *et al*., 2017). Different maritime pine populations have different responses to drought stress such as smaller height and needle growth or higher root growth, and that genetic variation plays a role in drought tolerance (Moran *et al*., 2017; Perdiguero *et al*., 2013). Genetic conservation efforts such as seed source selection, conservation stand *in situ* and *ex situ* with clonal and seed banks, and plantations with seeds of threatened populations, have been made to preserve this diversity and promote the sustainable use of the species (Alía & Martin, 2003).

Pinus pinea L. is commonly known as stone pine or umbrella pine, it belongs to the Pinaceae family and is distributed in all the Mediterranean Basin (Farjon, 2010). *Pinus pinea* is a valuable species for afforestation and reforestation due to its resistance to drought, wind, and fire (Fady *et al*., 2004). It is also an important species for biodiversity conservation, providing habitats for many species of insects, birds, and mammals (Abad Viñas *et al*., 2016). The seeds of *P. pinea*

are widely used in gastronomy, however, the over-harvesting of cones for pine nut production is one of the main threats to the species in Spain, Portugal, Italy, Tunisia and Turkey (Viñas *et al*., 2016b). It is a slow-growing tree species that can reach up to 20-35 meters height with a spreading, open-crowned, and umbrella-like shape (Farjon, 2010). *Pinus pinea* growth and development was shown to be more influenced by autumn temperatures, and by winter and spring precipitation (De Luis *et al*., 2009).

Pinus pinea is a drought-tolerant species adapted to the Mediterranean climate, where it grows in sandy or stony soils, often in coastal areas, but also in the mountains, up to an altitude of 1200 m (Abad Viñas *et al*., 2016; Manzanera *et al*., 2017). *Pinus pinea* is a genetically uniform species with no strong geographical structure in adaptive traits (Fady *et al*., 2004). It can tolerate drought, salt spray, and nutrient-poor soils (González, 2007), and has a relatively low water requirement and is known to reduce transpiration rates in response to water stress (Deligoz & Gur, 2015). The leaves are flattened and have a triangular cross-section, which is an adaptation for reducing water loss (Dörken & Stützel, 2012).

Pinus halepensis Mill., commonly known as Aleppo pine, belongs to the Pinaceae family (Farjon, 2013) and is widely distributed throughout the Mediterranean basin, including North Africa, the Middle East, and southern Europe, with the western most populations in Spain (De Luis *et al*., 2009). *Pinus halepensis* has been introduced in many other parts of the world, particularly in regions with similar climates, including southern Africa, South America, and the southwestern United States (Espelta, 2016). This species has a broad ecological amplitude and can grow in a wide range of habitats, from coastal sand dunes to mountain slopes. It is also a very resilient species, able to withstand harsh environmental conditions such as drought, fire, and extreme temperatures (Mauri *et al*., 2016). *Pinus halepensis* is an important species in the Mediterranean region, and its potential role in restoration efforts has been explored (Maestre & Cortina, 2004) due to its ability to survive in harsh environmental conditions, like drought (Manzanera *et al*., 2017). Efforts have been made to conserve the genetic diversity of *P. halepensis*, as it is an economically and ecologically valuable species (Fady *et al*., 2003).

Aleppo pine is adapted to withstand long periods of drought (Borghetti *et al*., 1998) and its growth and wood traits have been found to be influenced by both climate and provenance (Hevia *et al*., 2020). However, in southeastern Spain research has shown that drought can lead to growth decline in Aleppo pine (Gazol *et al*., 2017; Sánchez-Salguero *et al*., 2010). Moreover, size-mediated climate-growth relationships in *P. halepensis* have been investigated, highlighting

the importance of tree size in the growth response to climate (De Luis *et al*., 2009). Its hydraulic adjustments underlying drought resistance, involve a complex coordination between the water transport system and stomatal regulation (Klein *et al*., 2011). The species' resistance to embolism have been shown to be impacted by long-term drought (Borghetti *et al*., 1998). Wood traits and growth of *P. halepensis* are affect by both provenance and climate, with the former having a greater effect on wood density and the latter on growth rate (Hevia *et al*., 2020).

Overall these three pine species, although present adaptations to Mediterranean climate, namely to the summer drought, face several threats namely habitat fragmentation, overexploitation, and climate change-induced droughts, which can lead to significant declines in their growth and survival (Abad Viñas *et al*., 2016b; Calama *et al*., 2019; Sánchez-Salguero *et al*., 2010).

Biomass allocation and growth rates under drought

Biomass allocation and changes in growth rates can be important to manage resources under drought conditions (Pearson & D'Orangeville, 2022). The optimal partitioning hypothesis states that to maximize plant performance, plants allocate a greater proportion of resources, such as biomass, to the structure that captures the scarce resources such as water, nutrients, or light. For instance, when water is becoming scarce, plants devote more biomass to establishing roots (Eziz *et al*., 2017). A more modern perspective describes the development of the plants organs with different rates and related to plant body size, showing a variation between root, stem, leaf, and reproductive parts (Eziz *et al.*, 2017). *Pinus* species allometric analysis have shown a change in biomass allocation in response to water stress instead of plant size, where *P. halepensis* and *P. pinaster* had considerable population differences in their phenotypic response to the water stress treatment (Chambel *et al*., 2007). On the other hand, *P. pinea* showed consistent, high levels of allocational plasticity, indicating a wider ecological tolerance (Chambel *et al*., 2007). This reallocation strategy may enhance water uptake efficiency and reduce water loss through transpiration.

In the case of seedlings, like the ones used in our experiments, it has been shown that watering has more impact full on their second year or development. Showing how water in early stages can impact their development and later on their survival (Ruano *et al*., 2009).

Bachofen *et al*. (2019) studied the biomass partitioning in Scots pine (*Pinus sylvestris*) and European black pine (*Pinus nigra*) seedlings under different light conditions, water availability

and CO_2. It was observed that under shade and drought conditions, Scots pine seedlings showed a strong decrease in shoot and root biomass, while in European black pine biomass allocation was unaffected by the same treatments. These findings highlight the species-specific responses to drought stress and the importance of considering other environmental factors to understand biomass allocation.

Leaf anatomy under drought

The shape of the leaves can affect photosynthetic capacity and transpiration rates. The leaves of Gymnosperms are much more limited in shape compared to Angiosperms. Based on the width/thickness ratio (WTR) of the cross-section, Pinaceae leaves can be mainly divided into two types, needlelike (mean WTR < 2) and flattened (mean WTR > 2). Needlelike leaves appear to be more drought-resistant than flattened leaves, and also present a higher photosynthetic capacity (Du *et al*., 2020). Changes in leaf anatomy, such as reduced leaf area, increased leaf thickness, and altered stomatal density, can reduce transpiration but can also reduce the tree's ability to recover from drought stress (Binks *et al*., 2016). These anatomical changes can limit photosynthetic capacity and nutrient uptake, making trees more vulnerable to prolonged water scarcity. A comparison of leaf characteristics of *P. sylvestris*, *P. pinea* and *P. halepensis* under drought conditions showed different responses (Manzanera *et al*., 2017). *Pinus sylvestris* exhibited a decrease in leaf area and stomatal conductance, which helped reduce water loss through transpiration. *Pinus pinea*, on the other hand, displayed a similar reduction in stomatal conductance but maintained its leaf area. This indicated an adjustment to maintain photosynthetic activity while minimizing water loss. *Pinus halepensis* showed a significant decrease in leaf area and stomatal conductance, accompanied by a decrease in leaf water potential (Manzanera *et al*., 2017).

In pine species, such as *P. halepensis* and *P. pinaster*, specific adaptations of the needles help these trees withstand drought conditions. For instance, the primary needles of *P. halepensis* are covered by wax, have thick cuticles and sunken stomata, which reduce water loss (Boddi *et al*., 2002).

Xylem hydraulic traits and drought

A global meta-analysis of the leaf water potential at turgor loss point (TLP) of different species across different biomes showed that TLP was strongly correlated with water availability and can be used as a parameter to indicate drought tolerance (Bartlett *et al*., 2012).

Drought stress can lead to xylem embolism, impairing water transport and eventually causing tree mortality. Brodribb and Holbrook (2005) revealed how water stress deforms tracheid's peripheral to leaf veins in tropical conifers, further affecting water transport capacity.

Xylem functional traits can be used as indicators of tree drought tolerance. Species with higher hydraulic efficiency, represented by wider conduits and lower hydraulic resistance, are more susceptible to drought embolism (Borghetti *et al*., 2020). Thus xylem traits are crucial in determining a tree's ability to cope with water scarcity (Borghetti *et al*., 2020). However, the response of xylem traits to acute or prolonged drought can be different (Guérin *et al*., 2021). Pine trees under acute drought showed a reduction in the hydraulic conductivity, mainly due to a reduction of the tracheids lumen. However, under prolonged drought, the xylem was hydraulically more efficient, showing a lower $(t/b)^2$ value, where t is the cell double wall thickness and b is the lumen diameter (Hacke et al., 2001), but because the tracheid wall was thinner, and thus at a higher risk of implosion (Guérin *et al*., 2021).

Tracheids, specialized xylem cells responsible for water transport, have been extensively studied in relation to drought stress. In *P. pinaster*, drought stress altered the timing and intensity of xylem growth, indicating a tight coupling between tracheid development and water availability (Garcia-Forner *et al*., 2019). Another study showed that under water-limited conditions the plastic response of tracheids in *P. pinaster* was to adjust the lumen size rather than wall thickness (Carvalho *et al*., 2015). This adjustment strategy is a trade-off between water transport efficiency and embolism safety. The effects of drought on xylem anatomy and water-use efficiency in two co-occurring pine species, *P. nigra* and *P. sylvestris*, was translated in an increased conduit size but reduced tree-ring width, mainly due to a reduction in the number of tracheids (Martin-Benito *et al*., 2017). This response to drought indicates a trade-off between hydraulic efficiency and carbon assimilation. *Pinus halepensis*, growing in a semi-arid climate with long seasonal droughts, showed narrow tracheids and reduced hydraulic conductivity to adapt to the low soil water availability (Klein *et al*., 2011).

Objectives

This study aims to investigate the impact of an extreme drought event on growth, leaf anatomy and hydraulic traits of seedlings of three Mediterranean pine species: *Pinus pinaster, Pinus pinea,* and *Pinus halepensis*. By examining the adaptive strategies of these Mediterranean pine species, the study will shed light on their ability to withstand water scarcity and provide insights into their potential survival and growth under changing climatic conditions. The findings of this research will contribute to our understanding of species resilience in drought-prone environments, valuable information for forest management and conservation strategies.

Hypothesis

Considering the ecological distribution of the three pine species in the Iberian Peninsula along a drought gradient, from lower (*P. pinaster*), intermediate (*P. pinea)* to higher (*P. halepensis)*, we expect that:

1. Drought will reduce the growth in height and stem diameter of all pine species;
2. The biomass allocation to the roots will be higher in *P. halepensis* under high drought stress;
3. *Pinus halepensis* will be the most resistant species to cavitation;
4. The needle size and area will be lower for all species, in response to drought;
5. All pine species will show a lower number of tracheids, and will have a lower lumen area, under drought stress;

Materials and Methods

Species selection

  In November 2021 were acquired seedlings around 6 months of Maritime pine (*Pinus pinaster* Aiton) from two different provenances, Stone, or Umbrella pine (*Pinus pinea* L.) from one provenance, and Aleppo pine (*Pinus halepensis* Mill.) from one provenance. The two provenances of *P. pinaster* were classified as Qualified and Selected. According to *Decreto-Lei 205/2003* from 12th September *P. pinaster* Qualified type was obtained from seed orchards, parents of families, clones, or clonal mixtures, and they are phenotypically selected at the individual level and provenance from the National Forest of Escaroupim (Salvaterra de Magos, Portugal). The Selected type was obtained from a stand located within a single region of provenance, which has been phenotypically selected at the population level. Thus, we have *P. pinaster* Selected from the region of Ovar (Portugal), *P. pinea* Selected from Bêbeda de Cima (Sines, Portugal) and *P. halepensis* Selected from the East of the Iberian Peninsula (Spain).

  Pinus pinaster Qualified, P. pinaster Selected and P. pinea Selected were acquired from the nursery Veiga & Silva, Lda in Anadia (Aveiro, Portugal), and P. halepensis from VIVEROS LA DEHESA in Valdeobispo (Cáceres, Spain).

Experimental design

  A pot experiment was conducted using four groups: *P. pinaster* Qualified (PpiQ), *P. pinaster* Selected (PpiS), *P. pinea* Selected (PpnS), and *P. halepensis* Selected (PhaS).

  The experiment began with 120 plants (30 individuals per group). Seedlings were transferred to 8L pots in January 2022 with soil composed of pine bark hummus, Sphagnum blonde peat and heat-treated pine bark ("Siro-Strat Melhorado" by Siro) and mixed with sand, with a ratio of 2:1. All plants were placed in an open-air plot in InProPlant facilities in Coimbra (40°10'56.4"N 8°24'47.7"W) and watered until April 2022. By the end of April, the individuals were divided into two treatments: Drought (DT) and Control (CT). The DT treatment was a full water exclusion using plastic rain tarps to cover the plants' pots close to the soil.

  Three campaigns were planned to be carried out: April, June, and August 2022. However, due to a high mortality rate of the seedlings (see the results), the experiment ended in mid of June 2022. The measurements made in the first campaign (April) were used as baseline before the DT treatment was established.

In April 2022 a meteorological station was installed next to the plot (DL2e Data Logger; Delta-T Devices, Burwell, Cambridge, UK). Precipitation, temperature, air relative humidity, and solar radiation were measured every half an hour.

Mortality

Data was collected every week to assess the rate of mortality. A small strip of the bark was removed to evaluate if the phloem was alive, with green and brown color indicating alive or dead phloem, respectively.

Plant Growth, Biomass and Soil Water Content

In each campaign, stem diameter, plant height and soil water content (SWC) of all individuals were measured. Diameter was measured with a digital caliper, in the base of the main stem in a marked spot, in two different directions to account for variability in the stem circumference. Height was measured from the same marked spot used for diameter, using a ruler. SWC was measured using a moisture meter with 5 cm depth (HH2 Moisture Meter, Delta T Devices) and two points were taken to account for variability of moisture in the soil.

In each campaign, 3 individuals per species and treatment were cut to determine the Turgor Loss Point (see below), biomass and for needle and stem anatomical analysis. The individuals were separated into shoots and roots, and the roots were carefully cleaned. Afterwards the biomass of the shoots and roots was dried in an incubator at 60ºC for 48h and weighed.

Turgor Loss Point (TLP)

Pressure-volume curves are used to determine the plant's water status. Pressure volume curves describe the relationship between bulk water potential (Ψ_w, movement driven by a pressure gradient) and relative water content (RWC) measured on a drying leaf or shoots (Halbritter *et al*., 2020). The RWC is calculated using the following equation:

$$RWC = \frac{(fresh\ weight\ (g) - dry\ weight\ (g)) * 100}{turgid\ weigth\ (g) - dry\ weight\ (g)}$$

where the fresh weight is the weight taken before and after each Ψ_w measurement, the turgid weight is the weight of the sample taken before the first measurement, and the dry weight is the weight of the dried sample taken after all measurements are completed.

From the biomass of the tissues at different drying levels and the associated water potential, the turgor loss point (TLP) is calculated. TLP indicates the point when turgor pressure in cells is zero and can be used as an indicator of tolerance of leaves to drought stress (Halbritter *et al*., 2020). By plotting the inverse of the negative Ψ_w (water potential) against the relative water deficit (100 – RWC (relative water content)), the resulting graph shows a non-linear and linear portion (Figure 2). From this graph, TLP can be estimated from the inflection point where the non-linear portion meets the linear portion (Schulte & Hinckley, 1985). The linear portion can be extrapolated to the y-intercept, which will give the Ψ_o at full turgor pressure (Halbritter *et al*., 2020).
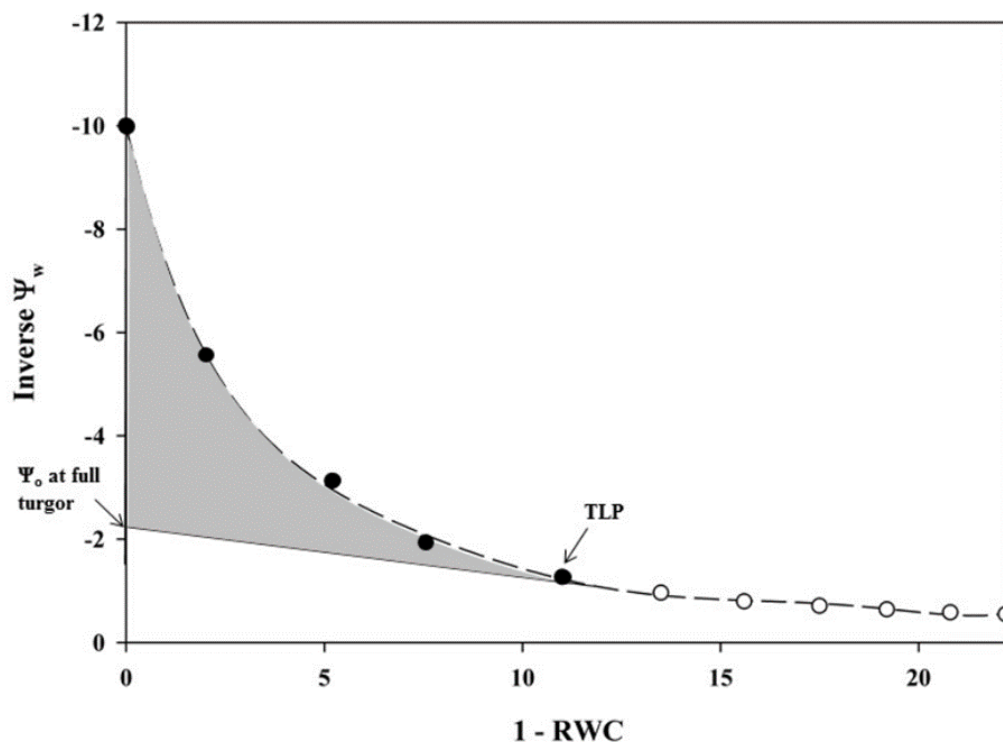

Figure 2. Example of a pressure-volume curve. The linear portion (solid line) shows the osmotic potential (Ψo), the curved portion (dashed line) is the water potential before the turgor loss point (TLP), and the shaded area is the turgor potential (Ψp). From Halbritter *et al*. (2020).

The Scholander pressure chamber (MANOFRÍGIDO) is used to obtain the water potential, by applying positive pressure to equal the tension of the water within the xylem (Figure 3, Pérez-Harguindeguy *et al*., 2013).

In each campaign, 3 individuals per group (PpiS, PpiQ, PpnS, PhaS) and treatment (CT and DT) were randomly selected, and the entire plant was collected. The day before the measurements, plants were collected at the end of the day, and wrapped in plastic bags to

decrease water loss and respiration. They were stored in the fridge overnight to have the maximum turgid weight, for a maximum of 12 hours to avoid over rehydration of tissues. In the following morning, the samples were removed from the fridge and left at room temperature on the bags to reduce air desiccation.

Figure 3. A Scholander pressure chamber used to measure water potential.

The shoot sample was placed in the gasket with the stem pointing up and a positive pressure was enforced gradually until water was visible with a magnifying glass on the stem cut. The positive pressure applied in the chamber is equal to the negative pressure within the stem. After the measurement of the water potential, the sample was weighted to assess fresh weight. The sample was then placed on the table to gradually desiccate, and the water potential and fresh weight was measured in intervals of 5, 10, 20 minutes, depending on the humidity of the room and the speed at which the sample loses water. After the sample had lost some water, sample intervals were increased from every 30 minutes to 2 hours. Measurements were taken up to 12 points to have enough data to calculate the TLP. After all measurements were done the samples were dehydrated in an incubator at 60 ºC for 48h to obtain the dry weight.

The pressure-volume curve fitting to find the inflexion point between the non-linear and linear portion of the curve that indicates the TLP, was made using the algorithms developed by Schulte

and Hinckley (1985). Due to a methodological error in the measurements of the samples of PpiS from April, the TLP data for that species and month was discarded.

<u>Tissue preparation for anatomical analysis</u>

Needles from the apical bud were removed and needle size was measured with a ruler longitudinally. The needles and a portion of the stem were collected and placed in histological cassettes and submerged in a solution of FAA (formaldehyde-acetic-alcohol) for 7 days, and then placed in a solution of ethanol 50% for 5 days. The step of dehydration of the samples is done using increasing ethanol concentrations (70% x2, 90% x2, 95% x2, 100% x2), followed by the clearing step using ClearRite™ 3 (Richard-Allan Scientific) (50% and 100% x2) and finally infiltration with paraffin at 65°C for two hours (x2). Afterwards, the samples were embedded in a paraffin block using a modular tissue embedding center (HistoDream by Milestone), and then sliced using a rotary microtome (Microm HM 340E). To obtain the microsections of the needles, since the tissue was soft, the paraffin blocks were submerged in ice for a minimum of 15 min to harden the tissues. The micro-sections of ~7 µm thickness were transferred to a water bath at 32°C to stretch the tissue and placed in a microscope slide pre-treated with glycerol albumin to enhance the section's adhesion. For the staining procedure, the paraffin needs to be removed. To start the deparaffination process, the slides were left in a heat chamber at 30 ºC overnight, followed by a succession of 100% ClearRite ™ 3 (x2) and 100% ethanol (x2) solutions to remove the paraffin. The slides were then immersed in Astra Blue and Safranin O (Sigma, 84120-25G) for 10 minutes, followed by a quick rinse in running water to remove excessive stain. Astra blue stains in blue the polysaccharides of cell wall's like cellulose and pectin's (Kraus *et al*., 1998), indicating living cells, and Safranin stains in red lignified cell walls (Vazquez-Cooz & Meyer, 2002), indicating dead cells. The definitive preparations were mounted with Eukitt (45% acrylic resin and 55% xylenes) and stored overnight in a heat chamber at 30°C. All steps were performed in a chemical hood, and waste materials were disposed of properly.

The anatomy of the needles and stems were observed using a microscope (LEIKA DM4000B), with magnification of 50x, for the cross-section of the needles, and 200x, for the cross-section of the stems. Images were obtained using a camera (LEICA DFC295) attached to the microscope. The obtained images were then analyzed using the software ImageJ.

In the cross section of the needles several measurements were obtained: needle width (µm), needle thickness (µm), epidermis thickness (µm), needle cross section total area (mm^2), central

cylinder area (mm^2), parenchyma area (mm^2) and vascular bundle area (mm^2) (Figure 4). The number of resin canals was also registered.
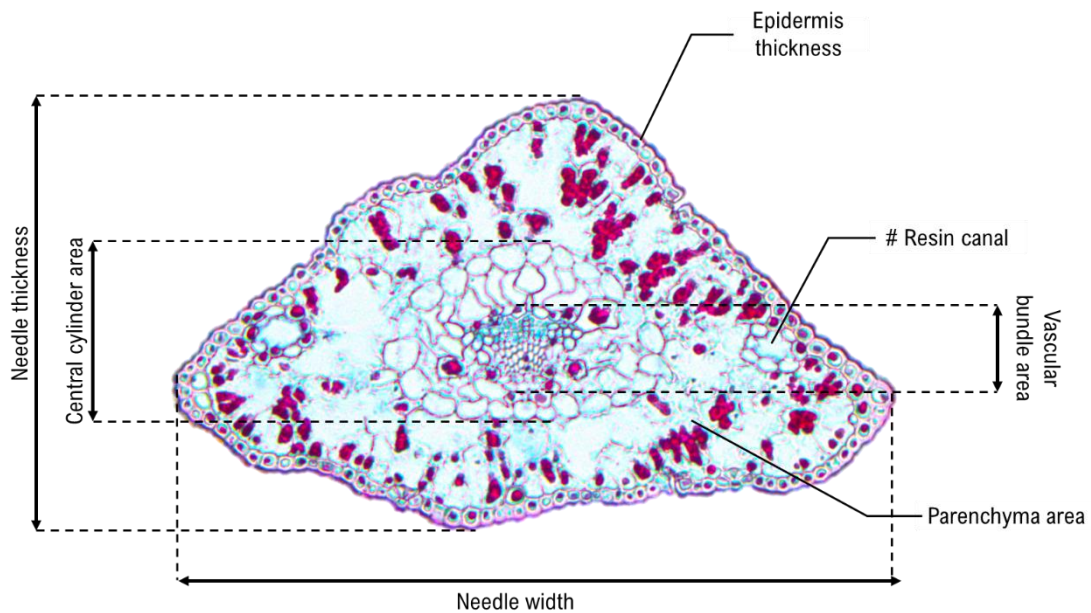

Figure 4. Cross section for needle of *Pinus pinaster* stained with Astra blue and Safranin to show the parameters measured in the anatomical assessment.

In the stem cross-section of April and June the total number of tracheids were counted and in the last campaign (June), the tracheids of the tree-ring formed in 2022 were measured (Figure 5). The cross-sectional tracheid anatomical features measured were lumen radial diameter [LRD] and cell wall thickness [CWT]. The ratio LRD/CWT was calculated to determine the earlywood and latewood type cells, with LRD/CWT$\geq$2 or <2, respectively, according with the Mork's law (Denne, 1988).

In each stem cross section, three radial rows were selected to measure tracheid features. When a radial row was not consecutive, due to deformed cells, the row was completed using another portion of the cross section, and then averaged into a representative radial file (tracheidogram) of the tree-ring using the R package tracheideR (Campelo *et al*., 2016) under the R programming environment (CRAN: http://cran.r-project.org). Mean tracheidograms (Cook & Kairiukstis, 1990; Vaganov *et al*., 2006) of LRD, CWT, and LRD/CWT were standardized by the "kCells" method using the tracheideR package, with the number of cells corresponding to the rounded average cell number for each species and treatment (Hothorn *et al*., 2006).
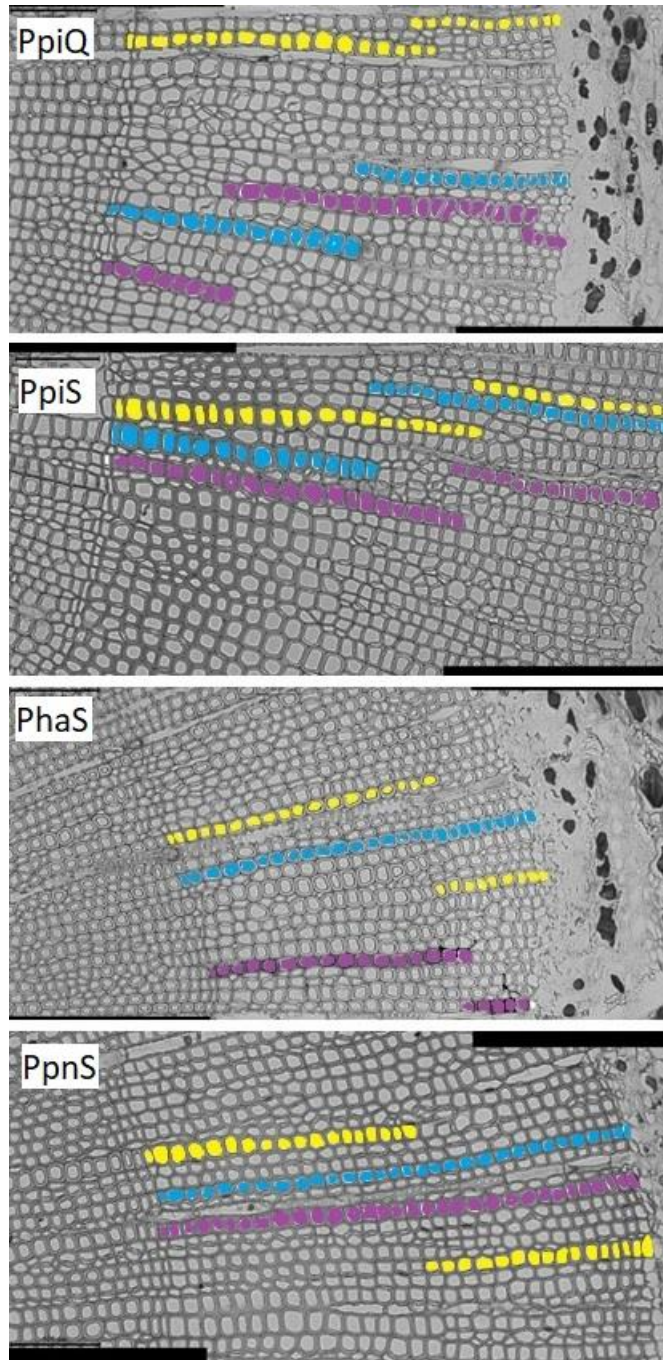
Figure 5. Examples of the selection of three radial files used to calculate the tracheidograms. PpiQ: *Pinus pinaster* Qualified; PpiS: *Pinus pinaster* Selected; PhaS: *Pinus halepensis* Selected; PpnS: *Pinus pinea* Selected. Each color represents a radial row.

Statistical Analysis

Survival rates were analyzed through R package survival (T. Therneau, 2023; T. M. Therneau & Grambsch, 2000), lubridate (Grolemund & Wickham, 2011) and ggsurvfit (Sjoberg *et al*., 2023). Differences were tested with analysis of variance between species and between treatments for variables like stem diameter, height, shoot biomass, root biomass and shoot/root biomass, TLP, needle size, epidermis thickness, needle cross section total area, central cylinder area, parenchyma area, vascular bundle area, LRD, CWT and ratio LRD/CWT using Linear Model (LM) using the function lm(). For plant height it was used the General Linear Model (GLM) with family distribution Gaussian and for the number of tracheids cells the GLM with family distribution Poisson using the function glm() from an R package lme4 (Bates *et al*., 2015). The package car (Hothorn *et al*., 2006) was used for the normality and homogeneity test using the functions shapiro.test() and leveneTest(). For the non-parametric tests Asymptotic Wilcoxon-Mann-Whitney Test was used with function wilcox_test() from the R package coin (Hothorn *et al*., 2006) for parameters such as Vascular Bundle T_{area}. Analysis was conducted using R version 4.1.2 (R Core Team, 2022), RStudio (Rstudio Team, 2020).

Results

Climatic Conditions and Soil Water Content

The total precipitation in April 2022 was 2.8mm, in May 2022 was 5.5mm and in the first half of June 2022 was 311mm (Figure 6). It was a year with a very dry spring, reflected in the high mortality rates of the seedlings (see results below). The average temperature in April 2022 was 14.6ºC, in May 2022 was 19.2ºC and in the first half of June 2022 was 21.3ºC (Figure 6). The relative humidity in April 2022 was 77.4%, in May 2022 was 70.1% and in the first half of June 2022 was 75.7% (Figure 6). The average daytime radiation in April, May and the first half of June was 1710, 1950 and 2002 $\mu mol\ m^{-2}\ s^{-1}$, respectively.

From April to May, and both in the CT and DT treatment, soil moisture at 5cm depth was below the detection limit of the equipment used, indicating that even for the control plants it was a very dry spring.
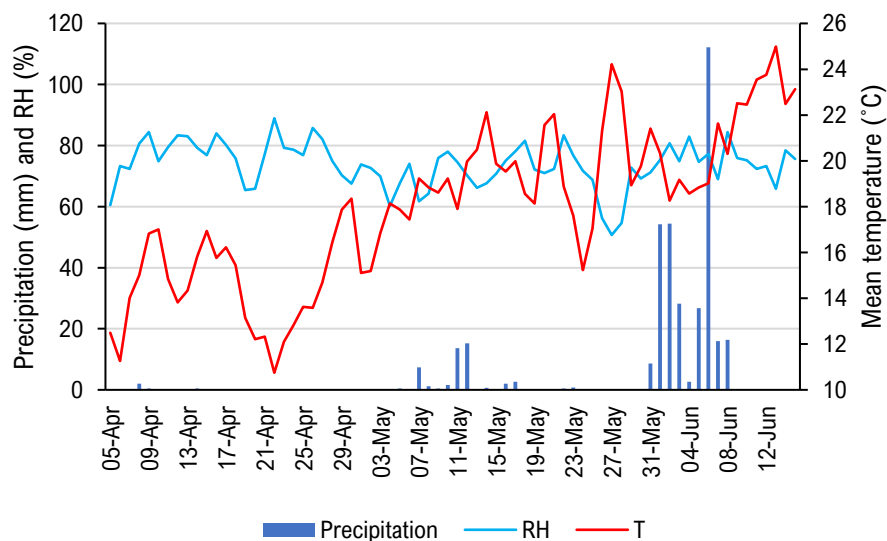
Figure 6. Daily precipitation, relative humidity (RH), and mean temperature (T) from April to mid-June 2022. The climatic data were acquired from a meteorological station installed in the experimental plot.

Survival rate

The survival rate was similar in the CT and DT treatment for PpnS (p-value=0.8) and PhaS (p-value=0.4). However, for PpiQ and PpiS the survival rate was significantly lower for the DT treatment with a p-value=0.005 and a p-value=5e-04, respectively (Figure 7).

Figure 7. Survival rates of *Pinus pinaster* Q (PpiQ), *P. pinaster* S (PpiS), *P. halepensis* S (PhaS) and *P. pinea* S (PpnS) between control and drought treatment.
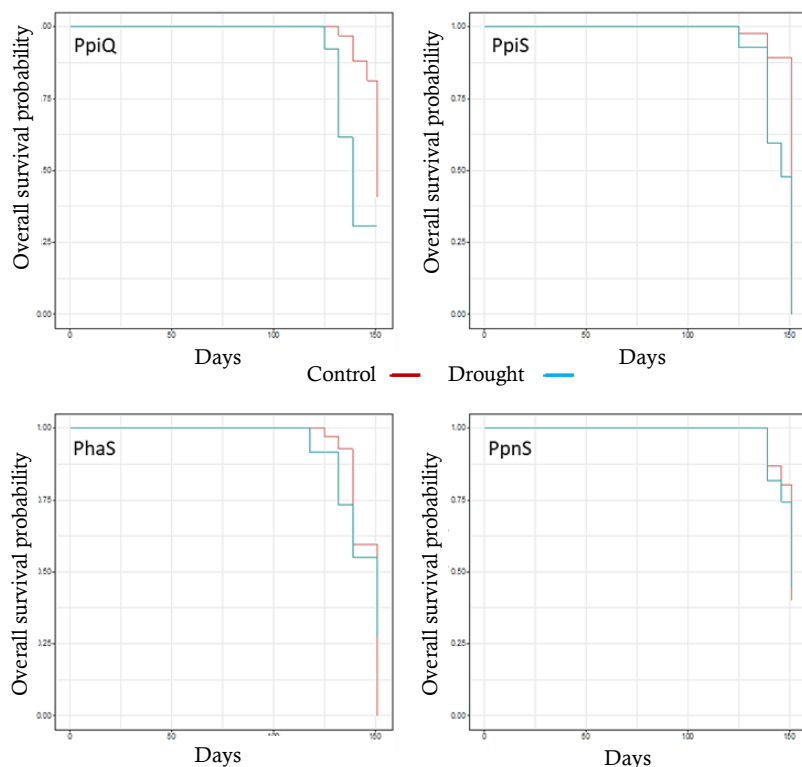
Diameter, Height, and Biomass

In April 2022, we have compared the diameter, height, and biomass of the different species. For the diameter there were significant differences between the species (p-value=0.00039), with *P. halepensis* showing the thinnest stem, and *P. pinea* the thickest stem (Table 1). In terms of height significant differences (p-value=2.2e-16) were registered between species, with both *P. pinaster* provenances significantly taller compared with PpnS and PhaS (Table 1).

Shoot biomass showed no significant differences (p-value=0.407) between species (Table 1). The root biomass was significantly higher in PhaS (p-value=0.00632) compared to PpnS and PpiS, but similar to PpiQ (Table 1). The ratio of shoot/root biomass of PpnS showed significantly higher values compared with PhaS (Table 1).

In June we compared CT and DT treatment, within each species. For the height, diameter, and shoot biomass there were no significant differences between the treatments for all the species (Table 1). The root biomass was significantly lower in the DT treatment in PhaS (Table 1). The ratio of shoot/root biomass was significantly higher in the drought treatment in PpiS (Table 1).

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Table 1. Diameter, height, shoot biomass, root biomass and shoot/root biomass of *Pinus pinaster* Q (PpiQ), *P. pinaster* S (PpiS), *P. halepensis* S (PhaS) and *P. pinea* S (PpnS) in April and June (control and drought treatment). Mean±SD.

Species\Treatment	Diameter (mm) April[1]	Diameter (mm) June[2] control	Diameter (mm) June[2] drougth	Height (cm) April[1]	Height (cm) June[2] control	Height (cm) June[2] drougth	Shoot biomass (g) April[1]	Shoot biomass (g) June[2] control	Shoot biomass (g) June[2] drougth	Root Biomass (g) April[1]	Root Biomass (g) June[2] control	Root Biomass (g) June[2] drougth	Shoot/Root Biomass April[1]	Shoot/Root Biomass June[2] control	Shoot/Root Biomass June[2] drougth
PpiQ	4.02 ± 0.49^{bc}	4.56 ± 0.62	4.34 ± 0.36	24.7 ± 2.6^{b}	25.0 ± 2.3	25.7 ± 3.3	2.849 ± 0.466	3.524 ± 0.892	4.121 ± 0.979	4.501 ± 0.563^{ab}	3.590 ± 0.625	4.254 ± 1.028	0.632 ± 0.050^{ab}	0.974 ± 0.078	0.970 ± 0.040
PpiS	3.78 ± 0.28^{ab}	4.34 ± 0.20	4.26 ± 0.39	24.7 ± 1.8^{b}	25.8 ± 1.8	24.8 ± 2.2	3.005 ± 0.353	3.728 ± 0.654	4.043 ± 0.369	3.533 ± 0.946^{a}	3.683 ± 1.044	2.680 ± 0.608	0.894 ± 0.252^{bc}	**1.031 ± 0.104***	**1.537 ± 0.188**
PhaS	3.70 ± 0.54^{a}	4.39 ± 0.68	4.07 ± 0.48	19.1 ± 2.1^{a}	20.0 ± 1.7	19.9 ± 2.3	2.433 ± 0.590	3.217 ± 0.741	2.289 ± 0.391	5.676 ± 1.067^{b}	**4.088 ± 0.479***	**2.896 ± 0.439**	0.428 ± 0.057^{a}	0.780 ± 0.093	0.789 ± 0.030
PpnS	4.10 ± 0.33^{c}	4.62 ± 0.33	4.52 ± 0.37	19.5 ± 1.0^{a}	20.6 ± 1.1	20.7 ± 1.2	2.612 ± 0.111	3.901 ± 0.184	3.630 ± 0.522	2.567 ± 0.207^{a}	2.979 ± 0.290	3.032 ± 0.407	1.020 ± 0.064^{c}	1.317 ± 0.129	1.201 ± 0.115

(1) different letters indicate statistical differences between species
(2) an asterisk and bold indicate statistical differences between treatments

Turgor Loss Point (TLP)

When comparing the species in April, there were significant differences in the TLP between PhaS and PpiQ, with PhaS showing a higher TLP (Figures 8). When comparing the TLP in June between CT and DT for each species, there were no significant differences.
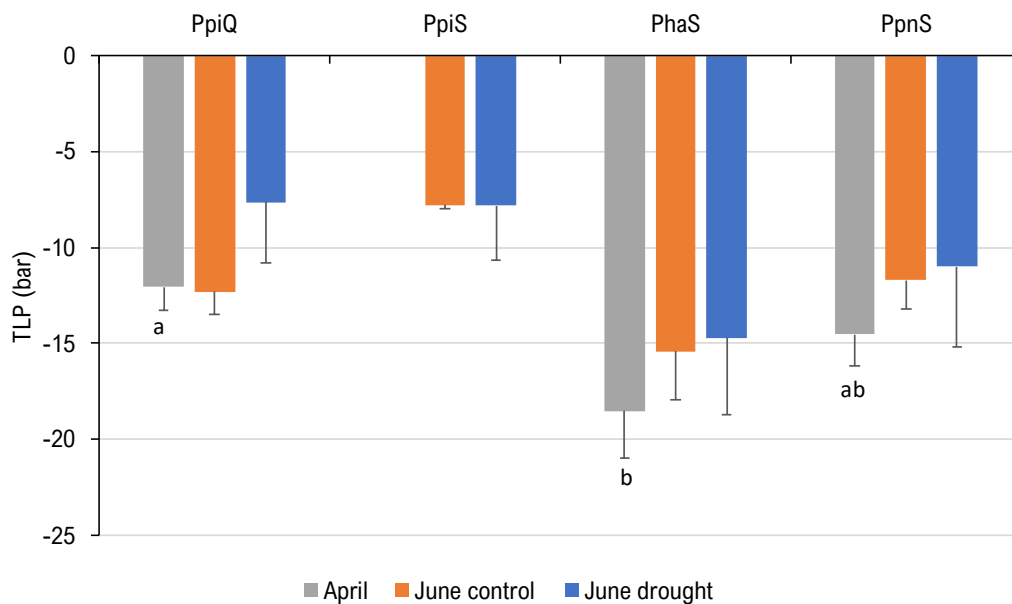

Figure 8. Turgor loss point (TLP) of *Pinus pinaster* Q (PpiQ), *P. pinaster* S (PpiS), *P. halepensis* S (PhaS) and *P. pinea* S (PpnS) in April and June (control and drought treatment). Mean±SD. Different letters show significant differences between species in April.

Needle Anatomy

All the anatomical parameters measured in the needles in April showed no significant differences between the species (Table 2). In June, the needle size was significantly lower in the drought treatment of PpiS (Table 2). The epidermal thickness was significantly higher in the needles of the drought treatment of PhaS (Table 2).

Table 2. Needle size, epidermis thickness, needle area, central cylinder area and vascular bundle area of *Pinus pinaster* Q (PpiQ), *P. pinaster* S (PpiS), *P. halepensis* S (PhaS) and *P. pinea* S (PpnS) in April and June (control and drought treatment). Mean±SD.

Species\Treatment	Needle Size (cm)			Needle T_{area} (mm^2)			Epidermis Thickness (μm)			Central cylinder T_{area} (mm^2)			Vascular bundle T_{area} (mm^2)		
	April [1]	June [2]		April [1]	June [2]		April [1]	June [2]		April [1]	June [2]		April [1]	June [2]	
		control	drougth		control	drougth		control	drougth		control	drougth		control	drougth
PpiQ	1.4 ± 0.2	1.6 ± 0.2	1.5 ± 0.2	0.363 ± 0.087	0.397 ± 0.052	0.354 ± 0.046	18.836 ± 1.363	25.477 ± 7.118	24.196 ± 3.138	0.064 ± 0.007	0.082 ± 0.010	0.082 ± 0.010	0.012 ± 0.002	0.013 ± 0.007	0.010 ± 0.001
PpiS	2.0 ± 0.4	**1.8 ± 0.1***	**1.5 ± 0.1**	0.354 ± 0.096	0.370 ± 0.042	0.418 ± 0.140	16.979 ± 3.036	21.097 ± 1.949	23.655 ± 1.623	0.064 ± 0.015	0.082 ± 0.005	0.082 ± 0.005	0.013 ± 0.003	0.009 ± 0.001	0.011 ± 0.005
PhaS	2.0 ± 0.2	1.5 ± 0.5	1.5 ± 0.3	0.255 ± 0.017	0.234 ± 0.021	0.222 ± 0.018	19.039 ± 0.750	18.325 ± 0.347	20.412 ± 0.419	0.057 ± 0.007	0.050 ± 0.004	0.050 ± 0.004	0.010 ± 0.001	0.009 ± 0.000	0.008 ± 0.001
PpnS	1.6 ± 0.5	1.4 ± 0.1	1.5 ± 0.2	0.347 ± 0.051	0.346 ± 0.014	0.352 ± 0.020	20.014 ± 2.973	**19.724 ± 2.061***	**21.756 ± 2.019**	0.083 ± 0.009	0.108 ± 0.006	0.108 ± 0.006	0.012 ± 0.002	0.012 ± 0.002	0.010 ± 0.004

(1) different letters indicate statistical differences between species
(2) an asterisk and bold indicate statistical differences between treatments

<u>Tracheidograms</u>

In April, the average number of tracheids formed was ~21±2, 19±2 , 18±4 and 15±1 in PpiQ, PpiS, PhaS and PpnS, respectively (Figure 9), but no significant differences were detected between species (p= 0.171). In June, no differences were observed in the number of tracheids between CT and DT for each species (Figure 9).
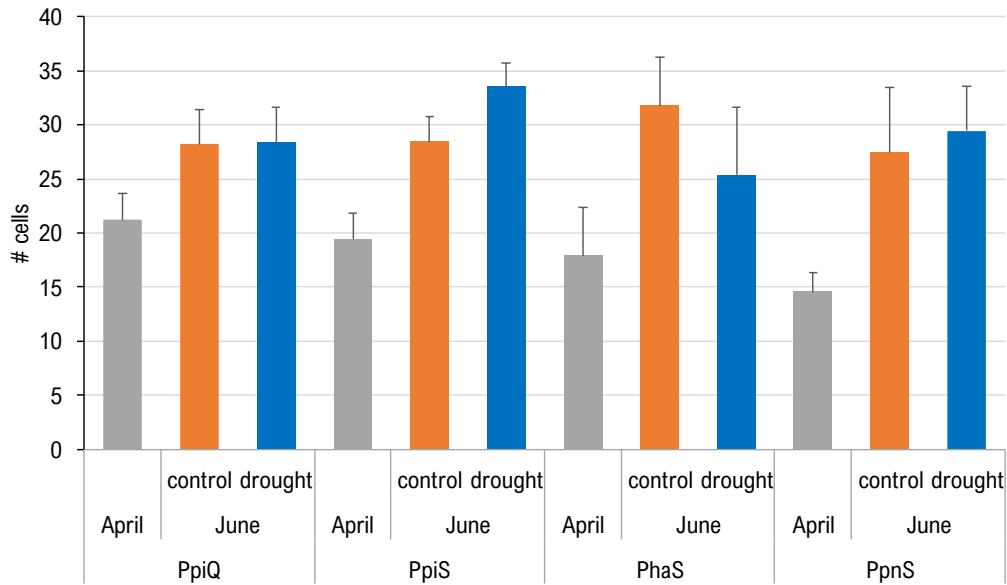

Figure 9. Number of tracheid cells (#cells) of *Pinus pinaster* Q (PpiQ), *P. pinaster* S (PpiS), *P. halepensis* S (PhaS) and *P. pinea* S (PpnS) in April and June (control and drought treatment). Mean±SD.

The lumen radial diameter (LRD)

For all species, and in both treatments, there was a general decline in LRD along the growing season (Figure 10). For each species, there were no significant differences in LRD between CT and DT, when comparing the average values until April and between April and mid-June 2022.
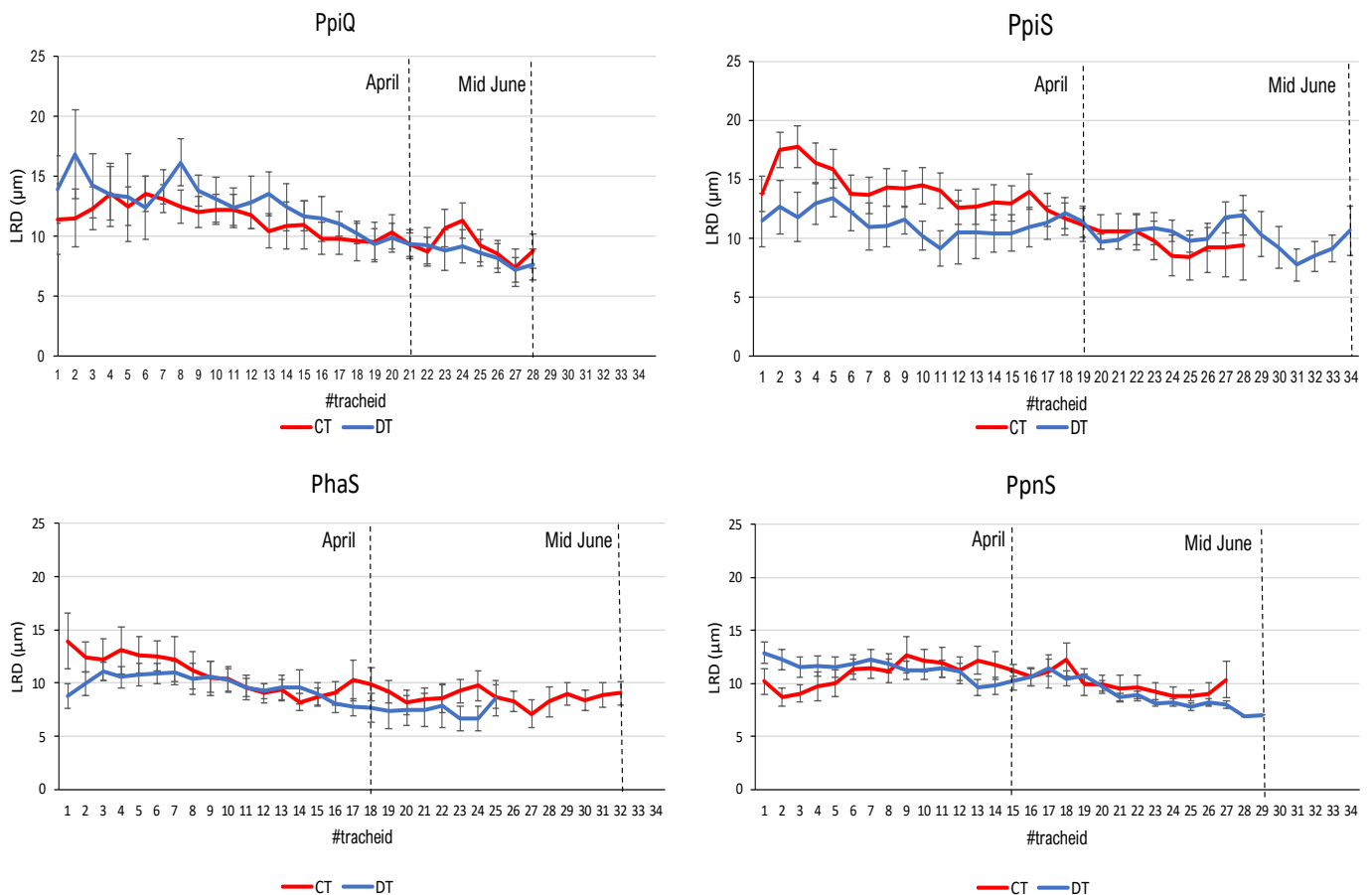

Figure 10. Lumen radial diameter (LRD) of *Pinus pinaster* Q (PpiQ), *P. pinaster* S (PpiS), *P. halepensis* S (PhaS) and *P. pinea* S (PpnS) of the tree-ring formed in 2022 of the individuals allocated to the control (CT) and drought (DT) treatment. The two-vertical dash lines show the number of tracheids in April and mid-June. Although all individuals were randomly allocated to the CT and DT from the beginning of the experiment, only by the end of April the drought treatment was applied. Mean±SE.

The cell wall thickness (CWT)

For all the species and treatments, CWT showed no clear trend during the growing season (Figure 11). However, PpnS seems to have a slight increasing trend of CWT along the growing season. By mid-June, for all species, and for both treatments, there was a decline in CWT (Figure 11). When comparing the average values of CWT until April and from April to mid-June 2022, between CT and DT, only PpiS showed a marginal significant value (p=0.0506), with trees allocated to the DT treatment showing higher values of CWT in April.
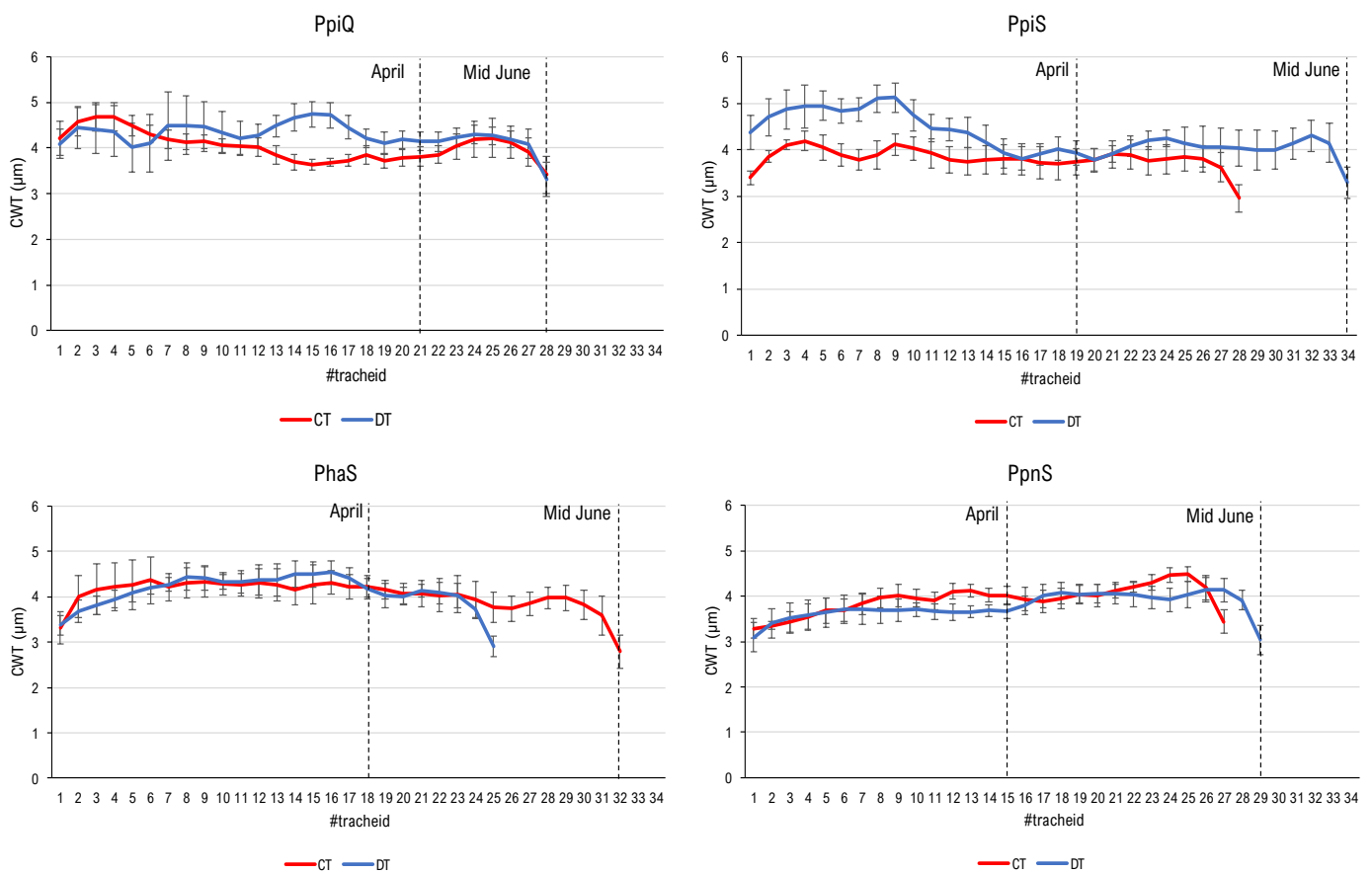

Figure 11. Cell Wall Thickness (CWT) of *Pinus pinaster* Q (PpiQ), *P. pinaster* S (PpiS), *P. halepensis* S (PhaS) and *P. pinea* S (PpnS) of the tree-ring formed in 2022 of the individuals allocated to the control (CT) and drought (DT) treatment. The two-vertical dash lines show the number of tracheids in April and mid-June. Although all individuals were randomly allocated to the CT and DT from the beginning of the experiment, only by the end of April the drought treatment was applied. Mean±SE.

The ratio between LRD/CWT

All species showed a declining trend of the ratio LRD/CWT along the growing season, although not so clearly visible for the DT treatment of PpiS (Figure 12). The ratio LRD/CWT was most of the times ≥2, meaning that the tracheids produced were earlywood-like cells. However, PhaS showed the lowest values of LRD/CWT and by the end of April, the DT treatment showed values <2, indicating latewood-like cells (Figure 12).
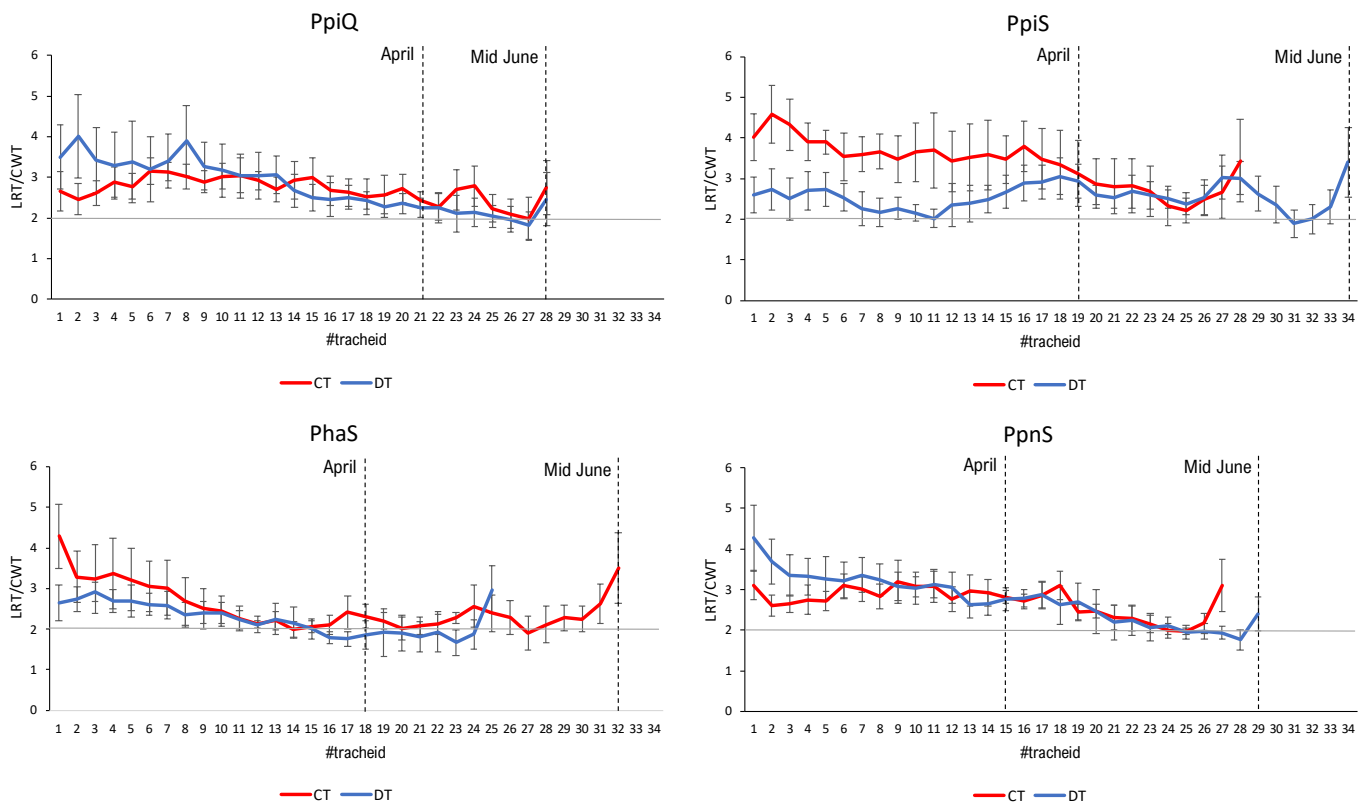

Figure 12. Ratio between LRD/CWT of *Pinus pinaster* Q (PpiQ), *P. pinaster* S (PpiS), *P. halepensis* S (PhaS) and *P. pinea* S (PpnS) of the tree-ring formed in 2022 of the individuals allocated to the control (CT) and drought (DT) treatment. The two-vertical dash lines show the number of tracheids in April and mid-June. The horizontal dash line shows the limit to consider an earlywood-like tracheid (≥2) and latewood-like tracheid (<2). Although all individuals were randomly allocated to the CT and DT from the beginning of the experiment, only by the end of April the drought treatment was applied. Mean±SE.

Discussion

The study aimed to understand the response of seedlings to water scarcity of three Mediterranean pine species, *P. pinaster*, *P. pinea* and *P. halepensis*. This knowledge is crucial in the context of climate change, and the associated risk of more frequent and severe drought events, especially in seedlings, a vulnerable stage of the tree's life. Seedlings were subjected to full water exclusion, and we have measured survival rates, growth (height, stem diameter, biomass), turgor loss point and anatomical parameters of the needles and woody stem.

The spring of 2022 was very dry, reflected in the high mortality rate of both the control and drought treatment, and for all the pine species. The three-pine species are considered to have a 'water-saving' strategy (Baquedano & Castillo, 2006; Pardos & Calama, 2018; Tognetti et al., 1997), and close the stomata before changes occur in leaf water potential. However, with the closure of stomata carbon assimilation can be completely inhibited, increasing the risk of oxidative stress and thus mortality (Alexou, 2013).

The first mortality events occurred in *P. halepensis* of the drought treatment, which was an unexpected result because this pine species has a high drought tolerance, compared with *P. pinaster* and *P. pinea* (Atzmon et al., 2004). However, mortality rates of seedlings of Aleppo pine under field conditions can reach 40-60% (Daskalakou & Thanos, 2004). In mid-June, the species with the most individuals alive was *P. pinea*. *Pinus pinaster* and *P. halepensis* are considered fast-growing species, while *P. pinea* is a slow growing species (Chambel et al., 2007). Maybe this can explain the fact that *P. pinea* seedlings survived for a longer time.

When comparing the survival rate between the control and drought treatment, *P. halepensis* and *P. pinea* showed no differences, while for *P. pinaster* the survival rate was lower in the drought treatment. Thus for *P. pinaster* the treatment made a difference, with a higher survival rate in the control treatment. *Pinus pinaster* occurs mainly near the coastal area, where fog occurs frequently. Foliar water uptake has been documented in some pine species and is related with the layer of wax that covers the leaves, with older leaves absorbing more water, due to erosion of the wax with age (Roth-Nebelsick et al., 2023). The experimental area was located near the Mondego river and frequent fogs occur during the morning, reflected in the high humidity values, even when precipitation in April and May was quite low. Maybe *P. pinaster* can profit from these fogs by foliar water uptake reducing the water stress. An experiment with irrigated and non-irrigated seedlings of *P. pinaster* showed that the needles from the non-irrigated treatment had a higher quantity of wax that can be a response to reduce the drought

stress (Le Provost et al., 2013). Thus, in our experiment, the wax layer of the needles might have increased in *P. pinaster* of the drought treatment, limiting the foliar water uptake. Concerning the other two species, *P. halepensis* and *P. pinea*, their natural distribution is associated with drier areas, and if the amount of wax represents an adaptation to drought stress, it can be a constitutional characteristic, precluding foliar water uptake, and partly explaining why there were no differences in the survival rates of the control and drought treatment.

Concerning the growth parameters and comparing the species before the application of the drought treatment, *P. pinaster* was taller, while *P. halepensis* and *P. pinea* were smaller. *Pinus halepensis* showed the highest root biomass reflected in the lowest shoot/root biomass compared with the other pine species. The higher root biomass of *P. halepensis* seedlings can represent an adaptation to dry conditions, prioritizing the part that scavenges for water (Eziz et al., 2017). Comparing the control and drought treatment, *P. halepensis* had a significant lower root biomass in the drought treatment, although the shoot/root ratio showed no significant differences. The *Pinus pinaster* Selected showed a significant increase of the shoot/root biomass in the drought treatment, meaning that invested relatively more in the shoot compared with the root biomass, that goes against the optimal partitioning theory (OPT). The OPT of root-shoot dynamics says that when a plant has low access to nutrients or water, it produces relatively more root biomass, allowing greater soil exploitation. Under low light conditions, shoot growth is favored to maximize light interception (Gedroc et al., 1996). However, the OPT is probably a very simple view of optimal resource allocation to optimize plant-growth rate, with similar growth rates possible with different allocations (Robinson, 2022).

Comparing the turgor loss point (TLP) between the pine species before the application of the drought treatment, the highest TLP was in *P. halepensis*, and the lowest in *P. pinaster*, with *P. pinea* in between those two species. A higher TLP value indicates that the species is much more drought tolerant, meaning that can withstand higher tensions before cavitation occurs. The TLP values are somehow associated with the ecological distribution of these pine species, with *P. halepensis* occupying drier areas and *P. pinaster* more humid areas (Gracia et al., 2002). This makes *P. pinaster* more sensible to drought events, and the first to be affected by heat waves and extreme drought events (Taïbi et al., 2017). The vulnerability of the hydraulic system to drought can be related with the morphological structure of tracheids, like the lumen area, pit membranes, that can differ between the pine species, rendering them more, or less tolerant to drought (Esteban et al., 2010). Comparing the control and drought treatment for each species,

there were no differences in TLP, indicating that the water conditions of the control and drought treatment were not so different, due to a very dry spring in 2022.

The several traits measured in the needles, from the size to anatomical traits like epidermal thickness and needle transversal area, showed no significant differences between the species. Comparing the control and drought treatment for each species, *Pinus pinaster* Selected showed a significant reduction in the needle size of the drought treatment. A reduction in the leaf size reduces the area exposed to evapotranspiration and can be a strategy to reduce the loss of water (Kuusk et al., 2018). *Pinus halepensis* showed a significant increase of the epidermis thickness in the drouth treatment. Increasing the epidermis thickness is another strategy that can reduce the evaporation from the leaf surface (Boddi et al., 2002).

The average number of tracheids produced in April, before the application of the drought treatment, showed no significant differences between the species. Nonetheless, *P. pinea* produced fewer tracheids, that might be related to the fact that it is considered a slow growing species. When comparing the total number of tracheids produced until mid-June, comparing the control and drought treatment for each species, again, no significant differences were found. However, in *P. pinaster* Selected, the number of tracheids produced in the drought treatment was slightly higher, compared with the control treatment, while the opposite occurred in *P. halepensis*. As mentioned before, the seedlings of *P. halepensis* from the drought treatment showed a lower root biomass, so maybe those seedlings were under more difficulties in obtaining water and/or having to close the stomata for longer periods. This would have an impact on the carbon acquisition, necessary to produce the tracheids (Anderegg et al., 2016). The higher number of tracheids of *P. pinaster* Selected in the drought treatment is somehow counterintuitive. Maybe it has to do with the characteristics of the tracheids, like LRD and CWT.

The average LRD of the tracheids produced until April 2022, and from April to mid-June, comparing the individuals allocated to the control and drought treatment for each species, showed no significant differences. Performing the same analysis for CWT, *Pinus pinaster* Selected showed an almost significant difference between the control and drought treatment, with the seedlings of the drought treatment showing a higher CWT and a lower LRD/CWT ratio until April. A higher LRD/CWT ratio indicates a lower hydraulic efficiency but a higher resistance to embolism, with a higher CWT reducing the risk of implosion due to high tensions. Narrower conduits and thicker cell walls can decrease the probability of cavitation and ultimately increase the hydraulic safety of the system (Hacke et al., 2001, 2004; Tognetti et al., 1997). It seems that

the seedlings of *P. pinaster* Selected allocated to the drought treatment showed, from the beginning, better hydraulic characteristics to withstand drought. This could explain why they have produced more tracheids. Although *P. pinaster* Selected showed a lower survival rate in the drought treatment, the individuals that survived until mid-June seem to have characteristics that allowed them to even produce more tracheids. Interestingly, the LRD/CWT ratio of *P. halepensis* allocated to the drought treatment by the end of April showed values <2, indicating latewood-like cells. It seems that *P. halepensis* was responding to stronger drought conditions by producing tracheids less prone to cavitation.

The CWT showed a general decrease in mid-June, and a slight increase of the LRD/CWT, occurring in all species and treatments. This was probably related to an increase of the tracheids turgidity in response to the precipitation that occurred in the first two weeks of June, 311mm, compared with a total of 8.3mm of April and May. It is known that plant cell expansion is due to the action of turgor pressure against the cell wall, when above a certain threshold (Cabon et al., 2020). Models of wood formation have long recognized the relationship between conduit enlargement and water availability (Cabon et al., 2020).

Conclusion

The very dry spring of 2022, and the decision of not irrigating the control seedlings, reduced the differences in water availability between the control and drought treatment, limiting the potential differences in the response of the species to the treatments. This also caused a high mortality, reducing the number of replicates, that also limits the statistical analysis. Nonetheless, despite the limitations imposed by the dry spring and reduced water availability, the study provides valuable insights into the response of Mediterranean pine species to water scarcity. The unexpected mortality of *P. halepensis* in the drought treatment highlights the vulnerability of a drought-tolerant species during critical stages of their life cycle.

Species showed differences in their growth patterns and biomass allocation, with *P. pinaster* being taller and *P. halepensis* exhibiting higher root biomass. The observed shift in resource allocation towards shoot growth in *P. pinaster* under drought conditions contradicted the optimal partitioning theory, highlighting the complexity of plant responses to limited resources.

In terms of physiological traits, the turgor loss point (TLP) reflected the species' ecological distribution, with *P. halepensis* exhibiting the highest TLP and *P. pinaster* the lowest. This suggests that *P. pinaster* is more susceptible to drought events, emphasizing its vulnerability in face of climate change.

The anatomical traits measured in the needles showed no differences between the pine species. *Pinus halepensis*, in response to drought, showed an increase of epidermis thickness, probably a strategy to reduce the loss of water. *Pinus pinaster* Selected showed a reduction in the needle size, a classical strategy to reduce the exposed area and thus reduce evapotranspiration.

The anatomical traits of the tracheids are linked to the hydraulic capacity of trees, with the lumen area and cell wall thickness representing an adjustment to the prevalent environmental conditions. In our study no differences were observed in the lumen radial diameter (LRD) and cell wall thickness (CWT) between the control and drought treatment, probably related to the fact that the soil water content of the control and drought treatment were not so different. However, *P. pinaster* Selected produced more tracheids in the drought treatment, and the tracheids formed until April showed a thicker cell wall and a lower ratio LRD/CWT. It seems that the individuals that were randomly allocated to the drought treatment and that survived to mortality along the experiment, the initial characteristics of the tracheids, before the application of the drought treatment, were better to withstand drought stress. As an additional analysis to be made in the future, as we have collected the stem of all the individuals that were dying along

the experience, it will be very interesting to characterize their tracheidograms to see if they died earlier because the characteristics of the tracheids made them more susceptible to cavitation.

This study highlights the importance of understanding the response of Mediterranean pine species to water scarcity in the context of climate change, particularly during the vulnerable seedling stage.

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